Publishing Sector of the Society of Physicians of Vojvodina of the Medical Society of Serbia, Novi Sad, Vase Stajica 9,

MEDICAL REVIEW

JOURNAL OF THE SOCIETY OF PHYSICIANS OF VOJVODINA OF THE MEDICAL SOCIETY OF SERBIA

THE FIRST ISSUE WAS PUBLISHED IN 1948

MEDICAL REVIEW is published two-monthly (six double issues per a year) in the circulation of 1000 copies. Payment for individuals from the territory of Serbia for the year 2014 is 3,000.00 dinars (the VAT being calculated in) and 4,000.00 dinars for the individuals outside the territory of Serbia, and 8,000.00 dinars (+ the VAT) for institutions. The payments are to be made to the account number 340-1861-70 or 115-13858-06, with the remark "Additional membership fee for the Medical Review".

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Printed by: Department for Joint Affairs of Provincial Authorities - Sector for Printing

Izdavačka delatnost Društva lekara Vojvodine Srpskog lekarskog društva, Novi Sad, Vase Stajića 9

MEDICINSKI PREGLED

ČASOPIS DRUŠTVA LEKARA VOJVODINE SRPSKOG LEKARSKOG DRUŠTVA
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Štamparija: Uprava za zajedničke poslove pokrajinskih organa - Odsek za poslove štamparije

M E D I C A L R E V I E W
JOURNAL OF THE SOCIETY OF PHYSICIANS OF VOJVODINA OF THE MEDICAL SOCIETY OF SERBIA
Novi Sad Vase Stajića 9 Serbia

Med Pregl 2014; LXVII (9-10): 273-356. Novi Sad: September-October.

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Novi Sad *Vase Stajića 9* *Srbija*

Med Pregl 2014; LXVII (9-10): 273-356. Novi Sad: septembar-oktobar.

SADRŽAJ

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Clinical Centre of Vojvodina, Novi Sad
Centre for Radiology

Original study
Originalni naučni rad
UDK 616.831-005.1-031-073
DOI:10.2298/MPNS1410277J

THE QUESTION IS WHETHER HEMIPARESIS IS MORE COMMON IN UNILATERAL THAN BILATERAL CHRONIC SUBDURAL HEMATOMA

PITANJE JE DA LI JE HEMIPAREZA ČEŠĆA KOD UNILATERALNOG NEGO BILATERALNOG HRONIČNOG SUBDURALNOG HEMATOMA

Mirela JUKOVIĆ, Kosta PETROVIĆ and Viktor TILL

Summary

Introduction. Chronic subdural hematoma is an intracranial hemorrhagic lesion that illustrates various expressions in clinical and radiological practice. The aim of this study was to emphasize the correlation between the brain site of chronic subdural hematoma and clinical symptoms/signs of disease. Furthermore, the study denotes the significance of hemiparesis occurrence in the patients with unilateral chronic subdural hematomas more than in those with bilateral ones, associated with time required to diagnose hematoma. **Material and Methods**: A three-year study included 72 patients with chronic subdural hematoma. According to their clinical and neurological symptoms on hospital admission, all patients underwent non-contrast brain computed tomography scan, which confirmed the diagnosis. The radiological parameters, inlcuding the site of chronic subdural hematoma, a hematoma width and midline shift were recorded to give precise data about the correlation with neurological symptoms. A special focus was put on the lag time between the onset of symptoms and signs to diagnosis of chronic subdural hematoma. **Results**. The study proved that the patients with unilateral chronic subdural hematoma had more frequent occurrence of hemiparesis than the patients with bilateral chronic subdural hematoma. It took the left-sided chronic subdural hematomas less time (about 200 hours earlier) than the right-sided ones to present its symptoms although the average hematoma diameter value was almost the same. **Conclusion**. The site and the form of intracranial lesion-chronic subdural hematoma could have a great influence on neurological and functional condition in a patient. Although the length of time required for making diagnosis as well as clinical symptoms greatly differ and the latter are not always so clear, physicians should maintain a high level of suspicion for this disease and thus contribute to prompt diagnosis and better clinical outcome of patients.

Key words: Paresis; Hematoma, Subdural, Chronic; Tomography X-Ray, Computed; Diagnosis; Signs and Symptoms

Sažetak

Uvod. Hronični subduralni hematom je intrakranijalna hemoragična lezija koja se različito ispoljava u kliničkoj i radiološkoj praksi. Cilj ove studije je da ukaže na povezanost strane nastanka hroničnog subduralnog hematoma i kliničkih simptoma/znakova bolesti. Pored toga, studija ukazuje na značajnije ispoljavanje hemipareze kod bolesnika sa unilateralnim nego sa bilateralnim hroničnim subduralnim hematomom, kao i povezanost sa vremenom koje je potrebno za dijagnostiku hroničnog subduralnog hematoma. **Materijal i metode.** U trogodišnjoj studiji evaluirano je 72 pacijenta sa hroničnim subduralnim hematomom. Prema kliničkim i neurološkim simptoma koje pacijenti sa hroničnim subduralnim hematomom ispoljavaju na bolničkom prijemu, svi su podvrgnuti nekontrastnom skeneru glave koji je potvrdio dijagnozu. Radiološki parametri: strana hematoma, širina hematoma i pomeranje u mediosagitalnoj liniji mereni su radi dobijanja preciznijih podataka o korelaciji sa neurološkim znacima i simptomima kod pacijenata. Poseban akcenat je stavljen na vreme od pojave simptoma i znakova do dijagnostike hroničnog subduralnog hematoma. **Rezultati.** Studija je pokazala da pacijenti sa jednostranim hroničnim subduralnim hematomom imaju češću pojavu hemipareze od bolesnika sa bilateralnim hroničnim subduralnim hematomom. Od svih unilateralnih hematoma, levostrani hronični subduralni hematomi su pokazali kraće vreme (oko 200 sati ranije) prezentacije simptoma kod pacijenata, iako je prosečna vrednost širine hematoma bila skoro jednaka kao i kod desnostranih. **Zaključak.** Mesto nastanka hroničnog subduralnog hematoma intrakranijalno kao i njegova forma može imati veliki uticaj na neurološko i funkcionalno stanje pacijenta. Iako vreme za dijagnostiku hroničnog subduralnog hematoma i simptomi i znaci bolesti variraju i nisu uvek tako jasni, lekari pri pregledu pacijenta moraju izraziti visok stepen sumnje na ovo oboljenje što će doprineti bržoj dijagnostici i boljem kliničkom ishodu lečenja pacijenta.

Ključne reči: Pareza; Hronični subduralni hematom; CT; Dijagnoza; Znaci i simptomi

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Abbreviations
CSDH – chronic subdural hematoma
CT – computed tomography
FA – fractional anisotropy
DTI – diffusion-tensor-imaging

Introduction

Chronic subdural hematoma (CSDH) is a common intracranial hemorrhagic lesion which could be presented on a brain computed tomography (CT) scan as unilateral **(Figure 1)** or bilateral form **(Figure 2)**. Patients with these extra axial crescent fluid collections show a spectrum of symptoms/signs in the clinical presentation of this disease. Although it is a relatively common disease in elderly, the prompt diagnosis is not usually so clear. In literature, the unilateral chronic subdural hematoma is more frequent than the bilateral one [1, 2]. The incidence of a bilateral CSDH is about 16-20% in population [3]. However, why some patients have unilateral and other bilateral hematoma or what determines the site of CSDH is not still quite clear. Lee at al. showed the influence of the cranial morphology on the formation of either unilateral or bilateral CSDH. Unilateral hematomas are more frequent in asymmetric cranium, but bilateral CSDHs are more common in the symmetric cranium [4]. According to Kim et al. asymmetric cranium is defined when the angles for both sides have difference bigger than 2 degrees [5].

Material and Methods

A prospective three-year study involved 72 patients with uni-or bilateral CSDH who had been diagnosed by CT scan at the Clinical Centre of Vojvodina, Centre for Radiology, and hospitalized and treated at the Deaprtment of Neurosurgery. All patients underwent clinical and neurological examination, which was followed by initial non-contrast brain CT scan. The CT brain protocol included axial slices parallel to the infraorbitomeatal line, from the foramen magnum to the apex of skull using *Somatom Emotion 16 and Cardiac Sensation 64 CT scan, Siemens, Germany.* Technical parameters for routine brain CT scan are: 120 kV, 400 mAs, detector collimation 0.75 mm, slice thickness 5 mm, with Kernel H31ms (www.ctisus.com). The brain CT findings included the side and the diameter of hematoma, as well as the midline shift and the angle of the cranium for both sides. The angle was measured by Picture Archiving Communication System (PACS) system using two lines: one passing through the median plane as the connection with the crista galli and occipital protuberance, and the other one touching the cranium tangentially at the point of its maximal convexity. The diameter of the bilateral hematoma was measured as the sum of both sides. The diameter of the midline displacement was measured as the perpendicular distance from the septum pellucidum to the line drawn from the crista galli to the occipital protuberance. The neurological symptoms on hospital admission varied from mild headache, nausea, vomiting, hemiparesis, psychoorganic syndrome, facial nerve paresis and impaired consciousness. A descriptive statistical (arithmetic mean, minimum and maximum values) and Fisher's exact test (nonparametric test) were used as statistical analysis for this study. The p value ($p<0.05$) was considered significant. Statistical software (Statistica 10.0) was used to evaluate these study data.

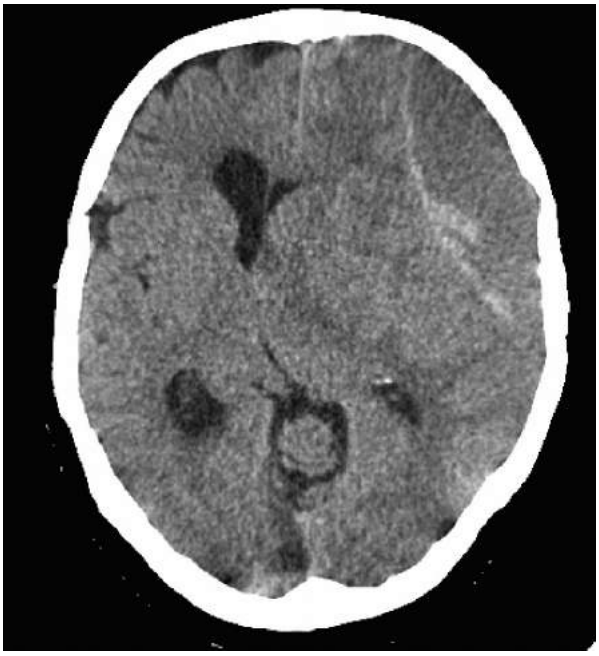


Figure 1. Unilateral left-sided chronic subdural hematoma with compressive effect on brain parenchyma and midline shift
Slika 1. *Unilateralni levostrani hronični subduralni hematom sa kompresivnim efektom na parenhim mozga i pomeranje u odnosu na mediosagitalnu liniju*

Results

The average age of the patients was 68.8, ranging from 39 to 90 years. Twenty five (35%) patients were women and 47 (65%) were men, the F:M ratio being 1:1.9. There were 29 patients younger than 65 years of age and 43 patients older than 65 years of age. The number of patients with unilateral CSDH was 44 (61%). There was an equal number (22 patients) with the left-sided and the right-sided CSDH. Twenty-eight (39%) patients had bilateral CSDH. Eighteen patients younger than 65 and 26 patients older than 65 had unilateral CSDH. Eleven patients younger than 65 and 17 patients older than 65 suffered from bilateral CSDHs. A trauma, which had preceded the hemorrhage, was known in about 66.6% of patients;

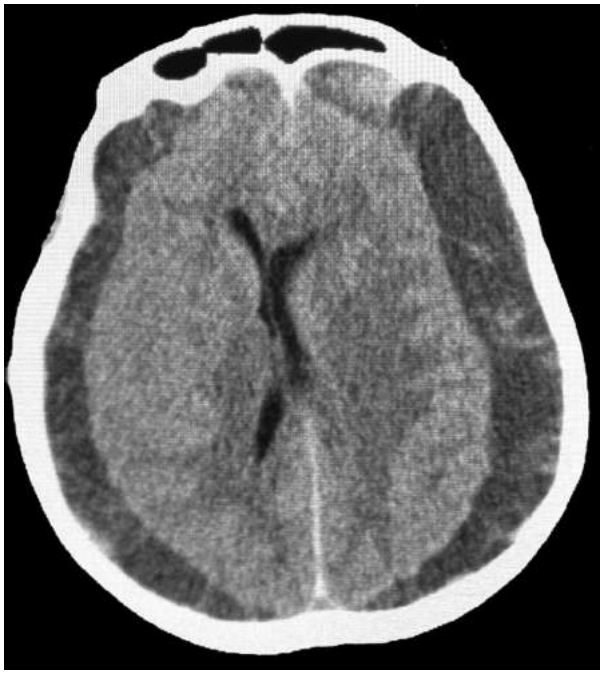
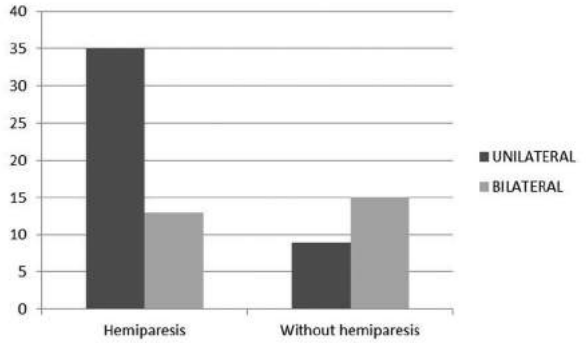


Figure 2. Bilateral chronic subdural hematoma
Slika 2. *Bilateralni hronični subduralni hematom*

however, the traumatic event was not reported in 33.4% of patients. The traumatic event was known in 30 patients older than 65 and in 18 patients younger than 65. There were no data about a trauma in 13 patients older than 65 and in 11 patients under the age of 65. In the group of patients with unilateral CSDHs, a trauma was present in 72%, while in the group of bilateral CSDHs, a trauma was present in 61%. The patients with unilateral CSDH had more frequent hemiparesis in comparison to the patients with bilateral CSDH. Thirty-five patients with unilateral presentation of CSDH had hemiparesis on hospital admission, but nine patients were without it. In the group of patients with bilateral CSDHs, 13 patients had hemiparesis, but 15 patients were without it. There were statistically significant differences between hemiparesis occurrence in these two hematoma types ($p < 0.05$) **(Graph 1).** Twenty-nine patients older than 65 had hemiparesis, whereas in the group of patients under the age of 65, hemiparesis was observed in 19 patients. Other symptoms and signs in the patients with unilateral and bilateral CSDH included headache, vertigo, the facial nerve paresis, a speech disturbance and a psychoorganic syndrome **(Table 1).** Some of the patients had two or more symptoms, but without significant differences in leading symptoms or signs between the group of patients under and above the age of 65. Many of these patients (58.8%) had arterial hypertension, four patients had anticoagulant therapy, five patients suffered from chronic obstructive pulmonary disease, one patient had thrombocytopenia and seizures were diagnosed in two patients. The majority of CSDHs were diagnosed in spring and summer months. The time interval from the onset of the first symptoms in the patients with CSDH to hospital admission and diagnosis was different between the left and the right-sided unilateral CSDH. On average, it took about 212 hours (about 9 days) and 433 hours (about 18



Graph 1. Presence/absence of hemiparesis in patients with uni- and bilateral CSDH
Grafikon 1. *Prisustvo/odsustvo hemipareze kod pacijenata sa unilateralnim i bilateralnim hroničnim subduralnim hematomom*

Table 1. Type of symptoms and signs in patients with chronic subdural hematoma (patient had more than one symptom and sign)
Tabela 1. *Vrsta simptoma i znakova kod pacijenata sa hroničnim subduralnim hematomom (pacijent je imao više od 1 simptoma i znaka)*

Symptoms and signs *Simptomi i znaci*	Number of patients Ages under 65 *Broj pacijenata* *Starost ispod 65 godina*	Number of patients Ages above 65 *Broj pacijenata* *Starost iznad 65 godina*
Hemiparesis/*Hemipareza*	19	29
Headache/*Glavobolje*	22	19
Speech disturbance/*Poremećaj u govoru*	11	24
Psychoorganic syndrome/*Psihoorganski sindrom*	0	6
Vertigo/*Vrtoglavica*	16	15
Facial nerve paresis/*Pareze facijalnog nerva*	2	8

Table 2. Cranial morphology of patients with CSDHs
***Tabela 2**. Kranijalna morfologija pacijenata za HSDH**

Cranial morphology *Kranijalna morfologija*	Unilateral CSDH *Unilaterlna HSDH*	Bilateral CSDH *Bilateralna HSDH*
Symmetrical/*Simetrična*	18 (25%)	14 (19.4%)
Asymmetrical/*Asimetrična*	26 (36%)	14 (19.4%)

**HSDH - hronični subdualni hematom*

days) the symptoms to develop in the patients with the left-sided CSDH and the right-sided CSDH, respectively; however, this difference is of no statistical significance ($p>0.05$). The average diameter of the left and right-sided CSDH was 23.5 mm and 21.3 mm, respectively. Regarding the presentation of the unilateral and bilateral CSDH, the mean time interval from the onset of symptoms to hospital admission was 310 hours and 293 hours, respectively, the average diameter of the unilateral and bilateral CSDH being 22 mm aned 31.6 mm, respectively. In the group of 28 patients with bilateral hematoma, 13 patients with hemiparesis had wider diameter of the hematoma on the left side than on the right side. The average diameter of the midline shift was 11 mm and 6 mm in the patients having the unilateral hematoma and in those having the bilateral one, respectively. The cranium morphology of patients with CSDH is given in **Table 2**. Although the asymmetry of the cranium was higher in the group of patients with the unilateral CSDH, there was no statistically significant difference ($p>0.05$) between the two groups.

Discussion

The clinical presentation of CSDH gives a wide spectrum of variety. It can be presented in the form of a mild headache with nausea and vomiting or with hemiparesis and psychoorganic syndrome that are more common in the elderly. All above mentioned neurological symptoms and signs could be interpreted as a stroke or functional decline during the physiological process of aging and would be, thus, misdiagnosed. Before the era of CT scans, a lot of CSDHs were diagnosed postmortem [6], but in daily routine CT imaging has become a more accessible method for the diagnosis of intracranial traumatic lesions. Moreover, specific clinical criteria in patients with minor head injuries were developed to identify indications for CT scan [7]. McFarlane et al. showed that CSDHs are more often in male than female patients [8]. According to them, the left-sided CSDHs were more common than the right-sided ones, with the possibility that the right-sided CSDHs were substantially more silent in the clinical presentation than the left-sided hematoma, or it could be that the right-sided CSDHs were diagnosed far more after the left-sided [5, 8]. Our study showed that CSDHs were more common in males and that the presence of the unilateral CSDH hematoma was more common than the bilateral one. There were a unique number of the left and right-sided unilateral CSDHs. Although the average diameter of the left-sided and the right-sided unilateral CSDH in our study was almost equal (being 23.5 mm and 21.3 mm, respectively), the time interval from the development of symptoms to the proper diagnosis was twice longer in the right-sided CSDH than in the left-sided hematoma (about 200 hours longer), but with no statistical significance ($p>0.05$). The hemiparesis was present in 18 patients with the left-sided CSDH and in 17 patients with the right-sided, but their clinical and neurological presentations were sooner discovered in the patients with the left-sided hematoma than in those with the right-sided one. One of the theories which can explain these phenomena is the functional asymmetry of the brain hemispheres. Ninety five percent of people have the left hemispheric dominance that is responsible for reading, speech, logic, noticing details. The left hemisphere controls the right hand movements and this could be the reason for rapid detection of neurological symptoms and signs in the patients with the left-sided form of CSDH [8, 9]. This study has also suggested that the development of hemiparesis is a significant sign of the unilateral CSDH presentation, more than in the bilateral CSDHs. The theory which could explain why patients with the unilateral CSDH have hemiparesis more often than those with the bilateral ones is a wider diameter of midline displacement and, consequently, the corticospinal tract compression, with the disturbance in the cerebral blood flow on the side of hematoma [10]. Moreover, Yokoyama et al. showed that the fractional anisotropy (FA) value in the pyramidal tract, measured by diffusion-tensor-imaging (DTI) on magnetic resonance was decreased on the side of the chronic subdural hematoma which could be responsible for the development of motor weakness in the patients. A reduction of FA value in the pyramidal tract was caused by the compressive effect of CSDH and vasogenic edema [11]. The average diameter of midline shift in the bilateral CSDH was smaller than in the unilateral one, but hemiparesis was manifested at lower values of midline shifts in the bilateral CSDH as compared to the unilateral CSDH. A possible explanation for this is probably the more substantial compression of pyramidal tracts and serious metabolic changes in specific regions of the brain parenchyma which are affected by the compressive effect of CSDH. In

addition, our study showed that previous trauma was a very important factor for the CSDH development, especially in the group of patients older than 65. In literature, the etiology of subdural hematoma is usually connected with the previous traumatic event [12, 13]. It may be interesting to state that CSDHs were diagnosed more frequently during spring and summer months. A possible mechanism which could explain the more frequent occurrence of CSDH in patients during these months is the greater mobility and higher temperature, which could be factors that may have affected a higher risk of injury. The comorbidity such as chronic obstructive pulmonary disease, seizures and coagulopathy are potential risk factors for intracranial hemorrhage event. In their study, Tsai et al. emphasized that bilateral hematoma was more common in patients with anticoagulant or antiplatelet therapy [1], but in our study only three patients with the bilateral CSDH and seven patients with the unilateral CSDH had coagulation disorders. The asymmetry of the cranium had a slightly higher frequency in our patients with the unilateral CSDH than in those with the bilateral hematoma, but with no statistical significance. One of the reasons for this fact might be the small sample size and future investigation could confirm or deny the hypothesis previously set by Lee et al. about the relationship between asymmetry of the skull and localization of CSDHs [4].

Conclusion

The site and the form of chronic subdural hematoma could have a great influence on neurological and functional condition in a patient. Although the length of time required for making diagnosis as well as clinical symptoms greatly differ and the latter are not always so clear, physicians should maintain a high level of suspicion for this disease and thus contribute to prompt diagnosis and better clinical outcome of patients.

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Rad je primljen 20. XII 2014.
Recenziran 11. V 2014.
Prihvaćen za štampu 22. V 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:277-281.

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Original study
Originalni naučni rad
UDK 616.2-022.1-053.2:615.33-03
DOI: 10.2298/MPNS1410282T

ANALYSIS OF ANTIBIOTIC CONSUMPTION FOR TREATING RESPIRATORY TRACT INFECTIONS IN CHILDREN AND COMPLIANCE WITH THE NATIONAL CLINICAL GUIDELINES

ANALIZA POTROŠNJE ANTIBIOTIKA ZA LEČENJE INFEKCIJA RESPIRATORNOG TRAKTA U DEČJOJ POPULACIJI I USKLAĐENOSTI SA NACIONALNIM VODIČIMA DOBRE KLINIČKE PRAKSE

Dragana LAKIĆ[1], Ivana TADIĆ[1], Marina ODALOVIĆ[1], Ljiljana TASIĆ[1], Ana SABO[2] and Aleksandra MEĆAVA[3]

Summary

Introduction. Respiratory infections are the most common infections in children. The aims of the study were to analyze the use of antibiotics for respiratory infections in the period 2008 – 2010 in children's population in region of Niš and to estimate the rational use of antibiotics in relation to the recommendations of the National Guidelines for physicians in primary care. **Material and methods.** Data source was a Pharmacy Niš database. Antibiotics prescriptions were selected for the following diagnoses: H65-H75 (acute otitis media, mastoiditis), J01 (acute sinusitis), J02-J03 (tonsillopharyngitis), J12-J18 (community acquired pneumonia), J20 (acute bronchitis), J32 (chronic sinusitis), J42 (chronic bronchitis). Antibiotic consumption was expressed in defined daily dose/1000 inhabitants/day. **Results.** The most widely prescribed antibiotic for the treatment of upper respiratory tract infections in children during the three years was amoxicillin (34.63; 32.50 and 31.00 defined daily dose/1000 inhabitants/day in 2008, 2009 and 2010, respectively). In the treatment of infections of the middle ear and mastoid, the combination of amoxicillin and clavulanic acid, was the most prescribed antibiotics (60% of total consumption of antibiotics for this indication). Azithromycin was the most widely prescribed antibiotic for the treatment of lower respiratory tract infections in children during the observed period (6.92; 8.20 and 7.18 defined daily dose/1000 inhabitans/day in 2008, 2009 and 2010, respectively). **Conclusion.** Recommendations of national guidelines are not complied with the treatment of upper and lower respiratory infections in the children population in region of Niš. This could be a sign of potentially irrational use of antibiotics that need to be further examined. Education of physicians can influence irrational use of antibiotics.

Key words: Anti-Bacterial Agents; Respiratory Tract Infections; Child; Physician's Practice Paterns; Practice Guideline; Drug Utilization

Sažetak

Uvod. Infekcije respiratornog sistema predstavljaju najčešće infekcije kod dece. Ciljevi ovog rada jesu analiza potrošnje antibiotika u lečenju infekcija gornjih i donjih respiratornih puteva kod dece u Nišavskom okrugu i procena racionalnosti upotrebe antibiotika u odnosu na preporuke Nacionalnog vodiča za lekare u primarnoj zdravstvenoj zaštiti. **Materijal i metode.** Kao izvor podataka korišćena je baza podataka Apoteke Niš za period 2008−2010. godine. Selektovani su svi izdati recepti antibiotika propisani deci starosti 0−19 godina za terapiju infekcija respiratornog trakta uključujući infekcije srednjeg uva ((H65-H75 (akutni otitis media i mastoiditis), J01 (akutni sinuzitis), J02-J03 (tonzilofaringitis), J32 (hronični sinuzitis) (J12-J18 (blaga do umerena pneumonija izazvana vanbolničkim uzročnikom), J20 (akutni bronhitis), J42 (hronični bronhitis)). Potrošnja je izražena u definitivnoj dnevnoj dozi/1 000 stanovnika/dan. **Rezultati.** Najpropisivaniji antibiotik za terapiju infekcija gornjeg respiratornog trakta kod dece u posmatranom periodu bio je amoksicilin (34,63; 32,5 i 31 definitivnoj dnevnoj dozi/1 000 stanovnika/dan tokom 2008, 2009. i 2010. godine). Za terapiju infekcije srednjeg uva i mastoidnog nastavka najčešće je korišćena kombinacija amoksicilina i klavulanske kiseline (60% ukupne potrošnje svih antibiotika za ovu indikaciju). Azitromicin je bio najpropisivaniji antibiotik za terapiju infekcija donjeg respiratornog trakta kod dece tokom posmatranog perioda (6,92; 8,2 i 7,18 definitivnoj dnevnoj dozi/1 000 stanovnika/dan tokom 2008, 2009. i 2010. godine). **Zaključak.** Preporuke nacionalnih vodiča nisu poštovane ni u slučaju terapije gornjih ni donjih respiratornih infekcija kod dece u Nišavskom regionu. To može biti znak potencijalno neracionalne upotrebe antibiotika koju je potrebno dodatno istražiti. Dodatna edukacija lekara mogla bi uticati na smanjivanje neracionalne upotrebe antibiotika.

Ključne reči: Antibiotici; Infekcije respiratornog trakta; Dete; Lekarska praksa; Vodiči; Korišćenje lekova

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Acknowledgement

This work has been supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, Project No. 41012, and Project No. 175035, 2011-2014.

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Abbreviations

RTIs – respiratory tract infections
WHO – World Health Organization
ICD – International Classification of Diseases
ATC – anatomical therapeutic chemical
DDD – defined daily dose
DID – defined daily doses per 1000 inhabitants per day
INN – international non-property name
H65-H75 – acute otitis media, mastoiditis
J01 – acute sinusitis
J02-J03 – tonsillopharyngitis
J32 – chronic sinusitis
J12-J18 – mild to moderate community-acquired pneumonia
J20 – acute bronchitis
J42 – chronic bronchitis

Introduction

Respiratory tract infections (RTIs) are the most frequent infections in all age groups of patients. According to the World Health Organization (WHO) Report for 2002, RTIs were reported to be the cause of 3.9 million deaths per a year [1]. The frequency of RTI in an adult is 3–5 episodes per a year, and even more in children. The frequency of RTI and the clinical state of a child with RTI depend on anatomical and functional characteristics of the organism, immunological state, age, gender, nutritional status of the child, as well as on the socio-economic factors [2–4].

The most extensive study on RTIs and associated risk factors in children was performed in Cleveland in 1964. The study results suggested that the frequency of RTI depended on the child's age. The authors emphasized a wide range of episodes of RTI in "healthy" children, the average number of episodes being 7.4 per a year in five-year-old children and the number ranging from 0 to 18. Assuming that an episode usually lasts one to two weeks, a five-year old child with 18 episodes of RTIs can be ill for 4.5-9 months per a year. According to the same study, the frequency of RTI is affected by the number of family members and the risk increases twice to eight times when the children attend kindergartens [5]. Fortunately, RTIs do not cause a high mortality rate; however, the morbidity rate is very high. Besides being the most frequent infections at children's age, these infections are the most frequent cause of children visiting general practitioners or paediatricians as well as of their hospitalization worldwide [6]. According to the data of the Institute for Public Health of Serbia for 2011, the total number of respiratory system diseases (diagnoses ICD-10, J00 – J99 according to the International Classification of Diseases, 10th Revision – (ICD-10) was over 1.6 million in children younger than age 6 years, that accounting for 55% of all recorded diseases, states and injuries.

At the same time, RTIs diagnosed in schoolchildren aged from 7 to 9 accounted for 48% of all recorded diseases, states and injuries in this population group [7].

Upper respiratory tract infections are more frequent than lower respiratory tract ones, representing 90% of all RTIs [8]. They were also more frequent in children and adolescents in Serbia, where tonsillopharyngitis accounted for 51% of all diseases in 2011 [7].

RTIs in children are caused by viruses, bacteria and other agents in 75, 15 and 10% of cases, respectively [9]. Therefore, the first line therapy of RTIs should be symptomatic therapy, while antibiotics should be the second line therapy. However, RTIs in children are the most common cause for prescribing antibiotics in the whole world. Prescriptions for antibiotics in treatment of RTIs amount to 75% of all prescriptions at the primary health care level [10, 11]. Concurrently, more than 50% of all prescriptions are for upper RTIs. Consequently, drug use in therapy of RTIs is often irrational. Unnecessary antibiotic treatment results in a number of unintended consequences, especially in the increase of bacterial resistance and enormous financial expenses. The most abused administration of antibiotics is in unnecessary treatment of viral infections, particularly in children. An additional problem is the consequent use of broad-spectrum antibiotics and the new generation antibiotics, which are more expensive [10–12]. Antibiotics should be chosen based on the identification of microbial pathogens, determination of antibiograms, pharmacokinetics of the drug, the age and general condition of the child, previous therapy, a possible allergic reaction to the applied antibiotic and drug prices [13].

The Ministry of Health of the Republic of Serbia adopted two guidelines for antibiotic prescribing in general practice within the primary health care of children and adults. The Guideline "The Selection and Use of Antibiotics in General Practice" [14] recommends therapy for several different infections, including RTIs and otorhinolaryngeal infections. It discourages administration of antibiotics in therapy of upper RTIs and mild symptoms of lower RTIs. Antibiotics should be used in therapy of community-acquired pneumonia; amoxicillin and azithromycin in children younger and older than age 5 years, respectively. Recommendations for therapy of acute otitis media are given in two guidelines "The selection and Use of Antibiotics in General Practice" and "Otitis Media" [15].

The aims of this study were to analyze antibiotic consumption in treatment of upper and lower RTIs in children population in region of Niš, and to analyze compatibility of the study results regarding drug consumption with the national guideline recommendation for treatment of RTIs.

Material and Methods

The source of data on outpatient antibiotic consumption issued on prescription was the anonymous

electronic database of Pharmacy Niš for the period from 2008 to 2010. The base anonymity included the anonymity of the patient, doctor and pharmacy. Criteria for database search were the patient's year of birth, the code of disease (ICD-10) for which antibiotic was issued, commercial drug name, the anatomical therapeutic chemical (ATC) code of drug, the number of issued packages, date of drug issue, etc. Criteria for data selection were the following disease codes: H65-H75 (acute otitis media, mastoiditis); upper RTIs: J01 - acute sinusitis, J02-J03 – tonsillopharyngitis, J32 - chronic sinusitis; lower RTIs: J12-J18 – mild to moderate community-acquired pneumonia, J20 - acute bronchitis, J42 - chronic bronchitis. The data on issued antibiotics were selected for each diagnosis according to the ATC classification: J01A (Tetracyclines), J01C (beta-lactam antibiotics), J01D (cephalosporins), J01E (sulphonamides and trimethoprim), J01F (macrolides, lincosamides), J01M (hinolons). Antibiotics consumption was followed in the period from 2008 to 2010.

Defined daily dose (DDD) was used in expression of antibiotics consumption. The DDD was suggested by the WHO as the *"*assumed average maintenance dose per day for a drug used for its main indication in adults". The unit DDD is an average dose for adult of 70 kg weight and it is independent of price and dosage form. It enables the researcher to assess trends in drug consumption and to perform comparisons between population groups. The DDD values were taken from the WHO web site [16]. Nowadays, drug consumption is expressed by DDD per 1000 inhabitants per a day (DDD/1000 inhabitants/day (DID)). The following formula was used to calculate drug consumption:

DID = (the amount of drug used in one year (mg) x 1000)/365 x the number of inhabitants x DDD (mg)

Antibiotic consumption in our study was expressed in DID for each antibiotic according to the international non-property name (INN), diagnosis, patient's age and the year of drug prescribing. The results were expressed as aggregated data for the children and adolescents aged 0-19 years. Due to the differences in guideline recommendations regarding treatment of community-ac-

Table 1. Consumption (expressed in DDD/1000 inhabitants/day) of the antibiotics groups (according to ATC classification) for the outpatient treatment of respiratory infections in children population during the period 2008 – 2010 year in region of Niš

Tabela 1. *Potrošnja (izražena u DDD/1 000 stanovnika/dan) grupe antibiotika (prema ATC klasifikaciji) za vanbolničku terapiju respiratornih infekcija kod dece u periodu 2008–2010. godine u Nišavskom regionu*

		Antibiotic consumption (DID)/*Potrošnja antibiotika (DID)*								
		Upper RTIs *Infekcije gornjeg respiratornog trakta* (J01, J02, J03, J32)			Complications of upper *Komplikacije infekcija gornjeg respiratornog trakta* RTIs (H65-H75)			Lower RTIs *Infekcije donjeg respiratornog trakta* (J12-J18, J20, J42)		
ATC classification *ATC klasifikacija*	Drug group *Grupa lekova*	2008	2009	2010	2008	2009	2010	2008	2009	2010
J01AA	tetracyclines	0.173	0.183	0.155	0.001	0.000	0.000	0.017	0.020	0.021
J01CA	broad spectrum penicillin	34.628	32.603	31.003	1.002	0.710	0.602	1.233	0.906	1.378
J01CE	beta-lactamase sensitive penicillin	10.868	10.598	7.520	0.041	0.029	0.040	0.327	0.264	0.237
J01CR	penicillin in combination with inhibitors of beta- lactamase	25.282	26.727	22.782	3.343	2.812	3.277	1.967	2.264	2.566
J01DB	1st generation of cephalosporins	13.893	12.408	11.669	0.001	0.000	0.001	1.214	1.168	1.236
J01DC	2nd generation of cephalosporins	3.439	3.863	3.170	0.342	0.428	0.648	1.368	1.888	1.484
J01DD	3rd generation of cephalosporins	5.612	6.829	5.893	0.222	0.257	0.241	6.409	6.422	5.711
J01EE	sulfonamids with thrimethoprime	1.382	1.114	0.811	0.029	0.014	0.009	0.075	0.067	0.059
J01FA	macrolides	26.971	29.317	23.338	0.209	0.382	0.228	11.466	12.564	10.605
J01FF	lincosamides	0.196	0.105	0.250	0.008	0.002	0.009	0.023	0.023	0.043
J01MA	fluoroquinolones	0.001	0.001	0.001	0.000	0.009	0.017	0.008	0.012	0.009
	Total/*Ukupno*	122.444	123.750	106.591	5.197	4.644	5.072	24.107	25.599	23.350

*DDD - definisana dnevna doza, **ATC - anatomsko-terapeutsko-hemijska, DID - DDD/1 000 stanovnika/dan*

Table 2. Consumption (expressed in DDD/1000 inhabitants/day) of the specific antibiotics (INN) for the outpatient treatment of respiratory infections in children population during the period 2008 – 2010 year in region of Niš
Tabela 2. *Potrošnja (izražena u DDD/1000 stanovnika/dan) pojedinačnih antibiotika (INN) za vanbolničku terapiju respiratornih infekcija kod dece u periodu 2008–2010. godine u Nišavskom regionu*

	Antibiotic consumption (DID)/*Potrošnja antibiotika (DID)*								
	Upper RTIs *Infekcije gornjeg respiratornog trakta* (J01, J02, J03, J32)			Complications of upper RTIs/*Komplikacije infekcija gornjeg respiratornog trakta* (H65-H75)			Lower RTIs *Infekcije donjeg respiratornog trakta* (J12-J18, J20, J42)		
INN	2008	2009	2010	2008	2009	2010	2008	2009	2010
Doxycycline	0.173	0.183	0.155	0.001	0.000	0.000	0.017	0.020	0.021
Amoxicillin	34.628	32.603	31.003	1.002	0.710	0.602	1.233	0.906	1.378
Phenoxymethylpenicillin	10.868	10.598	7.520	0.041	0.029	0.040	0.327	0.264	0.237
Amoxicillin. clavulanic acid	25.282	26.727	22.782	3.343	2.812	3.277	1.967	2.264	2.566
Cephalexin	12.538	11.279	10.442	0.001	0.000	0.001	1.153	1.088	1.145
Cefadroxil	1.355	1.129	1.227	0.000	0.000	0.000	0.061	0.080	0.091
Cefuroxime	0.194	0.570	0.668	0.000	0.000	0.000	0.150	0.237	0.296
Cefprozil	3.244	3.293	2.502	0.342	0.428	0.648	1.218	1.650	1.188
Cefixime	5.612	6.829	5.893	0.222	0.257	0.241	6.409	6.422	5.711
Thrimethoprim sulfamethoxazole	1.382	1.114	0.811	0.029	0.014	0.009	0.075	0.067	0.059
Erytromycin	10.865	11.405	10.066	0.039	0.046	0.005	1.512	1.280	1.600
Midecamycin	0.000	0.000	0.299	0.000	0.000	0.000	0.000	0.000	0.000
Roxithromycin	0.740	0.703	0.579	0.002	0.001	0.003	0.116	0.103	0.097
Clarithromycin	7.857	7.518	4.027	0.080	0.236	0.142	2.921	2.979	1.723
Azithromycin	7.509	9.692	8.366	0.088	0.099	0.079	6.917	8.203	7.185
Clindamycin	0.196	0.105	0.250	0.008	0.002	0.009	0.023	0.023	0.043
Ofloxacin	0.001	0.001	0.001	0.000	0.001	0.000	0.000	0.000	0.000
Ciprofloxacin	0.000	0.000	0.000	0.000	0.008	0.017	0.008	0.012	0.009
Total/*Ukupno*	122.444	123.750	106.591	5.197	4.644	5.072	24.107	25.599	23.350

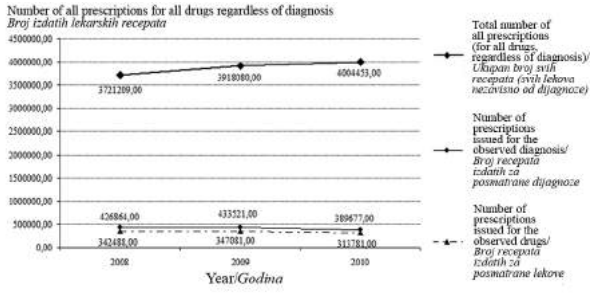
DID - DDD/1 000 stanovnika/dan

quired pneumonia in children under and above 5 years of age, subanalaysis was done for the diagnosis J12 – J18 for these two clusters of patients [14]. The number of inhabitants was taken from census 2002 for the population aged 0-19 years (80424) and the two mentioned clusters (16289 (0-4 years) and 64135 (5-19 years) [17]. Data on the number of inhabitants for each of observed years were not used in order not to compromise the accuracy of results.

Results

A low reduction in antibiotic prescribing was observed during the period 2008-2010 by comparing the total number of prescriptions (regardless of diagnosis) and the number of antibiotics prescribed for the observed respiratory diagnoses **(Graph 1).** The share of antibiotic prescriptions issued for RTI in the total number of issued prescriptions was 9.20%, 8.86% and 8.23% in 2008, 2009 and 2010, respectively.

The most widely prescribed antibiotics in treatment of upper RTIs was broad-spectrum penicillin followed by macrolides and penicillin in combination with inhibitors of beta-lactamase. These three groups of antibiotics accounted for more than 70% of total antibiotic consumption in the three observed years. The total antibiotic con-



Graph 1. The total number of the medical prescriptions in region of Niš for all medications and selected respiratory infections issued between 2008-2010 year
Grafikon 1. *Ukupan broj lekarskih recepata u Nišavskom regionu za sve dijagnoze i posmatrane dijagnoze respiratornih infekcija izdatih u periodu 2008−2010. godine*

sumption was 122.444, 123.750 and 106.591 DID in 2008, 2009 and 2010, respectively (**Table 1**).

The most widely prescribed antibiotic in therapy of upper RTIs according to INN was amoxicillin throughout the observed period, being 34.628, 32.603 and 31.003 DID in 2008, 2009 and 2010, respectively. The second place was taken by amoxicillin in combination with clavulanic acid. The share of amoxicillin, alone and in combination with clavulanic acid, accounted for almost 50% of the total antibiotic consumption. The consumption of almost all antibiotics was constant in the observed period, except for clarithromycin, whose consumption in 2010 was reduced by almost 50% in comparison with the consumption in 2009 (**Table 2**).

The most prescribed antibiotic in therapy of otitis media throughout the observed period was amoxicillin in combination with clavulanic acid. The share of this combination accounted for more than 60% in the total antibiotic consumption in therapy of otitis media in each observed year. Amoxicillin was on the second place, 1.002, 0.710 and 0.602 DID in 2008, 2009 and 2010, respectively (**Table 2**).

Antibiotics most widely prescribed in treatment of lower RTIs were macrolides. The consumption of cephalosporins, 3rd generation was less than half of macrolides consumption, while prescribing of other antibiotics was marginal. The total antibiotic consumption in treatment of lower RTIs was 24.107, 25.599 and 23.350 DID in 2008, 2009 and 2010, respectively (**Table 1**).

The most prescribed antibiotic by INN in therapy of lower RTIs throughout the observed period was azithromycin, 6.917, 8.203 and 7.185 DID in 2008, 2009 and 2010, respectively. Cefixime was on the second place. These two antibiotics accounted for more than 50% in the total antibiotic consumption in therapy of lower RTIs during the observed period (**Table 2**).

Some of the observed antibiotics were not prescribed for certain indications during the study period (e.g. midecamycin). Several antibiotics were not prescribed in each of observed years (e.g. doxycycline, cephalexin, ofloxacin) (**Table 2**).

The results of subanalaysis of antibiotic consumprion in treatment of community-acquired pneumonia in children under and above 5 years of age are given in **Graph 2**.

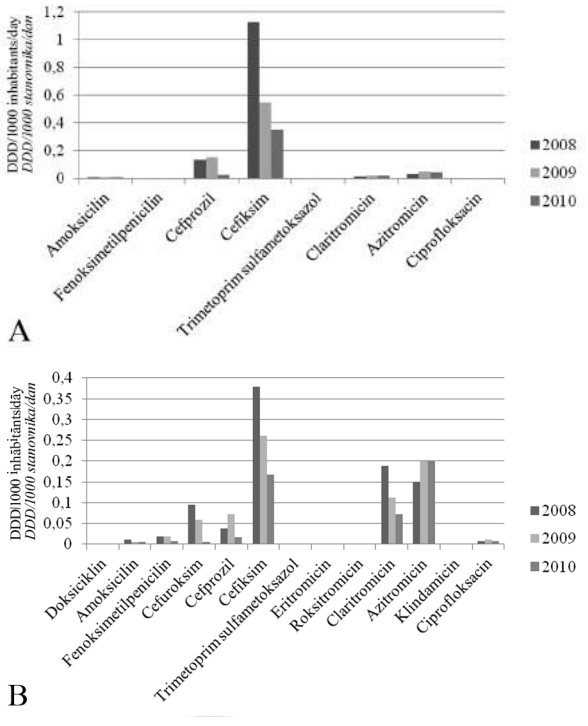
Cefixime and cefprozil were the most frequently prescribed antibiotics in treatment of community-acquired pneumonia in children 0-4 years old. Cefixime was also the most frequently prescribed antibiotic in therapy of observed indications in the population 5-19 years old in 2008 and 2009 (0.38 and 0.26 DID, respectively); but, in 2010, it was azithromycin (0.20 DID).

Discussion

The results of our study showed that the number of prescriptions for antibiotics given for RTIs decreased in 2010 comparing to 2009, in spite of increase in the total number of prescriptions regardless of diagnoses issued during the period 2008-2010.

Amoxicillin, alone or in combination with clavulanic acid, was the most widely prescribed antibiotic in therapy of upper RTIs throughout the observed period. The results showed that 12% of children consumed antibiotics prescribed for upper RTIs in region of Niš every day in 2008 and 2009, while it was 10% in 2010. Clinical practice showed non-adherence to National guidelines which recommended not prescribing antibiotics for upper RTIs, except for sinusitis and severe clinical state of the patient when the drug of choice should be amoxicillin. Serbian national guidelines are in line with the guidelines of the National Institute of Health and Care Excellence of Great Britain, which recommend the strategy of delayed prescribing [18].

Previous studies showed irrational antibiotic consumption in upper RTIs [11, 19]. As many as 46% of children with upper RTIs consumed an antibiotic in spite of its lack of efficacy in the therapy of upper RTIs. It was observed that antibiotics were more prescribed to children aged from 5 to 11 years



Graph 2. Consumption (expressed in DDD/1000 inhabitants/day) of the specific antibiotics (INN) for the outpatient pneumonia in children younger than 5 (A) and children older than 5 years (B)

Grafikon 2. *Potrošnja (izražena u DDD/1000 stanovnika/dan) pojedinačnih antibiotika (INN) za terapiju vanbolničke pneumonije kod dece mlađe od 5 godina (A) i populacije dece starije od 5 godina i adolescenata (B)*

than to younger children (Odds Ratio (OR): 1.94, 95% Confidence Interval (CI): 1.13 – 3.33) [19].

Our results also showed off-label antibiotic prescribing for some indications not approved by the regulatory authority. These were doxycycline and ofloxacine which are contraindicated for children and adolescents. What is even more worrying is the fact that doxycycline (capsule) were prescribed to children younger than 12 months.

The national guidelines recommend amoxicillin and amoxicillin in combination with clavulanic acid as the first and second line therapy, respectively, to treat otitis media and mastoiditis. The guideline for otitis media recommends delayed prescribing of antibiotics and non-prescribing of these drugs in the first 2-3 days due to spontaneous remission of symptoms and healing in 70-90% of cases. Contrary to these recommendations, the second line therapy, amoxicillin in combination with clavulanic acid, was the most widely prescribed antibiotic in treatment of otitis media, which confirmed the non-compliance with the guidelines in region of Niš.

Recommendations about therapy of otitis media have undergone significant changes over the years. Antibiotics were the standard therapy of otitis media in the majority of developed countries at the end of XX century [20, 21]. However, later studies showed that spontaneous remission of symptoms and healing eventually occurred in a great number of patients. Moreover, early administration of antibiotics could result in the alleviation of symptoms after 24 hours (not immediately), but it could also increase the incidence of diarrhoea in children. Therefore, the strategy of delayed antibiotic prescribing has been recommended as the strategy of choice. This strategy could contribute to the reduction in antibiotic prescribing by even 76% [22]. The last Cochrane Review (2009) also prioritized antibiotic non-prescribing [23] because 16 children should be treated by an antibiotic in order to prevent one otitis media with pain symptoms. Thus, it is recommended to prescribe antibiotics only in children younger than 2 years of age having bilateral inflammation or inflammation of one ear and otorrhea [24]. Identical recommendations are observed in Great Britain [18] and the United States [25].

About 2% of children in region of Niš consumed one of the antibiotics every day in therapy of lower RTIs. Azithromycin and cefixime (3rd generation of cephalosporins) were the most widely prescribed antibiotics in treatment of lower RTIs throughout the observed period. According to our results, antibiotics prescribed for community-acquired pneumonia accounted for less than 10% in the total antibiotic prescribing for lower RTIs. Since antibiotics should not be prescribed to treat lower RTIs except for community-acquired pneumonia, it is obvious that these recommendations have not been adhered to.

In addition, the guidelines recommend amoxicillin as the first choice in treatment of community-acquired pneumonia in children under 5 years of age, except for infections caused by mycoplasmas, when macrolides are recommended as the first choice. Azithromycin and clarithromycin are recommended as the first choice in children older than 5 years of age, as well as in adolescents. According to our results, 2nd and 3rd generations of cephalosporins, recommended as the second line therapy, were the most widely prescribed in treatment of lower RTIs in region of Niš. Therefore, it could be concluded that doctors did not completely comply with the national recommendations in therapy of community-acquired pneumonia in children under 5 years of age. However, they completely observed the recommendations given in 2010 for treatment of community-acquired pneumonia in children older than 5 years, bearing in mind that the most widely prescribed antibiotics were azithromycin and clarithromycin.

According to the recommendation of the World Society for Pediatric Infectious Diseases based on high level of evidence, prescribing of antibiotics should be avoided in pre-school children in routine clinical practice because of infections caused by viruses in this age group. If it is necessary to prescribe antibiotic, amoxicillin is recommended as the first choice in all age groups of children and adolescents, while macrolides are recommended in case of infections caused by untypical pathogens [26]. Unfortunately, the consumption of amoxicillin in treatment of community-acquired pneumonia in children in region of Niš was extremely low, being only 0.001%.

The results of our research showed that the per cent of antibiotic prescribed in case of a diagnosis of upper RTI was five times higher than in case of a diagnosis of lower RTI. Similar results were reported in previous studies [12].

Foreign guidelines as well as clinical studies have recommended the strategy of delayed antibiotic prescribing as useful even in case of lower RTIs [18, 27]. It is also advisable to inform patients and parents about long persistence of symptoms of infection, particularly cough which could last for as long as 4 weeks. Cough could persist in spite of antibiotic administration due to moderate impact of antibiotics on the severity of symptoms but not on the persistence of symptoms.

Several authors analyzed reasons for prescribing antibiotics for children. One of the reasons is that the patients expect to be treated by antibiotics and the other one is that the doctors themselves think that antibiotics are necessary. In addition, several studies reported that doctors were under pressure exerted by patients and parents who wanted to get antibiotic prescription [28, 29]. However, other studies showed that 90% of the patients who had expected an antibiotic from the doctor were satisfied with the explanation that the delayed pre-

scribing or administration of antibiotic would be a better option [30, 31]. These results showed that clinical practice could be improved by decreasing antibiotic consumption in infections which are "traditionally" caused by viruses.

Dutch authors also analyzed the reasons why the doctors do not comply with the guidelines [32]. They found that the majority of them reported medical reasons, such as the severity of disease, comorbidities, as well as habits, calming the patient, the patient's request and others.

Our study has several limitations. In spite of the fact that RTIs are among the most frequent reasons for antibiotic prescribing in children, it could not generalize all bacterial infections. The unit DID, being a well-accepted statistical unit for drug consumption, was used as a measure of antibiotic consumption. However, a DDD was defined as the assumed average maintenance dose per day in adults, as it was earlier stated [16]. In the situation of high variability in drug dosing regimes in children, a DDD for adult was recommended to be used even in children. In addition, our analysis included only oral dosing forms, while intramuscular forms were not included so a higher antibiotic consumption in children in the observed period could be expected.

Conclusion

The total antibiotic consumption in children in region of Niš decreased during the observed period. However, the total antibiotic consumption in children was very high, particularly in case of upper respiratory tract infections. Antibiotic prescribing is not in accordance with the national guidelines either in case of upper respiratory tract infections or lower ones, which suggests irrational antibiotic consumption. Prescribing practice in region of Niš is in line with the national guidelines only in case of community-acquired pneumonia in children older than 5. It often happens that the diagnosis becomes an excuse for antibiotic prescribing instead of being the main reason.

Excessive antibiotic consumption leads to antibiotic resistance, which results in consumption of newer antibiotics of broader spectrum, and even higher health care expenses. The strategy of delayed antibiotic prescribing should become a part of routine clinical practice in region of Niš.

Irrational antibiotic prescribing could be decreased by continuous professional education of doctors and strict adherence to guidelines regarding antibiotic prescribing.

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Rad je primljen 23. VIII 2013.
Recenziran 5. III 2014.
Prihvaćen za štampu 23. VI 2014.
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Original study
Originalni naučni rad
UDK 615.256-058.875(497.113 Novi Sad)
DOI: 10.2298/MPNS1410290M

HORMONAL CONTRACEPTION – HABITS AND AWARENESS FEMALE STUDENTS OF THE UNIVERSITY OF NOVI SAD, VOJVODINA, SERBIA

HORMONSKA KONTRACEPCIJA – NAVIKE I OBAVEŠTENOST STUDENTKINJA UNIVERZITETA U NOVOM SADU, VOJVODINA, SRBIJA

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Summary

Introduction. Despite a large number of modern contraceptive methods available in the market today, numerous studies have shown insufficient awareness of young women about these forms for birth control. The aim of this study was to compare characteristics of common use of contraceptives as well as the awareness of hormonal contraception among female students of the Faculty of Medicine and the Faculty of Technical Sciences in Novi Sad. **Materials and Methods.** In the study which was conducted in 2012 240 female students of the Faculty of Medicine and the Faculty of Technical Sciences participated. The average age of students of the Faculty of Medicine and the Faculty of Technical Sciences was 24.06 ± 3.24 and 22.72 ± 0.90 years, respectively. They completed an anonymous questionnaire, which consisted of three parts. The first part comprised general questions, the second part included characteristics of the sexual life of students and their contraceptive habits, while in the third part students were asked to evaluate the accuracy of statements about hormonal contraception. The obtained data were statistically processed by using appropriate methods. **Results.** The average age when the students of the Faculty of Medicine and the Faculty of Technical Sciences had the first sexual intercourse was 18.74 ± 2.61 and 18.75 ± 2.59, respectively. One third of students from both faculties (30.91% from the Faculty of Medicine and 35% from the Faculty of Technical Sciences) had only one sexual partner. Modern contraception was used by 83.64% of the medical students and by 80% of those from the Faculty of Technical Sciences. Contraception was used regularly by 54.54% of the medical students and 43% of those from the Faculty of Technical Sciences. The most frequently applied contraceptive method was condom (90% of the medical students and 93% of the Faculty of Technical Sciences students, respectively). Oral contraceptives were used by 24.54% of the medical students and 11% of those form the Faculty of Technical Sciences. There was a statistically significant difference in the knowledge level between the two groups since the medical students gave quantitatively higher range of expected responses than the students from the Faculty of Technical Sciences. **Conclusion**. It is necessary to increase the availability of adequate information on various types of contraception to student population in Vojvodina.

Key words: Contraceptives, Oral, Hormonal; Female; Students; Questionnaires; Health Knowledge, Attitudes, Practice; Sexual Behavior

Sažetak

Uvod. Pored velikog broja savremenih metoda kontracepcije koje postoje na tržištu, brojne studije su pokazale nedovoljnu obaveštenost mladih žena o ovim vidovima kontracepcije. Cilj rada je upoređivanje karakteristika korišćenja kontracepcije i informisanosti o hormonskoj kontracepciji studentkinja Medicinskog fakulteta i Fakulteta tehničkih nauka u Novom Sadu. **Materijal i metode.** U istraživanju sprovedenom 2012. godine učestvovalo je 240 studentkinja Medicinskog fakulteta i Fakulteta tehničkih nauka, odabranih metodom slučajnog izbora, koje su pristale da popune anonimni upitnik iz tri dela. Prosečna starost studentkinja Medicinskog fakulteta bila je 24,06 ± 3,24 godina, a Fakulteta tehničkih nauka 22,72 ± 0,90 godina. Prvi deo je obuhvatao opšta pitanja, drugi deo se odnosio na karakteristike seksualnog života ispitanica i kontraceptivne navike, dok su se u trećem delu ispitanice izjašnjavale o tačnosti tvrdnji o hormonskoj kontracepciji. Dobijeni podaci su statistički obrađivani odgovarajućim metodama. **Rezultati.** Prosečna starost pri stupanju u seksualne odnose studentkinja oba fakulteta bila je slična (18,74 ± 2,61 i 18,75 ± 2,59 godina). Trećina studentkinja oba fakulteta (30,91% sa Medicinskog fakulteta i 35% sa Fakulteta tehničkih nauka) imalo je jednog seksualnog partnera. Savremenu kontracepciju primenjivalo je 83,64% studentkinja Medicinskog fakulteta i 80% studentkinja Fakulteta tehničkih nauka, od čega ju je redovno primenjivalo 54,54% studentkinja Medicinskog fakulteta i 43% studentkinja Fakulteta tehničkih nauka. Najčešće zastupljeni savremeni metod kontracepcije bio je kondom (90% Medicinskog fakulteta, 93% Fakulteta tehničkih nauka). Oralne kontraceptive primenjivalo je 24,54% studentkinja Medicinskog fakulteta i 11% studentkinja Fakulteta tehničkih nauka. Poredeći znanja o oralnim kontraceptivima, studentkinje Medicinskog fakulteta dale su statistički značajno veći broj očekivanih odgovora od studentkinja Fakulteta tehničkih nauka. **Zaključak.** Potrebno je povećati dostupnost adekvatnih informacija o različitim vidovima kontracepcije među studentskom populacijom u Vojvodini.

Ključne reči: Hormonska kontracepcija; Žensko; Studenti; Upitnici; Znanje, stavovi i praksa o zdravlju; Seksualno ponašanje

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Abbreviations

FM – Faculty of Medicine
FTS – Faculty of Technical Sciences
IUDs – intrauterine devices

Acknowledgement

The Ministry of Education and Science of the Republic of Serbia (grant 41012) supported this research work.

Introduction

There are numerous and complex problems in the field of family planning and reproductive health of the population in the Republic of Serbia. One of them is the high prevalence of conservative birth control methods, among which interrupted intercourse (*coitus interruptus*) is dominant. Consequently, abortion is the most common solution in case of unintended pregnancy [1]. The overall abortion rate in Serbia was estimated to be 2.76 in 2007 (twice higher than the total fertility rate) and among the highest in Europe and the world [2].

Besides conservative methods of birth control, numerous modern contraceptive methods are nowadays available to sexually active people. They include not only barrier methods of contraception (condom, diaphragm), but also chemical contraception (spermicides), hormonal contraceptives (oral, vaginal, injection, patches, implants), intrauterine devices (IUDs) and emergency contraception.

Recently, there have been significant scientific improvements in the development of new contraceptive technologies, including the transfer from oral contraceptives with higher doses of estrogen to those with low doses as well as the implementation of new-generation progestogens with antiandrogenic properties. In addition, new ways of hormonal contraception application have been introduced - implants, IUDs, vaginal rings and patches [3]. Thus, the choice of new contraceptive methods has been expanded and their safety and effectiveness have been improved. In addition, hormonal contraception, apart from its basic properties – protection from unintended pregnancy, exerts various beneficial therapeutic effects [4].

Despite numerous methods of hormonal contraception, some studies have shown insufficient awareness of young women about this form of contraception [5]. Awareness of hormonal contraception is particularly important for medical staff because they play an essential role in health education of population. Consequently, their attitudes and knowledge about this issue can have a significant impact on the proper use of hormonal contraception in the general population [6].

According to recently published studies conducted in Europe, there is a direct correlation between the level of education of respondents and the use of modern contraceptive methods [7, 8]. It has also been observed that most of unintended pregnancies occur primarily in young women aged between 18 and 24 years [9]. Therefore, it is especially important to examine the use of modern methods of contraception in the student population because students belong both to the category of highly educated population and to the age group in which pregnancy occurs most often.

Bearing all the above mentioned in mind, the objective of this study was to compare the characteristics of contraceptives use as well as the knowledge on hormonal contraception in female students of the Faculty of Medicine (FM) and the Faculty of Technical Sciences (FTS) in Novi Sad.

Materials and Methods

Two hundred and forty randomly chosen female students of the 3rd, 4th and 5th year of the FM and the FTS, University of Novi Sad, participated in this study, which was conducted in October and November 2012. The participants filled in the questionnaire especially designed for this purpose. It was anonymous and approved by the Ethics Committee of the Faculty of Medicine in Novi Sad. All the respondents gave their consent to participate in this study.

The questionnaire consisted of three parts. The first part included general questions and was filled in by all the participants (i.e. the name of faculty, the year of study and the date of birth). The second part, which was filled in by sexually active students only, included questions related to the age when they had had the first sexual intercourse, the number of sexual partners as well as the characteristics of the contraceptive methods they used (i.e. types of methods, frequency of their use and who they had been recommended by). In the third part, all participants were asked to assess the accuracy of 10 statements regarding hormonal contraception. Three possible answers were offered (correct, incorrect and not known). The statements are listed below:

1. Contraceptive pill is reliable in unwanted pregnancy prevention.
2. Contraceptive pill protects from sexually transmitted diseases.
3. Contraceptive pill should be taken immediately before intercourse.
4. Regular and proper usage of oral contraceptives inhibits ovulation.
5. Periods are less painful and intense if oral contraception is used.
6. Oral contraceptives increases hirsutism of the body.
7. Infertility is one of the consequences of long-term administration of oral contraceptives.
8. If I use oral contraceptives, I may become promiscuous.
9. You should not use oral contraceptives if you are younger than 30.
10. Hormonal contraception is available in a form of transdermal patches.

If a certain statement was true (e.g. statement 1), the expected answer was *correct*, and the expected answer for a false statement (e.g. statement 2) was *incorrect*.

In addition, the female students expressed their opinion about the necessity to be educated on hormonal contraception.

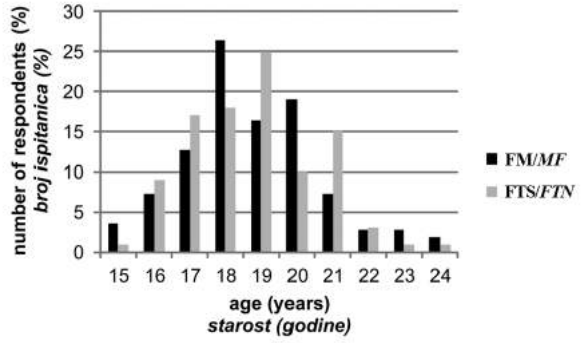
Data obtained from the questionnaires were statistically processed by using Microsoft Excel 2007. Mean value, standard deviation and percentage distribution were calculated. In order to test the statistical significance, Student t test and $\chi 2$ test were performed and the difference was considered statistically significant if $p < 0.05$.

Results

The study included 120 female students from FM and 120 female students from FTS, who attended the 3rd, 4th, and 5th year of the relevant faculty. No statistically significant difference in the structure between two studied groups was observed ($p = 0.25$). The average age of students from the FM was 24.06 ± 3.24 years while the average age of those from the FTS was 22.72 ± 0.90 years. There was no statistically significant difference between the groups regarding their age ($p = 0.06$). The vast majority of the students of both faculties (92% from the FM and 83% from the FTS) had sexual intercourses. No statistically significant difference in the number of sexually active students from the FM and the FTS was observed ($p = 0.05$).

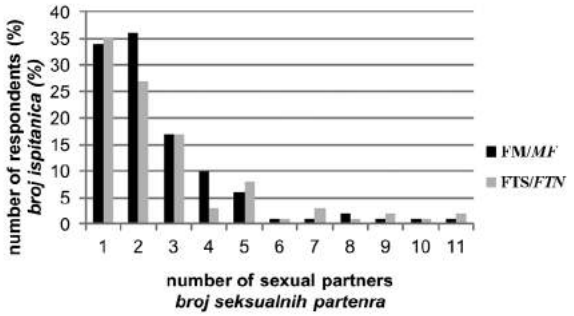
The FM and FTS students had the first sexual intercourse at the age of 18.74 ± 2.61 and 18.75 ± 2.59, respectively. There was no statistically significant difference in the average age of the respondents when they had had the first sexual intercourse between the two observed groups ($p = 0.99$). Fifty percent of medical students and 45% of those from the FTS had had sexual intercourses before the age of 19 years (adolescence) (**Graph 1**).

The percentage of students from the FM and the FTS having had one sexual partner was 30.91% and



Graph 1. Mean age of the study sample students when they had the first sexual intercourse

Grafikon 1. *Starost ispitanica sa Medicinskog fakulteta i Fakulteta tehničkih nauka prilikom stupanja u seksualne odnose*



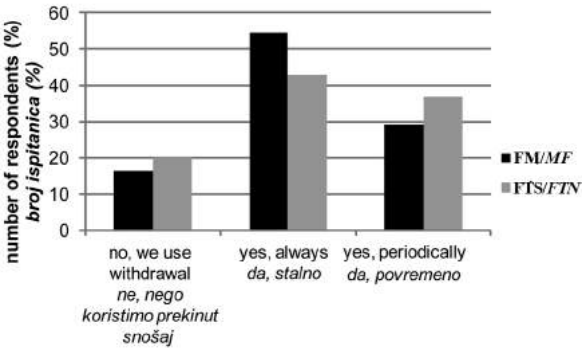
Graph 2. Number of sexual partners of the interviewed female students

Grafikon 2. *Broj seksualnih partnera ispitanica sa Medicinskog fakulteta i Fakulteta tehničkih nauka*

35%, respectively. Other participants had 3-11 sexual partners (**Graph 2**). There was no statistically significant difference in the number of sexual partners between the study groups ($p = 0.73$).

Of all sexually active female students who used some methods of contraception (including *coitus interruptus*), 83.4% of medical students and 80% of those from the FTS used one of the previously mentioned methods of modern contraception. Contraception was used regularly by 54.54% of medical students and 43% of those from the FTS (**Graph 3**). There was no statistically significant difference in the frequency of the use of contraceptive methods between the observed groups ($p = 0.25$).

The most frequently used contraceptive method was condom, which was used by 90% of the FM and 93% of FTS female students. A higher number of medical students used oral contraceptives than those from the FTS (24.54% *vs.* 11%), while more female students from the FTS used emergency contraception than the medical students (28% *vs.* 16.36%) (**Table 1**). There was no statistically significant differ-



Graph 3. Habits and choice of contraceptives among the interviewed sexually active female students according to their answers to the question: Do you use any modern method of contraception?

Grafikon 3*. Redovnost upotrebe i vrsta kontracepcije kod seksualno aktivnih studentkinja Medicinskog fakulteta i Fakulteta tehničkih nauka, procenjena na osnovu pitanja: Da li koristite neku od metoda savremene kontracepcije?*

Table 1. Types of methods of contraception used by the interviewed sexually active female students from the Faculty of Medicine and Faculty of Technical Sciences
Tabela 1*. Zastupljenost pojedinih vidova savremene kontracepcije kod studentkinja sa Medicinskog fakulteta i Fakulteta tehničkin nauka*

Contraception method *Vrsta kontracepcije*	Number (%)/*Broj (%)*	
	Faculty of Medicine *Medicinski fakultet*	Faculty of Technical Sciences *Fakultet tehničkih nauka*
Condom/*Kondom*	99 (90.00)	93 (93.00)
Urgent (postcoital) contraception *Urgentna (postkoitalna) kontracepcija*	18 (16.36)	28 (28.00)
Oral contraceptives/*Oralni kontraceptivi*	27 (24.54)	11 (11.00)
Local contraceptives/*Lokalna kontracepcija*	1 (0.91)	1 (1.00)
Intrauterine device/*Intrauterini uložak – spirala*	0	0

ence in the number of female students from both faculties who used a condom (p = 0.43), while there was a statistically significant difference in the number of participants who used emergency contraception (p = 0.04) and oral contraceptives (p = 0.01).

The respondents were not asked about the use of contraceptive patches because such contraceptive methods are not available on the market in the Republic of Serbia. Although the use of IUDs is not contraindicated in girls who have not given birth, none of the female students who participated in our study used this form of modern contraception.

The majority of medical female students (59.09%) consulted their partner when choosing the contraception method, while the female students from the FTS were advised by their gynecologist (55%). More than half of the participants from both groups (69.17% from the FTS and 65% from the FM) were interested in attending organized courses on modern contraceptive methods.

In terms of accurate awareness of oral contraceptives, the largest number of statements (statements 1, 2, 3, 5, 7, 8) were correctly assessed by more than 50% of female students from both faculties (*correct* for true statements and *incorrect* for false statements). However, more than 50% of medical female students and less than 50% of those from the FTS gave expected answers to three statements (statements 6, 9, 10).

The fact that regular and proper use of contraceptive pills inhibits ovulation (statement 4) was known by 74.17% of the medical students, but not by the 51.67% of participants from the FTS. A statistically significant difference in knowledge on oral contraceptives between the groups is shown in **Table 2**.

Taking into account all the items, there was a statistically significant difference in the knowledge level between the two groups, because the medical students produced a greater number of correct answers than those from the FTS (p = 0.00).

Discussion

Numerous studies conducted worldwide have examined contraceptive habits and knowledge on various forms of contraception among students [6, 10–18], and many of them have given special reference to students of medical faculties [6, 10–12, 15, 17]. Our study compared contraceptive habits and awareness of hormonal contraception among the medical female students, who had the opportunity to gain knowledge about various aspects of contraception during their regular studies, as well as the female students from the FTS, who did not have such an opportunity. In addition, the knowledge on contraception is of great importance especially for medical students who are expected to give reliable advice on contraceptive methods to their patients during their future practical work.

All sexually active female students included in our investigation used some contraceptive methods; however, 16.36% of medical students and 20% of those from the FTS used only coitus interruptus as the only form of contraception. Our results could be compared to the results of similar research conducted in Greece, which included female students of dentistry. In the latter study, all participants used contraception, but 20.5% of them used a condom or withdrawal, while 6.8% used only withdrawal [11]. A recent survey on contraceptive habits among young people from the Balkan Peninsula has reported that a relatively high percentage of respondents use some unsafe methods of contraception, which means that 4.3% of young men from the territory of Bosnia and Herzegovina, and even 19.7% of young men from Macedonia practice *coitus interruptus* [19]. No significant difference in contraceptive habits of young people from the neighboring countries could be explained by similar cultural characteristics. However, the comparison of the situation in countries from the Balkan Peninsula with Scandinavian countries, where health care system is highly developed, has shown that all sexually active students from Finland and Norway, regardless of socio-demographic characteristics, use reliable modern contraceptive methods such as hormonal contraceptives and condoms [13, 14].

As for the experience with reliable modern methods of contraception (condoms, hormonal contraceptives, IUDs), the majority of sexually active female

Table 2. Distribution of answers (correct. incorrect. not known) to the statements from the questionnaire given by the interviewed female students from the Faculty of Medicine and Faculty of Technical Sciences
Tabela 2. *Prikaz zastupljenosti tačnih, netačnih i ne znam odgovora na postavljene tvrdnje među ispitivanim grupama*

Statement *Tvrdnja*	Number (%)/*Broj (%)*					
	Medical Faculty *Medicinski fakultet*			Faculty of Technical Sciences *Fakultet tehničkih nauka*		
	Correct *Tačno*	Not known *Ne znam*	Incorrect *Netačno*	Correct *Tačno*	Not known *Ne znam*	Incorrect *Netačno*
1. Contraceptive pill is reliable in unwanted pregnancy prevention./*1. Kontraceptivna pilula je pouzdana zaštita od neželjene trudnoće.**	**95**	4.17	0.83	**88.33**	8.33	3.33
2. Contraceptive pill protects from sexually transmitted diseases./*2. Upotreba kontraceptivne pilule štiti od polno prenosivih bolesti.*	3.33	3.33	**93.33**	5.83	10	**84.17**
3. Contraceptive pill should be taken immediately before intercourse./*3. Kontraceptivna pilula se uzima neposredno pre polnog odnosa.**	2.5	6.67	**90.83**	9.17	18.33	**72.5**
4. Regular and proper usage of oral contraceptives inhibits ovulation. *4. Redovna i ispravna upotreba kontraceptivnih pilula sprečava ovulaciju.**	**74.17**	15	10.83	**29.17**	51.67	19.17
5. Periods are less painful and intense if oral contraception is used. *5. Menstruacije postaju manje obilne i manje bolne ukoliko koristim kontraceptivnu pilulu.*	**69.17**	20	10.83	**60**	30.83	9.17
6. Oral contraceptives increases hirsutism of the body./*6. Upotreba kontraceptivne pilule povećava maljavost na telu.*	27.5	18.33	**54.17**	20	33.33	**46.67**
7. Infertility is one of the consequences of long-term administration of oral contraceptives. *7. Jedna od posledica višegodišnje upotrebe kontraceptivnih pilula je neplodnost.**	8.33	21.67	**70**	5.83	37.15	**56.67**
8. If I use oral contraceptives. I may become promiscuous.*/*8. Upotrebom kontraceptivne pilule ću postati promiskuitetna.**	3.33	3.33	**93.33**	2.5	13.33	**84.17**
9. You should not use oral contraceptives if you are younger than 30.* *9. Ako ste mlađi od 30 godina ne bi trebalo da koristite kontraceptivne pilule.**	23.33	19.17	**57.5**	30	25	**45**
10. Hormonal contraception is available in the form of transdermal patches./*10. Hormonska kontracepcija je dostupna i u vidu flastera.*	**56.67**	39.17	4.17	**45.83**	52.5	1.67

Expected answers are bolded. * - a statistically significant difference in knowledge among respondents from the Faculty of Medicine and Faculty of Technical Sciences/*Zatamnjeni su očekivani odgovori; * - statistički značajna razlika u informisanosti među ispitanicama sa Medicinskog fakulteta i Fakulteta tehničkih nauka*

students from both faculties in our study stated to have had experience with condoms during their sexual life (90% of medical female students and 93% of those from the FTS), that being consistent with data obtained from studies conducted in the neighboring countries. While young people in the neighboring countries opt for condom of all available modern methods of contraception [10, 11, 15, 19], the majority of students from Scandinavian countries (Finland, Sweden), choose one of the methods of hormonal contraception as a protection from unintended pregnancy [14, 16]. Hence, condom is the most commonly used contraceptive method during the first sexual intercourse among female students from Finland, while 54% of female students who are in long-term relationship use oral contraceptives [14]. In our research, more medical female students (24.54%) had the experience with oral contraceptives than those from the FTS (11%). The medical female students had numerous opportunities to attend lectures (in pharmacology, gynecology) on hormonal contraception during their regular study, which probably made them more confident when choosing oral contraceptives. This is probably the reason why they consulted only the partner regarding birth control choices (59.09%), while the female students from the FTS primarily re-

lied on expert help when choosing a contraceptive method (55%).

Nevertheless, a recently published study which included female students from the Faculty of Medicine, University of Belgrade has reported the prevalence of oral contraceptives to be similar to the one among the female students from the FTS in our study (11.3%). In this study, 65.3% of respondents used contraceptive pills primarily because of other therapeutic effects of oral contraceptive such as treatment of dysmenorrhea, dermatological diseases (acne) etc. [6]. In addition, more than two thirds of sexually active female students from the Medical Faculty of Warsaw use oral contraceptives in order to regulate menstrual cycle [15]. A much lower rate of oral contraceptive administration was recorded among female students of the Faculty of Medicine in Greece (4.9%) and in Ukraine (4.5%) than in our study [11, 12].

A recent study on the level of knowledge on sexually transmitted infections among the students conducted by Nikolić and Kapamadžija has shown that the greatest number of female students believe that condom is the safest way of protection against sexually transmitted infections (up to 66.7%) while a much smaller number of them think that avoiding numerous sexual partners is also essential (to 38.8%) [20]. The majority of female students from our study sample had one or two sexual partners.

In the part of the questionnaire on the awareness of hormonal contraception, more than 50% of female students from both faculties gave the expected answers to the greatest number of statements. However, the overall awareness of hormonal contraception was better among the medical female students than among those from the FTS. It is not surprising because of broader medical education among the female students from the FM. According to our data, neither the age of engagement into sexual intercourses nor the number of sexual partners did influence the informedness about hormonal contraception. Namely, both groups of female students were of similar age when they had the first sexual intercourse (18.75 years) and they had the equal number of partners (1 or 2), while their overall awareness of hormonal contraception is different. A lower rate of oral contraceptive use among the female students from the FTS could be explained by the fact that more than a third of the participants (37.15%) believed that "infertility is one of the consequences of long-term usage of oral contraceptives", while 25% of female students from the FTS believed that "You should not use oral contraceptives if you are younger than 30". About half of the participants of both groups had little knowledge about the non-contraceptive benefits of oral contraceptives - it was unknown for most of them that oral contraceptives do not increase hirsutism of the body as well as that periods are less painfull and intense if oral contraception is used. The medical female students knew more about the mechanism of oral contraceptive actions, which is understandable taking into consideration their education. A study conducted in Norway as well as a large CHOICE (The Contraceptive Choice Project) study that involved 11 European countries has revealed that the knowledge on hormonal contraception is largely affected by gender (females have greater knowledge than males), and by the use of oral contraceptives because females who choose to use oral contraceptives are more motivated to be adequately informed about the effects and consequences of oral contraceptive use [13, 21].

The female students who participated in our study were interested in organized education about modern contraceptive methods. A study on the influence of socio-demographic factors on the use of contraception conducted by Bjelica et al. revealed that those female students who thought to have sufficient knowledge about contraception used it more frequently than those who believed that they had insufficient knowledge and needed additional information on that topic [18].

Conclusion

It is necessary to invest efforts to increase the availability of adequate information about the different types of contraception among the students in Vojvodina. The first steps have been made by the Provincial Secretariat for Sports and Youth by conducting a pilot study in order to introduce sexual education in schools. In addition, the subject "Health education on reproductive health" was taught in 10 secondary schools in Vojvodina during the school year 2013/14. Special attention should be paid to the education of future medical staff, who will contribute to reduce the number of unintended pregnancies and their termination by proper counseling of the patient in the future.

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Rad je primljen 30. XII 2013.
Recenziran 10. V 2014.
Prihvaćen za štampu 30. V 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:290-296.

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Original study
Originalni naučni rad
UDK 616.61-78:616.1]:577.2
DOI: 10.2298/MPNS1410297T

POLYMORPHISM OF ANGIOTENSIN CONVERTING ENZYME IN HEMODIALYSIS PATIENTS-ASSOCIATION WITH CARDIOVASCULAR MORBIDITY

POVEZANOST POLIMORFIZMA GENA ZA ANGIOTENZIN-KONVERTUJUĆI ENZIM SA KARDIOVASKULARNIM MORBIDITETOM BOLESNIKA NA HRONIČNOJ HEMODIJALIZI

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Summary

Introduction. Cardiovascular morbidity and mortality are the major concern in dialysis patients and many risk factors are thought to be involved in its pathogenesis. Apart from traditional and non-traditional risk factors, the genetic susceptibility may be of importance, including renin-angiotensin system gene polymorphism. The aim of this study was to analyse renin-angiotensin system polymorphism in our group of hemodialysis patients and to correlate the findings with cardiovascular morbidity. **Material and Methods.** The study included 196 patients on regular hemodialysis on polysulphone membrane three times per week for more than six months. Genetic analysis was performed by using polymerase chain reaction – restriction fragment length polymorphism method. **Results.** Out of 196 patients, 55% had I/D genotype, 35% had D/D and 10% had I/I, including angiotensin-converting enzyme polymorphism. It was shown that the patients with D allele genotype developed a significantly higher incidence of left ventricular hypertrophy and peripheral vascular disease. The angiotensin-converting enzyme polymorphism showed a significant association with the incidence of cerebrovascular accident and hyperlipoproteinemia in our group of hemodialysis patients. **Conclusion.** The angiotensin-converting enzyme gene polymorphism is associated with the development of cerebrovascular accidents and hyperlipoproteinemia. Allele D of this gene increases the risk for the development of left ventricular hypertrophy and peripheral vascular disease significantly in hemodialysis patients. A longer follow-up is needed to make the definitive conclusion about the influence of angiotensin-converting enzyme polymorphism on cardiovascular morbidity and its importance in everyday clinical practice.

Key words: Polymorphism, Genetic; Renal Dialysis; Cardiovascular Diseases; Risk Factors; Renin-Angiotensin System; Polymerase Chain Reaction

Sažetak

Uvod. Kardiovaskularne bolesti su glavni uzrok morbiditeta i mortaliteta bolesnika u terminalnoj fazi hronične bubrežne slabosti. Pored klasičnih faktora rizika, u poslednje vreme sve više se istražuju takozvani „netradicionalni" faktori rizika, uključujući i polimorfizam gena za angiotenzin-konvertujući enzim. Cilj ovog istraživanja bio je analiza kardiovaskularnog morbiditeta u odnosu na genski polimorfizam angiotenzin-konvertujućeg enzima kod bolesnika koji se leče hroničnim hemodijalizama. **Materijal i metode.** Studija je obuhvatila 196 bolesnika koji se nalaze na hroničnom programu lečenja hemodijalizama duže od šest meseci, tri puta nedeljno na polisulfonskim membranama. Genotipizacija je sprovedena metodom reakcije lančane polimerizacije. **Rezultati.** Od 196 ispitanika, njih 55% imalo je I/D, 35% D/D, a 10% njih imalo je I/I genotip. Pokazano je da samo prisustvo D-alela gena angiotenzin-konvertujućeg enzima povećava rizik za pojavu hipertrofije leve komore i periferne vaskularne bolesti. Genski polimorfizam angiotenzin-konvertujućeg enzima pokazao je značajnu povezanost sa nastankom cerebrovaskularnog insulta i hiperlipoproteinemije kod bolesnika na hemodijalizi. **Zaključak.** Polimorfizam gena za angiotenzin-konvertujući enzim povezan je sa nastankom cerebrovaskularnog insulta i hiperlipoproteinemije kod pacijenata na dijalizi. Alel D ovog gena značajno povećava rizik za nastanak hiperlipoproteinemije i periferne vaskularne bolesti. Genetske analize zahtevaju opsežnije analize radi definitivnog stava o povezanosti datog polimorfizma sa morbiditetom bolesnika na dijalizi i eventualnog značaja ovih nalaza u svakodnevnoj kliničkoj praksi.

Ključne reči: Genetski polimorfizam; Hemodijaliza; Kardiovaskularna oboljenja; Faktori rizika; Renin-Angiotenzin sistem; PCR

Introduction

Cardiovascular disease (CVD) is a leading cause of death in hemodialysis patients. The risk for developing cardiovascular event is 5-30 times higher in patients with end stage renal disease (ESRD) than in the general population [1–5]. Atherosclerosis has the principal role in cardio-

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Abbreviations

CVD	– cardiovascular disease
ESRD	– end stage renal disease
ACE	– angiotensin-converting enzyme
RAS	– renin-angiotensin system
RAAS	– renin-angiotensin-aldosterone system
ECG	– electrocardiogram
RNA	– ribonucleic acid
DNA	– deoxyribonucleic acid
PCR	– polymerase chain reaction
I	– insertion
D	– deletion

vascular events, and its pathogenesis is formulated as the response-to-injury model. Endothelial denudation, being the first step in atherosclerosis, can be the result of many different factors.

Traditional risk factors (smoking, hypertension, dyslipidemia, diabetes, hypoalbuminaemia, etc.), could not explain the high prevalence of cardiovascular disease in hemodialysis patients [1–8]. Therefore, recent reports have suggested a role of non-traditional risk factors in pathogenesis of CVD, such as gene polymorphism of angiotensin-converting enzyme (ACE) [9, 10]. Gene polymorphism means the presence of two or more variants of a gene, and the frequency of its rarest allele must be higher than 1% or equal to 1% to consider a gene locus polymorph.

Angiotensin-converting enzyme is a zinc metallopeptidase, distributed on the surface of endothelial and epithelial cells. It has a vital role in normal physiological conditions, as a part of renin-angiotensin-aldosterone system (RAAS), kinin-kallikrein system [11, 12]. ACE also takes part in *in vitro* degradation of amyloid beta peptide and in signal transduction in the central nervous system. Angiotensin-converting enzyme converts the inactive decapeptide, angiotensine I to the active octapeptide and potent vasoconstrictor angiotensin II, which is the main product of the renin-angiotensin system (RAS) [12].

The gene encoding ACE is located on the long arm of chromosome 17. The gene is 21 kilo bases (kb) long and comprises 26 exons and 25 introns. In mature sACE m ribonucleic acid (RNA), exons 1 to 26 are transcribed, except for exon 13, which is spliced. There is only one copy of ACE gene in human haplotype [13]. There are more than 160 ACE gene polymorphisms listed, most of which are single nucleotide polymorphisms (SNPs). Only 34 of them are located in coding regions. Others like insertion-deletion polymorphisms are less common [13].

Polymorphisms for different components of RAAS such as genes for renin synthesis, angiotensinogen and AT1 receptors, have been investigated as factors affecting the progression of cardiovascular and renal diseases. Investigators have shown the greatest interest for further studies on ACE gene polymorphism.

There are large interindividual differences in plasma ACE levels [13]. In 1990, Rigat et al. found a polymorphism involving either the presence (insertion – I) or absence (deletion – D) of a 287 bp sequence of deoxyribonucleic acid (DNA) in intron 16 of ACE gene [14]. The deletion is considered as a mutation. The insertion inside the regulatory region of ACE gene blocks, while the deletion of the same region activates the gene [14, 15].

There are three different genotypes, I/I, I/D and D/D, and each one of them might influence the ACE activity. Genotype DD indicates deletion of 287 bp in both alleles, while the presence of the insertion segment in both alleles indicates that the subject is II homozygote. Heterozygotes have ID genotype when the deletion of 287 kb is present in one allele. The highest levels of plasma ACE are found in DD homozygotes, homozygotes with I/I genotype have the lowest level, while those with I/D genotype have intermediate plasma levels of this enzyme [16, 17].

The aim of this study was to analyse ACE polymorphism in our group of hemodialysis patients and to correlate the findings with cardiovascular morbidity.

Material and Methods

This study was approved by the Ethical Committee of Zvezdara University Medical Centre and the written consent has been obtained from each patient.

The study included 196 patients on regular hemodialysis, on polysulphone membrane three times per week for more than six months at the Zvezdara University Medical Centre. The genetic analysis was performed by using polymerase chain reaction – restriction fragment length polymorphism method (PCR-RFLP).

The retrospective analysis included data collection from the patients' history regarding cardiovascular morbidity (myocardial infarction, cerebrovascular accident, coronary artery disease, heart arrhythmia, hypertension, left ventricular hypertrophy, peripheral artery disease). The collected data were correlated with the genetic polymorphism for ACE.

Heart arrhythmia was diagnosed by electrocardiogram (ECG) holter monitoring, hypertension was defined as blood pressure elevation over 140/90 mmHg in more than two repeated measurements, while the estimation of left ventricular hypertrophy was based on echosonography or ECG findings. Peripheral vascular disease was diagnosed by performing doppler echosonography or arteriography. Myocardial infarction and cerebrovascular accident was diagnosed by percutaneous coronary intervention and computerized tomography, respectively.

The data collected from patients' history included age, gender, cause of ESRD, duration of chronic hemodialysis programme and cardiovascular morbidity.

The whole blood with ethylendiamintetraacetic acid (EDTA) was used (5 ml of whole blood stored at +4^{o}C for less than 4 days, or at -20^{o}C for a longer period) for DNA extraction.

The extraction was performed by macro-method of genomic DNA isolation [18]. This method is based on cell lysis, which is commonly achieved by chemical and physical method-blending, grinding or sonicating the sample. The aim is to remove proteins, RNA and other macromolecules. Deoxyribonucleic acid is then precipitated by ice-cold ethanol or isopropanol.

Genotyping was performed by the method of polymerase chain reaction (PCR). The PCR was carried out in small reaction tubes (0.2 ml volumes) in a thermal cycler. The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the reaction. One cycle includes:

– denaturation of DNA by heating the reaction to 94-96°C;

– annealing of the primers to the single-stranded DNA template at the temperature of 50-65°C, and

– primer elongation - DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand at the temperature of 72°C.

PCR consists of a series of 25-45 repeated cycles, followed by the final product extension. To check whether the PCR generated the anticipated DNA fragment, agarose gel electrophoresis is employed for size separation of the PCR products. Positive samples are then used for further analysis by restriction enzymes.

Restriction enzymes can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a restriction digest.

The DNA sample is broken into pieces (digested) by restriction enzymes and the resulting restriction fragments are separated according to their lengths by gel electrophoresis.

The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

Genotypization of I/D polymorphism

DNA fragment of 287 pb, which represents a deletion fragment in introne 16, was amplified by PCR method. In the process of genotyping I/D polymorphism of ACE gene, the PCR products were fragments of 490 kb and 190 kb length and by using insertion specific set of primer products they were fragments of 335 bp length.

The reaction content volume was: 2,5 µl PCR buffer solution, 0,75 µl $MgCl_2$, 0,5 µl dNTP, 1 µl primer I, 1 µl primer II, 0,2 µl DNK polymerase, 5 µl genomic DNA in the whole volume of 25 µl. The reaction was performed in GeneAmp PCR System 2700, AB Applied Biosystem. The amplification consisted of 30 cycles, including denaturation at 94°C for 1 minute, primers annealing at 58°C for 1 minute and DNA extension/elongation at 72°C for 2 minutes. The fragments with no insertion (D alelle) and other with insertion (I alelle) of 190 bp i 490 bp respectively, were detected using 2% agarose gel electrophoresis with ethidium bromide.

To enhance DD genotyping specificity, the amplification was performed by using insertion specific set of primers in PCR conditions: 1 minute of denaturation at 94°C, followed by 30 cycles for 30 seconds at 94°C, 45 seconds at 67°C (annealing) and 2 minutes at 72°C (extension). Only the presence of I alelle generated the fragments of 335 bp length, which were identified by 3% agarose gel electrophoresis. An amplified sample with *Alu* sequence insertion represents a fragment of 490 bp (genotype II), while the DNA fragment of 190 bp represents a sequence deletion in alelle (genotype DD). The appearance of both fragments represents heterozygote (genotype ID).

The standard statistical analysis was performed in order to get the measures of variability and central tendency. Normal data were tested by parametric tools (Student's t test), while ordinal data were tested by non-parametric tools (chi-square test, Fisher's exact test). The correlation between genetic polymorphism and cardiovascular morbidity was tested by logistic regression analysis. Odds ratio was calculated to estimate the risk of ACE genotype for cardiovascular morbidity. Numeric values (patient's demographic characteristics) were analyzed by the Analysis Of Variance (ANOVA). The results are shown in tables.

Results

The patients' mean age was 62.3±11.4 years; 85 (43.4%) patients were women and 111 (56.6%) of them were men. The average duration of chronic hemodialysis was 8.4±5.2 years.

The causes of ESRD were as follows: hypertension in 104 (53%) patients, diabetes mellitus in 25 (13%) patients, chronic glomerulonephritis in 20 (10%) patients, polycystic kidney disease in 21 (10.5%) patients, chronic pyelonephritis in 16 (8%) patients, Balkan endemic nephropathy in five (2.5%) patients, and systemic disease, myeloma multiplex, tumors and unknown causes in six (3%) patients.

As for cardiovascular morbidity at the moment of collecting data, hypertension was present in 178 patients (90.8%); 30 patients (15.3%) had myocardial infarction; 31 patient (15,8%) had cerebrovascular accident; a coronary artery disease was diagnosed in 89 patients (45.4%); a peripheral vascular disease was present in 18 patients (9.2%); 66 patients (33.8%) and 114 patients (58.2%) were diagnosed to have left ventricular hypertrophy and hyperlipidemia, respectively; while heart arrhythmia was present in 48 patients (24.5%).

Out of 196 patients, 55%, 35% and 10% of our patients had I/D, D/D and I/I genotype, respectively.

Table 1 shows the influence of I/D genotype on coronary artery disease and odds ratio for its development, although without statistical significance (p=0,49). In addition, the results show that neither does the presence of D allele in the gene affect the development of coronary artery disease (p=0.86), nor

Table 1. Incidence of coronary artery disease regarding ACE polymorphism and logistic regression
Tabela 1. *Incidencija koronarne aterijske bolesti u zavisnosti od genotipova za polimorfizam angiotenzin konvertujućeg enzima i regresiona analiza*

			CAD/*KAB*		OR	95% C.I.	p
			No/*Ne*	Yes/*Da*			
ACE	I/I	N	10	9	0.794	0.298 - 2.111	0.643
		%	52.6	47.4			
	I/D	N	63	45	1.144	0.414 - 3.162	0.796
		%	58.3	41.7			
	D/D	N	34	35			0.491
		%	49.3	50.7			
	I/D+D/D	N	97	80	0.916	0.355 - 2.365	0.857
		%	54.8	45.2			

CAD - coronary artery disease/*KAB - koronarna arterijska bolest*

Table 2. Incidence of miocardial infarction regarding ACE polymorphism and logistic regression
Tabela 2. *Incidencija infarkta miokarda u odnosu na polimorfizam angiotenzin konvertujućeg enzima i regresiona analiza*

			MI/*IM*		OR	95% C.I.	p
			No/*Ne*	Yes/*Da*			
ACE	I/I	N	15	4	0.492	0.131 - 1.853	0.294
		%	78.9%	21.1%			
	I/D	N	90	18	0.750	0.223 - 2.542	0.750
		%	83.3%	16.7%			
	D/D	N	61	8			
		%	88.4%	11.6%			
	D/D+I/D	N	151	26	0.646	0.199 - 2.099	0.646
		%	85.3%	14.7%			

MI - miocardial infarction/*IM - infarkt miokarda*

does the risk for its development significantly vary among the three different genotypes of ACE gene.

Table 2 shows the influence of ACE gene polymorphism and D allele on myocardial infarction but without a statistical significance (p=0.5). The same table also shows the risk of the ACE genotype for the development of myocardial infarction (p=0.786).

The results did not show an association between D allele and cerebrovascular accident, although there was a significant correlation between the ACE gene polymorphism and the incidence of cerebrovascular accident (**Table 3**).

There was no significant correlation between left ventricular hypertrophy and ACE genotypes

Table 3. Incidence of cerebrovascular accident regarding ACE polymorphism in haemodialysis patients
Tabela 3. *Incidencija cerebrovaskularnog insulta u odnosu na polimorfizam angiotenzin konvertujućeg enzima kod bolesnika na hemodijalizi*

			CVA/*CVI*		p
			No/*Ne*	Yes/*Da*	
ACE	D/D	N	56	15	
		%	78.9%	21.1%	
	I/D	N	92	14	0.05
		%	86.8%	13.2%	
	I/I	N	19	0	
		%	100%	.0%	
	D/D+I/D	N	148	39	0.257
		%	12.3%	17.7%	

CVA - cerebrovascular accident/*CVI - cerebrovaskularni insult*

Table 4. Incidence of left ventricular hypertrophy regarding ACE polymorphism in haemodialysis patients
Tabela 4. *Incidencija hipertrofije leve komore u odnosu na polimorfizam angiotenzin konvertujućeg enzima kod bolesnika na hemodijalizi*

			LVH/*HLK*		p
			No/*Ne*	Yes/*Da*	
ACE	D/D	N	44	25	0.08
		%	63.8%	36.2%	
	I/D	N	69	39	
		%	63.9%	36.1%	
	I/I	N	17	2	
		%	89.5%	10.5%	
	D/D+I/D	N	113	19	0.025
		%	63.8%	36.2%	

LVH - left ventricular hypertrophy/*HLK - hipertrofija leve komore*

(p=0.08). However, the presence of D allele in ACE gene showed a significant association with the development of left ventricular hypertrophy (p=0.025) (**Table 4**).

The ACE polymorphism showed a significant association with the incidence of hiperlipoproteinemia in our group of hemodialysis patients (p=0,05), while the presence of D allele itself did not show any influence on its development (**Table 5**).

The highest risk for hiperlipoproteinemia was found in the patients with I/D genotype, being three times higher than in I/I homozygotes (**Table 5**).

It was shown that the patients with D allele genotype had a significantly higher incidence of peripheral vascular disease, being 2.4 times higher in the patients with I/D genotype than the D/D homozygotes (**Table 6**).

The presence of D allele was not proved to have a significant influence on hypertension (p=0.32). The correlation between the ACE gene polymorphism and hypertension could not be taken into consideration because hypertension was present in over 90% of patients.

Table 5. Incidence of hyperlipoproteinaemia regarding ACE polymorphism and logostic regression
Tabela 5. *Incidencija hiperlipoproteinemije u zavisnosti od genotipova za polimorfizam angiotenzin konvertujućeg enzima i regresiona analiza*

			HLP		P	OR	95% C.I.	p
			No/*Ne*	Yes/*Da*				
ACE	I/I	N	12	7				0.054
		%	63.2%	36.8%				
	I/D	N	37	70	0.05	3.056	1.092-8.553	0.033
		%	34.5%	65.5%				
	D/D	N	33	37		1.817	0.630-5.240	0.269
		%	47%	53%				
	D/D+I/D	N	69	107	0.067	2.473		0.075
		%	39.2%	60.8%				

HLP - hyperlipoproteinaemia/*hiperlipoproteinemija*

Table 6. Relative risk for development of peripheral vascular disease regarding the ACE polymorphism
Tabela 6. *Relativni rizik za nastanak periferne vaskularne bolesti u odnosu na polimorfizam angiotenzin konvertujućeg enzima*

ACE		PVD/*PVB*		OR	95% C.I	p
		No/*Ne*	Yes/*Da*			
D/D	n (%)	60 (88.2)	8 (11.8)			0.620
I/I	n (%)	18 (90)	2 (10)	1.636	0.195-13.717	0.650
I/D	n (%)	99 (90.8)	9 (9.2)	2.400	0.281-20.492	0.424
I/D+D/D	n (%)	159 (90.3)	17 (9.7)	1.925	0.242-15.326	0.005

PVD - peripheral vascular desease/*PVB - periferna vaskularna bolest*

The results showed that neither the heart arrhythmia incidence (p=0.67) was correlated with the ACE gene polymorphism.

Discussion

Although the effect I/D polymorphism of ACE gene is often studied with respect to cardiovascular and other complex disorders, its location in noncoding region makes it unlikely to be a function variant [11]. Since the nature and position of the functional polymorphism, which is responsible for plasma ACE levels, remains a mystery, investigators continue to use the I/D polymorphism as a valid marker for studying association between the unknown functional polymorphism and pathological conditions.

The results of this study have shown that I/D polymorphism of ACE gene is the most common genotype, that being in accordance with literature data, which shows that the distribution of ACE genotype in healthy population is almost the same in the population of dialysis patients. Odds ratio of ACE polymorphism for myocardial infarction was not significant. The first study reporting a positive association between the D allele and myocardial infarction was published in 1992, but later analyses showed different results [19]. While in one of the multicentre studies the DD genotype was found to be significantly more frequent in male patients with myocardial infarction than in the controls, particularly among low-risk individuals, this result was not replicated in a larger study done by Agerholm-Larsen et al. [16]. Three years later, the same research group published a meta-analysis, in which they included their own study and 21 other associated studies [16]. Five of these studies were large (over 600 patients), while the others were small. The result showed the positive association between allele D and myocardial infarction. The authors concluded that small studies showed a more pronounced effect on risk of myocardial infarction, so that it could be the reason for the overall result to be positive [14–16]. Despite the idea that small studies could be a reason for bias, because of lower level of quality control or study design, that might also indicate the importance of ACE gene polymorphism in certain subgroups.

The results of this study did not show an influence of ACE gene polymorphism on the development of coronary artery disease. In studies where coronary calcification were used as a measurement of atherosclerosis, investigators failed to find a significant association between ACE polymorphism and atherosclerosis [19–21]. In general, a moderate positive association between the D allele and atherosclerosis is expected, particularly in patients who have other cardiovascular risk factors. Investigators believe the D allele is not clinically important in coronary heart disease in the general population, but it may play an important role in certain groups of patients.

The results of this study showed a significant association of ACE gene polymorphism and hyperlipidemia. According to literature data, this kind of association has been studied only in patients with familial hyperlipidemia so far, where it has been shown that D allele might be an additional factor for the development of this hereditary disease. There are no literature data on the population of dialysis patients.

The results of this study showed a significant influence of ACE polymorphism on cerebrovascular accident. Two meta-analyses reported significant positive associations between the D allele and ischemic stroke [11]. Some studies found the association of D allele only with lacunar stroke, while others showed a positive association of D allele and carotid stenosis and cerebrovascular accident. The finding of an association of the D allele with a severe cerebrovascular disease may contribute to the understanding of the high prevalence of this complication in end stage renal disease. Many factors could contribute to these findings and they cannot be controlled because of dysregulated homeostasis in ESRD. Although the role of local and environmental factors (smoking) cannot be ruled out, larger studies will be needed to detect them.

This study showed that the patients with D allele had a significantly higher incidence of left ventricular hypertrophy. That may be the consequence of the action of localized RAS on the vascular function and structure, hypertrophic effects including extracellular matrix production. An unexpectedly small number of patients with left ventricular hypertrophy in our study might be ascribed to different diagnostic tools, where most of them were diagnosed using ECG instead of echosonography. That could also have affected our findings, which showed no association between ACE polymorphism and left ventricular hypertrophy.

Although it is known that a subject carrying D allele has higher plasma levels of ACE, which is usually associated with high blood pressure, the D allele has not been shown as a significant risk factor for hypertension in this study. Previous investigations came to controversial findings. The first meta-analysis on this topic, published by Staessen et al, included 23 studies with 6923 subjects and indicated a 10% increased risk of hypertension in DD versus II genotype, which was not statistically significant [15]. There was, however, a strong indication of heterogeneity among the reports. That is why the sensitivity analyses were performed in subgroups based on gender, ethnicity, mean age and genotyping method; and then, there was a significant relationship between the D allele and hypertension in women and Asians, whereas no association was found in all other subgroups. Another meta-analysis, done by Agerholm-Larsen et al, was published in 2000 and it was restricted to Caucasians. It included 19 studies with a

very little overlap with previous meta-analyses [16]. The results also indicated that ACE genotype did not affect hypertension. Of 26 association studies reviewed by Agarwal et al. in 2005, 12 reported positive results and 14 reported negative results [17].

The experiments in animals have shown that a 3-fold increase in plasma ACE level does not affect blood pressure. The reason of no association between ACE genotype and hypertension might be multifactorial causes of high blood pressure, which are always present in hemodialysis patients (hypervolemia, inadequate salt excretion) [22]. The carriers of the D allele seem to be less sensitive to sodium state than the I allele and could therefore be less responsive to sodium removal by ultrafiltration in dialysis [22]. In addition, antihypertensive therapy might have an influence on results regarding the association between ACE polymorphism and hypertension. It has been concluded that controversial results could be a consequence of cumulative influence of plasma ACE level, environmental factors and genetics.

Patients on chronic hemodialysis are constantly exposed to complex stimulation of RAS, whose activation affects many target organs. That is why patients who have genotype associated with a higher expression of RAS are more prone to organ damage and even higher mortality rate, especially when multiple interactions with other risk factors are included [23].

It has been shown so far that certain genes may cause some of cardiovascular diseases in interaction with other genes and environmental factors. Therefore, much more additional research is needed to make the definitive conclusion about the influence of gene polymorphism on cardiovascular morbidity.

Conclusion

In conclusion, the angiotensin-converting enzyme gene polymorphism is associated with the development of cerebrovascular accidents and hyperlipoproteinaemia. Allele D increases the risk for development of left ventricular hypertrophy and peripheral vascular disease significantly in hemodialysis patients. Longer follow-up is needed to reach the definitive conclusion about the influence of angiotensin-converting enzyme polymorphism on cardiovascular morbidity and its importance in everyday clinical practice.

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Rad je primljen 15. XII 2013.
Recenziran 2. V 2014.
Prihvaćen za štampu 12. VI 2014.
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Original study
Originalni naučni rad
UDK 616.441-008.61-085-037
DOI: 10.2298/MPNS1410305A

THYROID-STIMULATION HORMONE - RECEPTOR ANTIBODIES AS A PREDICTOR OF THYROSUPPRESSIVE DRUG THERAPY OUTCOME IN GRAVES' DISEASE PATIENTS

ANTITELA NA RECEPTORE TIROIDNOG STIMULIŠUĆEG HORMONA KAO PREDIKTOR ISHODA MEDIKAMENTNE TIROSUPRESIVNE TERAPIJE KOD PACIJENATA SA GREJVSOVOM BOLEŠĆU

Aleksandar Z. ALEKSIĆ, Željka ALEKSIĆ[2], Saška MANIĆ[3], Vladimir MITOV[1] and Aleksandar JOLIĆ[1]

Summary

Introduction. Graves' disease is autoimmune hyperthyroidism caused by pathological stimulation of thyroid-stimulation hormone – receptor antibodies. The decision on changing the therapy can be made on time by determining the prognostic factors of thyrosuppressive drug therapy outcome. The aim of the study was to determine the significance of thyroid-stimulation hormone-receptor antibodies level on the prediction of therapy outcome. **Material and Methods.** The study was prospective and involved 106 drug-treated patients with newly diagnosed Graves' disease. Thyroid-stimulation hormone - receptor antibodies level was measured at the beginning of therapy, during therapy and 12 months after it had been introduced. **Results.** No statistically significant difference in the level of thyroid-stimulation hormone - receptor antibodies was found at the beginning of disease and 12 months after the introduction of thyrosuppressive drug therapy among the patients who had been in remission and those who had not. Regardless of the outcome, thyroid-stimulation hormone - receptor antibodies level significantly decreased in all patients 12 months after the therapy had been introduced. **Conclusion**. The level of thyroid-stimulation hormone - receptor antibodies at the beginning of disease and 12 months after the introduction of therapy cannot predict the outcome of thyrosuppressive drug therapy.

Key words: Graves Disease; Receptors, Thyrotropin-Releasing Hormone; Antibodies; Treatment Outcome; Antithyroid Agents; Remission Induction; Recurrence; Prognosis

Sažetak

Uvod. Grejvsova bolest je autoimuni hipertiroidizam izazvan patološkom stimulacijom antitela na receptore tiroidnog stimulišućeg hormona. Utvrđivanje prognostičkih faktora za ishod medikamentne tirosupresivne terapije omogućuje pravovremeno donošenje odluke o promeni vrste terapije. Cilj rada bio je utvrđivanje značaja nivoa antitela na receptore tiroidnog stimulišućeg hormona na predviđanje ishoda terapije. **Materijal i metode.** Istraživanje je prospektivno i obuhvatilo je 106 pacijenata sa novootkrivenom Grejvsovom bolešću lečenih medikamentno. Na početku, tokom i 12 meseci po započinjanju terapije, praćen je nivo antitela na receptore tiroidnog stimulišućeg hormona. **Rezultati.** Nije nađena statistički značajna razlika u nivou antitela na receptore tiroidnog stimulišućeg hormona na početku bolesti i 12 meseci po započinjanju medikamentne tirosupresivne terapije, između pacijenata koji ulaze u remisiju i onih koji ne ulaze. Nivo antitela na receptore tiroidnog stimulišućeg hormona značajno opada 12 meseci nakon uvođenja terapije kod svih, bez obzira na ishod. **Zaključak**. Nivo antitela na receptore tiroidnog stimulišućeg hormona na početku bolesti i 12 meseci po započinjanju terapije ne može da predvidi ishod medikamentne tirosupresivne terapije.

Ključne reči: Grejvsova bolest; TSH Receptori; Antitela; Ishod lečenja; Tirosupresivna terapija; Remisija; Recidiv; Prognoza

Introduction

Graves' disease (GD) is an organ-specific autoimmune disorder characterized by hyperthyroidism with diffuse goiter and a possible presence of extra-thyroid manifestations such as eye changes (thyroid ophthalmopathy), skin change (dermopathy) and fingertips change (acropachy) [1, 2].

According to some estimation, GD is present in up to 1% of the population. In areas with adequate iodine supply, Graves' disease is the most frequent type of hyperthyroidism [1].

Etiology of GD is multifactorial: environmental factors, immune aberrations and minimal changes in the target organ probably interact with each other within the genetic predisposition. Among other things, vitamin D receptor gene polymorphism is associated with an increased risk for the occurrence of autoimmune thyroid disease. Possible external factors which play a role in the occurrence of disease are some of infectious agents

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Abbreviations

GD	– Graves' disease
TSH	– thyroid-stimulation hormone
TRAb	– TSH receptor antibodies
MTT	– thyroid suppressive therapy
ROC	– Receiver Operating Characteristic curve
MMD	– minimum maintenance dose
TSAb	– thyroid stimulating antibody
TBAb	– thyroid blocking antibody

(e.g. viruses), amount of iodine ingested via food, stress, smoking [1, 3, 4].

The presence of antibodies to the thyroid-stimulation hormone (TSH) – receptor of thyroid (TRAb) is of great pathogenic importance for the development and persistence of autoimmune hyperthyroidism. Using their own stimulatory effect upon binding to the TSH – receptor, these antibodies cause increased synthesis and secretion of thyroid hormones. GD patients may have stimulating, blocking and even neutral antibodies in the serum, and the clinical picture is a consequence of their relative potency and dominance [1, 2].

According to the reports on the achievements of long-term remission in patients treated with medical thyroid suppressive therapy (MTT), the percent of treated patients range from 14 – 80% [1, 2]. The overall recurrence rate of GD in patients treated with MTT after cessation of treatment is about 30 – 50% [5, 6].

Consensus about the TRAb importance, regarding the prediction of GD relapse and remission has not been reached yet. Studies conducted so far differ among themselves not only by the type of TRAb essay methodology applied but by the study design as well, some of them being retrospective and others prospective. They also differ in time when TRAb was tested during the disease. In addition, population genetics and iodine status could affect the results of studies because of different geographic areas where those studies were conducted [7].

The aim of this study was to determine the significance of TRAb level for predicting remission occurrence during thyrosuppressive drug therapy.

The hypothesis is that TRAb level at the beginning of the disease and during MTT is a predictive factor for the outcome of therapy.

Material and Methods

This prospective study included 106 patients with newly diagnosed GD who started treatment with MTT. After the diagnosis of hyperthyroidism had been made according to anamnestic data, clinical examination, signs of hypermetaboilism, suppressed TSH level and increased free fraction levels of serum thyroid hormones, TRAb level was determined in all patients. TRAb was measured by radio receptor method (DYNO test TRAK human Brahms Diagnostica GMBH) with normal values up to 1.5 IU/L. MTT was given using the process of titration. TRAb level was determined 6 and 12 months after the beginning of therapy. According to the response to thyrosuppressive drug therapy, the patients were divided into two groups: group A – patients who achieved remission, and group B – patients who did not achieve remission, non-responders, whereby the remission meant a clinical and biochemical euthyroid state which was maintained at least 12 months after MTT had been discontinued. TRAb level wass expressed as the mean value ± SD, median, as well as minimum and maximum value **(Table 1)**. T-test for two independent samples and analysis of variance of repeated measures were used to test differences between groups. The tests were done using transformed data. ROC analysis (Receiver Operating Characteristic curve analysis) was made in order to determine a specific TRAb level for the prediction of remission. The data are shown in tables and graphs.

Results

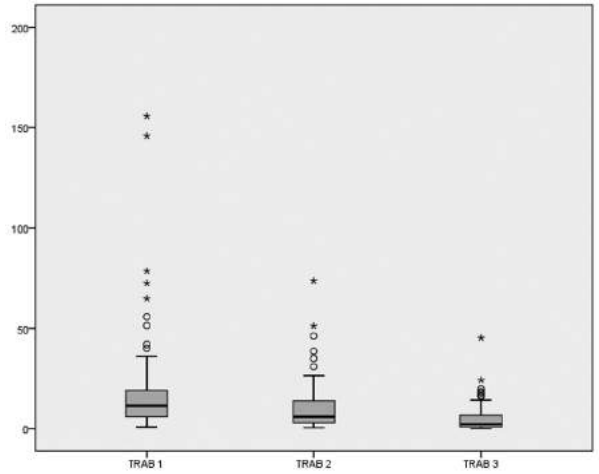
Out of 106 respondents, 21 (19.81%) were male and 85 (80.19%) were female. The average age of all the respondents was 44.27 ± 15.35 years (age range 14 – 74). The total follow-up of patients lasted 45 months. The average duration of MTT was 21.20 ± 14.01 (the median being 17 months), and the average follow-up after the therapy had been discontinued was 28.38 ± 27.21 (the median being 24 months).

Having been diagnosed, all our respondents underwent MTT titration regimen. Most of the patients were treated with methimazole (79%), while some of them (18%) were treated with propiltiouracil. Other patients (3%) started the treatment with methimazole and then continued with propiltiouracil, mainly because of some allergic manifestation to methimazole.

The average TRAb level in our patients at the beginning of disease was 11.42 U/L. Six months after the introduction of MTT, the average TRAb level in our patients was 5.9 U/L, and 12 months after the introduction of MTT, the average TRAb level was 2.3 U/L as shown in **Graph 1.**

Table 1. Structure of the patients according to thyrosuppressive drug therapy outcome 12 months after the introduction of therapy
Tabela 1. *Struktura ispitanika prema ishodu medikamentne tirosupresivne terapije nakon 12 meseci lečenja*

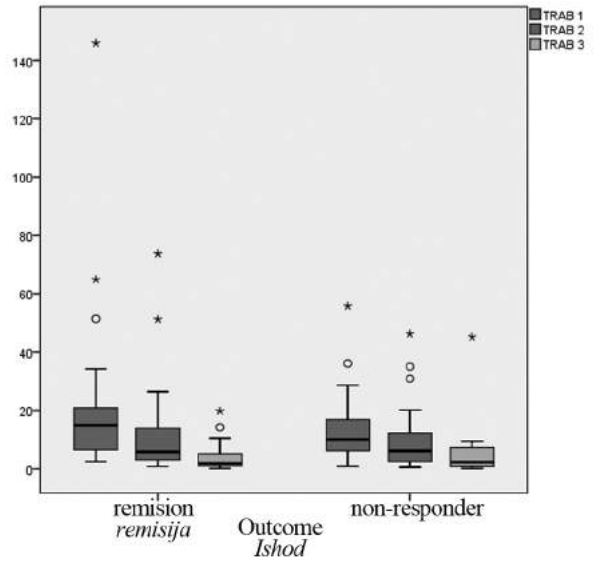
Outcome/*Ishod*	N	%
A Remission/*Remisija*	62	58.5
B – Non-responder/*Bez remisije*	44	41.5
Total/*Ukupno*	106	100



Graph 1. Mean TRAb level in patients. TRAb 1 – at the beginning of therapy; TRAb 2 – 6 months on therapy; TRAb 3 – 12 months after the introduction of therapy; *y axis – U/L*

***Grafikon 1**. Prosečan nivo TRAb u U/L kod ispitanika TRAb 1 – na početku terapije; TRAb 2 – 6 meseci na terapiji; TRAb 3 – 12 meseci po uvođenju terapije; y osa – U/L; TRAb - antitetla na receptore tiroidnog stimulišućeg hormona*

Graph 2 shows average TRAb level in patients in remission and in non-responders at the beginning of disease and 6 and 12 months after the introduction of therapy.



Graph 2. Mean TRAb level in patients according to the thyrosuppressive drug therapy outcome.

***Grafikon 2.** Prosečan nivo TRAb kod ispitanika prema ishodu medikamentne tirosupresivne terapije*

TRAb 1 – at the beginning of disease; TRAb 2 – 6 months on therapy; TRAb 3 – 12 months after the introduction of therapy; x axis – outcome; y axis– U/L

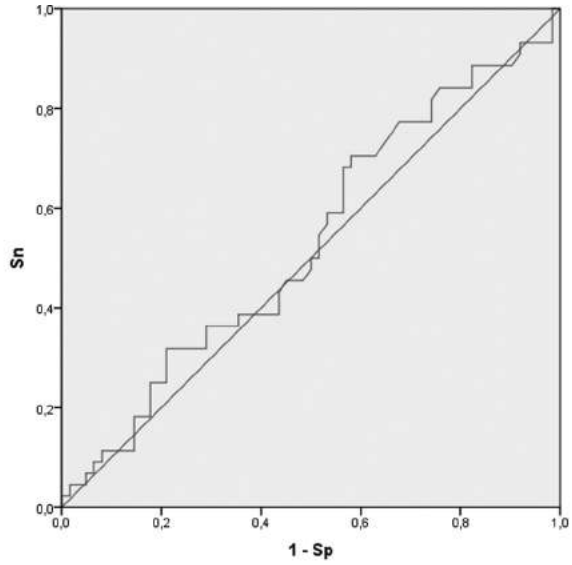
TRAb 1 – na početku bolesti; TRAb 2 – 6 meseci po započinjanju terapije; TRAb 3 – 12 meseci po uvođenju terapije; x osa – ishod/;y osa – U/L; TRAb – antitela na receptore tiroidnog stimulišućeg hormona

According to the statistical analysis of transformed data obtained by t-test for two independent samples, no statistically significant difference was found between the non-responders and the patients in remission regarding TRAb level at the beginning of disease (t=0.450; p=0.654), 6 months after the introduction of therapy (t=0.816; p=0.419) and 12 months after the introduction of therapy (t=1.467; p= 0.146).

The analysis of variances of repeated measures showed a highly statistically significant difference among these three measurements in the whole group of patients (F = 69.264; p< 0.001; Eta2part = 0.596), but no statistically significant difference in the change of TRAb level during the follow-up between the non-responders and the patients in remission (F=0.870; p=0.395; Eta2part = 0.018).

There was a statistically significant difference in TRAB level at the beginning of disease and 12 months after the introduction of therapy in the patients in remission ($p < 0.001$) as well as in the non- responders ($p < 0.001$).

In predicting the therapy outcome, receiver operating charaqcteristic curve (ROC) analysis was used to test TRAb sensitivity and specificity at the beginning of disease, 6 months and 12 months after the introduction of therapy. **Graph 3** shows ROC analysis for TRAb level at the beginning of disease and the therapy outcome. **Graphs 4 and 5** show ROC



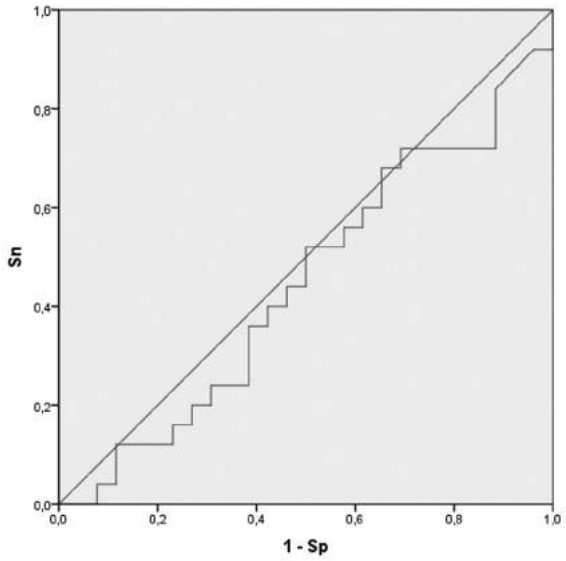
Sn – sensitivity; Sp – specificity

Sn – senzitivnost; Sp – specifičnost

Graph 3. ROC analysis for TRAb level at the beginging (TRAb 1) of disease and thyrosuppressive drug therapy outcome

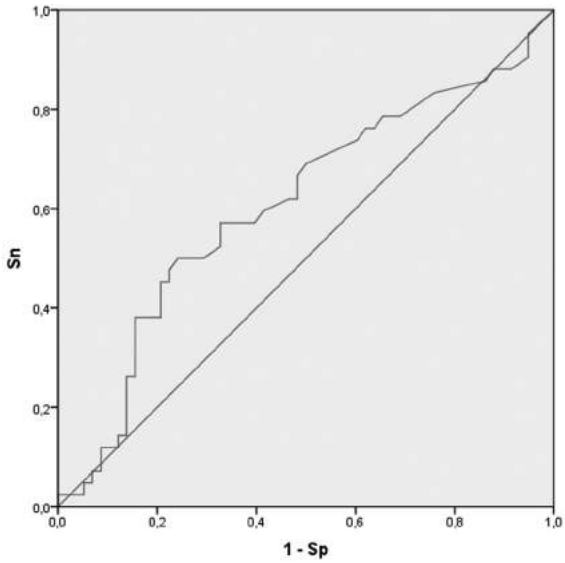
***Grafikon 3**. ROC analiza za nivo TRAb na početku bolesti (TRAb 1) i ishoda medikamentne tirosupresivne terapije*

TRAb – antitela na receptore tiroidnog stimulišućeg hormona



Sn – sensitivity; Sp – specificity
Sn – senzitivnost; Sp – specifičnost

Graph 4. ROC analysis for TRAb level 6 months after the introduction of thyrosuppressive drug therapy (TRAb 2) and therapy outcome
Grafikon 4. *ROC analiza za nivo TRAb 6 meseci po započinjanju medikamentne tirosupresivne terapije (TRAb 2) i ishod terapije*
TRAb – antitela na receptore tiroidnog stimulišućeg hormona



Sn – sensitivity; Sp – specificity
Sn – senzitivnost; Sp – specifičnost

Graph 5. ROC analysis for TRAb level 12 months after the introduction of thyrosuppressive drug therapy (TRAb 3) and therapy outcome
Grafikon 5. *ROC analiza za nivo TRAb 12 meseci po započinjanju medikamentne tirosupresivne terapije (TRAb 3) i ishod terapije*
TRAb – antitela na receptore tiroidnog stimulišućeg hormona

analysis for TRAb level 6 months after the introduction of therapy and the therapy outcome, and TRAb level 12 months after the introduction of therapy and the therapy outcome. It is obvious that the surface under the curve in ROC analysis for TRAb level at the beginning of disease and 6 months after the introduction of therapy was small, and that TRAb level at the beginning of disease and 6 months after the introduction of therapy did not have any significance in predicting the outcome of therapy. The surface under the curve was small (0.532; p = 0.577 and 0.446; p = 0.510 respectivly.

However, ROC analysis for TRAb level 12 months after the introduction of therapy and the therapy outcome showed that the surface under the curve was larger and p value was on the edge of significance.

The surface under the curve was a little bit larger than in previous analyses (0.607) and p value was near the conventional level of significance (p = 0.068).

Discussion

According to the therapy outcome, the our respondents are divided into those who achieved remission and non–responders (41.5%). The rate of remission ranges from 14 to 80% according to the published data [1, 2, 5, 6, 8–15]. A great number of studies show that at least 30 – 60% of the patients with GD relapse into disease within two years after MTT discontinuation; however, early prognostic parameters that would indicate long–term remission have not been determined so far [11–15].

The study published by Peixoto et al. revealed that a high initial TRAb level was associated with a reduced rate of remission in 46 patients with GD who were treated with MTT for 12 months and then followed up for 12 months after the discontinuation of drug [16].

In their study, Jonas et al. included 37 patients treated with thiamazole for 12 months to observe clinical and biochemical parameters of thyroid status after 1, 3, 6, 9 and 12 months from the introduction of therapy. The follow-up period after withdrawal of therapy was about 27 months. Out of the total number of patients, 32% had relapse of hyperthyroidism, usually 8 months after with drawal of therapy. It was noticed that the patients with TRAb levels higher than 14 IU/L after 3 months therapy, and those with TRAb levels higher than 8 IU/L after 6 months of therapy had relapse more often than those patients with lower TRAb levels, with sensitivity of 50% and specificity of 92% and 96% respectively. The authors have concluded that TRAb level in the early phase of thyrosuppressive therapy may be helpful in choosing an adequate therapy for GD, opting for more radical treatment with radioiodine or surgical treatment [12].

The median of TRAb level was about 11 IU/L in our patients, the range going from marginally positive to maximal 155 IU/L. During MTT, TRAb level decreased progressively and differed statistically significantly from the levels measured at the beginning of disease and 6 months after the introduction of MTT. Six months after the therapy had been introduced, the median TRAb level was about 6 IU/L, almost half of the initial value. The maximum measured TRAb value was 145.9 IU/L and 155.7 IU/L in the patients in remission and the non- responders, respectively. At the beginning of disease, the median of TRAb level did not differ significantly in the patients in remission and the non-responders, the value being about 11 IU/L. Six months after the introduction of therapy, the median of TRAb did not differ significantly in the patients in remission and the non-responders, the value being about 6 IU/L for both groups. Six months after the introduction of therapy, the maximum measured value was higher in the patients in remission (73.7. IU/L) than in the non-responders (46.2 IU/L).

In a prospective randomized clinical study, 47.7% of 218 patients with GD had a relapse of disease within two years after withdrawal of MTT. The patients who showed positive TRAb after 12 months of therapy had a higher relapse rate than TRAb negative patients [13].

Quadbeck et al. were determining thyroid status in 96 patients four weeks after discontinuation of thyrosuppresive drug therapy. The relapse rate was being assessed throughout the post-therapeutic two-year follow-up period. During the follow-up period, relapse of disease occurred in 49% of patients. Mean TRAb level at the end of therapy in the group of patients with relapse was significantly higher than in those who were in remission. Using cut off value of 1.5 IU/L, both the positive predictive value and the negative predictive value were low, being 49% and 54%, respectively (the specificity being 14%); however, taking 10 IU/L as a cut off values, the positive predicative value and the negative predictive value were improved to 83% and 62%, respectively (the specificity being 92%). Other factors such as age, sex, thyroid volume, smoking and the presence of ophtalmopathy did not have any influence on the relapse rate [11].

Okamoto et al. investigated the therapy outcome in 71 patients. The therapy was discontinued after euthyroid state had been achieved and maintained first during 6 months with a daily methimazol (MMI) dose of 5 mg, or 50 mg of propylthiouracil (PTU) and then during the next 3 months with the same dose but every other day. The follow-up continued one year after the therapy had been discontinued. During the follow–up, 37% of the patients had relapse. Mean TRAb level at the end of therapy in the group with relapse was significantly higher. All patients who had the same TRAb value or higher than 3 IU/L achieved relapse during the follow–up [15].

Contrary to the above mentioned results, a study with 129 patients found the positive predictive value and negative predictive value of only 55% and 62%, respectively for the prediction of relapse when TRAb cut off value was 1.5 IU/ml. The authors concluded that despite high diagnostic sensitivity and specificity of "TRAK human" assay, its predictive value for relapse of hyperthyroidism was not increased when measuring was done at the end of thyrosuppressive drug therapy [17].

One of rarely performed studies on this issue in our country reported high TRAb values in the group of non-responders after the first month of therapy, while the responders had a significantly lower TRAb level. The authors believe that the decision regarding treatment can be reached by obtaining either the positive or negative response to drug therapy, measured by the level of TRAb [18].

One of our earlier studies, which had followed the number of relapses and duration of remission in drug treated patients with GD, revealed that the duration of remission was longer and the relapse rate was lower in the patients with lower initial TRAb level and lower TRAb level at the end of the therapy. The initial TRAb level exceeding 5 IU/L increases the chance for remission shorter than 6 months by 18%, whereas the TRAb level exceeding 15 IU/L at the end of therapy makes this chance higher by 36% [19].

Persistently elevated TRAb values are associated with both a difficult clinical course and a low remission rate of hyperthyroidism, as it has been found in a study conducted by Eckstein et al. The authors have therefore suggested TRAb value cut off to be a prognostic factor during 6, 12 and 18 months of drug therapy as the basis for making an early decision on the final therapy [20].

Apart from the role of TRAb played in predicting the outcome of MTT, some authors emphasize the significance of TRAb in decision making regarding duration of the therapy as well as in the assessment of clinical course of thyroid ophtalmopathy [21]. Discontinuation of thyrosuppressive drug therapy is recommended when the normal concentration of free thyroxine and TSH is achieved and maintained during a certain duration of therapy with 5 mg of methimazole every other day, the so called minimum maintenance dose (MMD). The achieved rate of remission was 86.9% for 6 months, 73.8% for one year and 68.2% for two years after the discontinuation of MTT. The remission rate was higher in the patients with longer duration of MMD therapy, and significantly higher in the patients with MMD therapy for 19 months and longer than in those patients with MMD therapy for 6 months or shorter. The remission rate was also significantly lower in TRAb positive patients than in TRAb negative ones at the time of the discontinuation of therapy. It is recommended not to discontinue thyrosuppressive drug therapy in TRAb positive patients who have been using MMD therapy for 6 months or shorter [22].

Twelve months after the introduction of therapy, the median of TRAb level in our patients was about 2 IU/L, the maximum measured value being about 45 IU/L. Twelve months after the introduction of therapy, the median of TRAb level in the patients in remis-

sion was about 2 IU/L, the maximum measured value being 24.2 IU/L, while in the non-responders the value was twice as high, about 4 IU/L, the maximum measured value being 45.2 IU/L. Although no statistically significant difference was found between the patients in remission and the non-responders regarding TRAb level 12 months after the start of therapy, it is evident that TRAb level in the non-responders remained higher 12 months after the introduction of MTT, that being clinically significant in terms of predicting an adverse reaction to MTT.

The authors from Bern conducted a study which included 94 patients with the first episode of GD treated with MTT. The logistic regression analysis was done in order to determine factors predicting the beginning of remission and the occurrence of relapse. No correlation was observed among the size of goiter at the beginning of disease, TRAb level at the beginning of disease, TRAb level at the end of therapy and the remission rate or relapse [23].

ROC analysis was done at the beginning of disease and 6 months after the introduction of therapy in order to determine the sensitivity and specificity of TRAb for predicting MTT outcome in ou responders and no statistical significance was found. According to the sensitivity and specificity of TRAb for MTT outcome 12 months after the introduction of therapy, p value was close to the conventional level of significance; thus, TRAb level could have a predictive significance for further MTT outcome during a follow-up longer than 12 months after the introduction of therapy.

A small number of patients with autoimmune thyroid disease can experience changes in the types of TSH receptor antibodies, from thyroid stimulating antibody (TSAb) into thyroid blocking antibody (TBAb) and vice versa with the consequent changes in the thyroid function. During thyrosuppressive drug therapy, TSAb level falls causing predominance of TBAb. On the contrary, TSAb development during tyroxine supstitution therapy can be sufficient to annul the inhibitory effect of TBAb. This conversion can be one of the reasons which diminish the importance of TRAb for predicting remission and relapse [2].

Recent research has been focused on discovering drugs such as low molecular weight ligands which, by binding to a transmembrane allosteric pocket of TSH receptors, could block the pathological activation of receptor caused by TSH receptor antibodies [24–26].

Conclusion

Level of antibodies to thyroid-stimulation hromone receptors at the beginning of disease and 12 months after the start of therapy cannot predict the outcome of thyrosuppressive drug therapy. Persistently elevated level of antibodies to thyroid-stimulation hromone receptors for more than 12 months after the introduction of therapy could be a late predictive factor for an adverse outcome of thyrosuppressive drug therapy and could be clinically significant in making an early decision on changing the type of therapy.

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Rad je primljen 14. I 2014.
Recenziran 11. VI 2014.
Prihvaćen za štampu 20. VI 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:305-311.

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REVIEW ARTICLES
PREGLEDNI ČLANCI

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Review article
Pregledni članak
UDK 618.2:[613.2:577.164.1
DOI: 10.2298/MPNS1410313B

THE QUESTION IS WHETHER INTAKE OF FOLIC ACID FROM DIET ALONE DURING PREGNANCY IS SUFFICIENT

PITANJE JE DA LI JE UNOS FOLNE KISELINE SAMO ISHRANOM U TRUDNOĆI DOVOLJAN

Ines BANJARI[1], Vlatka MATOKOVIĆ[1] and Vedrana ŠKORO[2]

Summary

Pregnancy and Folic Acid. Pregnancy is the most important period in life of every woman, partially for the number of physiological adaptations she is going through, partially for the expectance of new life. In addition, pregnancy is the "critical window" for development later in childhood, as a period of foetal programming during which nutrition plays one of crucial roles. Despite the general belief that nutrition through pregnancy is adequate and characterized by better nutritional habits, a number of studies do not corroborate this belief. **Role of Folic Acid.** An adequate folate blood level is necessary for normal cell growth, synthesis of several compounds including deoxyribonucleic acid and ribonucleic acid, proper brain and neurologic functions; it is included in the regulation of homocysteine level, and closely related to the vitamin B_{12} metabolism. Folate deficiency in pregnancy is related to neural tube defects, other neurological disorders, preterm delivery and low birth weight. **Food sources.** A correlation between folate and the prevention of broad spectrum of chronic diseases has been confirmed. Emerging evidence from the epigenetic studies is now bringing even more light on the level of significance of folic acid. A wide range of plant and animal foods are the natural sources of folate; liver, yeast, mushrooms, and green leafy vegetables being the most significant. Different ways of food preparation influence the folate stability and its bioavailability varies from 25 to 50% from foods, 85% from enriched foods or 100% from supplements. **Conclusion**. A great amount of scientific results has led to official recommendations for folic acid supplementation in pregnant women as well as in a number of obligatory or voluntary fortification programmes in order to prevent the folate deficiency on the level of different population groups. Nevertheless, there must be a certain level of precaution for elderly because folate can mask the vitamin B_{12} deficiency with possible fatal outcomes.

Key words: Folic Acid; Pregnancy; Folic Acid Deficiency; Dietary Supplements; Food, Fortified; Food; Biological Availability

Sažetak

Trudnoća i folna kiselina. Trudnoća je najvažniji period u životu svake žene, što zbog fiziloških prilagođavanja kroz koje žena prolazi, što zbog iščekivanja novog života. Ujedno, trudnoća predstavlja „kritičan prozor" u kasnijem razvoju deteta, period fetalnog programiranja u kojem prehrana igra jednu od vodećih uloga. Uprkos prihvaćenom mišljenju kako je ishrana u trudnoći adekvatna i boljeg kvaliteta, sa boljim prehrambenim navikama, rezultati brojnih istraživanja to ne ukazuju. **Uloga folne kiseline.** Adekvatan nivo folne kiseline je neophodan za normalnu ćelijsku deobu, regulaciju sinteze različitih molekula, uključujući dezoksiribonukleinsku i ribonukleinsku kiselinu, za pravilno funkcionisanje mozga i neurološke funkcije, uključena je u regulaciju nivoa homocisteina, te je u uskoj vezi s metabolizmom vitamina B_{12}. Deficit folne kiseline u trudnoći je odgovoran za defekte neuralne cevi fetusa, druge neurološke poremećaje, prevremeni porođaj i malu porođajnu masu. **Izvori folne kiseline.** Potvrđena je i povezanost unosa folata sa prevencijom niza hroničnih bolesti. Rastući broj dokaza iz epigenetičkih istraživanja baca novo svetlo na stvarnu važnost folne kiseline. Folati su prisutni u različitoj biljnoj i hrani životinjskog porekla, a najznačajniji izvori su jetra, kvasac, gljive i zeleno lisnato povrće. Različiti načini pripreme hrane utiču na stabilnost folata, a njihova bioraspoloživost varira 25–50% u hrani, 85% u obogaćenoj hrani ili 100% iz suplemenata. **Zaključak**. Količina nepobitnih naučnih dokaza je rezultirala službenim preporukama suplementacije folnom kiselinom kod trudnica, kao i nizom mandatornih ili dobrovoljnih programa obogaćivanja hrane folnom kiselinom kako bi se prevenirao deficit na nivou različitih populacijskih grupa. Ipak, određena doza opreza je prisutna kada je u pitanju starija populacija jer folna kiselina može maskirati deficit vitamina B_{12} koji u ovoj populaciji može imati fatalan ishod.

Ključne reči: Folna kiselina; Trudnoća; Deficit folne kiseline; Suplementi; Obogaćena hrana; Hrana; Bioraspoloživost

Pregnancy and Folic Acid

Pregnancy is a truly very special condition for every woman. Throughout its duration of 280 days on average (or 40 weeks from the first day of the last menstrual cycle), women experience a number of deep adaptations of cardiovascular system, change in volume of body fluids, respiration, energy metab-

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Abbreviations

DNA – deoxyribonucleic acid
RNA – ribonucleic acid
NTD – neural tube defects
FH4 – tetrahydrofolate
C1 – carbon atom
eNOS – endothelial nitric oxide synthase
DFE – dietary folate equivalent
USA – United States of America

olism and nutrition [1, 2]. Different hormones induce these changes including the growth of uterus as the site of foetal growth and development [1].

For foetal metabolism, glucose is a main source of energy. Vitamin B complex, especially vitamin B_{12} and folic acid are necessary for proper synthesis and maturation of erythrocytes, development of nervous system and growth in general. Vitamin C is important for proper formation of tissues, especially bone matrix and connective tissues; vitamin D is essential for normal bone growth; whereas vitamin E is necessary for normal course of early stage of foetal development, and vitamin K is used by the foetal liver to synthesize the factors II, VII, IX, and X [2]. Due to growing needs of foetus during gestation, the need for nutritional intake of these nutrients increases. As pregnancy advances through trimesters, the daily need for energy increases [3]. From the aspect of vitamins and minerals, main deficiency usually develops for folic acid and iron. Suboptimal intake of both of these micronutrients has been the main focus of interest for pregnancy outcomes, developing foetus and foetal programming [4]. Problems related to iron intake during pregnancy have been discussed thoroughly earlier [5].

The increased need in pregnancy for folate partially reflects the need for blood cells production, growth in uterus tissue and placenta, growth of the foetus itself and increase in blood volume [1, 5, 6]. The need for folate is 5 to 10 times higher in pregnant women, which makes them susceptible to folate deficiency [6]. This results in a lower blood concentration of folate and even megaloblastic anaemia may develop in extreme cases [1, 5]. Maximal amount of folate is needed in the last trimester for the extensive growth of foetus and because foetus is stocking up folate stores [6]. In addition, haemodilution in the later stages of gestation and the way it modulates blood folate should be taken into consideration [1, 2, 7]. Fekete et al. [6] have confirmed that the increased intake of folate, even after the first trimester of pregnancy, is related to higher birth mass and lower risk for delivering low birth weight infant. Birth weight is one of the most important factors in infant death within the first 12 months of age. On the other hand, birth weight is a crude outcome measure of complex processes and is influenced not only by the nutrient status of the mother but also by a number of other factors, such as mother's anthropometry, the length of gestation, sex of the baby etc. [7].

Prolonged folate deficiency in pregnancy causes neural tube defects (NTDs) in a developing foetus. NTDs are congenital malformations leading either to death in case of anencephaly or disability for life in case of spina bifida [1, 5, 8]. Neural tube is a foetal structure from which the brain and spinal cord with nerves and their membranes develop [2]. The neural tube closes on the 24th day from conception or around that time [2], that being long before the woman even realizes she is pregnant. This is the reason why prophylaxis is recommended to all women of reproductive age, i.e. pregnancy planning should be the definitive aim.

Folic Acid

Folic acid or vitamin B_9 is one of eight vitamins of B complex. It is a water-soluble vitamin, isolated from spinach for the first time in 1940s. Its name originates from the Latin word *folium* meaning leaf, because of its high abundance in green leafy vegetables [9].

Folate or folacin is a generic name for folic acid. Folic acid (pteroylmonoglutamic acid) is the highest oxidized form and the most stable form used in supplements and for food fortification. The majority of folate to be found in nature are termed dietary folic acid, i.e. dietary folate, and they represent different derivates of glutamic acid (pteroylpolyglutamates), which have one joint name "folate" [8–10]. A subgroup of folate, 5,6,7,8-tetrahydrofolate (FH_4) has the highest biological importance. It acts as a coenzyme and an intermediate transporter of groups with one carbon atom (C1), e.g. methenyl and methylene groups [10].

Role of Folate in Organism

The function of folate in the organism is closely related to the function of vitamin B_{12}, other B complex vitamins (B_2 and B_6), and some minerals, iron being the most interesting of them from the aspect of pregnancy, because of their interrelation in the physiology of pregnancy [1, 4, 5, 11, 12]. In addition to the above-mentioned role in the neural tube closure and coenzyme activity in C1 metabolism, folic acid is a growth factor and it is also necessary for the synthesis of other compounds. Among these are deoxyibonucleic acid (DNA) and ribonucleic acid (RNA) synthesis and maintenance, cell division, and the regulation of homocystein levels [8, 9, 11]. The magnitude of the interrelation between folic acid and other vitamins of B complex, its importance in C1 metabolism and DNA methylation have been studied extensively in epigenetic studies (see the text below) [7, 13, 14]. **Table 1** shows some of the neurological and psychiatric disorders associated with folate deficiency [11].

From the aspect of public health significance, the involvement of folic acid in homocysteine levels is the most interesting one. An increased homo-

Table 1. Some of non-haematological manifestations of folic acid deficiency [11]
Tabela 1. *Neke od ne-hematoloških manifestacija nedostatka folne kiseline [11]*

Deficit consequences *Posledice deficita*	Deficit manifestations *Manifestacije deficita*
Neurological *Neurološke*	Depression, psychosis, peripheral neuropathy, subacute combined spinal cord degeneration, neural tube defects/*Depresija, psihoza, periferna neuropatija, subakutna kombinovana degeneracija kičmene moždine, defekti nervne cevi*
Metabolic *Metaboličke*	Increase of homocysteine and other metabolites levels *Povećanje nivoa homocisteina i drugih metabolita*
Cardiovascular *Kardiovaskularne*	Cardiovascular, peripheral vascular and cerebrovascular diseases with atherosclerosis and thrombosis/*Kardiovaskularne, periferne vaskularne i cerebrovaskularne bolesti sa aterosklerozom i trombozom*
Neoplastic *Neoplastične*	Preneoplastic alterations of cervix and uterus epithelium and gastrointestinal and pulmonary epithelium/*Pre-neoplatstične alteracije epitela cerviksa i uterusa i gastrointestinalnog i pulmonalnog epitela*
Developmental *Razvojne*	Congenital abnormality (of neural tube and others) *Kongenitalna anomalija (neuralne cevi i druge)*
Epithelial/*Epitelne*	Megaloblastic alterations/*Megaloblastne alteracije*

cysteine level resulting from vitamin B_{12} and folate deficiency is a significant risk factor for cardiovascular diseases [11]. Cardiovascular diseases are the number one death cause around the globe; Croatia, the Republic of Serbia and Montenegro are no exception [15]. Besides regulating homocysteine level, folic acid acts as a cofactor for endothelial nitric oxide synthase (eNOS) leading to improved vasodilatation and end-organ perfusion [14]. Additionally, the increased level of homocysteine in plasma can be embriotoxic. Homocysteine level shows a correlation with bone density; disturbances in the folate-homocysteine-methionine axis could be the basis of pathologies related to the loss of normal organization and strength of skeletal tissue [14], thus presenting a risk factor for osteoporotic bone fractures. Heppe et al. [16] have shown that an increased concentration of homocysteine in the mother in the first trimester of pregnancy shows a correlation to the lower mineral content and bone density in her child.

A significant number of studies have suggested that folic acid can prevent other congenital anomalies and low birth weight among infants, together with some chronic diseases such as cardiovascular diseases, stroke, different types of cancer, depression, dementia and osteoporosis [7–9, 14, 17, 18]. On the other hand, recent studies have found no effect of folic acid on the prevention of some of important chronic diseases, such as cardiovascular diseases or cancer [19, 20].

From the aspect of pregnancy, the main role of folic acid is observed through NTDs. The main risk factors are previous pregnancy with a neural tube defect, inadequate folate intake by the mother, diabetes, some drugs (which interfere with folate metabolism), obesity and inadequate intake of vitamin B_{12}. Several studies have confirmed an undoubted correlation between the consumption of folic acid supplements before conception and a lower risk for the development of neural tube defects [9, 21]. Intake of folic acid at the time of conception of 4 mg per day prevents the development of neural tube defects in women with the previous history of a defect by 72% [5, 11]. These results have led to the official recommendations for women of reproductive age: intake of folic acid in dose of 400 μg per day during pregnancy planning, 600 to 800 μg per day during pregnancy, and 4 mg per day for the prevention of recurrent defect [5, 8, 21–23]. The World Health Organization recommends supplementation with folic acid before conception until the 12th week of pregnancy [24]. A randomized prospective longitudinal observational study on pregnant women from Eastern Croatia has shown that women rarely use folic acid before pregnancy; however, as many as 81.1% of them take folic acid supplements from the time when the pregnancy was confirmed until the 12th week of gestation. Out of them, 59.9% of women continue to take supplements containing folic acid throughout gestation [12]. These data are in accordance with the research conducted by Vandevijvere et al. showing that less than one pregnant woman out of two take folic acid before pregnancy. The authors have shown that 39% of pregnant women from Belgium do not have adequate level of folate in the first trimester to prevent neural tube defects [25]. Vitale et al. conducted research on a group of parturient women from Zadar County and showed that although 75.2% of women consumed folic acid or multivitamin supplement containing folic acid, only 16.5% of these women took folic acid supplement before conception [22].

Folic Acid from the Aspect of Epigenetics

In order to be able to comprehend the overall complex role of folic acid in our organism we need to think „on top of genetics“. For the last 20 years or so, compiling evidence has been supporting the thesis on nutrition altering our epigenome. The un-

derlying changes on epigenome include a group of modifications to chromatin structure that do not alter the DNA sequence, but confer the transcriptional regulation over a range of timescales. The major epigenetic processes include DNA methylation, histone modification and non-coding RNAs. So far, most studies on the interaction between nutrition and epigenetic regulation of genes have been focused on DNA methylation although there are emerging findings about other epigenetic marks [26]. Even though the majority of existing evidence goes towards the correlation between maternal diet and offspring, paternal influence should not be neglected, especially when it comes to environmental factors such as smoking [27].

Maternal diet deficient in macronutrients during pregnancy affects metabolism of a growing foetus. This has been extensively reported both in animal models and humans, especially for protein restricted diets [13, 17, 26] and high-fat diets [26]. Probably one of the most famous studies showing the correlation between dietary restriction, the duration of such restriction and the impact on offspring's development is the Dutch famine cohort [28]. It should be noted that macronutrient and energy deficient diet during pregnancy is accompanied by the micronutrient deficiency [4, 12, 29]. As earlier mentioned, iron and folic acid (together with other B complex vitamins) have been put forward as the most important nutrients in the foetal programming. The role of iron is extensively covered elsewhere [27, 30, 31]. Attention is mainly oriented towards the DNA methylation reactions [26], bringing more attention on folic acid from the aspect of epigenetics.

Speaking of folic acid deficiency, low dietary intake of folate or genetic polymorphism alters the folate metabolism leading to the impaired purine and pyrimidine metabolism, DNA synthesis and/or cell proliferation; and affect foetal growth and result in teratogenesis and congenital malformations [7, 27]. Studies on rodents have shown that a low-protein diet supplemented with folic acid improves offspring's outcomes [13, 17]. In other words, marginally low protein intake and micronutrient insufficiency induce perturbations in C1 metabolism, which is the primary contributor to impaired foetal growth and associated long term consequences in the offspring [7, 27]. Nevertheless, findings are inconsistent. Several studies showed a positive correlation between the red blood cell or plasma folate levels and infant birth weight, while others did not find any relation in a well-nourished population of pregnant women between dietary folate, and plasma folate and the size of an infant at birth [7].

Elevated levels of homocysteine during pregnancy are associated with pregnancy-related disorders such as preeclampsia, early pregnancy losses etc. and the correlation has been found with the homocysteine effect on the vascular endothelial cell function and increased pro-oxidant activity [32]. Interesting epigenetic findings were reported by the Pune Maternal Nutrition study, the cohort study from India [33]. They found that a high level of circulating homocysteine was a predictor of intrauterine growth restriction, and showed a correlation with a low vitamin B_{12} status (folate deficiency in the studied population was rare). At 6 years of age, children born of mothers with high folate status in pregnancy were of higher adiposity and had insulin resistance. The combination of vitamin B_{12} deficiency and high folate status resulted in the most insulin resistant children [33].

Despite a limited number of available studies, transgenerational inheritance of epigenetic modifications induced by the exposure to macro- and micronutrients is getting more attention [27, 34], especially the gender-specific inheritance (Vanhees et al. 2014, Hussain 2012). For example, a study by Dunn and Bale [35] showed that the *in utero* exposure to a high-fat diet resulted in increased body size in third-generation (F3) female offspring, and the interesting part was that the inheritance seemed to be passed by the paternal line.

Cancer has recently gained a lot of attention due to a high increase in the incidence and mortality throughout the world [36], and the fact that the field of epigenetics has been studied largely in the context of tumorogenesis may be even more important [23]. This is especially interesting in cancers with a strikingly high correlation with diet, such as colorectal cancer [36]. There are clear DNA methylation pattern changes in tumors [23, 26, 37]. Folic acid has been suggested as both suppressor for some and promoter for other neoplasias [23]. It has been hypothesized that the early exposure to folic acid might prevent tumors through the provision of enough methyl groups to maintain proper methylation patterns and repair of DNA. In contrast, after the development of tumors, higher intake of folic acid might promote growth of existing tumors [23, 36]. According to some studies on rodents the tumor-suppressor gene *p53* seems to be less methylated in the intestines of adult mice born from mothers fed a diet low in folate during pregnancy. Importantly, this low folate intake by the mother during pregnancy resulted in global hypomethylation of the adult offspring, which is associated with a higher risk of developing cancer [27]. Still, folic acid is more likely to be taken in excess due to wide supplementation and fortification programmes. It is reasonable to mention dangers of the excess *in utero* exposure. Studies have shown that timing is crucial for epigenetic modulation by folic acid. In other words, a high-dose folic acid supplementation during early pregnancy is associated with increased neurodevelopment, resulting in enhanced vocabulary development, communicational skills and verbal comprehension at 18 months of age [18, 27]. However, the excessive exposure to folic acid during late pregnancy seems to have an association with childhood asthma and atopic dermatitis [27, 38]. Vitamin B_{12} in relation to folic acid shows the

potential in modulating epigenetic marks related to cancer, as well as for NTDs, impaired neurodevelopment, and insulin resistance [7, 33, 37].

Needs and Recommendations for Folate

Folate intake from foods, its metabolism, some drugs, certain life conditions and health status, as well as inter-individual genetic variations and gender differences all influence the level of folate [30]. Besides an inadequate folate nutritional intake, deficiency can develop due to malabsorption. Malabsorption can result from a number of disorders in digestive tract, from inflammatory bowel diseases, irritable bowel syndrome, coeliac disease or carcinoma. Inadequate intake from foods is common in elderly, alcoholics and people of low socioeconomic status. Different liver diseases, together with alcoholism can lead to folate deficiency because they intervene with C1 metabolism. In addition, the liver is the main storage organ for folic acid. As already mentioned, an imbalance between folate intake and increased need, as it happens in pregnancy, during lactation, in preterm infants, but also in haemolitic anaemia, inflammatory diseases and psoriasis, can lead to deficiency. A number of drugs also interfere with the metabolism and absorption of folate [11]. Recommended daily intakes and upper limits for folic acid are given in **Table 2** [39].

Nutritional intake of folate is believed to be the most relevant cause of deficiency; therefore, in order to accomplish the recommended intake, the use of supplements is strongly suggested, i.e. the consumption of foods fortified with folic acid. The majority of studies dealing with folic acid are focused on the folic acid deficiency. However, the question is: "Should we be more concerned with the excessive intake of folic acid?" When we observe general population, dietary habits seem to be unfavourable, suggesting the excessive intake of a number of macro and micronutrients. The result of such dietary habits is the ever-increasing rate of morbidity and mortality due to obesity, type 2 diabetes mellitus, cardiovascular diseases, and cancers. The questions of under- and over-nutrition elicit persistent attention in scientific community. The argument is especially acute when the use of supplements is on the table. As shown by the latest report on the supplements use among American adults, the prevalence range fluctuated from 64% to 69% in the period from 2007 to 2011 and the percent of regular users increased from 28% to 36% [40]. Multivitamins are the most commonly used supplements. For 58% of the study respondents, the main reason for supplement use are overall health and wellness, while for 42% of them it is the will to fill nutrient gaps in the diet [40]. These findings highlight one important fact. Despite the number of strong evidence from recent studies [19, 20], general public considers supplements almost as a crucial, key component of a daily diet, such as proteins, fats or folic acid.

In spite of the general belief that nutrition during pregnancy improves [41], compiling data show inadequate intake of energy as well as a wide range of nutrients throughout pregnancy [29, 42]. Nutrition of pregnant women is considered to be the most important external, environmental factor affecting the growth and development of foetus [43]. These external, environmental factors determine the final outcome of pregnancy and infant's birth weight by even 30% [1]. Some studies have shown that primaparae are highly motivated to change their nutritional habits for better during pregnancy [42, 44]. On the other hand, younger women, with lower education, low income, from rural areas, with more children and higher body mass index have nutrition of worse quality during pregnancy [42, 44]. Banjari et al. [29] have found that nutrition quality of pregnant women from Eastern Croatia shows the intake well below recommendations for most macro and micronutrients. In their randomized observational research on pregnant women from Czech Republic, Hronek et al. have shown that they have low intake of dietary folate, representing about 40% of the recommended daily intake [45].

Table 2. Daily need for intake of folic acid (μg/day) [39]
Tabela 2. *Dnevna potreba za unos folne kiseline (μg/day) [39]*

Age *Starost*	Infants (RDI) *Odojče*	Infants (UL) *Odojče*	Adults (RDI) *Odrasli*	Adults (UL) *Odrasli*	Pregnant women (RDI) *Trudnice*	Pregnant women (UL) *Trudnice*	Lactating women (RDI) *Dojilje*	Lactating women (UL) *Dojilje*
0 – 6 months/*meseci*	65	n/a	–	–	–	–	–	–
7 – 12 months/*meseci*	80	n/a	–	–	–	–	–	–
1 – 3 years/*godina*	150	300	–	–	–	–	–	–
4 – 8 years/*godina*	200	400	–	–	–	–	–	–
9 – 13 years/*godina*	300	600	–	–	–	–	–	–
14 – 18 years/*godina*	400	800	–	–	600	800	500	800
> 19 years/*godina*	–	–	400	1000	600	1000	500	1000

RDI – recommended daily intake, UL – upper limit, n/a – not defined
RDI – preporučeni dnevni unos, UL – gornja granica, n/a -nedefinisano

The emerging findings show that epigenetic plasticity may extend beyond the early development and include periods in the life course associated with rapid physiological change such as puberty and aging [26, 46]. In other words, we could modulate our predisposition for certain diseases gained *in utero* by specific nutritional interventions later in life. Gender differences, genotype, target gene, as well as timing of the exposure and the magnitude of the intervention all need to be taken into consideration for such nutritional interventions [26, 30, 34, 46]. A planned, specific supplementation is one of the examples.

Food Sources

Folate can be found in the variety of plant and animal foods. Liver, yeast, mushrooms and green leafy vegetables are the best food sources of folate. Vegetables like asparagus and green leafy vegetables are the excellent source of folate from food. Cereals (fortified breakfast cereals, whole grain products), meat (liver, eggs) and legumes (beans, sunflower seeds), and fruits (oranges, strawberries, melons) are good sources, whereas milk, yoghurt, cheese, fats and oils are considered poor sources of folate [9, 47, 48]. Some of the best food sources of folate are listed in **Table 3**.

Consumption of folate-poor diet is the main cause of folate deficiency. Similarly to vitamin B_{12} deficiency, lack of folate was noticed in poor regions around the world where food availability is restricted [48, 49]. Still, some types of diets have shown to be favourable in terms of folate intake both in pregnancy and in general conditions as well. Timmermans et al. [43] and Sotres-Alvarez et al. [50] have shown that the Mediterranean diet, which includes high intake of vegetables and plant oils, fibres and complex carbohydrates, moderate consumption of fish, poultry and alcohol, low intake of meat and high intake of unsaturated fats, is positively related to different characteristics of foetal growth. The Mediterranean diet also correlates to higher folate intake, as shown by Monteagudo et al. Their research included three different age groups of women from Spain and based on the Mediterranean Diet Score they have shown that women with a diet that shows a higher compliance to the Mediterranean dietary pattern have higher folate intake [51]. It is important to note that B complex vitamins, including folic acid, show better effectiveness with the Mediterranean diet because of the lower consumption of saturated fats, trans-fats and cholesterol [43, 50].

Folate Stability and Bioavailability

The majority of nutritional folate is easily oxidized and therefore they are not stable towards oxidation and aerobic conditions during storage and processing. Under these conditions (especially with additional presence of heat, light and/or metal ions), the physiologically inactive components can be formed due to complete or partial oxidation. In the presence of light, vitamin B_2 catalyses these reactions [9, 47].

Food preparation can significantly affect availability of food folate. Cooking can reduce the content folate by 50%, because it stays in water (overall loss of folate varies from 22% for asparagus to 84% for cauliflower). In addition, extreme pH leads to folate loss during food preparation [21, 47].

Bioavailability of food folate depends on numerous factors, including conditions of deconjugation in bowel, instability of several labile forms during digestion, cell structure of food itself and presence of specific compounds that can improve the stability of folate during digestion. Bioavailability of dietary folic acid is 25 to 50%, 100% from folic acid supple-

Table 3. The best food sources of folic acid [39]
Tabela 3. *Najbolji izvori folne kiseline u hrani [39]*

The best food sources/*Najbolji izvori u hrani*	Average amount (μg/100 g of food)/*Prosečan iznos*
Liver (raw)/*Jetra (sirova)*	141
Liver (cooked)/*Jetra (kuvana)*	1070
Tuna (canned)/*Tunjevina (konzervisana)*	15
Orange juice/*Sok od narandže*	220
Oranges/*Narandže*	24
Bananas/*Banane*	28
Broccoli (raw)/*Brokoli (sirov)*	169
Broccoli (cooked, without water)/*Brokoli (kuvan bez vode)*	65
Egg yolk (hard-boiled)/*Žumance (tvrdo kuvano)*	140
Bread (wholemeal)/*Hleb (integralni)*	54
Tomato (canned)/*Paradajz (konzervisan)*	43
Cabbage (raw)/*Kupus (sirov)*	30
Milk (fresh, cow's)/*Mleko (sveže, kravlje)*	5 – 12
Cheddar cheese/*Sir tvrdi*	20

ments (if taken on an empty stomach), and almost 85% from fortified foods [48]. This disproportion between the consumption and bioavailability can be more easily estimated via the so called dietary folate equivalent (DFE). DFE is adjusted with 50% worse use of folate from natural sources (foods) and synthetic folic acid from supplements and fortified foods (e.g. 1 μg of dietary folic acid = 0.6 μg of folic acid from fortified foods or supplements taken with foods; 1 μg of natural dietary folic acid = 0.5 μg of folic acid from supplements taken on an empty stomach). In other words, 1 μg of DFE equals to 1 μg of dietary folic acid, i.e. 0.6 μg of folic acid from fortified foods, i.e. 0.5 μg of folic acid from supplements [48].

Folic Acid Supplements and Food Fortification

In Europe, more than 40% of all pregnancies are not planned; therefore, prophylactic intake of folic acid is crucial for the successful prevention of neural tube defect. Socioeconomic conditions substantially influence availability of foods that are a significant source of folate. This kicked up food fortification programmes in foods widely consumed [8, 22, 23], so a large number of countries have introduced obligatory (mandatory) measures of food fortification with folic acid [8, 23, 52]. Mandatory fortification for cereal products such as wheat flour and bread is effective in 72 countries around the world, in both low- and high-income countries [53]. Croatia has no such programme in effect, but several years ago, wheat flower enriched with folic acid, some other B complex vitamins and iron was introduced into the market [4].

Studies have shown that not even general population has the recommended daily intake of 400 μg in most of the countries where fortification is not obligatory [8, 21, 22, 27]. Despite these findings, the main reason for controversy about fortification of foods widely consumed comes from the fact that folate, due to its interrelation to vitamin B_{12} metabolism, can mask deficiency of the latter. This is of special concern in elderly, who may develop severe neurological disorders caused by vitamin B_{12} deficiency [8, 21, 23, 54]. For example, the amount of folic acid used to fortify foods in the United States of America (USA) and Canada is balanced in a way that no one consumes more than 1 mg per day, that being the upper limit for daily intake. Still, a large part of scientific circles emphasize that this may not be the case in practice; the risk of potentially excessive intake does exist [21, 23, 30, 54].

National directives on food fortification intended for wide public consumption still differ significantly among the countries of the European Union. Voluntary fortification was implemented for several years in some member countries (the United Kingdom, Ireland, Spain, Portugal and Austria) as well as in Switzerland. In other member countries (Denmark, Finland and Sweden), voluntary fortification is restricted or forbidden. In Ireland, after stopping fortification of breakfast cereals, folate level in erythrocytes decreased by 111 nmol/L during 12 weeks, thus impressively showing what may be the consequences of such measures [8, 22, 23, 47].

The amount of folic acid used for fortification in the USA and Canada is 140 μg on 100 g of grain of cereal, assuming that this will lead to an increase in folate intake by approximately 100 μg a day in women of reproductive age. Fortification with folic acid significantly improved folate status in the USA. Data from the National Health and Nutritional Examination Surveys which had compared year 1999 with period from 1988 to 1994 showed an increase in the mean concentration of folate in serum of women of reproductive age, who did not take folic acid supplements, from $10x10^{-7}$ nmol/L on $28x10^{-6}$ nmol/L [8, 55].

Emphasis should also be put on the findings that the consumption of folic acid supplements can improve iron status significantly in pregnancy, thus partially compensating the lack of iron through gestation [56], and diminishing a possible adverse impact of iron deficiency anaemia on pregnancy outcomes [4, 12]. Christian et al. conducted a controlled intervention study on pregnant women from Nepal and they have shown that folic acid (in form of a supplement) in combination with iron increases haemoglobin level and reduces anaemia by 54%. On the other hand, the combination of folic acid, zinc, and iron resulted in 48% reduction, while the combination of folic acid, zinc, iron and additional 11 micronutrients led to a 36% reduction in anaemia. The same effect was found for food fortification. Ganji and Kafai [52] performed an analysis on the population level for the USA after the folic acid food fortification had been implemented. They found a significant decrease in the prevalence of iron deficiency anaemia in the periods from 1988 to 1994 and from 1999 to 2004, especially among women [57].

According to Mallard and Houghton [58], food fortification with folic acid is desirable because all social groups could achieve adequate intake of folic acid in that way. Studies have shown that women who are aware of the importance of folic acid before their pregnancy are more likely to use folic acid supplements. All the above mentioned strongly suggest the great necessity of education on this issue, which should be directed towards young women of lower educational background, lower income, primaparae and with unhealthy habits. In addition, women who happen to be single during their pregnancy or those with unplanned pregnancy should be encompassed as well [25]. All these women have been shown to have an inadequate folate status during pregnancy, inadequate intake of foods rich in folate, enriched foods or folic acid supplements [22, 25, 41, 45, 51, 53].

Conclusion

When observing pregnancy as the „critical window“ in foetal programming, or in other words as a milestone in setting up basis for a healthy life, additional intake of folic acid from supplements should

be a standard. Still, more effort should be put into the promotion of prophylactic use of folic acid by education or intervention programmes on the national level, which would eventually encourage women to plan their pregnancy. The final outcome of such politics would result in healthier population. On the other hand, if a person has no increased demand for folic acid, diet abundant with fresh fruits and vegetables can satisfy the need for folate. However, considering the aspect of fortified foods and the trend of wide supplement use among the general public, there is a level of caution. Demographic indicators on age of the population show a growing proportion of the population over 65 years of age and they are especially susceptible to the possible masking of vitamin B_{12} deficiency due to intake of folate enriched foods and/or folic acid from supplements.

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Rad je primljen 21. I 2014.
Recenziran 18. VI 2014.
Prihvaćen za štampu 21. VII 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:313-321.

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PROFESSIONAL ARTICLES
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University of Novi Sad, Faculty of Medicine[1]
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Professional article
Stručni članak
UDK 616.22-008.5
DOI: 10.2298/MPNS1410323V

ARTICULATION OF SOUNDS IN SERBIAN LANGUAGE IN PATIENTS WHO LEARNED ESOPHAGEAL SPEECH SUCCESSFULLY

ARTIKULACIJA GLASOVA SRPSKOG JEZIKA KOD PACIJENATA KOJI SU USPEŠNO NAUČILI EZOFAGUSNI GOVOR

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Summary

Introduction. Articulation of pronounced sounds during the training and subsequent use of esophageal speech is very important because it contributes significantly to intelligibility and aesthetics of spoken words and sentences, as well as of speech and language itself. The aim of this research was to determine the quality of articulation of sounds of Serbian language by groups of sounds in patients who had learned esophageal speech successfully as well as the effect of age and tooth loss on the quality of articulation. **Material and Methods.** This retrospective - prospective study included 16 patients who had undergone total laryngectomy. Having completed the rehabilitation of speech, these patient used esophageal voice and speech. The quality of articulation was tested by the "Global test of articulation." **Results**. Esophageal speech was rated with grade 5, 4 and 3 in 62.5%, 31.3% and one patient, respectively. Serbian was the native language of all the patients. The study included 30 sounds of Serbian language in 16 subjects (480 total sounds). Only two patients (12.5%) articulated all sounds properly, whereas 87.5% of them had incorrect articulation. **Conclusion**. The articulation of affricates and fricatives, especially sound /h/ from the group of the fricatives, was found to be the worst in the patients who had successfully mastered esophageal speech. The age and the tooth loss of patients who have mastered esophageal speech do not affect the articulation of sounds in Serbian language.

Key words: Speech Articulation Tests; Language; Speech, Esophageal; Age Factors; Tooth Loss; Articulation Disorders; Laryngectomy; Serbia

Sažetak

Uvod. Artikulacija izgovorenih glasova tokom edukacije i kasnije upotrebe ezofagusnog govora veoma je značajna jer znatno doprinosi razumljivosti i estetici izgovorenih reči i rečenica, odnosno govora i jezika. Cilj ovog istraživanja jeste da se ispita kakva je artikulacija glasova srpskog jezika po grupama glasova kod pacijenata koji su uspešno naučili ezofagusni govor kao i da li životno doba i gubitak zuba mogu uticati na kvalitet artikulacije. **Materijal i metode.** Ovo istraživanje je bilo retrospektivno-prospektivna studija koja je obuhvatila 16 pacijenata, kojima je urađena totalna laringektomija. Ovi pacijenti su završili rehabilitaciju govora i koriste se ezofagusnim glasom i govorom. Kvalitet artikulacije je bio ispitivan globalnim artikulacionim testom. **Rezultati.** Kod 62,5% pacijenata ezofagusni govor je ocenjen ocenom 5; ocenu 4 dobilo je 31,3%; a ocenom 3 ocenjen je govor jednog pacijenta. Svim pacijentima je maternji jezik bio srpski. Ispitivanje je obuhvatilo 30 glasova srpskog jezika kod 16 ispitanika (ukupno 480 glasova). Samo dva ispitanika, odnosno 12,5% imalo je pravilnu artikulaciju svih glasova, a ostalih 87,5% nepravilnu artikulaciju. **Zaključak.** Kod pacijenata koji su uspešno savladali ezofagusni govor, najlošija je artikualcija afrikata i frikativa a posebno glasa h koji je iz grupe frikativa. Životno doba kao i nedostatak zuba pacijenata koji su savladali ezofagusni govor ne utiču na kvalitet artikulacije glasova srpskog jezika.

Ključne reči: Testovi artikulacije govora; Jezik; Ezofagealni govor; Faktori godina; Gubitak zuba; Poremećaji artikulacije; Laringektomija; Srbija

Introduction

The quality of esophageal speech is assessed by rehabilitators, patients themselves and patients' interlocutors. There are still no uniform criteria for the estimated effectiveness of education, but the researchers themselves decide on what criteria will evaluate esophageal speech of patients [1].

Stanković [2] suggests the following five characteristics of esophageal speech to be evaluated: general impression of voice quality, roughness of voice, clarity, weakness and vocal strain. These characteristics can be evaluated descriptively as excellent, good, average, poor and very poor.

The general impression of the quality of esophageal speech is evaluated as excellent -when esopha-

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geal speech is fully and automatically produced; good - when esophageal speech is continuously produced but some syllables are occasionally voiceless; medium - when esophageal production is achieved, but without much continuity; weak - when esophageal speech is produced but in form of short, simple sentences; very bad - there is a production of esophageal speech just in the form of some words of two or multiple syllables.

The articulation of spoken sounds during training and later use of esophageal speech is very important. During the process of education, special attention is paid to articulation because it contributes significantly to the intelligibility and the aesthetics of the spoken words and sentences, as well as speech and language.

Articulation can be defined as the pronunciation or forming of sounds. Good articulation implies pure, clear and understandable pronunciation of all sounds in words. Articulation of sounds is composed of three main factors: 1. the quality of the spoken sound; 2. the position which speech organs occupy during the pronunciation of certain sound; 3. the ability to perceive pathological sound and its differentiation from other sounds [3]. Articulation division of speech sounds can be made by the place of articulation, which is determined by the position of speech organs, their relationship or touch. Thus, sounds can be divided into bilabials, labiodentals, dentals, alveolars, palatals, mediopalatals, and velars. According to the manner of articulation, which includes the type of movement and degree of opening or closing the speech organs, the sounds are divided into vowels (when the airflow passes freely, without obstacles) and consonants (when there is an obstacle, the narrowing of air column or redistribution between the nose and the mouth). According to their acoustic characteristics, they can be divided into voiced and voiceless sounds.

The articulation disorders represent an irregularity or failure of saying one or more of the sounds. The basic division of articulation disorders include omission of individual sounds, replacing certain sounds with different sounds (substitutions) and incorrect pronunciation of certain sounds (distortion). The causes of articulation disorders can be divided into organic and functional. The organic causes include congenital anomalies, deviations in the structure of the speech apparatus and hearing damage. Functional causes include inorganic or unknown causes [3].

The aim of this study was to examine the articulation of sounds of Serbian language by groups of sounds in patients who had successfully learned esophageal speech and whether age and tooth loss could affect the quality of articulation.

Materials and Methods

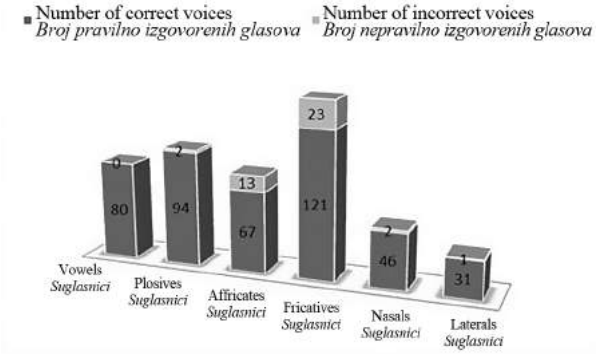
This retrospective - prospective study included 16 patients of the Department of Ear, Nose and Throat Diseases, Clinical Center of Vojvodina in Novi Sad, who, after having been diagnosed with a malignant tumor of the larynx, underwent total laryngectomy. These patients completed the rehabilitation and now they use esophageal voice and speech. When interviewed, the patients provided data on their age, sex, and the presence of teeth, the time when rehabilitation started, and its approximate length. The quality of articulation was examined by a competent speech therapist with years of experience in teaching esophageal speech using "Global test of articulation" which consists of giving scores for each sounds. The articulated sounds were rated with grades 1, 2, 3 depending on the level of their quality. Marginal sounds are those which are slightly devoiced or nasal and they get the grade 4, damaged sounds are rated 5 and 6 and missing sounds are rated 7. The software package Microsoft Excel 2003 was used for statistical analysis of data.

Results

The study sample included 15 men (93.8%) and one woman (6.3%) who had undergone total laryngectomy, who did not have local recurrence of the tumor and successfully completed training in esophageal voice and speech. Most patients belonged to two age groups: 51-60 years and 61-70 years, 37.5% of patients being in either of them. Rehabilitation of speech was introduced in 50%, 12.5% and 37.5% of patients 2 to 4 months after surgery, one month after surgery, and more than 6 months after the operation, respectively. Esophageal speech was learnt by 62.5% in one month, by 31.3% from 1 to 3 months and by only one patient in the period longer than 6 months.

The success of learning esophageal speech was evaluated by Stanković with grade 5 (excellent), 4 (good), 3 (moderate), 2 (weak), 1 (very bad). In 62.5% of patients, esophageal speech was rated as excellent (5), in 31.3%, as good (4), and as medium (3) in one patient. None of the patients received the grades 2 (weak) or 1 (very bad). Of all patients, 56.3% had all the teeth, there was a partial loss of teeth in 31.3% of patients, and 12.4% of the patients were edentulous. The Serbian language was the native language for all patients. In order to quantify the results of global articulation test as a continuous dependent variable, each correctly articulated sound was marked by coefficient 4, the border one by coefficient 3, the damaged and severely damaged ones by coefficients 2 and 1, respectively, and a missing sound by coefficient 0. Thus, the patient who articulated all sounds correctly was scored 120 on the "Global test of articulation". The study included 30 sounds in Serbian language in 16 patients (480 sounds in total).

Of the 16 subjects tested, only 2 respondents, or 12.50%, had the proper articulation of all sounds, while the remaining 87.50% had incorrect articulation.



Graph 1. The number of irregular voices by groups
Grafikon 1. *Broj nepravilnih glasova po grupama*

In the Serbian language, the group of fricatives includes the highest number of sounds, i.e. 9 (/f/, /v/, /s/, /z/, /š/, /ž/, /h/, /j/, /r/), whereas other groups include from 2 to 6 sounds. Of 14 patients with abnormal articulation of sounds, some articulated sounds incorrectly from only one or more groups of sounds. The group of fricatives was articulated incorrectly by 42.86% of patients and 21.43% of the patients articulated incorrectly both affricates (/c/, /ć/, /đ/, /č/, /dž/).

Incorrect articulation of only affricates; plosives and fricatives; plosives, affricates and fricatives; affricates, fricatives and laterals (/l/,/lj/); affricates, fricatives and devoicing of nasals /m/, /n/, /nj/) were recorded in one patient only for each of these groups of sounds. As for the type of sounds, fricatives took the first place among the incorrectly articulated sounds, then affricates, nasals and plosives, and laterals. None of the vowels was incorrectly articulated.

However, if one takes into account that 144 fricatives, 96 plosives, 80 affricates and 80 vowels, 48 nasals and 32 laterals were examined, it can be seen that the study subjects articulated incorrectly the group of affricates most frequently - 13 sounds (16.25%), then the group of fricatives - 23 sounds (15.97%), 2 nasals (4.17%), one lateral (3.13%) and 2 sounds from the group of plosives (2.08%) **(Graph 1)**. This result shows the percentage of sounds incorrectly articulated in a particular group in relation to the total number of sounds in that same group.

Of 16 patients, 11 patients (68.75%) had incorrect articulation of sound /h/ (/h/ as in the English word husband). The sound /h/ was not articulated at all by 31.25% of them, and the same number of patients articulated it correctly, wheareas the sound /h/ was borderline in 18.75% of patients and severely damaged in 18.75% **(Table 1)**.

Kruskal - Wallis -'s test did not show a statistically significant difference when comparing the results of global articulation test between different age groups. Based on the tested sample there is not enough evidence on which it could be argued that patients of different age vary in terms of regularity of articulation sounds. The same test found that the presence or absence of teeth did not affect the quality of articulation.

Discussion

In the present study, the ratio of male and female was 93.8% : 6.3%, showing that the incidence was in more frequent in men than in women. A similar relationship was also found in the research of other authors, Rosso et al. [5] - 91.5% : 8.5% in favor of men, Igissinov [6] 91.3% : 8.7% also in favor of men, Dragičević [7] 88.9% : 11.1%, again in favor of men. In his study [2], Stanković reported a significantly lower number of laryngectomized women than men, the ratio being 1:20; while Jović et al. [8] found that the ratio of men and women suffering from cancer of the larynx was 11.2 : 1. In the study sample, the most represented age group was the one from 51 to 70 years (75%), that being in line with the research of Dragičević [7], which shows that most of the patients were aged from 51 to 70 years, as well as with the study of Stanković [2] in which 70% of patients with total laryngectomy were aged from 51 to 70 years. Weisman et al. [9] state that in most countries the disease occurs between 55 and 65 years of age, and in the Scandinavian countries disease occurs later in life, for example in Norway between 70-74 yearsvof age. In the research of Jović et al. [8], the peak incidence both in men and women is between 65 and 69 years. In the present study, as many as 62.5% of patients had an excellent esophageal speech which is significantly higher than in the study of Sokal et al. [10], where only 20% of the patients mastered esophageal speech, 46.67% of patients were rated good, 13.33% of patients were rated sufficient and 20% of them used a whisper. Dragičević [7] reported in his research that 66.7% of patients mastered esophageal speech successfully and they were assessed with grades 5, 4, 3.

The results of global articulation test showed that the patients had the greatest problem in the articula-

Table 1. Quality of articulation of sound /h/ (/h/ as in the English word husband)
Tabela 1. *Kvalitet artikulacije glasa /h/ (/h/ kao u Engleskoj reči „husband")*

Mark for sound h/*Ocena za izgovor glasa /h/*	N	%
Properly/*pravilna*	5	31.25
Border/*granična*	3	18.75
Damaged/*oštećena*	3	18.75
Severely damaged/*jako oštećena*	5	31.25
Missing/*nedostaje*	16	100%

tion of fricatives (56.1%), then affricates (31.71%), while the articulation of vowels was correct by all patients. The biggest problem with the articulation of fricatives and affricates can be explained by the fact the some voiced sounds of these groups (/z/, /ž/, /đ/, /dž/) are produced by vibration of the vocal cords [11], which were removed with total laryngectomy in laryngectomized patients. The study subjects had the biggest problem in the articulation of one sound from group of fricatives and it was the sound /h/, which was incorrectly articulated by as many as 68.75% of patients, while 5 patients (31.25%) did not articulate the sound /h/ at all. Veselinović [1] also found that the sound /h/ was the most difficult sound to be learnt, or not learn at all, because it is a voiced sound, composed of pure noise, and it is articulated in a quite wide space between the back of the tongue and soft palate.

Christensen et al. [12] have used the dynamic palatometric assessment to examine lingua-palatal contact scheme and the duration of pronunciations of sounds /s/ and /z/ in people who use esophageal speech. It has been discovered that the narrowing of the groove, which created by the tongue of laryngectomized patients, is a physiological compensation for reduced air intake in esophageal speech. The average narrowing of the groove created by the tongue is 5-7 mm narrower for the sound /s/ than for the normal pronunciation when air passes through the larynx, pharynx, and all other parts needed for its final shaping. Narrow groove speech was interpreted as a significant articulation maneuver for limited intraoral air intake and affect on the length of duration of fricatives.

Crevier-Buchman [13] recorded the speech of 10 patients after partial supracricoid laryngectomy and recordings were presented to three expert listeners. They observed that the patients articulated the voiced consonants perceived as the production of voiceless consonants. It is believed that this is a direct consequence of the mechanical properties of pseudo-glottis, which is very different from the properties of the vocal cords.

Although no statistically significant results were obtained by comparing the age and the results of global articulation test, it was observed that the results tended to get worse in older patients. This can be attributed to the fact that aging leads to structural changes in general and degenerative changes in the resonant tract. In the elderly, the required volume and the oral cavity increases [14]. Auditory control of speech is worse in the elderly. Tests for functional magnetic resonance imaging show that aging primarily affects the posterior parts of the left upper temporal auditory cortex, whereby the elderly activate the prefrontal and ventral cortical zones compensatory by activating the zones for attention and working memory [15]. It leads to the deterioration of neural control for speech, articulation, and resonance [16]. Very old people have a cognitive impairment, a certain percentage of old people have signs of depression, which may be potentiated with response to malignant disease and the loss of the larynx.

Jokanović et al. [17] state that the teeth are of great importance for the creation of consonants, because sounds such as /c/, /ć/, /č/, /s/, /š/ cannot be properly created without teeth. In addition, these authors state that in the event of loss of teeth, there are a number of abnormalities that interfere with the proper articulation of consonants and normal communication. By comparing the results of global articulation test and the presence of teeth in patients in this study, it was concluded that the quality of articulation in this sample did not depend on the existence of the teeth. Both the patients who have all teeth and those who do not have them at all or have several teeth achieved nearly the same success in articulating sounds of Serbian language as the patients who have successfully mastered esophageal speech. It is possible that orolingual sensitivity and motility show the same compensatory adaptations as in the healthy people who have been edentulous for a longer time.

Conclusion

Cancer of the larynx, total laryngectomy, and subsequent learning of esophageal voice and speech are more common in men than in women in the age group from 51 to 70 years. Esophageal speech was rated excellent in more than half of patients and good in one third of patients. The articulation of affricates and fricatives especially of sound /h/, which is the sound belonging to the group of fricatives, was reported to be worse in the patients who mastered esophageal speech successfully.

Age and edentulousness in patients who have mastered esophageal speech does not affect the quality of articulation of sounds in Serbian language.

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Rad je primljen 24. II 2014.
Recenziran 30. III 2014.
Prihvaćen za štampu 13. V 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:323-327.

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Professional article
Stručni članak
UDK 616.14-005.6-055.2
DOI: 10.2298/MPNS1410328K

CLINICAL CHARACTERISTICS OF FIRST VENOUS THROMBOSIS AMONG WOMEN UNDER AND OVER 45 YEARS OF AGE

KLINIČKE KARAKTERISTIKE PRVE VENSKE TROMBOZE KOD MLADIH ŽENA I ONIH STARIJIH OD 45 GODINA

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Summary

Introduction. Venous thromboembolism is a multifactorial disease defined by multiple interactions between genetic and acquired risk factors. After coronary heart disease and stroke, venous thromboembolism is the most common cause of cardiovascular death and disability. **Material and Methods**. In order to investigate the clinical characteristics of first venous thromboembolism, 447 women younger than 45 and 174 over 45 years of age with confirmed venous thromboembolism, who had been tested for the presence of thrombophilia in the period 1998-2012, were included in the study. **Results**. Proximal deep vein thrombosis occurred most often among young women, while distal deep vein thrombosis was the most frequent in the older group. The most common reported risk for venous thromboembolism observed in 49.8% of the young women was pregnancy and puerperium, while 25.2% of them developed venous thromboembolism without any obvious cause. Among women over the age of 45, venous thromboembolism developed without an obvious cause in 38.5%, while malignant disease was identified as the most important risk factor in 23% of them. Thrombophilia was observed in 48.7% of the young women in comparison to 28.7% of the older ones ($p < 0.0001$). As for venous thromboembolism recurrence, it developed in 26.3% of young women and 17.8% of the older ones ($p = 0.03$). **Conclusion**. Younger women developed more severe forms of thrombosis than the older ones. Inherited risk factor for thrombosis was detected in almost half of all young women, and in every fourth elderly women. With the exception of factor V Leiden mutation, other types of congenital thrombophilia are almost negligible among older women. Therefore, thrombophilia testing in case of first thrombosis is fully justified only in young women.

Key words: Female; Adult; Aged; Venous Thrombosis; Thromboembolism; Signs and Symptoms; Risk Factors; Diagnostic Techniques and Procedures

Sažetak

Uvod. Venski tromboembolizam je multifaktorijalna bolest koja nastaje u interakciji genetskog i stečenog faktora rizika. Nakon bolesti koronarnih krvnih sudova i moždanog udara, najčešći je razlog kardiovaskularne smrti ili onesposobljenosti. **Materijal i metode.** Sa ciljem da se utvrde kliničke krakteristike prvog venskog tromboembolizma, u studiju je uključeno 447 žena mlađih od 45 i 174 žene starije od 45 godina, koje su testirane na prisustvo trombofilije u periodu 1998−2012. godine. **Rezultati.** Proksimalna duboka venska tromboza češće je zastupljena kod mladih žena, dok je distalna učestalija u grupi starijih. Najčešći faktor rizika za trombozu, koji je utvrđen kod 49,8% mladih žena je trudnoća i stanje posle porođaja, dok se kod 25,2% tromboza razvila bez jasno prepoznatljivog faktora rizika. U grupi starijih žena, tromboza nastaje kod 38,5% bez faktora rizika, dok je malignitet kao najznačajniji faktor rizika utvrđen kod 23%. Prisustvo trombofilije zabeleženo je kod 48,7% mladih, odnosno kod 28,7% starijih žena, $p < 0,001$. Razlika se beleži i u odnosu na ponavljane venske tromboze koje su zabeležene kod 26,3% mladih, odnosno kod 17,8% starijih žena, $p = 0,03$. **Zaključak.** Kod mladih žena se razvijaju klinički teže venske tromboze nego kod starijih. Urođeni faktor rizika otkriven je kod skoro polovine mladih ispitanica, odnosno kod svake četvrte starije žene. Sa izuzetkom faktora V Leiden mutacije ostali tipovi urođene trombofilije su gotovo zanemarljivi u grupi starijih žena. Stoga je testiranje na prisustvo trombofilije u slučaju prve tromboze, u potpunosti opravdano samo kod mlađih žena.

Ključne reči: Žensko; Odrasli; Stari; Venska tromboza; Tromboembolije; Znaci i simptomi; Faktori rizika; Dijagnostičke tehnike i procedure

Introduction

Venous thromboembolism (VTE) is a multifactorial disease and a major cause of morbidity and

Acknowledgements: This work was supported by grant 173008 from the Ministry of Education, Science and Technological Development, Serbia.

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Abbreviations

VTE – venous thromboembolism
CT – computed tomography
AT – antithrombin
PS – protein S
PC – protein C
F – factor
PE – pulmonary embolism
CVT – cerebral vein thrombosis
SVT – superficial vein thrombosis
OC – oral contraceptives
RIETE – Computerized Registry of Patients with Venous Thromboembolism
DVT – deep venous thrombosis

mortality, defined by multiple interactions between genetic (e.g. inherited thrombophilia) and acquired (e.g. age, malignant disease, autoimmune diseases or transient e.g. surgical interventions, fractures, trauma, prolonged immobilization) risk factors [1, 2]. After coronary heart disease and stroke, VTE is the most common cause of cardiovascular death and disability [3]. Transient risk factors for VTE are typical for females and render women more exposed than men to the risk of disease during their lifetime. These include oral contraceptive (OC) use, hormone replacement therapy and pregnancy/puerperium [2]. VTE occurs in one in every 1,000 individuals per year [4], the incidence being lower in individuals under 45 years of age (one of every 10,000) [2]. Survival after VTE is worse than expected, particularly for pulmonary embolism, and another important issue is the development of recurrent VTE [5]. Consequently, VTE has a significant impact on the quality of life since almost half of the patients may develop post-thrombotic syndrome and the cost associated with complications are high [6]. Therefore, the epidemiology of VTE related to the contribution of postulated risk factors and their interaction with VTE in the community has important implications for prevention and management of this serious disease.

Our study was aimed at investigating the clinical characteristics of the first thrombotic event among young women and those over the age of 45 in order to explore possible age dependent differences. Given the difference in the incidence of VTE in individuals up to 45 years of age [2], and the fact that the period of childbearing age in women is the same, statistical analyses were carried out related to the age of 45.

Materials and Methods

The study included 621 consecutive women who were referred from primary health care physicians to two thrombosis centers between January 1998 – June 2013, with the history of VTE to have their anticoagulant treatment followed or to be tested for thrombophilia.

All of them were registered and medical records collected at the first admission were used during the statistical analyses. All women had documented VTE (Doppler ultrasound, lung perfusion scan and helical tomography/computed tomography (CT), and they were tested for thrombophilia presence after anticoagulant therapy cessation. VTE was classified as "provoked" when occurring up to 3 months after the exposure to exogenous risk factors, which included surgery, trauma, immobilization for at least 7 days, OC use, pregnancy-postpartum up to 3 months and malignancy. Women with hepatic, renal or systemic disease were excluded from the study in order to minimize the influence of such diseases or treatment related to them on hemostatic parameters (activity of coagulation factors or natural inhibitors).

As for the age distribution, 447 were younger than 45 when first thrombosis occurred, while 174 were older than 45, their mean age being 29.5 years (ranging from 18 to 45 years) for the former group and 64.5 years (ranging from 45 to 75 years) for the latter one.

The laboratory work-up for thrombophilia included the following tests: biological activity of antithrombin (AT), protein C (PC), protein S (PS), presence of activated protein C resistance (APC-R) and lupus anticoagulants (LA) and factor (F)VIII activity. Deficiencies of natural anticoagulants were defined as less than 75%, 69% and 65% of normal activity for antithrombin, PC, and PS, respectively.

Factor VIII:C was measured by 1-stage clotting assay and levels above 150 IU/dL were considered increased. For the detection of thrombophilia, IL tests (Instrumentation Laboratory, Milan, Italy) were used, and analyses were performed on IL Coagulometers ACL 6000 and Elite Pro. Deoxyribonucleic acid (DNA) analyses for FV Leiden and FII G20210A mutations were conducted by polymerase chain reaction (PCR). The anticardiolipin (aCL) antibodies included determination of anticardiolipin and anti-β2glycoprotein-1 antibodies in both class immunoglobulin G (IgG) and immunoglobulin M (IgM) were determined by ELISA assay using Bindazyme Human Anti IgG and IgM (Binding Site, Birmingham, UK).

The following characteristics regarding the personal or family history, medical records and thrombophilia testing were assessed:

1. Age at time of first VTE
2. Type and localization of VTE - deep venous thrombosis (DVT) - distal or proximal, isolated pulmonary embolism (PE), DVT/PE, thrombosis in an unusual site. Distal thrombosis was considered as thrombosis below the trifurcation [7]
3. The presence of additional risk factors (acquired or transient)
4. The frequency of inherited thrombophilia
5. Time for the first recurrent event

Institutional approval for the study was granted by the Research Ethics Committee of the Blood Transfusion Institute of Serbia (REC number: 5063/3) in accordance with internationally accepted ethical standards and each patient signed the informed consent form.

The analyses were performed using MedCalc, Belgium. Differences between the two groups of women regarding the type/localization, risk factors

for the first VTE and time of the first recurrent event were estimated by the Chi square test, Fisher's test and Student's t-test. The probability value $p<0.05$ was taken into consideration to indicate statistical significance.

Results

The distribution of the type of VTE (distal, proximal, PE or thrombosis in unusual sites) was significantly different in the two groups of women. Thus, proximal DVT ($p<0.0001$) and thrombosis in unusual sites, such as the cerebral vein thrombosis (CVT; $p = 0.03$) were more often present in young women. On the contrary, a somewhat higher rate of distal vein thrombosis was observed in the group of older women, but this was not statistically significant, while the incidence of superficial vein thrombosis (SVT) was significantly higher among the older than among the younger women ($p = 0.04$; **Table 1**).

The rate of spontaneous VTE was found to be higher among the older women than in the group of young women ($p = 0.001$).

As for the acquired risk factors, the most important one affecting 49.8% of the young women was pregnancy/puerperium. Among the older women, malignancy was the most frequent risk for VTE. (**Table 1**). Thrombophilia was observed in 48.7% of the young women compared to 28.7% of the older ones ($p< 0.0001$). Concerning the frequency of congenital thrombophilia, statistically significant differences were recorded between the two groups for inhibitor deficiency ($p = 0.0009$), prothrombin G20210A mutation ($p = 0.04$) or combined thrombophilia ($p = 0.0006$). However, the FV Leiden mutation, as an inherited thrombophilic alteration, was equally represented in both groups of women ($p =0.277$; **Table 2**).

In three-quarters of young carriers of thrombophilia, thrombosis occurred during risk situations, such as pregnancy/puerperium or OC use. On the contrary, in older carriers of thrombophilia, VTE occurrence was equally distributed in groups with and without additional risk factors, ($p= 0.006$; **Graph 1**). Regarding the recurrence of VTE, a statistically significant difference was observed between the two groups, as 26.3% of the young women but only 17.8% of the older women developed recurrent VTE, ($p= 0.03$; **Table 3**).

Discussion

Our results showed that younger women developed proximal deep vein thrombosis or thrombosis in unusual sites such as the cerebral vein more

Table 1. Characteristics of study population
Tabela 1. *Karakteristike ispitanika*

	Young women/*Mlade žene* age/*starost* 18-45	Older women/*Starije žene* age/*starost* 45-75	p
Localization/*lokalizacija* n (%)			
Proximal DVT/*Proksimalna DVT**	120 (26.8)	15 (8.6)	< 0.0001
Distal DVT/*Distalna DVT*	159 (35.5)	75 (43.1)	0.06
DVT/*PE**	32 (7.1)	19 (10.9)	0.17
Isolated PE/*Izolovana PE*	35 (7.8)	20 (11.4)	0.198
Superficial/*Površinska*	34 (7.6)	23 (13.2)	0.04
Upper limb/*Ruke*	39 (8.7)	18 (10.9)	0.636
Splanchic/*Splanhička*	11 (2.4)	3 (1.8)	0.766
Sinus venous thrombosis/*Tromboza venskih sinusa*	17 (3.8)	1 (0.6)	0.03
Risk factor/*faktor rizika*			
Without/*Bez*	113 (25.2)	67 (38.5)	0.001
Pregnancy/*Trudnoća*	223 (49.8)	0	NA
Hormonal therapy/*Hormonska terapija*	28 (6.2)	1 (0.5)	NA
Infections/*Infekcija*	15 (3.3)	5 (2.8)	0.958
Surgical/*Operacija*	26 (5.8)	18 (10.3)	0.07
Malignancy/*Malignitet*	3 (0.7)	40 (23)	<0.000001
Trauma/*Povreda*	10 (2.2)	9 (5.1)	0.099
Obesity/*Gojaznost*	3 (0.7)	4 (2.3)	0.1
Varicose vein/*Varikoziteti*	6 (1.3)	9 (5.1)	0.01
Combine/*Kombinovano*	6 (1.3)	3 (1.7)	0.718
Comorbid disease/*Druge bolesti*	10 (2.2)	18 (10.3)	<0.0001
Physical effort/*Fizički napor*	4 (0.9)	0	0.580
	N	447	174

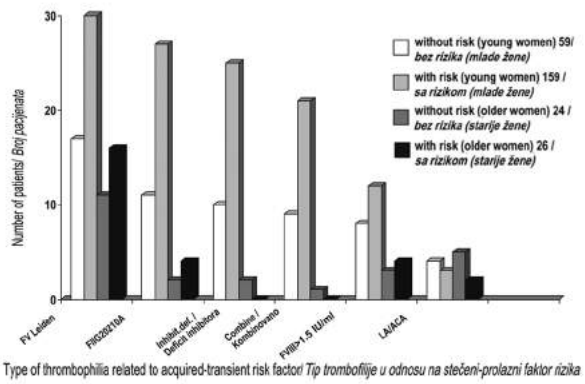
DVT - duboka venska tromboza, PE - plućna embolija

Table 2. Thrombophilia alterations
Tabela 2. *Prisustvo trombofilije*

	Young women/*Mlade žene* age/*starost* 18-45	Older women/*Starije žene* age/*starost* 45-75	p
Deficiency of natural inhibitors/*Nedostatak prirodnih inhibitora* n (%)			
(AT, PC, PS)	35 (7.8)	2 (1.1)	0.0009
FV Leiden mutation/*Mutacija*	88 (19.6)	27 (15.5)	0.277
Homozygous/*Homozigot*	1	2	
Heterozygous/*Heterozigot*	87	25	
FII G20210A mutation/*Mutacija*	38 (8.5)	6 (3.4)	0.04
Homozygous/*Homozigot*	4	1	
Heterozygous/*Heterozigot*	34	5	
Combined/*Kombinovani*	30 (6.7)	1 (0.6)	0.0006
FVIII > 1.5 iu/ml	20 (4.2)	7 (4.0)	0.977
Antiphospholipid syndrome *Antifosfolipidni sindrom*	7 (1.5)	7 (4.2)	0.144
n (%)	218 (48.7)	50 (28.7)	< 0.0001

AT - antitrombin, PC - protein C, PS - protein S, F - faktor

frequently than older women, who had distal or SVT more often. Regarding data from the Computerized Registry of Patients with Venous Thromboembolism (RIETE) study [8], distal DVT was less prevalent in women during pregnancy or puerperium, which was the most important risk factor among young women in our investigation. On the other hand, the most recent large observational study of patients with SVT has shown that this condition is typically diagnosed in outpatients, generally in women aged 60 years on average with high body weight, and/or a history of varicose veins [9]. This was confirmed among older women in our study. Data from both the RIETE [8] and our earlier study [10] showed that inherited thrombophilic alterations had no effect on the prevalence of distal DVT, which was confirmed in this study since the incidence of the inherited thrombophilia was almost double in the group of young women. That could have had an impact on the differences regarding the localization of thrombosis, especially in cases of thrombosis at unusual places (e.g. CVT). CVT was recently found to be strongly associated with congenital thrombophilia [11, 12]. The incidence of CVT is the highest in the third decade, and about 75% of all events occur in women with a strong association between CVT and two gender specific risk factors, such as the use of OC and pregnancy/postpartum [13], as confirmed in this study. In our group of young women, the development of CVT was associated with pregnancy/puerperium in 35%, or OC use in



Graph 1. Association of thrombophilia and acquired-transient risk factors
Grafikon 1. *Udruženost trombofilije i stečenog-prolaznog faktora rizika*

Table 3. Risk of recurrence
Tabela 3. *Rekurentne venske tromboze*

	Young women/*Mlade žene* age/*starost* 18-45	Older women/*Starije žene* age/*starost* 45-75	p
Number of women with RVTE/*Broj žena sa RVTE*	118 (26.3)	31 (17.8)	0.03
Time for the first recurrence */*Vreme za prvu rekurentnu trombozu*			
Mean (range)/*Prosek (raspon)*	5 (1-29)	4.8 (1-17)	0.588
N	447	174	

RVTE - recurrent venous thromboembolism/*rekurentni venski tromboembolizam*
*expressed in year/*izražen u godinama*

23% of cases and additionally potentiated with congenital thrombophilia in 41% of them. Contrary to that, only one CVT was observed among our older patients, in this case associated with a prothrombin G20210A mutation.

Among young women, the most important risk factor for first thrombosis was pregnancy and puerperium, while hormonal therapy was the second most important risk factor. The risk of occurrence of VTE in pregnant women is 5-fold higher than in non-pregnant women, and VTE is the most probable cause of death following delivery [14, 15]. On the other hand, hormonal therapy, especially in the presence of thrombophilia, is a strong risk for VTE [16]. These two transient risk factors inherent in women of reproductive age were found in 56% of our young patients. Spontaneous thrombosis without any particular risk occurred frequently among our older patients. More frequent occurrence of spontaneous thrombosis without any additional risk factors in this group could be explained by their age since the risk of thrombosis increases with age [17]. Regarding acquired risk factors associated with thrombosis in older women, a comorbid disease was the most important one, usually a malignant disease.

The frequency of congenital thrombophilia differed significantly between the two groups. Thus, incidences of an inhibitor deficiency, prothrombin G20210A mutation or combined thrombophilia were almost negligible in the older women. This difference results from the fact that a deficiency of natural inhibitors or combined thrombophilia, which are defined as severe thrombophilic conditions, are manifested very early and most carriers develop first thrombosis at an early age [18]. The patients with prothrombin G20210A were younger at their first VTE and had a higher rate of DVT accompanying PE than those with FV Leiden or no thrombophilia [10, 19]. One important observation from our current study relates to the finding that only the FV Leiden mutation, as an inherited thrombophilic alteration, was equally present in both groups of women. This mutation is a moderate thrombophilia alteration that often becomes clinically manifest in the presence of associated risk factors (e.g. pregnancy, surgery, trauma) and as such can appear for the first time in any period of life, as confirmed here. The second important observation concerning inherited thrombophilia is the finding that among young female carriers of thrombophilia, thrombosis occurred in three quarters of them during high-risk situations, such as pregnancy/puerperium or OC use. In their cases, FV Leiden and prothrombin mutations were the most frequent inherited thrombophilias combined with these risk factors, as shown previously [20–23], additionally emphasizing the multifactorial etiology of VTE. On the contrary, the effect of additional risk factors was much less pronounced among older female carriers of inherited thrombophilia.

Since 26.3% of the young women in our study group developed recurrent thrombosis in comparison with 17.8% of the older women, we assume that the higher prevalence of risk factors during the first event among young women may have an impact, especially given the higher incidence of inherited thrombophilia. It is also very important to note the influence of localization of thrombosis, bearing in mind the higher incidence of proximal DVT among young women. Similar findings regarding the localization and recurrence of thrombosis were observed earlier, where isolated distal DVT was associated with a lower risk of recurrence than proximal DVT or PE [24, 25]. On the other hand, inherited thrombophilic alterations, especially severe ones, such as deficiency of natural anticoagulants or combined thrombophilia are strong recurrence risk factors [26–30].

Among the limitations of our study that should be discussed is the fact that our study is retrospective, involving selected patients from two thrombosis centers, therefore the findings from our study should be confirmed in a further prospective studies.

Conclusion

Younger women developed more severe forms of thrombosis than the older ones. Inherited risk factor for thrombosis was detected in almost half of all young women, as opposed to less than 30% in elderly women. With the exception of factor V Leiden mutation, other types of congenital thrombophilia are almost negligible in the group of older women. Therefore, thrombophilia testing in case of first thrombosis is fully justified only in young women.

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Rad je primljen 13. I 2014.
Recenziran 29. VI 2014.
Prihvaćen za štampu 30. VI 2014.
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Professional article
Stručni članak
UDK 615.222.06:616.24
DOI: 10.2298/MPNS1410334V

DRUG INDUCED LUNG DISEASE - AMIODARONE IN FOCUS

LEKOVIMA IZAZVANA PLUĆNA BOLEST SA POSEBNIM OSVRTOM NA AMIODARON

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Summary

More than 380 medications are known to cause pulmonary toxicity. Selected drugs that are important causes of pulmonary toxicity fall into the following classes: cytotoxic, cardiovascular, anti-inflammatory, antimicrobial, illicit drugs, miscellaneous. The adverse reactions can involve the pulmonary parenchyma, pleura, the airways, pulmonary vascular system, and mediastinum. Drug-induced lung diseases have no pathognomonic clinical, laboratory, physical, radiographic or histological findings. A drug-induced lung disease is usually considered a diagnosis of exclusion of other diseases. The diagnosis of drug-mediated pulmonary toxicity is usually made based on clinical findings. In general, laboratory analyses do not help in establishing the diagnosis. High-resolution computed tomography scanning is more sensitive than chest radiography for defining radiographic abnormalities. The treatment of drug-induced lung disease consists of immediate discontinuation of the offending drug and appropriate management of the pulmonary symptoms. Glucocorticoids have been associated with rapid improvement in gas exchange and reversal of radiographic abnormalities. Before starting any medication, patients should be educated about the potential adverse effects of the drug. Amiodarone is an antiarrhythmic agent used in the treatment of many types of tachyarrhythmia. Amiodarone-caused pulmonary toxicity is a well-known side effect (complication) of this medication. The incidence of amiodarone-induced lung disease is approximately 5–7%.

Key words: Lung Diseases + chemically induced; Amiodarone; Diagnosis; Drug-Related Side Effects and Adverse Reactions; Risk Factors; Tomography, X-Ray Computed

Sažetak

Poznato je da više od 380 lekova može da ispolji toksični efekat na pluća. Lekovi za koje je pokazano da su značajni u tom smislu svrstavaju se u sledeće kategorije: citotoksični, kardiovaskularni, antiinflamatorni, antibiotici, nedozvoljeni lekovi i ostali. Neželjena dejstva mogu da se ispolje na plućnom parenhimu, pleuri, disajnim putevima, plućnom vaskularnom sistemu i medijastinumu. Lekovima izazvane plućne bolesti nemaju patognomonični klinički, laboratorijski, fizički, radiografski ni histološki nalaz. Njihova dijagnoza se obično postavlja metodom eliminacije drugih bolesti i često je zasnovana na kliničkom nalazu. Laboratorijske analize ne pomažu u postavljanju dijagnoze. U dijagnostičkom smislu, kompjuterizovana tomografija visoke rezolucije je mnogo senzitivnija od standardnog radiograma. Lečenje zahteva isključivanje leka čiji toksični efekat je doveo do plućnog oboljenja i primenu simptomatske terapije. Primena glikokortikoida dovodi do brzog poboljšanja gasne razmene i povlačenja radiografskih promena. Pre početka terapije svakim lekom sa potencijalnim toksičnim efektom na pluća, bolesniku treba da se predoče mogući neželjeni efekti leka. Amiodaron je antiaritmik koji se koristi u lečenju mnogih oblika tahiaritmije. Njime izazvana plućna toksičnost je dobro poznata komplikacija koju daje ovaj lek sa učestalošću 5–7%.

Ključne reči: Bolest pluća + hemijski indukovana; Amiodaron; Dijagnoza; Nuspojave i neželjene reakcije izazvane lekovima; Faktori rizika; CT

Introduction

More than 380 medications are known to cause pulmonary toxicity. Selected drugs that are important causes of pulmonary toxicity fall into the following classes: cytotoxic, cardiovascular, anti-inflammatory, antimicrobial, illicit drugs, miscellaneous. The adverse reactions can involve the pulmonary parenchyma, pleura, airways, pulmonary vascular system, and the mediastinum. Drug-induced lung diseases (DILD) have no pathognomonic clinical, laboratory, physical, radiographic or histological findings. DILD is usually considered a diagnosis of exclusion of other diseases. Pulmonary physicians are well aware of drug-induced acute and chronic pulmonary toxicities; inhaled or systemically administered drugs can affect airway tone and cause cough, dyspnoea due to airspace disease, diffuse alveolar damage, pulmonary capillaritis or interstitial lung fibrosis, to name a few presentations and tissue manifestations. Well-studied drugs that cause pulmonary toxicity include methotrexate, bleomycin and amiodarone, all of which can cause interstitial lung disease [1].

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Abbreviations

DILD – drug-induced lung diseases
APT – amiodarone pulmonary toxicity
BOOP – bronchiolitis obliterans organizing pneumonia
ARDS – acute respiratory distress syndrome
BAL – bronchoalveolar lavage
BPT – bleomycin pulmonary toxicity
NILT – nitrofurantoin-induced lung toxicity
CT – computed tomography

Amiodarone Pulmonary Toxicity

Amiodarone is the antiarrhythmic drug that is commonly used for the treatment of lifethreatening ventricular, ventricular premature beats and some other supraventricular arrhythmia. It contains a compound of iodine, which has a tendency to accumulate in certain organs, including the lungs [2]. Acute and chronic interstitial lung diseases are the most common manifestations of amiodarone pulmonary toxicity (APT). Patients receiving amiodarone often have associated cardiopulmonary disease as well, which may mask the toxic effect of drugs and the diagnosis is made too late, when the disease has already developed. Acute respiratory distress syndrome (ARDS) can rarely develop, especially in the perioperative period, in the patients on amiodarone therapy [3]. APT is the most common form of pulmonary toxicity with prevalence of 0.1-0.5% in patients taking amiodarone 200 mg/day, 5-15% of patients taking 500 mg/day or more, up to 50% of patients taking 1,200 mg/day or more. Risk factors for the development of APT are: daily doses greater than 400 mg (toxic drug reactions are more common in patients with amiodarone serum concentration higher than 2.5 mg/L), disrupted existing pulmonary disease (chronic obstructive pulmonary disease), previous lung surgery, treatment longer than 2 months, age, ethnic (racial) differences. (DILD is more common in Japanese population), exposure to high concentrations of oxygen, with or without mechanical ventilation. The two most important risk factors for the APT are age and duration of therapy. There is no safe dose. Most cases develop changes in the lungs 12 to 18 months from the start of taking the medication [3].

Amiodarone pulmonary toxicity should be taken into consideration, especially in elderly patients with pulmonary symptoms and changes even if low dose of the drug is administred for years [4]. There are two main hypotheses of the pathogenesis of APT: direct cytotoxicity and indirect immunological drug, hypersensitivity reactions. Direct cytotoxicity is associated with long elimination half-life and high affinity of amiodarone. The lung tissue hypersensitivity reactions are presented in some patients with lymphocytic infiltration of CD8 T-lymphocytosis and positive IgG immunofluorescence in the lung. In addition, the development of toxicity may be associated with the existing lung disease. The connection between the existing lung disease and APT may be masked because of the earlier limited pulmonary reserve [5]. Four forms of pulmonary toxicity caused by amiodarone are described: 1. Chronic interstitial pneumonitis is the most common presentation. Subacute attacks begin with nonproductive cough, dyspnea and weight loss, after two or more months of therapy. There are focal or diffuse interstitial fogginess on chest x-ray with foamy macrophages in the alveolar spaces. 2. Organizing pneumonia with or without bronchiolitis obliterans (Bronchiolitis obliterans organizing pneumonia – BOOP) accounts for about 25% of cases. It presents as a more acute condition with non-productive cough at the beginning, often with symptoms of pleurisy. Auscultation reveals crackles, and standard chest x-ray shows speckled shadows. Sometimes the signs of pleural affection appear and the condition can mimic infectious pneumonitis. 3. Acute respiratory distress syndrome occurs rarely, and it is of particular interest to anesthesiologists because it is characterized by fulminant flow especially in patients after surgery or pulmonary angiography. The incidence of ARDS after lung surgery is 11% in patients treated with amiodarone as compared with the 1.8% of those not treated in that way. Acute lung injury in surgical patients also develops one to four days after extubation. It is characterized by diffuse alveolar damage, with acute interstitial pneumonitis with hyaline membranes. It is assumed that amiodarone sensitizes patients who are at high oxygen (O_2) concentrations and high inspired oxygen (FiO_2), or increased sensitivity to iodinated contrast materials. Due to possible development of ARDS after surgery in patients receiving amiodarone, thoracoscopy, open lung biopsy is performed only after all other diagnostic modalities have been exhausted. 4. Solitary pulmonary mass is also shown as a complication of amiodarone therapy [6]. Radiology plays a central role in the diagnosis of APT. On chest radiographies, it appears as localized or diffuse speckled shadows, usually bilateral (**Figure 1**). Some infiltrates look like "ground glass". It was found that the right lung, especially in the right upper lobe, is more often affected than the left lung (**Figure 2**). Computed tomography (CT) more frequently reveals the disease compared to standard chest x-ray: bilateral interstitial, alveolar, or mixed interstitial and alveolar infiltrates could be seen (**Figure 3**). Initial radiographic APT findings follow ground glass pattern. It is crucially important to recognize it at the initial stage since the changes are potentially reversible [7]. Pleural thickening is commonly seen in the densest areas of infiltration. Pleural effusions have been described, but are less common. The appearance of one or more pulmonary nodules or tumor-like shadows is an unusual APT finding. If present, they are most commonly seen in the peripheral parts of upper lobes, attached to pleura. It is assumed that these nodes are the consequence of localized drug accumulation in areas of previous inflammation. Findings on chest radiograph can take up to 18 months to complete

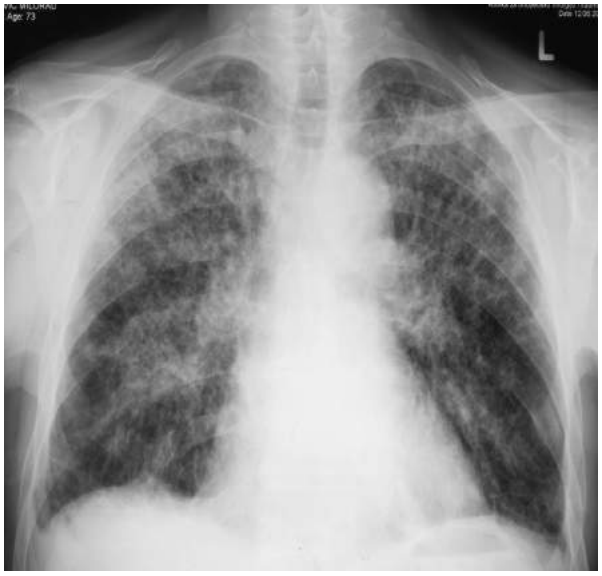

Figure 1. Chest x-ray shows thickened interstitium in both lungs
Slika 1. *Rendgentski snimak pluća pokazuje zadebljali intersticijum u oba plućna krila*

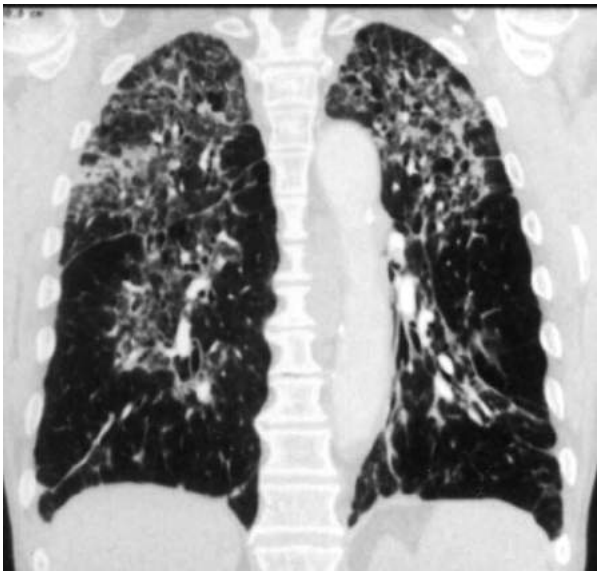

Figure 3. Chest CT in coronal view shows thickened intestitium more prominent in upper lobes
Slika 3. *CT snimak pluća, koronalni presek, pokazuje da je zadebljali intersticijum izraženiji u gornjim režnjevima*

withdrawal [2, 5]. Pulmonary function tests usually reveal a restrictive or mixed obstructive/restrictive model. Diffusing capacity of the lung for carbon monoxide (DLCO) is usually reduced. 20% decline in DLCO of the predicted value, or the value lower than 80% of the predicted one and reduces the total lung capacity (TLC) for more than 15% are the diagnostic criteria of APT. However, an isolated decrease in DLCO in the absence of clinical evidence of the disease is nonspecific and not diagnosed APT [8]. Fiberoptic bronchoscopy and bronchoalveolar lavage (BAL) are useful in excluding other interstitial lung problems. Polymorphonuclear leukocytosis and CD8 + T suppressor cells are predominant in BAL. The presence of "foamy" macrophages is consistent with the diagnosis, but these cells can be seen in up to one-half of patients receiving amiodarone and without signs of APT. However, in the absence of foam cells, the diagnosis of APT is unlikely [6]. Amiodarone discontinuation is the primary step in APT treatment approach. Due to accumulation in fatty tissues and the drug long half-life, pulmonary toxicity may initially progress despite drug intake discontinuation, and can even be repeated after withdrawal of steroids. Okayasi, et al. [9] found that obese patients with a higher body mass index (BMI) have more frequent relapses due to intensive accumulation of lipophilic amiodarone in the fat tissue. Discontinuation of amiodarone intake as the only form of therapy may be sufficient in the early and limited course of the disease. Corticosteroids should be used in patients who demonstrate a significant affection of the pulmonary parenchyma registered by various imaging methods with or without concurrent hypoxemia. Systemic corticosteroids are recommended (prednisolone 40 to 60 mg/daily) with gradually decreased dose for at least 4-12 months to avoid disease relapse. The benefits of this treatment strategy are earlier recovery and less parenchymal fibrosis. Irreversible pulmonary fibrosis develops in about 30% of patients. When treatment is started early, most cases of this disease are reversible and have a good prognosis. Later discovered, advanced disease can lead to poorer outcome, including pulmonary fibrosis and/or death, especially in cases where the ARDS develops [10].

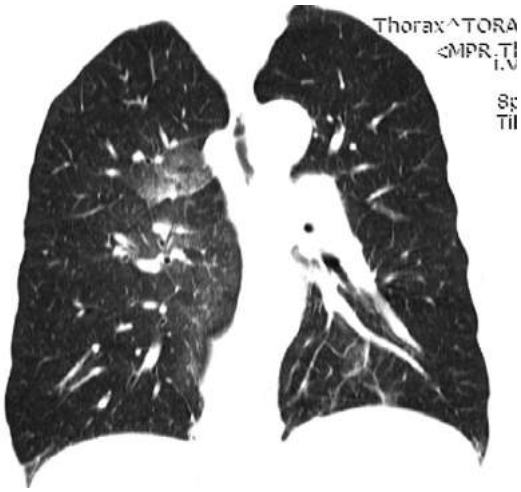

Figure 2. Chest CT in coronal view. Irregular ground glass opacities are present in posterior parts of the right lung
Slika 2. *CT snimak pluća – koronalni presek. Nepravilna zamućenja mlečnog stakla – prisutna su u zadnjim delovima desnog pluća*

Other Drug-Induced Lung Diseases-Cause Drugs

Methotrexate is a commonly prescribed antineoplastic and immune modulating compound that has gained wide acceptance in the management of rheumatoid arthritis, psoriasis, sarcoidosis and a number of neoplastic disorders. Although generally considered safe and easy to use, methotrexate has been associated with a number of adverse reactions. Pulmonary toxicity has been well-described and may take a variety of forms. Pulmonary infiltrates are the most commonly encountered form of methotrexate pulmonary toxicity and these infiltrates resemble hypersensitivity lung disease [11].

Bleomycin is a cytotoxic drug used in treatment of Germ Cell Tumours and is associated with pulmonary toxicity. Bleomycin pulmonary toxicity (BPT) manifests predominantly as pulmonary fibrosis, organizing pneumonia or nonspecific interstitial pneumonitis. The prevalence of BPT ranges from 0% to 46%, with mortality as high as 27%. Risk factors for the development of BPT include age, bleomycin regimen, bleomycin dose, renal insufficiency, radiation, underlying lung disease, smoking history, and granulocyte colony-stimulating factor (G-CSF) support [12].

Nitrofurantoin-induced lung toxicity (NILT) is relatively common. Patients usually use nitrofurantoin for urinary tract infections. Sometimes, histological patterns of lung damage are rare and may make the diagnosis difficult. The symptoms of NILT improve with cessation of nitrofurantoin, with steroids or without other therapy [13].

Leflunomide-induced pneumonitis (LEIP) usually occurs within the first 20 weeks of initiation of leflunomide, usually in patients with history of rheumatoid arthritis and either exposure to methotrexate or interstitial lung disease or both. Case mortality is about 20%. Poor prognostic indicators are diffuse alveolar damage on histological examination, pre-existing interstitial lung disease and ground glass shadowing on high resolution computerized tomography [14].

Conclusion

The drug-induced lung disease is an important and commonly neglected differential diagnosis in clinical practice. It may mimic a variety of pulmonary diseases. Amiodaron pulmonary toxity is found most frequently in pulmonology due to comorbidity treatment in these patients. The treatment of drug-induced lung disease consists of immediate discontinuation of the offending drug, and appropriate management of pulmonary symptoms. Drug-induced lung disease acute episodes usually disappear within 24-48 hours after the drug exclusion, but chronic syndromes may take longer to resolve. Complications, such as respiratory insufficiency, pulmonary thromboembolic disease, and pneumothorax, usually require hospital admission. Amiodaron pulmonary toxity should be taken into consideration in patients under long-term amiodarone use, especially in elderly patients with pulmonary symptoms, functional and radiographic changes even if low dose of the drug is administered for years.

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Rad je primljen 10. II 2014.
Recenziran 4. VII 2014.
Prihvaćen za štampu 111. VII 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:334-337.

SEMINAR FOR PHISICIANS
SEMINAR ZA LEKARE U PRAKSI

University of Novi Sad, Faculty of Medicine
Department of Pathological Physiology
Clinical Centre of Vojvodina
Centre for Laboratory Medicine

Seminar for phisicians
Seminar za lekare u praksi
UDK 616.379-008.64-074
DOI: 10.2298/MPNS1410339K

GLYCATED HEMOGLOBIN A1c AS A MODERN BIOCHEMICAL MARKER OF GLUCOSE REGULATION

GLIKOZILIRANI HEMOGLOBIN A1c KAO SAVREMENI BIOHEMIJSKI MARKER GLIKOREGULACIJE

Sunčica KOJIĆ DAMJANOV, Mirjana ĐERIĆ and Nevena EREMIĆ KOJIĆ

Summary

Glycated Hemoglobin Structure and Synthesis of Molecule. Glycated hemoglobin A1c, the major fraction of glycated hemoglobin, is formed by irreversible nonenzymatic glycation. Its concentration depends only on the life span of red blood cells and blood glucose levels. **Clinical Significance of Glycated Hemoglobin A1c.** It is the key parameter for monitoring the regulation of diabetes and for assessing the risk of microvascular complications. It is a diagnostic criterion for diabetes as well. Its concentration reflects the average value of blood glucose over the last two to three months. The estimated average glucose, a new parameter which facilitates the patient's self-monitoring of diabetes, can be calculated from its value. **Methods for Determining Glycated Hemoglobin A1c and their Standardization.** Immunoassay and ion-exchange chromatography are commonly used methods for the glycated hemoglobin determination in routine laboratory practice. The advantage of immunoassay is that there is no need for the sample pretreatment in order to eliminate unstable glycated hemoglobin A1c intermediary forms, and the possibility of false positive results is lower. The current program of standardization requires traceability to the International Federation of Clinical Chemistry and Laboratory Medicine reference method. **Reporting and Interpretation of Results of Glycated Hemoglobin A1c Determination.** Glycated Hemoglobin A1c can be reported as % or as mmol/mol. In our country, it is recommended to use the International Federation of Clinical Chemistry and Laboratory Medicine units (mmol/mol). When interpreting the results, the potential causes of falsely high or low values must always be taken into consideration. **Recommendations for Clinical Practice.** Periodic determinations of glycated hemoglobin A1c are recommended for monitoring of diabetes regulation. Additionally, the determination is recommended for the diagnosis of diabetes. The target value for the prevention of microvascular complications is < 7% and the diagnostic criterion for diabetes is ≥ 6.5%.

Key words: Hemoglobin A, Glycosylated; Biological Markers; Blood Glucose; Diabetes Mellitus; Risk Factors; Diabetes Complications; Diabetic Angiopathies; Diagnosis

Sažetak

Struktura i sinteza molekula glikoziliranog hemoglobina. Glikozilirani hemoglobin A1c, najzastupljenija frakcija glikoziliranog hemoglobina, nastaje ireverzibilnom neenzimskom glikacijom. Njegova koncentracija zavisi isključivo od životnog veka eritrocita i glikemije. **Klinički značaj glikoziliranog hemoglobina A1c.** Ključni je parametar za praćenje regulisanosti dijabetesa i procenu rizika od mikrovaskularnih komplikacija i dijagnostički kriterijum za dijabetes. Njegova koncentracija odražava prosečnu vrednost glikemije u protekla 2-3 meseca. Iz vrednosti hemoglobina A1c može se izračunati procenjena prosečna vrednost glikemije, novi parametar koji bolesnicima olakšava samokontrolu dijabetesa. **Metode određivanja glikoziliranog hemoglobina A1c i njihova standardizacija.** Imunoesej i jon-izmenjivačka hromatografija najčešće se primenjuju za njegovo određivanje u rutinskoj laboratorijskoj praksi. Prednost imunoeseja je da ne zahteva pretretman uzorka za uklanjanje labilnih intermedijera ovog hemoglobina, kao i manja mogućnost pojave lažno pozitivnih rezultata. Aktuelni program standardizacije zahteva usklađivanje sa referentnom metodom *International Federation of Clinical Chemistry and Laboratory Medicine*. **Izražavanje i interpretacija rezultata određivanja glikoziliranog hemoglobina A1c.** Glikozilirani hemoglobin A1c može se izražavati u % ili mmol/mol. U našoj zemlji je preporuka da se koristi mmol/mol. Prilikom interpretacije rezultata uvek se moraju razmatrati i potencijalni uzroci lažno visokih ili lažno niskih vrednosti. **Preporuke za kliničku praksu.** Periodično određivanje glikoziliranog hemoglobina A1c preporučuje se za praćenje regulisanosti dijabetesa. Uz to, preporučuje se i za postavljanje dijagnoze dijabetesa. Ciljna vrednost za prevenciju mikrovaskularnih komplikacija je < 7%, a dijagnostički kriterijum za dijabetes iznosi ≥ 6,5%.

Ključne reči: Glikozilizirani hemoglobin A1c; Biološki markeri; Glukoza u krvi; Diabetes mellitus; Faktori rizika; Dijabetesne komplikacije; Dijabetesne angiopatije; Dijagnoza

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Abbreviations

Hb	– hemoglobin
HbA1c	– glycated hemoglobin A1c
DCCT	– Diabetes Control and Complications Trials
UKPDS	– United Kingdom Prospective Diabetes Study
EPIC-Norflok	– European Prospective Investigation of Cancer and Nutrition
ADAG	– A1c-Derived Average Glucose Study
eAG	– estimated Average Glucose
NGSP	– National Glycohemoglobin Standardization Program
IFCC	– International Federation of Clinical Chemistry and Laboratory Medicine

Structure and Synthesis of Glycated Hemoglobin Molecule

Human hemoglobin (Hb) is not chemically homogenous. In erythrocytes of a healthy adult person, there are three different hemoglobins: fetal Hb (HbF) and two hemoglobins that belong to adults (HbA and HbA2), HbA being dominant. One pair of α-chains is same for all three fractions of Hb, while the other globin chain pair is different for every Hb: γ, β and δ-chain. In 1950s, HbA1 was isolated from HbA using chromatographic analysis and other three fractions HbA1a, HbA1b and HbA1c [1]. Glycation is the nonenzymatic addition of a sugar residue to amino groups of proteins. Glycated hemoglobins distinguish one from the other by the type of added carbohydrate and place of glycation. The place of glycation is usually N-terminal part of β-globin chain, but glycation can happen on other parts of β–chain or on α–chain [2, 3] **(Table 1)**.

HbA1c is the most important fraction of glycated HbA1, which is 75-80% of its total amount. It is formed irreversibly by nonenzymatic glycation, bonding two molecules of glucose for every N-terminal part of β-globin chain [2]. Chemically, NH_2-terminal valine residue is condensed with aldehyde glucose group. First unstable Schiff base is made (aldimine or pre-HbA1c). In normoglycemia, Schiff base (aldimine or pre-HbA1c) can dissociate again on glucose and HbA or in hyperglycemia, it can undergo the Amadori rearrangement to form stable ketoamine HbA1c, which cannot dissociate because human erythrocytes do not have enzymes which are necessary for its degradation. A similar process of glycation occurs on other plasma proteins [2, 3].

Clinical Significance of HbA1c

Over the last few years, HbA1c became a necessary parameter in following the regulation of diabetes mellitus, as well as a criterion for diagnosing this disease. In 1976, Koening et al. showed its strong correlation [4] to glucose levels. The studies were done on patients with diabetes type 1 and 2 [5–7] and they showed a good correlation with the degree of glycemic control. During the 1970s and 1980s [1, 3], kinetic studies showed that synthesis intensity and concentration of HbA1c depended exclusively on the previous glucose blood level and the lifespan of erythrocytes. A HbA1c concentration represents the integrated value for glucose over the preceding 6 to 8 weeks, compared to glycemia which represents the current glucose blood level [8]. That is why HbA1c has become firmly established as a biochemical marker of long-term blood glucose concentrations and as a measure of the success rate of diabetes treatment [9].

Besides that, big randomized studies such as the Diabetes Control and Complications Trials (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) have shown clearly that HbA1c is a predictor of diabetic microvascular complications. The DCCT study, which was carried out on 1400 patients with diabetes mellitus type 1 from 1983 to 1993 [5, 6], documented the direct relationship between HbA1c and the absolute risk for retinopathy, neuropathy and nephropathy. The risk for retinopathy continuously increased with increasing the HbA1c level. The HbA1c concentrations lower by 10% were associated with 45% lower risk for retinopathy development. In the UKPDS study conducted on more than 5000 subjects of both sexes with type 2 diabetes between 1977 and 1997 [7], 1% reduction in HbA1c was associated with the risk reduction of 37% for microvascular disease and 21% for deaths related to diabetes.

Individual studies [5–7, 10] showed that HbA1c could also be a risk marker for the development of macrovascular complications, primarily for cardiovascular disease although the obtained results were not significant.

A strong linear correlation between the levels of HbA1c and glycemia in the diabetic patients was found using the results from a big retrospective study, primarily the DCCT and the UKPDS [5–7], as well as from the studies of other authors [11–13], and it was the cause to perform the A1C-Derived Average Glucose (ADAG) study. This study included 507 healthy controls and patients with diabetes type 1 and 2 and stable glycemic control between 2006 and 2008 [14]. It confirmed a strong correlation between HbA1c and glycemia regardless of the sex, presence of diabetes or its type, which resulted in a mathematical equation of a new parameter showing glycemia in a three-month period: the estimated glyceamia value (eAG-estimated Average Glucose): 1.5944 x % HbA1c – 2.5944 (mmol/l). It is useful to calculate eAG because it makes it easier to understand the meaning of HbA1c as a marker of the long term glycemic control for patients [15–17] and expresses HbA1c in the same manner as when the patients perform their self-control. However, India and China, as the countries with lot of diabetic patients, were not the part of the ADAG study and neither were children or pregnant women, so the results cannot be applied generally and in all diabetic patient groups [14, 18].

Table 1. Hemoglobin species in adult erytrocites
Tabela 1. *Vrste hemoglobina u eritrocitima odraslih osoba*

Hemoglobin species *Vrsta hemoglobina*	Globin *Globin*	Part of total Hb *Udeo u ukupnom Hb*
Adult hemoglobin A/*Adultni hemoglobin A (HbA)*	α2 β2	90 – 97%
Adult hemoglobin A2/*Adultni hemoglobin A2 (HbA2)*	α2 δ2	2 – 5%
Fetal hemoglobin/*Fetalni hemoglobin (HbF)*	α2 γ2	2%
Total glycated hemoglobin *Ukupni glikozilirani hemoglobin (HbA1+A0)*	α2 β2 + sugar/*šećer*	3 – 9%
Glycated hemoglobin species/*Vrsta glikoziliranog hemoglobina*		
Glycated hemoglobin A0 *Glikozilirani hemoglobin A0 (HbA0)*	α2 β2-lysine or α-chain + sugar *α2 β2-lizin ili α-lanac + šećer*	1%
Glycated hemoglobin A1/*Glikozilirani hemoglobin A1 (HbA1 = HbA1a + HbA1b + HbA1c)*	α2 β2-valine + sugar *α2 β2-valin + šećer*	5 – 8%
Glycated hemoglobin A1a *Glikozilirani hemoglobin A1a (HbA1a)*	α2 β2-valine + fructose-1,6-diphosphate *α2 β2-valin + fruktoza-1,6-difosfat* or α2 β2-valine + glucose-6-phosphate *ili α2 β2-valin + glukoza-6-fosfat*	–
Glycated hemoglobin A1b *Glikozilirani hemoglobin A1b (HbA1b)*	α2 β2-valine + pyruvic acid *α2 β2-valin + piruvična kiselina*	–
Glycated hemoglobin A1c *Glikozilirani hemoglobin A1c (HbA1c)*	α2 β2-valine + glucose *α2 β2-valin + glukoza*	4 – 6% (80% HbA1)
Pre-HbA1c (unstable Schiff base, aldimine) *Pre-HbA1c (nestabilna Šifova baza, aldimin)*	labile intermediar of HbA1c *labilni intermedijer HbA1c*	5 – 8%

Hb – hemoglobin

Methods for Determining HbA1c and their Standardization

So far, there have been more than 30 different methods for determining glycated Hb. They differ one from another by the way of Hb separation from its glycated form. These methods are based on the charge differences (ion-exchange chromatography, high-performance liquid chromatography (HPLC), electrophoresis and isoelectric focusing), structural differences (immunoassay and affinity chromatography) or chemical analysis (photometry and spectrophotometry) [19]. The first methods determined the values of total glycated Hb and nowadays methods for measuring the largest fraction of HbA1c, whose concentration is a reliable marker for assessing the degree of glycation, are used.

In the beginning, there were numerous obstacles in HbA1c determination in routine practice because there were great differences between different methods and laboratory results due to the lack of method standardization. Therefore, a study work group of the DCCT and AACC (American Association for Clinical Chemistry) for HbA1c standardization was formed in 1993 and established the National Glycohemoglobin Standardization Program (NGSP). This group suggested measuring HbA1c using only reference method [1]. However, the NGSP was not accepted in all countries of the world especially in those which had their own program of standardization, such as Japan or Sweden. Besides that, the suggested reference method was not specific because it was used for measuring different forms of glycated Hb. Because of that, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) work group presented a new reference method for HbA1c determination in 1995, which was completely developed and accepted by all the IFCC members in 2001. This method is very specific and precise, but because of its complexity and high price, it cannot be used in routine clinical work. Therefore, the IFCC method of standardization, which has been supported by the development of the global reference laboratory network, makes it obligatory to standardize all laboratory instruments and tests for HbA1c determination according to the IFCC reference method, regardless of the applied methodology [16].

According to the European Union directive from 1998, all products distributed in Europe should be accompanied by the document stating that they are traceable with the IFCC reference method [20]. The correlation between the NGSP and IFCC method is strong, but the IFCC method is more specific and precise because it measures only HbA1c and not other fractions of glycated Hb. The values obtained by this method are about 1.5–2% lower than the ones obtained using NGSP method.

An immunoassay is the most frequently used method for determining HbA1c in routine laboratory practice. HbA1c is usually measured in venous blood using latex agglutination [19]. The test is based on the use of monoclonal antibody against HbA1c. These antibodies do not recognize unstable intermediary forms of glycation or other forms of glycated Hb (HbA1a or HbA1b) or other forms of Hb (HbF, HbS etc.), so there is no need for the sample pre-

Table 2. Clinical practice recommendations for HbA1c testing
Tabela 2. *Preporuke za određivanje glikoliziranog hemoglobina A1c u kliničkoj praksi*

HbA1c testing in adult patients with diabetes/*Određivanje HbA1c kod odraslih pacijenata sa dijabetesom*
•at least twice a year in patients who have achieved treatment goals and have stable glycemic control/*najmanje 2 puta godišnje kod pacijenata sa postignutim terapijskim ciljem i stabilnom glikemijskom kontrolom* • every 3 months in patients whose therapy has changed significantly or who have not achieved glycemic goals/*svaka 3 meseca kod pacijenata kod kojih je značajnije menjana terapija ili nisu postignute ciljne vrednosti glikemije*
Prevention of microvascular complications in diabetes/*Prevencija mikrovaskularnih komplikacija u dijabetesu*
• target value of HbA1c < 7% (53 mmol/mol), as close to the reference values as possible/*ciljna vrednost HbA1c < 7% (53 mmol/mol), što bliže referentnim vrednostima* • more or less stringent HbA1c target values may be appropriate in some patients/*kod određenih pacijenata mogu se primeniti više ili manje stroge ciljne vrednosti HbA1c*
HbA1c testing in pregnant women with diabetes/*Određivanje HbA1c kod trudnica sa dijabetesom*
• pre-existing diabetes:/*prethodno postojeći dijabetes:* every 4-8 weeks/*svakih 4-8 nedelja* target value of HbA1c < 6% (42 mmol/mol)/*ciljna vrednost HbA1c < 6% (42 mmol/mol)* • gestational diabetes/*gestacijski dijabetes:* not recommended/*ne preporučuje se*
HbA1c testing in children and adolescents with diabetes/*Određivanje HbA1c kod dece i adolescenata sa dijabetesom*
• at least 3-4 times a year, and in younger children up to 6 times a year/*najmanje 3-4 puta godišnje, a kod manje dece i do 6 puta godišnje* • HbA1c as a diagnostic criterion for diabetes: ≥ 6.5% (48 mmol/mol)* (as in adults)/*HbA1c kao dijagnostički kriterijum za dijabetes: ≥ 6,5% (48 mmol/mol)* (kao kod odraslih)*
HbA1c as a criterion for making diabetes diagnosis/*HbA1c kao kriterijum za postavljanje dijagnoze dijabetesa*
• HbA1c ≥ 6,5% (48 mmol/mol)* → diabetes mellitus • HbA1c 5,7 – 6,4% (39 – 46 mmol/mol)* → increased risk for diabetes (prediabetes)/*povećan rizik za dijabetes (predijabetes)* * results should be confirmed by repeating HbA1c testing/*rezultate je potrebno potvrditi ponavljanjem određivanja HbA1c*

treatment in order to eliminate them and the possibility of false positive result appearance is reduced to minimum [21]. In routine practice, besides the immunoassay, ion-exchange chromatography is frequently used. It requires the sample pre-treatment in order to remove labile intermediary forms of HbA1c. Despite that, there is a great possibility of getting false high results in case of the presence of great quantities of labile intermediary forms of HbA1c and in case of bonding non-carbohydrate compounds in uremia, alcoholism, different poisonings and chronic treatment with high doses of acetylsalicylic acid [21]. This has to be taken into consideration when interpreting the results. It is important to emphasize that fasting is not necessary for HbA1c determination.

The sample is taken from the venous blood, and ethylendiamintetraacetic acid (EDTA), oxalate or fluoride is used as an anticoagulant. The use of heparin is limited. If the analysis is not done immediately, it is recommended to store venous samples, not erythrocyte hemolysate [19].

Reporting and Interpretation of Results of HbA1c Determination

According to the NGSP standardization program, HbA1c values were expressed as the percentage proportion of HbA1c of the total Hb (%HbA1c). Since 2007, the IFCC has been recommending to show the HbA1c values in mmol of HbA1c on mol of total Hb (mmol/mol). It is possible to convert the results from the IFCC units to the NGSP ones by using a simple mathematical equation – the so called "master equation" [22]. Other conversion equations for IFCC transformation values into the values of HbA1c of other programs of standardization have been developed. However, it is still disputable which units should be used for the result presentation, whether mmol/mol or % HbA1c. According to the globally accepted consensus from 2007 [16], HbA1c should be presented in both IFCC and NGSP units, i.e. in mmol/mol and in % HbA1c, respectively; however, the final decision is up to each country individually. In the United States, the results are shown as %HbA1c together with calculated eAG [15], and in some other countries in % HbA1c and in mmol/mol with the tendency to adopt only the IFCC units. As recommended, the IFCC units (mmol/mol) have been used in our country since September 1st, 2011 [23].

HbA1c concentration in blood depends exclusively on the lifespan of erythrocytes and blood glucose level. Still, when interpreting the results , it is important to have on mind that glucose levels in the previous month determine about 50% of HbA1c level, while glucose levels in the first month determine

only 25% of its value [24]. The levels of HbA1c are not affected by daily glucose fluctuations, recent physical activity, food intake or an acute illness [3, 19]. Falsely high results can be obtained when the labile intermediary forms of HbA1c are present in the blood, especially when the methods of electrophoresis or ion-exchange are used [19]. In case of acute glucose level changes, the concentrations of labile intermediary forms change suddenly, while in cases of prolonged hyperglycemia, they bond irreversibly into HbA1c ketoamines. Therefore, labile intermediary forms reflect current glycemia. In healthy persons, they make 5–8% of total HbA1 and in diabetic patients up to 30% depending on the degree of glycemic control.

When interpreting the results, it is important to know whether the erythrocytes of tested person have the normal lifespan. The HbA1c values could be falsely low in the patients with hemolytic disease due to a large share of young erythrocytes or in other conditions where the lifespan of erythrocytes is shortened, as well as in recent significant blood loss. Falsely high values could be found in sideropenic anemia probably due to the enlarged share of old erythrocytes in blood. Falsely high or low values of HbA1c could be found in different hemoglobinopathies (HbF, HbS, HbC etc.) or in the presence of non-carbohydrate compounds bonded to Hb, such as in uremia, alcoholism, liver disease, different poisonings or applications of some medications [21]. In these cases, it is necessary to compare the HbA1c concentrations with the previous HbA1c results of the same person but not with the target values. Instead of HbA1c in these cases, some other glycated proteins, e.g. fructosamine, can be measured as an alternative indicator of glycemic control [15].

Recommendations for Clinical Practice

The current recommendations of the American Diabetes Association (ADA) for clinical practice [15] and the National Guidelines of Good Clinical Practice for Diabetes Mellitus of the Ministry of Health of the Republic of Serbia [25] recommend the HbA1c determination at least twice a year in patients with the achieved therapeutic goal and stabile glycemic control, and every three months in patients with a significant treatment change or when therapeutic goals have not been achieved **(Table 2)**.

For prevention of microvascular diabetic complications, the therapeutic target values of HbA1c in adults is below 7% HbA1c with the tendency to be as close to the reference values as possible.

In case of previously diagnosed diabetes in pregnancy, HbA1c should be measured every 4-8 weeks, and the therapeutic goal is less than 6% HbA1c, while in gestational diabetes, HbA1c determination is not recommended.

In order to diagnose diabetes mellitus, it is recommended to determine HbA1c, and the "cut-off" value is $\geq$ 6.5% HbA1c. It is necessary to confirm the diagnosis of diabetes mellitus by multiple determinations of HbA1c. *Point-of-care* tests should not be used for setting up the diagnosis of diabetes.

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Rad je primljen 20. I 2014.
Recenziran 10. IV 2014.
Prihvaćen za štampu 9. V 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:339-344.

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Seminar za lekare u praksi
UDK 612.173.08 i UDK 616.12:546.17
DOI: 10.2298/MPNS1410345S

MODULATORY ROLE OF NITRIC OXIDE IN CARDIAC PERFORMANCE

MODULATORNA ULOGA AZOT-OKSIDA NA SRČANE PERFORMANCE

Sonja SMILJIĆ[1], Vojkan NESTOROVIĆ[1] and Slađana SAVIĆ[2]

Summary
Nitric oxide is produced by almost all cardiac cells, endothelial cells, cardiomyocytes and nerve fibers. It is synthesized by an enzyme, a nitric oxide synthase, which occurs in endothelial, neural and inducible form. The distribution of nitric oxide synthase in the heart is characterized by a pronounced non-uniformity. Nitric oxide exerts its effects in physiological and pathophysiological conditions. The physiological effects of low concentrations of nitric oxide, which is released in the normal conditions under the influence of constituent enzymes, occur via cyclic guanosine monophosphate. The synthesized nitric oxide exhibits its effect in the cells where it is produced, in an autocrine manner, or by diffusing into the neighboring cells, in a paracrine manner. Nitric oxide acts by regulating the coronary vessel tonus, affecting the contractility of cardiomyocytes, generating an inotropic effect in a dose-dependent manner and controlling the cellular respiration. Other effects of nitric oxide in the cardiovascular system include the hyperpolarization of the smooth muscle cells in blood vessels, the inhibition of the monocyte adhesion, the inhibition of platelet migration, adhesion and aggregation and the proliferation of smooth muscle cells and fibroblasts. The anti-atherosclerotic effects of nitric oxide are based on these effects. Nitric oxide is a weak free radical in gaseous state, and the cytotoxic and/or the cytoprotective effects of the higher concentrations of nitric oxide are related to the chemical structure of nitric oxide as a free radical. The excessive production of nitric oxide by the activation of inducible nitric oxide synthase can lead to major irregularities in the function of cardiomyocytes and cardiac insufficiency. Understanding the nitric oxide molecular mechanisms of signaling pathways in the heart can provide a new strategic approach to prevention and treatment of cardiovascular diseases.
Key words: Nitric Oxide; Nitric Oxide Synthase; Cardiovascular Diseases; Free Radicals; Myocytes, Cardiac; Coronary Vessels; Cell Respiration

Sažetak
Azot-oksid proizvode gotovo sve ćelije srca, endotelne ćelije, kardiomiociti i nervna vlakna. Sintetiše ga enzim, sintaza azot-oksida koja se javlja u endotelnoj, neuralnoj i inducibilnoj formi. Distribuciju sintaza azot-oksida u srcu karakteriše izražena neuniformnost. Azot-oksid ima efekte u fiziološkim i patofiziološkim stanjima. Fiziološki efekti niskih koncentracija azot-oksida koji se oslobađa u normalnim uslovima pod dejstvom konstitutivnih enzima odvijaju se preko cikličnog guanozin-monofosfata. Sintetisan azot-oksid ostvaruje svoj efekat u ćelijama u kojima je proizveden, autokrino, ili difundujući u susedne ćelije, parakrino. Azot-oksid deluje putem regulacije tonusa koronarnih krvnih sudova, utiče na kontraktilnost kardiomiocita, na dozno-zavisan način ostvaruje inotropni efekat i kontroliše ćelijsku respiraciju. Drugi efekti azot-oksida u kardiovaskularnom sistemu su da dovodi do hiperpolarizacije ćelija glatke muskulature krvnih sudova, inhibira adheziju monocita, inhibira migraciju, adheziju i agregaciju trombocita i proliferaciju ćelija glatke muskulature i fibroblasta. Na ovim efektima se zasniva antiaterosklerotsko dejstvo azot-oksida. Azot-oksid je slab slobodni radikal u gasovitom stanju a citotoksična i/ili citoprotektivna dejstva viših koncentracija, u vezi su sa hemijskom strukturom azot-oksida kao slobodnog radikala. Prekomerna proizvodnja azot-oksida aktivacijom inducibilne sintaze azot-oksida može dovesti do velikih poremećaja funkcije kardiomiocita i srčane insuficijencije. Razumevanje molekularnih mehanizma azot-oksida u signalnim putevima u srcu može da obezbedi novi strategijski pristup u prevenciji i terapiji bolesti kardiovaskularnog sistema.
Ključne reči: Azot oksid; Azot oksid sintaza; Kardiovaskularna oboljenja; Slobodni radikali; Kardiomiociti; Koronarni krvni sudovi; Ćelijsko disanje

Introduction

Nitric oxide (NO) is an endogenous mediator with vasorelaxation properties and was originally named endothelium - derived relaxing factor (EDRF) [1]. The ensuing studies have shown that nitric oxide is an important physiological mediator and potent modulator of many biological functions. Today it is well known that NO is one of the key signaling molecules in cardiovascular and nervous system and that it plays a significant role in body's defense mechanism. NO has a prominent signaling function in macrophages, neurons, endocrine cells, skeletal muscle fibers and numerous other cell types. The most recent studies of cardiomyocytes have identified the function of NO in the regulation of cardiac growth and remodeling, contractile performance, rhythmicity and metabolic rates.

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Abbreviations

NO – nitric oxide
EDRF – endothelium-derived relaxing factor
NOS – nitric oxide synthase
eNOS – endothelial nitric oxide synthase
iNOS – inducible nitric oxide synthase
nNOS – neuronal nitric oxide synthase
MVE – microvascular endothelial
TGF – transforming growth factor
TNF-α – tumor necrosis factor
cGMP – cyclic guanosine monophosphate
ANG – angiotensin
EE – endocardial endothelium
ET-1 – endothelin-1
PGI_2 – prostaglandin I_2

Nitric oxide is a small non-polar molecule with one unpaired electron which makes it a weak free-radical [2]. It is a mediator acting as though it has opposite biological effects. The complexity of NO activity is manifested through the diversity of chemical reactions in which it participates and the characteristics of the tissue in which it operates. The level of synthesized NO is the key that determines the biological outcome of precise cellular responses to its various concentrations. In general, lower concentrations of NO promote the cell survival and proliferation, while higher levels favor the degradation of the cell, apoptosis and/or senescence. Interactions with free radicals affect the NO signaling pathways and reduce its bioavailability.

There are a number of regulatory mechanisms mediated by NO both under the physiological conditions in the healthy and pathological heart. The NO effect on the intracellular milieu and cell function is determined by its concentration, the time factor and by the kinetic determinants. Synthesis, diffusion and consumption rates, interaction with target tissues, free radicals and oxygen concentration contribute to the cellular and tissue-specific response to NO.

Biosynthesis of Nitric Oxide and its Control

NO can be synthesized from L-arginine using three different nitric oxide synthase (NOS) isoforms, two of which are constitutive, endothelial (eNOS, NOSIII) and neuronal (nNOS, NOSI) isoforms, manifested under physiological conditions, while the third isoform, inducible nitric oxide synthase (iNOS, NOSII), is biosynthesised only after the stimulation by a variety of stressors and cytokines [3]. Constitutive enzymes generate a small amount of NO, while the activity of iNOS is approximately one thousand times higher [2]. Endothelial NOS is associated with the caveolin in caveolae, specialized microstructures of the plasma membrane (**Figure 1**). The interaction between eNOS and caveolin is reversible and the release of eNOS from caveolin activates the enzyme. Neuronal and inducible NOS are more commonly found in their soluble form than the membrane-bound one. Constitutive enzymes are activated by Ca^{2+}-calmodulin and the control is carried out through the endothelium-dependent agonists, acetylcholine, bradykinin and substance P, which increase the concentration of intracellular calcium, thus increasing the Ca^{2+}-calmodulin. In contrast, iNOS is independent from Ca^{2+}. As iNOS has a Ca^{2+}-calmodulin binding domain, a high affinity of this ligand-binding domain means that iNOS is activated even at low calcium levels, which are present under basal conditions [2, 4].

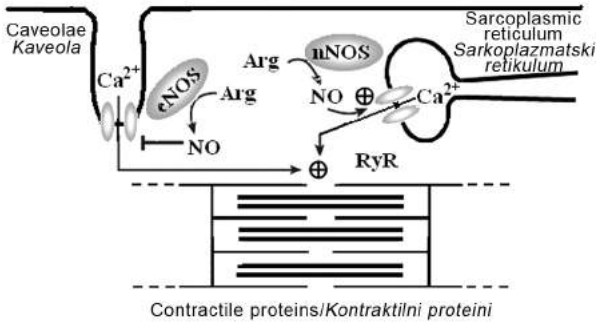

Figure 1. Biosynthesis of nitric oxide. Opposing roles for eNOS and nNOS in regulating heart contraction
Slika 1. *Biosinteza azot-oksida. Suprotne uloge endotelijalne i neuronske sintaze azot-oksida u regulaciji srčane kontraktilnosti*

The Distribution of NO Synthase in the Heart is Characterized by the Pronounced Non-uniformity

Endothelial NOS is mainly present in the coronary vascular and endocardial endothelium and to a lesser extent in cardiomyocytes. Neuronal NOS is present only in the subpopulation of intracardiac ganglia and nerve fibers in the atrial tissue, as well as in some of the perivascular nerve fibers of the ventricular myocardium. The manifestation of nNOS in cardiomyocytes and its physiological role are still being researched. A healthy heart does not usually exhibit iNOS under physiological conditions [5].

Despite the fact that eNOS is distributed in all cardiac endothelial cells, there is a significant disparity of eNOS activity between the endocardial endothelial, arterial, venous, and endothelial cells of myocardial capillaries with an exceptionally intense activity in the endocardial endothelial (EE) cells and endothelial cells of coronary arteries. EE cells have a more distinct Golgi complex than other endothelial cells in blood vessels of the heart. The size of the Golgi complex is most likely a marker of the eNOS synthetic activity; thus, these data indicate that the endothelial cells of the coronary arteries and EE cells have a higher synthetic

activity than microvascular endothelial (MVE) and venous endothelial cells. Immunohistochemical stainings of caveolin-1 have shown that the peripheral boundaries of EE cells are almost completely devoid of caveolin features. This indicates that the enzymatically active eNOS in EE cells, contrary to cardiomyocytes, can be connected to membrane components other than calveolin or to the cytoskeletal parts.

Immunohistochemical staining of the complete myocardial tissue has shown weak cytoplasmic activity of eNOS in MVE cells with a poorly developed Golgi complex. However, the staining of caveolin-1 is more pronounced in MVE than in EE or arterial endothelium. The increased levels of caveolin 1 in MVE has a cardioprotective effect in ischemia/reperfusion-induced damage, probably due to the increased endothelial NO release [5].

The reasons for the differences in eNOS distribution are still unknown. Experiments in the cultured endothelial cells showed that eNOS activity could be modified by a number of modulators, including the friction force, transforming growth factor (TGF-β), protein kinase C, tumor necrosis factor TNF-α, oxygen and proliferation. The differences in friction forces in the heart can explain the difference of the eNOS activity in the arterial, capillary and venous endothelial cells and the EE cells since different friction forces affect the endothelium. Friction forces in the laminar blood flow are not strong along the surface of the EE cells [6]. However, the EE cells exhibit eNOS activity almost as strong as that of the arterial endothelial cells. Endocardial endothelium may be exposed to the turbulent blood flow, but this type of flow does not increase eNOS activity or NO release. eNOS activity may be affected by the mechanical strain of the endocardium due to the three-dimensional changes of the inner wall during the cardiac cycle. Endothelial cells cultured on flexible substrates, which had been subjected to a cyclic tensile strain, displayed an enhanced production of NO. Some parts of the endocardium are subject to specific differences in the mechanical deformation during the cardiac cycle, such as tendon endings of ventricular papillary muscle and the atrioventricular valves. EE cells covering these elastic structures are smaller, and have a cytoskeletal organization which differs from the organizations of other endocardial fields, but they have not shown any consistent differences in eNOS characteristics or the size of the Golgi complex [6].

Endothelial NOS in cardiomyocytes is associated with caveolin-3, a muscle-specific isoform in caveolae protein layers [7–9]. However, the major physiological source of NO in a normal, adult, and unstrained cardiac tissue is most likely eNOS originating from EE and MVE cells, while NO originating from cardiomyocytes is probably negligible if they are not activated. Cardiomyocytes do not release NO in direct response to bradykinin or α_2 receptor agonists [10], which further indicates that NO originating from the cardiomyocytes is actually negligible in its basal, unstrained physiological condition. In contrast, the exposure of cardiomyocytes to β-adrenergic agonists increases the production of endogenous NO almost five times, indicating the regulation of eNOS in cardiomyocytes via β-adrenergic stimulation [11]. In a similar manner, cyclic guanosine monophosphate (cGMP), as a measure of NO activities, is almost 10 times increased in cardiomyocytes after the stimulation either by bradykinin, in contrast to previous studies [10], or by acetylcholine [12]. Myocardial stretching may participate in the activation of eNOS [13].

Accordingly, there is a significant non-uniformity in the distribution of eNOS in the cardiac endothelium, followed by a more intensive activity in EE and the endothelial cells of coronary arteries than in MVE and coronary venous endothelium. eNOS activity is only partially manifested in normal cardiomyocytes under the basal conditions and it can be affected by a number of mediators. Therefore, despite its role in many heart diseases, NO originating from cardiomyocytes is very likely to have an insignificant role in controlling the overall structure and function of the normal adult heart under unstrained physiological conditions. NO plays a role in the modulation of cardiac function in response to a specific stimulus, or in a state of myocardial stress, the release of parasympathetic and adrenergic neurotransmitters, muscle stretching, and the like.

Effects of NO on Cardiac Contractility

There is an enormous difference between the frequently conflicting effects of NO on myocardial contractility, depending on the animal species and experimental conditions, but most importantly on the experimental hierarchical level of research, no matter whether the individually isolated cardiomyocytes, multicellular preparations or in vivo intact heart are studied. Individual cardiomyocytes were extensively studied during the last 30 years. Despite the theoretical advantages of this experimental model, such as in the application of sophisticated molecular techniques for determining the specific signal molecules in cardiomyocytes, many uncontrolled artifacts induced by experimental isolating procedures can provide a distorted image of reality. This shows the limitations of such an approach in cardiac research, not only because of the isolation of cardiomyocytes from the inherent endothelial cells, but also of the neighboring cardiomyocytes [14, 15].

The positive inotropic response to NO on isolated cardiomyocytes has been obtained in several studies [16, 17]; however, there are also data on NO causing a negative inotropic effect but at higher concentrations [18]. Nevertheless, it remains unclear whether any response obtained in the indi-

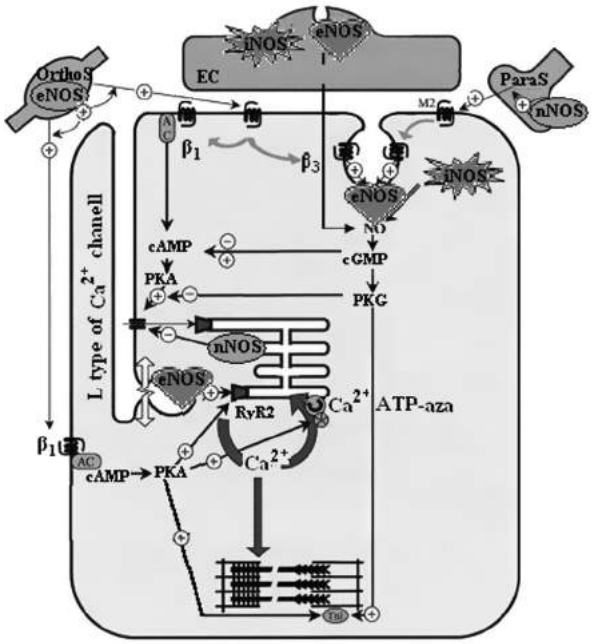

Figure 2. Autocrine and paracrine regulation of cardiomyocyte function by NO
Slika 2. *Autokrina i parakrina regulacija funkcije kardiomiocita azot-oksidom*

vidual cardiomyocytes can be beneficial or detrimental to cardiac function of the intact heart.

Data that are more relevant come from multicellular cardiac preparations. They show a typical dose-dependent biphasic inotropic response to NO. Low values of NO, which correspond to endogenously generated NO, cause a positive inotropic response, whereas higher concentrations cause a consistent negative inotropic response. The response to the increased concentrations of cGMP is similarly biphasic [19]. When the concentration of cGMP-inhibited phosphodiesterase is low, it increases the intracellular level of cyclic adenosine_monophosphate (cAMP), which can be considered a positive inotropy (**Figure 2**). Cyclic GMP at higher concentrations activates the cGMP-dependent protein kinase, which inhibits adenosine triphosphate (ATP) synthesis, and closes the voltage-dependent calcium channels, which can also be considered a negative inotropic response. Alternative interpretations, including the cGMP independent mechanisms, are possible due to the complexity of many NO signaling pathways and interactions [20, 21]. Although the modular interactions of NO with an autonomic nervous or other cardiomodulatory systems cannot be excluded in the human ventricle, no direct NO influence has been detected. The advantage of multicellular cardiac preparations is that they allow the complete tracking of both systolic (contraction and relaxation) and diastolic (tension at rest) phases of the cycle, both of which should not be ignored during the full assessment of the contractile function. For example, higher concentrations of NO cause a premature onset of relaxation during isometric contraction [19, 22]. NO-dependent effects on maximum rate of tension and peak tension can manifest themselves as decreased, unchanged or even increased.

It is very difficult to confirm the NO inotropic effects in the heart directly, in vivo, because of the confounding effects of varying loading conditions, coronary flow and neurohormonal control and interaction with β- adrenergic or cholinergic pathways as well as with the atrial natriuretic peptide. Several studies have provided indirect evidence of the less positive inotropic effects [23, 24]. The biphasic effect of NO, positive at low and negative at high concentrations of NO, is explained in transgenic mice in which the activity of eNOS was exaggerated. It is important to note that NO induces an earlier onset of ventricular relaxation in vivo, thus improving the ventricular relaxation, early rapid filling and diastolic compliance. This effect may be accompanied by a small decrease in the peak systolic pressure despite the frequently unchanged rate of pressure development and unchanged ejection fraction [25]. This effect is partially attributed to the NO-induced reduction of preload and afterload [24].

The negative inotropic effect can be considered potentially harmful. In fact, it should be considered potentially useful for the cardiac function since it acts as a compensatory feedback loop when the physiological effects on the duration of the contraction in the increased previous and/or subsequent ventricular load are superior to the pathologically prolonged duration of contraction in the ventricular hypertrophy. Pinsky et al. [26] have shown that there is a cyclic release of NO in the heart during heart rate, mainly in subendocardial zone, which reaches its peak during ventricular relaxation and early rapid chamber filling phase. This time convenient, short-term bursts of NO contribute to the important modulation of the chamber relaxation phases, especially in early filling and coronary perfusion during diastole. Subendocardial localization, as the principal area with the highest concentration of NO, indicates that EE cells are the main source of NO.

Accordingly, NO has a double effect on cardiac contractile performance. In response to the lower concentrations of the endogenously synthesized NO through eNOS activation, a positive inotropic effect is achieved, which contributes to the maintenance of cardiac contractility under basal physiological conditions. At higher NO levels, there is a negative response to peak contractile performance resulting from the iNOS activity or the pharmacological application of NO donors. Its impact on the onset of chamber relaxation, through the heart rate optimization and coronary perfusion is certainly more important, especially as a compensatory mechanism in the ventricular filling phase, when the latter is disrupted by disease.

NO has a significant effect on the time sequence of events and the very onset of the venticular relaxation and modulation of cardiac systolic function. By delaying the onset of relaxation, the heart prolongs and maintains systole, as a part of heterometric autoregulation (Starling law) under the conditions of increased capacity and load pressure. In contrast, the earlier onset of ventricular relaxation resulting from NO release, favors the ventricular relaxation, early rapid filling, diastolic compliance, and coronary perfusion during diastole, which all together represent a compensatory mechanism against the venticular load occurring under the conditions of severe maladaptive hypertrophy and/or tachycardia. When considering the impact of NO on contractility, we must take into account its interaction with endothelin-1 (ET-1), prostaglandin I_2 (PGI_2), angiotensin II (ANG II), β-adrenergic and cholinergic innervation, atrial natriuretic peptide (ANP) and aldosterone [25–28].

Effect of NO on Cardiac Metabolism

Endogenous and exogenous NO reduces the oxygen consumption of the myocardial tissue in both a healthy and diseased human heart [29, 30]. Similarly, a reduced production of NO leads to an increase in myocardial oxygen consumption in conscious dogs [31]. The inhibition of oxygen consumption by NO is also observed in the non-contractile cardiac muscle cells [32]. NO produced in the endothelial cells, and its inhibition of the myocardial oxygen consumption, is increased by angiotensin-converting-enzyme (ACE) inhibition, most probably by increasing the levels of bradykinin. Bradykinin-induced reduction in the myocardial oxygen consumption is reduced in eNOS of knockout mice [33]. The ability of NO to reduce the myocardial oxygen consumption indicates potential cardio-protective effects of NO as it partially reduces the heart rate during systole and leads to the increased myocardial metabolic efficiency. It seems to be achieved through a better use of the energy substrate (free fatty acids, as opposed to glucose), or by the regulation of the mitochondrial metabolism. NO originating from MVE and EE cells regulates the local myocardial metabolism directly. NO has a tendency to compete reversibly with oxygen for a common binding site on cytochrome-c oxidase, thus inhibiting the transfer of electrons to oxygen. It has recently been shown in cultured cardiomyocytes not normally producing NO in situations where they are not strained that cytokine-induced NO production by iNOS activity or exogenous NO provided by NO donors reduces the energy consumption, i.e. ATP production and its consumption of myocardial contraction. This is accomplished by the inhibition of the mitochondrial iron-sulfite reductase [34].

Nitric oxide contributes to cellular respiration and affects cell function due to its ability to bond the heme groups of the important biological proteins (such as cytochrome - c oxidase) in which it competes with oxygen [2].

Importance of NO in Embryonic Development of Heart

A congenital heart disease is the most common malformation in children at birth. Endothelial NOS is essential during the heart development. Lack of eNOS results in congenital septal defects, cardiac hypertrophy and postnatal heart failure. In addition, eNOS is essential for the morphogenesis of major coronary arteries and the development of myocardial capillaries. The effects of NO are mediated by the induction of the transcription of growth factors that are essential in angiogenesis. Insufficient eNOS results in a high incidence of bicuspid aortic valve, complicated by stenosis or regurgitation, endocarditis, aortic aneurysm and aortic dissection. Thus, NO produced by eNOS plays a critical role in the embryonic development of the heart, in the morphogenesis of the coronary arteries and the aortic valve [35].

As result of eNOS activity, NO contributes to the embryonic development of the heart as shown in the experimental model of mice lacking eNOS. These animals without eNOS survive until they reach maturity; however, they exhibit frequent malformations (bicuspid aortic valve) [36]. A study involving mice without eNOS reported a large volume of aortic and ventricular septal defect, and a pillowy deformity, resulting in death shortly after birth [37]. Pharmacological inhibition of NOS leads to slowing down or stopping cardiomyocytes differentiation, which suggests that cardiomyocyte-originated NO together with the endothelial NO may also play an important role in cardiomyogenesis at some stage, which probably corresponds to the later stage of the pillowy formation and early myocardial compactness. Brutsaert et al. [38] have come to the conclusion that eNOS is present in EE cells in the developmental stage of the rat heart even before eNOS occurs in MVE cells, and even when the endothelial cells in the ventricular myocardial capillaries have been detected by immunohistochemical staining [39]. At later stages of heart development, eNOS is not only present in EE cells but also in MVE and in the endothelium of coronary vessels, although it is absent from cardiomyocytes.

NO and Remodeling of Heart

Endothelial mediators such as NO, ET-1, PGI_2 and ANG II may affect the myocardial growth and remodeling, since they affect the growth of vascular smooth muscles [40]. Regardless of their origin, which may be myocardial or endothelial, NO and ET-1 can take part theoretically in reactions of the adult heart growth. Cardiac endothelum dysfunction is used to explain a maladaptive growth during the progression of heart failure [5].

Many experimental and clinical observations confirm the effect of NO on the growth and remodeling of the heart. Bradykinin-induced NO synthesis contributes to the negative effects on the growth and is an example of the intersection of endocardial-myocardial signaling pathways of the heart. Bradykinin directly stimulates the growth of cardiomyocytes in culture, and the anti-growth effect depends largely on the presence of endothelial cells in co-culture, and the release of their mediators NO and PGI_2 [40, 41]. In the experiment with mice, the dysfunction of bradykinin B_2 receptors leads to cardiac hypertrophy followed by the chamber dilation and reparative fibrosis, through the effects that can be prevented by ANG I receptor antagonists [40]. Kinins normally stimulate NO release through the activation of bradykinin B_2 receptors. Kallikrein/kinin system has a cardioprotective effect as it eases the process of remodeling by activating the cardiac endothelial/myocardial signaling pathways [42, 43].

NO may act as a molecular switch in the promotion and/or inhibition of the growth factors effects, such as basic fibroblast growth (bFG), vascular endothelial growth (VEGF) in TGF-β in an adult heart. The levels of eNOS activity in cardiac endothelial cells may, therefore, be valuable in controlling the growth and remodeling of the heart [44, 45].

NO and Heart Failure

Over the last couple of decades, we have witnessed an impressive progress in demystifying the pathogenesis of heart failure, which has emphasized the importance of a number of compensatory mechanisms, such as cardiac dilatation or hypertrophy, the participation of neurohumoral factors, synthesis of cytokines and the activation of endothelial cells. Compensatory mechanisms, either cardiac or non-cardiac ones, may be insufficient to adapt to the new conditions, with the resulting clinical manifestation of heart failure [46].

Endothelium, that is the endothelial activation and dysfunction, hold an important place in the pathogenesis of heart failure. Endothelial dysfunction in clinical practice is mainly related to the reduced production of endothelial NO and its bioavailability. It is believed that endothelial dysfunction occurs when vasodilatation is expected, mediated by endothelial NO in response to acetylcholine, bradykinin, substance P and/or serotonin deficient. The concomitant use of a NO donor, e.g. nitroprusside, would lead to the expected vasodilatation (**Figure 3**).

Measuring of the NOS levels during heart failure has given disappointingly inconsistent data, which may have resulted from the different etiology of heart failure (dilated, idiopathic, ischemic, hypertrophic). Thus, at the last stage of human heart failure, the level of eNOS is increased in the sub-endocardial cardiomyocytes, but it is decreased in the myocardial capillary endothelial cells [47]. In heart failure caused by septic shock, the activity of iNOS is increased, which

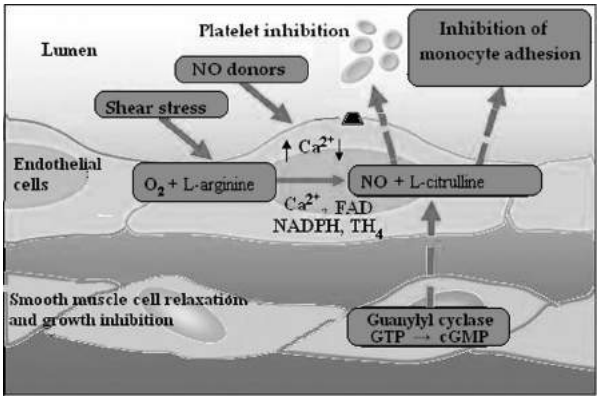

Figure 3. The role of NO in the vasorelaxation of blood vessels
Slika 3. *Uloga azot-oksida u vazorelaksaciji krvnih sudova*

results in the increased synthesis of NO with the consequent decreased strength of the heart contractions. Cytokines, such as TNF-α, whose plasma values are increased during the septic shock, are also considered strong inducers of iNOS [48].

There is a significant positive correlation between the activities of the endocardial and subendocardial iNOS and eNOS with ventricular contractile performance which suggests a beneficial effect of NO released from the endothelial cells in patients with dilated cardiomyopathy. Endogenous NO also has a cardioprotective effect in patients with idiopathic dilated cardiomyopathy by lowering the contractile response to β-adrenergic stimulation, thus saving myocardial oxygen [49].

Drugs used in the treatment of heart failure and angina pectoris, which are based on organic nitrates, act through NO in which they are metabolized. NO activates the soluble guanylate cyclase, increases the synthesis of cGMP, activates protein kinase A, and leads to the cascades of effects in the smooth muscle, ending with the dephosphorylation of light myosin chains, and the removal of $Ca^{2+}{}_i$ and muscle relaxation. NO donors cause a distinct venodilation with a reduction of central venous pressure and afterload. Its direct effect on coronary arteries decreases the spasm and increases the coronary flow. Moreover, drugs such as glyceryl trinitrate redirect the blood from normal to ischemic areas of the myocardium through the collaterals. In addition to their effect on smooth muscle, NO donors lead to the relaxation of the heart muscle.

Conclusion

The following conclusion can be made according to the aforementioned:

– nitric oxide synthesis in the heart is one of the factors that provides a normal cardiac activity under physiological conditions

– there is a significant non-uniformity in the distribution of endothelial nitric oxide synthase in

the cardiac endothelium and cardiomyocytes with the activity that is more intense in the endocardial endothelial cells

– endothelial nitrous oxide synthase activity is only partially expressed under the basal conditions in normal cardiomyocytes and can be affected by a number of mediators

– nitric oxide plays a significant role in modulating cardiac function in response to a specific stimulus, or in a state of myocardial stress, sympathetic or parasympathetic neurotransmitter releases, muscle stretching, and similar.

– endothelial nitrous oxide synthase-derived nitric oxide has a vasodilatation and atheroprotective effect, while nitric oxide synthesized by inducible nitrous oxide synthase in macrophages, which has a much higher capacity, has oxidative and anti-atherogenic function.

– it promotes or inhibits the growth factors which are important in controlling the growth and remodeling of the heart

– together with other pathogenic mediators, enhanced release or lack of nitric oxide may lead to heart failure.

The increased activity of inducible nitrous oxide synthase enzymes in the insufficient cardiac tissue leads to the increased nitric oxide synthesis, resulting in the decrease of contraction strength. However, measuring of inducible nitrous oxide synthase levels during heart failure has led to disappointingly inconsistent data due to different methods of determining the enzyme activity and different etiology of heart failure. The discovery of the modulatory effect exerted by nitric oxide on the contractility, rhythm, growth and remodeling of the heart has prompted considerable interest in the possibility of reversibility of cardiac dysfunction via nitric oxid synthase inhibitors.

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Rad je primljen 10. I 2014.
Recenziran 22. V 2014.
Prihvaćen za štampu 30. V 2014.
BIBLID.0025-8105:(2014):LXVII:9-10:345-352.

Štampu ovog časopisa ”Medicinski pregled” pomogla je **Vlada AP Vojvodine**

Printing of this journal ”Medical Review” has been supported by **the Government of the AP of Vojvodina**

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∗ *Sveska sa suplementom*

Gardos G, Cole JO, Haskell D, Marby D, Pame SS, Moore P. The natural history of tardive dyskinesia. J Clin Psychopharmacol 1988;8(4 Suppl):31S-37S.

∗ *Sažetak u Časopisu*

Fuhrman SA, Joiner KA. Binding of the third component of complement C3 by Toxoplasma gondi [abstract]. Clin Res 1987;35:475A.

Knjige i druge monografije:

∗ *Jedan ili više autora*

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

∗ *Urednik(ci) kao autor*

Danset J, Colombani J, eds. Histocompatibility testing 1972. Copenhagen: Munksgaard, 1973:12-8.

∗ *Poglavlje u knjizi*

Weinstein L, Shwartz MN. Pathologic properties of invading microorganisms. In: Soderman WA Jr, Soderman WA, eds. Pathologic physiology: mechanisms of disease. Philadelphia: Saunders;1974. p. 457-72.

∗ *Rad u zborniku radova*

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

∗ *Disertacije i teze*

Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [dissertation]. Mount Pleasant (MI): Central Michigan University; 2002.

Elektronski materijal

∗ *Članak u Časopisu u elektronskoj formi*

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 1 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htmArticle

∗ *Monografije u elektronskoj formi*

CDI, clinical dermatology illustrated [monograph on CDROM]. Reevs JRT, Maibach H. CMEA Multimedia Group, producers. 2nd ed. Version 2.0. San Diego:CMEA;1995.

∗ *Kompjuterski dokument (file)*

Hemodynamics III: the ups and downs of hemodynamics [computer program]. Version 2.2. Orlando (FL): Computeried Educational Systems; 1993.

6. Prilozi (tabele, grafikoni, sheme i fotografije).

Dozvoljeno je najviše šest priloga!

– Tabele, grafikoni, sheme i fotografije dostavljaju se na kraju teksta rukopisa, kao posebni dokumenti na posebnim stranicama.

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– Slike pripremiti u JPG, GIF TIFF, EPS i sl. formatu

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– Naslov, tekst u tabelama, grafikonima, shemama i legendama navesti na srpskom i na engleskom jeziku.

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Prijem rukopisa vrši se u elektronskoj formi na stranici: **aseestant.ceon.rs/index.php/medpreg/**. Da biste prijavili rad morate se prethodno registrovati. Ako ste već registrovani korisnik, možete odmah da se prijavite i započnete proces prijave priloga u pet koraka.

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Vase Stajića 9
21000 Novi Sad
Tel. 021/521 096; 063/81 33 875
E-mail: dlv@neobee.net

INFORMATION FOR AUTHORS

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4. Preliminary reports – up to 4 pages – contain scientific results of significant importance requiring urgent publishing; however, it need not provide detailed description for repeating the obtained results. It presents new scientific data without a detailed explanation of methods and results. It contains all parts of an original study in an abridged form.

5. Professional articles – up to 10 pages – examine or reproduce previous investigation and represent a valuable source of knowledge and adaption of original investigations for the needs of current science and practise.

6. Case reports – up to 6 pages – deal with rare casuistry from practise important for doctors in direct charge of patients and are similar to professional articles. They emphasize unusual characteristics and course of a disease, unexpected reactions to a therapy, application of new diagnostic procedures and describe a rare or new disease.

7. History of medicine – up to 10 pages – deals with history in the aim of providing continuity of medical and health care culture. They have the character of professional articles.

8. Other types of publications – The journal also publishes feuilletons, book reviews, extracts from foreign literature, reports from congresses and professional meetings, communications on activities of certain medical institutions, branches and sections, announcements of the Editorial Board, letters to the Editorial Board, novelties in medicine, questions and answers, professional and vocational news and In memoriam.

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The covering letter:

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– It must confirm that all the authors meet criteria set for the authorship of the paper, that they agree completely with the text and that there is no conflict of interest.

– It must state the type of the paper submitted (an original study, a review article, a preliminary report, a professional article, a case report, history of medicine)

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Use Microsoft Word for Windows to type the text. The text must be typed in font Times New Roman, page format A4, space 1.5 (for tables as well), borders of 2.5 cm and font size 12pt. The manuscript should contain the following elements:

1. The title page. The title page should contain a concise and clear title of the paper, without abbreviations, then a short title (up to 40 characters), full names and surnames of the authors (not more than 6) indexed by numbers corresponding to those given in the heading along with the full name and place of the institutions they work for. Contact information including the academic degree(s), full address, e-mail and number of phone or fax of the corresponding author (the author responsible for correspondence) are to be given at the bottom of this page.

2. Summary. The summary should contain up to 250 words, without abbreviations, with the precise review of problems, objectives, methods, important results and conclusions. It should be structured into the paragraphs as follows:

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– case reports should have the introduction, case report and conclusion

– review papers should have the introduction, subtitles corresponding to those in the paper and conclusion. It is to be followed by up to 10 Key Words from the list of Medical Subject Headings, MeSH of the American National Medical Library.

3. The summary in Serbian language. The summary in Serbian should be the translation of the summary in English, it should be structured in the same way as the English summary, containing up to 250 words, without any abbreviations.

4. The text of the paper. The text of original studies must contain the following: introduction (with the clearly defined objective of the study), material and methods, results, discussion, conclusion, list of abbreviations (if used in the text) and not necessarily, the acknowledgment mentioning those who have helped in the investigation and preparation of the paper.

– The text of a case report should contain the following: introduction (with clearly defined objective of the study), case report, discussion and conclusion.

– The text should be written in the spirit of Serbian language, without unnecessary abbreviations, whose first mentioning must be explained by the full term they stand for. Abbreviations should not be used in the title, summary and conclusion. Only commonly accepted abbreviations (such as DNA, MRI, NMR, HIV…) should be used. The list of abbreviations used in the text, together with the explanation of their meaning, is to be submitted at the last page of the manuscript.

– All measurements should be reported in the metric system of the International System of Units – SI. Temperature should be expressed in Celsius degrees (°C). and pressure in mmHg.

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Introduction contains clearly defined problem dealt with in the study (its nature and importance), with the relevant references and clearly defined objective of the investigation and hypothesis.

Material and methods should contain data on design of the study (prospective/retrospective, eligibili-

ty and exclusion criteria, duration, demographic data, follow-up period). Statistical methods applied should be clear and described in details.

Results give a detailed review of data obtained during the study. All tables, graphs, schemes and figures must be cited in the text and numbered consecutively in the order of their first citation in the text.

Discussion should be concise and clear, interpreting the basic findings of the study in comparison with the results of relevant studies published in international and national literature. It should be stated whether the hypothesis has been confirmed or denied. Merits and demerits of the study should be mentioned.

Conclusion must deny or confirm the attitude towards the problem mentioned in the introduction. Conclusions must be based solely on the author's own results, corroborating them. Avoid generalised and unnecessary conclusions. Conclusions in the text must be in accordance with those given in the summary.

5. References. References are to be given in the text under Arabic numerals in parentheses consecutively in the order of their first citation. Avoid a large number of citations in the text. The title of journals should be abbreviated according to the style used in Index Medicus (http://www.nlm.nih.gov/tsd/serials/lji.html). Apply Vancouver Group's Criteria, which define the order of data and punctuation marks separating them. Examples of correct forms of references are given below. List all authors, but if the number exceeds six, give the names of six authors followed by et 'al'.

Articles in journals

∗ *A standard article*

Ginsberg JS, Bates SM. Management of venous thromboembolism during pregnancy. J Thromb Haemost 2003;1:1435-42.

∗ *An organisation as the author*

Diabetes Prevention Program Research Group. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. Hypertension 2002;40(5):679-86.

∗ *No author given*

21st century heart solution may have a sting in the tail. BMJ. 2002;325(7357):184.

∗ *A volume with supplement*

Magni F, Rossoni G, Berti F. BN-52021 protects guinea pig from heart anaphylaxix. Pharmacol Res Commun 1988;20 Suppl 5:75-8.

∗ *An issue with supplement*

Gardos G, Cole JO, Haskell D, Marby D, Pame SS, Moore P. The natural history of tardive dyskinesia. J Clin Psychopharmacol 1988;8(4 Suppl):31S-37S.

∗ *A summary in a journal*

Fuhrman SA, Joiner KA. Binding of the third component of complement C3 by Toxoplasma gondi [abstract]. Clin Res 1987;35:475A.

Books and other monographs

∗ *One or more authors*

Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002.

∗ *Editor(s) as author(s)*

Danset J, Colombani J, eds. Histocompatibility testing 1972. Copenhagen: Munksgaard, 1973:12-8.

∗ *A chapter in a book*

Weinstein L, Shwartz MN. Pathologic properties of invading microorganisms. In: Soderman WA Jr, Soderman WA, eds. Pathologic physiology: mechanisms of disease. Philadelphia: Saunders; 1974. p. 457-72.

∗ *A conference paper*

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

∗ *A dissertation and theses*

Borkowski MM. Infant sleep and feeding: a telephone survey of Hispanic Americans [dissertation]. Mount Pleasant (MI): Central Michigan University; 2002.

Electronic material

∗ *A journal article in electronic format*

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [Internet]. 2002 Jun [cited 2002 Aug 12];102(6):[about 1 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htmArticle

∗ *Monographs in electronic format*

CDI, clinical dermatology illustrated [monograph on CD-ROM]. Reevs JRT, Maibach H. CMEA Multimedia Group, producers. 2nd ed. Version 2.0. San Diego:CMEA;1995.

∗ *Acomputer file*

Hemodynamics III: the ups and downs of hemodynamics [computer program]. Version 2.2. Orlando (FL): Computerized Educational Systems; 1993.

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