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Sanitetski major dr Stefan Nedok (1828–1878), prvi šef Unutrašnjeg (Internog) odeljenja Beogradske vojne bolnice i načelnik saniteta divizije i korpusa Srpske vojske u srpsko-turskim ratovima 1876. i 1877–78. godine, jedan je od lekara rođenih i odškolovanih van Srbije koji su, zadojeni idejom panslavizma, došli u siromašnu Srbiju da svojim znanjem i radom pomognu mladoj srpskoj državi, tek oslobođenoj od Otomanske carevine. Oni su bili utemeljivači sanitetske službe srpske vojske, čiji su predstavnici i dan-danas širom sveta poznati po stručnosti, humanosti i patriotizmu (vidi str. 792–6).
Ove godine, sanitetska služba Vojske Srbije proslavlja 175 godina svog organizovanog postojanja i rada (vidi Uvodnik, str. 711–3).

Medical Corps Major Dr. Stefan Nedok (1828–1878), the first Chief of the Internal Department, Belgrade Military Hospital and the Head of Medical Services of the Division and Corps of the Serbian Army in the Serbian-Turkish Wars in 1876 and 1877–1878 was one of the medical doctors born and educated out of the Kingdom of Serbia, but who, inspired with the idea of pan-slavism, came to the devastated Serbia to help with their knowledge and efforts to young Serbia, just liberated from the Ottoman Empire. They were the founders of the Medical Services in the Serbian Army. Their representatives still remain recognized worldwide for their exceptional medical skills, humanity and patriotism (see p. 792–6).
This year marks 175 years of the organized operation of the Medical Services in the Serbian Army (see Editorial, p. 711–3).

E D I T O R I A L / U V O D N I K

175 years of the Serbian Military Medical Corps (July 30, 1839 – July 30, 2014)

175 godina srpskog vojnog saniteta (30.7.1839 – 30.7.2014)

Aleksandar S. Nedok

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This issue of the *Vojnosanitetski Pregled* coincides with marking the Day of Medical Services of the Army of Serbia – the great jubilee of 175 years of the establishment of legally organized military medical services in the Army of Serbia. Namely, on July 30 (July 18 according to the old Julian Calendar) 1839 in the reborn Principality of Serbia the Decree was issued on the appointment of the members of the General Military Staff (*Glavni vojeni štab*) among which was the stuff Medical Doctor, the Head of the Military Medical Corps, as being the first time for this service to get the position of a separate unit within the military organization. That act was preceded by a few significant events, significant not only for the Army of Serbia, but also for the whole then Serbia.

On February 25, 1839 the new Serbian Constitution (sent from Constantinople, supported by Russian and Austrian governments, regulating the status of the state and law and inner organization of the autonomous Principality of Serbia) was red in the Tašmajdan Park. It resolved the inner clash between the autocratic Prince Miloš and the Serbian national representatives that used to shake the young state over the years. According to article 19 of that Constitution the Minister (*popečitelj*) of Internal Affairs "decides anything regarding the army" and "manages with any military medical affairs in the state".

Let us point out that both army and military medical corps existed even before this Constitution: that year in Serbia there were 8 physicians, 1 pharmacist, 1 MSc in Surgery, 3 medical practitioners, and 1 private pharmacist in Belgrade where there also were 1 physician and 1 MSc in Surgery in the service of Turkish pasha. Out of this number in the service of the Guard ("*gvardija*") of Prince Miloš there were 3 physicians (Lindenmeier, Beloni and Majnert, 1 MSc in Surgery – Slavuj, and 2 medical practitioners – Novaković and Kaparis).

According to these constitutional solutions and after passing the Law on "organizing central governing of the Principality of Serbia" (Government), in compliance with the Council of Serbian People on May 29 – June 10, 1839 Prince Miloš issued a Decree on "organizing a garrison army" to regulate inner af-

Izlazak ovog broja „Vojnosanitetskog pregleda" poklapa se sa obeležavanjem Dana sanitetske službe Vojske Srbije koja ove godine slavi veliki jubilej – 175 godina od uspostavljanja zakonom uređene sanitetske službe Srpske vojske. Naime, 30. jula (po starom julijanskom kalendaru 18. jula) 1839. godine, u obnovljenoj Kneževini Srbiji, donet je Ukaz o postavljanju članova Glavnog vojenog štaba, među njima i štab doktora, tj. načelnika vojnog saniteta, čime ova služba po prvi put zvanično dobija status posebne celine unutar vojne organizacije. Tom činu prethodilo je nekoliko važnih događaja, bitnih ne samo za srpsku vojsku, već za čitavu tadašnju Srpsku državu.

Dana 25. februara 1838. godine na beogradskom Tašmajdanu pročitan je novi srpski Ustav, poslat iz Carigrada i podržan od ruske i austrijske vlade, kojim je regulisan državnopravni status i unutrašnje uređenje autonomne Kneževine Srbije. Njime je rešen unutrašnji sukob između samovlasnog knjaza Miloša i srpskih narodnih predstavnika koji je nekoliko poslednjih godina potresao mladu državu. Prema čl. 19 toga Ustava popečitelj vnutreni djela (ministar unutrašnjih poslova) „raspoređuje sve što se tiče vojske" i „upravlja svim sanitetskim poslovima u državi".

Treba naglasiti da su i vojska i sanitet postojali i pre ovog Ustava; te godine u Srbiji je već bilo u državnoj službi osam lekara, jedan apotekar, jedan magistar hirurgije, tri lekara-empirika, kao i jedan privatni apotekar u Beogradu, gde su se u službi turskog paše, takođe, nalazili jedan lekar i jedan magistar hirurgije. Od ovog sastava, u službi kod Miloševe garde („gvardija") bila su tri lekara [Lindenmajer, Beloni i Majnert, jedan magistar hirurgije (Slavuj) i dva empiirika (Novaković i Kaparis)].

Na osnovu ovih ustavnih rešenja i posle donošenja zakona o „Ustrojeniju centralnog upravlenija Knjažestva srbskog" (Vlada), knjaz Miloš je, uz saglasnost Sovjeta naroda srbskog, 29. maja–10. juna 1839. doneo uredbu o „Ustrojeniju garnizone vojske" kojom se reguliše unutrašnja organizacija te, sada od turske Porte zvanično priznate, srpske unutrašnje vojske. Članom 6 predviđeno je formiranje Glavnog štaba i njegov na-

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fairs by internal army, then officially recognized by Turkish Porte. Article 6 anticipated the formation of General Staff and its Head "who cannot be under the rank of colonel and who will be directly under the orders of the Minister of Internal Affairs, and to whom any lower ranks in any military issues will be responsible". Article 7 anticipated 2 departments in the General Staff, 1 "of military profession" and another 2 "to meet the requirements of the army". Article 8 stated the personnel engagement of the General Staff including "1 physician".

Regarding the fact that Prince Miloš abdicated the position of Prince in favor of his undergraduate and already seriously ill son Milan as early as on 1 – 13 June 1839, further development of these legal regulations was overtaken by the "Deputy Office of His Royal Highness" by passing a Decree on appointing the members of the General Staff on 18 – 30 July, 1839, that at the list of the appointed personnel, item 7, indicated the name of "Dr. Emerich Lindenmeier for the staff physician". That was the way that the Serbian Military Medical Corps got its first chief!

Taken by his true devotion, Dr. Lindenmeier was directly at the head of the Military Medical Corps till 1845 when he was appointed to manage the whole Serbian Medical Corps, both civil and military, and indirectly till 1859, namely till coming back of old and vengeful Prince Miloš to the throne of Serbia, who dismissed him because he served under the rule of Advocates of Constitutionalism and the competitive family Karađorđević, although Dr. Lindenmeier always used to point out that he served to people and Serbia and not to the individuals and with no interest in politics. Dr. Lindenmeier showed his true devotion in 1862 when the Turks bombed Belgrade, and Prince Mihailo invited Dr. Lindenmayer to organize the complete management of the wounded with the help of the Belgrade physician Dr. Jovan Mašin, thus remaining totally loyal to his new homeland till his death in 1883!

On the basis of these documents, presented to the Military Health Department, Ministry of Defense, July 30 was accepted as the Day of the Serbian Army Medical Services.

All these 175 years the members of the Military Medical Corps were loyal to their homeland and people in peace and under war, always ready to help the wounded and ill, risking their own lives, proving that by their sacrifices and heroism in the wars of XIX and XX centuries. By that, but also by their high professionalism in any branches of medicine, pharmacy, dental medicine and veterinary, they were and remained known and recognized not only in Serbia but worldwide. Numerous peace and war medals, among which were those for heroism, were twinkled on their chests. In the Great War 2 physicians and 1 medical student were decorated with the Medal of Star and Sword of Karađorđe, historically the most brilliant medal for personal heroic deed.

This year's Day of the Serbian Army Medical Services, marking 175th birthday of its organized functioning, is the occasion to once more pay the deepest tribute to all its members starting from the founder to these days, from the numerous unknown heroes to the giants who carried out the glory of the Serbian Army and Military Medicine over the planet. Although they belonged to a so-called small nation and army, according to military medical procedures they put themselves among the

čelnik „koji ne može biti manje činom od polkovnika i koji će stajati neposredstveno pod nalozima popečitelja vnutrešni djela i kome će se svi niži činovi po svima strukama voenoga kruga odnositi". Član 7 je predvideo dva odeljenja u glavnom štabu, jedno „po struci službe vojene" i drugo „po struci nuždi potreba vojeni". Član 8 navodi personalni sastav Glavnog štaba u kome se nalazi i „jedan doktor".

Kako je već 1–13. juna 1839. knjaz Miloš odstupio sa mesta knjaza, prepustivši ga svome maloletnom i već teško bolesnom sinu Milanu, to je dalju razradu ovih zakonskih propisa na sebe preuzelo „Namesničestvo knjaževskog dostojanstva", donevši 18–30. jula 1839. godine Ukaz o postavljanju članova Glavnog vojenog štaba, u kome se na sedmom mestu spiska postavljenih lica navodi „doktor Emerih Lindenmajer za štab-doktora". Tako je srpski vojni sanitet dobio svoga prvog starešinu.

Doktor Lindenmajer je sa iskrenim zalaganjem bio na čelu vojnog saniteta direktno do 1845, kada je preuzeo rukovođenje celokupnim državnim sanitetom, građanskim i vojnim, a indirektno sve do 1859, do povratka ostarelog i osvetoljubivog knjaza Miloša na srpski presto, koji ga je smenio samo zato što je ostao da služi pod upravom Ustavobranitelja i konkurentske porodice Karađorđevića, iako je Lindenmajer uvek podvlačio da služi narodu i državi, a ne pojedincima i da ga ne interesuje politika. Svoju privrženost pokazao je 1862. u vreme turskog bombardovanja Beograda, kada je na poziv kneza Mihaila organizovao uz pomoć beogradskog fizikusa dr Jovana Mašina celokupnu brigu o ranjenicima, ostavši veran svojoj novoj domovini sve do svoje smrti 1883. godine.

Na osnovu ovih dokumenata koje smo predočili Sanitetskoj upravi u Ministarstvu odbrane, usvojen je 30. juli kao Dan saniteta Vojske Republike Srbije, što je objavljeno u „Službenom vojnom listu" br. 17 od 14. aprila 2008. godine.

Svih ovih 175 godina pripadnici vojnog saniteta bili su verno uz svoju državu i narod, u miru i u ratu, uvek spremni da pomognu povređenima i bolesnima, često rizikujući i sopstveni život, što su u ratovima XIX i XX stoleća svojim žrtvama i podvizima i dokazali. Po tome, ali i po visokoj stručnosti u svim granama medicine, farmacije, stomatologije i veterine, bili su i ostali poznati i uvažavani ne samo u zemlji, već i širom sveta. Veliki broj mirnodopskih i ratnih odlikovanja, među njima i brojna ratnička u vidu medalja i ordenja za hrabrost, krasila su njihove grudi. Samo u Velikom ratu dvojica lekara i jedan student medicine odlikovani su „Karađorđevom zvezdom sa mačevima", tim istorijski najsjajnijim srpskim ordenom za lični herojski poduhvat.

Poštujući međunarodne konvencije o brizi za neprijateljske ranjenike još od srpsko-turskih ratova 1876–1878, srpski vojni sanitet stekao je mnoge zvanične i lične zahvalnosti i pohvale. Sačuvani arhivski dokumenti srpskih sanitetskih jedinica, inostrana pisana svedočenja iz vremena ratova i pohvale Međunarodnog društva Crvenog Krsta rečito govore o tim plemenitim postupcima.

Ovogodišnji Dan sanitetske službe Vojske Srbije, kada se proslavlja 175. rođendan njenog organizovanog rada, prilika je da se još jednom oda duboko poštovanje svim njenim pripadnicima, od rodonačelnika do današnjih dana, od brojnih neznanih junaka do velikana koji su proneli slavu srpske

pioneers of world military medicine, and their heroic deeds are referred as such till today (sutures of blood vessels, direct blood transfusions under war conditions, prevention and care of night blindness to mention only some of them).

Next year, the first class of cadets of the Faculty of Medicine of Military Medical Academy, University of Defense, Belgrade will finish their 6-year studies as the first generation of new military physicians, bringing together the knowledge of modern medicine and the experience and deeds of their glorious predecessors, proud of them and responsible to continue their honorable and highly humane deeds by their own engagements in the years that are to come. The results shown till now in the studies of these future military physicians guarantee that they are on the right way to fulfill that aim.

VIVAT, FLOREAT, CRESCAT THE MILITARY MEDICAL CORPS OF THE ARMY OF SERBIA!

vojne i celokupne njene medicine širom planete. Iako pripadnici maloga naroda i male vojske, u nekim ratnomedicinskim postupcima svrstali su se među pionire svetske vojne medicine čija se dela i danas kao takva navode (šavovi krvnih sudova, direktne transfuzije krvi u frontovskim uslovima, prevencija i lečenje noćnog slepila...).

Sledeće godine prva klasa kadeta Medicinskog fakulteta Vojnomedicinske akademije, Univerziteta odbrane u Beogradu, završava šestogodišnje studije kao prva generacija novih vojnih lekara, sjedinjujući u sebi znanje savremene medicine sa iskustvima i delima svojih slavnih prethodnika, ponosna na njih i sa odgovornošću da svojim angažovanjem, u godinama koje slede, nastavi njihovo časno i duboko humano delo. Dosadašnji rezultati studija budućih vojnih lekara pokazuju da su na dobrom putu da to i ostvare.

VIVAT, FLOREAT, CRESCAT SANITETSKA SLUŽBA VOJSKE SRBIJE!

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ORIGINAL ARTICLE

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The A-test: assessment of functional recovery during early rehabilitation of patients in an orthopedic ward – content, criterion and construct validity

A-test: procena funkcionalnog oporavka tokom rane rehabilitacije bolesnika na ortopedskom odeljenju – validnost sadržaja, kriterijuma i konstrukcije

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Abstract

Background/Aim. The A-test was designed for assessment of functional recovery during early rehabilitation of patients in an orthopedic ward. This performance-based test consists of 10 items for assessing basic activities by a six level ordinal scale (0–5). Total scores can range from 0 to 50, i.e. from inability to perform any activity despite the help of therapists to complete independence and safety in performing all activities. The aim of this study was to examine the A-test validity. **Methods.** This prospective study was conducted in an orthopedic ward and included 120 patients [60 patients with hip osteoarthritis that underwent arthroplasty and 60 surgically treated patients with hip fracture (HF)] during early inpatient rehabilitation (1st–5th day). Validity was examined through 3 aspects: content validity – floor and ceiling effect, range, skewness; criterion validity – concurrent validity [correlation with the University of Iowa Level of Assistance Scale (ILAS) for patients with hip osteoarthritis, and with the Cumulated Ambulation Score (CAS) for patients with HF, Spearman rank correlation] and predictive validity [the New Mobility Score (NMS) 4 weeks after surgery, Mann-Whitney *U* test]; construct validity – 4 hypotheses: 1) on the fifth day of rehabilitation in patients underwent arthroplasty due to hip osteoarthritis, the A-test results will strongly correlate with those of ILAS, while the correlation with the Harris hip score will be less strong; 2) in patients with HF, the A-test results will be significantly better in those with allowed weight bearing as compared to patients whom weight bearing is not allowed while walking; 3) results of the A-test will be significantly better in patients with hip osetoarthritis than in those with HF; 4) the A-test results will be significantly better in patients younger than 65 years than in those aged 65 years and older. **Results.** The obtained results were: low floor (1%) and ceiling (2%) effect, range 0–50, skewness 0.57, strong correlation with ILAS for the patients with hip osteoarthritis ($r = -0.97$, $p = 0.000$) and with CAS for the patients with hip fracture ($r = 0.91$, $p = 0.000$) The patients with the A-test score 35 and more on the fifth day of rehabilitation ($n = 46$, $Md = 4$) had significantly higher NMS rank 4 weeks after surgery than the patients with the A-test score less than 35 ($n = 59$, $Md = 2$), ($U = 379$, $z = -6.47$, $p = 0.000$, $r = 0.63$). All 4 hypotheses were confirmed. **Conclusion.** The A-test is simple and valid instrument for everyday evaluation of pace and degree of functional recovery during early rehabilitation of patients surgically treated in an orthopedic ward.

Key words:
hip prosthesis; orthopedic procedures; postoperative period; physical therapy; recovery of function; predictive value of the tests.

Apstrakt

Uvod/Cilj. A-test je dizajniran za procenu funkcionalnog oporavka bolesnika na ortopedskom odeljenju. Ovaj *performance-based* test sastoji se od 10 stavki za procenu 10 bazičnih aktivnosti uz pomoć šestostepene ordinalne skale (0–5). Ukupni skor je u opsegu od 0 do 50, tj. od nesposobnosti bolesnika da izvede bilo koju osnovnu aktivnost uprkos pomoći fizioterapeuta do potpune samostalnosti i sigurnosti pri izvođenju svih osnovnih aktivnosti. Cilj ove studije bio je da se ispita validnost A-testa. **Metode.** Ova prospektivna studija sprovedena je na ortopedskom odeljenju i obuhvatila je 120 bolesnika (60 bolesnika sa osteoartritisom kuka kojima je učinjena artroplastika kuka i 60 hirurški lečenih bolesnika sa prelomom kuka) tokom rane rehabilitacije (1–5. dan). Validnost A-testa bila je ispitana kroz 3 aspekta: validnost sadržaja – efekat poda i plafona (*floor and ceiling effect*), opseg, asimetrija distribucije rezultata; validnost kriterijuma

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– konkurentna validnost [korelacija sa skalom *The University of Iowa Level of Assistance Scale* (ILAS) za bolesnike sa osteoartritisom kuka i sa skalom *The Cumulated Ambulation Score* (CAS) za bolesnike sa prelomom kuka, Spearman-ova *rank* korelacija] i prediktivna validnost [*The New Mobility Score* (NMS) 4 nedelje nakon operacije, Mann-Whitney *U*-test); validnost konstrukcije – ispitane su 4 hipoteze: 1) 5. dana rehabilitacije kod bolesnika kojima je urađena artroplastika zbog osteoartritisa kuka rezultati A-testa biće u jačoj korelaciji nego sa *Harris hip* skorom; 2) kod bolesnika sa prelomom kuka rezultati A-testa biće značajno bolji kod onih kojima je dozvoljen oslonac na operisanu nogu toikom hoda nego kod onih kojima to nije dozvoljeno; 3) rezultat A-testa biće značajno bolji kod bolesnika sa osteoartritisom kuka nego kod onih sa prelomom kuka; 4) rezultati A-testa biće značajno bolji kod bolesnika mlađih od 65 godina nego kod onih koji imaju ≥ 65 godina. **Rezultati.** Ustanovljen je nizak pod (1%) i plafon (2%) efekt, opseg 0–50, koeficijent asimetrije 0,57, kao i snažna korelacija sa ILAS-om za bolesnike sa osteoartritisom kuka (r = -0,97, p = 0,000) i sa CAS-om za bolesnike sa prelomom kuka (r = 0,91, p = 0,000). Bolesnici koji su imali skor A-testa veći od 35, petog dana nakon operacije (n = 46, Md = 4) imali su značajno veći NMS skor 4 nedelje nakon operacije od bolesnika sa skorom A-testa manjim od 35 (n = 59, Md = 2), (U = 379, z = -6.47, p = 0,000, r = 0,63). Sve četiri hipoteze su potvrđene. **Zaključak**. A-test je jednostavan i validan instrument za svakodnevno praćenje brzine i stepena funkcionalnog oporavka tokom rane rehabilitacije hirurški lečenih ortopedskih bolesnika.

Ključne reči:
kuk, proteza; ortopedske procedure; postoperativni period; fizikalna terapija; funkcija, povratak; testovi, prognostička vrednost.

Introduction

Patients in an orthopedic ward are heterogeneous, and this is the situation in all general hospitals. Surgical treatment is followed by early rehabilitation which usually lasts a short time, only a few days [1]. Adequate assessment of the functional recovery of patients in this period is important, not only for monitoring regaining functional ability, but also for an adequate (proper) dosage of physiotherapy and planning further rehabilitation. Simple instruments are needed to monitor the rehabilitation process, presenting the results of the work, and conducting clinical studies [2]. However, there are but a few tests that cover this period of rehabilitation [3].

A test that is the most adapted to the period of early rehabilitation is the University of Iowa Level of Assistance Scale (ILAS) [4, 5]. Its good to moderate validity, reliability and responsiveness were shown in the group of patients after hip and knee arthroplasty. The test assesses the four main activities through a seven-level ordinal scale (0–6) and walking speed. To calculate walking speed, stopwatch and path, length of exactly 13.4 m are required, which makes it complicated for everyday use, but also for clinical research. Thus, in an investigation of factors predictive of independence in transfers and ambulation after a hip fracture, only three basic functions of this test were used [6].

A few years ago, a Danish-Swedish team designed and tested the Cumulated Ambulation Score (CAS). This test is used to evaluate functional recovery in the first days after the surgical treatment of hip fractures [7–9]. The CAS measures the three main activities: getting up and returning to bed, sitting down and standing up from a chair and walking. In evaluating each activity, a three-level ordinal scale is used (0–2). The CAS has a good reliability [9, 10] and is a valid predictor of postoperative morbidity, mortality and rehabilitation [8]. The CAS is simple and applicable in daily practice. Although simplicity is a feature that attracted us to this test, it seemed to us that the test is too easy and that patients will quickly and easily reach the maximum score.

While the validity and reliability of the ILAS were tested in patients with hip and knee arthroplasty, CAS was examined in patients surgically treated for hip fractures. These two populations make up the majority of patients in our Orthopedic Ward. We needed one general test that would be valid and reliable in both populations, and whose simplicity would allow us to easely apply it every day. Therefore, we designed a test that could help in functional recovery assessment of patients in the Orthopedic Ward.

Ten years ago, we designed a test for assessment 9 basic activities that a patient needs to regain in this period. The tenth item of the test is walking endurance. Activities are evaluated using the six-ordinal scale (0–5). Total scores can range from 0 to 50, i.e. from inability to perform any activity despite the help of therapists to complete independence and safety in performing all the activities. The test was called A-test ("A" like Activity or Assessment). The A-test was first used in the study to assess the effects of preoperative physical therapy and education of patients who were scheduled for hip arthroplasty [10]. Then we continued to use it in everyday practice in the Orthopedic Ward.

The aim of this study was to examine the validity of the A-test through the evaluation of the functional abilities of patients who had been surgically treated for hip fractures and osteoarthritis.

Methods

Subjects

This prospective study was conducted in the Clinic for Orthopedic Surgery and Traumatology (COST), Military Medical Academy, Belgrade, and included 120 patients: 60 patients with acute hip fracture of both sexes who were, before the injury, able to walk with or without aids and up and down stairs (help of another person was allowed for this activity). This study did not include patients with dementia, pathological hip fracture, bilateral hip fractures, concurrent fracture in any other part of the body, and patients to whom surgical treatment was contraindicated; 60 patients who un-

derwent hip arthroplasty due to osteoarthritis, without significant mental disability, who were, before the operation, able to walk with or without aids and up- and downstairs (help of another person was allowed for this activity).

Exclusion criteria during the study were the occurrence of intraoperative or postoperative complications that prevented or delayed the beginning of rehabilitation, lethal outcome immediately after surgery and incomplete collected data for individual patient.

Procedure

All the patients were treated surgically. The modality of treatment depended on the type of fracture: osteosynthesis with dynamic hip screw was applied in patients with intertrochanteric fracture, and arthroplasty was performed in patients with fractures of the femoral neck (partial arthroplasty for older than 70 and total arthroplasty for younger than 70). All the patients admitted for arthritis of the hip underwent arthroplasty.

After the surgery, all the patients had the same rehabilitation treatment involving early mobilization of the patients at the bedside (from the first postoperative day, unless it does not allow the general condition of the patients), progressive verticalization (in accordance with the possibilities of the patient), walking with aids on the flat as well as up- and downstairs, practicing the basic activities of daily living (using the toilet, sitting down in a chair). Daily physical therapy treatment lasted 30 minutes, and it was implemented every day, except at the weekend. The modality of surgery determined allowable weight bearing when walking.

On admission, from all patients data on comorbidity and used drugs, mental and functional status before injury (for the patients with hip fracture) or on admission for the patients with hip osteoarthritis walking distance, the ability to walk up- and downstairs, use of walking aids, carrying out basic and instrumental activities, as well as socio-epidemiological data (marital status, housing conditions) were collected. Assessment of mental status was made using the Serbian version of shortened mental test score [11], while the functional status before injury was assessed by the New mobility score (NMS) [12]. Also, in the group of the patients with hip fracture assessment of general health status before arrival in hospital was done with Barthel index (BI) [13]. For the patients scheduled for hip arthroplasty, Harris hip score (HHS) [14] and Oxford hip score (OHS) [15] were used for estimation of osteoarthritis severity.

In the postoperative period, from the first day of rehabilitation until discharge, assessment of functional abilities of all patients was performed by the A-test, ILAS and CAS. In addition, the functional status of the patients who underwent arthroplasty due hip osteoarthritis was assessed with the HHS, as well as of the patients with surgically treated hip fracture with BI. In this report, we used only the results of the HHS gathered for the fifth day of rehabilitation.

By the protocol, postoperative complications that were slowed down the course of rehabilitation, the number of days of treatment and duration of hospitalization after surgery were recorded.

Finally, in order to investigate the predictive value of this test, four weeks after the surgery, the recovery of all the patients who underwent hip arthroplasty due hip osteoarthritis was assessed with the OHS, and for surgically treated patients with hip fracture assessment of recovery was done with BI. In addition, the functional status of all the patients was assessed with the NMS.

We conducted this research with the approval of the competent local Ethics Committee.

Measurement

The A-test is a performance-based test that assesses 10 activities necessary for everyday life that patients need to achieve in the first days after the surgery: turn to the side, from supine to sitting position, getting out of bed, go back to bed, standing, walking with aids, use of toilet and dining room chairs, walking up- and down stairs, walking endurance.

Depending on the success of performance, a patient is evaluated from 0 to 5 for each activity: score 5 – fully independent and secure; score 4 – completely independent but insecure (while performing activities, a patient needs the presence of another person, for example a family member); score 3 – activity performed with verbal suggestions of therapists; score 2 – requires adherence by a physiotherapist; score 1 – need full assistance of a physiotherapist; score 0 – activity is not achived.

Walking endurance is graded in a slightly different way: score 5 – a patient walks more than 100 meters; score 4 – a patient walks from 50 to 100 meters; score 3 – a patient walks from 20 to 50 meters; score 2 – a patient walks from 5 to 20 meters; score 1 – a patient walks across the room (up to 5 meters); score 0 – activity is not achieved.

For ease of grading walking endurance, we had a landmark in the hospital: score 5 – several times cross hospital corridor, score 4 – once cross hospital corridor, score 3 – two times cross ward hallway, score 2 – once cross ward hallway, score 1 – the patient walks across the room, score 0 – activity is not achieved.

The maximum sum is 50, which means that a patient is independent and secure in the performance of all activities envisaged in the early rehabilitation. The test is simple, convenient, taking no additional time and no additional equipment.

Validity

Validity or the extent to which an instrument measures what it intends to measure was examined from several aspects.

Content validity

The A-test was designed in 2002 in order to adequately assess and present the results of early rehabilitation in orthopedic ward. The form and content of the A-test were proposed by the physiatrist, while a group of experts, consisting of 4 physical therapists, 3 physiatrists and an orthopedic surgeon, supported the contents of the test with minor descriptions adjustments of each item. Initially, the A-test was used

for monitoring patients of interest for some studies. Since 2007 the A-test has been used in the routine practice of the rehabilitation team in the Orthopedic Ward. From January 2012 A-test has been an integral part of "Rehabilitation List" which is used for assessing the results of early rehabilitation of all patients in our hospital.

The A-test assesses the basic functions that a patient should regain during early rehabilitation in an orthopedic ward. Content validity is an extent to which an instrument contains items critical or appropriate to construct being measured [2]. Content validity is present when instrument fits for intended use and adequately covers measured domain with its items. An instrument that demonstrates content validity uses the full range of test results with a small asymmetry in the distribution of results and has a low ceiling and floor effect. We thought that the A-test would have a satisfactory content validity if its results collected from the first to fifth day of early rehabilitation (as it usually lasts in COHT) ranged from 0 to 50, if the distribution was such that the skew values were less than 1.00, and if less than 15% of the result had the minimum or maximum total scores of the test [2].

We examined, per days of rehabilitation, what ceiling and floor effect of the A-test, ILAS and CAS was for all patients and separately for patients with hip osteoarthritis and patients with hip fracture. By this, we intended to verify whether all these tests can be applied to all patients, and whether and when their measurable domain ends.

Criterion validity

Concurrent validity and predictive validity were examined within criterion validity.

Concurrent validity refers to the ability of an instrument to assess the current state of a patient. The instrument is compared with the existing measurement tool (the criterion). Since the validity of the ILAS was confirmed in patients after hip and knee arthroplasty, we examined the correlation between the A-test and ILAS in patients with hip osteoarthritis who had underwent arthroplasty. On the other hand, the validity of the CAS has been demonstrated in patients with hip fracture, so we correlated the A-test results with results of the CAS in this patient group. The magnitude and direction of the association between the results were calculated using the Spearman's rank correlation. The value of rho between 0.10 and 0.29 pointed to weak, from 0.30 to 0.49 to moderate, and from 0.50 to 1.00 to strong correlation. Statistical analyses were performed using SPSS version 10.0.

Predictive validity refers to the ability of the instrument to predict the future condition of a patient. In order to examine it, all the patients were evaluated using the NMS 4 weeks after surgery. Further, the overall condition of the patients surgically treated due to hip fracture was assessed with the BI, and patients who underwent hip arthroplasty due to osteoarthritis completed the OHS. The results of the OHS were calculated on http://www.orthopaedicscore.com/scorepages/oxford_hip_score.html, where the score range is from 0 to 48, a higher score indicating better function of the joint. Based on the A-test results of the fifth rehabilitation day, the patients were divided into two groups. The patients with A-test score 35 and higher made up the first group. These patients performed most activities independently and the help of the physiotherapist was reduced to verbal suggestion for some activities. The patients with the score less than 35 formed the group II. The physiotherapist's help in performing some or all activities from the early rehabilitation program was required by these patients. In this way, we divided all the patients and particularly the patients with hip fractures and hip osteoarthritis. We analyzed whether the two groups differ in the results of NMS, BI and OHS 4 weeks after operation. The differences between the groups were tested using the Mann-Whitney *U*-test.

Construct validity

Construct validity is present when there is a relationship between the instrument and various hypotheses. In order to confirm the construct validity, we set up several hypotheses to examine the relationship between A-test and other measures and parameters of the observed population.

The first hypothesis was: on the fifth day of rehabilitation in the group of patients who underwent arthroplasty due to hip osteoarthritis, the A-test results will strongly correlate with the results of ILAS, while the correlation with the Harris hip score will be less strong. To test this hypothesis, we used Spearman's rank correlation.

The second hypothesis was: for patients with hip fracture, the A-test results will be significantly better in patients with allowed weight bearing as compared to patients whom weight bearing is not allowed while walking.

The third hypothesis was: the A-test results will be significantly better for patients with hip osteoarthritis compared to the A-test results of patients with hip fracture.

The fourth hypothesis was: the A-test results will be significantly better in patients younger than 65 years compared to patients aged 65 years and older.

We tested the results between the groups for the second, third and fourth hypothesis by Mann-Whitney *U*-test.

Results

Out of 120 patients included in the study, 15 patients (10 with hip fracture and 5 with osteoarthritis of the hip) were excluded during the study: 2 patients with intertrochanteric fracture were excluded due to poor operative stabilization of the fracture and orthopedic surgeon recommendations to rest after surgery, 2 patients with hip fracture were excluded due to cardiac disorders and recommendations of cardiologists to delay mobilization, 3 patients (2 with hip fracture and one with osteoarthritis) were excluded because of the debilitating diarrhea, severe electrolyte imbalances and extreme hypotension so physiatrist recommended postponing initiation of early rehabilitation, in 1 patient with hip fracture and with symptoms of pulmonary embolism, early rehabilitation was interrupted in the first days after surgery as recommended by pulmonologists, 4 patients died in the first days after surgery (3 patients with hip fracture and one with

osteoarthritis of the hip), 3 patients with osteoarthritis had no completely collected data (hospital discharge was performed before the seventh day after surgery).

We did not delay the start of early rehabilitation because of complications occurred in other patients such were: confusion, gastric complaints, hypotension, urinary tract infection, short-term diarrhea, the occurrence of pressure ulcers in the sacral region and on the feet, vomiting.

Demographic characteristics, comorbidity, mental and functional status before admission (for the patients with hip osteoarthritis) or injury (for the patients with hip fracture), hospital stay and duration of early rehabilitation are shown in Table 1. Due to the large influx of patients in the Orthopedic Ward, patients were discharged relatively quickly, so most patients in both populations had only 5 days of early rehabilitation.

Content validity

All A-test results obtained from first to fifth day of early rehabilitation (105 patients × 5 days of rehabilitation = 525 measurements) were in the maximum possible range from 0 to 50, skewness was 0.57, 1% of the results had a minimum total score and 2% had the maximum total scores, which fulfilled our criteria for good content validity.

The A-test and ILAS had low floor and ceiling effect observed for the whole population, from the first to the fifth day of rehabilitation (Table 2).

The result was similar when analyzed separately the total scores of patients with hip fracture and osteoarthritis. From the third day of rehabilitation, CAS has expressed ceiling effect observed in the results of the entire patient population (21% on the third day – 40% on the fifth day of rehabilitation). A similar result was observed in the group of patients with osteoarthritis from the second day of rehabilitation (22% on the second day – 64% on the fifth day of rehabilitation). But even for the patients with hip fracture, ceiling effect has been increased on the fifth day of rehabilitation, when almost approaching the given criterion of 15%.

Criterion validity

Concurrent validity

Analyzing all data collected during early rehabilitation of patients undergoing arthroplasty due to hip osteoarthritis (55 patients × 5 days of rehabilitation = 275 measurements with each test) we noticed a strong negative correlation between total scores of the A-test and ILAS (r = -0.97, n = 275, $p = 0.000$). The correlation is negative because the higher the

Table 1

Demographic characteristics, comorbidity, mental and functional status before admission injury, living environment, hospital stay and rehabilitation duration

Parameters	The group of patients with osteoarthritis of hip (n = 55) [mean ± SD; median (range) or number, percent]	The group of patients with hip fracture (n = 50) [mean ± SD; median (range) or number, percent]	*p*
Age (years)	65 ± 12; 53 (32–85)	75 ± 10; 76 (47–89)	0.000*
Female	32 (58%)	37 (74%)	0.088†
Number of comorbid diseases	1 ± 1; 1 (0–4)	2 ± 1; 2 (0–4)	0.005*
Number of used drugs	2 ± 2; 2 (0–8)	3 ± 2; 3 (0–9)	0.083*
Shortened mental test score (Serbian version)	10 ± 0; 10 (10–10)	9.84 ± 0.51; 10 (8–10)	0.017‡
New Mobility Score	7 ± 2; 6 (2–9)	7 ± 2; 9 (1–9)	0.009‡
Limited walking distance	41 (74.5%)	26 (52%)	0.016†
Aids when walking	28 (51%)	16 (32%)	0.050†
Up and down stairs with difficulty:	51 (93%)	32 (64%)	0.000†
Lives in the flat without elevator	18 (33%)	14 (28%)	
Lives alone	7 (13%)	10 (20%)	
Hospital stay (days)	7.44 ± 1.08, 7 (7–12)	8.52 ± 3.40, 7 (7–24)	0.035*
Rehabilitation (days)	5.25 ± 0.78, 5 (5–10)	6.20 ± 2.28, 5 (5–16)	0.007*
5 days of rehabilitation	46 (84%)	33 (66%)	

***t*-test; †Pearson's Chi-Square; ‡Mann Whitney Test**

Table 2

Floor and ceiling effect of the A-test, the University of Iowa Level of Assistance Scale (ILAS) and the Cumulated Ambulation Score (CAS)

Patients	Score	Day of rehabilitation														
		1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th	1st	2nd	3rd	4th	5th
		A-test					ILAS					CAS				
All patients (n = 105)	Minimal (%)	3	1	1	1	0	0	1	2	3	3	12	3	2	3	1
	Maximal (%)	0	0	1	3	5	9	2	2	3	1	2	12	21	35	40
Patients with hip osteoarthritis (n = 55)	Minimal (%)	0	0	0	0	0	0	2	4	6	6	4	0	0	0	0
	Maximal (%)	0	0	2	6	9	4	0	0	0	0	4	22	36	60	64
Patients with hip fracture (n = 50)	Minimal (%)	6	2	2	2	0	0	0	0	0	0	22	6	4	6	2
	Maximal (%)	0	0	0	0	0	14	4	4	6	2	0	2	4	8	14

A-test the total scores indicate better functioning ability, which is reversed in the ILAS.

Also, all collected results of the A-test and CAS for patients after surgically treated hip fractures strongly correlated (r = 0.91, n = 250, $p = 0.000$).

Predictive validity

The patients with the A-test score 35 and more on the fifth day of rehabilitation (n = 46, Md = 4) had a significantly higher NMS rank 4 weeks after surgery than patients with the A-test score less than 35 (n = 59, Md = 2), ($U = 379$, z = -6.47, $p = 0.00$, r = 0.63).

In the group of patients who had been surgically treated due to hip osteoarthritis, patients with the A-test score 35 and more on the fifth day of rehabilitation (n = 37, Md = 36) had a significantly better OHS rank 4 weeks after the surgery than patients with the A-test score less than 35 (n = 18, Md = 29), ($U = 125$, z = -3.74, $p = 0.00$, r = 0.51).

Also, in the group of patients who had been surgically treated for hip fractures, Mann Whitney *U*-test revealed a significant difference in BI rank 4 weeks after the surgery in the patients with the A-test score 35 and higher (n = 9, Md = 95) compared with these with the A-test scores of less than 35 (n = 41, Md = 80) on the fifth day of rehabilitation, ($U =$ 19:50, z = -4.20, $p = 0.00$, r = 0.59).

Construct validity

All four hypotheses were confirmed.

The first hypothesis: on the fifth day of rehabilitation in the group of patients with arthroplasty due to hip osteoarthritis, the A-test results strongly correlated with the results of ILAS (rho = -0.94, n = 55, $p = 0.000$), while the correlation with the Harris hip score was moderate (rho = 0.49, n = 55, $p = 0.000$).

The second hypothesis: on the fifth day of rehabilitation, in the group of patients with hip fracture, the A-test scores rank was significantly better in patients with allowed weight bearing (n = 26, Md = 22) as compared to patients whom weight bearing was not allowed while walking (n = 24; Md = 7.5) ($U = 112.00$, z = -3.89, $p = 0.000$, r = 0,52).

The third hypothesis: the A-test scores rank was significantly higher for patients with hip osteoarthritis compared to the A-test scores rank of patients with hip fracture during early rehabilitation (Table 3).

The fourth hypothesis: the A-test results were significantly better in the patients younger than 65 (n = 33, Md = 44) compared to the patients aged 65 and older (n = 72, Md = 19.5) ($U = 449.50$, z = -5.10, $p = 0.000$, r = 0.50).

Discussion

This study investigated the validity of the A-test in the assessment of functional recovery of patients treated surgically due to hip fracture and osteoarthritis in an orthopedic department. In the Orthopedic Ward, the patients with different clinical entities were managed, but two large groups of patients dominated: the patients with arthroplasty due to osteoarthritis of the hip and the patients treated surgically for hip fractures. It may be noted that these two groups of patients are quite different in premorbid characteristics. The patients with hip fracture were significantly older and with more associated diseases. They also had occasional mild mental difficulties before injury, mostly related to the recall of new information, while patients scheduled for arthroplasty had perfectly satisfactory mental state. The patients with hip fracture had good mobility before the injury, even better than the patients with osteoarthritis. However, their stay in hospital lasted longer after the surgery, and therefore early rehabilitation was longer. These two groups also differed in the degree and pace of recovery after the surgery. Despite these differences, both groups of patients experienced the same type of functional disability after surgery. Our test was based on the assessment of their ability. Could they indeed be assessed by the same instrument?

The floor and ceiling effects that are usually considered within content validity [16, 17], provide part of the answer to this question. After analyzing the floor and ceiling effects of the A-test, ILAS and CAS results collected from the first to the fifth rehabilitation day, we can say that the content of the A-test and ILAS can cover a period of early rehabilitation in a heterogeneous patient population in an orthopedic ward. In patients with osteoarthritis of the hip, floor effect of the ILAS and ceiling effect of the A-test slightly higher than 5% appears on the fourth and the fifth day of rehabilitation. This indicates that these patients by the end of the fifth day of rehabilitation were ready for some more sophisticated tests. It can be noted that no patient in the group with hip fracture reached the maximum score during the first five days of rehabilitation. That would mean that the A-test could be used for assessing functional ability of these patients during further subacute rehabilitation. This area of the A-test application could be the subject of future research.

The CAS is primarily intended for assessment of the functional recovery of patients with hip fracture. Therefore, the expressed ceiling effect of the results of patients with hip osteoarthritis, already on the second day of rehabilitation, was not surprising. But for patients with hip fracture, ceiling

Table 3

The A-test scores from the first to the fifth day of rehabilitation – differences between patients with hip osteoarthritis and patients with hip fracture

Rehabilitation day	The group of patients with osteoarthritis of hip (n = 55) [mean ± SD, median (range)]	The group of patients with hip fracture (n = 50) [mean ± SD, median (range)]	Mann Whitney *U*-Test			
			U	z	*p*	r
1st	10 ± 9; 8 (1–42)	5 ± 5; 2 (0–17)	774.5	-3.880	0.000	0.379
2nd	22 ± 13; 18 (2–48)	10 ± 8; 7,5 (0–36)	549.0	-5.307	0.000	0.518
3rd	30 ± 13; 29 (7–50)	13 ± 10; 12 (0–43)	389.0	-6.630	0.000	0.647
4th	36 ± 12; 42 (11–50)	15 ± 13; 11 (0–47)	343.5	-6.622	0.000	0.646
5th	38 ± 12; 43 (12–50)	18 ± 14; 13,5 (1–47)	392.5	-6.308	0.000	0.615

effect also increased on the fifth day of rehabilitation, and almost reaches 15%. Therefore the application of this test remains limited to the elderly and frail patients with hip fractures with delayed recovery [9].

Strong correlations between the A-test and ILAS and similar content validity provide a recommendation to use both tests in the early period of rehabilitation. The differences in structure between the A-test and ILAS significantly affect the feasibility of these two tests. The A-test includes more activities, but has a simpler scale. The scale is designed to remind on the grades that pupils receive during elementary and secondary school in the region. Also, the program of early rehabilitation is called "school of walking". The scoring is close to the patient and they can easily monitor their progress in the "school of walking". But in the case of the A-test, as performance-based test, it is important that the scale is not complex for the physiotherapist and does not affect the reproducibility of the results. However, what makes the ILAS difficult for everyday practice is not more complex scale, but the assessing and calculation of walking speed. To test and calculate the speed, a stopwatch, the path length of exactly 13.4 m and extra time are required. We marked the path in the hallway of the ward for research purposes. Patients with hip fracture often do not have sufficient duration when walking to get to the start of the track or to cross the entire path. There were no many complaints and observations of patients who participated in the survey for this item, but some patients sometimes simply were not willing to undergo this test.

Unlike the ILAS, during evaluation of the activities by the A-test, patients are unaware that their activities are assessed. Each activity of the A-test is an integral part of early rehabilitation program. And most importantly, the therapist, who conducts the assessment, has no additional obligation during the session. After treatment, the physiotherapist records the degree of autonomy which the patient has achieved for a particular activity from the early rehabilitation program in the A-test form. And for this activity the physiotherapist does not need more than a minute. And when the two tests have similar reliability and validity, this difference between the tests becomes very important [18].

The predictive validity of our instrument is very important for planning further rehabilitation of patients and rational use of health facilities. This study showed that based on the A-test total score on the fifth day of rehabilitation, we can predict what the functional ability of patients 4 weeks after the surgery will be.

In addition, the A-test showed that it has satisfactory discriminative ability, convergent and divergent validity, which gives it a good recommendation for clinical use. The A-test and ILAS measure the same construct – patient's functional ability, and the HHS beside function, estimate pain and range of motion in the hip. Therefore we are not surprised that the A-test results strongly correlate with the results of the ILAS, while the correlation with the results of the HHS has a moderate magnitude on the fifth day of rehabilitation.

Although we tried to examine all the recommended aspects of validity [2, 19–21], what's missing in this study is Rasch analysis of the A-test. This will be our future task.

Conclusion

Early rehabilitation in an orthopedic ward usually lasts for a short time. However, it is an important period for the restoration of patient's functional capacity and determination of further objectives and modalities of rehabilitation. The lack of simple and easy tests for assessing functional recovery of heterogeneous population of patients in an orthopedic ward is noticeable. The results of this study show that the A-test could be a valid instrument for evaluation monitoring the pace and degree of functional recovery of surgically treated patients for hip fractures and osteoarthritis of the hip during early rehabilitation.

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Diagnostic value of combined magnetic resonance imaging examination of brachial plexus and electrophysiological studies in multifocal motor neuropathy

Značaj kombinovane primene elektrofizioloških ispitivanja i ispitivanja magnetne rezonance brahijalnog pleksusa za potvrdu dijagnoze multifokalne motorne neuropatije

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Abstract

Background/Aim. Multifocal motor neuropathy (MMN) is an immune-mediated disorder characterised by slowly progressive asymetrical weakness of limbs without sensory loss. The objective of this study was to investigate the involvement of brachial plexus using combined cervical magnetic stimulation and magnetic resonance imaging (MRI) of plexus brachialis in patients with MMN. We payed special attention to the nerve roots forming nerves inervating weak muscles, but without detectable conduction block (CB) using conventional nerve conduction studies. **Methods.** Nine patients with proven MMN were included in the study. In all of them MRI of the cervical spine and brachial plexus was performed using a Siemens Avanto 1.5 T unit, applying T1 and turbo spin-echo T1 sequence, axial turbo spin-echo T2 sequence and a coronal fat-saturated turbo spin-echo T2 sequence. **Results.** In all the patients severe asymmetric distal weakness of muscles inervated by radial, ulnar, median and peroneal nerves was observed and the most striking presentation was bilateral wrist and finger drop. Three of them had additional proximal weakness of muscles inervated by axillar and femoral nerves. The majority of the patients had slightly increased cerebrospinal fluid (CSF) protein content. Six of the patients had positive serum polyclonal IgM anti-GM1 antibodies. Electromyoneurography (EMG) showed neurogenic changes, the most severe in distal muscles inervated by radial nerves. All the patients had persistent partial CBs outside the usual sites of nerve compression in radial, ulnar, median and peroneal nerves. In three of the patients cervical magnetic stimulation suggested proximal CBs between cervical root emergence and Erb's point (prolonged motor root conduction time). In all the patients T2-weighted MRI revealed increased signal intensity in at least one cervical root, truncus or fasciculus of brachial plexus. **Conclusion.** We found clinical correlation between muscle weakness, prolonged motor root conduction time and MRI abnormalities of the brachial plexus, which was of the greatest importance in the nerves without CB inervating weak muscles.

Key words:
peripheral nervous system diseases; diagnostic techniques and procedures; brachial plexus; magnetic resonance imaging; electrophysiology.

Apstrakt

Uvod/Cilj. Multifokalna motorna neuropatija (MMN) je imunski posredovano oboljenje perifenih nerava koje karakteriše sporo napredovanje asimetričnih slabosti mišića ekstremiteta, bez poremećaja senzibiliteta. Cilj rada bio je da se ispita značaj kombinovane primene magnetne stimulacije u cervikalnom nivou i magnetne rezonance (MR) brahijalnog pleksusa u potvrdi proksimalnih blokova provođenja kod obolelih od MMN. Takođe, želeli smo da utvrdimo da li postoje znaci oštećenja nervnih korenova koji grade nerve koji inervišu klinički slabe mišiće, kod kojih konvencionalnim ispitivanjem provodljivosti perifernih nerava nije registrovano postojanje blokova provođenja (BP). **Metode.** U studiju je bilo uključeno devet bolesnika sa klinički i elektrofiziološki potvrđenom dijagnozom MMN. Svim bolesnicima urađen je

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MR vratnog dela kičme i brahijalnog pleksusa u T1 i turbo spin-eho T1 sekvenci, aksijalnoj turbo spin-eho T2 sekvenci i koronarnoj turbo spin-eho T2 sekvenci sa saturacijom masti, pomoću aparata Simens Avanto jačine 1.5 T. **Rezultati.** Kod svih bolesnika registrovana je izražena asimetrična distalna slabost mišića inervisanih radijalnim, ulnarnim, medijalnim i peronealnim nervima, sa najupečatljivijom kliničkom prezentacijom viseće šake i prstiju. Analizom cerebrospinalnog likvora zabeležena je blaga proteinorahija kod većine obolelih. U serumu šest bolesnika nađena su poliklonska anti-GM1 antitela. Elektromioneurografija (EMG) pokazala je znake neurogene lezije, predominantno u distalnoj muskulaturi inervisanoj radijalnim nervom. Kod svih bolesnika registrovan je parcijalni BP van uobičajenih mesta kompresije radijalnog, ulnarnog, medijalnog i peronealnog nerva, a MR pregledom detektovane su zone pojačanog intenziteta signala u najmanje jednom cervikalnom korenu, trunkusu ili fascikulusu brahijalnog pleksusa. Kod tri bolesnika kod kojih standardnim elektroneurografskim pregledom nije registrovano postojanje BP primenom magnetne stimulacije u cervikalnom nivou sugerisani su proksimalni BP, što je bilo u korelaciji sa MR promenama odgovarajućih cervikalnih korenova. **Zaključak.** Rezultati ispitivanja pokazuju korelaciju između mišićne slabosti, produženog vremena provođenja kroz motorne korenove i promena na MR brahijalnog pleksusa, što je od posebnog značaja za nerve koji inervišu klinički slabe mišiće, a kod kojih primenom konvencionalne elektroneurografije nije moguće detektovati BP.

Ključne reči:
živci, periferni, bolesti; dijagnostičke tehnike i procedure; plexus brachialis; magnetna rezonanca, snimanje, elektrofiziologija.

Introduction

Multifocal motor neuropathy (MMN) is a chronic, slowly progressive immune-mediated neuropathy, characterized by progressive, predominantly distal, asymmetric limb weakness, mostly affecting upper limbs, minimal or no sensory impairment, and the presence of multifocal persistent partial conduction blocks (CB) on motor, but not sensory nerves [1]. It is a rare disorder with an estimated prevalence of 1–2/100,000 individuals, more frequently present in men. MMN predominantly affects young people and almost 80% of patients develop first symptoms between 20 and 50 years of age [2]. Evidence of CBs is the electrophysiological hallmark of MMN and must be found at sites distinct from common entrapment or compression syndromes [3]. CBs may occur in any motor nerve, but have been more frequently reported in the distal segment of upper limb nerves [4].Very proximal CBs located in the sites above Erb's point cannot be detected by routine nerve conduction studies (NCS) [3, 5]. Transcranial magnetic stimulation (TMS) technique combined with peripheral conduction time can detect CB between root emergence and Erb's point (motor root conduction time) [5]. Proximal CB may be also confirmed by incresed signal in cervical roots, truncus or fasciculus of the brachial plexus by magnetic resonance imaging (MRI) [6]. Supportive diagnostic criteria include elevated serum anti-GM1 antibodies [7].

The purpose of this study was to investigate the involvement of cervical roots, truncus or fasciculus of brachial plexus by MRI examination in patients with the proven diagnosis of MMN, especially in the cases without conventional electrophysiological proof of the CB. Our hypothesis was that MRI of the brachial plexus combined with magnetic stimulation at cervical level could give valuable contribution to confirmation of proximal CB. These are not accesible to evaluation by conventional nerve conduction studies.

Methods

The clinical diagnosis of MMN was based on the presence of a chronic or stepwise progressive asymmetric limb weakness with a multineuropathic distribution affecting the muscles of at least two distinct motor nerves lasting at least two months, and minimal or no sensory loss or symptoms and absence of clinical upper motor neuron signs [2, 8, 9].

Electrophysiology

The standard methods of motor and sensory NCS and concentric needle EMG were performed. Motor NCS included distal motor latencies, compound muscle action potential (CMAP) amplitudes, conduction velocities and F wave latencies. Motor NCS were performed up to Erb's point in the median (recording: *m. abductor pollicis brevis*), ulnar (recording: *m. abductor digiti minimi*), radial (recording: *m. extensor indicis*) nerves, and up to the popliteal fossa in the deep peroneal (recording: *m. extensor digitorum brevis*) and tibial (recording: *m. abductor hallucis brevis*) nerves. CB was defined according to Europen Federation of Neurological Society/Peripheral Nerve Societies guidelines [10]. It was defined as definite motor CB: negative CMAP area reduction on proximal versus distal stimulation of at least 50% whatever the nerve segment length (median, ulnar and peroneal). Negative CMAP amplitude on stimulation of the distal part of the segment with motor CB must be > 20% of the lower limit of normal and > 1 mV (baseline negative peak) and an increase of proximal negative peak CMAP duration must be ≤ 30%; probable motor CB was defined as a negative CMAP area reduction of at least 30% over a long segment of an upper limb nerve with an increase proximal negative peak CMAP duration ≤ 30%, or negative CMAP area reduction of at least 50% (the same as definite) with an increase in proximal negative peak CMAP duration > 30%, and normal sensory nerve conduction in upper limb segments with CB and normal sensory nerve action potential amplitudes, evidence for conduction block must be found at sites distinct from common entrapment or compression syndromes [10]. Antidromic sensory NCS were investigated in the median, ulnar, radial and sural nerves. Concentric needle EMG was performed in proximal and distal upper and lower limb muscles.

In addition to conventional NCS, paravertebral magnetic stimulation at cervical level *via* a round coil (outer dia-

meter 90 mm) centered over the spinous process of C7 was applied in order to detect the most proximal conduction blocks. Motor root conduction time (MRCT) was calculated by subtracting latency of motor evoked potentials evoked by cervical magnetic stimulation and total peripheral motor conduction time (PMCT) obtained by electrical stimulation of corresponding nerve. PMCT was estimated from the latencies of the CMAPs and F-waves as follows (latency of CMAPs + latency of F-waves − 1)/2. MRCT was accepted as prolonged only for the peripheral nerves along which conductive block in more distal segments has not been previously found. MRCT was considered normal if the latency was shorter than 1.46 msec. We have decided to accept as possible proximal conductive blocks only cases with clear MRCT prolongation, since a possible inability to achieve supramaximal stimulation by magnetic stimulation leaves the least impact on latencies, contrary to significantly lower amplitudes and areas in such cases [5, 11].

Serum and cerebrospinal fluid analyses

Serum IgG and IgM antibodies to ganglioside GM1 were measured by enzyme linked immunosorbent assay (ELISA) before imunoglobulin treatment [12, 13]. The presence of monoclonal gammopathy was investigated by serum immunoelectrophoresis and immunofixation. CSF was examined for total protein concentration and cell count by standard procedures and by isoelectric focusing agarose gel electrophoresis for oligoclonal bands [8].

MRI of cervical spine and brachial plexus was performed in all patients using a Siemens Avanto 1.5 T unit. The following sequences were applied: an axial turbo spin-echo T1 sequence (FoV 280 mm, slice thickness 3.0 mm, TR 561 ms, TE 11 ms, flip angle 150 degree, acquisition number 1, base resolution 320), an axial turbo spin-echo T2 sequence (FoV 280 mm, slice thickness 3.0 mm, TR 3600 ms, TE 127 ms, flip angle 170 degree, acquisition number 1, base resolution 512), a coronal fat-saturated (FS) turbo spin-echo T1 sequence (FoV 350 mm, slice thickness 2.5 mm, TR 550 ms, TE 11 ms, flip angle 150 degree, acquisition number 1, base resolution 256) and a coronal fat-saturated (FS) turbo spin-echo T2 sequence (FoV 350 mm, slice thickness 2.5 mm, TR 7500 ms, TE 157 ms, flip angle 170 degree, acquisition number 1, base resolution 384). The T1 sequences were also made after application of paramagnetic contrast [14].

Results

Nine patients (5 male, 4 female), the mean age of 38 (range 22–53) years had the history of MMN (Table 1). The mean duration of the disease was 6.3 (range 3–12 years). In 7 of 9 (78%) patients the first symptom was asymmetric weakness of the finger extensors without wasting of the arm muscles (Figure 1). The course of the disease in all the patients was stepwise progressive, resulting in severe, asymmetric, distal weakness of muscles inervated by radial, ulnar, median and peroneal nerves and the most striking presentation was bilateral wrist and finger drop. Three of them (No 5, 7, and 8) had additional proximal weakness of muscles inervated by axillar and femoral nerves. In one patient (No 5) there was the facial nerve involvement with mental muscle myokimia and in one patient also the occurence of seropositive myasthenia gravis [6].

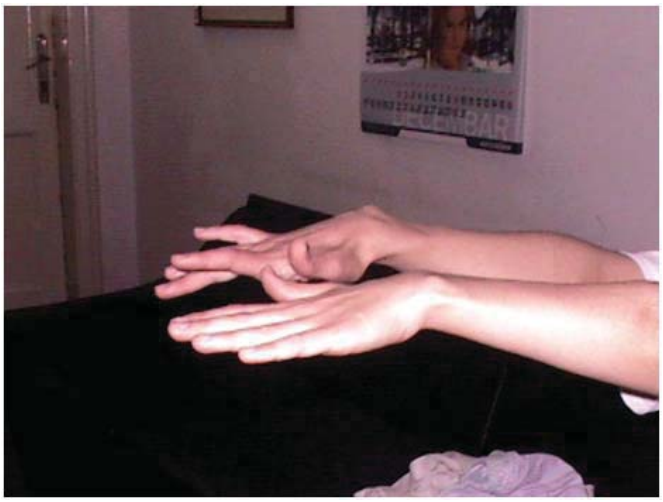

Fig. 1 – Typical presentation at the onset of the disease with asymmetric finger extensor weakness (the patient No 8). Different degrees of finger drop (III and IV fingers) imply differential conduction block in the terminal motor branches of the posterior interosseous nerve.

In six patients (No 1, 2, 5, 6, 7, 8) polyclonal IgM anti-GM1 antibodies were detected. Eight patients had a slightly elevated CSF protein content, ranging from 0.46 to 0.84 g/L (mean 0.58 g/L). In the patient No 5 with the very severe clinical presentation, oligoclonal IgG bands were detected in CSF.

Neurogenic EMG pattern was found in all affected muscles. Demyelination of the motor nerves and CBs were detected in all the patients at sites distinct from common entrapment syndromes, most frequently in the radial nerve (7/9), suggesting the diagnosis of MMN. In all of the analyzed patients MRI of the brachial plexus revealed asymmetric increased signal intensity on T2-weighted ima-

Table 1

Clinical, electrophysiological and magnetic resonance imaging (MRI) features of patients with multifocal motor neuropathy (MMN)

Patients' characteristics	Patient's number								
Sex / age (years) at onset	Male / 49	Male / 25	Male / 30	Male / 45	Male / 53	Female/ 37	Female / 52	Female / 26	Female / 22
Muscle weakness related to the distribution of individual nerve	Bilateral radial, bilateral ulnar, bilateral median, left peroneal	Bilateral radial, right median, bilateral peroneal, right tibial	Bilateral radial, bilateral ulnar	Left median, bilateral radial	Bilateral median, left ulnar, bilateral radial, left axilar	Bilateral radial, bilateral median, left ulnar	Right axillar, left radial, right median, right peroneal	Bilateral radial, bilateral peroneal, left femoral	Bilateral radial, left ulnar, right median
Motor nerves with CB (% of CMAP amplitude reduction)	Right ulnar (53%), left radial (90%), left peroneal (53%)	Right peroneal (66%), left peroneal (69%), right tibial (50%)	Right radial (70%), right median (50%)	Left ulnar (80%) left median (50%) rigt radial (60%)	Right radial (75%) left radial (80%) left median (60%)	Left radial (80%), right radial, (40%), left ulnar (45%)	Left radial (45%), left ulnar (56%)	Left radial (70%)	Left radial (45%)
MRI changes of brachial plexus	Increased signal intensity in median trunk right and C6 root left	Increased signal intensity in both C7 roots	Increased signal intensity in brachial plexus lower trunk left and inferior fasciculus right	Increased signal intensity in inferior fasciculus right and C8 root left	Increased signal intensity in C6 root left and truncus inferior right	Increased signal intensity in upper trunk left and C6 root right	Increased signal intensity in C6 and C7 roots on the right side	Increased signal intensity in inferior fasciculus right and C7 root left	Increased signal intensity in fasciculus medialis left and middle trunk right
TMS	Prolonged MRCT*	Extremely prolonged MRCT*					Prolonged MRCT*		

***MRCT – Motor root conduction time; CB – conduction block; CMAP – compound muscle action potential.**

ges as well as increased signal intensity on T1-weighted images after gadolinium enhancement. However, three patients (No 1, 2 and 7) with severe muscle weakness related to distribution of right median nerve, but without CB on coventional electroneurography on this nerve, were subjected to cervical magnetic stimulation. In all of them substantial MRCT prolongation (2–5 times longer) was obtained when motor evoked potentials was registered in corresponding thenar muscles, indicating the presence of proximal CB (Table 2). In those patients MRI of the brachial plexus also revealed high signal intensity in roots from which arises median nerve, corresponding to proximal CBs detected by paravertebral cervical magnetic stimulation (Figures 2 and 3).

Table 2

The results od magnetic paravertebral stimulation in patients with multifocal motor neuropathy (n = 9)

Patient's number	Registration site	MEP latency				
		cortical	spinal	$CMCT^M$	$CMCT^F$	MRCT
1	median right	30.75	19.03	9.64	4.74	4.73*
	ulnar right	27.63	20.64	6.99	5.57	1.42
2	median right	32.3	17.02	15.28	7.54	7.74*
	ulnar right	31.02	21.51	9.51	7.82	1.69
	median left	25.05	17.39	7.66	5.82	1.84
	ulnar left	28.07	20.16	7.91	5.87	2.04
7	median right	23.41	15.08	8.33	5.29	3.05*

All the values represent the absolute/relative latences of evoked responses expressed in msec.
MEP – motor evoced potential; PMCT –peripheral motor conduction time;
$CMCT^M$ (central motor conduction time) = Cortical MEP latency – spinal MEP latency;
$CMCT^F$ = Cortical MEP latency – PMCT; MRCT (motor root conduction time) = PMCT – spinal MEP latency
Prolonged MRCT indicates by asterixes.

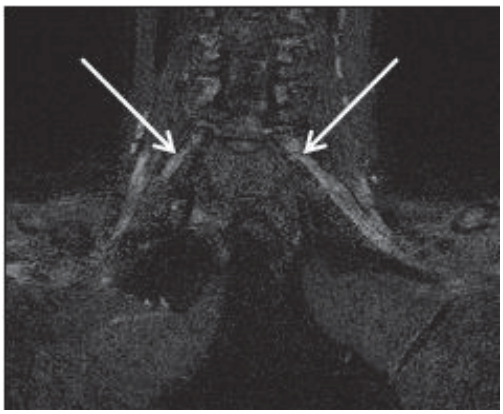

Fig. 2 – T2 FS sequence magnetic resonance imaging (MRI) shows increased signal intensity in both C7 roots in patient with multifocal motor neuropathy (the patient No 2).

Discussion

The clinical presentation and electrophysiological studies fulfilled criteria for the diagnosis of definite MMN in all the presented patients, also supported by serum and CSF findings. Similarly to many other reports, the upper limb onset with finger extension weakness as the first clinical manifestation of the disease was present in two-thirds of our patients [2, 15–17]. Three patients of our cohort had additional proximal weakness of muscles inervated by axillar and femoral nerves. On the contrary, some authors show that lower limb onset is present in 27% of patients with MMN [3].

In the majority of the analyzed patients in our study, CSF analyses show a slightly elevated protein content (0.58 g/L), which is in line with the data from the literature [16–18].

In six out of nine (66.7%) patients of our cohort polyclonal IgM anti-GM1 antibodies were detected. This result is in agreement with previous ones in which these antibodies were found in 22–85% of patients with MMN, but its relationship to the pathogenesis of the disease still remains unclear [19, 20]. Although there was no relationship between the antibody finding and the disease severity and there was no decline in anti-GM1 titer with immunomodulatory treatment [17], a consensus statement of the American Academy of Electrodiagnostic Medicine included anti-GM1 antibody in the possibly supportive laboratory criteria for MMN diagnosis [3]. It is especially important in situations where a definite diagnosis of MMN cannot be made on clinical and neurophysiological grounds [2, 12].

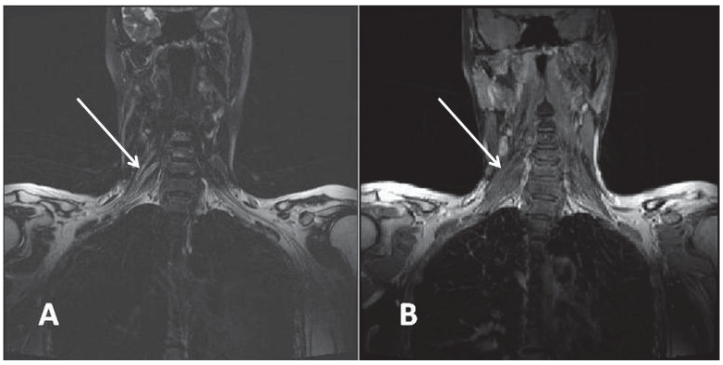

Fig. 3 – T2 FS sequence of magnetic resonance imaging (MRI) shows increased signal intensity in C6 and C7 roots on the right side in a patient with multifocal motor neuropathy (the patient No 7): a) without contrast; b) with contrast.

It is well known that the neurophysiologic hallmark of the MMN is the presence of CBs in motor nerves, which is supposed to be the underlying cause of muscle weakness [3, 8, 10]. Accordingly, CBs are most commonly found in long arm nerves that innervate weak muscles. One of the possible explanations for this is that motor axons in the arm nerves have slower potassium conductance than motor axons in leg nerves [21]. These differences could contribute to the greater susceptibility of arm motor axons for developing CB [15, 21, 22]. In line with this results we found CBs in all patients included in the study at sites distinct from common entrapment syndromes, most frequently in radial nerve. In the majority of patients with MMN the distribution of muscle weakness correlated with the CBs detected by NCS [2, 16, 23]. However, CB may be localized in proximal nerve segments and may be difficult to be detected by conventional NCS [5, 24], as was the case in three patients [1, 2, 7] of our cohort. The significance of MRI in these cases is valuable, because the pattern of signal alteration in brachial plexus closely correlates with the distribution of muscle weakness and together with magnetic stimulation at cervical level could be helpful in detection of proximal CB in the affected nerve [6, 14, 25, 26].

In addition, MRI may give some contribution to differential diagnosis of inflamatory neuropathies – MMN and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and lower motor neuron disease (LMND). In MMN MRI shows asymmetrically increased signal intensity on T2-weighted images sequences with evidence of nerve swelling in 40–50% of patients, while in CIDP MRI usually depicts diffuse and homogenous signal enhancement. However, in patients with LMND there are no such findings [6].

Our results suggest that in all the examined patients MRI showed asymmetric focal lesions in the cervical roots and structures of brachial plexus. This finding was particularly significant in our three patients in whom MRI showed high signal intensity in the C6 and C7 roots corresponding with the affection of right median nerve and proximal CBs detected by magnetic paravertebral stimulation. A similar finding was reported by Arunachalam et al. [5]. Using triple stimulation technique, they found the presence of proximal CBs in 7 out of 10 analyzed MMN patients indicating proximal demyelination.

Conclusion

The involvement of cervical roots, truncus or fasciculus of brachial plexus detected by MRI examination, along with the prolonged motor root conduction time detected by cervical magnetic stimulation could be valuable in detection of proximally localized CB in patients with clinically highly suspected MMN. In that way both methods are noninvasive tools providing assesment of proximal nerve segments integrity in patients with MMN without CB on routine NCS.

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Distribution and characteristics of molar-incisor hypomineralization

Rasprostranjenost i karakteristike hipomineralizacije na kutnjacima i sekutićima

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Abstract

Background/Aim. Developmental disorders of teeth are the problems that are becoming more present in pediatric dentistry, especially on first permanent molars and incisors. Molar Incisor Hypomineralization (MIH) is proposed term for this phenomenon. The aim of this study was to establish the MIH prevalence in children living in the Foča municipality (Bosnia and Herzegovina) as well as to assess characteristics and expression of hypomineralization within the tested population. **Methods.** A total of 141 children from the Foča municipality, 8 years of age, were included in this study. Criteria according to Weerhejm have been used for diagnosis of hypomineralization: demarcated opacity (DO), post-eruptive breakdown (PEB), atypical restoration (AR), extracted molars due to MIH (E-MIH) and unerupted tooth (UT). Level and the prominence of color changes have been determined for patients with DO, PEB and AR. **Results.** MIH in this area was present in 12.8% of children. The prevalence of MIH changes expressed in percentages was as follows: DO was at 9.2%, PEB in 3.5%, AR in 5.6%, while E-MIH was 5.6%. A total of 9.9% of the examinees had mild, 5.6% moderate, and 7.8% severe form of MIH. White form of MIH defects was found in 9.9% of the examinees, white-yellow one in 5.6% and yellow-brown color in 3.5% of the examined children. These changes were more often present in the lower jaw (60.3%). In total, 6.4% of children had these changes present only on molars, while 6.4% of them both on molars and incisors simultaneously. **Conclusion.** A total of 12.8% of the examinees with MIH is not to be disregarded. With timely diagnosis, prevention and therapy complications could be avoided or mitigated.

Key words:
tooth demineralization; dentition, permanent, child; bosnia-herzegovina; molar; prevalence.

Apstrakt

Uvod/Cilj. Razvojni poremećaji zuba sve su prisutniji problem u dječjoj stomatologiji, posebno na prvim stalnim kutnjacima i sekutićima. Za ovu pojavu, predložen je termin molar-incizor hipomineralizacija (MIH). Cilj istraživanja bio je da se utvrdi stepen rasprostranjenosti MIH kod djece iz Foče, te procijene karakteristike i stepen izraženosti hipomineralizacije kod ispitivane populacije. **Metode.** Ispitivanjem je obuhvaćeno 141 dijete iz opštine Foča (Bosna i Hercegovina), starosti osam godina. Za dijagnozu oboljenja korišteni su kriterijumi po Weerhejm-u: ograničena zamućenost gleđi (OZG), poseruptivni prekid gleđi (PPG), atipične restauracije (AR), vađenje kutnjaka zbog MIH (E-MIH), i retencija zuba (RZ). Ispitanicima kojima je evidentirana OZG, PPG i AR određen je stepen i boja izraženosti promjene. **Rezultati.** Molarna i incizorna hipomineralizacija na ovom području iznosila je 12,8%. Distribucija MIH promjena po stepenima bila je sledeća: OZG iznosila je 9,2 %, PPG pronađen je kod 3,5 % ispitanika, AR pronađene su kod 5,6 % ispitanika, dok je E-MIH utvrđena kod 5,6% ispitivane djece. Blagu formu imalo je 9,9% ispitanika, umjerenu 5,6% ispitanika, a tešku 7,8%. Bijela boja MIH defekata konstatovana je kod 9,9% ispitanika, bjeložuta kod 5,6%, a žutobraon kod 3,5% djece. Rezultati pokazuju da je donji desni prvi stalni kutnjak najčešće izvađeni zub zbog MIH, kao i da su ove promjene prisutnije u donjoj vilici (60,3%). Ukupno 6,4% djece imalo je promjene samo na kutnjacima, a 6,4% djece na kutnjacima i sekutićima istovremeno. **Zaključak.** Procenat od 12,8% ispitanika sa MIH promjenama nije zanemarljiv. Ranom dijagnozom, te blagovremenom prevencijom i terapijom, znatno se mogu spriječiti i ublažiti komplikacije.

Ključne reči:
zub, demineralizacija; denticija, stalna; deca; bosna i hercegovina; molari; prevalenca.

Introduction

In addition to dental caries and its complications, developmental disorders of the teeth are becoming increasingly common problem in dentistry. Tooth decay is the most widespread disease of the modern era, but in many developed countries where the level of health education is at an enviable level, significant results were achieved with prevention

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of caries and its complications. The obvious decline in caries prevalence was registered in Switzerland in early sixties, in the Scandinavian countries in the late sixties, and in Denmark in late seventies [1]. Another problem was noted in these countries regarding the disease of hard dental tissue, and that is a growing percentage of people with the onset of tooth enamel mineralization disorders, especially on the first permanent molars and incisors.

In Denmark, for example hypomineralized enamel defects in first permanent molars are more frequent than caries on the occlusal surfaces of these teeth [2]. Weerheijm et al. [3] were the first to observe and describe clinical picture of idiopathic enamel hypomineralization. They also pointed to the significance and inconvenience caused by these teeth. The authors have proposed the term Molar Incisor Hypomineralization (MIH) for this phenomenon, and defined it as systemic origin hypomineralization of one or more first permanent molars, often associated with changes in the maxillary and mandibular incisors. Literature data indicate a different distribution of this phenomenon in the world, from 2.9% to 25% [4–9], suggesting a different, yet undetermined etiology, and uneven methodology for estimation of these defects.

Clinically, MIH is presented as a limited demarcated opacity (enamel opacity) of irregular shape, different color and abnormal translucency. Enamel opacities can be soft with frequently observed enamel discontinuity. These changes are especially expressed in the first permanent molars immediately after the eruption [10]. Irregular patches of different colors are usually observed at central incisors labial surfaces, and they are rarely accompanied by enamel discontinuity [11]. Common for the incisors and molars (affected with MIH), in most of the cases, is sensitivity to thermal, chemical and mechanical stimuli [3]. Children with these changes tend to avoid washing teeth due to painful sensations they feel. This creates a greater amount of dental plaque, which is followed by rapid progression of carious lesions that lead to the destruction of the crown and eventually to tooth loss [12].

In children with MIH, dental treatment need is multiply increased, considering the fact that these teeth, especially molars, depending on the degree of hypomineralization are brittle, fragile and easily susceptible to caries [13, 14]. Although numerous studies have been conducted on caries prevalence in children, MIH was not monitored and its distribution was not defined in this area. MIH patients care requires teamwork and individual approach to each patient as well as well-designed plan of preventive and therapeutic measures.

The aim of this study was to determine the prevalence of molar incisor hypomineralization in children living in the Foča municipality, Bosnia and Herzegovina as well as to assess the characteristics and severity of hypomineralization in studied population.

Methods

In accordance with the objectives set, observational, descriptive and cross-section studies were undertaken. This was a retrospective study. There are two primary schools attended by about 2,000 students in Foča municipality. This survey is scheduled to include all children aged 8 years. There were a total of 147 such children, in 2005/06 school year. This age was chosen because the first permanent molars and central incisors relatively soon erupted, caries prevalence is still low, therefore ability of carious lesions to mask hypomineralization is reduced. Prior to examinations the parents were informed in writing of research purpose and methodology to be applied. The parents gave written consent for children participation in the study. There were no written consents for 6 children, so they were not included in the study.

Dental mirror, probe and common lighting were used for examinations of children. Probes were applied only as needed in order to remove dental plaque. All changes found were photographed with a digital still camera (Canon A 520). Examinations of children in urban schools took place in the school dental clinic, while the children of suburban schools were inspected in the brightest classroom. The study was approved by the Ethics Committee of the Medical Faculty in Foča and was conducted according to principles of the Helsinki Declaration.

Criteria used in this study and commonly used in the literature, are those proposed by Weerhejim et al. [15]. These are: demarcated opacity (DO); post-eruptive enamel breakdown; (PEB); atypical-restoration (AR); extracted molar due to MIH (E-MIH); unerupted teeth (UT).

Severity and color of changes were determined to patients with DO, PEB and AR. There are 3 degrees of severity as follows: mild form, moderate form and severe form. Mild form of tooth mineralization disorder is characterized by tooth enamel color changes (white, yellow or brown). Moderate form is characterized by discoloration and minimal loss of tooth substances without the need for restoration. Damaged enamel and dentin loss that require restoration are marked as a severe form. In the case when there is more than one defect on the tooth, toughest change was recorded as valid. Teeth with more than half of the crown arisen were included in the study while tooth lesion smaller than 1 mm were not included in the study.

Color of hypomineralized changes were characterized as: white, white-yellow and yellow-brown. Color of hypomineralized changes can be masked by dental fluorosis, and it is important to note the amount of fluoride in drinking water in the area where the children are grown. There are two water sources in Foča that supply households. One source has 0.00019 ppmF and the second 0.0025 ppmF, which means extremely low concentrations of fluoride in drinking water.

Reliability of the researchers for this type of research (extra-intra-examiners reliability) was performed in 10% of the planned sample (10% of respondents, 14 children, were examined twice with a minimum interval of 4 hours between hits) and Kappa value obtained in this way indicates the reliability of examiners (Kappa = 0.85). SPSS 11.0 was program used for data analyzing.

Results

The study included 141 subjects, with approximately the same percentage of boys (50.4%) and girls (49.6%). All

hypomineralized changes of teeth were observed in 18.4% of the children. The mentioned changes were slightly more represented in the group of boys (21.1%) compared to the group of girls (15.7%). Data analysis showed that the difference was not statistically significant ($\chi^2 = 0.687$; $p > 0.05$). Hypomineralized changes were more common in children from the urban area (20.4%), compared to children who were from suburban municipalities (13.2%), but data analysis did not find a statistically significant difference between these two groups ($\chi^2 = 0.965$; $p > 0.05$). However, hypomineralized changes that affect only the incisors without affecting the first permanent molars can be caused by other disorders, therefore they should not be counted as real MIH changes.

According to the results of this study, genuine MIH was present in 12.8% of the studied children in this area. MIH was found in 14.0% of the boys and 11.4% girls. Out of the total percentage of children with MIH defects 10.93% of the children had changes in two teeth, 39.07%, in three teeth, 39.07%, in four teeth and 10.93% in five teeth (Figure 1).

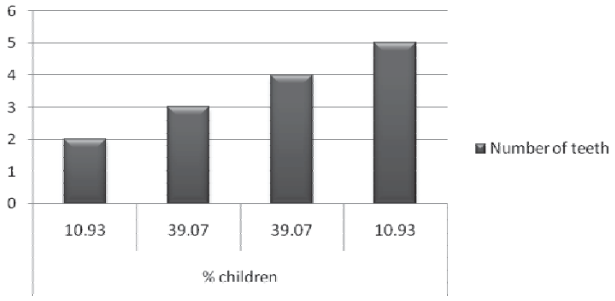

Fig. 1 – Molar-incisor hypomineralization (MIH). Prevalence according to the number of affected teeth.
E-MIH – extracted molars due to MIH

The prevalence of MIH changes according to the levels was as follows: demarcated opacity (DO) was present in 9.2%, post-eruptive breakdown (PEB) 3.5% of patients, atypical restorations (AR) were found in 5.6% of respondents, extraction of teeth due to hypomineralization (E-MIH) was found in 5.6% of children or 19.0% of the total number of MIH teeth and they were all molars (Table 1). Teeth that had not erupted due to MIH were not registered in this study. The obtained results indicate that demarcated opacity is the most common one. Molars were the most affected by all changes. Changes in molars regarding PEB were more common, as confirmed by the statistical significance ($\chi^2 = 6.414$; $p < 0.05$). The study found a high statistical difference when it comes to AR ($\chi^2 = 16.414$; $p < 0.01$) and E-MIH ($\chi^2 = 14.204$; $p < 0.01$) between the observed types of teeth. It should be, particularly noted, that relatively high percentage of teeth extracted due to MIH was found.

The mild form of hypomineralized change was the most common. It was found in 9.9% of respondents, or 52.4% of teeth with MIH. Moderate form of hypomineralized changes was found in 5.6% of respondents what was confirmed by significant statistical difference ($\chi^2 = 6.567$; $p < 0.05$). The severe form of MIH had 7.8% of the respondents, and all the changes were in molars ($\chi^2 = 12.654$; $p < 0.01$). If subjects with extracted teeth due to MIH were considered as severe form (MIH signs present on the other teeth), it could be noted that eleven children had a severe form of expression. Out of 16 totally affected teeth, four teeth were still present in the mouths of patients, and 12 teeth had already been removed (Table 2).

White color of hypomineralized changes was the most common. It was found in 9.9% of respondents. Out of 51 teeth with MIH (extracted teeth were not included in this analysis), the white color was present in 56.8% of teeth, mainly in molars, but there was no statistically significant difference ($\chi^2 = 0.471$; $p > 0.05$). White-yellow color was seen in 5.6% and yellow brown in hypomineralized changes 3.5% of the respondents. All these changes were more present in the molars, which is a highly statistically significant difference ($\chi^2 = 8.931$; $p < 0.01$ and $\chi^2 = 12.820$; $p < 0.01$) (Table 3).

Data analysis showed that the most frequently affected molar by MIH changes is the lower left first permanent molar – 36 (26.9%). When it comes to the incisors, most frequently affected by MIH changes, is the lower right central incisor – 41 (6.3%). The central incisors in the upper jaw

Table 1

Prevalence of molar-incisor hypomineralization (MIH) criteria

MIH criteria	Children (%)	Teeth with MIH (%)	Incisives (%)	Molars (%)
Demarcated opacity	9.2	44.4	46.4	53.6
Post-eruptive enamel breakdown	3.5	12.7	37.5	62.5*
Atypical-restoration	5.6	23.8	0.0	100†
Extracted molar due to MIH	5.6	19.0	0.0	100 †
Unerupted teeth	0.0	0.0	0.0	0.0

***Statistically significant difference ($p < 0.05$); †Highly statistically significant difference ($p < 0.01$).**

Table 2

Prevalence of molar incisor hypomineralization (MIH) expression

MIH expression level	Children n (%)	Present teeth with MIH n (%)	Incisives n (%)	Molars n (%)
Mild form	14 (9.9)	33 (52.4)	13 (39.4)	20 (60.6)
Moderate form	8 (5.6)	14 (22.2)	3 (21.4)	11 (78.6)*
Severe form	3 (2.1)	4 (6.3)	0 (0)	4 (100)
(present + extracted teeth)	3 + 8 = 11 (7.8)	4 + 12 = 16 (25.4)	0 (0)	16 (100)†

***Statistically significant difference ($p < 0.05$); †Highly statistically significant difference ($p < 0.01$).**

Table 3

Prevalence of colour in molar-incisor hypomineralization (MIH) changes

Colour of MIH changes	Examined children (%)	Present teeth with MIH (%)	Incisives (%)	Molars (%)
White	19.9	56.8	48.3	51.7
White-yellow	5.6	29.4	13.3	86.7*
Yellow-brown	3.5	13.7	0.0	100*

***Highly statistically significant difference ($p < 0.01$).**

were equally affected by the aforementioned changes. In this study, the changes mentioned before were not observed in the left lateral incisors (22, 32). The results show that the lower right first permanent molar (46) is the most frequent tooth extracted due to MIH, and that these changes were more present in the lower jaw (60.3%). If we analyze side of the jaw, MIH was more present on the right (52.37%), compared to the left side of the jaw – 47.63% (Figure 2).

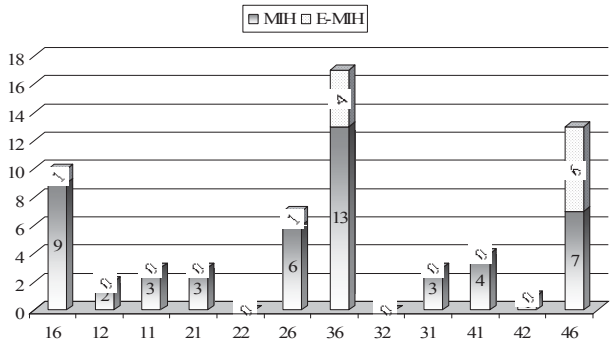

Fig. 2 - Teeth with molar-incisor hypomineralization (MIH) changes.

E-MIH – extracted molars due to MIH

Discussion

Over the recent years the literature often describes hypomineralization in permanent first molars and incisors as a clinical problem, in the same time indicating the necessity of conducting studies on the prevalence of these in various countries [5, 7, 10, 12, 16].

Hypomineralized changes can affect all the teeth in each dentition. This study examined the prevalence of hypomineralization in permanent first molars and incisors. A total of 6.4% of the children had changes in only the molars and 6.4% of the children in both molars and incisors, respectively. Statistical analysis showed no significant differences in MIH incidence for children of different gender, which is consistent with all studies on this issue [6, 7].

A study conducted in Italy (Lissone), also on eight year olds, showed the approximate results (13.7%) [7]. In Turkey, 14.8% of MIH changes were registered in the test group of children aged 7–12 years [17]. A significantly higher percentage of MIH was found in Finland in 2001, 19.3% [18]. In Sweden in 2001, a study performed in children aged 7–8 years, showed the prevalence of MIH of 18.4% [6]. More than 5,000 children, aged 7 years, were examined in Denmark in 2003, and the percentage of MIH was in the range of 15–25% [10]. A slightly lower percentage of MIH, compared to our results, was found in the Netherlands [19] in 2001, and Lithuania in 2007 9.7% [20]. Even smaller values (6%) were recorded in Greece in 2006. [21]. Two separate studies were conducted in Germany, in Dresden in 2003 [5] and in Giessen in 2006 [4], in different population groups, and similar percentages of MIH were registered. In Dresden MIH was 5.6% for the ages 10–17 years, in Giessen 5.9% in children from 6–12 years. A study conducted in Libya in 2006 in children between 7–9 years, found a significantly lower percentage of MIH (2.9%) [9].

The analysis of these epidemiological studies can lead to two conclusions: firstly, the percentage of MIH is not negligible although not all studies used the same criteria and, secondly, data from other areas of Europe and the world are missing in order to obtain a clearer picture and eventually discover a possible etiologic factor.

A review of the literature show that there is little data on individual MIH criteria representation. In a study conducted in Lithuania, limited representation was present in 54.8% of respondents with MIH changes [20]. Generally, the prevalence of individual criteria is difficult to compare because, despite the fact that they are clearly determined, detailed criteria are not precisely defined. One of the first concerns is the size of the lesion (demarcated opacity). Therefore, all the defects larger than 1 mm in diameter, were included in the study. Some studies have included only those defects that are larger than 2 mm [6, 7, 16, 18].

Analyzing the previous studies, we can conclude that the size of the lesions itself is not crucial because "small" lesions are usually seen only on incisors. Because incisors that are not followed by changes in molars at the same time are not included in MIH, not altering the end result. It is important to note that in many analyzes the color of a change is more important than the size itself. The white color and all its nuances can be differently noticeable on teeth. Undoubtedly, the chalky-white or yellow brown blur, on the tooth is clearly observed, although only 1mm sized [21].

Post-eruptive enamel breakdown was found in 12.7% of teeth. In a study conducted in Lithuania in 2007 [20], post-eruptive enamel breakdown was present in 28.2% of respondents with MIH. There is no information about what teeth were affected by post-eruptive enamel breakdown. Atypical restoration was found in 23.8% of MIH teeth, all of which were molars. In a study from Lithuania, atypical restorations were in 16.9% MIH patients. Teeth extracted due to MIH were all molars (19.0%). Having in mind that patients are 8 years old and the fact how important the first permanent molars are and that they have erupted quite recently percentage of extracted teeth was really great. In a study from Lithuania there were no teeth extracted due to MIH [20]. No erupted teeth due to hypomineralized changes were found in this research. This data is in agreement with the most of studies on MIH [5, 7, 16].

Mild form of hypomineralized changes is the most common in this study and is 52.4% of teeth with MIH. Compared to all MIH studies, we confirmed that a mild form prevailed. Moderate form of hypomineralized changes was found in 22.2% of teeth with MIH. The highest percentage was represented at the molars (78.6%). In the Italian study [7] no patients with moderate form of MIH changes were found. In Germany, Preusser et al. [4], found moderate forms in 25.4% of teeth with MIH present, while Dietrich et al. [5] found a smaller percentage of 6.1%. However, it is difficult to make comparisons when it comes to expression form of MIH changes, because only a small number of studies analyzed and shared the defects as mild, moderate and severe forms.

Severe form was found in 6.3% of teeth, or with extracted teeth counted a total of 7.8% of the respondents (what is 25.4% of teeth affected by MIH changes). Severe form was not found in the incisors of children examined. A similar percentage was found in a study from 2001 in Sweden, where the presence of severe forms was found in 6.4% of respondents [6], while a much smaller percentage was found in the group of children living in the Italian city Lissone (0.4%) [7]. Higher values were found in Finland [18] where 8.4% of respondents had the mentioned changes. A smaller percentage of severe form, on teeth with the mentioned changes, was established in Germany in 2003 (9.4%) [5] and 2006 (7.4%) [4]. It can be concluded, by analyzing these studies, that a relatively high proportion of severe form categorizes MIH as an important problem in pediatric dentistry. The authors did not specifically describe the color of hypomineralized changes in literature review.

MIH changes were more present in the lower (60.3%) compared to the upper jaw (39.7%). The first permanent molars in the lower jaw (36 and 46) are teeth with the most commonly diagnosed MIH changes. These results are in agreement with the results obtained by Kalderara et al. [7]. As for the incisors, in this study, MIH is equally present in the upper and lower jaw. Literature data indicate that changes are more present in the maxillary incisors [5, 6, 8].

Conclusion

Today, molar-incisor hypomineralization presents a problem in pediatric dentistry to children, parents and to dentists, as well, because of hypersensitivity that these patients experience, minor or major esthetic problems, the rapid occurrence of caries and its complications. Besides, etiology is still unknown. Early diagnosis and timely prevention and treatment can significantly reduce and prevent complications. These patients require a multidisciplinary approach and close cooperation between pediatric dentists, orthodontists, parents and patients themselves.

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Characterization of deciduous teeth stem cells isolated from crown dental pulp

Karakterizacija matičnih ćelija izolovanih iz zubne pulpe mlečnih zuba dece

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Abstract

Background/Aim. The last decade has been profoundly marked by persistent attempts to use *ex vivo* expanded and manipulated mesenchymal stem cells (MSCs), as a tool in different types of regenerative therapy. In the present study we described immunophenotype and the proliferative and differentiation potential of cells isolated from pulp remnants of exfoliated deciduous teeth in the final phase of root resorption. **Methods.** The initial adherent cell population from five donors was obtained by the outgrowth method. Colony forming unit–fibroblast (CFU-F) assay was performed in passage one. Cell expansion was performed until passage three and all tests were done until passage eight. Cells were labeled for early mesenchymal stem cells markers and analysis have been done using flow cytometry. The proliferative potential was assessed by cell counting in defined time points and population doubling time was calculated. Commercial media were used to induce osteoblastic, chondrogenic and adipogenic differentiation. Cytology and histology methods were used for analysis of differentiated cell morphology and extracellular matrix characteristics. **Results.** According to immunophenotype analyses all undifferentiated cells were positive for the mesenchymal stem cell markers: CD29 and CD73. Some cells expressed CD146 and CD106. The hematopoietic cell marker, CD34, was not detected. In passage one, incidence of CFU-F was 4.7 ± 0.5/100. Population doubling time did not change significantly during cell subcultivation and was in average 25 h. After induction of differentiation, the multicolony derived cell population had a tri-lineage differentiation potential, since mineralized matrix, cartilage-like tissue and adipocytes were successfully formed after three weeks of incubation. **Conclusion.** Altogether, these data suggest that remnants of deciduous teeth dental pulp contained cell populations with mesenchymal stem cell-like features, with a high proliferation and tri-lineage differentiation potential and that these cultures are suitable for further *in vitro* evaluation of cell based therapies.

Key words:
dental pulp; stem cells; tooth, deciduous; child, preschool; cell differentation; adipogenesis; chondrogenesis; osteogenesis.

Apstrakt

Uvod/Cilj. Prošla dekada je bila posebno obeležena naporima na polju korišćenja *ex vivo* razvijenih i usmeravanih mezenhimskih matičnih ćelija (MSCs), kao sredstva za različite tipove regenerativne terapije. Cilj ove studije bio je da se utvrdi imunofenotip i potencijal za proliferaciju i diferencijaciju ćelija izolovanih iz zubne pulpe mlečnih zuba dece eksfoliranih u periodu kada je koren zuba bio u poslednjoj fazi resorpcije. **Metode.** Primarna adherentna populacija ćelija poreklom od pet donora dobijena je metodom eksplanta. Prisustvo progenitorskih ćelija koje obrazuju kolonije fibroblasta (CFU-F) pokazano je u prvoj pasaži. Do treće pasaže ćelije su ekspandirane, a potom korišćene za analiziranje. Imunofenotip je određen korišćenjem protočne citometrije. Proliferativni potencijal i vreme udvajanja ćelija (PDT) u kulturi je definisano na osnovu apsolutnog broja ćelija na početku i na kraju svake pasaže. Posle tronedeljne kultivacije ćelija u komercijalnim medijumima za stimulaciju osteogeneze, hondrogeneze i adipogeneze, citološkim i histološkim metodama je određena morfologija ćelija i karakteristike vanćelijskog matriksa. **Rezultati.** Antigeni koji karakterišu mezenhimske matične ćelije CD29 i CD73 su bili eksprimirani na svim nediferenciranim ćelijama, dok su antigeni CD146 i CD106 bili eksprimirani na ograničenom broju ćelija. Antigen CD34 (karakterističan za ćelije hematopoetske loze) nije bio eksprimiran. Incidencija CFU-F bila je 4,7 ± 0,5/100 ćelija. PDT se nije menjao tokom osam pasaža i u proseku je iznosio 25 h. Posle tronedeljne stimulacije diferencijacije u kulturama sa adipogenim medijumom došlo je

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do stvaranja ćelija sa masnim kapljicama, a u kulturama sa osteogenim medijumom došlo je do formiranja vanćelijskog matriksa sa deponovanim kalcijumovim solima. U kulturama sa hondrogenim medijumom došlo je do stvaranja tkiva sličnog hrskavici i vanćelijskog matriksa sa glikozaminoglikanima i kolagenom II. **Zaključak**. Zubna pulpa mlečnih zuba dece sadrži ćelijsku populaciju koja odgovara mezenhimskim matičnim ćelijama prema svojim karakteristikama, ima visok proliferativni potencijal i potencijal da se diferencira u tri ćelijske linije što je čini pogodnom za dalje *in vitro* analize i evaluaciju ćelijske terapije.

Ključne reči:
zub, pulpa; ćelije, matične; denticija, mlečna; deca, predškolska; ćelija, diferencijacija; adipogeneza; hondrogeneza; osteogeneza.

Introduction

The last decade has been profoundly marked by persistent attempts to use *ex vivo* expanded and manipulated mesenchymal stem cells (MSCs), as a tool in different types of regenerative therapy. Most of them were focused on healing diseases and injuries of the musculoskeletal system [1], as well as solving problems in dental medicine [2].

Research targeting cell therapy and tissue engineering in regeneration of tooth structures, was stimulated after Gronthos and coworkers [3] described dental pulp stem cells (DPSC) isolated from impacted third molars of adult donors. Clinically interesting populations of cells have also been isolated from deciduous teeth. Thus, Miura et al. [4] described stem cells from human exfoliated deciduous teeth (SHED) and Kerkis et al. [5] obtained immature DPSC (IDPSC) from the same source. Subsequently, several more papers confirmed these findings and enlarged our knowledge about stem cells that could be isolated from deciduous dental pulp [6–11]. In the review of Kerkis and Caplan [12] all isolated cell populations were named deciduous teeth stem cells (DTSC) with the conclusion that they have a higher colony forming cell score and a higher proliferation rate than DPSC, and therefore are more primitive than their counterparts isolated from permanent teeth. The pluripotent nature of DTSC, and the fact that teeth develop from oral ectoderm and neural crest-derived mesenchyme, leaded some investigators to conclude that these cells display developmental potential similar to embryonic stem cells [13]. *In vivo*, SHEDs generate a tissue with morphological and functional properties that closely resemble those of human dental pulp [14] and strongly induce bone formation [4]. Besides stem cells from dental pulp, periodontal ligament stem cells [15], dental follicle progenitor cells [16], stem cells from apical papilla [17] and even stem cells from periapical lesions have been described [18]. Development in the field of biomaterials and tissue engineering, together with stem cell research, has shown promising results for the development of optimal restorations to replace lost tooth structures.

Special interest in characterization of stem cell populations that can be found in dental pulp of human exfoliated deciduous teeth is underlined by the fact that these cells are easly obtained. Instead of being discarded, they could be cryopreserved, and if necessary, expanded and used for autologeous or allogeneous treatment.

The aim of this study was to test the proliferation and differentiation potential of cells isolated from dental pulp of human exfoliated deciduous teeth in the final phase of root resorption, and to describe their colony forming capacity, population doubling time, immunophenotype in the undifferentiated state and their tri-lineage differentiation capacity. The term DTSC will be used subsequently for the cell population isolated in this work.

Methods

Isolation of the initial cell population

Deciduous incisor teeth from children aged 6 and 7 years (5 patients) were obtained after extraction due to orthodontic reasons, under local anesthetic, with informed consent of their parents and ethical comity approval. Teeth roots were in the final phase of resorption with viable pulp tissue. Only crowns that contained gelatinous pinkish pulp tissue were used. Dental pulp was pulled out with a barbed Nervbroach, washed twice with sterile phosphate buffered saline (PBS) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and antimycotic (2.5 μg/ml amphotericin B) – AA solution (antibiotic/antimycotic). Pulp tissue was minced into 1–2 mm fragments, transferred to 35 mm Petri dishes and cultivated using Dulbecco's modified Eagle's medium (DMEM) / Ham's F12 (1:1, Invitrogen, Carlsbad, California, USA) supplemented with 10% FBS MSC qualified (Invitrogen, Carlsbad, California, USA) and AA solution. Cultures were incubated at 37°C in a humified atmosphere with 5% CO_2.

The growing culture of the initial cell population (passage 0) was maintained for 10 to 15 days, dissociated in a 0.05% TrypLE™ Express (Invitrogen, Carlsbad, California, USA), and seeded at 1×10^5 cells per 25 cm^2 flasks. Cultures initiated as multicolony culture systems were maintained semi-confluent in order to prevent premature senescence. Thus, the cells were passed every 5 days, while the medium was replaced every 2–3 days. Cells were used from passages three to eight.

Colony forming unit fibroblast (CFU-F) assay

After harvesting cells from passage 0, single-cell suspensions (1×10^4 cells) designated passage 1, within DMEM/F12 containing 10% FBS were seeded into T 25 tissue culture flasks (BD Falcon, Becton, Dickinson and Company – BD, NJ, USA). After 10 days, cultures were fixed with 10% methanol, and then stained with Crystal violet solution. Aggregates containing 50 or more cells were counted as CFU-F under the microscope.

For assessment of colony-forming efficiency (CFE), cells in the fourth passage were plated at a density of 500

cells in six-well plates and colony formation was inspected under a microscope after 7 days of culture. The CFE index was calculated by dividing the number of colonies formed by the number of cells plated and multiplying with the factor 100.

Population doubling time

For analysis of population doubling time (PDT), cells were seeded at a density of 1×10^4 cells/well in six-well plates. The cell number was assessed after 4 days, with a hemocytometer, after collecting cells from the wells by trypsinization (3 replicates for each time point). PDT was calculated by the formula: PDT = [ln (Nt / N0) / ln (2)] / t (t = the time period, Nt = number of cells at time t and No = initial number of cells).

Flow cytometry

After harvesting, cells (third to sixth passage) were washed in cold PBS supplemented with 0.5% BSA (Sigma-Aldrich, Saint Louis, MO, USA). Aliquots of 5×10^5 cells were labeled (30 min in the dark at 4°C) with monoclonal antibodies specific for human markers associated with mesenchymal and hematopoietic lineages. Namely, mouse anti-human antibodies against the following antigens were used: CD34 (PE conjugated), CD29 PECy5 conjugated, CD73 and CD146 (PE conjugated) and CD106 (FITC conjugated), all purchased from BD Biosciences. To determine the level of nonspecific binding, fluorochrome conjugated isotype control antibodies were used. Flow cytometry was performed using a CyFlow CL (Partec, Münster, Germany).

Differentiation

Complete commercial media (StemPro Osteogenesis, Chondrogenesis and Adipogenesis Kits, Gibco-Invitrogen, Carlsbad, CA, USA) were used to induce osteogenesis, chondrogenesis and adipogenesis of DTSCs from the third to sixth passage. Characteristic features of differentiated cells were visualized by cytochemical and/or histochemical methods.

After 3 weeks in complete medium (changed every 2 days) for osteogenesis, calcium depositions were demonstrated in the extracellular matrix. Cell layer was washed twice in PBS and fixed with 10% neutral buffered formalin (NBF) for 1 h at room temperature (RT). Cultures were then stained with 1% Alizarin red S solution (Sigma-Aldrich, Saint Louis, MO, USA), pH 4.2, for 20 min at RT, followed by rinsing three times with deionized water. After 3 weeks in complete medium for adipogenesis (changed every 2 days), cells were fixed in 4% paraformaldehyde for 8 h, rinsed twice with PBS, then treated with 60% isopropanol (until evaporation), stained with a fresh 0.35% Oil Red O solution for 10 min, followed by washing twice with deionized water. The chondrogenic differentiation potential of the expanded cells was investigated by micromass culture. The cell solution of 2×10^5 viable cells was prepared in chondrogenic or control medium. Tubes were centrifuged at 1,000 rpm for 6 min allowing cells to aggregate at the tube bottom. Pellets were formed after 24 h. After 2 and 3 weeks (medium changed every 2 days), pellets were fixed in 4% non-buffered formaldehyde for 24 h, embedded in paraffin and 5 μm thick sections were prepared. Sulfated glycosaminoglycans (GAG) were demonstrated with 0.1% Alcian blue (Sigma-Aldrich, Saint Louis, MO, USA) counterstained with 0.1% Fast nuclear Red (Sigma-Aldrich). The presence of collagen type II was detected immunohistochemically using rabbit polyclonal antibodies to collagen type II (Abcam, Cambridge, MA, USA).

All the quantitative data are presented as mean ± standard deviation. Data were processed in Excel for Windows program.

Results

Our study demonstrated that after adhesion of dental pulp explants to plastic, initial cell migration was obtained in 2 to 3 days, followed by rapid cell proliferation. Initial cell growth was designated passage 0. The number of cells harvested after passage 0 was 0.3×10^5 to 3×10^5. In passage 1, the incidence of CFU-F was 4.7 ± 0.5 per 100 cells (Figure 1).

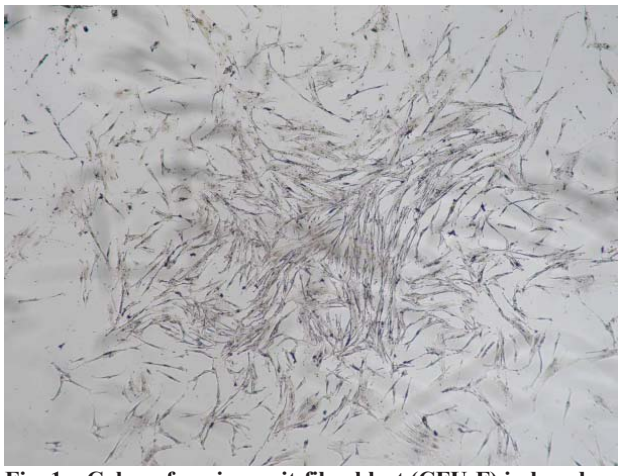

Fig. 1 – Colony forming unit-fibroblast (CFU-F) in low density culture at passage 1 (Crystal violet staining, magnification objective 4 ×).

In our experiment, in all time points, the PDT was approximatly 25 h (Figure 2a). Colony forming efficiency (CFE) in passage four was 80.4 ± 7.5% on average (Figure 2b).

Using flow cytometry, we demonstrated that all cells expressed CD29 and CD73 (Figures 3a and 3b), 88% of cells expressed CD146 (Figure 3c), and 5% of cells expressed CD106 (Figure 3d). We also confirmed that CD34 was not expressed on the cell population examined (Figure 3e). Figure 3f demonstrate mean expression of the analyzed markers from five donors.

The differentiation potential of isolated cells is important when considering their potential to regenerate specified tissues, like bone, cartilage or adipose tissue. After 3 weeks of cultivation in adipogenic medium, the cells became more round and filled with fat droplets (Figure 4a) while cells

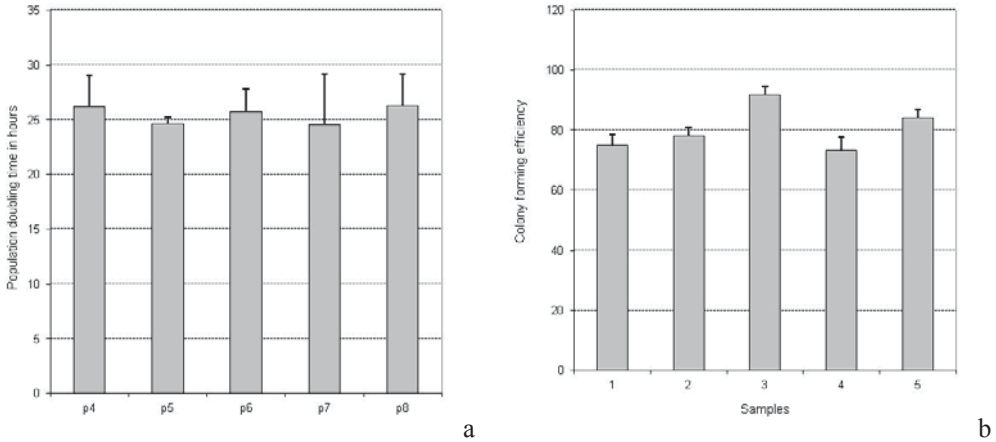

Fig. 2 – a) Deciduous teeth stem cells (DTSC) population doubling time from passage four to passage eight did not change significantly (n = 5, mean ± standard deviation); b) Colony forming efficiency was similar between different samples at passage four (values are mean ± standard deviation of experiments done in triplicate).

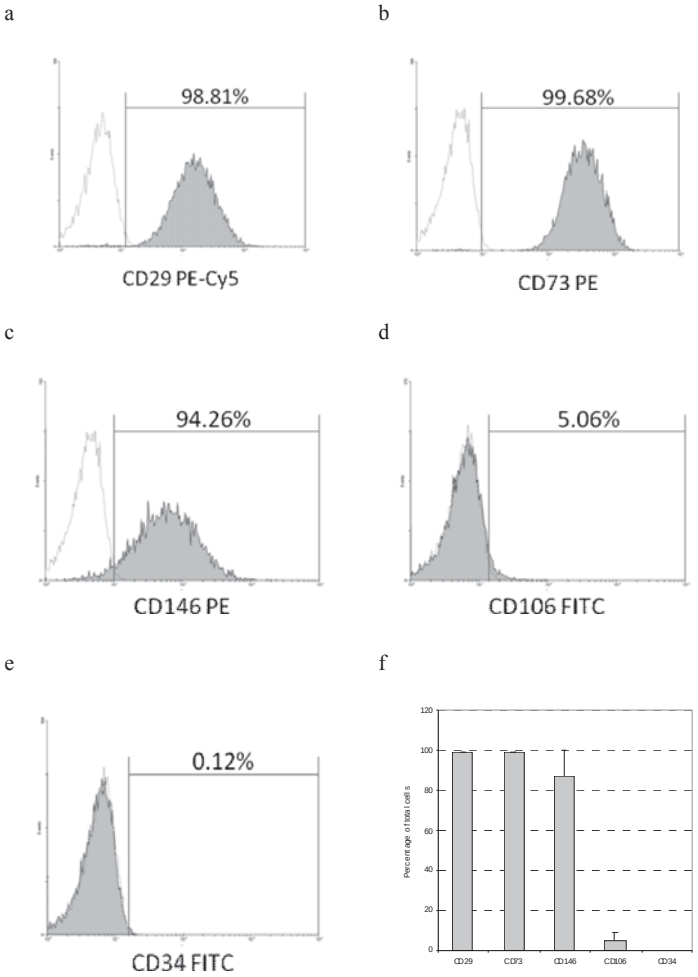

Fig. 3 – Representative flow cytometry plots (passage four) showing expression of a) CD29; b) CD73; c) CD146; d) CD106; e) CD34 f). The histogram gives the mean (± standard deviation) expression of the analyzed markers from five donors.

in the control media had scarce and small fat droplets (Figure 4b). Highly induced calcium deposition in ECM was demonstrated with Alizarin red staining after 3 weeks of culture (Figure 4c). In control media no Alizarin red staining was noted (Figure 4d). After 2 and 3 weeks in chondrogenic medium, small, compact pellets rich in cells were formed. Cells produced the ECM with postive GAGs (Figure 4e) and collagen type II (Figure 4f) staining. Due to the loose tissue structure, pellets in controle medium were decomposed during paraffin embedding protocols.

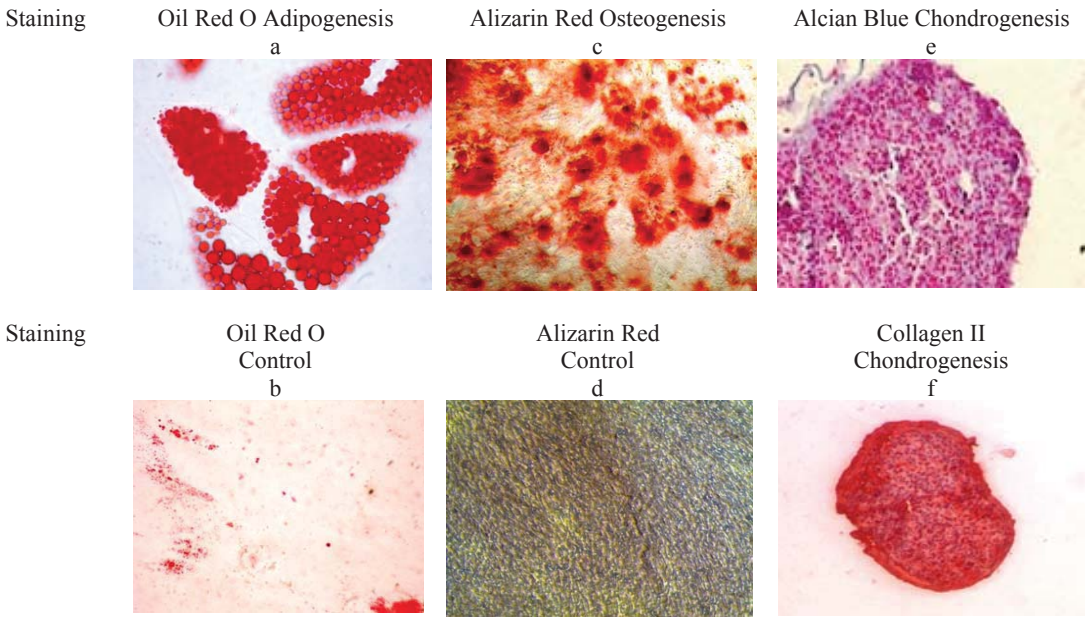

Fig. 4 – Representative images demonstrating: a) Formation of lipid droplets stained with Oil Red O in adipogenesis medium after 3 weeks of cultivation (magnification objective 100 ×); b) Oil Red O staining in a well with cells cultivated in the control media (magnification objective 100 ×); c) Mineralized matrix stained with Alizarin red in osteogenesis medium after 3 weeks of cultivation (inverted microscope, magnification objective 10 ×); d) Alizarin red staining in a well with control media (inverted microscope, magnification objective 10 ×); e) After 2 weeks in chondrogenesis medium, pellets contained sulfated glycosaminoglycans stained light blue with Alcian Blue (magnification objective 20 ×); f) Immunocytochemistry for collagen type II in pellets formed in chondrogenesis medium after 3 weeks of cultivation (magnification objective 10 ×).

Discussion

Our study demonstrates that remnants of crown dental pulp of exfoliated deciduous teeth contain a population of cells that migrate *in vitro*, form CFU-F and have high colony forming efficiency. The CFU-F assay is a useful tool to demonstrate, among primary isolated cells, single ones with sufficient proliferative potential to form colonies of several hundred to a thousand cells. In analogy with the hematopoietic system hierarchy, those cells may form a population of progenitor cells with tri-lineage, bi-lineage or uni-lineage potential. The CFU-F frequency could represent the tissue potential for generating enough cells for tissue engineering or cell therapy. In our study the CFU-F frequency was comparable with earlier published data about culture of deciduous teeth pulp cells and those isolated from other tissues connected with tooth development [19]. Calculating on the basis of 10^5 cells, dental pulp contains at least 10 times more CFU-F than bone marrow (BM), but the total number of CFU-F in one digested remnant of crown dental pulp is about 12 to 20 [4], much below the total number of CFU-F that can be obtained after BM aspiration [20]. From the clinical point of view, the low initial number of CFU-F is a disadvantage. However, DTSC can exert three times more population doublings than BM MSCs [4], so their proliferation potential is higher and they are naturally more primitive. Short PDT reveals a high proliferative activity of cells isolated in our experiment. This is consistent with similar findings of other authors [4, 21], but much shorter than the average PDT reported by Suchaneck et al. [22]. This inconsistency could be explained by diverse culture conditions in different laboratory protocols, which could lead to isolation or expansion of different cell populations. The other possibility is that PDT could be influenced by different FBS lots containing different amounts of stimulators or inhibitors of cell proliferation. Besides fundamental stem cell biology, our data concerning the proliferative potential of DTSCs are important for cell therapy protocols. Namely, a small number of cells harvested from a primary source is a limitation for therapeutic use [23, 24]. We showed that, although the initial number of 0.3×10^5 to 3×10^5 cells harvested from dental pulp tissue explants was insufficient for clinical use, expansion was fast and the final number of cells after the fourth passage (calculating PDT ~ 25 h) was around 100×10^6. Also, their CFE was high, leading to the conclusion that most cells have the important proliferative potential necessary for tissue engineering strategies.

It is known that remnants of dental pulp contain extracellular matrix, odontoblasts, fibroblasts, endothelial cells, pericytes and MSCs [4]. Among them, MSCs, endothelial cells and pericytes are migratory cells that at the same time have high proliferative potential. A heterogeneous phenotype for CD146 and CD106 antigens in multicolony culture of MSCs is a common finding [4, 5] and not all of the markers are specific for putative mesenchymal stem cells. CD146 is expressed in pericytes and endothelial cells in culture [4, 25, 26]. CD106 is a vascular cell adhesion molecule (VCAM) expressed in endothelial cells and also in smooth muscle cells and proliferating pericytes [27]. DPSC and SHED were found positive for CD106 but less strongly than BM MSC [3, 28, 29]. Since CD146 and CD106 molecules are expressed on endothelial cells and dental pulp stem cells easily differentiate

into endothelial cells [10, 30], we cannot exclude that a small portion of cells positive for CD106 in our cultures could be endothelial cells. Based on markers expression, the majority of cultivated cells could be pericytes. Indeed, multiple studies have recognized pericytes as MSCs [31, 32]. Therefore, we can conclude that using the outgrowth method to yield cells from remnants of deciduous teeth dental pulp, results in isolation of cells that do not belong to hematopoietic cell lineages but have markers indicative for pericytes that are also indicated as markers for MSCs [32].

The differentiation potential of harvested cells is important when considering their potential to regenerate specified tissues, like bone, cartilage or adipose tissue. We demonstrated that cells isolated in our multicolony culture system are able to differentiate in cells that from large lipid droplets, depose ECM with calcium salts and from cartilage like tissue that contains GAGs and collagen II. It was previously shown that SHED, obtained by enzymatic digestion of dental pulp, underwent adipogenic, osteogenic, dentinogenic and neurogenic differentiation *in vitro* [4], while chondrogenic potential [8] and embryonic stem cell markers were demonstrated later [33]. IDPSC obtained by the outgrowth method formed adipocytes, osteoblasts, chondrocytes, skeletal and smooth muscles as well as neurons [5]. OCT-4 and other embryonic stem cell markers were also detected pointing to the very primitive nature of these cells [5]. Considering all these findings, it seems that SHED and IDPSC, first claimed to have separate characteristics [5], do not differ from each other. The spectrum of the differentiation potential of DTSC was enlarged when these cells were found to differentiate into a pancreatic cell lineage resembling islet-like cell aggregates [34], endothelial [10] and epithelial-like cell types [9].

Previously, important results about the differentiation potential of DTSC multicolony and clonal cell cultures were collected from experiments *in vivo*, mostly using immunocompromised mice. Thus, one quarter of SHED clones generated dentine-like tissue [10]. Also, multicolony derived cells generated ectopic dentine-like tissue equivalent to that produced by clonal cells [4] indicating that multicolony derived cell populations have the same differentiation capacity as clonal cells but are more convenient to use in a clinical setting.

Conclusion

In our experimental conditions, after 10 to 15 days of explant culture, the harvested cell population was able to expand for up to 1 month, when the cultures were stopped. Cells were positive for mesenchymal cell markers typically found on expanded stem cell populations, produced CFU-F and successfully formed a mineralized matrix, cartilage-like tissue and adipocytes, so showing multipotency. Taking together, the approach using dental pulp tissue explants yield high number of cells with MSC properties and is convenient for further investigations *in vitro* and work on tissue engineering protocols.

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Prospective study of perinatal outcome in pregnancies with primary antiphospholipid syndrome

Prospektivna analiza perinatalnih ishoda kod trudnica sa antifosfalipidnim sindromom

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Abstract

Background/Aim. Pregnancies complicated with antiphospholipd syndrome are associated with the increased perinatal mortality and morbidity. The aim of this study was to assess perinatal outcome in pregnancies with primary antiphospholipd syndrome. **Methods.** This prospective study evaluated perinatal outcome in 25 pregnant women with antiphospholipid syndrome. After establishing vital pregnancy all the patients were treated with low-molecular-weight heparin and aspirin. The perinatal outcome was measured by rates of miscarriages, preterm deliveries, live births and neonatal complications. **Results.** Of the 25 pregnancies, 20 (80%) resulted in live birth, 3 (12%) in spontaneous abortion and 2 (8%) were stillbirths. The mean gestational age at delivery was 37.2 ± 1.0 weeks, mean neonatal birth weight was 2,930.4 ± 428.0 g. Prematurity occurs in 4 (20%) live births, and there were 4 (20%) intrauterine growth restriction with mean birth weight 2,060 ± 210.6 g. Neonatal complications were present in 6 (30%) newborns. Adverse perinatal outcome was significantly associated with anticardiolipin IgG antibodies ($p < 0.01$) and development of hypertension during pregnancy ($p < 0.01$). **Conclusion.** Despite a high incidence of adverse perinatal outcomes in pregnancies with primary antiphospholipid syndrome, early treatment with aspirin and low-molecular-weight heparin, combined with meticulous fetomaternal monitoring could be associated with a relatively high probability of favorable perinatal outcome.

Key words:
antiphospholipid syndrome; pregnancy outcome; aspirin; heparin, low-molecular-weight.

Apstrakt

Uvod/Cilj. Trudnoća komplikovana antifosfolipidnim sindromom udružena je sa povećanim perinatalnim morbiditetom i mortalitetom. Cilj rada bio je procena perinatalnog ishoda u trudnoći sa primarnim antifosfolipidnim sindromom. **Metode.** U prospektivnoj studiji analizirali smo perinatalni ishod kod 25 trudnoća sa antifosfolipidnim sindromom. Po utvrđivanju vitalnosti trudnoće sve ispitivane bolesnice dobijale su niskomolekularni heparin i aspirin. Procena perinatalnog ishoda bazirana je na učestalosti pobačaja, prevremenih porođaja, živorođenosti i neonatalnih komplikacija. **Rezultati.** Ishod 25 analiziranih trudnoća bio je sledeći: 20 (80%) živorođenih, 3 (12%) spontana pobačaja i 2 (8%) mrtvorođenih. Prosečna gestacijska starost na rođenju iznosila je 37,2 ± 1,0 nedelja, a prosečna telesna masa novorođenčadi 2 930,4 ± 428,0 g. Prevremeni porođaj registrovan je kod 4 (20%) živorođenih, bilo je 4 (20%) slučaja intrauterinog zastoja u rastu ploda sa prosečnom težinom na rođenju od 2 060 ± 210,6 g, a neonatalne komplikacija bile su prisutne kod 6 (30%) novorođenčadi. Nepovoljan perinatalni ishod bio je značajno povezan sa antikardiolipinskim IgG antitelima ($p < 0,01$) i razvojem hipertenzije tokom trudnoće ($p < 0,01$). **Zaključak.** Uprkos visokoj incidenciji nepovoljnog perinatalnog ishoda trudnoća sa antifosfolipidnim sindromom, rano započinjanje tretmana sa niskomolekularnim heparinom i aspirinom, uporedo sa intenzivnim nadzorom majke i fetusa, moglo bi biti udruženo sa relativno velikom verovatnoćom povoljnog perinatalnog ishoda.

Ključne reči:
antifosfolipidni sindrom; trudnoća, ishod; aspirin; heparin, niskomolekulski.

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Introduction

Antiphospholipid syndrome (APS) is an immune-mediated thrombophilia, presenting as recurrent vascular thrombosis and pregnancy morbidity, in association with positive tests for antiphospholipid antibodies [1]. Antiphospholipid syndrome is classified as secondary if there is present underlying autoimmune disease such as systemic lupus (SLE) or rheumatoid arthritis [2]. In contrast, in primary APS thrombosis and/or pregnancy failure occur in isolation.

Women with APS have an unusually high proportion of pregnancy losses within the pre-embryonic, embryonic and fetal period [1]. Pregnancies with APS can also be complicated by preterm delivery due to pregnancy-associated hypertensive disease and placental dysfunction [1].

Pathophysiological mechanism present in this syndrome includes antibody-mediated interference with coagulation homeostasis, platelet and endothelial cell activation, placental tissue injury, T-cell immune response and complement activation [3].

Since this syndrome has a tremendous impact on maternal and fetal morbidity and mortality, there has been continued interest and efforts to better define therapy for the condition.

A combination of heparin and aspirin represents the most frequently applied therapeutic protocol, resulting in a live birth rate of up to 70–80 % of cases [4–6].

Steroids have also been used during pregnancy in patients with APS, however a significant maternal and fetal morbidity reported discourage this treatment modality [7].

Intravenous immunoglobulin (IVIG) is a form of therapy usually combined with heparin and low-dose aspirin, especially in women with unfavorable obstetric history or recurrent pregnancy loss during heparin treatment [4]. However, a randomized study of IVIG treatment during pregnancy in unselected APS cases found no benefit of this expensive therapy compared to heparin and low-dose aspirin [8]. It is currently unclear whether IVIG may have therapeutic significance in refractory APS cases, and it would be vise to limit its use to patients with APS who have had poor pregnancy outcome despite treatment with aspirin and heparin [8].

A recent update of the classification criteria for definite APS introduced the concept of stratification of APS patients on the basis of laboratory and clinical characteristics [9]. According to these reports, different therapeutic regimens should be used in the various subsets of APS patients [9].

Methods

In this study we followed 25 monofetal pregnancies in women who had been diagnosed preconceptionally with APS according to the International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome [9]. Clinical criteria were: 1) one or more clinical episodes of arterial, venous or small vessel thrombosis; 2) pregnancy morbidity that included: a) one or more unexplained deaths of morphologically normal fetus before 10th week of gestation; b) one or more premature births of morphologically normal neonate before 34th week of gestation because of eclampsia or severe pre-eclampsia, or recognized features of placental insufficiency; c) three or more unexplained consecutive spontaneous abortions before 10th gestation. Laboratory criteria were: 1) Lupus anticoagulant (LA) present in plasma, on two or more occasions at least 12 weeks apart, detected according to the guidelines of the International Society on Thrombosis and Hemostasis; 2) Anticardiolipin (aCL) antibody of IgG or IgM isotype in serum or plasma present in medium or high titer on two or more occasions at least 12 weeks apart measured by standard ELISA; 3) Anti-β2 glycoprotein-I antibody of IgG or IgM isotype in serum or plasma present on two or more occasions, at least 12 weeks apart measured by standardized ELISA.

The diagnosis of antiphospholipid syndrome requires the combination of at least one clinical and one laboratory criterion.

Women with uterine abnormality and multiple pregnancies were excluded from the study on the basis of ultrasound examination. Other thrombophilias such as activated protein C resistance, protein C/S deficiency, G 20210A prothrombin mutation that may mimic APS were diagnosed using appropriate hematological laboratory testing and were also excluded from the study [10]. Patients with abnormal karyotype detected on standard cytogenetic analysis were not included in the study.

All women in our study had the history of recurrent miscarriage and persistently positive results for antiphospholipids antibodies.

The study was approved by the Ethics committee of the Belgrade University Clinical Center.

All the patients received aspirin 100 mg/daily when they had positive serum β-hcG (> 50 IU/mL) indicating that they are pregnant. When fetal heart activity was detected on vaginal ultrasound all women received additional therapy of subcutaneous low-molecular-weight heparin (deltaparin sodium) 5000 IU 24 hourly. The last heparin injection was made 12 hours before planned caesarean delivery and was begun again 12 hours after delivery. Aspirin was stopped three weeks prior to labor.

During first hospitalization clinical history, physical examination, complete blood count, routine biochemistry were evaluated. Coagulation screening included prothrombin time, partial thromboplastin time, fibrinogen, anti-thrombin III and D-dimer levels. Patients were hospitalized every four week until the delivery.

From the 24th week of gestation Doppler analysis of fetal uteroplacental and cerebral circulation was performed during hospitalization, and from 32nd weeks of gestation cardiotocography was also introduced as a part of fetal monitoring.

Uterine artery mean pulsatility index (PI) values ≥ 1.45 were considered abnormal, and umbilical artery Doppler PI value > 95th for gestation or absent/reversed end-diastolic flow were considered abnormal [11].

Definitions of adverse perinatal outcome were: fetal deaths defined as stillbirths ≥ 22 weeks of gestation or an infant weighting ≥ 500 gr; neonatal death defined as the death of a liveborn infant before the 28th day of life; perinatal mortality comprised both fetal and neonatal deaths; spontaneous abortion was defined as spontaneous loss of a fetus before 24 weeks of gestation; preterm delivery comprised delivery < 37 weeks of gestation; newborn were considered small (intrauterine growth restriction – IUGR) when their

birth weight was below the tenth percentile, on the basis of standard growth and development for Serbian population [12].

Anticardiolipin antibodies (IgM and IgG) were identified with an enzyme-linked immunosorbent assay (ELISA) and lupus anticoagulant (LA) was detected with standard activated partial thromboplastin time (aPTT) followed by the dilute Russell`s viper venom time (dRVVT) [13]. Data for anticardiolipin antibodies were expressed as Immunoglobulin G Phospholipid (GPL) or Immunoglobulin M Phospholipid (MPL) units using international reference material [13]. Cutt-off values for medium/high titers were 15 GPL and 16 MPL, respectively [13].

Statistical analysis was performed using SPSS statistical software (SPSS for Windows, release 10.0, Chicago, IL). Descriptive statistics are presented as mean values ± standard deviation (SD), frequency and percentage. The differences between the groups were compared with parametric Student's *t*-test or χ^2-test. A statistical significance was set up at *p* less than 0.05 ($p < 0.05$).

Results

The clinical characteristics of patients and laboratory parameters are presented in Table 1.

Treatment with aspirin began at the mean gestational age of 4.2 ± 1.2 weeks and treatment with heparin at the mean gestational age of 7.33 ± 1.73 weeks. Eight (32%) women developed hypertension during pregnancy, of whom 2 (8%) had pre-eclampsia.

Abnormal uterine artery PI was detected in 5 (20%) pregnancies, and abnormal umbilical artery PI in 3 (12%) pregnancies. There was 1 (4%) patient with absent umbilical artery end-diastolic velocity.

The parameters of perinatal outcome are presented in Table 2. The live birth rate was 20 (80%), 3 (12%) pregnancies ended in spontaneous abortion and there were 2 (8%) stillbirths. Spontaneous abortion occurred at the mean of 12.0 ± 4.1 weeks. The mean gestational age at delivery was 37.2 ± 1.0 weeks, mean neonatal birth weight was 2,930.4 ± 428.0 g. Prematurity occurs in 4 (20%) live births, and there were 4 (20%) intrauterine growth restriction with the mean birth weight 2,060 ± 210.6 g.

Six (30%) newborns were admitted to the neonatal intensive care unit (three with respiratory distress, two with perinatal asphyxia and one with intraventricular hemorrhage grade I/II) of whom none had fatal outcome or did developed permanent disability.

Poor perinatal outcome was significantly associated with anticardiolipin IgG antibodies ($p < 0.01$) and development of hypertension during pregnancy ($p < 0.01$).

Discussion

In the last three decades many efforts have been made to define the best approach for treatment and monitoring pregnancies with primary APS.

In patients with primary APS treated with heparin and aspirin, according to the different studies, live births ranges between 70–80%, early fetal loss between 10–20%, IUGR between 15–30%, preterm deliveries between 10–25%, maternal complications other than preeclampsia are present in 8–13%

Table 1

Clinical characteristics and antiphospholipid antibody profile of patients with primary antiphospholipid syndrome (APS)

Patients characteristics	Values
Age (year), $\bar{x}$ ± SD	31.0 ± 4.95
Number of previous abortion, $\bar{x}$ ± SD	2.41± 0.87
Lupus anticoagulant positive, (%)	58.82
Anticardiolipin IgM positive, (%)	35.29
Anticardiolipin IgG positive, (%)	29.41
Antikardiolipin IgM + IgG positive, (%)	17.64
Anticardiolipin IgM titre (MPL), $\bar{x}$ ± SD	12.46 ± 4.72
Anticardiolipin IgG titre (GPL), $\bar{x}$ ± SD	19.43 ± 8.31
Abnormal uterine artery pulsatility index (PI), (%)	5 (20)
Abnormal umbilical artery pulsatility index (PI), (%)	3 (12)
Absent umbilical artery end-diastolic velocity, n (%)	1 (4)

GPL – immunoglobulin G phospholipid units;
MPL – immunoglobulin M phospholipid units.

Table 2

Perinatal outcome in pregnancies with primary antiphospholipid syndrome (APS)

Perinatal outcome	Values
Live birth, n (%)	20 (80)
Spontaneous abortions, n (%)	3 (12)
Stillbirth, n (%)	2 (8)
Preterm delivery, n (%)	4 (20)
IUGR, n (%)	4 (20)
Neonatal complications, n (%)	6 (30)
Neonatal gestational age at birth (weeks), $\bar{x}$ ± SD	37.2 ± 1.0
Neonatal birth weight (g), $\bar{x}$ ± SD	2,930.4 ± 428.0

IUGR – intrauterine growth restriction.

and preeclampsia develops in 11–60% of patients [3, 13–16]. In our group of patients with primary APS live birth rate was 80%, early fetal loss was 3%, preterm delivery rate was 20%, IUGR was present in 20%, preeclampsia developed in two patients, and the rate of neonatal complications were 30% of whom neither had fatal outcome or did result in permanent disability. This relatively high rate of live births and moderate level of preterm deliveries could be regarded as good results despite small number of patients that were enrolled in the study. The explanations for these outcomes may be sought in fact that lupus anticoagulant antibodies were positive in 60% of patients and that anticardiolipin antibodies titers were moderately elevated without higher titres of IgG anticardiolipin antibodies present. Therefore, the group of patients analyzed in our study could represent a milder form of antiphospholipid syndrome.

Primarily, anticardiolipin (aCL) antibodies are not as strong risk factor for development of trombosis and other complications as lupus anticoagulant antibodies (LA). LA is considered the most powerful predictor of thrombosis [14]. Antibodies titer and isotype are also important: IgG aCL is more strongly associated with clinical manifestations than IgM aCL, ant the risk of thrombosis and other complications increases with high titers (> 40 U) [15].

However, there are still present controversies about the significance of the titer of anticardiolipin antibodies (particulary IgG) and its contribution to maternal morbidity and perinatal outcome that deserves further comprehensive studies. The current problem of laboratory standardization and the clinical heterogeneity inherent in the antiphospholipid syndrome have resulted in difficulties to interprete the significance of various factors for the clinical outcome.

Uterine artery Doppler blood flow analysis provides a noninvasive indirect method of screening women with risk of uteroplacental insufficiency. In our study abnormal uterine artery Doppler waveform pattern was present in 20% of pregnancies, and abnormal Doppler waveform, in umbilical artery in 12% of pregnancies. All these pregnancies developed hypertension, and 60% of these women had some unfavorable perinatal outcome. Despite a high incidence of adverse perinatal outcome in pregnancies with hypertension and abnormal uterine or umbilical artery Doppler waveforms because of a small number of patients in this subgroup we could not find any statistically significant association between these two variables.

Overall, our findings indicate that poor perinatal outcome was significantly associated with anticardiolipin IgG antibodies ($p < 0.01$) and development of hypertension during pregnancy ($p < 0.01$).

Conclusion

The results of our study show that despite a high incidence of adverse perinatal outcomes in pregnancies with primary antiphospholipid syndrome, early treatment with aspirin and low-molecular-weight heparin combined with meticulous fetomaternal monitoring could be associated with a relatively high probability of favorable perinatal outcome. Accurate preconceptional counseling and multidisciplinary approach are essential to achieve these results.

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Lipodystrophy induced by combination antiretroviral therapy in HIV/AIDS patients: A Belgrade cohort study

Lipodistrofija indukovana kombinovanom antiretrovirusnom terapijom kod HIV/AIDS bolesnika

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Abstract

Background/Aim. Highly active antiretroviral therapy (HAART) has led to dramatic reductions in mortality and morbidity of HIV/AIDS-patients. Lipodystrophy, a syndrome including peripheral fat wasting and central obesity, is well-documented side effect of HAART. The aim of this study was to evaluate the incidence of lipodystrophy, and to determine its risk ratios in a HIV/AIDS-cohort. **Methods.** This cross-sectional study included all the antiretroviral-naive HIV/AIDS patients commencing HAART from October 1, 2001 to October 1, 2010, at the HIV/AIDS Center, Institute of Infectious and Tropical Diseases, Belgrade, Serbia. Univariate and stepwise multivariate logistic regression analyses were used to determine the odds ratios (OR) with the confidence interval (CI) of 95%, in order to establish the relative risk for lipodystrophy. The Kaplan-Meier-method was used to determine the probability of development lipodystrophy over time. All statistical analyses were performed using SPSS software version using 0.05 as a *p*-treshold for the significance. **Results.** This study included 840 HIV/AIDS patients, 608 women and 232 men, followed for 5.6 ± 2.8 years. The prevalence of lipodystrophy was 69.2%. Univariate and stepwise multivariate regression analysis identified that the female gender, hepatitis C coinfection, AIDS diagnosis prior to HAART initiation, nucleoside-reverse-transcriptase-inhibitors and protease-inhibitors based regimens had a high risk for developing lipodystrophy in HIV/AIDS-patients (OR = 1.6, 95% CI = 1.1–3.49, $p = 0.04$; OR = 3.31, 95% CI = 1.3–6.8, $p < 0.01$; OR = 3.7, 95% CI = 1.7–6.1, $p < 0.01$; OR = 2.1, 95% CI = 1.7–3.3, $p < 0.01$; OR = 6.1, 95% CI = 4.1–9.7, $p < 0.01$, respectively). **Conclusion.** Despite much greater life expectancy of HIV/AIDS-patients, treatment-related toxicities still remain a major concern. Monitoring of lipodystrophy, as side effect of HAART, is particularly important.

Key words:
lipodistrophy; incidence; risk factors; hiv; antiretroviral therapy, highly active; sex; hepatitis c.

Apstrakt

Uvod/Cilj. Visokoaktivna antiretrovirusna terapija (HAART) dovela je do značajnog sniženja mortaliteta i morbiditeta kod bolesnika sa HIV/AIDS-om. Lipodistrofija, sindrom definisan perifernim gubitkom masnog tkiva, praćen istovremenom centralnom gojaznošću, predstavlja dokumentovani neželjeni efekat HAART–a. Cilj ovog istraživanja bio je da se utvrdi incidencija lipodistrofije i da se determinišu potencijalni faktori rizika od njenog nastanka i razvoja kod pacijenata sa HIV/AIDS-om koji su lečeni HAART-om. **Metode.** Retrospektivnom studijom bili su obuhvaćeni svi HIV/AIDS pacijenti koji nikada ranije nisu bili lečeni HAART-om i koji su započinjali naznačenu terapiju u periodu od 01.10.2001. do 01.10.2010. u Centru za HIV/AIDS Instituta za infektivne i tropske bolesti, Beograd, Srbija. Univarijantna i naknadna multivarijanta logistička regresiona analiza primenjene su da bi se utvrdio odnos šansi (OR) i interval poverenja od 95% (95% CI) u cilju procene relativnog rizika od razvoja lipodistrofije. Kaplan-Majerova analiza korišćena je u cilju procene verovatnoće razvoja lipodistrofije tokom vremena. Sve korišćene statističke analize rađene su primenom SPSS softverskog paketa, pri čemu je $p < 0.05$ smatrano statistički značajno. **Rezultati.** U studiju je bilo uključeno ukupno 840 HIV/AIDS-pacijenata, 608 muškog i 232 ženskog pola, koji su praćeni 5,6 ± 2,8 godina. Prevalencija lipodistrofije iznosila je 69,2%. Univarijantna i naknadna multivarijantna regresiona analiza identifikovale su ženski pol (OR = 1,6; 95% CI = 1,1–3,49, $p = 0,04$), istovremenu infekciju he-

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patitis C virusom (OR = 3,31, 95% CI =1,3–6,8, $p < 0,01$), AIDS pre započinjanja HAART-a (OR = 3,7; 95% CI = 1,7–6,1, $p < 0,01$), nukleozidne inhibitore reverzne transkriptaze (OR = 2,1, 95% CI = 1,7–3,3, $p < 0,01$) i proteazne inhibitore (OR = 6,1, 95% CI = 4,1 – 9,7, $p < 0,01$), kao nezavisne prediktore za razvoj lipodistrofije kod pacijenata inficiranih HIV-om. **Zaključak.** Uprkos značajnom produženju života pacijenata sa HIV/AIDS-om, toksičnost indukovana primenom HAART-a i dalje predstavlja značajan problem. Upravo zato, praćenje pojave i razvoja lipodistrofije, kao neželjenog efekta HAART-a, jeste od izuzetne važnosti.

Ključne reči:
lipodistrofija; incidenca; faktori rizika; hiv; lečenje antiretroviroticima, visokoaktivno; pol; hepatitis c.

Introduction

The use of highly active antiretroviral therapy (HAART), in treatment of human immunodeficiency virus (HIV) has led to dramatic reductions in mortality and morbidity of patients [1]. From a very bad prognosis in its beginnings, HIV infection and acquired immunodeficiency syndrome (AIDS) meanwhile became the chronic disease, which can be treated, controlled and supervised.

HAART usually consists of the combination of two drugs from the nucleoside reverse transcriptase inhibitors (NRTI) group, and a single drug from the protease inhibitors (PI) group (2 NRTIs + 1 PI) or of one non-nucleoside reverse transcriptase inhibitor (NNRTI). The latter combination (2 NRTIs + 1 NNRTI) is equally efficient as the first one, so that the PIs are often reserved for the later stages of the treatment due to their higher resistance barrier [2].

Lipodystrophy, a syndrome including peripheral fat wasting and central obesity, which in some patients may be followed with metabolic changes such as hyperlipidemia and glucose intolerance, or diabetes mellitus, is well-documented side effect of HAART [3, 4]. Changes in body composition include lipoatrophy, a complete or partial loss of adipose tissue predominantly in limbs and face and lipohypertrophy, which is pathological accumulation of adipose tissue in the omentum, mesenterium, retroperitoneum and pelvis, dorsocervical region, or breast enlargement in women [5]. Lipoatrophy and lipohypertrophy may or may not coexist. Reported prevalence rates of lipodystrophy range from a few percent to over 80% [4, 5].

The aim of this study was to evaluate the incidence of lipodystrophy, and to determine the relative risk rates of this side effect of HAART, in HIV/AIDS cohort of antiretroviral-naive patients commencing HAART in Belgrade, Serbia.

Methods

This cross-sectional study included all eligible HIV-infected patients receiving HAART at the HIV/AIDS Center of Institute of Infectious and Tropical Diseases, Clinical Center of the Republic of Serbia, Belgrade.

To be eligible in the study, a patient had to be 18 or older, with documented HIV infection [6], antiretroviral-naive patients commencing HAART from October 1, 2001 to October 1, 2010, and with collected data about the occurrence of lipodystrophy. The clinical diagnosis of lipodystrophy was made on the basis of physical examination, by recognizing changes such as fat loss from face, arms and legs, and accumulation of fat in the abdominal and/or dorso-cervical region, including breast enlargement in women, as described in the European AIDS Clinical Society (EACS) guidelines [7].

The exclusion criteria were: simultaneous therapy with corticosteroids, anabolic steroids, as well as the treatment of some of the AIDS-related opportunistic infections, within 3 months before the lipodystrophy diagnosis.

During the study period, 25 antiretroviral drugs were registered worldwide, of which the following were also registered in Serbia: zidovudine, didanosine, lamivudine, stavudine, zalcitabine and abacavir (from the NRTI group), nevirapine and efavirenz (from the NNRTI group), saquinavir, nelfinavir, indinavir, fosamprenavir, lopinavir/ritonavir, and ritonavir for boosting other PIs (from the PI group). Nelfinavir and indinavir were withdrawn in 2008. Enfuvirtide was introduced in 2007 for salvage regimens. Newer drugs, such as tenofovir/emtricitabine, tipranavir, atazanavir, etravirine, raltegravir, as well as maraviroc, were not included among the drugs reimbursed by the national health insurance system during the study period.

The immunological and virological responses to HAART were evaluated every 4–6 months by measuring plasma viral loads (pVL) and CD4+ T-cell counts. Due to shortages, HAART monitoring was not performed regularly, which led to the delay in the measurements of CD4+ T-cell counts and viral loads. CD4 cells were quantified by flow-cytometry. Plasma HIV-1 RNA loads were measured by quantitative reverse transcriptase polymerase chain reaction (ultrasensitive assay version 1.5, Roche Molecular Systems, Branchburg, NJ, USA), with a lower limit of detection of 50 copies/mL (1.7 $\log_{10}$).

All statistical analyses were performed using SPSS software version using 0.05 as a p treshold for the significance. Possible associations between lipodystrophy and gender, age above 40, coinfection with hepatitis C virus, AIDS [7] at HAART initiation and the type of HAART regimen, were tested. Univariate and stepwise multivariate logistic regression analyses were used to determine the odds ratios (OR), with the confidence interval (CI) of 95%, in order to establish the relative risk for the occurrence of lipodystrophy. The Kaplan-Meier product limit method were used to determine the probability of developing lipodystrophy over time.

The study was approved by the Clinical Center of Serbia Ethics Committee, Belgrade, Serbia.

Results

This study included 840 HIV/AIDS patients, followed for a mean period of 5.6 ± 2.8 years. There were 608 (72.4%) men and 232 (27.6%) women. The median age was 43.5 years (range: 22–58 years). Along with HIV, 216 (25.7%) patients were coinfected with hepatitis C virus (HCV), and 52 (6.2%) patients with hepatitis B virus (HBV). The median CD4+ cell count was 347 cells/mm^3 (range: 183–455 cells/mm^3).

HAART regimens included the following: combinations of two NRTIs as a treatment backbone, with one or two PIs (taken by 14.3% of all the patients), or one NNRTI (33.3%), and multiple combinations of drugs from all the classes (52.4%).

The prevalence of lipodystrophy was 69.2%. The overall estimated probability of developing lipodystrophy increased with time, reaching 100% after ten years of treatment, with median time of 7 years for the development of lipodystrophy in 50% of patients (Figure 1). In the subgroup of patients coinfected with HCV, the prevalence of lipodystrophy was 41%, as opposed to 25.8% among those without HCV ($p < 0.01$).

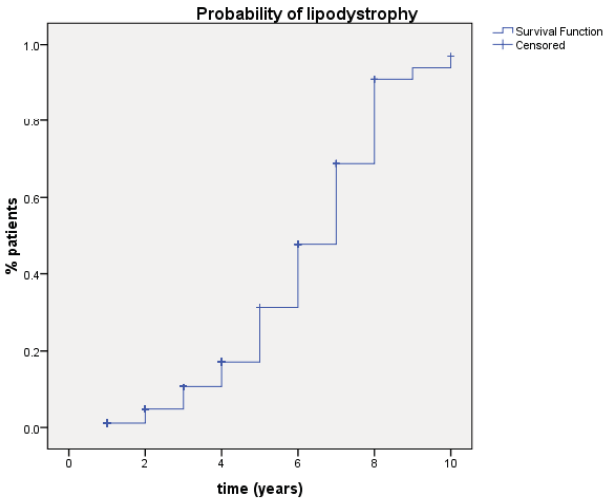

Fig. 1 – The estimated probability (Kaplan-Meier product limit method) of developing lipodistrophy over time.

Univariate logistic regression analysis (Table 1) showed that female gender, age above 40, HCV coinfection, AIDS at HAART initiation, and prolonged usage of NRTIs, were all associated with lipodystrophy. Among the NRTIs, multivariate logistic regression analysis indicated that highest risk for lipodystrophy was associated with the usage of stavudine, didanosine and zidovudine. In contrast with this, lamivudine and abacavir were not significant predictors (OR 2.1, 95% CI 1.6–3.9, $p = 0.09$; OR 3.3, 95% CI 1.7–4.1, $p = 0.6$, respectively). The usage of NNRTI based regimens carried a lower risk for lipodystrophy (OR 2.1, 95% CI 1.2–3.3, $p < 0.01$). The same analysis showed that the PI regimens were also associated with the development of lipodystrophy (OR 5.9, 95% CI 3.7–9.6, $p < 0.001$). The stepwise multivariate regression analysis identified that the PI based regimens had together with NRTI drugs, female gender, HCV coinfection, and AIDS diagnosis prior to HAART initiation, a high risk for developing lipodystrophy in HIV/AIDS patients (Table 1).

Discussion

Within the recognition that HIV production could be suppressed, but not eradicated, the focus of antiretroviral therapy in the mid-1990s was to convert the infection from uniformly fatal disease into a long term, manageable condition. This accomplishment was reported in 1998, with the seminal publication from the Multicenter AIDS Cohort Study (MACS), associating a dramatic drop in death rates from AIDS with the widespread application of HAART in the USA [1]. However, as patients may potentially be exposed to HAART for decades, treatment-associated toxicities, such as lipodystrophy, remains a concern.

The frequency of lipodystrophy in the Belgrade cohort was 69.2%, which was in correlation with the results of Miller et al. [8]. Some previous studies had shown a lower prevalence of lipodystrophy, but that was probably in connection with the lack of precise definition of the syndrome, and the fact that only the most severe cases had been registered [9–11].

We also showed that the probability of developing lipodystrophy increases with time, reaching 100% by 10 years of HAART and with the median time of 7 years for the development of lipodystrophy. Lipodystrophy identification had

Table 1

Risk factors for lipodystrophy

Variable	Univariate analysis			Multivariate analysis		
	OR	(95% CI)	*p*	OR	(95% CI)	*p*
Female gender	1.6	(2.12–4.8)	< 0.01	1.6	(1.1–3.49)	0.04
Age above 40 (years)	1.9	(1.4–3.8)	0.01	–		–
HCV coinfection	2.4	(1.8–4.3)	0.01	3.31	(1.3–6.8)	< 0.01
AIDS at HAART initiation	1.6	(1.1–3.9)	0.02	3.7	(1.7–6.1)	< 0.01
NRTIs (prolonged usage)	2.9	(1.4–5.1)	< 0.01	2.1	(1.7–3.3)	< 0.01
d4T	6.7	(1.8–8)	< 0.01	4.8	(1.61–5.59)	< 0.01
ddI	4.6	(2.2–6.8)	< 0.01	4.2	(2.82–5.71)	< 0.01
AZT	2.2	(1.2–3.9)	0.02	2.1	(1.51–3.86)	< 0.01
PIs	5.9	(3.7–9.6)	< 0.01	6.1	(4.1–9.7)	< 0.01

HCV – hepatitis C virus; AIDS – acquired immunodeficiency syndrome; HAART – highly active antiretroviral therapy; NRTI – nucleoside reverse transcriptase inhibitors; d4T – stavudine; ddI – didanosine; AZT – zidovudine; PIs – protease inhibitors; OR – odds ratio; CI – confidence iterval.

profound implications for the management of HIV/AIDS. Its recognition as a side effect of HAART led to the reevaluation of the appropriate time to start the therapy and also to various modifications in therapy of HIV.

Numerous cross-sectional studies and many prospective cohort studies analyzed the occurrence of lipodystrophy, and attempted to determine the factors that cause it. In our study, both the age above 40 and AIDS diagnosis at HAART initiation were associated with lipodystrophy, but the age above 40 was not an independent predictor. Several studies have demonstrated that the older age and AIDS diagnosis prior to HAART initiation are the risk factors for lipodystrophy [12–14]. There are conflicting data regarding the risks associated with gender. Martinez et al. [15], in their study, found that the female gender is in higher risk for lipodystrophy. Thiebaut et al. [16], on the other hand, came to the contrary conclusions, that the female gender was not in higher risk for lidodistrophy. Several other studies established HCV coinfection as an independent predictor of lipodistrophy, demonstrating the association of HCV co-infection with higher rates of lipodystrophy [17–19].

NRTIs, especially thymidine analogues, are known to cause mitochondrial toxicity [20]. Given the similarities in function between HIV reverse transcriptase and human DNA polymerase, it is not surprising that nucleoside analogues are competitive inhibitors of human DNA polymerase-gamma, a key enzyme for mitochondrial DNA (mtDNA) replication. The accumulation of mtDNA deficits induces a deficient production of molecules devoted to the intramitochondrial synthesis of adenosine triphosphate (ATP). Once ATP production drops below a certain threshold, sudden mitochondrial and then cellular damage occurs that can lead to cell death. Tissues and organs cannot function properly, and the damage becomes clinically apparent [21]. The clinical use of NRTI has been associated with adverse effects caused by mitochondrial dysfunction, such as acute pancreatitis, myopathy, peripheral neuropathy, anemia, neutropenia, hepatic toxicity and hyperlactataemia/lactic acidosis [20–22]. Our data show that the prolonged usage of NRTIs, especially thymidine analogues such as stavudine, didanosine and zidovudine, is likely to be associated with the development of lipodystrophy, which is in accordance with numerous other studies [23–25]. NRTIs induce lipodystrophy by way of mitochondrial toxicity, which is not the case with PIs and NNRTIs. Moreover, NNRTI based HAART is less likely to induce lipodystrophy than the PI based HAART [26].

Lipodystrophy syndrome, together with the insulin resistance, type 2 diabetes mellitus and hyperlipidemia develop a cluster of metabolic abnormalities, referred to as the metabolic syndrome. Metabolic syndrome, increases the cardiovascular risk and has great impact on life expectancy in HIV/AIDS patients [27].

Life style modifications and exercise training, antiretroviral switch strategies, pharmacological management (rosiglitazone, metformin, human growth hormone, human growth hormone–releasing factor, leptin, etc) and reconstructive surgery could be possible treatment options for lipodystrophy syndrome [14].

The initial enthusiasm of clinicians with HAART success, primarily because of the possibility of the long-term control of HIV replication, is suppressed with the new knowledge about the potential toxicity of antiretroviral drugs. For some patients lipodystrophy is only a cosmetic problem, but for others the very same problem may affect their future decisions on the treatment, making them cease the therapy or significantly reduce treatment compliance. As the result, the patients may experience rebound in viral load or the development of viral resistance. This would eventually lead to disease progression and even to an increased risk of disease transmission, possibly by a drug resistant viral strain [28].

Conclusion

Despite much greater life expectancy of HIV/AIDS patients, treatment related toxicities still remain a major concern. Monitoring of the side effects of highly active antiretroviral therapy, such as lipodystrophy is, particularly important.

Acknowledgments

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Medical students` health-related quality of life – A comparative study

Kvalitet života povezan sa zdravstvenim stanjem studenata medicine – komparativna studija

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Abstract

Background/Aim. Previous studies on medical students' subjective perception of health and health-related quality of life (HRQoL) showed inconclusive results. Moreover, there are no published studies to compare HRQoL of medical students to non-medical university students. The aim of the study was to assess subjective perception of health-related quality of life (HRQoL) in medical students' sample, to compare it with non-medical university stu-dents and to ascertain predictors of better perception of HRQoL in medical students. **Methods.** Scores of all domains on the Mental and Physical Component Summary subscales and total score of the Short Form Health Survey (SF-36), used for assessment of HRQoL in samples of 561 medical and 332 non-medical university students were assessed and compared. In addition, linear regression to identify predictors of better perception of mental and physical components of HRQoL and overall HRQoL in the sample of medical students was used. The dependant variables were subscores and total score with the SF-36, and independent variables were certain sociodemographic and academic characteristics of the students. **Results.** Medical students had statistically significantly higher scores on the Mental Component Summary and total SF-36 score compared to non-medical students. Linear regression analysis demonstrated that higher scores of Physical Component Summary were associated with age, male sex and the year of studies. The Mental Component Summary were associated with age, male sex, the year of studies and marital status. The total SF-36 score was associated with age, male sex and the year of studies. **Conclusion.** Medical students perceive their health much better than other university students do, but female, older and second grade medical students have worse perception of their HRQoL. Those points should be potential target areas for specific prevention and treatment in order to achieve better HRQoL.

Key words:
quality of life; students, medical; students; serbia; health; psychiatric status rating scales; questionnaires

Apstrakt

Uvod/Cilj. Dosadašnje studije subjektivne percepcije zdravlja i kvaliteta života povezanog sa zdravstvenim stanjem (HRQoL) studenata medicine pokazale su kontradiktorne rezultate. Štaviše, ne postoje objavljene studije koje su poredile HRQoL studenata medicine i studenata nemedicinskih fakulteta. Cilj ove studije bio je da se proceni subjektivna percepcija HRQoL na uzorku studenata medicine, da se uporedi sa percepcijom HRQoL studenata nemedicinskih fakulteta i da se utvrde prediktori bolje percepcije HRQoL kod studenata medicine. **Metode.** Uzorak za istraživanje obuhvatio je 561 studenta medicine i 332 studenta ne-medicinskih fakulteta Univerziteta u Beogradu. Istraživanje je obavljeno uz pomoć *Short Form Health Survey* (SF-36) upitnika koji procenjuje mentalnu i fizičku komponentu, kao i ukupan skor subjektivne procene kvaliteta života vezanog za zdravstveno stanje. Upoređeni su skorovi sa zbirne skale i supskala dve grupe studenata. Pored toga, korišćena je i linearna regresija da bi se procenili prediktori boljeg sagledavanja ukupnog skora i mentalne i fizičke komponente HRQoL na uzorku studenata medicine. Zavisne varijable bile su supskorovi i ukupan skor sa SF-36 upitnika, a nezavisne varijable sociodemografske i akademske karakteristike ispitanika. **Rezultati.** Studenti medicine imali su statistički značajno više skorove na supskali mentalnog zdravlja i na ukupnom skoru SF-36 upitnika u odnosu na studente ne-medicinskih fakulteta. Linearna regresija pokazala je da su viši skorovi fizičke komponente povezani sa godinama starosti, muškim polom i godinom studija; viši skorovi mentalne komponente povezani sa godinama starosti, muškim polom, godinom studija i brač-

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nim statusom. Ukupan SF-36 skor povezan je sa godinama starosti, muškim polom i godinom studija. **Zaključak.** Studenti medicine gledaju na svoje zdravstveno stanje mnogo bolje nego studenti ne-medicinskih fakulteta. Ipak, devojke, stariji studenti i studenti druge godine medicine imaju lošiju percepciju svog kvaliteta života koji se vezuje za zdravlje. Ovo bi trebalo da budu fokusi za specifičnu prevenciju i eventualnu terapiju u cilju postizanja boljeg kvaliteta života studenata medicine.

Ključne reči:

kvalitet života; studenti medicine; studenti; srbija; zdravlje; psihijatrijski status, određivanje, skale; upitnici.

Introduction

The development of modern medical practice puts the focus on achieving high quality of life, although there are different approaches in interpretation of this term. The World Health Organization defines quality of life as individuals' perception of their position in life in the context of culture and value systems in which they live and in relation to their goals, expectations, standards and concerns [1]. The health-related quality of life (HRQoL) is a relatively new term in medical literature, receiving more attention in recent years. The HRQoL is developed as a narrower term than the comprehensive "quality of life" term, adequate for use in medical science and as an additional indicator of an individual's health. Unlike conventional indicators of a person's health, the quality of life related to health attempt to assess a person's health by the person him/herself. In case of a person with deteriorated health, the HRQoL is defined as his/her perception of the way illness and treatment affect his/her physical and working abilities, physical health as well as psychological condition and social communication [2].

The common impression is that the student population is a part of the general population with a relatively high level of good health. However, it is also a population that is constantly being exposed to a great number of stressors and there are studies that show that students' mental health worsens during the study [3–5]. For example, students of medicine are exposed to the amount of stressors which can be significant and as a consequence can lead to negative effects in achieving academic results, occurrence of emotional problems or deterioration of physical health [6, 7]. Moreover, a subjective evaluation of the HRQoL can be more negative.

The aim of the study was to assess the subjective perception of the HRQoL in medical students' sample, to compare it with non-medical university students and to identify important predictors of the HRQoL by analyzing a number of sociodemographic and academic characteristics in a sample of medical students.

Methods

Participants

The research was conducted among the student population of the Belgrade University, Serbia. The Belgrade University is a public university, located in a large metropolitan area. With 89,482 students, it is the biggest and oldest higher education institution in the Balkan region, consisting of 31 faculties divided into four sections: social sciences and humanities (with 41,231 students), medical sciences (with 12,857 students), nature sciences and mathematics (with 6,873 students), and technology and engineering sciences (with 28,521 students) [8, 9].

The participants were recruited during the introductory lesson in amphitheatres of the faculties, at the begging of the winter semester 2010, which is mandatory for all students. The students were selected in order they appeared.

The sample consisted of 902 students divided into two groups based on the school they were attending. The number of recruited students was about 1% of all Belgrade University students. Nine (1.0%) students were not included in the study sample because they provided invalid data – six medical students and three students from the control answered incorrectly. Thus, the final sample included 893 (99%) participants.

The first group consisted of 561 students of the Medical Faculty. The Belgrade University Medical Faculty has traditional semester curriculum which takes 6 years, including 2 years of basic sciences, 3 years of clinical training and 1 year of clinical internship. During the winter semester of 2010, the number of students that enrolled to the first year was 552, on the second year there was 512, on the third year 511, fourth 494, fifth 525, and final, sixt year, 509 students.

The control group consisted of 332 students from a variety of non-medical schools from the same university: 178 (53.6%) students of social sciences and humanities, 30 (9.0%) students of nature sciences and mathematics and 124 (37.4%) students of technology and engineering sciences. The participants from the control group reflected the proportions of students in these faculty sections (0.43%). Students of veterinary medicine, dentistry and pharmacy were excluded from the research.

Participation was anonymous and voluntarily. All the students who agreed to participate gave their written informed consent. The approval for the study was obtained from the Ethics Committee of the Faculty of Medicine of the University of Belgrade.

Instruments

In all the students, the HRQoL was assessed using Medical Outcomes Study Short Form 36-Item Questionnaire (SF-36 Questionnaire, Serbian translation). The SF-36 Questionnaire is a general health multidimensional survey with 36 questions [10–15]. It is used to evaluate both negative (illness or incapability) and positive (well-being) aspects of health. This questionnaire provides scores in eight domains of life quality which are organized into two summary subscales: Physical Component Summary – PCS (which consists of the following domains: Physical Function, Role Limitations due to Physical Health, Body Pain, General Health) and

Mental Component Summary – (MCS) (which is comprised of the following domains: Energy/Fatigue, Social Functioning, Role Limitations due to Emotional Problems, Emotional Well-being). Each domain was scored out of 100, where a higher score indicate less limitation, better functioning or less pain. The total score of the questionnaire was obtained by calculating the average in each score in all domains, which was also transferred into grades from 0 to 100. Better perception of HRQoL was defined as a higher total score of the SF-36 Questionnaire and its two dimensions – Physical Component Summary and Mental Component Summary, defined as higher scores of those dimensions.

Information concerning age, sex, marital status, parenthood and place of residence and academic data (year of studies, the number of failed years and the average grade of the studies) were gathered through the sociodemographic and academic questionnaire.

Statistical analysis

We used several different methods: descriptive summary statistics for the sociodemographic and academic characteristics and SF-36 scores; parametric (*t*-test) and non-parametric statistic tests ($\chi 2$ and Fisher exact test) to determine sociodemographic and academic characteristics and differences between students according to the school; non-parametric statistics (Mann–Whitney test), in comparison with the SF-36 scores according to the faculty of studying because data were not normally distributed; regressive multivariable analysis (linear regression) to identify the predictors of a better perception of HRQoL in the sample of medical students. Better perception of HRQoL is defined as a higher score of the Physical Component Summary, Mental Component Summary and total SF-36 score. These were dependent variables, and the independent variables were: demographic data (age, sex, marital status, parenthood and place of residence) and academic data (year of studying, average grade, history of failed grade years). A statistical significance was set at $p < 0.05$.

Results

Sociodemographic and academic characteristics data for the medical students (n = 561) and the control group of students (n = 332) are presented in Table 1. The results showed that the medical and the control group of students did not differ on marital and parental status and the history of failed grade year, while the medical students were statistically significantly older, had higher average grade, and female gender dominated. The two groups of students, also, differed on frequency of grade year and on living place.

Table 1

Sociodemographic and academic characteristics of medical and non-medical students

Characteristics	Medical students n = 561	Control group n = 332	*p*
Sex, n (%)			
male	192 (34)	158 (48)	< 0.001*
female	369 (66)	174 (52)	(Fisher exact test)
Age (years), median (range)	23 (18–35)	22 (18–31)	< 0,001 (t = 3.74)
Average grade, $\bar{x} \pm SD$	8.21 ± 0.78	7.97 ± 0.92	0.001 (t = 3.46)
Grade year, n (%)			
1	76 (14)	73 (22)	
2	85 (15)	72 (22)	
3	111 (20)	71 (21)	< 0.001
4	81 (14)	66 (20)	($\chi 2$ = 78.38)
5	110 (20)	50 (15)	
6	98 (17)	0 (0)	
Failed year, n (%)	185 (33)	97 (29)	0.264 (Fisher exact test)
Marital status, n (%)			
single/separated/divorced	544 (98)	328 (99)	0.190
married	13 (2)	3 (1)	(Fisher exact test)
Parenthood, n (%)	10 (2)	4 (1)	0.588 (Fisher exact test)
Place of living, n (%)			
with parents	202 (36)	114 (34)	
in university dormitories	118 (21)	120 (36)	< 0.001
in rented apartments	158 (28)	64 (19)	($\chi 2$ = 27.75)
in own apartment	71 (13)	29 (9)	
other	11 (2)	5 (2)	

Note: Four medical students and one from the control group failed to respond when asked for marital status and one medical student failed to respond when asked for place of living;

The scores and significance of differences of all SF-36 Questionnaire domains for the medical and the control group of students are presented in Table 2. The results indicated that medical students in comparison to the control group had

Table 2

The scores and significance of differences of all domains, Physical Component Summary, Mental Component Summary and total SF-36 score for medical and non-medical students

Significant predictors	Medical students (n = 561)		Control group (n = 332)		*p*
	Mean (SD)	Median	Mean (SD)	Median	
Physical Function	95.20 (9.89)	100	94.08 (11.69)	100	0.026
Role limitations due to physical health	80.70 (28.99)	100	76.81 (29.48)	100	0.012
Body Pain	75.34 (22.48)	74	76.55 (20.98)	77	0.530
General Health	74.87 (16.96)	77	74.47 (16.48)	77	0.582
Energy/ fatigue	55.60 (18.91)	55	52.42 (19.41)	50	0.025
Social Functioning	74.73 (22.31)	75	70.93 (23.65)	75	0.019
Role limitations due to emotional problems	63.87 (40.59)	67	54.08 (42.61)	67	0.001
Emotional well being	66.12 (18.88)	68	60.61 (20.25)	64	< 0.001
Physical Component Summary	76.32 (13.51)	79	74.86 (13.66)	76	0.058
Mental Component Summary	67.07 (17.87)	69	62.53 (18.87)	64	< 0.001
Total SF-36 score	73.31 (15.23)	76	70.02 (15.94)	72	0.001

Multiple ANOVA test with Tukey comparison.

significantly higher scores on the following variables: Physical Function, Role limitations, Energy/Fatigue, Social functioning, Role limitations due to emotional problems and Emotional well being. Also, medical students had significantly higher scores on Mental Component Summary and total SF-36 score than the control group.

Multiple ANOVA test with the Tukey comparison was used to compare the total score of the SF-36 Questionnaire according to the grade year of study. The ANOVA was statically significant (F = 3.24; $p < 0.007$) and there was a statistically significant difference between second and fourth grade year (mean difference = -7.03; $p = 0.033$) and between the second and the sixth year (mean difference = -6.42; $p = 0.048$) indicating statistically significant lower scores in the second year. The other comparisons did not provide statistically significant differences.

Linear regression analysis identified significant sociodemographic and academic predictors of PCS [F (8.445) = 3.19; $p = 0.002$)], MCS [F (8.45) = 3.54; $p = 0.001$], and total SF-36 score [F (8,45) = 3.95; $p < 0.001$]. Significant predictors for PCS in medical students were: male sex, younger age and higher grade year of studies; predictors for MCS were: male sex, younger age, higher grade year of studies and marital status; and predictors for total SF-36 score were: male sex, younger age and higher year of studies (Table 3).

Discussion

Previous studies on medical students` subjective perception of health and HRQoL show inconclusive results. One of them indicates that medical students rate their own health as very good, with no significant differences to the general population aged 20–30 [16] but the others indicate that medical students have lower mental and physical quality of life scores than population norms [13] and that medical students perceive themselves as less healthy and more likely to become ill than general population aged 25–34 years [14]. Also, there are studies which indicate that major impairments in medical students` HRQoL were observed among the third year, in students with depressive symptoms and in female sex [15].

The results of this study indicate that medical students had higher scores of HRQoL than non-medical counterparts. Clearly, the results of this study indicate that medical students generally perceive their HRQoL better than other university students. The difference is more obvious on mental health domain but it is also noticeable in some aspects of physical health dimension of HRQoL. This in contrast to Pekmezovic et al. [8], which may reflect different sample sizes used in our studies (195 *vs* 561 students, respectively), a different methodological approach (consecutive approach *vs* distribution from all years) and different sample participants ("medical sciences" *vs* only medical students, respectively).

Table 3

Results of linear regression to identify sociodemographic and academic predictors of Physical Component Summary, Mental Component Summary and total SF-36 score from medical students (n = 561)

Sociodemographic and academic predictors	Physical Component Summary				Mental Component Summary				Total SF-36 score			
	Unstandardized coefficients		*t*	*p*	Unstandardized coefficients		*t*	*p*	Unstandardized coefficients		*t*	*p*
	B	Std. Error			B	Std. Error			B	Std. Error		
(Constant)	85.56	12.10	7.07	0.000	69.06	16.29	4.24	0.000	82.37	13.71	6.00	0.000
Sex	2.47	1.30	1.90	0.058	4.90	1.75	2.80	0.005	4.03	1.47	2.74	0.006
Age (years)	-1.32	0.43	-3.11	0.002	-1.42	0.57	-2.48	0.013	-1.53	0.48	-3.18	0.002
Study year	2.50	0.65	3.85	< 0.001	3.22	0.87	3.68	< 0.001	2.98	0.73	4.04	< 0.001
Average grade	0.42	0.87	0.48	0.630	0.09	1.18	0.08	0.936	0.39	0.99	0.39	0.693
Failed year	0.32	1.68	0.19	0.847	0.27	2.27	0.12	0.905	0.24	1.91	0.13	0.896
Marital status	8.27	6.00	1.38	0.169	17.23	8.08	2.13	0.034	11.50	6.81	1.69	0.091
Parenthood	-6.50	6.81	-0.95	0.340	-10.27	9.18	-1.12	0.264	-6.47	7.72	-0.83	0.402

In comparison to non-medical students, medical students recognize their HRQoL much enhanced on almost all dimensions, which indicate better perceiving of their mental and physical health and overall well-being. Therefore, it seems that most medical students have either better (mental or physical) health or they have the significant resilience in the face of great demands on their inner resources, time, and health during the studies. Our findings could be explained as follows. It appears that the people attracted to medicine are more likely to possess the necessary resiliency that could lead to better perceiving the HRQoL. This means that medical students might have notable potential for successful adaptation, despite challenges and difficult circumstances and that they may better cope with all types of stress, whether it comes from academic, psychosocial and health circumstances. Further, it is possible that the medical school selection process identifies and picks individuals with great resilience and good health and that students attracted to medical studies are more likely to have the basic resiliency.

Although the results indicate that medical students have better perception of the HRQoL than non-medical students, we assume that some students are inherently more resilient while others are more susceptible for mental and physical health problems and that they may experience even small stressors as major threats or crises. The overall results, similarly to recent one [15], indicates that male, younger and higher grade year students better perceive their HRQoL. In other words, female, older and second year medical students have negative perception of their HRQoL, which correlates with the study of Ray et al. [14].

The results of several previous studies indicate that male students scored better compared to female students in almost all dimensions and overall score of the SF-36 quality of life instrument [8, 17]. The reason could be that academic stress among university students varies across gender with higher levels of depression [8] and anxiety [18, 19] perceived by female students. The result that the students from lower grade years, especially in the second year, had worse perception of overall HRQoL and both its` subdimensions related to mental and physical health is in concordance with other studies [20] and it could be due to stress related to specifics of medical study curriculum [21]. Possible explanations of this may be that first two years of the curriculum at the Belgrade Faculty of Medicine are related to basic sciences, which are taught mainly by subject specialists without medical education or experience or that clinical study years give better perception of health compared to pre-clinical years or that the students at clinical years have some strategy to cope with the stress of the medical education/profession [20]. The result that younger age students had better perception of HRQoL, at a glance, stands out against previous one. But, the truth is that there are many very young and ambitious medical students who have or who develop resilience and who can successfully face up with problems with real life circumstances.

We assume that students that posses predictors of worse perception deserve more attention, prevention activities and, maybe, support in order to improve their resilience and consequently to get better HRQoL. A previous report indicates several measures that should be addressed to vulnerable population of medical students but also to medical schools in order to achieve better quality of life of medical students [22]. The measures include: improvement of medical school selection criteria, support of positive social relationship and mentoring process, self-care skills training, facilitation of strategies for coping with examination stress and modelling of self care [22].

The results of this study should be interpreted with caution because of several limitations. Firstly, there are limitations associated with the reliance on a self-report instrument (SF-36) for collection of information about HRQoL. Secondly, we did not assess possible mental and physical health problems as (negative) predictors of HRQoL, so we cannot discuss about them as factors that influence the HRQoL. Thirdly, the compared medical students and other university students differed significantly in some demographic data but this is inevitable consequence of different length of studies between medicine (6 years) and other study programs (4 to 5 years of study). And finally, we assessed HRQoL in the sample of students at one university with traditional medical curriculum, so interpretation of these results should be done with caution due to distinctive features of educational programs and curricula in different universities.

Conclusion

Medical students perceive their health better than other university students. We found several predictors of worse perception of HRQoL among medical students, which may be useful for specific prevention and treatment in order to achieve better quality of life of this population. Finally, we assume that assessment of specific predictors of better perception of HRQoL might help to potentiate those factors in order to get healthier life satisfaction. Furthermore, the overall findings of the study might have implications for more accurate and specific treatments and prevention activities in medical students` population.

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Conflict of interest

The authors declare no conflict of interest.

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Melanoma risk prediction models

Modeli za procenu rizika obolevanja od melanoma

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Abstract

Background/Aim. The lack of effective therapy for advanced stages of melanoma emphasizes the importance of preventive measures and screenings of population at risk. Identifying individuals at high risk should allow targeted screenings and follow-up involving those who would benefit most. The aim of this study was to identify most significant factors for melanoma prediction in our population and to create prognostic models for identification and differentiation of individuals at risk. **Methods.** This case-control study included 697 participants (341 patients and 356 controls) that underwent extensive interview and skin examination in order to check risk factors for melanoma. Pairwise univariate statistical comparison was used for the coarse selection of the most significant risk factors. These factors were fed into logistic regression (LR) and alternating decision trees (ADT) prognostic models that were assessed for their usefulness in identification of patients at risk to develop melanoma. Validation of the LR model was done by Hosmer and Lemeshow test, whereas the ADT was validated by 10-fold cross-validation. The achieved sensitivity, specificity, accuracy and AUC for both models were calculated. The melanoma risk score (MRS) based on the outcome of the LR model was presented. **Results**. The LR model showed that the following risk factors were associated with melanoma: sunbeds (OR = 4.018; 95% CI 1.724–9.366 for those that sometimes used sunbeds), solar damage of the skin (OR = 8.274; 95% CI 2.661–25.730 for those with severe solar damage), hair color (OR = 3.222; 95% CI 1.984–5.231 for light brown/blond hair), the number of common naevi (over 100 naevi had OR = 3.57; 95% CI 1.427-8.931), the number of dysplastic naevi (from 1 to 10 dysplastic naevi OR was 2.672; 95% CI 1.572–4.540; for more than 10 naevi OR was 6.487; 95%; CI 1.993–21.119), Fitzpatricks phototype and the presence of congenital naevi. Red hair, phototype I and large congenital naevi were only present in melanoma patients and thus were strongly associated with melanoma. The percentage of correctly classified subjects in the LR model was 74.9%, sensitivity 71%, specificity 78.7% and AUC 0.805. For the ADT percentage of correctly classified instances was 71.9%, sensitivity 71.9%, specificity 79.4% and AUC 0.808. **Conclusion.** Application of different models for risk assessment and prediction of melanoma should provide efficient and standardized tool in the hands of clinicians. The presented models offer effective discrimination of individuals at high risk, transparent decision making and real-time implementation suitable for clinical practice. A continuous melanoma database growth would provide for further adjustments and enhancements in model accuracy as well as offering a possibility for successful application of more advanced data mining algorithms.

Key words:
melanoma; risk factors; factor analysis, statistical; predictive value of tests.

Apstrakt

Uvod/Cilj. Nedostatak efikasne terapije za kasni stadijum melanoma upućuje na značaj preventivnih mera i praćenja (testiranja) populacije pod rizikom. Izdvajanje osoba pod visokim rizikom trebalo bi da omogući ciljano ispitivanje i dalje praćenje osoba koje bi imale najviše koristi od toga. Cilj ove studije bio je da identifikuje najznačajnije faktore rizika od melanoma u našoj populaciji i napravi modele za procenu rizika. **Metode.** Ova anamenestička studija uključila je 697 ispitanika (341 bolesnik operisan zbog melanoma i 356 ispitanika kontrolne grupe) koji su bili pregledani i intervjuisani o faktorima rizika od melanoma. Nakon univarijantnog poređenja grupa urađena su dva prognostička modela bazirana na statistički značajnim faktorima rizika: model logističke regresije (LR) i alternativno stablo odlučivanja (ADT). Oba modela su procenjena i utvrđena je njihova tačnost u proceni rizika od obolevanja od melanoma. Procena slaganja modela sa podacima za model LR urađena je pomoću Hosmer-Lemeshow testa, dok je za ADT korišćena desetostruka unakrsna procena. Za oba modela data je procena senzitivnosti, specifičnosti, tačnosti i AUC. **Rezultati.** Logistička

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regresija ukazuje na značajnost sledećih faktora rizika za melanom: korišćenje solarijuma (OR = 4,018; 95% CI 1,724–9,366 za osobe koje ponekad koriste solarijum), solarno oštećenje kože (OR = 8,274; 95% CI 2,661–25,730 za osobe sa teškim znacima oštećenja kože), boja kose (OR = 3,222; 95% CI 1,984–5,231 za svetlo braon/plavu kosu), ukupan broj mladeža (više od 100 mladeža karakteriše OR = 3.57 95% CI 1,427-8,931), broj displastičnih mladeža (od 1 do 10 displastičnih mladeža OR je bio 2.672, 95% CI 1,572-4,540; za više od 10 displastičnih mladeža OR je bio 6.487; 5% CI 1,993–21,119), fototip kože po Fitzpatricku i kongenitalni mladeži. Crvena kosa, fototip I i veliki kongenitalni mladeži bili su prisutni samo u grupi melanoma te su zato i pokazali visoku značajnost u predviđanju rizika. Procenat ispravno klasifikovanih osoba u modelu LR bio je 74,9%, senzitivnost 71%, specifičnost 78,7% i AUC 0,805. Za stablo odlučivanja procenat ispravno klasifikovanih osoba bio je 71,9%, senzitivnost 71,9%, specifičnost 79,4% i AUC 0,808. **Zaključak.** Primena različitih modela za procenu rizika obolevanja od melanoma treba lekarima da pruži efikasno, jednostavno i standarizovano sredstvo za testiranje rizika. Predloženi modeli nude brzo otkrivanje osoba pod visokim rizikom, transparentan algoritam odlučivanja i identifikovanja u realnom vremenu, pogodan za kliničku praksu. Dalja poboljšanja moguća su sa porastom baze podataka o obolelima, što će omogućiti ne samo poboljšanje tačnosti predloženih modela već i primenu naprednijih algoritama mašinskog učenja.

Ključne reči:
melanom; faktori rizika; testovi, prognostička vrednost; statistička analiza faktora.

Introduction

Considering the continuons trend of increasing incidence of melanoma in the last 50 years, with the fastest growing incidence of all malignant diseases in the United States, melanoma is becoming one of the most urgent problems of medicine today. Epidemiological data indicate a constant increase in the melanoma incidence, ranging from 4% to 6% *per* year [1, 2]. A good indicator of our inability to control this disease is the lifetime risk of getting melanoma. In the United States in 1935 it was 1: 1,500, in 1980 1 : 250, in 2000 1 : 74, in 2009 1 : 58 and in 2015 the lifetime risk is expected to be 1:50 [3–5]. Melanoma makes about 4% of all malignant tumors of the skin, but is responsible for about 75% of deaths caused by malignancies of the skin. Despite numerous achievements in the areas of etiology, pathology, diagnosis and therapy in different fields of medicine, lack of effective therapy for advanced stages of melanoma emphasizes the importance of preventive measures, risk factors and screenings of population at risk. Identifying persons at risk of getting melanoma is a prime goal of all preventive strategies. Persons at risk could be educated in risk factors and involved in follow-up programs in order to avoid getting melanoma. Also, targeted screenings of potentially high risk groups in general population should lead to early detection of the disease *in situ* when it is expected to have high survival rate.

There are many factors influencing the melanoma incidence and several meta-analysis have contributed significantly to their understanding [7–10]. In order to be able to reduce melanoma incidence we have to be aware of those factors, the way they influence melanoma development and the modalities to keep them under control. Most epidemiological studies highlight the following as key factors for the development of melanoma: intermittent UV exposure, sunbeds, blistering sun burns in childhood, fair skin phototype (Fitzpatrick I and II), a great number of common naevi, the presence of atypical naevi, blond hair, blue eyes, freckles, melanoma in family. These days there are also contradictory data about the association between melanoma and obesity [11, 12] Parkinson's disease [13], vitamin D [14] , immunosuppressive therapy [15–17], ionizing radiation [18] and oral contraceptives [19, 20].

The application of predictive models in medicine developed as a part of the strategies for the prevention of different malignancies, including melanoma. Many studies deal with this problem trying to create a model with good sensitivity and useful in clinical practice [21–24]. Models are based on well recognized risk factors for specific disease. Usually they summarize results of different meta-analyses or multicentric studies that involve great number of participants from different regions in order to overcome bias of some specific constitutive features in one population or specific environmental characteristic. Universal prognostic models aim at good generalization emphasizing common melanoma risk factors. However, the significance and relevance of some constituting risk factors largely depend on geographic region, different latitudes and different races. For these reasons, analysis of risk factor in smaller scale regions yields more accurate predictive models encompassing both demographic and regional characteristics. Such smaller scale studies give an insight into the differences, allowing for the identification of risk factors that are most important for specific population as in a study of Fargnoli et al. [25] on Italian population, Ballester et al. [26] on Spanish population, Bakos et al. [27] on Brazilian population, Fears et al. [28], Williams et al. [24] and Cho et al. [8] on North American population, Mar et al. [22] on Australian population and others. Application of the prognostic models enables efficient and rapid screening and, therefore, focuses further diagnostic measures on a small group of high-risk individuals.

The aim of this study was to identify risk factors in our population, to measure their respective importance and determine the most significant risk factors for melanoma prediction. Based on the selection of the most important risk factors, we created two prognostic models based on logistic regression (LR) and alternating decision trees (ADT) and assessed their usefulness for identification of patients at risk to develop melanoma. In order to avoid relying on an expert knowledge, experience and ability to estimate impact of all environmental and constitutive factors in a patient the proposed predictive models would standardize screening process and focus the surveillance programs to those who would benefit most. Both models are intuitive and computationally

efficient offering transparent and understandable decision making. Model dissemination and its simple usage could lead to recognition and prevention of undesirable behavioral habits and consecutively the reduction in the incidence and mortality from melanoma.

Methods

Study population

This case-control study included patients operated on for skin melanoma at the Department of Plastic and Reconstructive Surgery, Clinical Center of Vojvodina, Novi Sad, during a 12-year period, 2001–2012. From 542 patients that were operated on during that period we managed to reach 341 that agreed to participate in this study. All the patients were Caucasians, both genders, over 18, with histologically verified diagnose of skin melanoma. The controls were patients consecutively presenting at the same department that were Caucasians, both gender, over 18, personal history of melanoma. The controls were matched with patients by gender and age.

All the participants underwent extensive interview and skin examination. The interview provided data on gender, age, education level, medical history (previous skin cancers), melanoma in family (first-degree relatives), exposure to ultraviolet radiation (exposure to sunbeds, intermittent outdoor UV exposure, occupational UV exposure), use of sunscreens, blistering sunburns in different periods of life (before 14 years, 15–19 years, after 19 years), hormonal contraceptive therapy (HCT), immunosuppressive therapy. Intermittent UV exposure was defined as exposure to UV radiation during recreational (outdoor activities in warmer weather such as sport practicing or gardening) and vacation activity.

A single physician interviewed and examined all individuals and assessed skin phototype (Fitzpatrick), natural hair color (black/dark brown, blond/light brown, red), eye color (black/brown, blue/green), the presence of freckles, a number of common naevi (whole body count), a number of dysplastic naevi (none, 1–10, more then 10), level of solar damage on the skin of the shoulders and back (four category scale-none, mild, moderate, severe).

This study was approved by the Ethical Committee of Clinical Center of Vojvodina. All the participants signed informed consent.

Statistical analysis

The statistical package SPSS for Windows (version 21) was used for statistical analysis. To test the significance of differences between the two groups of patients we used $\chi 2$ test and Fisher exact test. Statistical significance was accepted at the level of $p < 0.05$. Logistic regression modeling was done in SPSS, offering full model description, significance of coefficients and model validation. Weka 3.6.9, freely distributable machine learning software, was used for alternating decision tree modeling and validation.

Upon pairwise univariate comparison, logistic regression analysis was done using the factors with a statistically significant difference in distribution among patients and controls: level of education, intermittent UV exposure, number of dysplastic naevi (DN), number of common naevi, congenital naevi, use of HCT, Fitzpatrick phototype, level of solar damage of the skin, natural hair color, eye color and use of sunbeds. Logistic regression was used to evaluate prediction level attributable to every risk factor. When building LR model all of the selected variables entered the model simultaneously. Odds ratio (OR), confidence interval (95% CI), coefficient of regression (β) and two-tailed *p*- value were calculated for every variable (risk factor). Use of OR as an indicator of relative risk is acceptable in case-control studies, especially where an outcome (disease) can be considered rare ("rare disease assumption") as in case of melanoma [29]. Wald test was used for evaluation of statistical significance of a regression coefficient resulting in a two-tailed *p*-value. Validation of regression model was done by Hosmer and Lemeshow (HL) test. The percentage of correctly classified instances, sensitivity, specificity and the area under the ROC curve (AUC) were calculated. Sensitivity presents a true positive rate reflecting the probability that subject is classified correctly as high risk individual. The higher the sensitivity the bigger chances to identify high-risk subjects. Specificity reflects the ability of a model to correctly classify low risk patients. If AUC is 0.5, classifier performance is on the level of random classification, which makes the model useless, AUC > 0.7 indicates good classification, AUC > 0.8 indicates excellent classification, while the model with AUC above 0.9 is considered extraordinary classifier.

Melanoma risk score (MRS) is defined as likelihood (*p*) of getting melanoma given the subject's specific attributes according to the obtained logistic regression model. Values of probability ranges from 0 – meaning that the chance of getting melanoma is none (minimal) to 1 – chance of getting melanoma is reaching 100%. The participants were classified according to the risk level into three categories: low risk (MRS < 0.25), standard risk ($0.25 \leq MRS \leq 0.5$) and high risk (MRS > 0.5).

The ADT is built in Weka by using the boosting method to combine decision trees. The basic ADT elements are decision nodes containing the prediction condition, i.e. certain attribute value and prediction nodes containing only the number. For each subject all the paths, depending on prediction condition, are explored and the resulting decision is brought by summing up the values in prediction nodes. The input variables to the ADT algorithm were the same selected variables as in logistic regression. The number of variables is further reduced by ADT, leaving the eight most important attributes for decision-making. The model was validated using 10-fold cross-validation. The achieved sensitivity, specificity, accuracy and AUC are provided.

Results

There were 697 participants in this study: 341 patients and 356 controls. The melanoma patients included 165 (48.39%) females and 176 (51.91%) males; the mean age was 56.44 ± 15.21 years (ranging from 19 to 87 years). The controls included 180 (50.56%) females and 176 (49.43%)

men; the mean age was 55.5 ± 15.15 years (ranging from 18 to 88 years). There were no statistically significant differences between these groups considering age and gender distribution ($p > 0.05$). The distribution of risk factors among patients and controls, with calculation of statistical significance by $\chi 2$ test ($p < 0.05$) is shown in Table 1.

Table 1

Distribution of risk factors in the patients and controls and statistical significance analysis

Risk factors	Groups of participants				χ^2	df	*p*
	Patients		Controls				
	n	(%)	n	(%)			
Level of education							
primary school	59	17.3	22	6.2			
secondary school	185	54.3	244	68.5	24.967	3	< 0.0001
college	16	4.7	15	4.2			
university degree	81	23.8	75	21.1			
Occupational UV exposure							
yes	59	17.3	129	36.2	31.699	1	< 0.0001
Intermittent exposure							
yes	233	68.3	206	57.9	8.179	1	0.005
Use if sunbeds							
never	298	87.4	336	94.4			
sometimes	35	10.3	16	4.5	10.371	2	0.006
often	8	2.3	4	1.1			
Other malignant tumors							
yes	6	1.8	3	0.8	1.149	1	0.284
Malignant tumors of the skin							
yes	20	5.9	48	13.5	11.481	1	0.001
Melanoma in family							
yes	2	0.6	0	0	2.094	1	0.148
Immunosuppressive therapy							
yes	3	0.9	8	2.2	2.097	1	0.148
HCT							
yes	14	4.1	4	1.1	6.156	1	0.013
Sunburns							
< 14 years	19	5.6	99	27.8	61.240	1	< 0.0001
14–19 years	32	9.4	78	21.9	20.560	1	< 0.0001
> 19 years	32	9.4	72	20.2	16.123	1	< 0.0001
Solar damage of the skin							
none	68	19.9	88	24.7			
mild	117	34.3	144	40.4	51.678	3	< 0.0001
moderate	98	28.7	119	33.4			
severe	58	17	5	1.4			
Use of sunscreens							
never	156	45.7	193	54.2			
sometimes	103	30.2	60	16.9	37.978	3	< 0.0001
often	61	17.9	42	11.8			
always	21	6.2	61	17.1			
Fitzpatrick phototype							
type I	7	2.1	0	0			
type II	157	46	189	53.1	9.932	2	0.007
type III	177	51.9	167	46.9			
Hair color							
black/brown	100	29.3	163	45.8			
light brown/blond	230	67.4	193	54.2	29.018	2	< 0.0001
red	11	3.2	0	0			
Eye color							
black/brown	167	49	217	61	10.106	1	0.002
blue/green	174	51	139	39			
Freckles							
yes	22	6.5	23	6.5	0.000	1	0.996
Number of common naevi							
<50	112	32.8	263	73.9			
50–100	182	53.4	81	22.8	120.085	2	< 0.0001
>100	47	13.8	12	3.4			
Number of dysplastic naevi							
none	220	64.5	310	87.1			
1–10	79	23.2	41	11.5	56.147	2	< 0.0001
> 10	42	12.3	5	1.4			
Congenital naevi							
none	315	92.4	293	82.3			
small	14	4.1	50	14	31.293	3	< 0.0001
medium	5	1.5	13	3.7			
large	7	2.1	0	0			

HCT – hormonal contraceptive therapy; df – degree of freedom.

The factors that showed up to be significant in melanoma patients based on $\chi 2$ test calculation ($p < 0.05$) are: level of education, intermittent UV exposure, use of sunbeds, HCT, level of solar damage (severe), Fitzpatrick phototype (type I), hair color (red, light brown/blond), eye color (blue/green), the number of common naevi (over 50), the number of dysplastic naevi (any), congenital naevi (large). The factors that were significant for controls in our sample are occupational UV exposure, blistering sunburns, other skin cancers, and use of sunscreens. Risk factors, such as melanoma in the family, freckles, use of immunosuppressive therapy or other malignant tumors did not show a statistically significant difference between the two groups.

Risk factors significant for the melanoma patients were further included in the logistic regression model. For every variable coefficient of regression (β), standard error (SE), *p*-value, OR and 95% CI for OR were calculated (Table 2).

The HL test showed that the observed and expected values were not significantly different ($p > 0.05$), meaning that the model effectively describes data ($\chi 2 = 7.880$; df = 8; $p = 0.445$; $p > 0.05$). The percentage of correctly classified subjects was 74.9%, sensitivity 71%, specificity 78.7% and AUC was 0.805.

LR analysis showed that the following risk factors were associated with melanoma: sunbeds, solar damage of the skin, Fitzpatrick's phototype, hair color, number of common naevi, number of dysplastic naevi, and the presence of congenital naevi. A 4-fold increase in melanoma risk was observed for those that sometimes used sunbeds compared with those who never used them (OR = 4.018, 95% CI 1.724–9.366). The participants with severe solar damage of skin had 8.3-fold increase in melanoma risk compared with those that did not have signs of solar damaged skin (OR = 8.274; 95% CI 2.661–25.730). Factors like red hair, phototype I, and large congenital naevi showed expectably high

Table 2

Logistic regression model of risk factors for melanoma prediction

Risk factors	β	SE	Wald	*p*	OR	95% CI
Level of education						
primary school*	–	–	–	–	–	–
secondary school	-1.309	0.350	13.973	<0.0001	0.270	0.136–0.536
college	-1.295	0.564	5.270	0.022	0.274	0.091–0.829
university degree	-1.351	0.398	11.534	0.001	0.259	0.119–0.565
Intermittent exposure						
yes	-0.065	0.204	0.102	0.749	0.937	0.627–1.398
Use if sunbeds						
never*	–	–	–	–	–	–
sometimes	1.391	0.432	10.378	0.001	4.018	1.724–9.366
often	0.957	0.808	1.403	0.236	2.603	0.535–12.680
HCT*						
yes	0.987	0.708	1.946	0.163	2.683	0.670–10.739
Solar damage of the skin						
none*	–	–	–	–	–	–
mild	0.104	0.255	0.168	0.682	1.110	0.674–1.830
moderate	-0.319	0.275	1.342	0.247	0.727	0.424–1.246
severe	2.113	0.579	13.325	< 0.0001	8.274	2.661–25.730
Fitzpatrick phototype						
type I	18.096	$1.3x10^4$	0.000	1	$7.2x10^7$	0.000
type II	-1.248	0.251	24.652	< 0.0001	0.287	0.175–0.470
type III*	–	–	–	–	–	–
Hair color						
black/brown*	–	–	–	–	–	–
light brown/blond	1.170	0.247	22.380	< 0.0001	3.222	1.984–5.231
red	21.271	10.2×10^3	0.000	1	1.73×10^9	0.000
Eye color						
black/brown*	–	–	–	–	–	–
blue/green	0.165	0.234	0.495	0.482	1.179	0.745–1.866
Number of common naevi	–	–	–	–	–	–
< 50*	1.668	0.213	61.373	< 0.0001	5.301	3.493–8.047
50–100	1.273	0.468	7.399	0.007	3.570	1.427–8.931
> 100						
Number of dysplastic naevi	–	–	–	–	–	–
none*	0.983	0.271	13.197	< 0.0001	2.672	1.572–4.540
1–10	1.870	0.602	9.641	0.002	6.487	1.993–21.119
> 10						
Congenital naevi						
none*	–	–	–	–	–	–
small	-1.148	0.378	9.215	0.002	0.317	0.151–0.666
medium	-2.191	0.708	9.586	0.002	0.112	0.028–0.448
large	20.501	1.36×10^4	0.000	1	8×10^8	0.000

***Reference category; β – coefficient of regression; SE – standard error; OR – odds ratio; 95% CI – confidence interval; HCT –hormonal contraceptive therapy.**

large congenital naevi showed expectably high association with melanoma as were only present in the patients. A large associated standard error is due to the small number of patients with these attributes. Light brown or blond hair individuals compared with black/brown hair subjects as reference category showed 3.2-fold increase in melanoma risk (OR = 3.222; 95% CI 1.984–5.231).The number of common naevi over 100 marked 3.6-fold higher melanoma risk over individuals with less than 50 common naevi (OR = 3.57, 95% CI 1.427–8.931). Also, a subject with 50 to 100 common naevi had high OR of 5.3 compared with the reference category of < 50 (OR = 5.301; 95% CI 3.493–8.047). Subjects with following categories: over 10 and 1-10 DN, had 6.5-fold (OR = 6.487, 95% CI 1.993–21.119) and 2.7-fold (OR = 2.672, 95% CI 1.572–4.540) increase in melanoma risk respectively compared with a subject without DN.

No remarkable association with melanoma risk was found for intermittent UV exposure with OR of 0.937 although previously calculated univariate $\chi 2$ test showed statistically significant difference between the cases and the controls ($p < 0.05$). HCT showed OR of 2.683 but as 95% CI contains 1 this difference could not be considered significant. Also, subject with blue/green eyes had OR of 1.179 compared to reference category of black/brown eyes, but 95% CI included value 1 meaning that the association is not significant.

Based on the obtained logistic regression model the likelihood (p) of getting melanoma was calculated for each participant. Distribution of probabilities in the controls is presented in Figure 1.

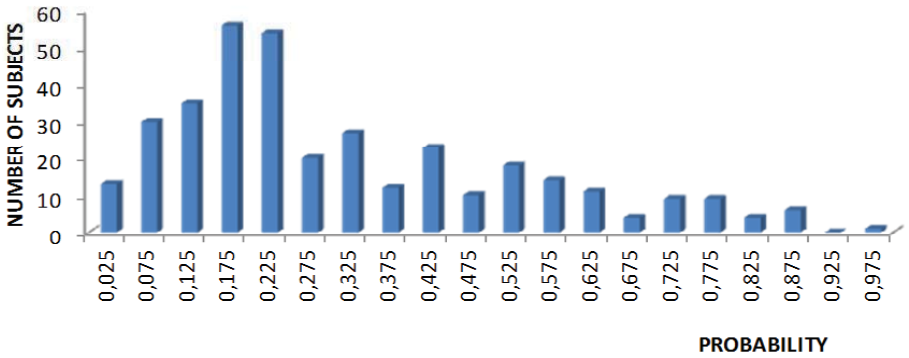

Fig. 1 – Distribution of probabilities in controls

The LR model was built based on both controls and melanoma patients in order to identify the risk factors and behavioral habits that lead to melanoma development. If the attributes of the control subjects match the typical melanoma patients, it is indicative that those subjects are at high risk of developing melanoma. According to distribution of MRS (individual likelihood of getting melanoma) controls were classified in three groups: low risk (MRS < 0.25) - 188, (52.81%) standard risk (0.25 ≤ MRS ≤ 0.5) - 92, (25.84%) high risk (MRS > 0.5) - 58, (21.35%). The sensitivity of this model, defined as the percentage of individuals among the patients that the model classified correctly, was 71%. The specificity of this model, defined as the percentage of individuals in the controls that the model classified correctly was 78.7%.

All the risk factors included in logistic regression analysis were included in construction of alternating decision tree. The selected attributes in decision nodes of ADT and respective prediction nodes form the possible decision making paths.

Based on the subject's specific attribute values, there are several paths from the root to the leaves, and the final decision depends on the sign of the sum of all the prediction nodes passed. The more negative value implies the higher risk of melanoma (Figure 2).

To illustrate the decision making based on ADT we give two typical examples. A subject X, that has many risk factors: primary education, 60 common naevi, 5 dysplastic naevi, severe sun damage of the skin, Fitzpatrick I phototype, blond hair, blue eyes, never use sunbed and has none congenital naevi, would have final score of -3.608 as the sum of all the prediction nodes passed. A subject Y, who does not have many risk factors: secondary education, black hair, brown eyes, Fitzpatrick phototype III, 20 common naevi, no dysplastic naevi, never use sunbeds, has none congenital naevi and mild level of sun damaged skin, would have the final score 1.237. The negative final score means high risk for getting melanoma, whereas the higher positive prediction score means the lower risk.

The percentage of correctly classified instances by the ADT tree is 71.9%, average sensitivity 71.9%, specificity 79.4% and AUC was 0.808. It could be noticed that the ADT achieved almost the same sensitivity and AUC with a significant attribute reduction. Decision making in ADT is done based on eight attributes, offering fast and easily implementable algorithm for efficient population screening.

Discussion

Our study included 697 participants which is comparable to other case-control studies: Fargnoli et al. [25] study on Italian population with 300 participants, Ballester et al. [26] study on Spanish population with 415 subject or study of Fortes et al. [23] with 609 participants. Limitations of our study, like other case-control studies, should be considered when interpreting results. Reporting and recall bias in participants is limiting possibility to estimate correctly associations of some risk factors for melanoma. In our study we

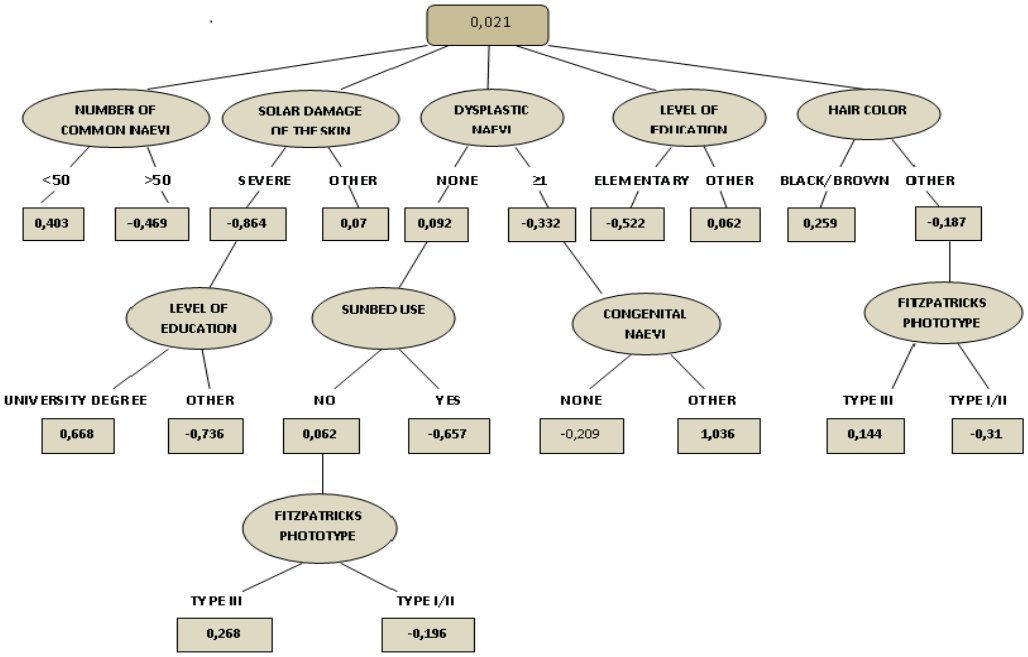

Fig. 2 – Alternating decision tree

faced that problem while interviewing the participants about sunburns and sunscreen use.

Although blistering sunburns are considered risk factor for melanoma our data failed to confirm that, showing no association with higher melanoma risk. Lack of this association was also seen in some other studies [25, 26]. There could be several explanations for this result. We have to keep in mind that no objective method exists for retrospective assessment of age-specific sunburns and that this factor is subject to recall bias. Also, the patients already diagnosed with melanoma when interviewed by their surgeon about risk behavior modalities that possibly led to tumor development tended to report differently in order to deflect blame from themselves. This problem could be overcome with different study design, as in prospective cohort studies.

Use of sunscreens was also excluded from further creation of predictive models as participants were often guessing about this factor and the answers did not seem reliable. Reporting bias should not be underestimated, as the patients are aware of the fact that they should have avoided exposure to UV radiation and should have used sunscreens. The extent to which the use of sunscreen can be considered protective was difficult to estimate because we had no information about how often they use sunscreens, they often did not know which SPF usually had sunscreen they use or the type of sunscreen (against UVB radiation or including UVA and UVB protection). The level of education was significantly associated with the use of sunscreens in both groups as we expected. In the cases and the controls patients with primary education mostly answered that never use sunscreens, while in the group of participants with university degree this percentage was much lower.

Other group of factors, like melanoma in the family, freckles, use of immunosuppressive therapy and other malignant tumors did not appear to be significantly different between the two groups. We decided not to include them in risk models as, besides the fact that there were no significant difference in distribution between the patients and the controls, the number of patients with these factors was very small so further analyzes would not be reliable. This does not mean that melanoma in the family is not important factor, but rather that our sample was small for analyzing this specific factor. Immunosuppressive therapy was also excluded as besides the fact that this factor was also present in few participants, data from the literature about this factor are limited to specific groups in population such as transplant patients [15–17].

The factors that were more significant in the controls such as: occupational UV exposure, blistering sunburns, other skin cancers and use of sunscreens were excluded from further model creation.

In our sample occupational UV exposure was more prevalent in the controls and thus not significant for melanoma. Similar results could be seen in other studies that emphasize importance of occupational UV exposure dominantly for non-melanoma skin cancers (NMSC) [30–33]. This causal relation between chronic UV exposure and NMSC coincides with our results where more NMSC was detected in controls where occupational UV exposure was dominant. A study of Chang et al. [34], which included 5,700 patients with melanoma at different latitudes, confirms the importance of occupational UV exposure in the development of melanoma only in low latitudes and in the cases of melanoma localized on exposed parts of the body. Bearing this in mind, it is expected that in central Europe, which is a zone of high latitude, occupational exposure may not be as important for the development of melanoma as intermittent exposure. Intermittent UV exposure was more present in patients, but in multivariate logistic regression setting the distribution of this

feature among the subjects did not lead to strong association with increased risk of melanoma. This could be explained partly by a greater prevalence of subjects with primary education than in the cases. They are expected to have lower economic status, thus traveling to warmer climates, vacations with sunbathing and intermittent UV exposure in general are not as achievable for them. This bias could be overcome if the controls and the patients were matched also according to economic status.

Logistic regression on the selected factors showed strong association between the use of sunbeds and melanoma risk in those who reported to sometimes use sunbeds compared to those that never used them. This coincides with results of some other studies in the literature. Meta-analyzes of Boniol et al. [35] based on 27 studies showed 20% higher risk for ever use of sunbeds. Lazovich et al. [36] confirmed this results showing 74% greater risk in those who ever used sunbeds with differentiating between UVB devices and primarily UVA devices. In many studies on melanoma risk prediction this factor was not included as data about association between artificial UV radiation and melanoma in literature were inconsistent. International Agency for Research on Cancer (IARC) published a large review in 2007 based on 19 studies considering carcinogenic effect of indoor tanning where they underline that ever use of sunbeds is associated with melanoma risk [37]. If exposure was before 35 years, risk to get melanoma was 75% higher. This study led to a decision of IARC to classify sunbeds as group I devices (carcinogenic to humans) so we can expect that this factor is going to be addressed more in further studies.

Considering constitutive features like hair color, eye color and phototype we marked red or blond/light brown hair and phototype I as strongly associated with increasing risk of melanoma. Data in the literature coincide with our results. Although there were statistically significant differences in distribution of blue/green eyes in the patients and the controls, based on $\chi 2$ test analyzes, association with melanoma risk could not be considered significant according to logistic regression as they were underrepresented in data sets which caused problems in associated β coefficients estimation. Freckles were also one of the features evenly distributed between patients and controls (6.5%), so in our sample did not appear to be important predictor. This could be explained with specific phenotypic characteristics of nations present in Vojvodina (Hungarian, Slovakian) which typically have fair hair, blue/green eyes, pale skin, so this features were not so specific for melanoma patients. Data confirming these specific phenotypic characteristics of Hungarian and Slovakian population we saw in a study of Csoma et al. [38] on schoolchildren population in South Hungary, which included 1320 participants. In this study phototype I/II was represented in 76.61% and blue/green eyes in 38.9% of children. According to other study on Hungarian population made by Fehér et al. [39] fair skin is present in 42.2%, blue/green eyes in 47.3% and blond/red hair in 66.3% of school children participating in the study. In Pesch et al. [40] study on Slovakian population, dealing with NMSC, blue/green eyes was noticed in 47.2% of men and 48.6% of women in the control group. In a Ballester et al. [26] study on Spanish population phototype I/II was present in 29.4%, blond/red hair in 40.5%, blue/green eyes in 31.8%. In Fargnoli et al. [25] study on Italian population phototype I/II was present in 30% of participants, fair hair in 12.5% and blue/green eyes in 33.5%. These data on Hungarian and Slovakian population differ from phenotypic characteristics seen in Spanish or Italian population and we consider this important in analyzing phenotypic characteristics in our multinational population in Vojvodina. The absence of association of blue eyes and freckles with melanoma risk was also seen in a Ballester et al. [26] study.

The number of common naevi and dysplastic naevi in our data coincide with results of other studies confirming that the higher number of naevi, the higher risk of melanoma. One of the largest meta-analysis on naevi as a risk factor done by Gandini et al. [41] and based on 47 case-control and cohort studies highlights DN as one of the most important predictor of increased risk for melanoma. They presented data on increased risk that ranged from RR = 1.6 for one DN, to RR = 10.5 for more than 5 DN. Our results also confirm this observation showing that less that 10 DN mark 2.7 increase in risk, while for subjects that have more than 10 DN we noticed 6.5-fold increase in risk. Considering the number of common naevi Fortes et al. [23], as Gandini et al. [41], had RR of 6.89 for more than 100 naevi, while we had 3.6-fold increase in melanoma risk. Comparing data about moles as risk predictor can be confusing as there are studies where the count of common naevi is limited on specific parts of body (trunk, arms) and those where answers are only dichotomous without a precise number of DN, but they all agree that DN and more than 50 common naevi increase risk for melanoma.

Both models for prediction of melanoma risk showed good classification performances with AUC over 0.8. Based on the learned classification scheme, they are successfully utilized for melanoma risk prediction. These results were better than results seen in some risk models in the literature: AUC = 0.79 in Fortes et al. [23], 0.77 in training set model of Williams et al. [24], 0.62 in Cho et al. [8], but lower than AUC in Bakos et al. [27] five-variable model that achieved AUC of 0.85. Using data mining techniques in cancer prediction has proven to be a helpful tool in identifying persons at risk in many diseases such as lung cancer [42], glioblastoma multiforme [43], hepatocellular carcinoma in chronic hepatitis C [44], stroke [45], Alzheimer's disease [46] and others. According to our knowledge, so far only LR was used for melanoma risk prediction, so proposed ADT based on eight variables could be seen as a useful contribution to the screening process in melanoma detection.

As multiple studies showed that specific combinations of risk factors are associated with elevated risk for melanoma, further analysis could be oriented on increasing the melanoma patients database and follow-up studies in order to verify the relations between some factors. Also, in order to analyze the association of some age specific factors like use of tanning devices or HCT, future studies should be focused on specific age groups (younger) as analyzing those factors in general population often leads to underestimation of their association with melanoma.

The advantage of our study is the use of different approaches in melanoma risk prediction and a wide range of assessed risk factors. So far, to our knowledge, no study was done on melanoma risk prediction on Vojvodina population based on data mining techniques. For these reason, inclusion of ADT as prediction tool is important contribution of our study. Additionally, this study offers scoring system based on probability of getting melanoma (MRS) that allows good discrimination of individuals at risk and could be readily used in clinical practice.

The main limitations are related to case-control design that is prone to recall bias. Better selection of controls could be done by avoiding patients from plastic surgery department in order to avoid bias of reporting due to preselection of subjects. As Gandini et al. [41] concluded, after comparing different study designs, when controls were drown from hospitals calculated risks were lower than in population-based studies. Also, as noticed in the same study, ORs from case-control studies were significantly lower than RRs from cohort studies. Other limit to consider is failure to estimate association between sunburns and melanoma. This could be attributed to dichotomous answer modality (ever/never) as most studies that confirm the strength of this association are limiting this association to higher number of sunburn episodes.

Conclusion

Facing the rising melanoma incidence and considering that dealing with this disease in advanced stages is rather difficult with not so favorable results, medicine turns its focus to prevention and to risk factors. Application of different models for risk assessment and prediction of the disease should provide efficient and standardized tool in the hands of doctors. The presented models offer effective discrimination of individuals at high risk, transparent decision making and real-time implementation suitable for clinical practice. Further model improvement is possible by increasing the melanoma database, which will allow for better representation of all attributes. Bigger sample sizes would enable successful use of more advances data mining algorithms. Control subjects identified as high risk according to the proposed models could be followed which might offer the insight into some risk factor associations of special importance for melanoma development.

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Influence of bile acid derivates on morphine analgesic effect in mice

Uticaj derivata žučne kiseline na analgetski efekat morfina kod miševa

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Abstract

Background/Aim. It is known that bile acids improve the absorption, bioavailability and pharmacodynamic characteristics of some drugs. Morphine analgesia is produced by activation of opioid receptors within the central nervous system (CNS) at both spinal and supraspinal levels. Since a morphine molecule contains 3 polar groups and therefore hard to transfer through the blood-brain barrier, the aim of the study was to examine the potential influence of bile acids derivates, namely sodium salt of monoketocholic acid (MKH-Na) and methyl ester of monoketocholic acid (MKH-Me), on analgesic effect of morphine. **Methods.** White male mice of NMRI-Haan strain, with body weight of 20–24 g, were used in this study. The analgesic effect of morphine (administered by subcutaneous and intramuscular route in a dose of 2 mg/kg), with and without pretreatment with MKH-Na (4 mg/kg) and MKH-Me (4 mg/kg) was estimated by the hot plate method. **Results.** Administration of MKH-Me prior to subcutaneous administration of morphine increased the morphine analgesic effect but the increase was not statistically significant. At the same time administration of MKH-Na did not affect morphine analgesic effect. The analgesic effect of morphine increased when administered intramuscularly 20 min after MKH-Me administration. When compared with the group of animals treated only with morphine, a statistically significant increase in analgesic effect was detected 10, 30, 40 and 50 min after morphine administration ($p < 0.05$). Pretreatment with MKH-Na did not affect morphine analgesic effect. **Conclusion.** According to the results of this study it can be presumed that after intramuscular morphine administration methyl ester of monoketocholic acid increases morphine transport into the central nervous system and consequently the analgesic effect, as well. Further research on bile acids-morphine interaction both *in vitro* and *in vivo* is necessary to completely elucidate the mechanism of this interaction and increase in the morphine analgesic effect.

Key words:
morphine; mice; bile acids and salts; blood-brain barrier.

Apstrakt

Uvod/Cilj. Poznato je da žučne kiseline poboljšavaju apsorpciju, povećavaju biološku raspoloživost i poboljšavaju farmakodinamske osobine nekih lekova. Budući da je analgezija izazvana morfinom posledica aktivacije opioidnih receptora u centralnom nervnom sistemu na spinalnom i supraspinalnom nivou, i da molekul morfina sadrži tri polarne grupe zbog čega teško prolazi kroz hematoencefalnu barijeru, cilj studije bio je da se ispita potencijalni uticaj derivata žučnih kiselina, natrijumove soli monoketoholne kiseline (MKH-Na) i metil estra monoketoholne kiseline (MKH-Me), na analgetski efekat morfina. **Metode.** Studija je sprovedena na belim miševima NMRI-Haan soja, muškog pola, telesne mase 20–24 g. Analgetski efekat morfina (primenjenog supkutano i intramuskularno u dozi 2 mg/kg) sa i bez pretretmana MKH-Na (4 mg/kg) i MKH-Me (4 mg/kg) procenjivan je metodom vrele ploče. **Rezultati.** Primena MKH-Me pre supkutane primene morfina pojačala je analgetski efekat morfina bez statistički značajne razlike. Primena MKH-Na nije uticala na analgetski efekat morfina primenjenog supkutano. Analgetski efekat intramuskularno primenjenog morfina, 20 minuta nakon primene MKH-Me, bio je pojačan. U poređenju sa grupom životinja kod kojih je primenjen samo morfin, statistički značajna razlika u analgetskom efektu zabeležena je 10, 30, 40 i 50 minuta nakon njegove primene ($p < 0,05$). Pretretman sa MKH-Na nije uticao na analgetski efekat morfina primenjenog intramuskularno. **Zaključak.** Na osnovu rezulata studije može se pretpostaviti da nakon intramuskularne primene morfina metil estar monoketoholne kiseline povećava transport morfina u centralni nervni sistem i posledično dovodi do pojačanja analgetskog efekta morfina. Dalja istraživanja interakcija žučnih kiselina i morfina *in vitro* i *in vivo* neophodna su da bi se u potpunosti rasvetlio mehanizam interakcije, a time i mehanizam pojačanja analgetskog efekta morfina.

Ključne reči:
morfin; miševi; žučne kiseline i soli; krvno-moždana barijera.

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Introduction

The application of bile acids in human medicine dates back to the thirties of the 20th century. It has been shown that bile acids improve the absorption, bioavailability and pharmacodynamic characteristics of some drugs [1, 2]. Bile salts form micelles, which increase the permeability of the mucosal membrane by overcoming resistance at the aqueous diffusion layer [3]. They are also capable of enhancing drug delivery by interacting with membrane lipids and proteins that affect membrane fluidity [4]. The effects of bile acids on biological membranes are similar to those of detergents and they appear to have the potential to aid intestinal, buccal, transdermal, ocular, rectal and pulmonary absorption [1].

Morphine is the prototypical opioid analgesic. It interacts with μ and κ opioid receptors to exert its analgesic effect [5]. Analgesia is produced by activation of opioid receptors within the central nervous system (CNS) at both spinal and supraspinal levels [6]. Morphine is a weak base that is relatively water soluble and poorly lipid soluble. After oral administration it is predominantly absorbed in the proximal small bowel and has a poor and variable bioavailability (20–40%) [7]. In humans, following absorption, 90% of drug is metabolized, principally by glucuronidation in the liver, to form morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). Morphine is widely distributed within the body. Excretion of morphine occurs predominantly in urine in the form of morphine glucuronides with unchanged morphine representing between 2% and 10% of the total, independently of the dose. Some 70–80% of an administered dose is excreted within 48 h of administration and most of this appears within the first 24 h [8].

Since morphine analgesia is produced by activation of opioid receptors within the CNS at both spinal and supraspinal levels, the concentration of morphine at any point in time at active sites in the CNS will depend on the systematic and the CNS disposition of the drug. Alteration at either of these locations may influence morphine CNS concentrations and thus the degree of antinociception.

Having in mind characteristics of bile acids and their derivates as well as the characteristics of morphine, the aim of this research was to determine the influence of bile acids derivates, namely sodium salt of monoketocholic acid (MKH-Na) and methyl ester of monoketocholic acid (MKH-Me), on analgesic effect of morphine detected by antinociceptive hot plate method.

Methods

White male mice of NMRI-Haan strain, with body weight of 20–24 g, were used in this study. Each control and experimental group was formed of 6 animals. Animals had free access to food and water, and subjected to 12-h light and dark cycles, at a room temperature of 22°C. Laboratory animals were under human care in accordance with the criteria given in the 'Guide for the Care and Use of Laboratory Animals' [9]. The study was approved by the Ethics Committee on Laboratory Animal Welfare of the University of Novi Sad, approval No. IV-2011-05.

For the experiment we used: morphine from Sigma-Aldrich; MKH-Na and MKH-Me – synthesized at the Department of Chemistry, Faculty of Sciences, University of Novi Sad, according to the procedure by Miljkovic et al. [10]; hot/cold plate – Ugo Basile (kept at 53 ± 1°C); chronometer.

The study groups included the following: C1 – the control group without treatment (n = 6); C2 – the control group given morphine intramuscularly (*im*) at 2.0 mg/kg (n = 6); C3 – the control group given morphine subcutaneously (*sc*) at 2.0 mg/kg (n = 6); C4 – the control group given MKH-Na subcutaneously (*sc*) at 4.0 mg/kg (n = 6); C5 – the control group given MKH-Me subcutaneously (*sc*) at 4.0 mg/kg (n = 6); E1 – the experimental group given MKH-Na *sc* 4.0 mg/kg 20 min before morphine *im* at 2.0 mg/kg (n = 6); E2 – the experimental group given MKH-Me *sc* 4.0 mg/kg 20 min before morphine *im* at 2.0 mg/kg (n = 6); E3 – the experimental group given MKH-Na *sc* 4.0 mg/kg 20 min before morphine *sc* at 2.0 mg/kg (n = 6); E4– the experimental group given MKH-Me *sc* 4.0 mg/kg 20 min before morphine *sc* at 2.0 mg/kg (n = 6).

The doses and time interval of administration of bile acid derivates were determined according to previously published studies in which they were determined to be optimal for the analgesic model used in this study [11–13].

The analgesic effect of morphine was estimated by the hot plate method [14]. Mice were gently restrained and placed on the hot plate surface (53 ± 1°C). The latency for paw withdrawal from the heated surface was manually recorded with a chronometer. Only the clear withdrawal of the either rear paw was taken into account, discarding the nonspecific generalized struggle observed in some cases. Three measures at 10-min intervals were taken at the beginning of the study or before the treatment (groups of animals other than the control one) and their means were considered as basal (control) latencies. The mean basal values were sought around 11–13 s and animals showing any latency equal to or higher than 20 s were rejected. A maximal latency value (cut-off) of 30 s was determined.

The latency for paw withdrawal from the heated surface (reaction time in seconds) was recorded as follows: control (basal) reaction time for each animal in all the study groups; the study group C1 – experimental reaction time was recorded at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after CRT measurement; study groups C2, C3, E1, E2, E3 and E4 – ERT was recorded 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after morphine administration; study groups C4 and C5 – reaction time measurement started 20 min after MKH-Na and MKH-Me administration (control reaction time). The experimental reaction time was recorded at 10, 20, 30, 40, 50, 60, 80, 100 and 120 min from this time point.

The morphine dose for both routes of administration was 2.0 mg/kg. The analgesic effect determined in seconds was expressed as a percentage of prolongation of measured experimental reaction time (ERT) compared to control reaction time (CRT) according to the equataion:

$$\text{Analgesic effect (\%)} = \frac{(\text{ERT} - \text{CRT})}{\text{CRT}}$$

Statistical analysis of collected data was performed by MedCalc 9.2.0.1. The statistical significance was determined using Student's *t*-test. A *p*-value of 0.05 was considered statistically significant.

Results

Figure 1 shows that MKH-Me administration prior to subcutaneous administration of morphine increased the morphine analgesic effect, but this increase was not statistically significant. At the same time MKH-Na administration did not affect morphine analgesic effect.

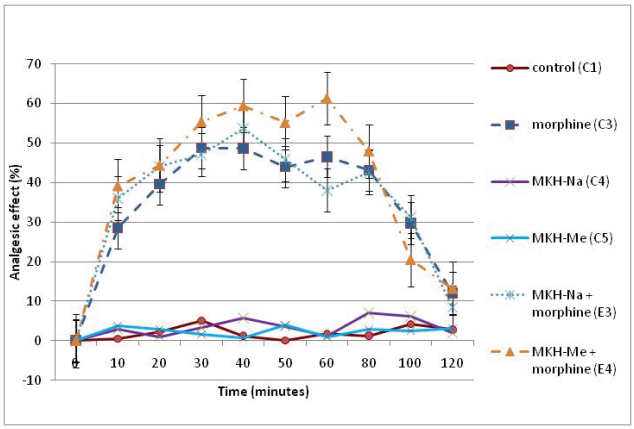

Fig. 1 – Analgesic effect after subcutaneous administration of morphine, morphine + sodium salt of monoketocholic acid (MKH-Na) and morphine + methyl ester of monoketocholic acid (MKH-Me) before morphine administration and 10, 20, 30, 40, 50, 60, 80, 100 and 120 minutes after morphine administration for the groups C3, E3, E4 and at the corresponding time points for the groups C1, C4 and C5.

Figure 2 shows the increased analgesic effect of morphine when morphine was administered intramuscularly 20 min after MKH-Me administration. When compared with the group of animals treated only with morphine, a statistically significant increase in analgesic effect was detected 10, 30, 40 and 50 minutes after morphine administration ($p < 0.05$). Pretreatment with MKH-Na did not affected morphine analgesic effect after its intramuscular administration.

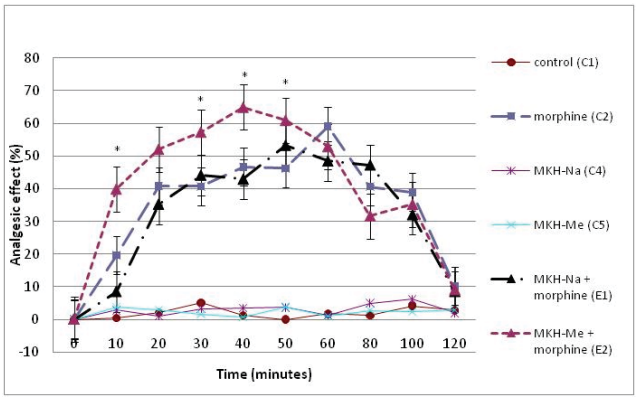

Fig. 2 – Analgesic effect after intramuscular administration of morphine, morphine + sodium salt of monoketocholic acid (MKH-Na) and morphine + methyl ester of monoketocholic acid (MKH-Me) before morphine administration and 10, 20, 30, 40, 50, 60, 80, 100 and 120 min after morphine administration for the groups C2, E1, E2 and at the corresponding time points for the groups C1, C4 and C5. (*$p < 0.05$).

Discussion

Since it is known that morphine molecule containing 3 polar groups hardly passes through the blood-brain barrier (BBB), the potential influence of bile acids derivates on blood-brain barrier permeability and the influence on analgesic effect of morphine was examined.

In this experiment hidrosoluble sodium salt of monoketocholic acid did not affect morphine analgesic effect regardless the route of morphine administration. Similar findings were obtained in the study of Vasovic et al. [13], in which no significant interaction of sodium salt of monoketocholic acid and tramadol was detected regarding the antinociceptive effect in healthy mice.

This finding is in accordance with some authors suggesting that liposoluble derivates of bile acids are more successful in promoting drug transport through biological barriers [1, 15]. The results published by Posa et al. [12, 16], who studied lidocaine and bile acid derivates interactions, also support this assertion. However the potential of hidrosoluble salts of bile acids to promote drug transport was also verified [11, 17, 18].

In our study methlyester of monoketocholic acid (liposoluble), given subcutaneously 20 minutes before subcutaneous morphine administration, enhanced morphine analgesic effect although the increase in analgesic effect was not statistically significant when compared to experimental group treated only with morphine. On the other hand methylester statistically significantly increased the analgesic effect of morphine when morphine was administered intramuscularly. This increase in analgesic effect in the period from 10 to 50 min after morphine administration was statistically significant compared to animals treated only with morphine except at the time point of 20 min after morphine administration.

According to Kuhajda et al. [19], this could be attributed to the formation of two different types of the molecular aggregates between bile acids and morphine. Molecular aggregate of morphine and one molecule of 12-monoketocholic acid (12-MKC) in which morphine binds to the side of the steroid skeleton of 12-MKC, results in a molecular aggregate that is more hydrophobic than the morphine itself what accelerates the ingression of morphine to the lipid phase of the membrane and increases its analgesic effect. The additional increase in analgesic effect results from the formation of the hydrophilic aggregate of the two bile acid molecules with one molecule of morphine in the intracellular space. Since hydrophilic aggregate is less lipophilic than morphine itself the morphine flux directed towards the extracellular space is reduced.

Another possible mechanism to which the increase in analgesic effect could be attributed is the inhibition of P-glycoprotein (P-gp) mediated morphine efflux from the CNS. P-gp, located in brain capillary endothelial cell membranes, may function as a component of the blood-brain barrier [20, 21].

Morphine may be a substrate for a P-gp, as demonstrated *in vitro* and in knockout mice studies [22, 23]. Disruption of P-gp in brain capillary endothelial cells would in theory result in enhanced accumulation of morphine in the brain. Since it is known that bile acids are also substrates for the P-gp the possible mechanism could involve competition for the P-gp transport system between bile acids and morphine [24, 25].

One fact in this study remains to be further studied. Although the increase in analgesic effect was detected after subcutaneous administration of methyl ester of monoketocholic acid it was not statistically significant. At the same time a detected increase in analgesic effect was statistically significant after intramuscular administration of methyl ester of monoketocholic acid. Whether this occurred due to higher standard deviation of the measured values in the experimental group treated subcutaneously or there exist some differences in pharmacokinetics related to different routes of administration, it remains to be further studied. According to most of the studies there is no significant difference in absorption of morphine after intramuscular and subcutaneous administration [26, 27]. However some studies suggest there exists a difference in plasma levels 15 min after administration, with higher levels detected after subcutaneous administration. It is possible that due to intense liver metabolism better part of subcutaneously administered dose of morphine is metabolised to morphine-6-glucuronide which posses a weaker analgesic effect than morphine [28, 29], or morphine-3-glucuronide which is without analgesic effect at all [30, 31], and even has the potential of antagonising morphine analgesic effect [32, 33]. In that manner smaller amount of morphine would be available for possible interaction with bile acids at the level of blood-brain barrier. Consequently lower amount of morphine would be present in CNS resulting in weaker analgesic effect. On the other hand, some sources state that the peak analgesia of morphine occurs within 50–90 min following subcutaneous administration and 30–60 min after intramuscular injection without explaining what is the background of this difference [34]. If this is maybe a consequence of slower or weaker absorption of morphine after subcutaneous administration remains to be further studied.

Conclusion

According to the results of this study it can be presumed that after intramuscular morphine administration methyl ester of monoketocholic acid increases morphine transport into the central nervous system and the analgesic effect, as well. Further research on bile acids-morphine interaction both *in vitro* and *in vivo* is necessary to completely elucidate the mechanism of this interaction and increase in the morphine analgesic effect.

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CURRENT TOPIC

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Osteonecrosis of the jaw as a serious adverse effect of bisphosphonate therapy and its indistinct etiopathogenesis

Osteonekroza vilica kao ozbiljan neželjeni efekat terapije bisfosfonatima i njegova nejasna etiopatogeneza

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Ključne reči:
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Introduction

Bisphosphonates (BPs) represent a class of drugs that are applied in therapy of different pathological conditions related to bone. Their main role in bone metabolism is to inhibit osteoclast function, so these drugs act as potent devices in suppression of the bone resorption process. Considering the presence of two phosphonate groups with a high affinity for calcium ions in their chemical structure, BPs have the ability to accumulate predominantly in bones [1]. According to the differences in side chain, related to the presence or absence of nitrogen atom, BPs are classified in two different groups: nitrogen containing (aminoBPs) and non-nitrogen containing (non-aminoBPs) drugs. These two groups of bisphosphonates also differ in mechanism by which they inhibit osteoclast action. Aminobisphosphonates (pammidronate, neridronate, olpadronate, ibandronate, risedronate, zoledonate) act directly on HMG-CoA reductase (mevalonate) pathway by binding and blocking enzyme farnesil diphosphate synthetase (FPPS) [2]. Non-amino bisphosphonates (etidronate, clodronate, tiludronate) are metabolised within osteoclasts to analogues of adenosine triphosphate (ATP) that accumulate within the cells, which leads to inhibition of numerous metabolic enzymes, cytotoxicity and apoptosis [3–6]. Nitrogen addition greatly increases the potency of bisphosphonate, so aminoBPs are claimed to be 10–10,000 times more potent comparing non-aminoBPs [7].

As they act as inhibitors of osteoclast function in order to suppress bone resorption and improve bone mineral density, during the past three decades, bisphosphonates have been increasingly used in therapy of diferrent pathologic conditions related to bone. Intravenous BPs are principally used for treatment of metastatic bone lesions, multiple myeloma and hypercalcemia of malignancy. Oral BPs take part in therapy of osteoporosis, Paget disease and paediatric osteogenesis imperfecta. Positive effects of bisphosphonates in these conditions are: significant reduction of bone pain, osteolytic lesions and fracture risk, and improvement of bone mineral density.

BPs are considered as drugs of certified efficiency, with rare negative side effects, such as gastrointestinal intolerance, headache, hypocalcemia, hypophosphatemia, bone pain, dizziness, fever, fatigue etc. [8], probably due to their low serum concentration and rapid accumulation in bone matrix.

History and definition of bisphosphonate-related osteonecrosis of the jaw

In 2003 Marx [9] described non-healing and painful exposure of jaw bone after intravenous administration of potent aminobisphosphonates in patients with multiple myeloma and metastatic bone lesions, and soon, this adverse effect was named bisphosphonate-related osteonecrosis of the jaw (BRONJ).

BRONJ in a short time became the main and most speculated adverse effect of BPs therapy [10]. In 2009 the American Association of Oral and Maxillofacial Surgeons (AAOMS) defined criteria for BRONJ: the presence of exposed necrotic bone in maxillofacial region for more than 8

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weeks in patients that currently take, or used to take bisphosphonates, with no history of radiation therapy to the jaws [11]. AAOMS also proposed staging system for BRONJ according to symptoms, clinical and radiographic findings, and recommended treatment strategy for each stage (Table 1) [11]. Risk factors included in developing BRONJ are pointed out in many studies on this subject [3, 11–14]. Now it is known that BRONJ mostly occur in patients who used to receive more potent, nitrogen containing BPs, in the treatment of multiple myeloma or metastatic bone lesions. Long-term therapy and intravenous administration of BPs are associated with increased risk for developing BRONJ. Local risk factors include oral-surgery interventions, such as tooth extraction, implant placement etc., but also chronic irritations (inadequate dentures), and chronic periodontal disease. In the majority of reported cases, local trauma, particularly tooth extraction, appear to be the direct cause, in fact, the trigger factor in developing BRONJ, yet there are reported cases that occur spontaneously, with no previous dental treatement, or trauma [12, 15]. Futhermore, there are also areas within the jaws, that show greater predilection to this complication. Lesions are found more commonly in the mandible than in the maxilla (ratio 2 : 1), and more often in areas where thin oral mucosa overlies bony prominences and ridges [11, 15].

Table 1

Bisphosphonate-related necrosis of the jaw (BRONJ) risk staging

BRONJ stages	Clinical features	Treatment strategy
Patients at risk	No apparent necrotic bone in asymptomatic patients treated with IV or oral bisphosphonate.	Patients should be informed on the risks of developing BRONJ.
Stage 0	No clinical evidence of necrotic bone, but the presence of nonspecific symptoms or clinical and radiographic findings that address osteonecrosis.	Symptomatic treatment and conservative management of local factors, such as caries and periodontal disease.
Stage I	Exposed and necrotic bone in asymptomatic patients with no evidence of infection.	Antimicrobial rinses, such as chlorhexidine 0.12%.
Stage II	Exposed and necrotic bone in patients with pain and clinical evidence of infection.	Antimicrobial rinses in combination with antibiotic therapy.
Stage III	Exposed and necrotic bone in patients with pain, infection and one or more of the following: exposed necrotic bone extending beyond the region of alveolar bone; pathologic fracture; extraoral fistula; oral antral/ oral nasal communication; osteolysis extending to the inferior border of the mandible or sinus floor.	Surgical debridement, including resection in combination with antibiotic therapy.

Incidence of bisphosphonate-related osteonecrosis of the jaw

According to the current literrature review [16] the incidence of BRONJ associated with parenteral administration, showed a high variation from 0.00% to 27.5%, mean value 7% (in studies reported from 2003 to March 2010), but these reviewed studies showed also high variance in duration and design (retrospective, prospective, letters to editor and review).

The overall incidence of BRONJ associated with oral bisphosphonate therapy was estimated to be 0.12%, ranging from 0.00% to 4.3%, also with variations in study type and its duration [16].

The importance of investigation of biphosphonate therapy

While the clinical presentation of BRONJ is well-known and described, the exact etiology and pathogenesis still remains an enigma, despite a number of suggested theories that tried to give an appropriate explanation. Besides, the question that has not been completely answered yet is: Why are the jaws almost the only affected area? There were rare reports in the literature of bisphosphonate-related osteonecrosis affecting other bones [17–21], so jaws remain, certainly the main target for this complication of BPs therapy.

Cognition of the exact etiology and pathogenesis of this adverse effect may make it be predictable, help its prevention and facilitate its treatment, which is often without an adequate response.

Etiopathogenesis

While there is more or less concordance among reports in clinical presentation and risk factors related to BRONJ, a concrete etiology and pathogenesis are still confusing and therefore, there is a tendency in the literature to give an appropriate explanation for this adverse effect of bisphosphonate therapy.

Suggested hypotheses are related to bone turnover suppression, angiogenesis suppression, soft tissue toxicity, infection and local pH value changes, immune system deficiency, and genetic predisposition [22–26].

Via osteoclast inhibition (what is actually their mechanism of action), BPs definitely, on many levels, disturb communication and signaling pathways among cells included in bone remodelling, which leads to supression of this process or at least to its defective enactment. As jaws have a high remodelling rate, they would be the most affected area [27]. Whereas the osteoclasts are, undoubtly, the main target cells for BPs, there are also speculations and studies about BPs' effect on other bone cells: osteocytes and osteoblasts, which affection could play a role in pathogenesis of BRONJ, too.

BPs cause acumullation of avital bone matrix with non-viable osteocytes, which has been already proved on animal model [28], but it has not been clarified yet whether it is a consequence of their indirect action, through remodelling supression, or direct effect on osteocytes' viability, when these cells are exposed to high concentrations of BPs.

In *in vitro* studies of BPs' action, the direct effect of BPs on cells of the osteoblast lineage is confirmed and appeared to be dose-dependent, so it seems that BPs may inhibit bone formation process, too [29, 30].

Subramanian et al. [31] actually believe that combined reduction in bone formation and bone resorption leads to signifficant attenuation of bone remodelling response to physiological stimuli such as bone aging, microdamage and mechanical stress, so bone matrix with apoptotic osteocytes persists unresorbed and unrepaired. Finally, BRONJ develops when local remodelling aparature is not able anymore to maintain homeostasis and respond to bone damage subsequent to dentoalveolar infection, local trauma, or , the most frequent, tooth extraction.

The effect of BPs treatement on vasculature has been speculated in some studies [22, 23]. It is familiar that BPs act as potent devices in suppression of angiogenesis associated with tumor growth, and their antiangiogenic effect has been documented. Exposed bone subsequent to BPs therapy does not bleed and it is visiably avascular. However, more potent substances with antiangiogenic action, that are in clinical use, do not lead to osteonecrosis of the jaw, except reported cases of treatement with bevacizumb- monoclonal human antibody that through inhibition of vacular endothelial growth factor A (VEGF-A) achieves antiangiogenic potential [32]. Since these reports are extremly rare, we cannot make a conclusion of antiangiogenic effect of BPs as the main causal factor included in etiopathogenesis of BRONJ. In 2010, Yin, Bai and Luo [22] established a hypothesis of addititious, indirect antiangiogenic effect of BPs, *via* inhibition of osteoclasts, that impact this process, and further suppression of angiogenesis as in the study of Cackowski et al. [33].

Furhermore, it has been suggested that BPs accumulated in bone after tooth extraction play with direct toxic effect on oral epithelium, keratinocytes and fibroblasts, compromiting soft tissue healing, so the exposed bone becomes necrotic [25, 34, 35]. Otherwise, reported cases of BRONJ that develops without prior invasive intervention, such as tooth extraction, confront this theory. Besides, an open question is whether or not oral mucosa is exposed to enough concentrations of BPs, which are known to accumulate, predominantly, in bone?

Otto et al. [24, 35] hypothesized that local infection and subsequent changes of local pH value have important role in pathogenesis of BRONJ. AminoBPs are known to bind to bone matrix in neutral pH, but their relase and activation take place in acid environment, which starts cascade of pathways and leads to BRONJ. Since jaws, especially mandible, are acessible to infecton, despite other area of the skeleton, this theory could give an atrractive explanation of the pact that osteonecrotic process predominanatly affects jaws. This pathophysiological mechanism has been proved *in vitro*, on cellular level, where it has been adjusted that nitrogen containing BPs act as more toxic in acid environment, in contrast to non-nitrogen containing BPs [36]. Having in mind this study, the fact that BRONJ mostly occures in cases with *iv* administration of aminobisphosphonates, might not be surprising. Recent studies pointed at *Atctinomyces* colonisation associated with BRONJ [37] and one metagenomic study revealed *Proteobacteria*, *Furmicutes* and *Actinobacteria* being the domminant phylotypes in BRONJ patients, but also detected associated viruses [38].

It is still unclear, yet, whether extraction or infection is the real trigger for BRONJ development. This theory, however, related to pH value changes, explains why preventive measures before and during BPs therapy are very important and successful, which has been proved in some studies [39, 40]. Tooth extraction is always associated with loss of integrity of the soft and hard intraoral tissue. This procedure enables direct invasion of intraoral microorganisms into extraction socket. Because of abundance of microorganisms in the oral cavity, also regarding the previous fact, it is difficult to consider extraction without infection.

None of these theories could give a complete, conspicuous explanation for etiopathogenesis of BRONJ. It seems that all these theories are complementary to each other, and the majority of promoted mechanichms could be included in this process, although none of them has been experimentally confirmed.

A relatively new bone antiresorptive agent, denosumab, that is approved by the Food and Drug Administration for use in patients with osteoporosis and metastatic bone disease, has been associaeted wit osteonecrosis of the jaw, too. Nevertheless, according to its different pharmacology characterisics and more rapidly reversible impact on bone turnover comparing to bisphosphonates, as it was explained in a study by Malan et al. [41], osteonecrosis of the jaw related to denosumab might resolve in a shorter drug holiday period.

Treatement strategy and outcome

The treatment strategy for managing BRONJ depends on the stage of this condition (Table 1) and consists of preventive measures, antibiotic medication, surgical debridement/resection and sometimes even discontinuation or modification of bisphosphonate therapy. The last mentioned should be done only if systemic conditions permit and in obligate consultation with the treating physician or oncologist and patient about risks and benefits of continuing bisphosphonate therapy [42].

There is an agreement among all experts about treatment difficulties concerning BRONJ, because of frequent relapse after conservative or surgical therapy. Doubtless, implementation of adequate prevention measures in patients treated with bisphosphonates are very important and require a multidisciplinary approach.

Clinical manifestation of BRONJ could be very similar to many pathological conditions of maxillofacial region. Considering differential diagnosis, it is very important for clinical to get detailed anamnesis from patients (previous

BPs administration, malignancy or osteoporosis). Clinic have to distinguish BRONJ from other lesions in maxillofacial region (oral carcinoma, cysts, chronic irritation of oral mucosa, alveolitis after tooth extraction, malignant ameloblastoma) because the treatment strategies of these pathologies are completely different [43, 44].

Conclusion

It could be concluded that BRONJ is a serious negative side effect of bisphosphonate therapy, that impacts negatively on patients' quality of life since it is painful, nonhealing and often without adequate response to the applied therapy, especially when it has not been recognised on time. BRONJ certainly requires attention and further investigation. Effective treatment could be achieved only if ethiopathogenesis was clarified.

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CASE REPORTS

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Coronectomy of two neighbouring ankylosed mandibular teeth – A case report

Koronektomija dva susedna ankilotična mandibularna zuba

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Abstract

Introduction. Intentional partial odontectomy or coronectomy is primarily advocated in situations with intimate relationships between the roots of the tooth indicated for extraction and the inferior alveolar nerve (IAN). The aim of this report was to present a patient with two neighbouring infraoccluded teeth in the right mandible indicated for extraction prior to prosthetic rehabilitation, which were coronectomied as to prevent injuring of the IAN and causing iatrogenic fracture of the mandibular body. **Case report.** Coronectomy of both teeth was performed as recommended in the literature. The patient had no special discomfort after the operation or deficit in sensitive nerve function, and the wound healed uneventfully. The patient was followed regularly during a 2- year period. **Conclusion.** The presented case suggests coronectomy as quite beneficial solution for avoiding serious problems (injuring of the IAN and possible fracture of the mandible) and making feasible forthcoming prosthetic rehabilitation.

Key words:
tooth ankylosis; mandible; oral surgical procedures; treatment outcome.

Apstrakt

Uvod. Parcijalna odontektomija, ili koronektomija, prvenstveno se preporučuje u situacijama u kojima postoji blizak odnos korenova zuba indikovanih za ekstrakciju i donjeg zubnog nerva (DZN). Cilj ovog rada bio je da se prikaže bolesnica sa dva susedna infraokludirana zuba u mandibuli sa desne strane, indikovanih za ekstrakciju pre protetske rehabilitacije, koji su parcijalno ekstrahovani radi prevencije povrede DZN i jatrogene frakture tela donje vilice. **Prikaz bolesnice.** Koronektomija oba zuba izvedena je u skladu sa preporukama iz literature. Bolesnica nije imala nikakve posebne nelagodnosti posle operacije niti poremećaj funkcije DZN, a rana je zarasla bez komplikacija. Bolesnica je redovno kontrolisana tokom dve godine. **Zaključak.** Kod prikazane bolesnice, koronektomija se pokazala kao korisno rešenje kojim su izbegnute ozbiljne komplikacije (povreda donjeg zubnog nerva i moguća fraktura mandibule) čime je omogućena sledstvena protetska rehabilitacija.

Ključne reči:
zub, ankiloza; mandibula; hirurgija, oralna, procedure; lečenje, ishod.

Introduction

Modern dentistry is based on conservative thinking and use of minimally invasive procedures [1]. In oral surgery, intentional partial odontectomy (coronectomy) could be an example of such a principle. This procedure has raised a special attention in the last decade of previous century and at the beginning of this century after several reports on its benefits [2–5]. It is primarily advocated in situations with intimate relationships between the roots of the tooth indicated for extraction (mainly lower third molars) and the inferior alveolar nerve (IAN) [3, 6].

However, there are still some controversies about the procedure that does not follow a customary principle of oral surgery practice – to finish tooth extraction removing all parts of the tooth. These controversies are related to the lack of knowledge on the long-term outcome of root remnants and possible complications of the procedure, for example apical periodontitis associated with the pulp necrosis [7]. There are, also, some other possible problems with root remnants, such as postoperative pain, dry socket or root migration after some elapse of time [8].

It is interesting that coronectomy has been primarily recommended for protecting the IAN when there is a need

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for removal the impacted lower third molar. Coronectomy is recommended for treating of ankylosed teeth too, but mainly as a method for preservation of alveolar ridge for subsequent prosthetic rehabilitation [9]. The aim of this report was to present a patient with two neighbouring ankylosed and infraoccluded teeth in the mandible indicated for extraction prior to prosthetic rehabilitation, which were coronectomied to prevent injuring of the IAN and causing iatrogenic fracture of the mandibular body.

Case report

A 42-year-old female patient was referred by her private dentist to the Clinic of Oral Surgery, Faculty of Dental Medicine in Belgrade, for extraction of two infraoccluded teeth in the right mandible, the second premolar and first molar, prior to prosthetic rehabilitation. The patient was warned by her dentist to a possibility of injuring the IAN during tooth extraction, and even causing an iatrogenic fracture of the mandible. That doubt was confirmed by the panoramic X-ray, which pointed out a close relationship of the mandibular canal to the first molar roots (Figure 1) – the fact that was confirmed by computed tomography (CT). Even more, a close relationship of the second premolar and the IAN could also be noticed at CT. We decided to perform coronectomy of both teeth to prevent both prospective unfavorable outcomes, and explained that proposal to the patient, which she accepted.

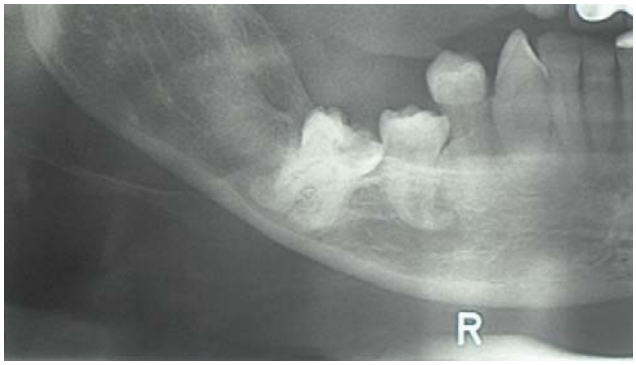

Fig. 1 – Closed relationship of the first molar roots to the mandibular canal.

Coronectomy was performed as recommended, sectioning the crowns of both teeth and part of the roots, approximately 3 mm below the buccal and the lingual bony margins (Figure 2). In the region of the mental foramen the nerve was completely visible, but it was not injured. Wound debridement was done as usual, closing the wound primarily. The patient did not have any special discomfort after the operation, only slight pain and swelling, and did not have any deficit in sensitive nerve function; the wound healed uneventfully and sutures were removed after a week.

The patient was followed regularly and three digital panoramic X-rays, one of those presented at Figure 3, were made in 6-month intervals in order to control the position of root remnants. The last panoramic x-ray, done two years postoperatively did not show any movement of the roots left in the mandible; the molar roots were fully covered by bone and premolar roots only partially (Figure 4).

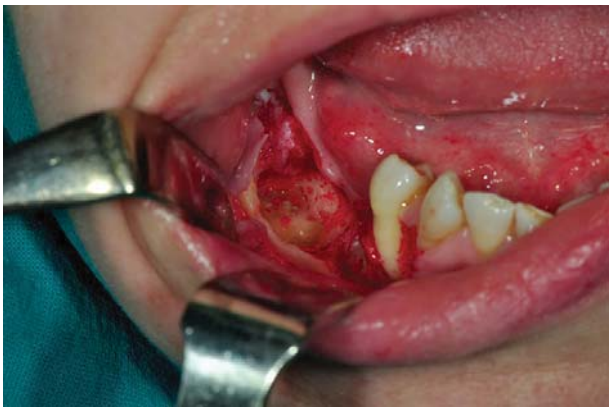

Fig. 2 – Intraoral view of the coronectomied teeth.

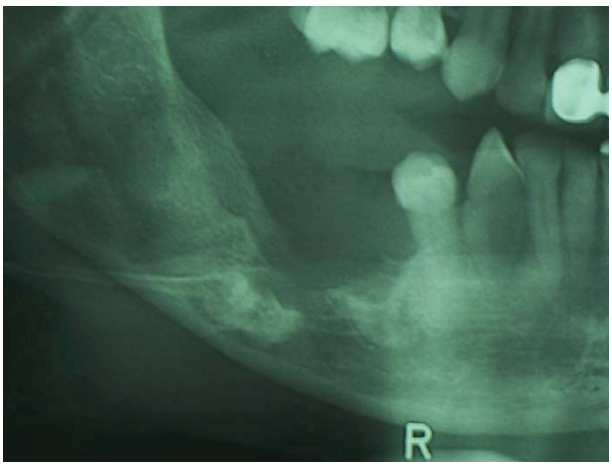

Fig. 3 – Panoramic view of the right mandible and the coronectomied teeth six months after the operation.

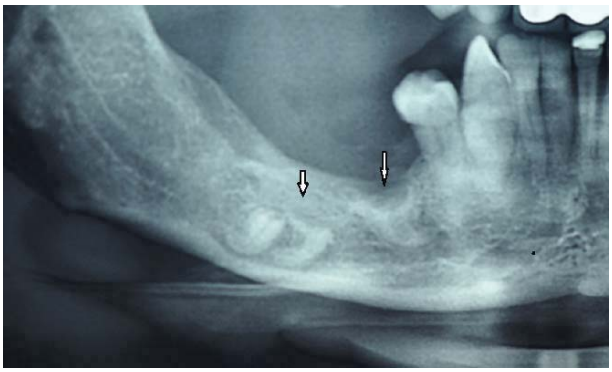

Fig. 4 – Panoramic view of the right mandible and the coronectomied teeth two years after the operation.

Discussion

Removal of impacted or partially impacted lower third molars is a very common procedure in oral surgery, accompanied with a number of temporary and mainly non-serious complications. One of the most embarrassing complications is IAN injury during the procedure, which may impair the

nerve function for an unpredictable period of time, even permanently [10]. To avoid such outcome when there is an intimate relationship between tooth roots and the IAN, intentional partial odontectomy or coronectomy is recommended for protecting the IAN [3, 5, 6]. In the presented patient, this procedure showed to be quite beneficial not only for avoiding IAN injury, but also a probable fracture of the mandible, as both ankylosed teeth occupied almost the whole diameter of otherwise tiny mandibular corpus.

Ankylosis of two mandibular permanent neighbouring teeth, the first molar and the second premolar, is not a common situation. Ankylosis usually affects teeth after dental trauma [11]. We could not claim what had caused ankylosis of those teeth; trauma, however, could not be disclosed in the patient's medical or dental history. Ankylosed teeth seem to be very difficult to extract without extensive bone removal. Therefore, coronectomy of ankylosed teeth was recommended as a suitable method for alveolar ridge preservation prior to prosthetic reconstruction or implant placement [9, 12].

In the presented patient, insisting to complete extraction of both ankylosed teeth (the first lower molar and the second premolar) would almost certainly provoke an injury of the mandibular canal content, with concomitant bleeding and future neural deficit, and probably cause a mandibular fracture. Moreover, extensive bone removal needed for complete removal of ankylosed teeth would definitely interfere with forthcoming prosthetic rehabilitation. Choosing coronectomy, the patient was submitted to shorter surgery and much lesser surgical stress than if the complete extraction was attempted, both serious complications (impairment of nerve function and mandibular fracture) were avoided and subsequent prosthetic rehabilitation was enabled.

Conclusion

Although we cannot report on long-term data of the undertaken procedure, not any of the cited questionable outcomes (postoperative pain, dry socket, root migration) has been encountered till now. However, having in mind the fact that long-term data concerning the outcome of the roots left *in situ* still lack, we believe that real indication for coronectomy exists primarily in situations with a clear benefit of avoiding serious complications. We consider that the presented patient just stands for such a case.

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CASE REPORT

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Asymptomatic flow of Rosai-Dorfman disease

Nesimptomatični tok bolesti Rosai-Dorfman

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Abstract

Introduction. Sinus histiocytosis with massive lymphadenopathy is a rare benign self-limiting disease of unknown etiology. The salivary gland involvement, indicating the extranodal character of the disease, often presents a diagnostic dilemma requiring immunohistochemical staining of surgically removed tumor to confirm the diagnosis. **Case report.** We report a 43-year-old man presented with an asymptomatic mass in the left mandibular angle. On physical examination, the lesion was described as a painless, mobile, firm-elastic consistency nodule, which measured 4 × 3 cm in diameter, with normal overlying skin. A mass with the same characteristics, dimensions 2 × 2 cm, was also noted in the right parotid region. No other changes in regional lymph nodes were detected. On macroscopic examination the lesion was firm, multilobulated, yellowish and rounded, while on microscopic examination the lesion was composed almost entirely of polygonal histiocytes with abundant cytoplasm, emperipolesis, plasma cells arranged in sheets, and lymphocytes scattered or within clusters. The observed histiocytes were found to be CD68 and S100 protein positive. **Conclusion.** Rosai-Dorfman disease is a beningn and frequently overlooked clinical and pathological entity that may be misinterpreted as a neoplastic disease.

Key words:
histiocytosis, sinus; diagnosis, differential; immunohistochemistry; treatment outcome.

Apstrakt

Uvod. Sinusna histiocitoza sa masivnom limfadenopatijom je retka benigna bolest nepoznate etiologije. Zahvaćenost pljuvačnih žlezda ukazuje na ekstramodularni karakter bolesti i često zahvata imunohistohemijsko bojenje hirurški odstranjenog tumora za potvrdu dijagnoze. **Prikaz bolesnika.** U radu je prikazan bolesnik, star 43 godine, sa bezbolnim tumorom u predelu levog ugla donje vilice. Kliničkim pregledom ustanovljena je bezbolna, pokretna, elastična čvoričasta struktura, promera 4 × 3 cm sa neizmenjenom kožom. Promena istih karakteristika, promera 2 × 2 cm, takođe je primećena u desnoj parotidnoj regiji. Regionalni limfni nodusi su bili urednog izgleda. Makroskopski, promena je bila čvrste konzistencije, kružna, multilobulirana, žućkaste prebojenosti. Mikroskopski, promena je bila uglavnom izgrađena od histiocita svetle citoplazme sa emperipolezom, plazma ćelija i limfocita, koji su bili raspoređeni difuzno i u grupama. Imunohistohemijski, histiociti su bili CD68 i S 100 protein pozitivni. **Zaključak.** Rosai-Dorfman je benigna bolest, na koju se često ne misli, te može biti pogrešno interpretirana kao neoplastična bolest.

Ključne reči:
histiocitoza, sinusna; dijagnoza, diferencijalna; imunohistohemija; lečenje, ishod.

Introduction

Rosai-Dorfman disease (RDD) which is widely recognized as a sinus histiocytosis with massive lymphadenopathy (SHML) is a benign self-limiting disease, originally described by Juan Rosai and Ronald F. Dorfman in 1969 [1]. The disease was initially reported as a bilateral cervical lymph nodes enlargement, usually less than 5 cm, with relatively infrequent involvement of other groups of nodes. All the patients had typical histopathological findings of polygonal histiocytes with abundant cytoplasm, emperipolesis, plasma cells arranged in sheets, and lymphocytes scattered or within clusters. Typically, the histiocytes in RDD are positive for S-100 protein as well as CD163 and CD68 proteins. Moreover,

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histiocytes are negative for CD1a, CD34, CD15, CD30, CD3, CD20, keratin, EMA, SMA, desmin and HMB45 [2]. Other clinical features commonly include fever, malaise and weight loss, frequently accompanied with elevated sedimentation rate (ESR) as well as hypergammaglobulinemia. Of note is that salivary gland involvement, indicating the disease extranodal character, frequently poses a diagnostic dilemma necessitating immunohistochemically staining to separate it from malignant neoplasms [3].

Case report

We reported a 43-year-old man, presented with an asymptomatic mass in the left mandibular angle of one year history. There was no history of fever, pain, respiratory tract infections or any other symptoms related to ear, nose or throat. There was also no family history of tuberculosis. On physical examination, the lesion was described as a painless, firm-elastic consistency nodule, which measured 4 × 3 cm in diameter that was freely mobile with normal overlying skin.

On further inspection, the right parotid gland region showed oval, painless mobile mass of soft consistency, measuring 2 × 2 cm and no other changes in regional lymph nodes (Figure 1). Intraoral examination revealed no significant findings.

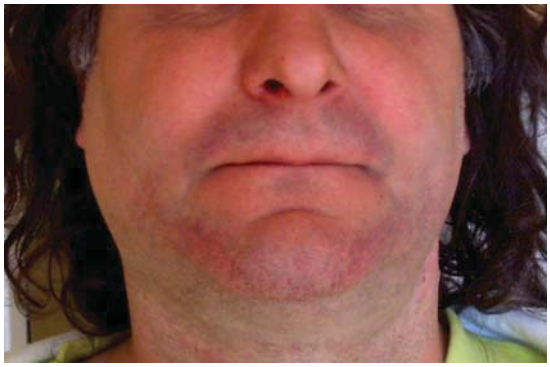

Fig. 1 – Tumor in the right parotid region.

Ultrasound examination revealed oval hypoechogenic mass in the left parotid area, at the largest site measuring 28.2 mm (Figure 2A). In the right parotid region the lesion measured 24.4 mm (Figure 2B). The results of standard biochemical analysis of blood including: glycemia, urea, creatinine, heapatogram, lactate dehydrogenase (LDH), creatine phorphokinase (CPK), transaminases, electrolytes as well as peripheral, blood smear were normal.

After routine medical consultation we decided to surgically remove tumor which was grossly multilobulated and partly surrounded by adipose tissue. On the macroscopic examination we found a homogenous, yellowish tumor, dimension 6 × 4 × 3 cm.

On light microscopic examination, the sections were stained with hematoxylin-eosin, and a lymph node with an infiltrate consisting of many histiocytic cells admixed with lymphocytes in salivary gland parenchyma was observed.

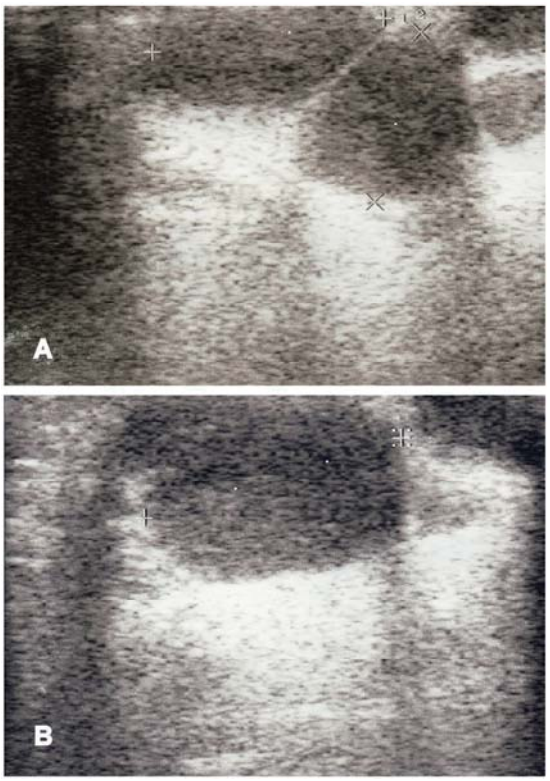

Fig. 2 – A) Ultrasound finding of the right parotid region; B) Ultrasound finding of the left parotid region.

Histiocytes of the infiltrate were large and contained of vesicular nuclei with delicate nuclear membranes, distinct nucleoli, and abundant pale-to-eosinophilic cytoplasm, with emperipolesis (Figure 3).

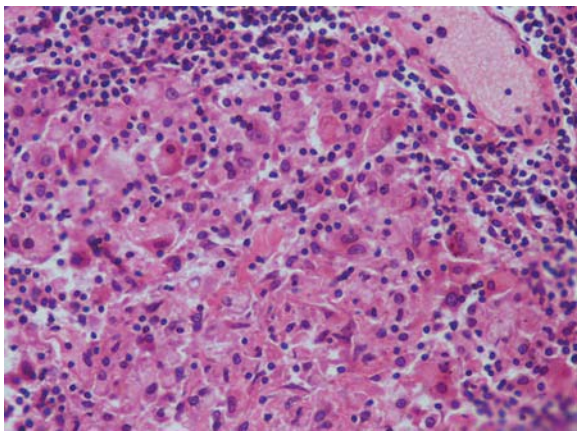

Fig. 3 – Lymph node sinuses filled with pale histiocytes, showing emperipolesis [hematoxylin and eozin (HE) staining, × 20].

Immunohistochemical staining was performed on formalin-fixed paraffin-embedded tissue sections using the Dako-autostainer link 48 (Dako, Ontario, Canada) and the color was developed by EnVision Flex Target Retrieval Solutions (Dako, Burlington) using diamnobezidene (DAB) as the chromogen. The following antibodies were applied: anti-CD1a (O10, Predilute, DAKO, Carpinteria, CA, USA), anti-S-100 (S-100, Predilute, DAKO, Glostrup, Denmark), anti-

CD68 (PG-M1, Predilute, DAKO, Glostrup, Denmark), anti-CD3 (polyclonal rabit, Predilute, DAKO, Carpinteria, CA, USA), anti-CD30 (Ber-HL, Predilute, DAKO, Glostrup, Denmark), anti-CD34 (QBEnd 10, Predilute, DAKO, Glostrup, Denmark), anti-CD20 (L26, Predilute, DAKO, Glostrup, Denmark), anti-Ki67 (MiB-1, Predilute, DAKO, Glostrup, Denmark), anti-CKHMW (34BE12, 1 : 50, DAKO, Carpinteria, CA, USA), anti-CD23 (MHM6, 1 : 50, DAKO, Glostrup, Denmark), anti-EMA (E29, Predilute, DAKO, Glostrup, Denmark) and anti-CD138 (MI15, Predilute, DAKO, Glostrup, Denmark).

Immunohistochemical assessment revealed follicular dendritic cells (CD23 positive), rare plasmocytes (CD138 positive), epithelial cells of salivary ducts and plasmocytes (EMA positive), basal cells (CKHMW positive), rare lymphocytes, epitheloid histiocytes and germinal centre of lymph follicles that were slight Ki67 positive, histiocytes CD68 positive (Figure 4A) and S-100 protein positive (Figure 4B). The Mantle zone of lymph follicles was also CD20 positive. In addition, numerous T-lymphocytes were CD3 positive, endothelial cells were also CD34 positive and staining for CD1a and CD30 was completely negative.

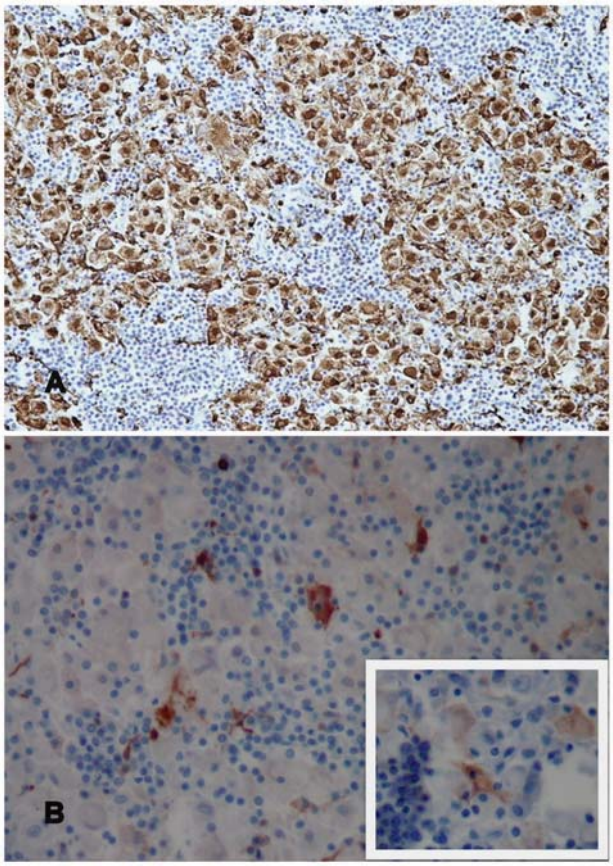

Fig. 4 – A) Histiocytes strongly expressing CD68 (× 20); B) – Histiocytes expressing S-100 protein [× 10 (inst × 20)].

The postoperative course of the presented patient was uneventful, the patient subjectively felt good, and occasional control visits were advised to him (Figure 5).

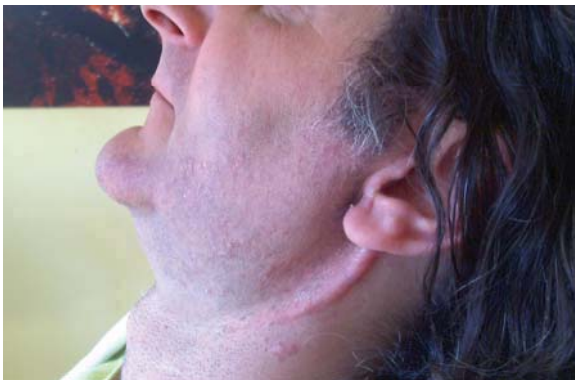

Fig. 5 – The left side of the face and neck of the patient, postoperatively.

Discussion

The patient presented with an asymptomatic mass in the left mandibular angle of one year history and no other exceptional clinical findings. What made this case somewhat unusual was the fact that the patient was 43-year-old male (an atypical age group) and the salivary gland involvement thus presenting us a challenge to separate it from other malignant neoplasms. However, further microscopical finding as well as immunohistochemical assessment confirmed the disease.

Rosai-Dorfman disease is a benign clinical entity which is characterized by over-production and accumulation of a specific type of white blood cell (histiocyte) in the lymph nodes of the body, most often those of the neck (cervical). Literature reviews reported till 2004 show about 600 cases of RDD of which 81% were diagnosed in the first and second decades [4].

The etiology of the disease is not known and the pathogenesis is speculated to have been related to an unidentified infectious agent or an altered immune response [5]. The course of the disease spans over a few to many years, characterized by episodes of waxing and waning in the size of lesion before it undergoes complete resolution.

Microscopically, the lesions have sheets of polygonal histiocytes with abundant cytoplasm, emperipolesis, plasma cells, and lymphocytes scattered and within clusters.

The most common clinical presentation of the disease is painless and bilateral cervical lymphadenopathy (87.3% of cases), affecting one or all cervical ganglion chains. The initial stages are characterized by lymph nodes which are isolated, mobile, and small, but during disease progression they become adherent and form a multinodular mass. The axillary (23.7%), inguinal (25.7%), and mediastinal (14.5%) regions can also be affected, but always to a lesser extent than cervical involvement [6, 7]. Rarely the extranodal disease may be the initial and the only manifestation of the disease what was our case [8].

The cause of the disease has not yet been established, but two theories exist. In the first theory, SHML is caused by a specific infectious process based on the generally infectious process seen at the onset of the disease (localized

lymphadenopathy, fever, leukocytosis with neutrophilia, increased ESR, and hypergammaglobulinemia), which tends to spontaneously regress after some time. In the second theory, the disease is attributed to an abnormal immunologic response, because depression of immunologic cells can be observed [6]. However, in our case, no laboratory evidence points to an etiologic agent.

The patients with extranodal disease confined to head and neck regions. Nodal involvement was not observed, although nodal involvement may have occurred during an earlier phase. Fever occurs in up to 30% of cases but was absent in our patient. In 85% of cases, patients with RDD are in good general health without significant symptoms of the disease [6].

Identification of SHML at an extranodal site (salivary gland) without associated lymphadenopathy raises the suspicious of other diagnoses including Langerhan's cell histiocytosis, Kuttner's tumor, malignant histiocytosis, Hodgkin's disease, and metastatic carcinoma.

Large histiocytes with intracytoplasmic lymphocytes are also cytological features of other diseases. Lack of eosinophils is substantial in differentiating SHML from Langerhan's cell histiocytosis, malignant histiocytosis and T-cell lymphomas. The absence of necrosis and mitotic activity is also important in differential diagnosis from Hodgkin's disease, which is characterized by classical Reed–Sternberg (RS) cells seen in the background consisting of neutrophils, lymphocytes, plasma cells and eosinophils. Though a large number of foamy macrophages can mimic mononuclear variants of RS cells, eosinophils and lymphophagocytosis are not seen [3].

Immunohistochemical stains help a lot in diagnosing SHML since SHML histiocytes are strongly positive for CD68, negative for CD1a and variably positive for S-100 protein. On the other hand, Langerhans cell histiocytosis is positive for both S-100 protein and CD1a, and the cells of Langerhan's cell histiocytosis ultrastructurally reveal characteristic rod-shaped Birbeck granules.

Conclusion

Sinus histiocytosis with massive lymphadenopathy (SHML) is a benign disease usually characterized by spontaneous or steroid induced diminution of all clinical symptoms. It is an often overlooked clinical and pathological entity that may be misinterpoeted as a neoplastic disease.

Acknowledgments

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CASE REPORT

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Giant esophageal fibrovascular polyp with clinical behaviour of inflammatory pseudotumor: A case report and the literature review

Džinovski fibrovaskularni polip jednjaka sa kliničkim ponašanjem inflamatornog pseudotumora

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Abstract

Introduction. Esophageal fibrovascular polyps are rare, benign, intraluminal, submucosal tumor-like lesions, characterized by pedunculated masses which can demonstrate enormous growth. The most frequent symptoms are dysphagia, vomiting and weight loss. Fibrovascular polyps with long stalks can regurgitate into the airways and cause asphyxia. Esophageal inflammatory pseudotumor is extremely rare lesion accompanied with various systemic manifestations as fever, anemia and thrombocytosis. **Case report.** We presented a 29-year-old man complaining of a long-lasting fever and dysphagia. He was found to have huge pedunculated submucosal tumor of esophagus, surgically completely resected. Histopathological examination showed that this giant tumor, 24 × 9 × 6 cm, was a fibrovascular polyp. The postoperative course was uneventful. The preoperative fever, anemia and thrombocytosis disappeared and did not recur in the postoperative course. **Conclusion.** We reported a patient with giant esophageal pedunculated tumor with clinical manifestations of inflammatory pseudotumor and histopathological picture of fibrovascular polyp, that we have not found described in the literature before.

Key words:
polyps; esophagus; granuloma, plasma cell; diagnosis; endosonography; histological techniques; surgical procedures, operative; treatment outcome.

Apstrakt

Uvod. Fibrovaskularni polipi jednjaka su retke, benigne, intraluminalne, submukozne neoplazme na peteljci, koje mogu postići džinovski rast. Najčešći simptomi fibrovaskularnih polipa jednjaka su otežano gutanje, povraćanje i mršavljenje. Fibrovaskularni polipi sa dugom peteljkom se mogu povratiti u disajne puteve izazivajući asfiksiju. Inflamatorni pseudotumor jednjaka je veoma redak tumor, često udružen sa različitim sistemskim manifestacijama kao što su febrilnost, anemija i trombocitoza. **Prikaz bolesnika.** Prikazali smo bolesnika starog 29 godina, sa dugotrajnom febrilnošću i disfagijom. Tokom ispitivanja kod bolesnika je nađen veliki submukozni tumor jednjaka na peteljci, koji je hirurški odstranjen u celosti. Histopatološki pregled ovog velikog tumora, 24 × 9 × 6 cm, pokazao je da se radilo o fibrovaskularnom polipu. Postoperativni tok je protekao bez komplikacija, a preoperativna febrilnost, anemija i trombocitoza su nestale. **Zaključak.** Ovo je prikaz slučaja bolesnika sa velikim polipoidnim tumorom jednjaka koji se klinički ponašao kao zapaljenski pseudotumor, a mikroskopska slika je odgovarala fibrovaskularnom polipu, što nismo našli opisano u literaturi do sada.

Ključne reči:
polipi; jednjak; granulom, plazmocelularni; dijagnoza; endosonografija; histološke tehnike; hirurgija, operativne procedure; lečenje, ishod.

Introduction

Fibrovascular polyps (FPs) are rare, benign, intraluminal, submucosal, tumor-like lesions characterized by the development of pedunculated, intraluminal masses, which in the esophagus can demonstrate an enormous growth. Dysphagia, vomiting, weight loss, and respiratory symptoms are the most frequent complaints of patients with FP, and these with long stalks can regurgitate into the pharynx or mouth and cause asphyxia [1].

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Inflammatory pseudotumors (IPTs) are benign and rare lesions, forming a group of etiologically, histologically, and biologically heterogeneous lesions that are histologically characterized by prominent inflammatory infiltrates [2]. IPT has been described in various organs but esophageal localization is extremely rare. These quasineoplastic lesions may mimic a malignant tumor clinically and radiologically [3]. Clinical presentation of patients with IPTs tends to be with varying degrees of fever, iron-refractory anemia, and thrombocytosis [4].

We presented a 29-year-old male with fever of unknown origin, malaise, anemia and thrombocytosis, who was diagnosed and operated for a really giant esophageal polypoid tumor. The clinical manifestations of this unique lesion with behavior of IP and histopathological picture of FP were described with a review of the literature.

Case report

A 29-years-old Caucasian male, without any previous medical history, was referred to the Military Medical Academy (MMA) Belgrade, Serbia, for further investigation due to throat pain, dysphagia, and fever up to 39°C, malaise, anemia and thrombocytosis, present for few months. The patient had nonselective anorexia and lost 7 kg of his body mass for the last 4 months.

In the moment of the patient referral to MMA, his status during physical examination was normal. There was no organ enlargement, ascites or edema. Body temperature showed high fever, up to 39°C.

Laboratory investigations showed some increased values: the erythrocyte sedimentation rate (ESR) was 98 mm/h (normal: < 15 mm/h), C-reactive protein (CRP) 16.72 mg/L (normal range 0–3 mg/L), fibrinogen 8.9 g/L (normal range 2–4 g/L) platelets 833×10^9/L (normal range $140–450 \times 10^9$/L) and gamma-glutamyl transferase (GGT) 78 U/L (normal range 0–38 U/L). There was a decreases in the levels of red blood cell (RBC) 3.6×10^{12}/L (normal range $3.9–5.7 \times 10^{12}$/L, hemoglobin (Hgb) 83 g/L (normal range 120–180 g/L) and hematocrite (Hct) 0.27 L/L (normal range 0.36–0.503 L/L). A blood film showed a microcytic, hypochromic picture with hypersegmented neutrophils. Urine sample was normal and repeated blood, urine and stool cultures were negative. There was no serological evidence of infection due to cytomegalovirus (CMV), Ebstein-Barr virus (EBV), adenovirus, brucellosis, psittacosis, mycoplasma, legionella, toxoplasma or Q fever. Thyroid hormones were within normal range. Bone marrow smear showed reactive hyperplasia. The purified protein derivative (PPD) skin test was negative. Rheumatoid factors and antinuclear antibodies (ANA) were negative. Serum immunoglobulins were within normal limits.

Electrocardiogram (ECG) and echocardiogram showed a normal heart function without evidence of vegetations. Chest x-ray was normal as abdominal echosonogram.

Initial upper endoscopy demonstrated a large pedunculated mass starting from cervical part of esophagus, on 21st cm from the front teeth there was a stalk with 3 cm in diameter, originating from the right esophageal wall. The tumor stretched across the entire esophagus, up to gastric cardia. Tumor was covered with overlying mucosa and had mucosal erosion on the top, without actual bleeding. The stomach and duodenum were without any pathological changes on mucosa. Initial biopsies were inconclusive.

Endoultrasonography (EUS) confirmed endoscopic findings. Pedunculated submucosal tumor was starting from lamina muscularis propria of cervical esophagus, the upper diameter was 3 cm and the distal 6–9 cm. The tumor was echoheterogenic, dominantly hypoechogenic.

Double-contrast barium meal examination demonstrated normal deglutition act and giant pedunculated intraluminal mass, which completely filled the esophagus, up to the gastric cardia, causing significant dilatation and obstruction (Figure 1). Tumor starts in the cervical part of esophagus and contrast normally passes in the stomach (Figure 2A and B).

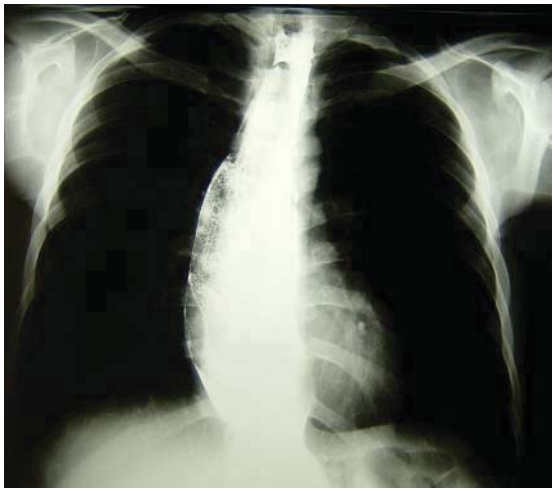

Fig. 1 – Barium meal examination shows esophageal enlargement with a huge tumor inside.

A computed tomographic (CT) scan of the chest showed an intraluminal esophageal mass of $21 \times 9 \times 6$ cm, arising from the cervical part of the esophagus and reaching the cardiac orifice of the stomach. Tumor mass fulfilled and dilate the complete esophagus but without wall infiltration. There was no evidence of metastasis or lymphadenopathy in the thorax and abdomen (Figure 3).

After investigation was completed, operative treatement was suggested to the patient and he accepted it.

In general anestesia we reached the esophagus through the left lateral neck incision. A left esophageal wall was incised in the length of 4 cm. Through this incision a huge pedunculated tumor with smooth wall was visible. The stalk of this tumor was 3 cm in its basis, originating from the right esophageal wall. The stalk was cut and the basis checked by frozen section, and no malignancy was found. The basis of the stalk was sutured by absorbable sutures. An attempt to remove the tumor through esophageal incision failed due to its huge calibar and the esophagotomy was sutured in a two-layered fashion. Median laparotomy was done and exploration did not show any pathological findings in abdomen. After

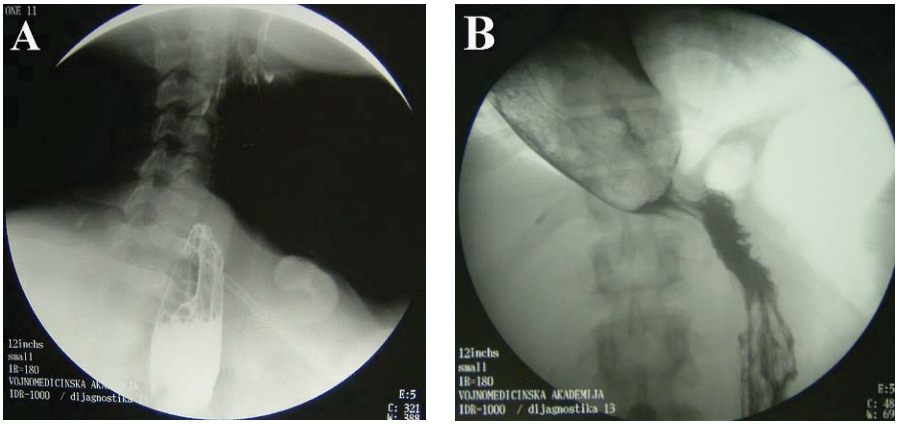

Fig. 2 – Barium meal examination: A) The tumor basis starts in the cervical esophagus; B) The top of the tumor is in front of the gastric cardia.

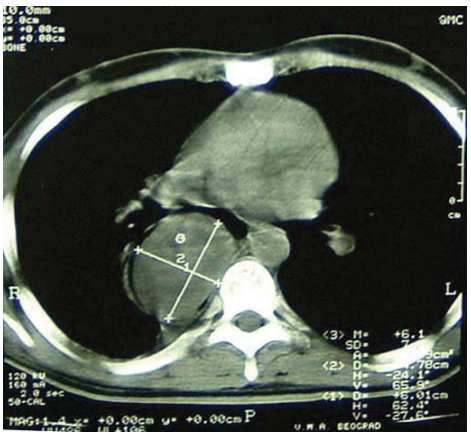

Fig. 3 – Thorax computed tomography – tumor fulfills the esophagus without wall infiltration.

gastrotomy we found no pathological content in the stomach. The tumor apex was prominent through the gastric cardia and the tumor was completely removed, "delivered", through gastric incision (Figure 4). Gastrotomy was closed by a layer of running absorbable sutures, abdominal cavity drained with rubber drain and laparotomy closed.

Gross pathology demonstrated that the removed polipoid tumor was penile – like in shape, 21 × 9 × 6 cm (Figure 5). The surface of the tumor was mainly smooth except on the top where mucosal erosion was present (Figure 6). It was open longitudinally; tissue on the cutting surface was homogeneous, white color, smooth and shiny with foci of mixoid degeneration and with tough and elastic consistency (Figure 7). The proximal part of polyp ended with the stalk, 2 cm long.

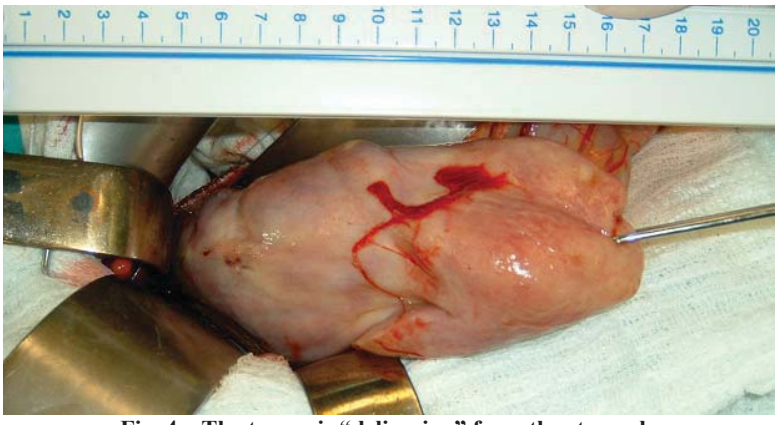

Fig. 4 – The tumor is "delivering" from the stomach.

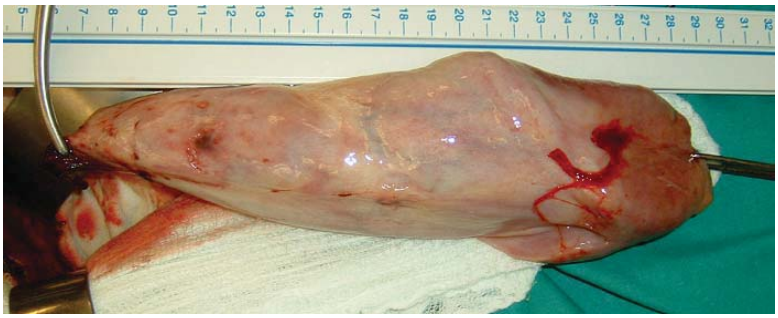

Fig. 5 – A complete tumor with a stalk.

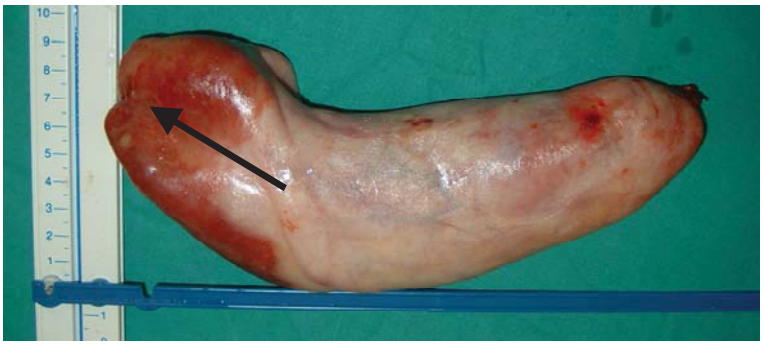

Fig. 6 – The tumor from the other side with mucosal ulceration.

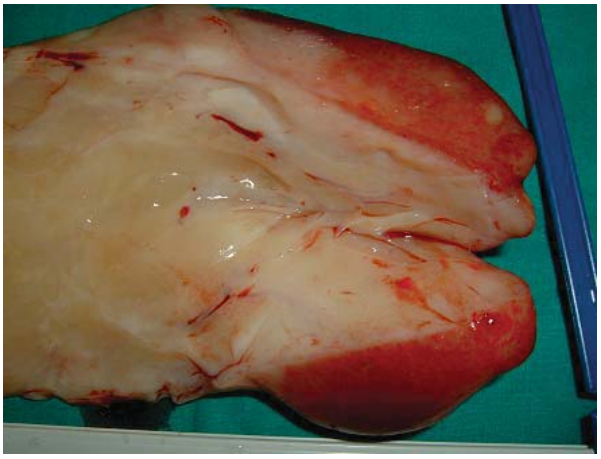

Fig. 7 – Cutting surface of the tumor.

Histologic sections were fixed in formalin, routinelly processed, embedded in paraffin and stained with hematoxylin-eosin (H E). The immunohistochemistry analysis was routinely processed on formalin fixed paraffin embedded (FFPE) tissue sections thickness of 4 μ. After deparaffinization and blocking of endogenous peroxidase activity by 3% hydrogen peroxide, the sections were incubated with primary antibodies with a Dako EnVision system. The following antibodies were used: smooth muscle actin (SMA), CD34, CD68, Ki-67 and S-100 protein. After incubation with secondary antibodies, the sections were visualized with 3-3'-diaminobenzidine and were counterstained with Mayer's hematoxylin.

Microscopicaly, the polyp was mainly covered with mature squamous epithelium, exept on the top (Figure 8A and B), where mucosal erosion was present and covered by necrotic detritus with underlying granulation tissue with diffuse infiltration of mixture of lymphocytes, plasma cells and neutrophyls. In the base of granulation tissue few scattered atypical fibroblasts were found, mitotically inactive and seeming regenerative. The tumor was composed from the loose, focally mixoid and edematous collagen connective tissue with foci of hyalinization. Inside the tumor lobules of mature adipose tissue without lipoblasts were found (Figure 8C). The polyp was very well vascularized (Figure 8D and E) with multiplied blood vessels of small and medium calibar, venous and arterial type. Foci of inflammatory infiltrates, mainly lymphocytes and plasma cells, were present not only around ulceration but also throughout entire polyp, especially perivasculary. Cappilary and bigger blood vessels showed a positive reaction of endothelial cells on CD34 (Figure 8F) and SMA, S-100 protein was negative. Inside the tumor there were histiocytes with immunoreactivity on CD68. In rare spindle cells Ki-67 showed positive reaction.

The postoperative cours was uneventful. A double-contrast barium meal examination on 7th postoperative day showed no extravasation and normal passage through the esophagus and stomach and the patient started oral feeding. Postoperatively, the patient's body temperature was normal, anemia was corrected well as thrombocytosis (value of RBC was 4.1×10^{12}/L, Hgb 105 g/L, Hct 0.322% L/L, platelets 440×10^{9}/L).

Follow-up within 5 years showed that the patient was without any complaints, without dysphagia and fever, with laboratory findings in the normal range. Control upper endoscopy showed normal status on esophagus and stomach and there was no tumor recurrence.

Discussion

Fibrovascular polyps of the esophagus are rare benign tumors, comprising about 1% of all benign esophageal tumors. However, they are the most common submucosal tumor-like lesions of the esophagus, characterized by the development of pedunculated, intraluminal masses that, in the esophagus can demonstrate enormous growth. Giant FPs are defined as polyps larger than 5 cm in maximal diameter. They are slow growing, pedunculated tumor masses that often arise from the upper esophagus, at the pharyngo-esophageal junction, Laimer's triangle [5].

Inflammatory pseudotumors are benign and rare lesions, forming a group of etiologically, histologically, and biologically heterogeneous tumefactive lesions that are histologically characterized by prominent inflammatory infiltrates [4]. IPT has been described in various organs, most commonly involving the lungs and orbit, but also found in nearly every site in the body [6]. These lesions may mimic a malignant tumor clinically and radiologically [7]. Recently there have been reports about multicentric localization of IPT [8]. Tumor size of IPT varies and can be very huge. Esophageal localization is very rare as their size over 20 cm[9].

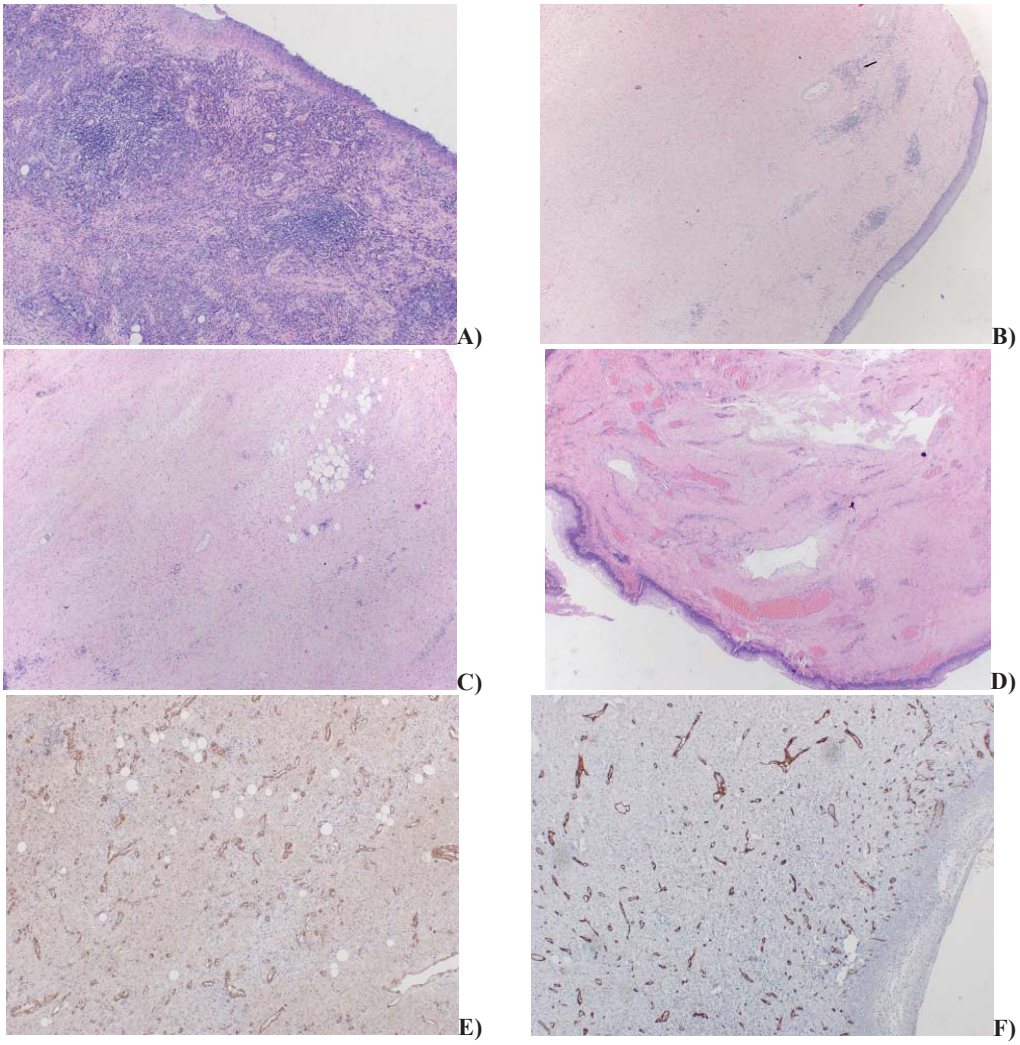

Fig. 8 – A) Mucosal erosion on the top of the polyp with the underlying granulation tissue infiltrated with lymphocytes, plasma cells, neutrophyl and eosinophyl granulocytes (HE, ×20); B) Regulary stratified squamous epithelium covering surface of the polyp out of ulceration (HE, ×4); C) Fat tissue lobulus in the connective tissue (HE, ×10); D) Multiplied blood vessels, small and medium calibar (HE, ×10); E) Vascular spaces collored by actin (immunohistochemical reaction, LSAB+, ×10); F) Endothelial cells with positive reaction on CD34 (immunohistochemical reaction, LSAB+, ×10).

The majority of FPs occur in elderly people, aged 60–70 years, but they have been reported also in a 5 months old infant [10]. Although IPT can occur at any age, in both genders, it is most commonly present in children and young adults [11].

The pathogenetic origin of FP is from the loose and redundant submucosal tissue near the Laimer's triangle. Due to the lack of muscular support, this relatively mobile tissue through years of esophageal peristalsis, traction and swallowing, is dragged along, elongated and enlarged intraluminally [12]. The etiology and pathogenesis of IPT remain unclear, reactive-infectious, immunological and idiopathic factors might play a role in their initiation and growth [13]. Speculated etiology includes viral infection, focal parenchymal necrosis with hemorrhage secondary to trauma, coagulopathy or surgical trauma [14]. An immune-autoimmune mechanism has also been implicated [15].

Fibrovascular polyps are covered with normal mucosa and containing different amounts of fibrous, vascular, and adipose tissue. Based on their histological composition, these polypoid lesions have been termed as lipomas, fibromas, fibrolipomas, or fibroepithelial polyps in the literature. More recently, the World Health Organization has classified them as FPs, in their international histological classification system [16]. Malignant transformation is rare but has been reported in esophageal polyps. The lipomatous components can undergo sarcomatous changes, the squamous mucosa can develop into squamous carcinomas and small polyps can develop into adenocarcinoma [17].

Inflamatory pseadotumors can present as a single mass or multiple masses with polymorphous inflammatory cell infiltrates and variable amounts of fibrosis, necrosis, granulomatous reaction, and myofibroblastic spindle cells. The term IPT denotes a histologically similar group of tumors, characterized by a spindle cell proliferation with a fibroin-

flammatory appearance that has been reported under a variety of additional descriptive terms, such as atypical fibromyxoid nodule, inflammatory fibroid polyp, inflammatory pseudotumor, plasma cell granuloma, and pseudosarcomatous myofibroblastic proliferation. The gross features of esophageal IPT vary from polypoid to diffuse non-polypoid, and are likely to occur in the distal esophagus or the esophagocardial junction [18].

Since polyp is slowly growing, it may remain asymptomatic for years until it reaches a large size. Dysphagia, vomiting, chronic gastrointestinal bleeding, weight loss and respiratory symptoms are the most frequent complaints of patients with FPs. Though biologically benign, these giant FPs can have dramatic and even life-threatening presentations because these polyps with long stalks, however, can regurgitate into the pharynx or mouth and cause death from asphyxiation if the larynx is occluded [19].

A distinguishing feature of IPT, in up to 50% of cases, is the presence of a varying degree of inflammatory syndrome consisting of persistent fever, weight loss, malaise, iron-refractory anemia, moderate leukocytosis, thrombocytosis, polyclonal hyper-globulinemia and elevated erythrocyte sedimentation rate. Many of these features can be related to the production of inflammatory mediators such as cytokines and particularly Interleukin-1, which has a wide range of local and systemic effects, as tumor-specific inflammatory response [10]. Our patient demonstrated several aspects of this syndrome, i.e. fever, malaise, weight loss, and thrombocytosis. Both clinical and laboratory manifestations tend to resolve rapidly after surgery [4], as was the case in the presented patient.

Usually, the diagnosis is made by imaging and endoscopic studies. Barium double-contrast examination of the esophagus usually shows a sausage-shaped mass with multiple filling defects, which originates in the cervical esophagus and extends to the lower esophagus [20].

Endoscopy usually shows an intraluminal mass that is mobile and covered with normal mucosa. The presence of easily bleeding ulcer on the top can be observed by endoscopy, leading to the suspicion of malignancy. Careful examination of the upper esophageal sphincter may reveal the stalk of pedunculated mass. EUS has been reported as a method to demonstrate the submucosal origin of polyps [21]. EUS also provides information on a diameter of a polyp, as well as its vascularity at insertion point [22]. The submucosal location can make endoscopic or tru-cutt biopsies difficult to obtain good specimens and this histopathological specimens are often inconclusive or misdiagnosed. The definitive diagnosis is often made based on histopathological analysis of surgically removed specimens of FP or IPT [23].

CT scanning and magnetic resonance imaging (MRI) can be useful to diagnose FP. In particular, MRI of the neck and thorax might be decisive in the choice of treatment by demonstrating the origin of the pedicle and the composition of a polyp. If the mass consists predominantly of fat with a minimal blood supply, the risk of bleeding during an endoscopic treatment is small. In case the polyp is rich with vascular structures, endoscopic resection can be troublesome due to uncontrollable bleeding. Preoperative identifying the place and site of a polyp's stalk, planning of cervical incision opposite to the origin is possible [24].

Histological differentiation of FP from IPT is somewhat difficult [25]. This difficulty is partly due to the lack of exact histological definition of each lesion that gives rise to the nosological confusion [26]. Key histological findings in establishing the diagnosis of IPT are the co-existence of variable numbers of inflammatory cells and spindle cells, consisting of fibroblasts and myofibroblasts and with varying degrees of fibrosis. This variation in the extent of inflammatory infiltrate and fibrosis suggests that this is a dynamic and evolving inflammatory process [27].

The first choice of therapy for this giant, pedunculated, intraluminal masses is surgical excision [28]. Surgical management is necessary because malignancy can not be excluded preoperatively and this is the only way to got definitive diagnosis and to allow oral feeding. In addition, surgical therapy is recommended because of the progressive nature of the lesion and the underlying risk of asphyxiation and sudden death [29].

Management can be complex and varies from endoscopic removal to total esophagectomy, usually is a combination of different types of endoscopical and surgical techniques. Endoscopic removal should be reserved for small, pedunculated tumors without evidence of muscularis propria involvement on EUS. Endoscopic removal has rarely been reported for giant esophageal inflammatory fibrous polyps because the procedure is technically demanding and hemostasis is difficult to ascertain, but it is possible [30]. Surgical excision is preferable by a left-sided cervical approach and, when tumor is too big, removal should be done through gastrotomy by open access or in laparoscopic way [9, 31, 32]. In case of a large-size polyp, a thoracotomy may be necessary [33]. If there is no stalk, the operative enucleation should be recommended [34]. Complete surgical resection by esophagectomy, whether partial or total, should be the procedure of choice for large and obstructing esophageal IPTs or any tumor with muscularis propria involvement, decreasing the risk of recurrence[35]. Nowadays, a minimally invasive approach is more often used in esophageal surgery for these challenging cases [36]. In our case, we succeed to remove complete tumor without esophagectomy, using a bi-approach.

Local excision of FP is curative and recurrence after resection is very rare. However, there are reports on recurrent FP that recurred within years. Some authors believe that residual tissue around the pedicle's base may cause recurrent polyp formation, which hypothetically can be the reason for recurrence. Local recurrence of giant IPS is rare but may occur if there is incomplete resection of the lesion [37]. Due to the risk of recurrence, patients should undergo to endoscopical and radiological surveillance for several years [38].

In the literature, there are several reports in which corticosteroids were successfully used for the treatment of IPT [39]. The use of chemotherapy and radiation for IPT treatment is still controversial [40].

Conclusion

The presented case illustrates the complexities involved in diagnosing and the management of giant esophageal pedunculated tumors. This unique lesion starts in the upper esophagus and has histopathological picture of FP with clinical manifestations and behavior of IPT, i.e. young ages, fever, malaise, weight loss, and thrombocytosis that resolved after operation. To our knowledge, such a case has not been described in the literature before.

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I S T O R I J A M E D I C I N E

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Sanitetski major dr Stefan Nedok (1828–1878), prvi šef Unutrašnjeg odeljenja Beogradske vojne bolnice, načelnik saniteta divizije i korpusa u ratovima sa Turskom 1876. i 1877–78.

Dr. Stefan Nedok (1828–1878), Medical Corps Major, the first Head of the Internal Department of the Belgrade Military Hospital, the Head of Medical Services of the Division and Corps in wars with Turkey (1876 and 1877–78)

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Uvod

Srpska Narodna skupština donela je 1859. godine odluku da se u cilju poboljšanja zdravstvene službe u svim srezovima postave sreski lekari, ukoliko se narod istih izjasni da li želi sopstvenog lekara koji bi bio plaćen iz sredstava dobijenih povećanjem poreza. Pošto je do sledeće godine 11 srezova (od 21) prihvatilo predlog, Popečiteljstvo unutrašnjih dela je juna meseca 1861. godine objavilo konkurs u austrijskim listovima [1].

Osmog oktobra 1861. iz Praga na konkurs se prijavio dr Stefan Nedok, doktor medicine i hirurgije, trenutno na mestu aspiranta za višeg lekara Garnizone vojne bolnice br. 1 [2]. Primljen na mesto lekara Sreza kragujevačkog, sporazumno je odložio dolazak zbog postojećih obaveza i na dužnost je stupio tek 4. februara 1862. godine [3].

Slika 1 – dr Stefan Nedok

Stefan Nedok, Slovenac rođenjem, rođen je 23. decembra 1828. godine u selu Vogričevci kod Ljutomera (Donja Štajerska) [4] u siromašnoj porodici vinogradarskih radnika, usled čega se teško i sa prekidima školovao. Osnovnu školu završio je u susednom selu Cezanjevci, a gimnaziju u Cesarskoj velikoj gimnaziji u Varaždinu 1850 [5]. Tokom 1852. i 1853. podnosio je pokrajinskoj upravi u Gracu molbe za upis u dvogodišnju bečku Vojnu medikohiruršku akademiju „Jozefinum“ [6]. Po njenom završetku je bio mlađi vojni lekar, za šta govori jedan dopis dr Belonija iz 1864. ('...jer je dr Nedok pri vojsci austrijskoj nekoliko godina služio... ' [7], a od 1857. do 1860. nastavio je studije medicine na Karlovom Univerzitetu u Pragu. Godine 1860. promovisan je za doktora medicine, a 1861. za doktora hirurgije [8]. Između dve promocije bio je godinu dana sreski lekar u Ljubnom (Slovenija) [9], a od jula 1861 vojni lekar u Pragu, u Vojnoj bolnici br. 1 [2].

Iz kojih razloga je napustio perspektivnu vojnu službu u Pragu, jednom od najlepših gradova Evrope, svoju užu domovinu slovensku Štajersku, samohranu majku i brata i odlučio da se uputi u zaostalu tursku vazalnu državicu o kojoj nije znao skoro ništa, osim da su se Srbi, njen slovenski narod, u dva krvava ustanka oslobodili ropstva i da je sada mukotrpno izgrađuju tražeći inteligenciju iz inostranstva, jer sopstvene još nemaju dovoljno?

Polovinom XIX veka (1848) širom Evrope, pa i u Austrijskoj carevini, desili su se veliki revolucionarni potresi koji su uzdrmali temelje feudalnih država i probudili slobodarske težnje porobljenih naroda. Tada su se, kao njihov odraz, u dva velika univerzitetska grada Austrije, u Beču i Pragu, pojavili studentski panslovenski i južnoslovenski pokreti koji su u maloj Srbiji videli svetionik slobode slovenskih naroda. Dr Stefan Nedok, kao privrženik tih ideja, iskoristio je kon-

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kurs da ih životom i radom u Srbiji ostvari. Sa njime se na konkurs javio i drug sa studija Čeh dr Josif Holec, takođe panslavista, koji je dobio mesto sreskog lekara u Paraćinu [10].

Došavši u Srbiju dr Nedok je od februara 1862. u Kragujevcu preuzeo dužnost sreskog lekara za platu od 500 srebrnih talira godišnje, a po potrebi je odlazio u susedne srezove, lepenički i gružanski. Zbog potreba službe 20-og septembra 1863. „Knjaz srbski, na predlog popečitelja vnutrešnjih dela, 'odlučio je' da se dr Stevan Nedok, lekar sreza kragujevačkog, pod kontrakt i po uobičajenim uslovima primi za lekara okružija podrinskog“ [11]. Ni u Loznici se na dužnosti okružnog fizikusa nije dugo zadržao, već 7. novembra 1864. je „od dojakošnje dužnosti razrešen pošto ga g. Ministar voeni za vojenog lekara postavi“ [12].

Tadašnji ministar vojni, francuski artiljerijski major i srpski pukovnik Ipolit Monden, koga je Knjaz Mihailo doveo da mu pomogne u izgradnji savremene vojske, želeo je, pored ostalih mera, da ojača vojni sanitet lekarima koji su imali neko vojno iskustvo, pa je zato 10. aprila 1864. pismom upućenom Popečiteljstvu vnutrešnjih dela „ moli (o) da mu se javi da li bi (se) mogo skoro od dužnosti okružnog fizikusa razrešiti lekar okružija podrinskog dr Stefan Nedok koga bi on bio nameran uzeti za lekara voenog“ [13]. Posle prepiske između ministarstava, dat je odgovor da će ga Popečiteljstvo „od dojakošnje dužnosti razrešiti pošto ga g. Ministar voeni za vojenog lekara postavi“, [14] i uz svoj pristanak dr Nedok je prešao u vojnu službu za manju platu i veće obaveze.

Vojna služba

Od 1. decembra 1864. pa sve do svoje smrti 4/16. maja 1878, za vreme primirja u Drugom srpsko-turskom ratu, dr Nedok je proveo u vojnoj službi [15] u kojoj je dotle bilo samo četiri lekara (dr Herman Kraus, dr Karl Kiko, dr Sava Dimitrijević i dr Maksim Nikolić-Miškovičev), jedan apotekar (Venceslav Švarc), 5 priučenih lekarskih pomoćnika (Karalić, Radovanović, Ilić, Lomigorić, Đorđević) i dva empirika (Dimitrije Kaparis i Jovan Petrović /pokršteni Jozef Šauengel/). Do kraja te godine u službu su primljena još tri lekara: dva Slovenca (dr Stefan Nedok i dr Jovan Kovač) i jedan Poljak (bivši ruski vojni lekar dr Aleksandar Verminski). Tadašnji vojeni lekari, bez oficirskog čina, a u rangu od kapetana 2. klase do potpukovnika, imali su godišnju platu od 450–600 srebrnih talira i „11 hvati drva za ogrev“ [16].

Od 1. decembra 1864. do 31. marta 1875. dr Nedok je bio na službi u Beogradu vršeći dužnosti trupnog lekara u Topčideru kod artiljerije i konjice, a najduže (1865–1875) bio je lekar u vojnoj bolnici. U međuvremenu bio je član nekoliko vojnih komisija, predavač na bolničarskim kursevima i učesnik na vojnim manevrima [14].

Godine 1867. primljen je u srpsko državljanstvo ('podanstvo') odrekavši se austrijskog.

Beogradska garnizona bolnica u to vreme imala je obično do dva lekara koji su istovremeno vršili službu dežurnih garnizonih lekara u ciklusu, zajedno sa ostalim vojnim lekarima. Komesar bolnice, trupni oficir, bio je nadređeno lice celokupnom osoblju, osim lekarima po stručnoj liniji, što je dovodilo do čestih interpersonalnih problema u svakodnevnom radu. Da bi se ti problemi razrešili, ministar vojni je, na predlog načelnika vojnog saniteta dr Karla Belonija, 1867. oslobodio lekare komesarskog tutorstva, a 3. decembra 1871, na predlog Belonija, ukazom postavio „... u beogradskoj garnizonoj bolnici za šefa bolnice glavnog vojenog lekara 2. klase g. dr Jovana Mašina, za lekara Odeljenja unutrašnjih bolesti vojenog lekara 2. kl. g. dr Stevana Nedoka, a za lekara Odeljenja spoljnih bolesti g. dr Vladana Đorđevića. To je bilo prvo imenovanje jednog lekara za opšteg šefa bolnice i imenovanje stručnih šefova bolničkih odeljenja. Od tada '...svi redovi, podoficiri i oficiri bolničarske struke stoje pod neposrednom upravom dotičnih šefova vojnih bolnica.... '. [17], iako je tek 29. januara 1875, uoči ratova sa Turskom, izmenjen član 31. 'Zakona o ustrojstvu vojske' iz 1864. da glasi: ' Bolničari stoje pod komandom komandira bolničara, oficira stajaće vojske, koji je komesar bolnice i koji je pod neposrednim nalozima i zapovestima šefa dotične bolnice'. U isto vreme, izmenjenim članom 32, lekari su dobili oficirske činove, postupno od kapetana 2. klase do potpukovnika, lekarski pomoćnici/magistri hirurgije/nediplomirani studenti medicine činove potporučnika i poručnika, a apotekari i pomoćnici od potporučnika do kapetana 1. klase [18].

U toku reorganizacije državne uprave 13. maja 1868. godine knjaz Mihailo Obrenović III je svojim Ukazom promenio sastav „Stalne lekarske komisije“, najviše zdravstvene institucije Kneževine Srbije, postavivši nove članove: dr Pavla Šafarika, dr Mladena Jankovića, dr Đorđa Klinkovskog, dr Josifa Holeca, dr Stefana Nedoka i magistra farmacije Pavla Ilića [19]. Sastav je ostao nepromenjen za života dr Nedoka [14].

Iste godine Knjaz ga je 'za zalaganje u vršenju vojnolekarske službe' odlikovao 'Zlatnom medaljom za revnosnu službu' [20].

Kada su 1875. u novoj kragujevačkoj bolnici, koja je bila u rangu beogradske, ali sa polovinom broja njenog osoblja, iskrsli organizacioni i kadrovski problemi, ministar vojni je, posle izvršene komisijske inspekcije, naredio 31. marta 1875.„... da se u kragujevački garnizon pošalje jedan viši sanitetski oficir, major dr Stefan Nedok, za šefa tamošnje bolnice i da ujedno vodi nadzor nad trupnim lekarima (kapetani dr Kufas i dr Lontkijević), da u bolnici zavede odeljenje za unutrašnje i spoljašnje bolesti i da premesti ambulatoriju iz bolnice u kasarnu“ [21].

Prvi srpsko-turski rat 1876

Na novoj dužnosti dr Stefana Nedoka zatekle su pripreme za rat sa Turskom i mobilizacija. Svojim Ukazom od 13. marta 1876. knez Milan postavlja dr Nedoka za načelnika saniteta Timočke divizije [22] čiji se štab nalazio u Zaječaru. Tu je vršena koncentracija divizije i uvežbavanje jedinica. Rat je počeo 20 juna 1876.

Srbija je u ovaj rat ušla sa narodnom vojskom milicijskog tipa, nepripremljenom i neopremljenom i malom 'stajaćom vojskom'. Ta, većinom loše opremljena i obučena vojska od 123 000 vojnika i nedovoljnim brojem oficira, tre-

balo je da se bori protiv 180 000 regularnih turskih vojnika, dobro naoružanih i uvežbanih. U pomoć joj je došlo oko 2 500 ruskih dobrovoljaca i nekoliko stotina drugih (Bugari, Rumuni, Italijani-garibaldinci). Ništa bolje nije bilo ni u sanitetu, magacini prazni, oprema oskudna, a ceo sanitet je činilo 19 lekara, aktivnih oficira, 5 lekarskih pomoćnika, 1 apotekar i 4 apotekarska pomoćnika. Pored ovih, u građanskom sanitetu radio je još 41 diplomirani lekar, koje je vojska većinom mobilisala [23]. U to vreme, uprkos zalaganju dr Belonija, 'vojni sanitet srpske vojske još nemađaše ustrojstva za ratnu službu, a kamoli detaljnog uputstva za njeno vršenje'. Tek uoči rata su brže-bolje izdate dve uredbe ('o uređenju' i 'o službi u vojnom sanitetu') kojima je pokušano da se taj propust ispravi. I da ne beše tek osnovanog Srpskog društva Crvenog Krsta i velikog priliva inostrane medicinske pomoći, materijalne i kadrovske (118 lekara, većinom Rusa, 26 apotekara i pomoćnika, 25 ruskih felčera, 147 studenata medicine, 13 milosrdnih sestara) [24] sanitet bi još u samom početku rata potpuno podbacio.

Timočka divizija (25 000 vojnika) pod komandom pukovnika Milojka Lešjanina imala je da brani celu istočnu granicu Srbije i da sprečava prodor Turaka u Moravsku dolinu. Njen štabni sanitet se sastojao iz 3 lekara (načelnik major dr Stefan Nedok, komandir sanitetskog odeljenja kapetan 2 kl. dr Julius Lenk, komandir poljske bolnice kapetan 2. kl. dr Jovan Danić, 1 apotekara (Hristifor Dimitrijević) i nešto priučenih bolničara. Sanitetskog materijala je bilo vrlo malo, a bolnica je mogla da primi jedva tridesetak ranjenika [25].

Sanitet brigada u sastavu divizije bio je različitog lekarskog sastava: Krajinska (dr Patricije Černi, dr Anton Sobotik, Franc Bihele, dr Pavle Katanić, medicinar Marko Milosavljević), Crnorečke 1. i 2. kl. (dr Stevan Mačaj i medicinar Laza Ilić), Knjaževačka (dr Alojz Kumer), Požarevačke 1. i 2. kl. (dr Sava Dimitrijević, dr Kazimir Staniševski, dr Julius Petiko i dr Vladislav Jasnjevski), Braničevska (dr Ilija Jovanović i dr Milan Milovanović/eks dr Bernhard Pas) [26]. Posle poraza kod Velikog Izvora dodeljena joj je i Beogradska brigada 2 kl. (kapetan 2 kl. dr Periša Šljivić i medicinar Svetozar Atanasijević [26], dr Mačaj pominje i dr Panajota Papakostopulosa [27]. Zbog velikog broja ranjenika i obolelih (tifus, dizenterija) diviziji je u julu dodeljena još jedna, valjevska poljska bolnica [27].

Već u prvom sukobu sa nadmoćnim turskim trupama 23. juna kod Velikog Izvora divizija je bila potučena i morala je da se povlači, ostavivši neprijatelju Knjaževac i Zaječar. Pošto je i južna grupa divizija pretrpela neuspeh i velike gubitke u ljudstvu i teritoriji krajem jula izvršena je reorganizacija cele vojske spajanjem jedinica istočne i južne grupacije u Moravsko-timočku vojsku pod komandom ruskog generala Mihaila Grigorjeviča Černjajeva sa 4 korpusa. Potpukovnik dr Vladan Đorđević postavljen je za načelnika saniteta vojske, dok je dr Nedok postavljen za načelnika lukovskog korpusa sa sedištem u Lukovu i Izvoru kod Paraćina. Za načelnike saniteta ostalih korpusa postavljeni su kapetani 1. kl. dr Aleksandar Verminski (aleksinački), dr Pavle Stejić (deligradski) i dr Leonardo Lontkijević (banjski) [28]. Dok su Lukovski i Banjski korpus branili prilaz Moravskoj dolini sa istoka, Aleksinački i Deligradski su to činili sa juga.

Iako nadmoćni, Turci nisu dugo uspevali da postignu odlučujući uspeh, nisu uspeli, sa istoka i posle više bojeva da prodru u Moravsku dolinu, a na jugu su vođene teške borbe na Krevetu, Šumatovcu i Deligradskom šancu. Srbi su čak odneli pobede kod Šumatovca i Bobovišta. Ni zapadne srpske vojske (Drinska i Javorska) nisu imale trajnijih uspeha, mada su vođene borbe u Semberiji oko Bijeljine. Najzad, turska nadmoć u ljudima i materijalu odlučila je rat: Đuniski uspeh otvorio im je put ka Aleksincu, Kruševcu i centralnoj Srbiji, ruski dobrovoljci skoro su svi izginuli braneći do kraja đuniske položaje (od 1.000 boraca poginulo je 650). Pritiskom evropskh sila, Srbija je prošla bez teritorijalnih gubitaka.

„Srbija je puna četiri meseca odoljevala moćnoj Turskoj carevini koja je protiv nje poslala jezgro svoje ubojne snage. Ona u ovom ratu nije pobedila, ali je uz podršku Rusije zaključila častan mir. Krv srpska iz svih krajeva pomešala se sa krvlju naše moćne slovenske braće sa severa“ (Jovan Ristić u Velikoj narodnoj skupštini) [29]. Ljubav velikog slovenskog naroda prema svome manjem bratu bila je u toliko vrednija, jer je organizovana isključivo van učešća zvaničnih vlasti, čak, što se tiče vojničkog učešća i protiv njihove volje, potajno, zbog njihovog protivljenja iz političkih razloga.

„Posle objave rata i posle prvih ratnih operacija sanitetska služba otkazala je na svim stranama, pokazujući kako svoju slabu organizaciju, tako i oskudno brojno stanje lekara i bolničara, njihovu nepripremljenost....., oskudicu i nemaštinu sanitetskog i apotekarskog materijala i prevoznih sredstava“ [30]. Što do njenog potpunog sloma nije došlo, ima se zahvaliti obilnoj materijalnoj pomoći Srpskog društva Crvenog Krsta i obilnoj pomoći iz inostranstva, kako dobrovoljnoj (Rusija), tako i društava Crvenog Krsta iz više evropskih zemalja. Ruski sanitetski 'atrjadi', vođeni poznatim profesorima i docentima sa mnogih njenih univerziteta, đacima slavnog Pirogova, držali su u svojim rukama skoro celokupnu bolničku službu u pozadini, ali ih je bilo i na nekim istaknutim položajima. Svojim učešćem oni su ne samo spasli mnoge živote, mnoge rešili invalidnosti, već su preneli i znanje njihove medicinske, posebno vojnohirurške pirogovske škole.

Bilans rata bio je: oko 6 000 mrtvih i 20 000 onesposobljenih i državni dug od oko 30 000 000 zlatnih dinara. Od registrovanih 13 342 ranjenika umrlo je 428, a od 20 000 bolesnika umrlo je 356, mada su faktičke cifre morale biti mnogo veće.

Za ispoljenu hrabrost, zalaganje i požrtvovanost knjaz Milan je mnoge sanitetske oficire nagradio odlikovanjima. Dr Nedok je odlikovan „Takovskim krstom na prsima“ i „Srebrnom medaljom za hrabrost“ [31, 32].

Između dva rata

Neposredno po primirju ukinuta je ratna komanda i doneta nova formacija vojske, koja je podeljena na 5 korpusa sa po 4 brigade i u miru se sastojala iz „aktivne“ i „rezervne“ vojske. Za načelnika saniteta Vrhovne komande postavljen je potpukovnik dr Vladan Đorđević, a za pet načelnika saniteta korpusa potpukovnik dr Jovan Mašin (moravski), majori dr Stevan Nedok (timočki), dr Josif Holec (drinski), dr Lazar Dokić (šumadijski) i kapetan 1 kl. dr Petar Ostojić (javorski) [15].

Na zahtev dr Vladana Đorđevića svi korpusni i brigadni načelnici saniteta dostavili su svoje zabeleške o iskustvima iz proteklog rata i predloge o unapređenjima službe [33]. Na osnovu njih dr Vladan trebalo je da napiše drugu svesku druge knjige svoje 'Istorije' o radu saniteta tokom rata. Nažalost, knjiga nije napisana, a beleške su zagubljene u zaostavštini dr Vladana, koji se u međuvremenu okrenuo drugim delatnostima. Naše traganje u zemlji i u inostranstvu po rasturenim delovima njegove zaostavštine u raznim arhivima ostalo je uzaludno. Zna se da je na osnovu tih zabeleški maja 1877. jedna vojnolekarska komisija (Tajsić-Dokić-Holec) sačinila „Izmene i dopune uputstva za službu vojnog saniteta srpske narodne i stajaće vojske“ koje su donele višestruka poboljšanja, a naročito je značajno bilo to što je sanitetskim oficirima dat mnogo veći značaj i veća samostalnost u radu [34]. To će se već u sledećem ratu pozitivno odraziti na rad saniteta.

Dr Nedok je ostao sa divizijom u opustošenom Zaječaru sa praznim magacinima sanitetsko-apotekarske spreme. Na njegovo traženje da se izvrši popuna i preraspodela raspoloživog materijala ministar vojni je odgovorio „da se sa sanitetskim stvarima postupi prema predlogu....a da se nove ne mogu nabaviti“. U toku nekoliko meseci zdravstveno stanje dr Nedoka, uopšte slabo kao posledice teškog detinjstva i mladosti, sada je usled pretrpljenih ratnih napora popustilo, pa je više puta molio ministarstvo da mu se odredi mirnija služba, pretpostavljajući da će uskoro ponovo doći do rata [35]. U čekanju odgovora došlo je do novog rata.

Drugi tursko-srpski rat 1877–1878.

I u ovome ratu, pored materijalnog siromaštva i ispražnjenih depoa, glavni problem bio je nedostatak stručnog osoblja: umesto potrebnih 139 lekara na raspolaganju je bilo 64, umesto 165 lekarskih pomoćnika – 22, umesto 75 apotekara – 49. Pokušaji dr Vladana Đorđevića da tokom putovanja po Austriji nađe izvestan broj potrebnih kadrova završio se neuspehom. Jedino se uspelo da se poveća broj bolničara. Ni Rusija nije mogla da pruži kadrovsku sanitetsku pomoć jer je već nekoliko meseci vodila rat protiv Turske na Kavkazu i Balkanu, ali je ovoga puta pružila veliku novčanu pomoć izdržavajući celu srpsku vojsku [15].

Plevna je bila osvojena, kada se knez Milan rešio da napadne. Timočki korpus 11/12 decembra bio je u boju na Babinoj glavi kada je iz Ministarstva vojnog došla naredba da načelnici saniteta Drinskog (dr Holec) i Timočkog korpusa (dr Nedok) zamene mesta [36] čime je sa zakašnjenjem udovoljeno molbi dr Nedoka za 'mirnijim mestom'. Drinski korpus (12 500 vojnika i 54 oficira) u ovome ratu nije imao ofanzivnu, već defanzivnu ulogu, odbranu granice duž Drine, od Višegrada do ušća u Savu, i Šapca. Sastav njegovog saniteta bio je sledeći [37]: načelnik saniteta major dr Stevan Nedok, komandir sanitetskog odeljenja kapetan dr Milutin Popović, komandir sanitetskog odeljenja Šabačke brigade 1. kl. dr Milan Novaković v.d. lekara Šabačke rezervne vojske, medicinar Laza Lazarević, komandir sanitetskog odeljenja Podrinske brigade dr Josif Svetič, komandir 1. poljske bolnice dr Antonije Groder, komandir 2. poljske bolnice dr Franja Kopše, v. d. lekara vlaškonjivske posade medicinar Bogoslav Zavađil, v.d. lekara ljubovijske posade medicinar Mihailo Leščinski, komandir Valjevske rezervne bolnice dr Herman Kraus. Velika dužina fronta, loše komunikacije, a uz to hladna i vlažna zima, stavili su korpusni sanitet, ionako kadrovski slab, na velika iskušenja. Koliko su uslovi ratovanja bili teški najbolje se vidi iz broja obolelih u korpusu: skoro polovina sastava korpusa (44,32%) bolovala je tokom rata i primirja, a u poljskim bolnicama korpusa lečeno je 2 189 vojnika, većinom od bolesti disajnih organa, reumatičnih i zaraznih bolesti [38].

Za Srbiju rat je bio uspešan: oslobođena je cela jugoistočna Srbija (Niš, Pirot, Leskovac, Prokuplje, Kuršumlija, Vranje), vojska je stupila na Kosovo polje kod Gnjilana gde je zastala zbog primirja, ali je za sanitet bio poguban: usled izloženosti velikim naporima i nepovoljnim radnim uslovima umrlo je 8 lekara: majori dr Stevan Nedok i dr Aleksandar Verminski, kapetani dr Aleksa Đukić, dr Adam Đerman, dr Radivoj Petrović, dr Andrej Bihl, dr Periša Šljivić i nešto kasnije dr Ilija Milijić [39]. Njihova smrt je najbolji izraz požrtvovanosti koju su lekari srpske vojske u radu pod najnepovoljnijim okolnostima pokazali.

Dr Nedok se razboleo tokom obilaska sanitetskih jedinica u gornjem toku Drine, verovatno od tifusa, pa je sanitetskim kolima preko Loznice i Šapca prevožen 5 dana do Beograda, pred čijim je kapijama umro u noći 4/5. maja 1878. u 50. godini života, [33, 40] za sobom ostavivši suprugu Mariju i tri maloletna deteta: kći Nadeždu (11 godina) i sinove Josifa (8 godina) i Stevana (5 godina). Na radnom stolu kneza Milana stajao je 5. maja spremljen za potpis Ukaz kojim se sanitetski major dr Stefan Nedok unapređuje u čin sanitetskog potpukovnika [32, 40, 41].

Tako se prerano završio život jednog lekara koji je, nošen slobodoumnim i južnoslovenskim idejama, izabrao Srbiju za domovinu svoju i svoje dece. U sačuvanom personalnom listu ostala je službena zabeleška o njemu: 'Prirodnog dara je dobrog, sposoban je lekar, dobar hirurg i valjan nastavnik bolničara, u svojoj struci za sve je sposoban i vrlo upotrebljiv. U vršenju službe vrlo revnostan, tačan, uredan i iz sopstvene volje zauzimljiv. U privatnim svojim poslovima dobar je ekonom. U službi i izvan službe vladanja je dobrog, ponašanja uljudnog i u svemu odgovara stanju i položaju. Zaslužuje da se unapredi. Konduitirao glavni vojni lekar 2 kl. dr Filip Taisić' [41].

U istom dokumentu je svojeručno zapisano da je znao, pored maternjeg slovenačkog, srpski, nemački, mađarski, italijanski i latinski jezik [41].

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3. ***Tekst članka***

Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Uvod.** Posle uvodnih napomena, navesti cilj rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo važne podatke iz literature a ne opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

Metode. Jasno opisati izbor metoda posmatranja ili eksperimentnih metoda (ispitanici ili eksperimentne životinje, uključujući kontrolne). Identifikovati metode, aparaturu (ime i adresa proizvođača u zagradi) i proceduru, dovoljno detaljno da se drugim autorima omogući reprodukcija rezultata. Navesti podatke iz literature za uhodane metode, uključujući i statističke. Tačno identifikovati sve primenjene lekove i hemikalije, uključujući generičko ime, doze i načine davanja. Za ispitivanja na ljudima i životinjama navesti saglasnost nadležnog etičkog komiteta.

Rezultate prikazati logičkim redosledom u tekstu, tabelama i ilustracijama. U tekstu naglasiti ili sumirati samo značajna zapažanja.

U **diskusiji** naglasiti nove i značajne aspekte studije i izvedene zaključke. Posmatranja dovesti u vezu sa drugim relevantnim studijama, u načelu iz poslednje tri godine, a samo izuzetno i starijim. Povezati zaključke sa ciljevima rada, ali izbegavati nesumnjive tvrdnje i one zaključke koje podaci iz rada ne podržavaju u potpunosti.

Literatura

U radu literatura se citira kao superskript, a popisuje rednim brojevima pod kojima se citat pojavljuje u tekstu. Navode se svi autori, ali ako broj prelazi šest, navodi se prvih šest i *et al*. Svi podaci o citiranoj literaturi moraju biti tačni. Literatura se u celini citira na engleskom jeziku, a iza naslova se navodi jezik članka u zagradi. Ne prihvata se citiranje apstrakata, sekundarnih publikacija, usmenih saopštenja, neobjavljenih radova, službenih i poverljivih dokumenata. Radovi koji su prihvaćeni za štampu, ali još nisu objavljeni, navode se uz dodatak „u štampi“. Rukopisi koji su predati, ali još nisu prihvaćeni za štampu, u tekstu se citiraju kao „neobjavljeni podaci“ (u zagradi). Podaci sa *Interneta* citiraju se uz navođenje datuma pristupa tim podacima.

Primeri referenci:

Đurović BM. Endothelial trauma in the surgery of cataract. Vojnosanit Pregl 2004; 61(5): 491–7. (Serbian)

Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u desnom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fusnoti, ne u zaglavlju. Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

Ilustracije

Slikama se zovu svi oblici grafičkih priloga i predaju se kao dopunske datoteke u sistemu **aseestant**. Slova, brojevi i simboli treba da su jasni i ujednačeni, a dovoljne veličine da prilikom umanjivanja budu čitljivi. Slike treba da budu jasne i obeležene brojevima, onim redom kojim se navode u tekstu (**Sl. 1; Sl. 2** itd.). Ukoliko je slika već negde objavljena, obavezno citirati izvor.

Legende za ilustracije pisati na posebnom listu, koristeći arapske brojeve. Ukoliko se koriste simboli, strelice, brojevi ili slova za objašnjavanje pojedinog dela ilustracije, svaki pojedinačno treba objasniti u legendi. Za fotomikrografije navesti metod bojenja i podatak o uvećanju.

Skraćenice i simboli

Koristiti samo standardne skraćenice, izuzev u naslovu i apstraktu. Pun naziv sa skraćenicom u zagradi treba dati kod prvog pominjanja u tekstu.

Detaljno uputstvo može se dobiti u redakciji ili na sajtu:

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3. Virmanom po prijemu profakture.

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