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CONTENTS / SADRŽAJ

Dr. Harriet MacMillan Cockburn (1873–1948), a Canadian woman physician, is one of a number of medical workers who as a part of the foreign medical and humanitarian aid arrived in Serbia during World War I to help its army and people. Along with them went the Golgotha retreat through Albania and along the way she met an orphan, adopted him after the war and educated in Canada. In the book, presented in this issue of the *Vojnosanitetski Pregled*, is her upseting testimony about the horrors that the Serbian people were exposed in World War I (see p. 700–1).
This year, the 100th anniversary of the beginning of World War I is marking around the world.

Dr Harijet Koburn (1873–1948), kanadska lekarka, jedna je od brojnih medicinskih radnika koji su u sklopu inostrane medicinske i humanitarne pomoći došli u Srbiju za vreme Prvog svetskog rata da pomognu njenoj vojsci i narodu. Zajedno sa njima prošla je golgotu povlačenja preko Albanije i na tom putu srela jedno siroče, usvojila ga i po završetku rata odvela sa sobom i školovala u Kanadi. U knjizi čiji prikaz dajemo u ovom broju VSP-a, nalazi se njeno potresno svedočanstvo o strahotama kojima je srpski narod bio izložen u Prvom svetskom ratu (vidi str. 700–1).
Ove godine, širom sveta obeležava se 100. godišnjica od početka Prvog svetskog rata.

Note: This photo of Dr. Cockburn is located in the University of Toronto Library.
Napomena: Ova fotografija dr Koburn nalazi se u biblioteci Univerziteta u Torontu.

SHORT COMMUNICATION

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Congenital cholesteatoma of mastoid origin – A multicenter case series

Kongenitalni holesteatom mastoida – multicentrična studija slučajeva

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Abstract

Background/Aim. The mastoid is the rarest site for the onset of congenital cholesteatoma (CC). The symptoms are atypical and minimal. The aim of this multicenter retrospective descriptive study was to define this extremely rare condition and its clinical presentation, diagnosis and management. **Methods.** We analyzed data files for a 15-year period in 4 tertiary otology centers and discovered 6 patients with the diagnosis of CC of the mastoid. **Results.** The clinical presentation of CC varied from incidental findings in patient to patient with otogenic meningitis. The most common findings during surgical procedures were mastoid cortex erosion, sigmoid plate dehiscence, dural exposure and external canal wall destruction. **Conclusion.** CC of mastoid origin tends to occur in adult patients probably because of minimal symptoms and the delayed diagnosis. It can exist for years in a nonaggressive state and develop to giant sizes. In children it is almost incidentally diagnosed. Early imaging is necessary in order to prevent serious complication.

Key words:
cholesteatoma; congenital abnormalities; diagnosis, differential; otologic surgical procedures; recurrence.

Apstrakt

Uvod/Cilj. Mastoid je najređe mesto za nastanak kongenitalnih holesteatoma (KH). Simptomi su netipični i minimalni. Cilj ovog multicentričnog, retrospektivnog, deskriptivnog istraživanja bio je da se definiše ovo izuzetno retko oboljenje i prikaže njegova klinička prezentacija, dijagnostika i lečenje. **Metode.** Analizirali smo baze podataka za 15 godina, u četiri tercijarna otološka centra i otkrili šest bolesnika sa dijagnozom KH mastoida. **Rezultati.** Klinička prezentacija KH varirala je od slučajnih nalaza do bolesnika sa otogenim meningitisom. Najčešći intraoperativni nalazi bili su erozija mastoidnog korteksa, dehiscencija sigmoidne ploče, ekspozicija dure srednje lobanjeske jame i destrukcija spoljašnjeg zida kanala. **Zaključak.** Kongenitalni holesteatom mastoida uglavnom se javlja kod odraslih verovatno zbog minimalne simptomatologije i odložene dijagnoze. Može da postoji godinama u neagresivnoj formi i da se razvije do gigantske veličine. Kod dece uglavnom se slučajno dijagnostikuje. Neophodna je rana "radiografska" dijagnostika da bi se sprečile ozbiljne komplikacije.

Ključne reči:
holesteatom; anomalije; dijagnoza, diferencijalna; hirurgija, otološka, procedure; recidiv.

Introduction

Congenital cholesteatoma (CC) is an epidermoid cyst arising from congenital remnants of keratizing squamous epithelium in the temporal bone [1]. The rarest site of origin within temporal bone is mastoid process and may go undiagnosed for years [2]. The symptoms are atypical and in reported cases the most common presentation was an incidental finding during radiologic evaluations [3]. CC of mastoid origin could be diagnosed if the patient had intact tympanic membrane, intact skin of external canal, no ossicular erosion and no attic involvement of cholesteatoma in intraoperative and imaging findings. CC of mastoid origin can exist for years in a nonaggressive state, develop to gigantic size and, in some cases, rapidly cause bone destruction and serious complication [4, 5].

The purpose of this multicenter study was to better define this rare condition and its clinical presentation, diagnosis and surgical treatment.

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Methods

Data files in six otology tertiary referral centers from Serbia were analyzed and patients with the diagnosis of CC of the mastoid origin were identified. All the patients were operated on by one of the authors of this article. Clinical information were compiled from the their histories. Demographics, symptoms and signs, otomicroscopic findings, audiometric data, and findings at the time of surgery were also analyzed. Computed tomography (CT) and magnetic resonance imaging (MRI) images were reviewed, as well. Follow-up length and recurrence of the disease were recorded. Audiometric data were presented as pure tone average air bone gap (ABG) preoperatively and postoperatively in four frequencies (500, 1000, 2000, 4000 Hz).

Results

Analyzing patient's data for the period of 15 years we recognized 6 cases of CC of the mastoid region in 4 otologic centers (Table 1). The mean age of patients was 23 years (range from 7 to 60 years). The clinical presentation of the disease varied. One of the cases (case 1) was with minimal and atypical otologic symptoms (retroauricular pain and mild hearing loss). Two of the cases (cases 2 and 5) were with ear discharge with normal tympanic membrane, but with swelling of the skin of meatus. One child (case 3) underwent MRI due to bilateral sensorineural hearing loss, and cholesteatoma was incidentally found. The oldest patient (case 6) was with otogenic meningitis and CC was displayed with CT scan only in the mastoid. There were 5 cases of the patients with few months' symptoms and the patient with a complication had one day history of headache, fever, dizziness and vomiting. All the patients had normal tympanic membrane findings.

Five out of 6 patients underwent CT scanning, whereas 2 of 6 patients underwent MRI. In the CT scan results, 2 of 5 patients had a well-circumscribed, expansive lesion into mastoid portion of the temporal bone. Three of 5 patients had external auditory canal wall destruction and 3 had sigmoid plate dehiscence and dural destruction, as well (Figure 1 and 2).

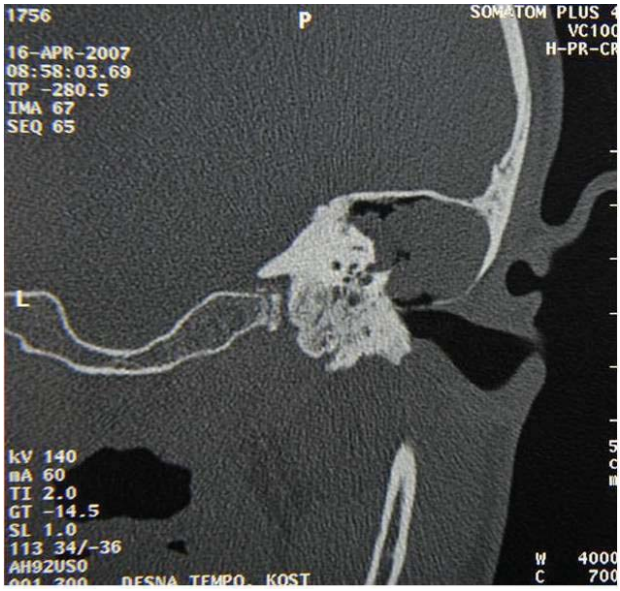

Fig. 1 – Case 1: Preoperative computed tomography of the right mastoid area. Coronal view of temporal bone showing the lesion destroying the posterior canal wall of auditory canal.

Table 1

Characteristics of patients with congenital cholesteatoma

Caracteristics	Cases					
	1	2	3	4	5	6
Age (years)	29	22	7	13	8	60
Side	Right	Left	Left	Right	Left	Right
Symptoms and signs	retroauricular pain, hearing loss	Ear discharge	Hearing loss in both ears	Hearing loss	Ear discharge	Headache, earache, fever
Duration of symptoms	3 months	6 months	1 month	4 months	3 months	1 day
Otomicroscopic findings	Normal tympanic membrane	Normal tympanic membrane, swelling of skin of meatus	Normal tympanic membrane	Normal tympanic membrane	Swelling of skin of meatus	Normal tympanic membrane
Imaging	CT	CT	MRI	CT	CT, MRI	CT
Preoperative ABG	20 dB	0 dB	0 dB	30 dB	0 dB	No data
Postoperative ABG	20 dB	0 dB	0 dB	30 dB	0 dB	No data
Surgical approach	Retroauricular transmastoid-perilabyrinthi-nemastoidectomy wall down	Retroauricular mastoidectomy wall down	Retroauricular mastoidectomy wall up	Retroauricular mastoidectomy wall up	Retroauricular, mastoidectomy wall down	Retroauricular mastoidectomy wall down
Follow-up period	3 years	2 years	2 years	3 years	2 years	3 years
Recurrence	no	no	no	no	yes	no

ABG – air bone gap; L – left, R- right; CT – computed tomography; MR – magnetic resonance imaging.

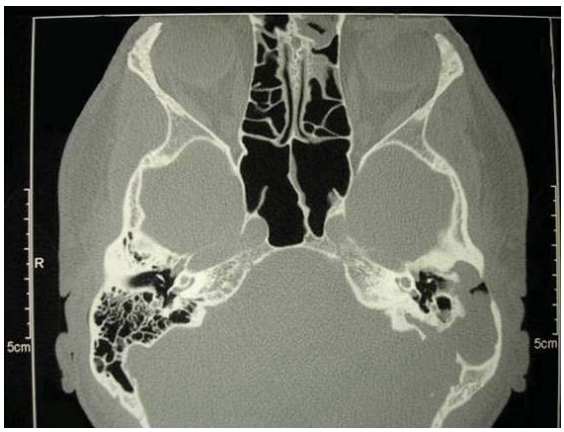

Fig. 2 – Case 2: Preoperative computed tomography of the left mastoid area. Axial view showing an expansive mass eroding cortex of mastoid and the bony plate of the posterior fossa and the bony plate covering the sigmoid sinus.

In the MRI results, 2 of 2 patients had sharply circumscribed mass into mastoid process (Figure 3). Surgical approaches were 4-canal wall down mastoidectomies, and 2-canal

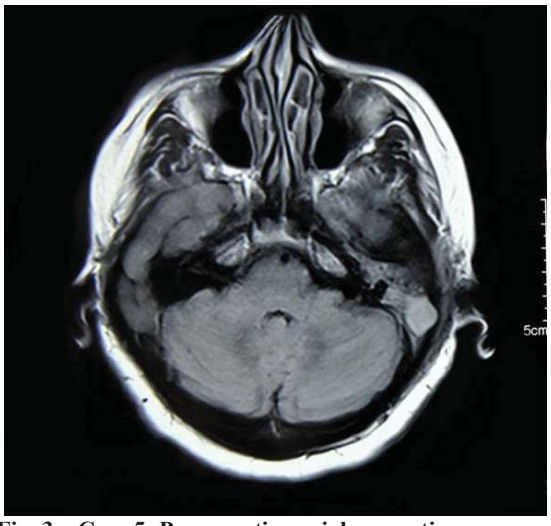

Fig. 3 – Case 5: Preoperative axial magnetic resonance imaging of the left mastoid area. T1 weighted image showing isointense, well delineated lesion of mastoid portion.

wall up. The patients with sigmoid plate dehiscence had erosion of bony plate of the posterior fossa, as well (Table 2). Only one of the patients had exposed mastoid portion of the facial nerve. Two of the cases had spread into epitympanum without ossicular erosion. Two of the cases with hearing loss with intact ossicular chain had slight fixation of incudomalleolar and incudostapedial joint. None of them had erosion of the osseous labyrinth and 3 had external canal wall destruction but with intact skin of meatus. The most common findings in surgery were mastoid cortex erosion, sigmoid plate dehiscence, dural exposure and external canal wall destruction.

Five of 6 patients had audiometric data. Preoperative ABG ranged from 0 to 30dB and postoperative ranged from 0 to 20dB. Follow-up period was from 2 to 3 years, and only one patient had recurrence during this time.

Discussion

CC of mastoid origin is an extremely rare disease [2]. In the literature there are a few case reports about CC of mastoid origin [1, 2, 4, 5] and one case report with analysis of radiologic data in nine patients [3]. The symptoms are atypical and include neck pain, retroauricular swelling and pain, and dizziness [2]. CC of mastoid origin was diagnosed if a patient had intact tympanic membrane, intact skin of external canal, no ossicular erosion neither attic involvement of cholesteatoma. The most common presentation in our series was pain and ear discharge. Erosion of the mastoid cortex could be the reason of pain because the disease involves the periosteum. Intermittent ear discharge was a symptom in 2 cases due to inflammation of intact meatus skin. Hearing loss is an unusual symptom for CC and in our series was due to fixation into incudomalleolar and incudostapedial joint as another congenital anomaly in two cases. All the patients had normal otomicroscopic findings on ear drum. Everyone had a relatively short duration of symptoms.

Although CC of mastoid origin has a congenital origin, it tends to occur in adulthood probably because of minimal symptoms and delayed diagnosis.

Two of 3 children had only hearing loss as a symptom (case 3 – sensorineural loss and case 4 – conductive loss) and radiological imaging procedures were performed to differentiate reasons of hearing loss. Mastoid CC was the incidental finding in these cases.

The oldest, 60-year-old, patient (case 6) had expansive destructive mass into mastoid portion of temporal bone with 1-day duration headache, neck stiffness, vomiting and fever. Blood analysis and lumbar puncture confirmed the meningitis and CT scan showed a huge mass of the mastoid.

Table 2

Intraoperative findings in patients with congenital cholesteatoma

Intraoperative findings	Cases					
	1	2	3	4	5	6
Mastoid cortex erosion	Yes	Yes	No	No	No	Yes
Sigmoid plate dehiscence	Yes	Yes	No	No	No	Yes
Dural exposure	Yes	Yes	No	No	No	Yes
Facial nerve exposure	Yes	No	No	No	No	No
Spread into epitympanum	No	No	No	No	No	No
Ossicular erosion	No	No	No	No	No	No
Erosion of osseous labyrinth	No	No	No	No	No	No
External auditory canal destruction	Yes	Yes	No	No	Yes	No

The data of one patient in this descriptive case series (case 1) had already been published as a case report [5]. That patient had a giant cholesteatoma with complete exposure of the facial nerve with no facial weakness. Also, there was erosion of the perilabyrinthine space with no history of dizziness, and dural and jugular bulb exposures without intracranial or extracranial complication.

CC of mastoid origin can exist for years in a nonaggressive state and develop to giant size. It is possible that CC saves fine tissue of temporal bone and is very destructive for bone tissue, or the mastoid portion is silent region.

Early CC diagnosis is essential to prevent delayed treatment and serous complications. The most important tools in the diagnosis are CT and/or MRI [3, 6]. CC gives minimal symptoms that might seem mild and nonspecific. It is not unusual to incidentally find CC on imaging [3]. Imaging is of essential importance for preoperative surgical planning as well.

Surgical management of these lesions is primarily directed by intraoperative and imaging findings. Surgical approaches were 4-canal wall down mastoidectomies, and 2-canal wall up. Where the lesion was gigantic and where posterior canal wall was destructed, the canal wall down mastoidectomy was done. Giannuzzi et al. [7] considered that the management of the sigmoid sinus and the jugular bulb is the most demanding surgical key point.

Conclusion

Congenital cholesteatoma of mastoid origin can develop to huge sizes, can have minimal clinical presentation, and can be found not before causing serious complications. Sometimes the diagnosis is incidental. Otologists should keep this disease in mind in cases with normal otomicroscopic findings, unusual retroauricular pain and ear discharge.

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ORIGINAL ARTICLES

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Treatment of obstructive sleep apnea patients using oral appliances – our experiences

Lečenje opstruktivne apneje u snu pomoću oralnih aparata – naša iskustva

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Abstract

Background/Aim. Obstructive sleep apnea (OSA) is one of the most prevalent sleep disorders. It is recognized as a serious risk factor for car and workplace accidents due to daytime sleepiness, and factor for coronary heart diseases and stroke. The aim of this study was to examine the effectiveness of oral appliances for mandibular advance in treating mild to moderate OSA. **Methods.** A total of 15 patients were included in this study, all diagnosed with mild or moderate OSA. Oral appliances were custom made for each patient in protrusive position at 50% of maximum mandibular advancement. The patients were given instructions not to sleep on their backs and avoid alcohol consumption during the study as these are the factors that can contribute to symptoms progression. **Results.** Complete and partial treatment success was achieve in 14 of the patients. Apnea-hypopnea index values were significantly lower ($p < 0.05$) at the end of a 6-month observation period compared to those at the treatment beginning. A great improvement in symptoms was observed, with daytime sleepiness index values significantly reduced already within the first month of the treatment. **Conclusion.** Treatment of obstructive sleep apnea with oral appliances has proven successful. Patients were comfortable using oral appliances and were ready to wear them for prolonged period of time. Use of oral appliances is very common in the world and should not be discarded. They are also very comfortable, practical and affordable comparing to continuous positive airway pressure (CPAP) apparatus, not to mention surgery. Use of oral appliances is safe and very well tolerated, and ought to be offered to patients with OSA.

Key words:
sleep apnea, obstructive; orthodontic appliances; treatment outcome.

Apstrakt

Uvod/Cilj. Opstruktivna apneja u snu je jedan od najčešćih poremećaja spavanja. Dokazano je da su bolesnici koji pate od ovog poremećaja skloniji saobraćajnim nezgodama i povredama na radu, kao i da su izloženi većem riziku od nastanka koronarne bolesti srca i moždanog udara. Cilj ovog istraživanja bio je da se utvrdi uspešnost terapije blage i umerene opstruktivne apneje u snu oralnim aparatima. **Metode.** Ukupno 15 bolesnika sa prethodno uspostavljenom dijagnozom blage ili umerene opstruktivne apneje u snu bilo je uključeno u istraživanje. Oralni aparati su individualno urađeni za svakog bolesnika, sa mandibulom u protruzionom položaju 50% od maksimalnog. Bolesnicima je preporučeno da izbegavaju spavanje na leđima i unošenje alkohola tokom trajanja studije, jer ovi faktori mogu uticati na pogoršanje simptoma. **Rezultati.** Potpuni i delimični uspeh terapije postignut je kod 14 bolesnika. Vrednosti apneja-hipopneja indeksa bile su statistički značajno niže ($p < 0,05$) od početnih vrednosti na kraju 6-mesečnog perioda posmatranja. Registrovano je i značajno poboljšanje simptoma, kao i značajno umanjenje vretnosti indeksa dnevne pospanosti već posle prvog meseca terapije. **Zaključak.** Oralni aparati su bili uspešni u lečenju opstruktivne apneje u snu. Bolesnici su iskazali veliku lagodnost i bili su spremni da nose oralne aparate i duži vremenski period. Upotreba oralnih aparata je uobičajena u svetu, i ne treba da bude zanemarena. Vrlo su praktični, lagodni za nošenje i pristupačniji u poređenju sa aparatom za kontinuirani pozitivni pritisak u disajnim putevima (CPAP), a pogotovo sa hirurškom intervencijom. Upotreba oralnih aparata je bezbedna i trebalo bi je ponuditi bolesnicima kao jedno od mogućih rešenja.

Ključne reči:
apneja u snu, opstruktivna; ortodontski aparati; lečenje, ishod.

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Introduction

One third of human life is spent sleeping, so the need for a healthy and undisturbed night sleep is of essence. There are many sleep disorders described so far [1], obstructive sleep apnea (OSA) being one of the most common, affecting at least 1–5% of adult population [2, 3]. Due to structurally small or highly collapsible upper airways, patients suffer from abnormal ventilation during sleep, manifesting in a complete or partial blockade of breathing, repeatedly during the night. Fragmented sleep affects daytime concentration and presents significant problem for the patients. It is described that people suffering from OSA are prone to work place accidents, and more often involved in car crashes due to overt sleep and inattention due to sleepiness [4, 5]. OSA is recognized as a serious risk factor for hypertension, coronary heart disease and stroke [6–8], and as such, it needs to be properly diagnosed and treated accordingly. In addition, patients are not well educated and informed about symptoms and risks of obstructive sleep apnea, and are not aware that they suffer from this serious condition. Furthermore, patients who are diagnosed are mainly offered only continuous positive airway pressure (CPAP). CPAP is the use of continuous positive pressure to maintain a continuous level of positive airway pressure but apparatus used for this is highly uncomfortable and patients can not adequately adjust [9]. Another treatment modality for OSA patients is surgery but, as all surgery options, it represents a risk for patients, and thus, alternative treatments that are safe, effective, and acceptable are needed.

Oral appliances for mandibular advance are used for treating mild and moderate OSA, and are proved very successful [10–12]. In addition, patients reported high levels of comfort, and were prepared to use oral appliances for longer period of time [12]. Varieties of oral appliances are used for treating OSA, and are all quite effective [13]. It is important to present oral appliances for mandibular advance as treatment modality to both patients suffering from OSA and medical practitioners treating them.

The aim of this study was to examine effectiveness of oral appliances for mandibular advance (Thornton adjustable positioner – TAP) in treating mild to moderate OSA.

Methods

A total of 15 patients (ten men and five women) were included in this study. All of the patients were diagnosed with mild or moderate obstructive sleep apnea by sleep medicine specialist during nocturnal polysomnography (Stardust II Sleep Recorder, Philips Respironics, Amsterdam, The Netherlands) using a standard method [14]. All the patients had an apnea / hypopnea index (AHI) > 10. Before enrolling in the study dental exam was performed. All of the patients were found to have 10 periodontally healthy teeth needed to adequately wear the oral appliance. In addition, none of them was found to suffer from craniomandibular dysfunctions. Oral appliances were custom made for each patient (Figure 1).

After taking the impressions maximal lower jaw advancement was determined. Using specially designed bite registrator (Figure 2) occlusal impressions were taken in protrusive position at 50% of maximum mandibular advancement.

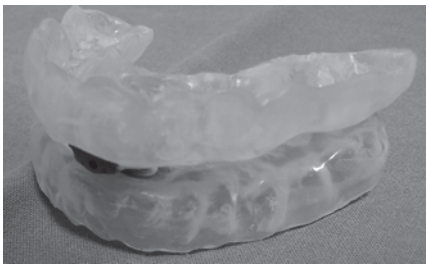

Fig. 1 – Thornton adjustable positioner (TAP) oral appliance for mandibular advance

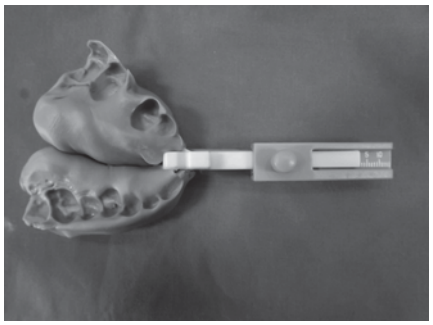

Fig. 2 – Bite registrator

This position was described comfortable by the patients. Before receiving appliances patients were given rubber spatulas to chew on for a period of one week to accommodate temporomandibular joints to protrusive position of the lower jaw (Figure 3). After receiving oral appliances patients were given instructions not to sleep on their backs and avoid alcohol consumption during the study, as these are the factors that can contribute to symptoms progression [15].

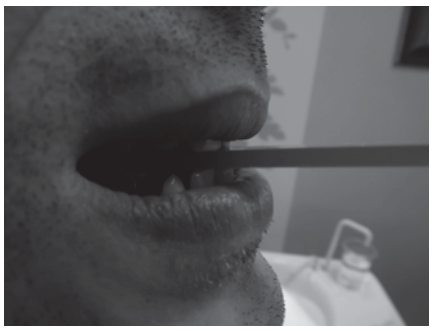

Fig. 3 – Chewing spatula

During observational period that lasted for 6 months, follow-up exams were conducted on the first, third and sixth month. At the beginning of the observational period and at the follow-ups patients were asked to fill questionnaires for subjective assessment of the frequency and intensity of snoring, and sleepiness index, designed by the author specifically to help access treatment success, already used in previous study [12]. The sleepiness index contained 6 questions: 1) “Do you wake up rested in the morning?”; 2) “Does

the sleep invigorate you?"; 3) "Do you continuously wake up during night sleep?"; 4) "Do you have trouble performing your daytime activities due to sleepiness"; 5) "Do you ever fall asleep while waiting at the doctors, bank etc.?"; 6) "Do you regularly take naps during the day?". The answers for each question were graded from 0 to 4, 0 being with no symptoms or problems at all, while 4 being fully expressed symptoms or problems. Scores were designed in the way that 0–10 points meant normal range, 10–12 borderline and 12–24 abnormal. Also, data about snoring and quality of life was gathered from participants' partners. At the end of observational period patients had once again undergone polysomnography, and AHI values were recorded.

Success of the treatment was defined according to recommendations of American Academy of Sleep Medicine. Complete success of the treatment was defined as reduction in AHI index to ≤ 5 with the resolution of symptoms, while partial success was defined as improvement in symptoms and reduction of AHI index for more than 50% compared to baseline measurement but AHI remaining ≥ 5. Treatment failure was defined as ongoing clinical symptoms with AHI reduction less than 50% compared to the baseline. Severity of OSA was defined based on the AHI: mild from 5 to less than 15, moderate from 15 to 30 and severe over 30 apneic episodes *per* hour of sleep [16].

Data were analyzed using a statistical package (SPSS version 17.0, SPSS Inc. Chicago, IL, USA). Paired *t*-tests were used to compare AHI values before and after the treatment and daytime sleepiness index before the treatment and after 1, 3 and 6 months within the group. A *p*-value of < 0.05 was used to assign statistical significance for all tests. All descriptive statistics are presented as mean ± SD.

Results

All of the patients involved in the study completed the protocol. The average age of the patients was 49 (SD ± 7), and there was no statistical difference in age between male and female participants. Baseline AHI measurements were between 15 and 28 apnea/hypopnea episodes per hour (h^{-1}) (average 20.87 ± 3.64) with no statistical significance between genders. Average protrusion value was 5.46 mm (SD ± 1.06). TAP appliance was well tolerated by all of the patients, with three of them reported transient excessive salivation. On the first follow-up patients and their partners reported substantial reduction in symptoms, 80% of the patients snored less, 73% slept better. After the third month these percentages raised, and on the second follow-up all of the patients reported improvement in sleeping quality and decline in snoring. In addition, the patients were quite comfortable wearing oral appliances. The baseline sleepiness index results showed abnormal results regarding daytime sleepiness, and sleep quality with average value of 17.3 (SD ± 2.4), without statistical significance between male and female patients. A statistically significant difference was observed between the values for baseline sleepiness index measurement and measurements after the first, third and sixth month ($p < 0.05$), but not between follow-ups (Figure 4).

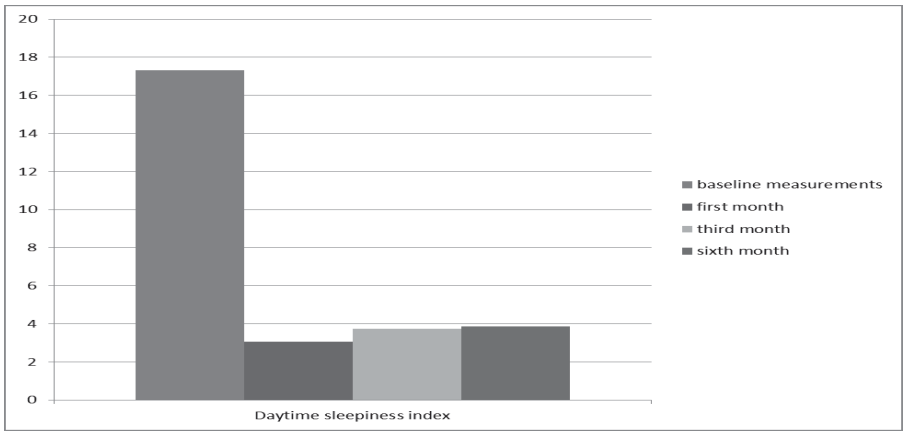

Fig. 4 – Daytime sleepiness index values

Also, the AHI values after observational period were significantly lower than baseline ($p < 0.05$), averaging 6.1 ± 3.5 (Table 1). No difference could be observed regarding gender. In seven patients a complete treatment success was achieved, partial success in seven patients and treatment failure was reported for only one patient. All of the patients reported that they were abiding by the instructions regarding alcohol consumption and sleeping on their side.

Discussion

A complete therapy success was recorded in 7 out of 15 (47%) patients who participated in the study. In addition to 7 patients with partial success rate, 14 out of the 15 patients included in the study reported substantial improvement in their condition and regression if not elimination of symptoms. Treatment failure was recorded in only one patient, but with noticeable subjective improvements. Subjective parameters regarding the success of the treatment show that all of the participants in the study slept better, snored less, and functioned more efficiently during the day, thus improving their quality of life. These findings should be used with caution, as any subjective data should be. Data regarding sleepiness index showed

an improvement and reduction in the values, showing the reduction of daytime sleepiness, and related problems. These results correspond with the findings in other studies [17, 18]. It is important to emphasize that patients were comfortable wearing oral appliances, without any noticeable side effects.

The proper diagnose of obstructive sleep apnea is very important, if we are to prevent risks associated with this condition. Snoring is one of the main symptoms of OSA. Every patient that suffers from OSA snores, but not everyone who snores suffers from OSA. Patients should be properly informed about the symptoms, risks and treatment modalities of OSA. Oral appliances are proven to reduce collapsibility of upper airways, thus reducing objective and subjective symptoms of OSA [19]. They are also very comfortable, practical and affordable comparing to CPAP, not to mention surgery.

Conclusion

Oral appliances use is very common in the world and should not be discarded. Oral appliances are not novelties, but proven and efficient treatment for people suffering from mild or moderate obstructive sleep apnea, and should be used accordingly. Oral appliances use is safe and very well tolerated, and ought to be offered to patients with OSA.

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Association of renin-angiotensin system genes polymorphism with progression of diabetic nephropathy in patients with type 1 diabetes mellitus

Udruženost polimorfizma gena renin-angiotenzin sistema u razvoju dijabetesne nefropatije kod bolesnika sa dijabetesom tipa 1

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Abstract

Background/Aim. Diabetic nephropathy (DN) as a major microvascular complication of diabetes mellitus (DM) include a progressive increase in urinary albumin excretion in association with an increase in blood pressure and to end stage renal failure. Hypertension connected with renin-angiotensin system (RAS) hyperactivity and corresponding genotypes, angiotensinogen (AGT), angiotensine-converting enzyme (ACE) and angiotensin II type 1 receptor (AT1R), predispose the increasing risk of DN. The aim of this study was to assess the distribution of AGT, ACE and AT1R gene polymorphisms in patients with type 1 DM according to the level of DN and patients clinical characteristics. **Methods.** The study included 79 type 1 diabetic patients. Inclusion criteria were: age between 20–40, duration of diabetes > 5 years, and no other severe diseases. Clinical characteristics were gained from interviewing the patients. Polymorphism was detected by polymerase chain reaction (PCR) and restriction fragment length polymorphism using restriction enzymes Psy I (Tth 111 I) and Hae III. **Results.** The patients with proteinuria compared with normo- and microalbuminuric patients, highly differed in age, diabetes duration, blood pressure level, hypertension, rethynopathy and urinary albumin excretion values ($p < 0.001$). No statistically significant difference between the groups was found for the ACE and AT1R gene polymorphisms distribution. The presence of TT genotype of the M235T polymorphism was significantly higher in the group with proteinuria ($p < 0.05$). The patients with hypertension raised nephropathy 5.2 times higher (OR = 5.20, $p < 0.05$) while carriers of TT allel developed nephropathy 28.38 times higher (OR = 28.389, $p < 0.01$) than those with MM genotype. **Conclusion.** Increased association of hypertension and TT angiotensinogen gene polymorphism in patients with diabetes mellitus with proteinuria could be a significant marker of diabetic nephropathy.

Key words:
diabetic nephropathies; renin-angiotensin system; diabetes mellitus, type 1; polymorphism, genetic.

Apstrakt

Uvod/Cilj. Dijabetesna nefropatija (DN) kao jedna od najznačajnijih komplikacija dijabetesa melitusa (DM) uključuje progresivno povećanje urinarne ekskrecije albumina koja udružena sa hipertenzijom, dovodi do terminalne bubrežne insuficijencije. Hipertenzija i povećana aktivnost renin-angiotenzin sistema (RAS) uz prisustvo određenih polimorfizama gena za RAS, angiotenzinogen (AGT), angiotenzin-konvertujući enzim (ACE) i angiotenzinski receptor tipa 1 (AT1R) mogu da ukažu na povećanu sklonost ka razvoju DN. Cilj rada bio je ispitivanje distribucije polimorfizma gena AGT, ACE i AT1R u grupi bolesnika sa DM tipa 1 u odnosu na stepen razvoja nefropatije, definisane kliničke parametre i faktore rizika. **Metode.** Ispitivanjem je bilo obuhvaćeno 79 bolesnika sa DM tipa 1, starosti 20–40 godina, trajanjem dijabetesa duže od 5 godina i bez drugih hroničnih bolesti. Polimorfizam gena RAS ustanovljen je metodom PCR-RFLP korišćenjem restrikcione endonukleaze Psy I (Tth 111I) i Hae III. **Rezultati.** Bolesnici sa proteinurijom u odnosu na normo- i mikroalbuminurične ispitanike značajno su se razlikovali po godinama starosti, trajanju dijabetesa, vrednosti krvnog pritiska, hipertenzije, retinopatije i urinarne ekskrecije albumina ($p < 0,001$). U ispitivanim grupama, razlike u učestalosti genskih polimorfizama za ACE i AT1R nisu bile statistički

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značajne. Prisustvo TT genotipa u M235T polimorfizmu gena za AGT bio je značajno viši u grupi bolesnika sa proteinurijom ($p < 0,05$). Učestalost nefropatije bila je 5,2 puta veća (OR = 5,20, $p < 0,05$) kod dijabetičara sa hipertenzijom, dok je TT genotip ukazivao na 28.38 puta veću sklonost (OR = 28,389, $p < 0,01$) ka razvoju nefropatije u odnosu na MM genotip. **Zaključak.** Povećanje učestalosti hipertenzije i TT alelske forme u angiotenzinogen genskom polimorfizmu kod bolesnika u terminalnom stadijumu nefropatije mogu imati značaj za određivanje markera procene rizika od nastanka komplikacija u dijabetesu melitusu tipa 1.

Ključne reči:
dijabetičke nefropatije; renin-angiotenzin sistem; dijabetes melitus, tip 1; polimorfizam, genetički.

Introduction

Diabetic nephropathy (DN) is an important microvascular complication of a long-standing type 1 and type 2 diabetes mellitus (DM) associated with considerable morbidity and mortality. In spite of the main risk factors for the development of DN as hypertension and poor glycemic control, genetic susceptibility in both type 1 and type 2 DM is also of the great importance. Other risk factors that relate to the development of nephropathy are smoking, obesity and dyslipidemia [1]. Once DN is present, risk factors favorise evolution to more advanced stage. Recent studies suggest that antihypertensive drugs (angiotensine converting enzymes – ACE-inhibitors) should be used in prevention of DN even in normotensive patients.

DN is defined as increased protein excretion in urine. Early stage is characterized by a small increase in urinary albumin excretion (UAE), also called microalbuminuria or incipient DN. More advanced disease is defined by the presence of macroalbuminuria or proteinuria. The latter is named overt or "manifested" DN.

Patients with elevated level of urinary albumin excretion are at high risk of cardiovascular complications and need frequent and carefull examination for early detection of nephropathy and cardiovascular and lipid abnormalities [2].

Hemodynamic changes in DN, as systemic and glomerular hypertension, lead to the initiation and progression of nephropathy and may be explained by alteration in the renin-angiotensine system (RAS).

The prominent role of the component genes of the RAS in cardiovascular regulation sugest the possibility that polymorphism of the genes of the RAS might be involved in the genetic predisposition to develop DN. Among the candidate genes of the RAS, the angiotensinogen (AGT), ACE and angiotensin II type 1 receptor (AT1R) genes seem to be particulary relevant to renal disease and related with predisposition for renal complications [3, 4]. The genetic polymorphisms of these key components of RAS provide a basis for studying the relationship between genetic variants and the development of vascular and renal damage in patients with DM.

Several polymorphisms were identified in the AGT gene wich are linked to essential hypertension. Of those, M235T polymorphism (methionine substituted by threonine) resulting in M and T alel forms, was extensively studied in cardiovascular and renal diseases [4, 5]. A TT genotype is associated with the progression of limited hypertension into manifested hypertension as well as with increasing risk for the diabetic nephropathy level [6].

Polymorphism of the ACE gene has a frequent insertion-deletion (I/D) polymorphism characterized by the 278-bp insertion (allele I) or deletion (allele D) variant in intron 16 associated with serum and tissue ACE levels. The I/D polymorphism, wich determine most of ACE individual variance was proposed as a genetic marker for DN. The level of ACE in plasma is in direct correlation with the genotype, and plays a critical role in determining intrarenal angiotensin and kinin concentrations [7].

The AT1R polymorphism located at the position 1166 (A/C) in 3´ untranslated region has been considered as a risk factor for hypertension and cardiovascular disease [5].

In the present study we examined the distribution of the AGT, ACE and AT1R gene polymorphisms in patients with type I DM devided into three groups according to the level of DN – normoalbuminuric, microalbuminuric or incipient nephropathy and proteinuric or macroangiopathy level. Also, polymorphisms of three studied genes were correlated with clinical characteristics of patients with DN.

This study was approved by the Ethic Committee of the Institute of Diabetes and Metabolic Diseases, Clinical Center of Serbia, Belgrade.

Methods

All the patients with type 1 DM were presented with certain criteria and hospitalised in the Institute of Diabetes and Metabolic Diseases from 2008 to 2010.

The prospective observational study included 79 type 1 diabetic patients, among them 33 had normoalbuminuria, 21 were with microalbuminuria, while 25 patients had proteinuria. The patients were classified according to the amount of albuminuria using the median result of the three 24 h urine collections. Urinary albumin excretion (UAE) was determined with the nephelometric technique. Normoalbuminuric level was defined as UAE < 30 mg/24 h, microalbuminuria as UAE 30–300 mg/24 h and macroalbuminuria or manifested proteinuria as UAE over 300 mg/24 h. Inclusion criteria were age (20–40 years), duration of diabetes (> 5 years) and the absence of other severe diseases.

Clinical characteristics were gathered from interviewing the patients (duration of diabetes, family history for hypertension, cardiovascular diseases, diabetes, smoking duration). The defined risk factors (arterial blood pressure and complications) were measured on the first day of visit. Blood samples were drawn for measurement of serum cholesterol, HbA1c and for the determination of genotypes.

Determination of genotypes

Individual genomic DNA samples were extracted from periferal blood with Applied Biosystems 6100 Nucleic Acid prep Station instrument.

The AGT (M235T) gene polymorphism and AT1R (A1166C) gene polymorphisms were analysed by polymerase chain reaction (PCR) and subsequent restriction-endonuclease digestion (Tth 111I and Hae III) [8]. To determine the ACE I/D genotype, DNA (100 ng) was amplified using two insertion-specific primers described by Bonnardeaux et al. [9]. The reaction products were visuelised on 2% agarose gel stained with ethidium bromide. Final analysis of the genotype was performed after 10% PAGE stained with silver nitrate.

Statistical analysis

All statistical analyses were performed in SPSS 12.0 (SPSS Inc, Chicago, Illinois) statistical package). The results are presented as frequency, percent and mean ± SD. The χ^2, Kruskal-Wallis and ANOVA test were used to compare the 3 groups, while Mann-Whitney *U*-test was used to compare the 2 groups. A logistic regression model was performed to asses associations of nephropathy and other variables. All *p*-values that were less than 0.05 were considered significant.

Results

Patient characteristics (total number 79) are given in Table 1.

crovascular complication showed high statistically significant difference in the group of patients with proteinuria (Table 1).

We genotyped all the patients for the AGT, ACE and AT1R gene polymorphisms. The distribution of genotype and allele frequencies in the examined groups are presented in Table 2.

No statistically significant difference among the examined groups was found in the genotype and allele distribution for the ACE and AT1R gene polymorphisms.

The carriers of the MT and TT genotype of the AGT gene polymorphism were more frequent in the group of patients with elevated UAE. The distribution of homo- and heterozygous genotypes for the T allele (MT and TT) was significantly higher in the group of patients with proteinuria when compared to normoalbuminuric patients.

The presence of homozygous TT genotype of the AGT M235T polymorphism, compared among the 3 devided groups according to UAE level, was significantly higher in the group of patients with high albumin excretion.

The genotype and allele distribution for the ACE and AT1R gene polymorphisms, among the examined groups according to elevated albumin excretion, showed no statistically significant difference.

We found the tendency for statistical significance between the pressence of TT and I/D gene polymorphisms and increased blood pressure in patients with proteinuria. The pressence of MT/TT genotype of AGT M235T gene and AC/CC genotype of AT1R gene did not reveal a significant association with retinopathy in the group of patients with

Table 1

Characteristics of diabetes mellitus (DM) patients

Patients characteristics	Normoalbuminuria (n = 33)	Microalbuminuria (n = 21)	Proteinuria (n = 25)	*p*-value
Age (years), $\bar{x}$ ± SD	28.1 ± 5.8	25.8 ± 6.8	34.2 ± 6.8	< 0.001[a]
Duration of DM (years), $\bar{x}$ ± SD	9.6 ± 4.6	11.3 ± 4.4	21.9 ± 5.8	< 0.001[a]
Family history of HTA, n (%)	22 (42.3)	14 (26.9)	16 (30.8)	ns[c]
Family history of DM, n (%)	11 (52.4)	14 (19)	6 (28.6)	ns[c]
Family history of CVD, n (%)	16 (43.2)	13 (35.1)	8 (21.6)	ns[c]
Blood pressure (mmHg), $\bar{x}$ ± SD				
systolic	121 ± 12	126 ± 14	148 ± 26	< 0.001[a]
diastolic	79 ± 7	85 ± 9	92 ± 15	< 0.001[a]
Hypertension, n (%)	9 (24.3)	9 (24.3)	19 (51.4)	< 0.001[c]
Polineuropathy, n (%)	13 (30.2)	14 (32.6)	16 (37.2)	ns[c]
Retinopathy, n (%)	7 (18.9)	11 (29.7)	19 (51.4)	< 0.001[c]
HbA1c (%)	9.3	9.7	10.4	ns[c]
Cholesterol, n (%)	11 (39.3)	7 (25.0)	10 (35.7)	ns[c]
HDL cholesterol (mmol/L), $\bar{x}$ ± SD	1.25 ± 0.39	1.18 ± 0.44	1.28 ± 0.38	ns[a]
LDL cholesterol (mmol/L), $\bar{x}$ ± SD	3.14 ± 1.06	3.05 ± 1.07	4.00 ± 1.58	ns[b]
UAE (mg/24 h)	13.1 ± 5.2	128.1 ± 87.3	368.3 ± 223.8	< 0.001[b]
Smokers, n (%)	14 (34.1)	12 (29.3)	15 (36.6)	ns[c]

HTA – hypertension; CVD – cardiovascular disease; BP – blood pressure; HbA1c – glycohemoglobin A1c; HDL – high density lipoprotein; LDL – low density lipoprotein; UAE – urinary albumin excretion; n – number of patients in examined groups; ns – not significant; values are calculated by [a]ANOVA, [b]Kruskal Wallis; [c]χ^2 test.

The patients with proteinuria, compared with normo- and microalbuminuric patients, highly differed in age and diabetes duration, blood pressure level and hypertension.

There was no statistically significant diference in the occurence of polyneuropathy among the examined groups of patients, while the presence of rethinopathy as a frequent miproteinuria, but the homozygous DD genotype of the ACE gene polymorphism showed an increasing frequency in the proteinuric group compared with normoalbuminuric patients (Table 3).

Using the Backward logistic regression model, after obtaining all predictors associated with renal failure, we

Table 2

Distribution of genotypes and allele frequencies of AGT, ACE nad AT1R genes in the examined patients

Genotype	Normoalbuminuria (n = 33)	Microalbuminuria (n = 21)	Proteinuria (n = 25)	*p*-value
AGT (M235T), n (%)				
M allele	37 (56)	20 (47.6)	19 (38)	ns[a]
T allele	29 (44)	22 (52.4)	31 (62)	
MM	7 (21.2)	5 (23.8)	3 (12.0)	
MT	23 (69.7)	10 (47.6)	13 (52.0)	< 0.05[b]
TT	3 (9.1)	6 (28.6)	9 (36.0)	
MM+MT	30 (90.1)	15 (71.4)	16 (64)	< 0.05[c]
TT	3 (9.9)	6 (28.6)	9 (36)	
ACE (I/D), n (%)				
I allele	34 (51.5)	25 (59.5)	24 (48)	ns[a]
D allele	32 (48.5)	17 (40.5)	26 (52)	
II	11 (33.3)	7 (33.3)	6 (24.0)	
ID	12 (36.4)	11 (52.4)	12 (48.0)	ns[ab]
DD	10 (30.3)	3 (14.3)	7 (28.0)	
II+ID	23 (69.7)	18 (85.7)	18 (72)	ns[c]
DD	10 (30.3)	3 (14.3)	7 (28)	
AT1R (A1166C), n (%)				
A allele	47 (71)	28 (67)	33 (66)	ns[a]
C allele	19 (29)	14 (33)	17 (34)	
AA	16 (48.5)	12 (57.1)	10 (40.0)	ns[b]
AC	15 (45.5)	4 (19)	13 (52)	
CC	2 (6.1)	5 (22.8)	2 (8.0)	
AA+AC	31 (93.9)	16 (76.2)	23 (92)	ns[c]
CC	2 (6.1)	5 (23.8)	2 (8)	

[a]Mann-Whitney U test; [b]Kruskal-Wallis test; [c]χ^2 test;
AGT – angiotensinogen; ACE – angiotensin-converting enzyme; AT1R – angiotensin II type I receptor; n – number of patients in examined groups according to albuminuria (percentages are shown in parentheses); ns – not significant.

Table 3

Genotype distribution according to hypertension, polyneuropathy and rethinopathy in the examined groups of patients with diabetes mellitus

Genotype distribution	Yes, n (%)	No, n (%)	*p*-value
Hypertension			
AGT			
MM	8 (53.3)	7 (46.7)	ns
MT/TT	34 (53.1)	30 (46.9)	ns
ACE			
II	13 (54.2)	11 (45.8)	ns
ID/DD	29 (52.7)	26 (47.3)	ns
AT1R			
AA	20 (52.6)	18 (47.4)	ns
AC/CC	22 (53.7)	19 (46.3)	ns
Polyneuropathy			
AGT			
MM	8 (53.3)	7 (46.7)	ns
MT/TT	28 (43.8)	36 (56.3)	ns
ACE			
II	10 (41.7)	14 (58.3)	ns
ID/DD	26 (47.3)	29 (52.7)	ns
AT1R			
AA	16 (42.1)	22 (57.9)	ns
AC/CC	20 (48.8)	21 (51.2)	ns
Rethynopathy			
AGT			
MM	5 (33.3)	10 (66.7)	ns
MT/TT	37 (57.8)	27 (42.2)	ns
ACE			
II	14 (58.3)	10 (41.7)	ns
ID/DD	28 (50.9)	27 (49.1)	ns
AT1R			
AA	19 (50.0)	19 (50.0)	ns

χ^2 test; AGT – angiotensinogen; ACE – angiotensin-converting enzyme; AT1R – angiotensin II type I receptor; n – number of patients in examined groups according to albuminuria (percentages are shown in parentheses); ns – not significant.

found that duration of diabetes, high blood pressure, rethinopathy and AGT polymorphism were highly associated with nephropathy in patients with DM (Table 4).

Table 4

Correlation of diabetes mellitus (DM) duration, hypertension (HT), rethinopathy, AGT and ACE gene polymorphism with nephropathy in the examined group of patients

Predictor	*p*-value	OR (95% CI)
Duration of:		
DM	< 0.01	1.192 (1.057–1.345)
HT	< 0.05	5.200 (1.301–20.790)
rethinopathy	< 0.05	2.840 (1.288–6.261)
AGT polymorphism		
MM	< 0.05	-
MT	ns	1.358 (0.234–7.885)
TT	< 0.01	28.389 (2.422–332.724)
ACE polymorphism		
II	ns	-
ID	ns	2.113 (0.435–10.258)
DD	ns	0.307 (0.053–1.780)

AGT – angiotensinogen; ACE – angiotensin-converting enzyme; ns – not significant; OR – odds ratio; CI – confidence interval.

It was shown that as the duration of diabetes increases by one unit, the chances of developing nephropathy were 1.192 times higher being statistically significant. If a patient had hypertension, the likelihood for nephropathy was 5.2 times higher. A patient with TT allele proned to develop nephropathy 28.38 times higher than the carrier of MM genotype. If a patient had the MT allele, the chance to develop nephropathy was 1.358 times greater, but this was not statistically significant. In fact, only the TT genotype showed notable importance.

The univariate analyses did not show a statistically significant association for ACE/II genotype according to increasing renal complications. The DD genotype in our study is associated with 0.307 lower risk for nephropathy.

Discussion

We examined the association between the DN level and genotype distribution of the RAS genes in normoalbuminuric, micro and proteinuric groups of patients with DM type 1. Also, we analysed main clinical complications in patients with DM type 1 and correlated them with the studied gene polymorphisms.

Patients with microalbuminuria are at high risk of cardiovascular complications and need frequent and careful examination for early detection of nephropathy, retinopathy, cardiovascular and lipid abnormalities. Identification of factors related to the development of microalbuminuria leads to the development of strategies to prevent nephropathy and reduce the occurence of new cases [8].

Our data suggest a statisticaly significant correlation between blood pressure level (systolic and diastolic) and rethinopathy as more prominent microvascular complication in patients with terminal renal insuficiency. Both systolic and diastolic hypertension accelerate the progression of diabetic nephropathy, and agressive antihypertensive management successed to decrease the rate of fall of glomerular filtration rate (GFR) [10].

In patients with type 1 diabetes, the median life expectancy can significantly increase using appropriate antihypertensive intervention, with the reduction of mortality from 94% to 45%, and the reduction in the need for dialysis and transplantation from 73 to 31% 16 years after the development of overt nephropathy [11]. For patients with nephropathy, treatment with ACE inhibitors is indicated as a part of initial therapy concerning previous genotyping analysis of the ACE gene polymorphism.

In the present study, due to a rather small number of patients, T allele of the M235T polymorphism and D allel of the ACE I/D polymorphism tend to be increased in patients with elevated blood pressure in the proteinuric type 1 diabetes group. Furthermore, current literature suggests that the ACE D/D genotype predicts poor renal response to ACE inhibitors and to agents that do not block the RAS in subjects with DN. Two longitudinal observational studies pointed out that GFR decreased rapidly in proteinuric type 1 DM patients with the DD genotype [12, 13]. In the other study, the albumin excretion rate of type 1 DM patients with the II genotype increased more rapidly than in those with the DD genotype, while the response to ACE inhibitor was better in patients with II than in those with the DD genotype [14].

In some follow-up studies, implementation of RAS blockade therapy in type 1 diabetic patients reduced the progression to overt DN to 3.4% *per* year. It should be notified that 45% of patients in progression of DN decrease microalbuminuria by intensified antihypertensive treatment [15].

Several data suggest that hyperglicaemia may be very important factor for the increasing incidence of cardiovascular complications and progression from microalbuminuric to DN. Despite previous observational studies findings about association of poor glycaemic control and progression in albuminuria and the rate of progression to DN, we found no statistically significant correlations in HbA1c values and quality of glycoregulation between the normoalbuminuric and microalbuminuric patients. There was no significant increasing serum levels of total cholesterol, low density hipoprotein (LDL), high density lipoprotein (HDL) cholesterol in our examined group although it has been reported that dia-

betic patients could manifest alterations in serum lipid level ranging from 30 to 90% [16].

Polymorphisms for different components of RAS have been described with controversal results according to the different ethnic backgrounds of the study populations, for example Asian carriers of 235T allel appear to have a higher risk of hypertension than white carriers [17].

On the basis of the previous studies, we cannot conclude that there is a link between the ACE I/D polymorphism and DN. The results differ in a number of studies and data do not exclude a pathogenic role of mutations somewhere else in the ACE gene, but prevaling conclusion that the ACE I/D polymorphism is not a reliable marker for the prediction of DN in patients with type 1 diabetes [18]. Our data do not indicate an independent effect of ACE and AT1R polymorphism on the risk for DN, so we are unable to confer the interaction between the ACE I/D and M235T polymorphism, suggesting that geneticaly determined AGT levels can cause risk for DN through Ang I generation.

Previous results in type 1 diabetic patients showed an association between DN and the T allele of AGT M235T polymorphism. In the article of Walder et al. [18], the autors therefore found that the TT genotype was 3 times more common in the group of 98 nephropathic than in 98 normoalbuminuric type 1 DM patients, indicating a link to this genotype and elevated serum angiotensinogen with the possible risk for DN conferred by this genotype. In the another study group of hypertensive patients at a start of dialysis, the carriers of the T allele of the AGT gene were more frequent in the group of patients compared to controls [6, 19]. The results of our study showed that in patients with hypertension, a likelihood for nephropathy is 5.2 times higher, while in the group of patients with TT allele risk to develop nephropathy is 23.38 times higher than in the carriers of MM genotype.

Our data designate that the T allele occurs more frequently among patients with proteinuria compared with those with normoalbuminuria, and the level of evidence shows statistically highly significant difference.

In our study, the T allele of the AGT-M235T polymorphism and CC genotype of the AT1-A1166C polymorphism was not associated with retinopathy.

A recent meta-analysis, including two studies with type 1 diabetic patients, did not find corellation between the AGT M235T gene polymorphism and diabetic retinopathy. Nevertheless, the associations between RAS activity and retinopathy have been previously reported [20–23].

Local changes as a result of the presence of different polimorphisms of RAS are manifested as changes in renal hemodynamics, increased intraglomerular pressure and glomerular filtration rate, as the major determinants of renal function. The interactions between the alleles of the RAS probably play a major role in determining the development of DN. Experimental data might allow identification of groups with high risk of developing diabetic nephropathy and, therefore, the analysis of RAS system genes plays an important role in providing novel therapeutic targets or individualized treatment strategies for both the prevention and treatment of these complications.

Conclusion

The increased association of hypertension and the TT AGT gene polymorphism in patients with diabetes mellitus with proteinuria could be a significant marker of diabetic nephropathy.

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Evaluation of the quality of life in adolescents with acne

Procena kvaliteta života kod adolescenata sa aknama

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Abstract

Background/Aim. Acne is well recognized condition that adversely affects quality of life (QoL) of the patients. The aim of this study was to determine the self-reported prevalence of acne and its main characteristics in high school pupils, and to assess their QoL. **Methods.** The cross-sectional study conducted in May 2011 comprised 440 pupils from Serbia (281 from Belgrade and 159 from Užice). Participation in the study was voluntary and anonymous. Two questionnaires were administered: a short demographic questionnaire, and Children's Dermatology Life Quality Index (CDLQI). Internal consistency was assessed using the Cronbach's alpha while item-total score correlations were assessed using Spearman's correlation analysis. **Results.** The majority of the study population (84.3%) were girls, and 15.7% were boys. The total mean age of the pupils was 16.48 ± 0.55 years. A total of 228 (51.8%) pupils self-reported their acne with significantly higher prevalence in Užice (73.6%) than in Belgrade (39.5%). The mean CDLQI score was 3.55 with the similar quality of life impairment in adolescents from the two cities. The mean Cronbach's alpha was 0.83. There was a statistically significant positive correlation between the mean overall CDLQI score and CDLQI subscale scores that ranged from 0.401 to 0.841. **Conclusion.** Our study confirmed that acne is associated with impairment in QoL that is in accordance with previous studies performed on teenagers. The CDLQI is a reliable and valid measure, and can be used as a practical tool for measuring the impact of acne on patients' QoL.

Key words:
acne vulgaris; quality of life; adolescents; questionnaires.

Apstrakt

Uvod/Cilj. Poznato je da su akne bolest koja pogoršava kvalitet života obolelih. Cilj ove studije bio je da se utvrdi prevalencija akni i sagledaju glavne karakteristike ove bolesti među učenicima srednje škole, kao i da se proceni kvalitet života obolelih od akni. **Metode.** Studijom preseka sprovedenom u maju 2011. godine obuhvaćeno je 440 učenika iz Srbije (281 učenik iz Beograda i 159 učenika iz Užica). Učešće u studiji bilo je dobrovoljno i anonimno. Primenjena su dva upitnika: kratak upitnik o demografskim karakteristikama i dermatološki indeks kvaliteta života kod dece (CDLQI). Unutrašnja konzistentnost upitnika procenjena je pomoću Kronbahovog alfa koeficijenta, dok je korelacija između skorova za pojedina pitanja i ukupnog skora upitnika procenjivana Spirmanovom korelacionom analizom. **Rezultati.** Devojke su činile većinu ispitivane populacije (84,3%), dok je mladića bilo 15,7%. Prosečan uzrast učenika bio je 16,48 ± 0,55 godina. Ukupno 228 učenika (51,8%) izjasnilo se da ima akne, a prevalencija je bila statistički značajno viša u Užicu (73,6%) nego u Beogradu (39,5%). Srednja vrednost CDLQI skora iznosila je 3,55 sa sličnim oštećenjem kvaliteta života kod učenika iz oba grada. Srednja vrednost Kronbahovog alfa bila je 0,83. Statistički značajna pozitivna korelacija između ukupnog CDLQI skora i skorova subskala ovog upitnika bila je u rasponu od 0,401 do 0,841. **Zaključak.** Našom studijom potvrđeno je da su akne udružene sa narušenim kvalitetom života obolelih, što je u skladu sa ranijim studijama u populaciji tinejdžera. CDLQI kod dece je pouzdan i validan upitnik koji se može koristiti kao praktična alatka za merenje uticaja akni na kvalitet života obolelih.

Ključne reči:
akne; kvalitet života; adolescent; upitnici.

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Introduction

Acne is a chronic inflammatory, multifactorial skin disorder of the pilosebaceous unit that usually occurs in adolescence affecting nearly all teenagers to some degree [1]. The clinical picture can vary significantly from mild comedonal acne to fulminant systemic disease.

It is well recognized that acne adversely affects quality of life (QoL), and that can leave physical and emotional scars that can persist throughout the life of the affected person [2]. Loney et al. [3] showed dermatological-related social anxiety of acne patients to be negatively associated with intention to participate in sport/exercise, self-esteem and health related QoL.

As there is not always a correlation between the severity of acne and its impact on QoL, it can be helpful for dermatologists to use either global or specific QoL measures to determine the psychological impact of acne on patients [4].

Within the last few decades health-related QoL of patients has become an important factor of patient care, and several dermatologic and acne-specific measures have been created to assist in acne research, management, and care [5].

The aim of this study was to determine the self-reported prevalence of acne and its main characteristics in high school pupils, and to assess their QoL.

Methods

The cross-sectional study conducted in May 2011 comprised 440 pupils from Serbia (281 from Belgrade and 159 from Užice). We used convenience sampling. Participation in the study was voluntary and anonymous. The informative consent in the written form was obtained from pupils' parents. The response rate was 74.7% (281/376) in Belgrade and 75.7% (159/210) in Užice. Two questionnaires were administered – a short questionnaire including questions about demographic and disease characteristics, and Children's Dermatology Life Quality Index (CDLQI).

CDLQI is a skin disease specific questionnaire that was used to assess acne-related QoL in pupils [6]. It is a one-page questionnaire comprising 10 questions covering all aspects of quality of life. The questions are graded from 0–3, with a possible maximum score of 30 with higher scores representing worse QoL. According to the instructions for use if two or more questions are left unanswered the questionnaire is not scored. Questions about symptoms, feelings, embarrassment, clothes, hobbies, daily activities at school and leisure time, as well as issues related to sleep, personal relationships and treatment were asked. A total of 199 out of 228 (87.3%) pupils with acne filled the questionnaire properly and were included in the analysis. We used the Serbian version of the CDLQI [7].

All statistical analysis was performed using the Statistical Package for the Social Sciences, SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A two-tailed probability value of 0.05 or less was considered significant. The differences between variables were assessed by χ^2 or *t*-test. Internal consistency was assessed using the Cronbach's alpha while item-total score correlations were assessed using Spearman's correlation analysis.

Results

A total of 440 pupils from the two high medical schools in Serbia (one from Belgrade and another from Užice) participated in this study. Demographic characteristics of the pupils are presented in Table 1.

Table 1

Demographic characteristics of adolescents in the study sample, and acne prevalence

Parameter	Participants			*p*
	Total (n = 440)	Belgrade (n = 281)	Užice (n = 159)	
Males, n (%)	69 (15.7)	39 (13.9)	30 (18.9)	
Females, n (%)	371 (84.3)	242 (86.1)	129 (81.1)	n.s.†
Age (year), $\bar{x} \pm SD$	16.48 ± 0.55	16.37 ± 0.51	16.66 ± 0.58	< 0.001†
Acne prevalence, n (%)	228/440 (51.8)	111/281 (39.5)	117/159 (73.6)	< 0.001*
Males, n (%)	41/69 (59.4)	22/39 (56.4)	19/30 (63.3)	n.s.*
Females, n (%)	187/371 (50.4)	89/242 (36.8)	98/129 (76.0)	< 0.001*

***χ^2 test; †*t*-test; $\bar{x}$ – mean value; SD – standard deviation; ns – non significant.**

The majority of the study population (84.3%) were girls, and 15.7% were boys. The total mean age of the pupils was 16.48 ± 0.55 years, with significantly older pupils from Užice ($p < 0.001$). A total of 228 (51.8%) pupils self-reported acne with significantly higher prevalence in Užice than in Belgrade for the entire sample and for the females.

Disease characteristics of the high school pupils who reported acne and answered questions are presented in Table 2.

The difference between two cities was observed in acne duration and problems related to skin appearance. Acne duration less than 1 year had 51.4% of adolescents and 1 year more 48.6% with significantly longer duration among pupils from Belgrade compared to those from Užice. The most of adolescents had problems caused by skin appearance with significantly higher levels of concern among those from Belgrade than those from Užice.

The mean overall CDLQI score in acne self-reported pupils was 3.55 ± 4.14 with the similar quality of life impairment in adolescents from the two cities.

There was a statistically significant positive correlation between the mean overall CDLQI score and CDLQI subscale scores that ranged from 0.401 to 0.841 (Table 3).

The highest correlation with the mean overall CDLQI score was observed for subscales "symptoms and feelings"

Table 2

Disease characteristics of adolescents with acne in Belgrade and Užice

Characteristics	Participants			
	Total (n = 228)	Belgrade (n = 111)	Užice (n = 117)	*p*
Family history of acne, n (%)				
yes	101 (44.7)	53 (48.6)	48 (41.0)	n.s*
no	125 (55.3)	56 (51.4)	69 (59.0)	
Duration of acne, n (%)				
< 1 year	112 (51.4)	46 (43.8)	66 (58.4)	
≥ 1 year	106 (48.6)	59 (56.2)	47 (41.6)	< 0.05*
Problems related to skin appearance, n (%)				
yes	197 (93.8)	95 (99.0)	102 (89.5)	
no	13 (6.2)	1 (1.0)	12 (10.5)	< 0.01*
Therapy for acne, n (%)				
yes	157 (70.1)	80 (74.8)	77 (65,8)	n.s.*
no	67 (29.9)	27 (25.2)	40 (34.2)	
Presence of other skin disease, n (%)				
yes	22 (9.8)	11 (10.3)	11 (9.4)	n.s.*
no	202 (90.2)	96 (89.7)	106 (90.6)	
CDLQI score, $\bar{x} \pm SD$	3.55 ± 4.14	4.00 ± 4.36	3.18 ± 3.94	n.s.†

CDLQI – Children's Dermatology Life Quality Index; ***χ^2 test; †*t*-test;**
$\bar{x}$ – mean value; SD – standard deviation; ns – non significant.

Table 3

Children's Dermatology Life Quality Index (CDLQI) subscale and overall scores

Subscale	$\bar{x} \pm SD$	Min/Max Possible	Min/Max	Subscale total correlation*
Symptoms and feelings (items 1 and 2)	1.35 ± 1.22	0/6	0/6	0.841
Leisure (items 4, 5 and 6)	0.80 ± 1.40	0/9	0/7	0.746
School or holidays (item 7)	0.41 ± 0.77	0/3	0/3	0.616
Personal relationships (items 3 and 8)	0.31 ± 0.78	0/6	0/5	0.548
Sleep (item 9)	0.17 ± 0.53	0/3	0/3	0.401
Treatment (item 10)	0.47 ± 0.73	0/3	0/3	0.673
Overall score (n = 199)	0.36 ± 4.14	0/30	0/24	1.00

***Spearman's rho; $\bar{x}$ – mean value; SD – standard deviation.**

(0.841) and "leisure activities" (0.746) and the lowest correlation was observed for "sleep" (0.401).

The value of the Cronbach's alpha for CDLQI was 0.83.

The vast majority (74.4%) of acne affected adolescents had CDLQI score < 5 as presented in Table 4, and even in 93% of pupils quality of life impairment was mild (CDLQI < 10). Only 3.5% of the pupils had CDLQI score > 15 with the maximum reported CDLQI score 24 (of possible 30) in one affected individual.

Table 4

Children's Dermatology Life Quality Index (CDLQI) scores distribution

CDLQI score	Participants	
	n	(%)
< 5	148	74.4
5–9	37	18.6
10–14	7	3.5
> 15	7	3.5
Total	199	100.0

Discussion

Acne vulgaris is a common, chronic, inflammatory skin condition that affects nearly all adolescents to some degree [8]. Although acne is considered as a trivial skin disorder, it has great impact on psychologically fragile period of adolescence. The interaction of acne and psychosocial issues is complex and can elicit negative emotional reactions including depression, anxiety, helplessness and frustration that can lead to impaired functional status and decreased quality of life [2].

The prevalence of acne in teenagers differs from study to study and ranges from 41.7% to 93.3% [9–14]. It could be explained by methodological differences (such as definition and grading of acne) and population characteristics. In our study 51.8% of the pupils self-reported acne what is in accordance with the findings of Smithard et al. [11] and Rigopulous at al. [9]. The regional difference (Belgrade *vs.* Užice) in acne prevalence, disease duration and levels of concern caused by skin appearance could perhaps be explained by the cultural differences. The appearance of the skin affects the

patients' overall body image [15], and therefore they are prone to embarrassment and social withdrawal, depression, anxiety, anger, and even suicidal ideation [16, 17].

Acne has long been recognized to have a significant effect on the QoL of patients. However, no significant correlation between patients' and dermatologists' assessments of acne severity was observed [18, 19]. Acne patients usually see their disease as more troubling than their physicians do. The use of standardized subjective QoL measures in routine clinical practice in conjunction with clinical assessment can provide additional information on QoL impairments. It is of great importance to recognize depressive symptoms in acne patients, which sometimes may be concealed or masked by aggression or disruptive behavior [20] and to evaluate the psychological impact of acne and its repercussion on QoL.

We used the CDLQI to evaluate the impact of acne on QoL in adolescents. Ten questions encompass different aspects of a child's life that could be affected by their skin disease, including physical symptoms, such as itching and sleep loss, as well as psychosocial questions regarding friendships, bullying, school performance, sports participation, and enjoyment of vacation. The total mean score of the CDLQI in our study was 3.6 which is somewhat lower in comparison with recently conducted Serbian study [14], but twice higher than in Scottish study [10]. Relatively lower scores of CDLQI could possibly be explained by the nature of the population studied (population from the community with a predominantly mild form of acne). In addition, the fact that all the pupils completed questionnaires together in the same classroom with their classmates being able to read their answers, might prevent some of them to express their true emotions.

Although the overall mean score of CDLQI is rather low, our study confirms that acne is associated with impairment in QoL.

Internal consistency reliability (Cronbach's alpha = 0.83) and item-to-total correlation (Spearman's rho = 0.40–0.84), were found to be sufficient, indicating that the Serbian version of CDLQI is a reliable questionnaire with good consistency between the items. It provides information about aspects of acne treatment that could be of great assistance to dermatologists to provide effective clinical intervention which could be crucial for maintenance or restoration of psychological well-being of affected individuals.

The strength of our study was a large number of adolescents surveyed from the general population, thus excluding the possibility of referral bias and overestimation of psychometric morbidity with hospital-based data. Cross sectional type of study, however, may introduce biases associated with self-reporting, such as recall bias, and under or over-reporting of information.

Conclusion

The present study confirmed that acne is associated with impairment in QoL that is in accordance with previous studies performed on teenagers. We did not find statistically significant difference in QoL between pupils from Belgrade and Užice.

Our results also demonstrated that the Serbian version of Children's CDLQI is a reliable and valid measure, a practical tool for measuring the impact of acne on patients' QoL.

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The A-test – reliability of functional recovery assessment during early rehabilitation of patients in an orthopedic ward

A-test – pouzdanost procene funkcionalnog oporavka bolesnika tokom rane rehabilitacije na ortopedskom odeljenju

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Abstract

Background/Aim. There are few tests for evaluation of functional abilities of patients surgically treated for hip fractures or osteoarthritis during early rehabilition period. The aim of this study was to investigate reliability (interobserver reproducibility and internal consistency) of the A-test, an original test for functional recovery evaluation during early rehabilitation of patients in an orthopedic ward. **Methods**. The investigation included 105 patients (55 patients with hip osteoarthritis that underwent arthroplasty and 50 surgically treated patients with hip fracture). It was conducted in an orthopedic ward during early inpatient rehabilitation (from 1st to 5th day). For their functional recovery evaluation during early rehabilitation we used the A-test, a performance-based test with 10 items for assessing basic activities by six level ordinal scale (0–5). For internal consistency of the test the Cronbach coefficient alpha was calculated for the A-test results collected during early rehabilitation for all patients (105 patients x 5 days = 525 measures) and separately for the results of patients with hip osteoarthritis (275 measures) and hip fracture (250 measures). Values of this coefficient > 0.7 imply good internal consistency of the test. Interobserver reproducibility was estimated as follows: two physiotherapists together conducted physical therapy with the patients, and then, separately, rated the performance of each activity from the test (78 measures). The agreement between their estimations was expressed by the linear weighted kappa coefficient (for very good agreement values of kappa coefficeent have to be in the range 0.81–1). **Results.** The Cronbach coefficient alpha was 0.98 (the results of all the patients and the results of the patients with hip osteoarthritis) and 0.97 (the results of the patients with hip fracture). The values of kappa coefficient were in the range 0.81–0.92 for all items. **Conclusion.** The A-test is a reliable instrument for everyday evaluation of functional recovery during early rehabilitation of patients surgically treated in an orthopedic ward.

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

Apstrakt

Uvod/Cilj. Postoji malo testova za procenu funkcionalne osposobljenosti bolesnika hirurški lečenih zbog frakture kuka ili osteoartritisa tokom ranog rehabilitacionog perioda. Cilj ove studije bio je ispitavanje pouzdanosti (interopservacione reproducibilnosti i interne konzistentnosti) A-testa, originalnog testa za procenu funkcionalnog oporavka bolesnika tokom rane rehabilitacije na ortopedskom odeljenju. **Metode.** Istraživanje je obuhvatilo 105 bolesnika (55 sa osteoartritisom kuka kojima je učinjena artroplastika i 50 sa prelomom kuka koji su lečeni operativno) i sprovedeno je na ortopedskom odeljenju tokom rane rehabilitacije (od 1. do 5. dana). Kao merni instrument korišćen je A-test (*performance based* test) sa 10 stavki kojima se procenjuju osnovne aktivnosti uz pomoć šestostepene ordinalne skale (0–5). Za procenu njegove interne konzistentnosti izračunat je Cronbach-ov koeficijent alfa za rezultate prikupljene tokom rane rehabilitacije za sve bolesnike (105 bolesnika × 5 dana = 525 merenja) i posebno za rezultate bolesnika sa operativno lečenom osteoartritisom kuka (275 merenja) i prelomom kuka (250 merenja). Vrednost ovog koeficijenta veća od 0,7 ukazuje na dobru konzistentnost. Interopservaciona reproducibilnost procenjivana je na sledeći način: dva terapeuta zajedno su sprovodili fizikalnu terapiju sa bolesnikom, a zatim su odvojeno ocenjivali izvođenje svake aktivnosti iz testa (78 merenja). Slaganje njihove procene

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bilo je izraženo *kappa* koeficijentom (za veoma dobru interopservacionu reproducibilnost vrednosti *kappa* koeficijenta treba da se nalaze u rasponu 0,81-1). **Rezultati.** Izračunata vrednost Cronbach-ovog koeficijenta alfa za rezultate svih bolesnika, kao i za rezultate bolesnika sa osteoartritisom kuka iznosila je 0,98, a za rezultate bolesnika sa prelomom kuka 0,97. Vrednosti *kappa* koeficijenta za sve stavke bile su u rasponu 0,81–0,92. **Zaključak**. A-test jeste pouzdan test za svakodnevno praćenje funkcionalnog oporavka bolesnika koji su operativno lečeni na ortopedskom odeljenju, tokom ranog perioda rehabilitacije.

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

Introduction

Early rehabilitation period usually lasts only a few days [1], and it is particularly present in an orthopedic ward. In this period patients experience greater or lesser degree of functional disability followed by restoration efforts to return to premorbid activity level. Regardless of the short duration, reliable tests for monitoring and evaluating the functional recovery of patients and presenting the results of work are required in the period of early rehabilitation [2]. However, there are few tests that have been created for the period of early rehabilitation.

The Cumulated Ambulation Score (CAS) with a simple three-level ordinal scale was designed to monitor functional recovery of older people who were surgically treated for hip fractures and in this population it has excellent reliability [3–5]. The University of Iowa Level of Assistance Scale (ILAS) [6, 7] has a complex seven-level scale and demonstrated moderate reliability in a population of patients who underwent arthroplasty for osteoarthritis of the hip and knee. The existing tests are aimed to specific clinical entities and cannot be applied in another domain without any modification. All three tasks from the CAS are too simple for patients who underwent arthroplasty while some tasks from ILAS are too demanding for patients after surgically treated hip fracture. Thus, in an investigation of factors predictive of independence in transfer and ambulation of patients after hip fracture, only 3 from 5 tasks of the ILAS were used [8]. In the absence of a single test that could be easily applied in a heterogeneous population of patients in an orthopedic department we established an original test that could be a solution to this problem. The test was called A-test (simple test and simple name "A" like Assessment or Activity).

We have 10 years of experience with the A-test. We use it in the orthopedic ward to assess the recovery of all patients surgically treated for diseases and injuries of the lower extremities. Unlike the CAS and the ILAS which were designed for estimation of only 3 and 5 activities, respectively, this performance based test consists of 10 items for assessing 9 basic activities and walking endurance by a six-level ordinal scale (0–5). In this way, a more reliable picture of a patient's physical ability is obtained. Total scores can range from 0 to 50 (inability to perform any activity despite the help of therapists until complete independence and safety in performing all activities). We designed the A-test in 2002 and used it initially for monitoring patients of interest for some studies [9, 10]. The study population consisted of patients with arthroplasty for osteoarthritis of the hip in the first study [9]. In the second study, we observed functional recovery in patients surgically treated for hip fracture [10]. Since 2007, the A-test has been used in routine practice of the rehabilitation team in our Orthopedic Ward. This test has proved to be a useful and practical measuring tool in routine and research work, but we did not have a solid evidence that this test is reliable.

Reliability refers to error of an instrument. Reliability is best represented by reproducibility and internal consistency [11]. The aim of this study was to examine the reliability of the A-test through the evaluation of the functional abilities of patients surgically treated for hip fractures and osteoarthritis.

Methods

Subjects

This prospective study was conducted at the Clinic for Orthopedic Surgery and Traumatology (COST), Military Medical Academy, Belgrade, and initially included 120 patients: 60 consecutive patients with acute hip fracture of both sexes who before the injury were able to walk with or without aids and up- and downstairs (help of another person was allowed for this activity; patients with dementia, pathological hip fracture, bilateral hip fractures, concurrent fracture in any other part of the body, and patients to whom surgical treatment were not included), and 60 consecutive patients who underwent hip arthroplasty due to osteoarthritis, without significant mental disability who were able to walk with or without aids before the operation and up- and downstairs (help of another person was allowed for this activity).

Exclusion criteria during the study were the presence of intraoperative or postoperative complications that prevented or delayed the beginning of rehabilitation, lethal outcome immediately after the 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, which involved early mobilization of the patients at the bedside (from the first postoperative day, un-

less their general condition did not allow it) and activities such as getting out of bed (in accordance with the possibilities of the patients), walking with aids on the flat, as well as up- and downstairs, practising 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.

Data on comorbidity and used drugs, mental and functional status before injury for patients with hip fracture or on admission for patients with hip osteoarthritis (walking distance, the ability to walk up- and downstairs, the use of walking aids, carrying out basic and instrumental activities), as well as socio-epidemiological data (marital status, housing conditions) were collected from all the patients. Mental status assessment was made using the Serbian version of shortened mental test score [12], while the functional status before injury was assessed by the New mobility score (NMS) [13].

In the postoperative period, functional abilities assessment of all the patients was performed by the A-test, the ILAS and the CAS from the first day until the fifth day of rehabilitation (this was the day of discharge for the majority of patients).

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

We conducted this research with the approval of the Ethics Committee of our hospital.

Measurement

The A-test is a performance-based test that assesses 10 activities necessary for everyday life that the patient needs to achieve in the first days after the surgeries: (1) turning to side, (2) transition from supine to sitting position, (3) getting out of bed, (4) return back to bed, (5) standing, (6) walking with aids, use of (7) toilet and (8) dining room chairs, (9) walking up- and downstairs, (10) walking endurance.

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

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

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

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, does not take additional time and requires no additional equipment.

Reliability

We examined the reliability or the ability of the instrument to measure something twice or more in the same manner, through assessing internal consistency and reproducibility of the A-test.

Internal consistency

Internal consistency is a measure of how homogenous or consistent items in the scale are and it gives us information to what extent they measure the same thing [10]. As an indicator of internal consistency, we calculated the Cronbach coefficient alpha for the A-test results collected during early rehabilitation for all the patients and separately for the results of the patients with hip osteoarthritis and hip fracture. Cronbach alpha coefficient greater than 0.7 was considered feature of good internal consistency [14].

Reproducibility

There are two forms of reproducibility: interobserver and test-retest [10].

Test-retest reproducibility or intratester reliability indicates the agreement in measurements over time [15]. This approach assumes that there is no substantial change in the construct being measured between the two occasions [10]. In our case, the A-test evaluates the patient's functional status which changes daily during early rehabilitation (usually improves day by day), so we could not examine this form of reproducibility.

Interobserver reproducibility or intertester reliability is the consistency of measurement when the measurement is performed independently by two or more examiners and indicates the agreement of measurements performed by different examiners. It was tested in the following way: two physiotherapists conducted together physical therapy with the patient, and then, separately, rated the performance of each activity from the test. The first physiotherapist in the team had extensive experience in rehabilitation of patients in the COST (29 years of work experience). Also, the first physiotherapist was involved in collecting data in our previous studies. The A-test form was known to this physiotherapist. Another physiotherapist had 12 years of work experience, but until this research did not work in orthopedic rehabilitation team. The second physiotherapist had no experience in completing the A-test form. We did not organize specific training for the use of the A-test for another therapist. We planned to examine the reliability in the whole population of respondents, one day during each patient's rehabilitation (105 measurements).

In order to compare the reproducibility of the A-test with the CAS and the ILAS, we also examined the reliability of the ILAS and the CAS. There is the same item in all three tests which assesses walking, but with different scales. We

considered that the reproducibility of this item, as representative of the test, could be adequately compared with 3 tests by the same methodology.

In addition to assessing the reliability of all tests, physiotherapists were doing their usual job. Thus, we created a situation which corresponds to real everyday work of physiotherapists.

Agreement of the results of each item between the examiners is expressed by kappa coefficient with corresponding 95% confidence interval that was calculated using the linear weighted kappa for ordinal scale [16]. We considered that the discrepancy by one ordinal category was less than the discrepancy by two or more ordinal categories and that was the basis for weighting. Kappa coefficient was evaluated according to widely accepted interpretation by Landis and Koch [17]. The agreement between the examiners is good if the kappa coefficient is between 0.61 and 0.80, very good if the kappa coefficient is between 0.81 and 1.00. Linear weighted kappa was calculated according to the procedure given on the website: http://vassarstats.net/kappa.html.

nologists, 4 patients died in the first days after the 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 beginning of early rehabilitation because of complications occurred in other patients like: 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 for early rehabilitation.

Analyzing all the A-test results collected from the first to the fifth day of rehabilitation, Cronbach alpha coefficient was

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)]
Age (years)	65 ± 12; 53 (32–85)	75 ± 10; 76 (47–89)
Female	32 (58%)	37 (74%)
Number of comorbid diseases	1 ± 1; 1 (0–4)	2 ± 1; 2 (0–4)
Number of used drugs	2 ± 2; 2 (0–8)	3 ± 2; 3 (0–9)
Shortened mental test score (Serbian version)	10 ± 0; 10 (10–10)	9.84 ± 0.51; 10 (8–10)
New Mobility Score	7 ± 2; 6 (2–9)	7 ± 2; 9 (1–9)
Limited walking distance	41 (74.5%)	26 (52%)
Aids when walking	28 (51%)	16 (32%)
Up and down stairs with difficulty:	51 (93%)	32 (64%)
Lives in the flat without elevator	18 (33%)	14 (28%)
Lives alone	7 (13%)	10 (20%)
Hospital stay (day)	7.44 ± 1.08, 7 (7–12)	8.52 ± 3.40, 7 (7–24)
Rehabilitation (day)	5.25 ± 0.78, 5 (5–10)	6.20 ± 2.28, 5 (5–16)
5 days of rehabilitation	46 (84%)	33 (66%)

Results

Out of a total 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 due to poor operative stabilization of the fracture and orthopedic surgeon recommendations to rest after surgery, 2 patients with hip fracture due to cardiac disorders and recommendations of cardiologists to delay mobilization, 3 patients (2 with hip fracture and one with osteoarthritis) because of debilitating diarrhea, severe electrolyte imbalances and extreme hypotension, so the 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 pulmo-

0.98, indicating excellent internal consistency (Table 2). Table 2 presents the results of correlation between all items, as well as between each item and total score. A strong correlation exists between all the items and total score, and the removal of any of the items does not contribute to increasing alpha.

A similar result was obtained when the A-test was used for observation of the patients with hip fracture (n of cases = 250, n of variables = 10, alpha = 0.97) and the patients with osteoarthritis of the hip (n of cases = 275, n of variables = 10, alpha = 0.98).

We planned 105 measurements to test the interobserver reproducibility (one measurement for each patient during rehabilitation), but due to unplanned absence of the second examiner, 78 measurements were done. The agreement between examiners for each of the A-test items is shown in Table 3. The kappa coefficient was 0.81 and

Table 2

The A-test internal consistency

Reliability analysis – scale (alpha); n of cases = 525, n of variables = 10					
Interitem correlations					
Mean	Minimum	Maximum	Range	Max/Min	Variance
0.83	0.61	0.99	0.37	1.61	0.01
Item-total statistics					
Items	Scale mean if item deleted	Scale variance if item deleted	Corrected item-total correlation	Squared multiple correlation	Alpha if item deleted
From supine to side lying	17.30	193.56	0.88	0.82	0.98
From supine to sitting	17.26	194.30	0.94	0.91	0.98
From sitting to standing	17.54	188.25	0.96	0.98	0.97
Standing	17.51	188.07	0.95	0.96	0.97
Back to bed	17.61	188.08	0.96	0.98	0.97
Walking	17.75	188.19	0.95	0.94	0.97
Use of toilet	18.73	188.93	0.85	0.85	0.98
Sit on and get up a chair	18.39	187.31	0.92	0.89	0.98
Up and down stairs	19.36	208.15	0.72	0.63	0.98
Walking endurance	18.34	203.12	0.89	0.83	0.98
Alpha = 0.98			Standardized item alpha = 0.98		

Table 3

Reproducibility of the A-test's 10 items

Items	Observed kappa	Std. error	95% confidence interval	
			lower limit	upper limit
From supine to side lying	0.86	0.04	0.79	0.94
From supine to sitting	0.81	0.04	0.73	0.90
From sitting to standing	0.89	0.03	0.84	0.94
Standing	0.88	0.03	0.81	0.94
Back to bed	0.87	0.03	0.81	0.93
Walking	0.86	0.03	0.81	0.93
Use of toilet	0.91	0.03	0.85	0.97
Sit on and get up a chair	0.84	0.04	0.77	0.92
Up and down stairs	0.92	0.04	0.84	1.00
Walking endurance	0.85	0.04	0.78	0.92

higher for all items, indicating very good inter-observer reproducibility.

For the item that assesses walking, kappa coefficient was very high and almost equalized in all three tests (Table 4).

strongly correlated with the total score, it is evident that the correlation magnitude of the item that estimates walking up- and downstairs with a total score is slightly lower than the others. This is the most difficult activity in early rehabilitation program. A large percentage of patients live in an apartment with no elevator so this activity becomes an important criterion of whether a patient can be discharged home from the hospital. Therefore, it is important that estimation of walking up- and downstairs is an integral part of the A-test. However, the analysis shows that, by removing this item, alpha does not increase.

Table 4

Reproducibility of items that assess walking with three different scales of the A-test, the University of Iowa Level of Assistance Scale (ILAS) and the Cumulated Ambulation Score(CAS)

Tests (score range)	Observed kappa	Std. error	95% confidence interval	
			lower limit	upper limit
1. A-test (0–5)	0.86	0.03	0.81	0.93
2. ILAS (0–6)	0.86	0.03	0.80	0.91
3. CAS (0–2)	0.88	0.04	0.79	0.97

Discussion

This study investigated the reliability of the A-test in the assessment of functional recovery of patients treated surgically due to hip fracture and osteoarthritis in an orthopedic department. Internal consistency analysis is an integral part of estimating the test reliability [11, 18, 19]. According to the Cronbach alpha values, the A-test has excellent internal consistency (alpha = 0.98). Although all the A-test items

Kappa coefficient is an appropriate measure of reliability for data from an ordinal scale [16]. In our study, kappa coeffi-

cients were greater than 0.81 for all items. We expected high reliability due to the results of the study that was conducted in 2003 when we also examined one aspect of reliability (interobserver reproducibility) of the A-test. Then we calculated the correlation between the results of 80 measurements that were performed by two therapists in a population of patients with hip arthroplasty for osteoarthritis. Based on the obtained values of the correlation coefficient (r = 0.99), we concluded that the A-test had good interobserver reliability.

The results of this study were presented at the 14th European Congress of Physical and Rehabilitation Medicine (Vienna, 2004) but only in the form of abstract [20]. However, a disadvantage of this study is that we used the Pearson's correlation coefficient for statistical analysis and presentation of the results. In addition, we did not examine another form of reliability – internal consistency.

Now we find that interobserver reproducibility of the A-test is very good (kappa coefficient was 0.81–0.92). By interpretation of Landis and Koch, the A-test is found in the same gradation of reproducibility as the CAS, which has the simplest scale and strong evidence of highly reliable test for recovery assessment of elderly patients with hip fracture [5]. In their intertester reliability study of the CAS, kappa coefficient was very high for all three items (0.92–0.97) [5]. On the other hand, the results of reliability for the A-test in this study seem to have the advantage over moderate interobserver reliability of the ILAS which was demonstrated in patients after hip and knee arthroplasty (0.48–0.78) [7]. The scale of the A-test is simpler than the scale of the ILAS and slightly more complex than the scale of the CAS which could partly explain the differences in reliability obtained in these three studies. However, it is known that Cohen's kappa, weighted for ordinal data does not allow comparability between studies and scales [21]. That is why we have selected an item that assessed walking. This activity is assessed in all three tests, but with different scales. Interestingly, the agreement between the two examiners was not much affected by the complexity of the scale and kappa coefficient was almost the same for all three tests.

There are methodological limitations in this paper that we emphasize on this occasion. In analyzing the data, we calculated Cronbach's alpha. Due to the nature of the data obtained by the A-test measuring, this is not the most appropriate statistical method [21]. However, this indicator of reliability is requested in estimates used to determine whether a test was examined adequately from all aspects [11, 18, 19]. This was why we showed it in this paper. Also, in examining interobserver reliability we limited our study to the assessment of only one pair of examiners. We were not able to avoid this limitation of the study in the situation when the research was adjusted with the possibilities of routine work in the department. However, the results showed that estimates agreement of an experienced therapist and other therapists with no special preparation for the use of the A-test was very good. This situation corresponds to a real everyday practice for which we recommend the A-test.

Patients with various injuries and diseases of the lower extremities are treated surgically in an orthopedic ward of a general hospital. But, after surgery, they have a similar form of physical disability by International classification of functioning, disability and health (ICF). Therefore, there is a need for a single test that would facilitate functional recovery monitoring of patients in an orthopedic ward. Two large groups of patients with surgical treatment distinguish in the heterogeneous population of patients in our Orthopedic Department: patients with hip fracture and patients with hip osteoarthritis. We chose this mixed study population to test reliability of the A-test because we wanted to show that the A-test could be a reliable tool in this situation. Instead of using separate tests for different clinical entities, one can use a single test that is reliable in both cases.

Each test should be evaluated from several aspects [2] and we are preparing the results of validity, diagnostic test accuracy and practical applicability of the A-test in this same study population. However, we believe that future research should focus on other clinical entities present in an orthopedic ward. Moreover, the recovery of every patient who experienced sudden functional disability due to illness or injury could be monitored by the A-test during early rehabilitation. This year, we started to use the A-test outside the orthopedic department. Future research could be focused on the usefulness of this test in early rehabilitation of patients in departments of neurology, neurosurgery, cardiology and cardiac surgery, plastic and vascular surgery.

Conclusion

This study showed that the A-test could be a reliable instrument for monitoring functional recovery of patients surgically treated for hip fractures and osteoarthritis of the hip during early rehabilitation in an orthopedic ward.

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Immunolocalization of different neuropeptides in human interthalamic adhaesion indicates its functionality

Imunolokalizacija različitih neuropeptida u intertalamičkoj adheziji čoveka ukazuje na njenu funkcionalnost

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Abstract

Background/Aim. The interthalamic adhaesion (IA), gray matter connecting both thalami, is absent in about a quarter of human brains. Controversies are present about the nature and functional significance of the human IA. **Methods.** In six adult human brains we investigated the expression of different neuropeptides: somatosatin (SOM), neuropeptide Y (NPY), ghrelin, neurotensin (NT), adrenocorticotropic hormone (ACTH), substance P (SP) and L-enkephalin (L-Enk) in neurons and/or neuropil of the IA, using immunohistochemistry (streptavidin-biotin technique). **Results.** In neurons, as well as in fibers, we found immunoreactivity for ghrelin, SOM, L-Enk and NT. However, reactivity for NPY, SP and ACTH was present only in fibers within the IA. Fusiform neurons were immunoreactive for SOM, Ghrelin, L-Enk, and NT, neurons with oval perikaryon for SOM, and L-Enk, triangular neurons showed immunoreactivity mainly for NT and multipolar neurons for NT and L-Enk. **Conclusion.** These findings can contribute to the understanding of the function of interthalamic adhaesion, and to resolving the question whether it is a vestigial structure. No mather if the interthalamic adhaesion is vestigial structure or not, its presence or absence could be a marker for other, genetic or functional differences between human brains. Our findings indicate the presence of certain neuronal organization in the human interthalamic adhaesion which could have functional significance, and do not support its vestigial nature.

Key words:
brain; humans; thalamus; neuropeptides; immunohistochemistry.

Apstrakt

Uvod/Cilj. *Adhaesio interthalamica* (AI), siva masa koja povezuje oba talamusa, odsutna je kod oko jedne četvrtine ljudi. Postoje različita mišljenja o prirodi i funkcionalnom značaju AI čoveka. **Metode.** Na šest mozgova odraslih osoba imunohistohemijskim metodama (tehnika streptavidin-biotin) istraživali smo prisustvo različitih neuropeptida: somatosatin (SOM), neuropeptid Y (NPY), grelin, neurotenzin (NT), adrenokortikotropni hormon (ACTH), supstanca P (SP) i L-Enkefalin (L-Enk) u neuronima i/ili neuropilu AI. **Rezultati.** Našli smo pozitivnu imunoreaktivnost na grelin, SOM, L-Enk i NT u neuronima i vlaknima. Reaktivnost na NPY, SP i ACTH bila je prisutna samo u vlaknima unutar AI. Fuziformni neuroni su bili imunoreaktivni na SOM, grelin, L-Enk i NT, neuroni sa ovalnim perikarionom na SOM i L-Enk, triangularni neuroni su, najvećim delom, bili imunoreaktivni na NT, dok su multipolarni neuroni bili imunoreaktivni na NT i L-Enk. **Zaključak.** Dobijeni nalazi mogu da doprinesu razumevanju funkcije *adhaesio interthalamica* čoveka, kao i razmatranju pitanja da li je *adhaesio interthalamica* vestigijalna struktura ili ne. Nezavisno od toga da li je *adhaesio interthalamica* vestigijalna struktura ili ne, njeno prisustvo ili odsustvo može, takođe, biti i marker za druge, genetske ili funkcionalne razlike između ljudskih mozgova. Rezultati ukazuju na postojanje određene neuronske organizacije u *adhaesio interthalamica* čoveka koja bi mogla imati funkcionalni značaj, i ne govore u prilog njene vestigijalne prirode.

Ključne reči:
mozak; ljudi; talamus; neuropeptidi; imunohistohemija.

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Introduction

Interthalamic adhesion (massa intermedia; middle commissure; gray commissure), as a specific part of human diencephalon connects the medial surfaces of the left and right thalamus within the third ventricle. Specific is the fact that the human interthalamic adhesion (IA) is variable in the presence (absent in 22–30% of subjects), localization, size, and density, as well as in the number of its neurons [1]. Males without the IA die earlier than males with it, but such finding has not been reported for females [2]. Male neurological patients with the IA have relatively lower nonverbal factor scores [3]. Identification of nuclei within the human IA based on their homology to mammalian thalamic midline nuclei is not clearly established, so that the controversial and non-uniform terminology complicates the studies of IA. Various midline nuclei in the IA (paratenial, paraventricular, reuniens, rhomboidal, median central and intermediodorsal nucleus) [4, 5], are actually very small and difficult to demarcate in humans [2, 6–9]. Human IA contains only nucleus reuniens and should not be unconditionally compared with the IA of animals and can thus be considered as a specific finding [2, 8]. Within the IA there are four types of neurons on Golgi sections: fusiform (most characteristic for human IA), oval, triangular, and multipolar [10]. In addition, the presence or absence of the IA in humans can be an indicator for some other genetic and/or functional differences between the persons with and without the IA. For example, the absence of the IA in schizophrenia could be a marker of developmental abnormalities in the neural network including the thalamus and connected amygdala regions [11].

The aim of this study was to investigate the pattern of the expression of different neuropeptides in the human IA, in order to contribute to better understanding of this structure in the human brain.

Methods

In this study 6 human brains with the IA (4 males and 2 females; age 45 to 65 years), and without any visible pathological changes or neuropsychiatric history were examined. The brains were obtained during the routine autopsies at the Institute of Pathology of Faculty of Medicine in Belgrade with postmortem intervals from 4 to 7 hours. The tissue blocks of thalamus containing the IA with adjacent paraventricular regions were dissected and fixed by immersion (10% formalin solution in isotonic phosphate buffer) during 3 weeks, dehydrated, and embedded in paraffin. Frontal serial sections (4 mm thick) for immunohistochemistry were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol. Afterwards, the slides were immersed in citrate buffer (pH 6.0) (Target Retrieval Solution, ready-to-use; DAKO) and heated for 21 min in a microwave oven at 680 w (except for neurotensin and L-Enk). After cooling, slides were rinsed in distilled water and treated with 3% H_2O_2 in distiled water for 10 min to reduce endogenous peroxidase activity. Immunostaining was performed by incubating tissue sections with appropriate sera for 60 min at room temperature in a humid chamber or overnight at +4°C, using the streptavidin-biotin technique (LSAB+ Kit, Peroxidase Labeling, K0690, DAKO Cytomation, Denmark). The list of the primary antibodies is shown in Table 1.

Table 1

Antibodies used in the present study

Antibody	Monoclonal (Mo)/Polyclonal (Po) Antigen unmasking technique	Manufacturer Code No. or received from	Dilution	Detection system
Mo mouse anti-hu Adrenocorticotropin (ACTH), Clone 02A3	Mo Not recommended	DAKO Cytomation Denmark M3501	1:50–1:75	LSAB
Po goat anti-Ghrelin	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	Santa Cruz Biotechnology, INC Sc-10368	1:100	LSAB+
Po rabbit anti-hu Substance P, 4-11	Po Not recommended	MP Biomedicals, USA 11845	1:400–1:800	LSAB+
Po rabbit anti-hu Somatostatin	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	DAKO A/S Denmark A 0566	1:200–1:300	LSAB+
Po rabbit anti-hu neuropeptid-y (NPY)	Po Microwave-20min, 0.01M citrate retrieval solution pH6.0 or DAKO cytomation target retrieval solution No. S1700	Euro-Diagnostica B 48-1	1:400–1:800	LSAB+
Neurotenzin (NT)		R. L. Eskay, Bethesda, MD, USA	1: 15 000	
L-enkephalin		R. L. Eskay, Bethesda, MD, USA	1:10 000	

After washing in 0.01M phosphate buffered saline (PBS, pH 7.2) specimens were incubated with biotinylated anti-mouse, anti-rabbit and anti-goat immunoglobulins for 30 min at room temperature in a humid chamber, and subsequently incubated with peroxidase-conjugated streptavidin–biotin for another 30 min. After incubation, the sections were rinsed in 0.01M PBS. Antigen-antibody complexes were visualized with 3-amino-9-ethylcarbasole (AEC, No. K3469, DAKO Cytomation, Denmark) or diaminobenzidine hydrochloride (DAB, No. K3468, DAKO Cytomation, Denmark) substrate solution and afterwards washed in distillated water. The cell nuclei were contrastained with Mayer's haematoxylin. Control stainings included omission of the primary antisera and replacement of the primary antibody by non-immune serum diluted 1:10 and by the diluent alone.

Immunoreactive neurons and fibers were studied and photographed on the light microscope (Olympus) under different magnifications.

Results

Both, immunoreactive fibers and neurons were found for NT, ghrelin, L-Enk and SOM. However, immunoreactivity to SP, NPY and ACTH was found only in fibers, and not in neurons of human IA.

Neurons in the human IA immunoreactive for SOM, NT, ghrelin, and L-Enk

Ghrelin immunoreactivity (IR) was found in medium sized oval and fusiform neurons, but IR granules, accumulated in one part of soma opposite to the nucleus were sparse (Figure 1 A). Ghrelin IR fibers were found in all cases.

Somatostatin IR of ependyma was intense, while there was no reaction in the subependimal region. SOM IR neurons were numerous and large, and their bodies were fusiform or oval (Figure 1 B and C). Somatostatin IR fibers were closely related to non-reactive neurons.

L-Enk IR neurons were very rare and of fusiform, oval and multipolar shape, with some reactivity present also in their dendrites (Figure 1 D). In human IA also L-Enk imunoreactive fibers were present.

NT IR neurons were grouped (Figure 1 E and F), but not all neurons in such groups were immunoreactive. The majority of NT IR neurons were triangular, but NT IR was found also in fusiform neurons. Varicose NT fibers were related to other non-reactive neurons (Figure 1G).

Fiber networks in the IA immunoreactive for NPY, SP and ACTH

ACTH IR fibers were rare, branched and often cut in their course directed supraependymaly. In IA they were located around cell bodies and ramified around the neurons (Figure 2A).

SP – long varicose fibers were commonly located around non-reactive neurons (mainly around fusiform neurons) (Figure 2B).

NPY – varicose fibers were relatively rare and when present they were not distant from each other (Figure 2C).

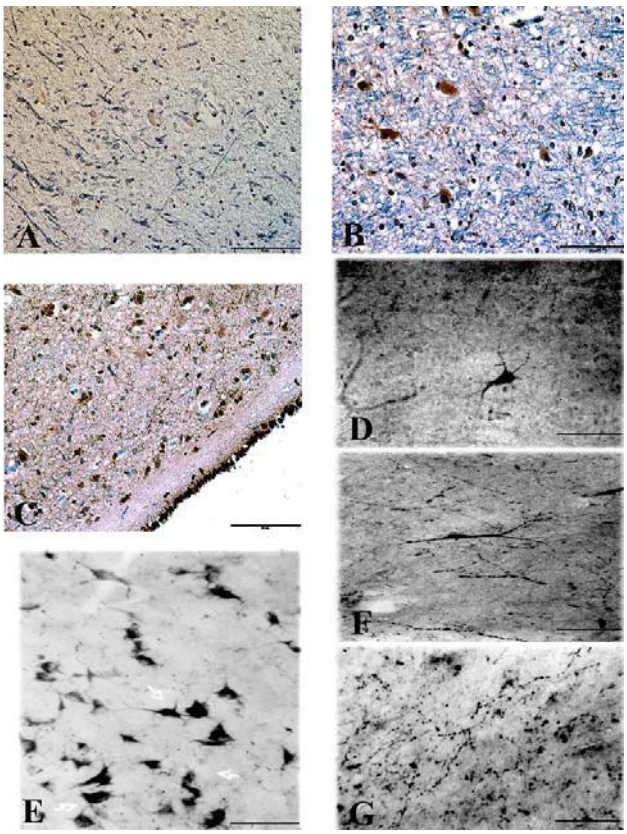

Fig. 1 – Human interthalamic adhesion, frontal section: immunoreactivity both in neurons and fibers.
A – ghrelin; B and C – somatostatin; D and F – L-enkephalin; E – Neurotensin (NT) positive neurons in groups, and G – NT positive fibers. (Scale bars in A and C = 50 μm; B = 40 μm; in D = 100 μm; E and = 100 μm; G = 20 μm).

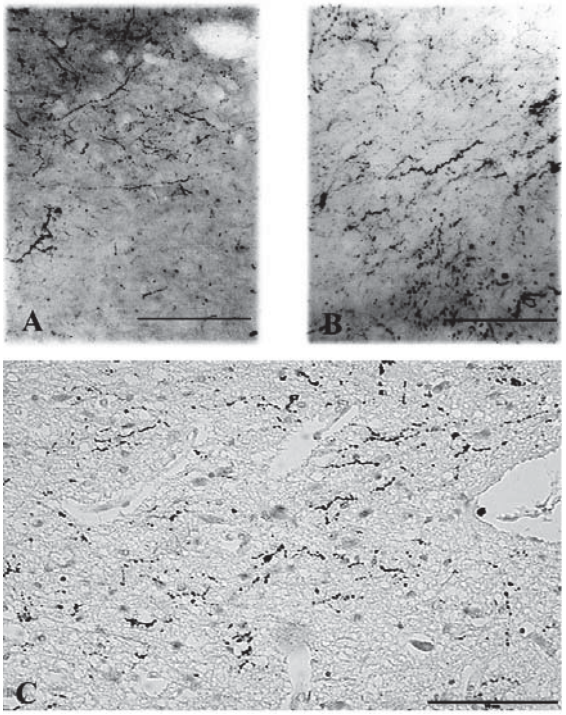

Fig. 2 – Human interthalamic adhesion, frontal section.
Immunoreactivity for adrenocorticotropic hormone (ACTH), substance P (SP) and neuropeptide Y (NPY) is present exclusively in fibers. A – ACTH; B – SP; C – NPY. (Scale bars in A and B= 50 μm; C= 100 μm).

Specific IR and different neuronal types in the human IA

Considering potential neuronal forms representing specific neuropeptide IR, we can conclude that fusiform neurons showed IR for SOM, ghrelin, L-Enk, and NT, neurons with oval perikaryon for SOM and L-Enk. Grouped triangular neurons showed IR mainly for NT, and multipolar neurons for NT and L- Enk (Figure 1).

Discussion

Comparing to other mammals, the human IA is specific in its considerably smaller size, and in its variability, including its absence in a considerable percent of cases [12]. Contrary to the human brain, IA in diversity of mammalian species is generally of considerable size and leaves only smaller and narrow space of the third ventricle [4, 13, 14]. The question is whether IA in humans, with its neurons and fibers, is simply vestigial structure or not. The midline nuclei, variously defined and designated by different investigators, include the diverse *nn. reunientes*, and seem particularly related to the periventricular fiber system, as well as to other intrinsic diencephalic connections. While some authors interpret these grisea as phylogenetically "very ancient", the mammalian *nn. reunientes* may also be interpreted as manifestations of "progressive differentiation" [12]. Even if IA is a vestigial structure, its significance for the human brain cannot be necessarily ascribed exclusively to its specific function, but its presence or absence can somehow indicate different genetics, development or different modes of human brain functioning. However, it should not be neglected that the comparative small size of the IA structure, is not the exclusive indicator of its function, and that even small structures in the brain can have important functions [15].

For a long time the midline thalamic nuclei were considered nonspecific, but their designation as a part of "diffuse", "nonspecific", "generalized" or "commissural" systems, institutes misleading simplifications [16]. Dense nerve cell and/or neuropil immunoreactivity of human midline nuclei characterized by calbindin-D–28K, and calretinin indicate their limbic connections [17]. Executive deficits in humans may arise from combined lesion of several structures, including midline nuclei and in monkeys IA is involved in motor functions [18, 19]. Anatomical relationships, combined with functional studies in animals and in humans, lead to propose that the midline, and intralaminar nuclei of the thalamus, as a whole, play a role in awareness, with each of the groups having a role in a different aspect of awareness [5].

In previous studies [20] we found the differences in modalities of functioning of human intelligence in persons with and without IA. In subjects with the IA, the complex simultaneous processing (the ability of spatial visualization in particular, which means the capacity for 3D mental manipulation of objects) is more developed. On the other hand, the simpler perceptive processing (which includes perceptive search, identification, visual attention and 2D object manipulation) is more developed in subjects without IA [20]. The presence of neuropeptides reported here in specific circular formations of IA neurons, together with the uncertain vestigial nature of IA in human, suggest the necessity for further comparative studies.

During this study we were not able to clearly delineate any of nuclei on frontal sections of the human IA what corresponds to the statement of Gottschick [21]. Within the human IA, in addition to four types of neurons, five fiber systems were found: from paramedian, dorsomedial, and ventral posterolateral nucleus of thalamus, from nucleus centromedianus, intralaminar, and neighboring nuclei [10, 22]. Very high densities of histamine immunoreactive fibbers mostly oriented sagittally were found in the human thalamus midline nuclei [23]. It cannot be excluded that the circular formations that we previously described in the human IA represent a kind of "bed nucleus" for some of fiber tracts within the human IA [24]. The modulatory neuropeptides as modulatory transmitters are released from both synaptic terminals and axonal varicosities, providing not only 'point to point' contact (pre- to postsynaptic), but also extending integrative potential, 'volume control', which could be considerably different in human brains with and without IA [25].

Conclusion

Contributing to the elucidation of the function of the human interthalamic adhaesion, we can conclude that even if interthalamic adhaesion is a vestigial structure in humans, our previous and current results do not exclude a certain degree of neuroanatomical organization within the human interthalamic adhaesion.

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Manufacturing and use of human placenta-derived mesenchymal stromal cells for phase I clinical trials: Establishment and evaluation of a protocol

Proizvodnja i upotreba humanih mezenhimskih stromalnih ćelija izolovanih iz placente za klinička istraživanja prve faze: uspostavljanje i procena protokola

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Abstract

Background/Aim. Mesenchymal stromal cells (MSCs) have been utilised in many clinical trials as an experimental treatment in numerous clinical settings. Bone marrow remains the traditional source tissue for MSCs but is relatively hard to access in large volumes. Alternatively, MSCs may be derived from other tissues including the placenta and adipose tissue. In an initial study no obvious differences in parameters such as cell surface phenotype, chemokine receptor display, mesodermal differentiation capacity or immunosuppressive ability, were detected when we compared human marrow derived-MSCs to human placenta-derived MSCs. The aim of this study was to establish and evaluate a protocol and related processes for preparation placenta-derived MSCs for early phase clinical trials. **Methods.** A full-term placenta was taken after delivery of the baby as a source of MSCs. Isolation, seeding, incubation, cryopreservation of human placenta-derived MSCs and used production release criteria were in accordance with the complex regulatory requirements applicable to Code of Good Manufacturing Practice manufacturing of *ex vivo* expanded cells. **Results.** We established and evaluated instructions for MSCs preparation protocol and gave an overview of the three clinical areas application. In the first trial, MSCs were co-transplanted *iv* to patient receiving an allogeneic cord blood transplant as therapy for treatment-refractory acute myeloid leukemia. In the second trial, MSCs were administered *iv* in the treatment of idiopathic pulmonary fibrosis and without serious adverse effects. In the third trial, MSCs were injected directly into the site of tendon damage using ultrasound guidance in the treatment of chronic refractory tendinopathy. **Conclusion.** Clinical trials using both allogeneic and autologous cells demonstrated MSCs to be safe. A described protocol for human placenta-derived MSCs is appropriate for use in a clinical setting, relatively inexpensive and can be relatively easily adjusted to a different set of regulatory requirements, as applicable to early phase clinical trials.

Key words:
stromal cells; therapeutics; clinical protocols; clinical medicine.

Apstrakt

Uvod/Cilj. Mezenhimske matične (stromalne) ćelije (MSCs) trenutno se koriste u velikom broju kliničkih istraživanja za različite kliničke indikacije. Iako je koštana srž uobičajeni izvor početnog materijala za kultivaciju ovih ćelija, količina ćelija koja se pri tome dobija i dalje predstavlja ograničavajući faktor. Alternativno, MSCs sve više se izoluju iz drugih tkiva kao što su placenta novorođenih beba ili masno tkivo. U inicijalnom istraživanju nije otkrivena nikakva razlika u osnovnim fenotipskim karakteristikama ćelijskih receptora, hemokinskih receptora ili sposobnosti ćelija za normalnu mezodermsku diferencijaciju između MSCs izolovanih iz placente i koštane srži. Cilj ovog rada bio je uspostavljanje i procena protokola za kultivaciju i odabir MSCs izolovanih iz placente i adekvatno pripremljenih za upotrebu u kliničkim istraživanjima prve faze. **Metode.** U ovoj studiji korišćena je placenta beba rođenih carskim rezom nakon normalne trudnoće. Izolacija, zasejavanje, inkubacija, krioprezervacija i kriterijumi za proizvodnju humanih MSCs bili su u skladu sa složenim regulatornim principima koji se u ovom trenutku primenjuju u Australiji. **Rezultati.** Uspostavljen je i procenjen detaljan protokol za pripremu MSCs i dat je pregled njihove upotrebe u tri različite kliničke studije. U prvoj studiji MSCs su date *iv* putem, pre alogene transplantacije matičnih ćelija krvi, u lečenju akutne mijeloidne

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leukemije refraktorne na terapiju. U drugoj studiji, MSCs su date *iv* u lečenju idiopatske fibroze pluća, bez ozbiljnih neželjenih efekata. U trećoj studiji, MSCs su injektovane direktno u mesto oštećenja tetive pod kontrolom ultrazvuka, u lečenju hronične refraktorne tendinopatije. **Zaključak.** Kliničke studije, bazirane na primeni ćelija alogenog i autologog porekla, demonstrirale su bezbednu upotrebu MSCs. Prikazani protokol je pogodan za rane faze kliničkih istraživanja, relativno je pristupačan i može se lako prilagoditi različitim kliničkim uslovima i zakonskim regulativama.

Ključne reči:
ćelije, stromalne; lečenje; protokoli, klinički; medicina, klinička.

Introduction

Although stem cells and their differentiated progeny offer great promise for treatment of many congenital and acquired human diseases, the optimal type and source remain unclear [1, 2]. Mesenchymal stromal cells (MSCs), derived from rare mesenchymal stem cells, are fibroblastoid cells that are present in the bone marrow and virtually all other tissues of the body and which can be readily isolated and expanded *ex vivo* [3–10]. MSCs are able to differentiate *in vitro* into cells of the mesodermal lineage such as osteoblasts, chondrocytes and adipocytes, but their ability to differentiate to cells outside the mesodermal lineage such as hepatocytes, endothelial cells and neuronal cells is controversial [1, 2, 6]. MSCs are also able to modulate the activity of cells of the immune system. Preclinical studies to date have provided a significant amount of information on MSCs indicating that they can deliver therapeutic proteins in a paracrine fashion or participate in the repair of defects by mesodermal differentiation [3–10]. The same studies emphasize the potential of MSCs *in vivo* and their capacity to home to sites of tissue injury and inflammation. MSCs have the potential for cell banking [5, 11]. With no need to match a donor and intended recipients for the antigens of the major histocompatibility complex (MHC), MSCs from a single manufacturing campaign can be utilised in numerous clinical trials and for a number of patients [5, 11]. However, MSCs manufacture is a highly specialized, rather complex, time consuming and labour-intensive exercise [11–13]. The placenta is a natural "waste product" at birth and can provide a practically unlimited supply of donor tissue [6, 11, 12]. Human placenta-derived MSCs (also referred to as hpMSCs, in further text MSCs) need to be isolated, cultured and cryopreserved according to stringent regulatory requirements [11, 12] including the current Code of Good Manufacturing Practice (cGMP) standard or its close equivalent [11, 12]. The manufacturing process includes collection of source tissue (placenta), isolation of MSCs, and their subsequent processing, storage and transport [2, 5, 11, 12, 14]. A set of policies and procedures is required to support the manufacturing process; this process needs to be based on the Quality Management System (QMS) principles, the International Organization for Standardization (ISO) standard and other complex regulatory requirements [13–17]. MSC from various sources have been used extensively in different disease models [18–20] and continue to be investigated for safety, efficacy and potency [21–24], their lifespan [25] as well as manufacturing protocol variations, improvements and adjustments [26–28].

The primary aim of this paper was to establish a protocol for manufacturing/ preparation of clinical-grade human placenta-derived MSCs that could be cell banked and used for various clinical applications on an "as needed" basis. Additionally, we aimed to evaluate the protocol and its feasibility in a clinical trial programme, in the areas of hematology (co-transplantation of human MSCs and allogeneic cord blood cells as therapy for treatment-refractory hematological malignancies), pulmonary medicine (intravenous administration of MSCs for idiopathic pulmonary fibrosis), and in a musculoskeletal application (local administration of MSCs for chronic refractory tendinopathies). The key parameters for evaluation of the protocol were: 1) manufacturing considerations of MSCs for use in clinical settings; 2) regulatory considerations related to MSCs manufacturing (*eg* could the protocol be relatively easily adjusted to comply with a different set of regulatory requirements as mandated for applications in early phase clinical trials in Australia or other comparable sets of regulations), and 3) safety, accessibility and usability of human placenta-derived MSC.

Methods

The material used for cell isolation, culture and cryopreservation is presented in Table 1. The placenta was collected from a healthy mother (the donor) during a routine term elective Caesarean section birth [5, 6, 11]. A full informed consent was attained several weeks prior to the delivery [5, 6, 11]. The donor selection guidelines of both the Australian Red Cross Blood Services and the Australian Cord Blood Registry (AusCord) were fulfilled [11, 14]. A complex set of quality assurance system policies was applied to each step of the collection, processing, storage and transport of MSCs [5, 6, 11].

The placenta (Figure 1) was collected and double-bagged aseptically in the operating suite and transferred in a cool box to the processing laboratory [5, 6, 11]. The external membranes and the umbilical cord were removed from the placenta in a biosafety flow-cabinet using an aseptic technique. The placental tissue was divided into smaller pieces (up to 10 g each) [5, 6, 11]. The small pieces of placental tissue were washed with 500 mL/100 g of Hanks Balanced Saline Solution (HBSS, Invitrogen) [6] and this process was repeated with the same volumes of HBSS for several times.

Additional details on MSCs isolation, seeding, incubation and cryopreservation are described in Table 2. Each passage consisting of 90 tissue culture flasks (T175) yielded between 4×10^8 and 1×10^9 cells. At the end of each passage (P2 to P5), cells were reseeded and also cryopreserved for clinical trial use and further testing [6]. The MSCs intended for cryopreservation were washed with 50 mL HBSS twice, centrifuged and the pellet was resuspended in 65% Plasma-

Table 1

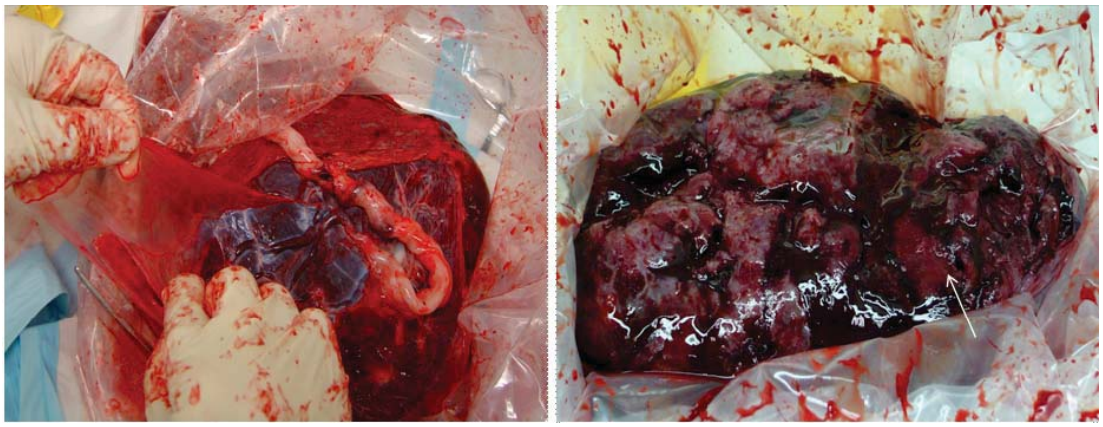
Materials used for human placenta-derived mesenchymal stromal cells isolation, culture and cryopreservation [5, 6, 11].

Equipment and solutions	Media and reagents (1)	Media and reagents (2)
- On-demand label printer (Birch) - Weighing balance (Sartorius) - Heat Sealer CRG (Ljungberg and Kogel AB) - Bench Centrifuge (Sigma) - Cryocyte Freezing bags, 50 mL (Baxter) - Controlled-rate freezer (Planar) - Sterile surgical gowns, face masks, over-shoes and hair nets (Kimberley-Clark) - 0.2 µm sterile filter (Becton Dickinson) - Incubated Orbital Shaker (Bioline) - 70 µm cell strainer (BD Pharmingen) - Centrifuge tubes, 50 mL, conical bottom (Nunc) - FACS tubes (BD Pharmingen) - Syringes (60 mL) (Terumo) - Mixing canula (Unomedical) - Seriological pipettes; 5, 10 and 25 mL (Sarstedt) and motorized pipetter. - Tissue culture flasks (175 cm^2 and 25 cm^2) (Nunc) - Sterile 70% ethanol (Pharmacia) - Sterile water (Baxter)	- Hanks Balanced Salt Solution (HBSS) (Invitrogen) - TrypLE Select (recombinant trypsin-like substitute) (Invitrogen). Dulbecco's Modified Eagle Medium, low glucose 1g/100 mL (DMEM-LG, Invitro gen) - Trypan Blue (Invitro gen) - Gentamicin, 40 mg/mL (Pharmacia) - Ficoll-Paque Premium (GE Healthcare) - Fetal calf serum, Australian sourced (Invitrogen) - Digest Media: 50 mL Collagenase I stock (Worthington (stock is 0.2 µm filter sterilised 2000 U/mL colla genases in sterile RO-grade water) - Pulmozyme (recombinant cGMP grade DNase I) (Genentech) - Albumex 40 (40% human serum al bumin, Australian Red Cross Blood Service) - Tissue culture (TC) medium (TCM): DMEM-LG supplemented with 20 % fetal bovine serum (Invitrogen) and 50 µg/mL gentamicin (40 mg/mL stock)	- Bovine serum albumin (BSA)(Sigma) - Plasma-Lyte 148 Replacement In travenous Infusion (Baxter) - CryoSure-Dimethyl-sulphoxide (DMSO) (Wak Chemie) - Cryopreservation media: 65 mL Plasmalyte, 25 mL Albumex-20 and 10 mL DMSO (Note: to avoid pro tein precipitation always add Albumex-20 to the Plasmalyte and mix before adding DMSO) - 7-Amino-actinomycin D (7AAD) (BD Pharmingen) - mAbs: CD45APC-Cy7, CD73PE, CD105FITC CD45APC-Cy7, CD73PE, CD105FITC (BD Pharm ingen) - Calibrite fluorescent beads (BD Pharmingen) - Sterile phosphate buffered saline (PBS) (Invitrogen)

Table 2

Methods used for human placenta-derived mesenchymal stromal cells isolation, cell seeding, incubation and cryopreservation [5, 6, 11]

Cell isolation	Cell seeding	Cell incubation and cryopreservation
- The pieces of tissue were finely diced and transferred to 50 mL centrifuge tubes (approximately 10 g per tube). The 16 x 50 mL tubes were used. Dulbecco's modified Eagle's medium-low Glucose (DMEM-LG; SACF Biosciences) with 100 U/mL collagenase, type I (Worthington Biochemical Corporation) and 5 µg/mL DNase I (Pulmozyme) was added to each tube up to a total volume of 25 mL.	- The cells were pelleted by centrifugation (540 g, 5 min, 4°C), the supernatant discarded and the cell pellets resuspended in 30 mL HBSS. 12 mL Ficoll-Paque™ Premium (1.073 g/mL) was then under-layered. Samples were centrifuged (540 g, 20°C, 20 min, no brake).	- The cells were removed by pipetting HBSS across the monolayer. The cells were pelleted by centrifugation (350 g, 5 min, 4°C) and cell pellets resuspended in DMEM media. The cells were equally divided between 90 T175 flasks, each in a final volume of 60 mL DMEM media and further incubated (as above). This equated to an approximate cell concentration of 0.7–1.4 x 10^4/mL (in our first two production runs).
- Tubes were placed in an incubated shaker (37°C, 2 hr), then pulse spun at (540 g, 5 sec, 4°C) to remove large particulate matter and the cell suspensions were collected and filtered into 16 x 50 mL centrifuge tubes using 70 µm filters (BD Falcon).	- The cells at the interface of were transferred to 12 x 50 mL centrifuge tubes, HBSS as added to 50 mL and the cells were pelleted by centrifugation (540 g, 20°C, 10 min). The cells were resuspended in 10 mL DMEM media: (DMEM (1g/l glucose), 20 % FCS (Invitrogen, cat # 10099-141, Australian sourced), 50 µg/mL gentamicin (Pharmacia).	- The relatively large volume of 60 mL was used because the media was left on the cells for 6–7 days without being changed. When these were confluent after approximately 6–7 days, the cells were detached from the flasks.
- 15 mL HBSS was added to the original tubes containing large particulate placental matter and the tubes were inverted several times to elute remaining loose cells into suspension6. Tubes were pulse centrifuged as before to pellet large particulate matter (540 g, 5 sec, 4°C), and cell suspensions were transferred to fresh tubes through 70 µm filters.	- Cells were initially seeded into 8 T175 cm^2 tissue culture flasks (T175). The cells were cultured in 30 mL DMEM media (37°C, 5% CO_2, humidified incubator). The relatively high FCS concentration was used to maximise cell expansion rate. When the cells were about 90–99% confluent, the DMEM media was removed and flasks washed with 20 mL HBSS. 5 mL TrypLE select (a GMP grade trypsin-like substitute) (Invitrogen), was added to each flask and incubated (15 min, 37°C).	- The majority of cells were then cryoreserved, whilst a fraction of the cells were used to seed a further 90 T175 flasks. This procedure was repeated a further three times.



(A) (B)

Fig. 1 – The human term placenta. (A) The fetal portion of the human placenta with the umbilical cord still attached. Mechanical separation of the fetal membranes, the amniotic membrane and the chorionic membrane is shown; (B) The portion of the placenta that embeds into the mother's uterus, the decidua. /Reproduced with permission from Heazlewood et al. [14]/.

lyte (Baxter Healthcare), 25% human serum albumin (Australian Red Cross) and 10% DMSO (Wak-Chemie Medical GmbH). The cells were frozen either in 50 mL freezing bags (Baxter Healthcare) or in 1.8 mL cryovials (Nunc) [6]. A set of release criteria [6] is shown in Table 3.

Table 3

The production release criteria used for manufacturing human placenta-derived mesenchymal stromal cells [5, 6, 11, 14]

Test	Pre-donation	Passage 0	Passage 1	Passage 2	Passage 3	Passage 4	Passage 5	Day 180**
Gram Stain	N/A	N/A	N/A	✓	✓	✓	✓	N/A
14 day microbiological culture	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Mycoplasma detection	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Endotoxin detection	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Purity (by FACS phenotype)	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Karyotype (by cytogenetic analysis)	N/A	N/A	N/A	✓	✓	✓	✓	N/A
Donor serology*	✓	N/A	N/A	N/A	N/A	N/A	N/A	✓
Donor health questionnaire	✓	N/A	N/A	N/A	N/A	N/A	N/A	✓

*** For infectious disease markers; ** Donor/mother and the baby follow-up; ✓– applicable; N/A – not applicable.**

The osteogenic differentiation was obtained while confluent MSCs were cultured for three weeks (solution: DMEM-HG, 10% FCS, 0.1 μM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, 10 mM β-glycerol phosphate disodium salt pentahydrate and 0.3 mM sodium phosphate) [2, 5, 6, 11]. It was assessed by staining cells in wells with Alizarin Red S [6]. The chondrogenic differentiation was also obtained and the cells (5×10^5 MSCs) were cultured over a 3-week period (solution: DMEM-HG, 0.1 μM dexamethasone, 1 mM sodium pyruvate, 50 μM L-ascorbic acid-2-phosphate, 35 mM L-proline, 10 ng/mL $TGF\beta_1$ (R&D Systems) and 50 mg/mL ITS Premix (insulin, human transferrin and selenious acid; BD Biosciences) [6]. Chondrogenic differentiation was evaluated by staining the cell pellets with periodic acid Schiff (PAS) [6].

Adipogenic differentiation was initiated and confluent MSCs were cultured for three weeks (solution: DMEM-HG, 1 μM dexamethasone, 5 μg/mL insulin, 60 μM indomethacin and 0.5 mM 3-Isobutyl-1-methylxanthine, IBMX) [6]. Level of the adipogenic differentiation was measured as cells were stained with Oil Red O [6]. Reagents used for differentiation techniques were mostly obtained from Sigma [6] while mAb were manufactured by BD Pharmingen and prepared in PBS with 2% Human Serum Albumin (1 : 5 dilution) [6].

MSCs were detached from flasks using TrypLE select (Life Sciences), washed and added to FACS tubes in order to prepare them for flow cytometry analysis [6]. MSC were incubated as follows: 1×10^6 cells in 100 μL of mAb mix (15 min at 4ºC) with directly conjugated mouse anti-human CD45-FITC, CD14-APC-Cy7, CD73-PE and CD105-APC antibodies [6]. An additional tube with MSC was prepared and it was stained with 5 μg/mL isotype control mAb (IgG1 FITC, IgG1 PE, IgG1 APC and IgG1 APC-Cy7) [6]. The excess antibody was removed and cells washed with the phosphate buffered saline (PBS). After the last wash, cells were resuspended in the solution consisting of 200 μL PBS and 7-Amino-actinomycin D(7AAD) (BD Pharmingen) (1 : 40 dilution) in order to exclude the dead cells. An LSRII flow cytometer (Becton Dickinson) was used and the data were analysed by FCS Express Version 3 software (DeNovo) [6]. A defined threshold for fulfilling our MSCs purity criterion was set (> 85% $CD73^+/CD105^+$(double positive), < 1% $CD45^+$) [5, 6, 11].

Results

Establishing the protocol

Manufactured human placenta-derived MSCs were very similar to human bone marrow-derived MSCs (hbmMSCs) [2, 5, 6]. We were unable to find major differences between human placenta-derived MSCs and bone marrow-derived MSCs in terms of morphology (Figure 2) and cell surface

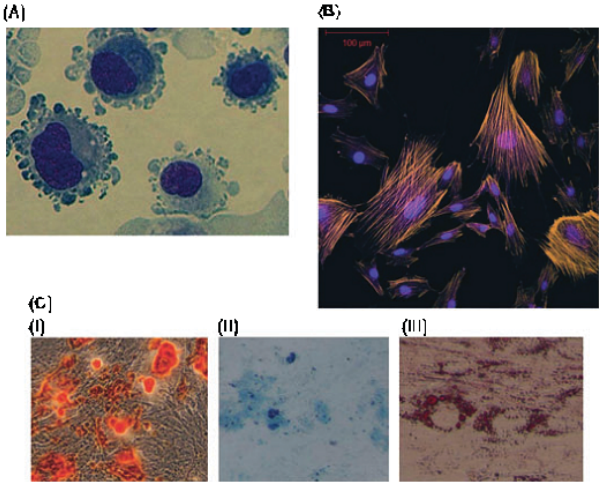


Fig. 2 – Photomicrograph showing morphology (Giemsa stain and Phalloidin stain) and mesodermal differentiation of human placenta-derived mesenchymal stromal cells (MSC). (A) Giemsa stain of human placenta-derived MSCs; (B) Morphology of human placenta-derived MSCs when stained with Phalloidin-AF546; (C) Osteogenic (i), chondrogenic (ii) and adipogenic (iii) differentiation and histological staining of human placenta-derived MSCs by alizarin red, Periodic acid schiff and Oil Red O, respectively.
Original magnification x1000 (A); ×400 (B) and (Ciii); ×100 (Ci) and (Cii).
/Reproduced with permission from Brooke G, et al. [6]/.

phenotype [2, 5, 6] (Figure 3), chemokine receptor display [4], mesodermal differentiation [2], or immunosuppressive capacity on alloreactive T cells [29]. Yen et al. [17] established in their study that human placenta-derived MSCs expressed the early antigens SSEA4, Tra1-60 and Tra1-81, while the bone marrow derived MSCs lacked those antigens [6]. Conversely, we discovered a low level expression of SSEA4 and Tra1-60 on the human placenta-derived MSCs from our manufacturing process [4, 6].

All release from manufacturing criteria were fulfilled at the time of passage cryopreservation [5, 6, 11] as presented in Table 3, including pre-donation serology screening, donor serology for infectious disease markers screening on day 180, pre-donation health questionnaire for the mother, and the day 180 health questionnaire for the mother and the baby [5, 6, 11, 14].

Evaluation of the protocol

Human placenta-derived MSCs manufactured at the Mater Medical Research Institute were used for three phases I (safety) clinical trials. These are briefly presented below.

First trial was a phase I multicentre open label dose-escalation study of unrelated, MHCs unmatched placenta-derived MSCs in recipients of unrelated umbilical cord blood hematopoietic stem cell transplants.

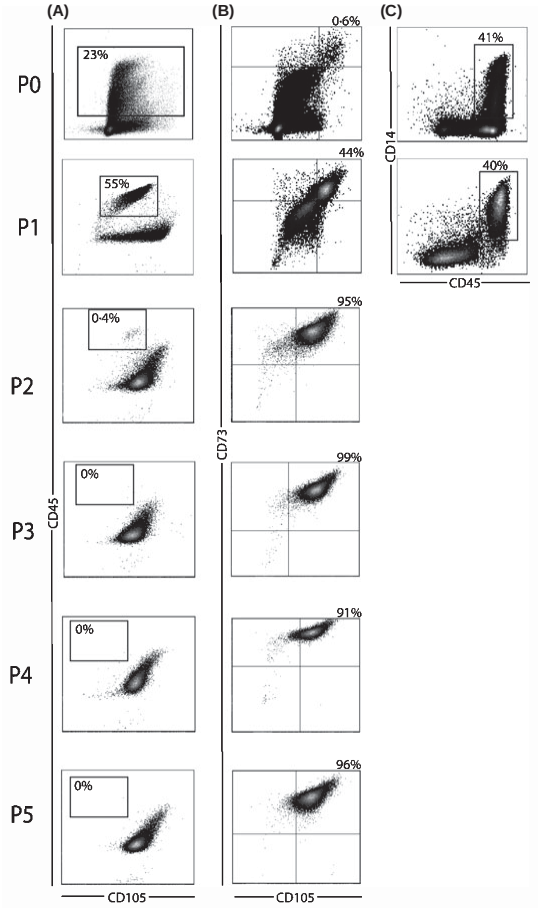


Fig. 3 – The flow cytometric phenotype of human-placenta derived mesenchymal stromal cells (MSCs) during isolation and passaging. (A) $CD45^+$ frequency at each passage; (B) $CD73^+/CD105^+$ frequency at each passage; (C) $CD14^+/CD45^+$ frequency at early passage.
/Reproduced with permission from Brooke G, et al. [6]/.

In this phase I trial of safety and feasibility initiated in 2007 we co-transplanted human MSCs in a subject receiving an allogeneic cord blood transplant as therapy for treatment-refractory hematological malignancy. The trial was performed in collaboration with Professors Ken Bradstock and David Gottlieb of Westmead Hospital, Sydney. MSCs at a dose of 1×10^6 MSCs/kg were infused into the cord blood recipient 5 hours before infusion of cord blood cells [6], in order to obtain a 5-hour window for observation of early adverse reactions due to MSCs (for example, infusional toxicity) unclouded by any possible side-effects from subsequent infusion of cord blood cells. The time from submission of the application until approval was received from the two institutional Human Research Ethics Committee (HRECs) involved was one year in total [6]. Two-way clinical trial agreements were put in place with an appropriate indemnification for each of the participating sites and for any adverse events attributed to MSCs. During this time, a request was made by one of the institutional HRECs for an external audit to be

conducted in the manufacturing processes outlined in the study protocol. This was performed by the staff of the Australian Red Cross Blood Service. We have conducted the two were external audits since then.

Clinical course [6]: 24 hours after the pre-transplant myeloablative conditioning regimen completion (cyclophosphamide plus a total body irradiation), 100×10^6 human placental MSCs (1×10^6/kg) were given intravenously to a 20-year-old Caucasian male with acute myeloid leukaemia in second remission [6]. The cells were suspended in 30 mL and were infused over 7 min using a 200 μm in-line filter. No adverse events were noted. According to the study protocol, the patient received two allogeneic cord blood units five hours later. The total nucleated cell dose from two cord blood units was, post-thaw, 3.6×10^7/kg while the total $CD34^+$ cell dose was 1.2×10^5/kg [6]. The placenta-derived MSCs given to the patient were MHC-unmatched with both the recipient and the two cord units [6]. MSCs donors and cord blood donors were unrelated to each other [6]. At the day 70 posttransplant the patient developed cytomegalovirus (CMV) infection and he subsequently died from multiorgan failure [6]. Of note, MSCs had been procured from a CMV-seronegative maternal donor. There were no reported serious adverse events related to the MSCs, including infusional toxicity or ectopic tissue formation. This trial was ceased when Westmead Hospital decided to no longer perform cord blood transplants in adult subjects. This was the first time, to our knowledge, that placenta-derived MSCs have been trialled in humans [6, 11].

Second trial was a phase I study to evaluate the potential role of MSCs in treatment of idiopathic pulmonary fibrosis.

In 2010 we initiated a phase I trial of MSCs in collaboration with Drs Daniel Chambers and Peter Hopkins of the Prince Charles Hospital in Brisbane. Idiopathic pulmonary fibrosis is a chronic, fibrosing lung disease of unknown cause predominantly occurring in middle-aged people that is characterized by severe, refractory and progressive breathlessness. There is no effective treatment for this disease except for lung transplantation, and this approach is limited by a shortage of lung organ donors. The rationale for this trial was based on literature reports of a beneficial effect noted in mice given murine MSCs after experimental pulmonary fibrosis was induced by the cytotoxic drug bleomycin.

Clinical course: MSCs were administered *iv* as follows: 1×10^6 MSCs/kg (first cohort of 4 subjects) and 2×10^6 MSCs/kg (second cohort of 4 subjects). A validated dry shipper was used to transport MSCs to the collaborating hospital. The cells were kept cryopreserved until required for administration to subjects in the trial. Subsequently, the cells were thawed and infused intravenously. There were no reported serious adverse events related to MSCs, including infusional toxicity or ectopic tissue formation. The results of this trial were of interest since it involved subjects with impaired pulmonary function. MSCs injected intravenously immediately home to the lungs where they remained for approximately three days and could theoretically cause further pulmonary function compromise. This did not happen.

Third trial was a phase I study to evaluate the potential role of MSCs in treatment of chronic refractory tendinopathy.

In 2011 the phase I was initiated in subjects with Achilles tendinopathy refractory to conventional treatments. In contrast to the first two trials MSCs were injected directly into the site of tendon damage using ultrasound guidance. The Principal Investigator on this trial was Dr Mark Young, Visiting Medical Officer in Sports Medicine at Mater Private Hospital, Brisbane. Tendinopathy is a common condition associated with pain and diminished function in tendons. It occurs in active young people, and the incidence increases with age. The incidence of tendinopathy increases along with the life expectancy, which in turn places larger costs on the health system. The initial management of all tendinopathies is conservative, including activity modification, medications, corticosteroid injections and/or exercises. Surgical treatments are considered if a prolonged conservative management fails, but these interventions are costly and involve periods of immobilisation.

Clinical course: All the patients received their *iv* MSCs injections under the ultrasound guidance. The subjects were monitored for a period of at least 4 weeks before the next patient is treated so that any early adverse effects from the previous MSCs application could be assessed – both clinically and by ultrasound examination. The 3 patients of the first cohort received a single dose of 1.0×10^6 placenta-derived MSCs (1 mL of solution with a total of 1.0×10^6 cells per mL) each. The next cohort of 3 patients received a total of 4.0×10^6 MSC (1.0 mL of a solution with a total of 4.0×10^6 cells per mL) each.

Each of the above trials was subjected to oversight by a Data Safety Monitoring Committee. The Data Safety Monitoring Committee carried out an interim safety analysis after each cohort of patients had received their injections and no adverse events attributed to MSCs administration were noted. The overall safety outcomes were encouraging since these phase I trials were not designed to assess efficacy of the biologic drug but its safety. Clinical trial design and clinical outcomes (other than safety), patient inclusion and exclusion criteria, patient follow-up, relevant controls and clinical parameters are beyond the scope of this paper.

Discussion

In this study we presented our experience in establishing a protocol for manufacturing/ preparation of clinical-grade human placenta-derived MSCs that can be cell banked and used for various clinical applications on an “as needed” basis. We also evaluated the protocol based on manufacturing considerations of MSCs for use in clinical settings, regulatory considerations related to MSCs manufacturing (*eg* could the protocol be relatively easily adjusted to comply with a different set of regulatory requirements as mandated for applications in early phase clinical trials in Australia or other comparable sets of regulations), and safety, accessibility and usability of human placenta-derived MSCs. There are several issues to be discussed.

Firstly, there are manufacturing protocol considerations. These include, but are not limited to, the amount and type of tissue used as a source of cells, choice of manufacturing reagents (*eg* clinical grade reagents) and variations of the laboratory protocol, as well as the conditions of the *ex vivo* cell expansion procedure (*eg* "open" *vs* "closed" system). In our first manufacturing campaign, we used a part (300–500 g) of one placenta (placenta 1). This represented over a half of the total placenta mass and was used to seed 6 × T175 flasks (P0) [6]. It yielded approximately 40×10^6 cells at first passage (P1) [6] which were then split between 90 flasks at 4.38×10^5 cells/flask and plated at the initial density of 2500 cells/cm^2. The average yield for each subsequent passage was 742×10^6 cells (with a standard deviation of 82.6×10^6) [6]. At each passage, 40×10^6 cells were held back for the next passage and seeded to a new set of flasks at 4.38×10^5 cells/T175, and the remaining cells were cryopreserved [6]. It was noted that cell recovery at P4 and P5 of the first processed placenta was only 60% although cell recoveries were generally excellent [6]. The noted variability may have been due to the use of small aliquots of cells stored in cryovials for counting purposes and testing only [5, 6]. The yield from cryovials, in our experience, tends to be slightly lower than that from cryobags [6]. The placentas (1 and 2) processed in the first two manufacturing campaigns using clinical grade manufacturing protocols yielded a total of 4.5×10^9 cryopreserved cells. MSC were released from production [11] as 97% viable, Gram stain negative, endotoxin test < 2 EU/mL, sterile on 14 day microbiological culture, with an appropriate cell surface phenotype CD45$^-$ and > 85% CD73$^+$/CD105$^+$ (*eg* in P2 the results of cell purity were: 0.4% CD45$^+$, 95 % CD73$^+$/CD105$^+$; in P3: 0.0% CD45$^+$, 99%CD73$^+$/CD105$^+$; in P4: 0.0% CD45$^+$, 91% CD73$^+$/CD105$^+$; and in P5: 0.0% CD45$^+$, 96% CD73$^+$/CD105$^+$) demonstrated by flow cytometry, and with normal karyotype [5, 6, 11].

A two-stage release process was applied with a set of criteria for each stage. Release from manufacturing included tests performed on each batch of cells immediately following manufacturing of each passage (*eg* sterility, mycoplasma and endotoxin test, purity, viability, maintenance of normal karyotype). Release from cryopreservation included tests performed on a cryovial corresponding to each bag planned for administration to a patient; the test-vials were thawed several weeks before the planned administration to a patient and sterility, viability and mycoplasma tests were performed.

In both first two manufacturing campaigns, a collagenase I based tissue digestion protocol was utilised. One of the major obstacles in the initial phase of MSCs manufacturing was a lack of cGMP grade reagents or clinical-grade reagents in the cell isolation steps [6]. Hence, the human recombinant DNase was used for digestion in our manufacturing process, which was produced by Roche under the cGMP conditions. It is intended for clinical use in cystic fibrosis treatment [6, 11].

Additionally in both our first two manufacturing campaigns the cell preparation (following tissue dissociation and digestion) was initially purified using density gradient centrifugation with Ficoll-Paque Premium™ (GE Healthcare). This is an alternative to Percoll and it is a clinical-grade reagent [6, 11].

Use of media with fetal calf serum for cell expansion may increase the theoretical risk of bovine spongiform encephalitis transmission. The Australian regulatory body for medicines, the Therapeutic Goods Administration, (TGA, equivalent of the Food and Drug Administration [FDA] in the USA and the European Medicine Agency [EMA] in Europe [15]) approves in principal the use of fetal calf serum in clinical grade material production – as long as it is sourced from a country free of bovine spongiform encephalitis, such as Australia or New Zealand [6, 11]. Most of the protocols use 10–20% fatal calf serum (FSC) growth media [24–28] but serum-free media is a preferred option from the safety perspective.

At this stage our process of MSCs manufacture is still conducted in an "open" system due to the multiple steps required in MSCs extraction and culture. The extended period of cell expansion (up to 6 weeks) introduces the risk of microbial contamination although class II biosafety cabinets and/or clean rooms are utilised, [6, 11]. Hence, an extensive in-process and end-product testings are utilised prior to release of the cell product for clinical use [6, 11].

Secondly, there is consideration of regulatory requirements related to MSCs manufacturing for use in early stage clinical trials. All stages of the manufacturing process were completed in accordance with cGMP principles and internal Quality Management System policies and procedures. A Quality Management System includes cGMP (or equivalent standard)-compliant policies, procedures and extensive documentation applies, but is not limited, to the following: facilities, equipment, materials, staff, monitoring, validation, process change, record generating and record keeping [5, 6]. Production scientists had to work closely with the regulatory compliance staff to ensure a safe cellular therapy product [11, 13, 14]. Staff education and ongoing training were crucial components of regulatory compliance. Overall, biologic drugs (also referred to as biologics, biologicals or Advanced Therapy Medicinal Products) regulations have been widely developed by mature regulatory agencies such as the Australian framework administered by the TGA, the European Union's EMA levied framework and the framework of the United States of America (USA) imposed by the FDA [15]. All of these biologic drugs regulations are multilayered and complex [15, 16]. However, common underlying principles of cGMP, provide a useful guide in each regulatory domain applicable to biologic drugs, including cell and tissue based therapeutics [15, 16].

Finally, there are considerations related to the safety, accessibility and usability of human placenta-derived MSCs. Adult MSCs can be derived from different source-tissues and can be expanded in culture while maintaining their characteristics. MSCs are currently extensively used in preclinical and clinical studies, including tissue engineering. There is a considerable promise for the use of MSCs in rebuilding damaged or diseased mesenchymal tissues in different tissue-engineered models [8–10]. The fact that MSCs secrete a large spectrum of bioactive molecules was intriguing [10]. The im-

munosuppressive molecules produced by MSCs, especially those affecting the T-cell immune response, are now considered to be one of the mechanisms by which MSC mediate their therapeutic effect in some disease settings [18–23]. MSC-secreted bioactive molecules may be able to provide a regenerative microenvironment for a variety of injured adult tissues and limit the area of tissue damage [10]. Hence, a number of clinical trials currently use allogeneic MSCs for the treatment of Crohn's disease, myocardial infarcts, graft-versus-host disease, cartilage and meniscus repair, spinal cord injury, stroke and other clinical indications [18–23].

The placenta and other pregnancy-related tissues appear an attractive source of MSCs [7, 11–14, 17, 21, 25, 28]. Cells can be prepared in advance, for a number of patients or clinical trials, on a therapeutic scale manufacturing and cell banking process. Placenta, for example, is normally discarded after the birth of the baby, thus providing a practically unlimited supply of source material. Placental MSCs exhibit the classical MSCs surface phenotype, differentiation potential and have potent immunosuppressive properties [2, 6, 14, 17]. Placental MSCs have been utilised by a number of other research groups worldwide [12].

Conclusion

Clinical trials using both allogeneic and autologous cells have demonstrated MSCs to be safe and have targeted diseases in areas such as orthopaedic, cardiovascular, degenerative and inflammatory diseases. Phase I clinical trials are a challenging step in this process due to limited funding, laborious and long manufacturing procedures and the need for a multidisciplinary team with a unique skill set. We described a manufacturing protocol for human placenta-derived MSCs that is appropriate for use in a clinical setting, relatively inexpensive and can be relatively easily adjusted to a different set of regulatory requirements, as applicable to early phase clinical trials.

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Women's demand for late-term abortion – A social or psychiatric issue?

Zahtev žena za kasnim abortusom – socijalni ili psihijatrijski problem?

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Abstract

Introduction/Aim. Induced termination of unwanted pregnancy after 12th gestational week (late-term abortion) is legally restricted in Serbia as well as in many other countries. On the other hand, unwanted pregnancy very often brings women into the state of personal crisis. Psychiatric indications for legally approved late-term abortion on women's demand include only severe psychiatric disorders. The aim of this paper was to compare sociodemographic, psychological characteristics and claimed reasons for abortion in the two groups of women with late-term demand for abortion – the group of women satisfying legally prescribed mental health indications, and the group of women not satisfying these indications. The aim of the study was also to determine predictive validity of the abovementioned parameters for late-term abortion as the outcome of unwanted pregnancy. **Methods.** A total of 62 pregnant women with demand for late-term abortion were divided into two groups according to the criteria of satisfying or not satisfying legally proposed psychiatric indications for late-term abortion after psychiatric evaluation. For the assessment of sociodemographic and psychological parameters sociodemographic questionnaire and symptom checklist – 90 revised (SCL-90®) scale were used, respectively. The outcome of unwanted pregnancy was followed 6 months after the initial assessment. **Results.** The obtained results showed a statistically significant difference between the groups in educational level, satisfaction with financial situation, elevated anxiety and distress reactions. Unfavorable social circumstances were the main reason for an abortion in both groups and were predictive for an abortion. A 6-month follow-up showed that women had abortion despite legal restrictions. **Conclusion.** Pregnant women with psychiatric indication for late-term abortion belong to lower socioeconomic and educational level group compared to women without this indication who have more frequently elevated anxiety and distress reactions to unwanted pregnancy. It is necessary to have more accurate guidelines for mental health indications for legally approved late-term abortion, respecting social circumstances. Preventive measures are of great importance in order to lower the risk of illegally performed late-term abortions.

Key words:
pregnancy, unplanned; abortion, legal; abortion, criminal; mental disorders; psychiatric status rating scales; demography; social conditions.

Apstrakt

Uvod/Cilj. Arteficijelni abortus kod neželjene trudnoće nakon 12. gestacione nedelje (kasni abortus), zakonski je ograničen u Srbiji kao i u mnogim drugim zemljama. Na drugoj stani, neželjena trudnoća često je uzrok lične krize. Indikacije za zakonski dozvoljen kasni abortus na zahtev žene, podrazumeva samo teške psihijatrijske poremećaje. Cilj našeg rada bio je da uporedimo sociodemografske, psihološke karakteristike i navedene razloge za abortus kod dve grupe žena sa zahtevom za kasni abortus: grupe žena koje zadovoljavaju zakonom propisane psihijatrijske indikacije za kasni abortus i grupe žena koje ne zadovoljavaju ove indikacije. Cilj rada bio je i da utvrdimo prediktivnu vrednost navedenih parametara istraživanja za kasni abortus kao ishod neželjene trudnoće. **Metode.** Ukupno 62 gravidne žene sa zahtevom za kasni abortus podeljene su u dve grupe prema kriterijumu zadovoljenja zakonom propisanih psihijatrijskih indikacija za kasni abortus nakon psihijatrijske procene. Sociodemografski i psihološki parametri evidentirani su pomoću sociodemografskog upitnika i *Symptom checklist-90-revised* (SCL-90 R®) skale kojom se procenjuje revidirana lista simptoma. Ishod neželjene trudnoće praćen je šest meseci od početne procene. **Rezultati.** Naše istraživanje pokazalo je da postoji statistički značajna razlika između grupa u odnosu na nivo obrazovanja, zadovoljstvo finansijskom situacijom, povišeni nivo anksioznosti i distresne reakcije. Nepovoljne socijalne okolnosti bile su glavni razlog zahteva za abortusom u obe ispitivane grupe i imale su prediktivni značaj za kasni abortus. Šestomesečno

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praćenje pokazalo je da se neželjena trudnoća u 2. trimestru završavala abortusom, uprkos zakonskom ograničenju. **Zaključak.** Gravidne žene sa psihijatrijskom indikacijom za kasni abortus imaju niži socioekonomski i obrazovni nivo u poređenju sa ženama koje ne zadovoljavaju ove indikacije, a koje imaju povišeniju anksioznost i distresne reakcije na neželjenu tudnoću. Potrebne su preciznije smernice psihijatrijskih indikacija za zakonski dozvoljen kasni abortus, koje bi uvažavale i socijalne okolnosti. Neophodno je preventivno delovanje u cilju sniženja rizika od ilegalnih kasnih abortusa.

Ključne reči:
trudnoća, neželjena; abortus, legalni; abortus, ilegalni; psihički poremećaji; psihijatrijski status, testovi; demografija; socijalni faktori.

Introduction

Late-term abortion often refers to an induced abortion procedure that occurs after the 12th week of gestation [1]. However, the exact point when pregnancy becomes late-term is not clearly defined. Different countries define it by law, and ban late-term abortion after particular gestational age. In the East and the South Europe 12th week is a limit, in Italy 13th week, in the Central Europe 14th week, in Sweden it is 18th week. In Danemark, it is possible to terminate late pregnancy for socioeconomic, not only for medical reasons. Some countries, like Canada, China (Mainland only) and Vietnam have no legal limit on when abortion can be performed [2].

Unwanted pregnancy is often personal crisis. Induced abortion in Serbia is a legally restricted service. It is permitted on women's demand up to 12th gestational week. Induced termination of unwanted pregnancy is allowed up to 20th week for law determined reasons considering physical and mental health of women, fetal aberration and pregnancies resulted from rape [1]. After 20th week, it is possible under limited circumstances and has to be approved by Ethical committee of the regional medical center (The abortion law: Official Gazette of the Republic of Serbia no. 16/95 and 101/2005.).

Psychiatric assessment of pregnant women who demand abortion after 12th gestational week is required to determine whether their condition satisfies indications due to mental health reasons for abortion, prescribed by the law.

In our clinical practice the problem arises when the woman's demand for an abortion is in contradiction with law – when the reasons are more social rather than medical or psychiatric in nature.

Little is known about the reasons for second trimester (late) abortions in Serbia. In everyday practice, providers have noticed adverse emotional reactions of pregnant women, after they had realized that late abortion is not allowed without medical or psychiatric reasons.

There are no published studies that provide comparative analyses of what happens when access to second trimester abortion is restricted by the law. Psychological neurotic reactions and adverse socioeconomic circumstances are not legally approved indications for abortion in the second trimester. These women could be in a position to continue unwanted pregnancy or terminate it in spite of legal restrictions. It means that woman is forced to continue an unwanted pregnancy with the risk of rejection of the child and the emergence of unfavorable conditions for the psychological development of the offspring. The other option is unsafe, illegal or self-induced abortion, which is a serious risk for her health.

The aim of this paper was to compare sociodemographic, psychological characteristics and claimed reasons for induced abortion in the two groups of women with late-term demand for abortion – the group of women satisfying legally prescribed mental health indications for abortion, and the group of women not satisfying these indications. The aim of the study was also to determine predictive validity of parameters under study for late-term abortion as the outcome of unwanted pregnancy after a 6-month follow-up.

Methods

In 2010, 62 pregnant women with demand for late-term abortion were referred by their gynecologist to the Clinic for Mental Health for psychiatric evaluation. This evaluation had to determine the presence of mental disorder as psychiatric indication for late-term abortion in the absence of the other medical indications. Psychiatric evaluation was undertaken by the team of two psychiatrists who did evaluation independently for every woman and after joint discussion made consensus about the indications for termination of pregnancy. When minors have been involved, psychiatrist for children and adolescents was invited to the team. The mental state of women was evaluated in order to determine the presence of disorders which influence sexual behavior and decision making about termination of pregnancy in the first trimester.

The presence of psychotic disorder, major depression, mental retardation, addiction, and the age under 16 were criteria for psychiatric consent for late-term induced abortion. Women with distress reaction and minor depression did not get approval for abortion in the 2nd trimester, according to the abortion law.

For the overall psychiatric evaluation unstructured psychiatric interview was applied just after the admission to the Clinic. Clinical diagnosis was made in accordance with the criteria of the International Classification of Mental Disorders – 10 (ICD 10) [3] and using semi-structured Mini International Neuropsychiatric Interview (MINI Version 5.0.0) [4]. The Symptom Check list-90R (SCL-90 R®) [5] was used for the evaluation of clinical symptomaology. This is a 90-item self report inventory for the assessment of 9 psychological dimensions: somatization, obsessive-compulsiveness, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation and psychoticism. A score over 63 suggests clinically significant expression of certain dimension. Sociodemographic questionnaire, made for the purpose of this study, contained data about age, gestational age, education level, marital status, employment, subjective satisfaction with

financial situation, the number of household members. At the end of the questionnaire, there was an open ended question: "What is the reason for your request for late-term abortion?"

After the assessment, the patients were divided into two groups. The women in the group A (n = 32) did not satisfy criteria of psychiatric indication for the 2nd trimester abortion and did not get psychiatric consent for termination of a pregnancy. The women in the group B (n = 30) received psychiatric consent due to the presence of the diagnosis of mental disorder or the age under 16.

The outcome of unwanted pregnancy was followed 6 months after the psychiatric assessment. Women from both groups were phone called by the psychiatrist for a short interview about the outcome of their unwanted pregnancy.

All the participants give their written consent to participate, so the data could be explored. Confidentiality and anonymity were ensured. Ethical approval was obtained from the Ethics Committee of the Clinical Center Niš.

We compared the 2 groups by sociodemographic data, psychological dimensions (score on SCL- 90R > 63), presence of the mental disorder, gestational age of pregnancy at the time of demand for abortion, and stated reasons for late-term demand for abortion.

Statistical Package for Social Sciences (SPSS 15.0) was used for statistical analysis.

The difference between the parameters was calculated using the Student *t*-test and χ^2-square test, *p* values < 0.05 were considered statistically significant. Univariate logistic regression was used to calculate predictive values of the parameters for late abortion.

Results

The youngest woman in our sample was 15, and the oldest one 43 years old. Gestational age was from 15 to 25 gestational weeks. The average age in the group A was 28.7 years and in the group B 28.4 years. The average gestational age in the group A was 19.1 gestational weeks, and in the group B 18.6. gestational weeks. There was no statistically significant difference between the two groups in the mean age, or in the gestational age of pregnancy (Table 1).

The analysis of sociodemographic characteristics of women in our sample showed a statistically significant difference between the two groups in relation to the level of education and subjective satisfaction with financial situation.

Nineteen women in the group A had 12 years of education, significantly more than women in the group B. More than 12 years of education had only 4 women in the group A, and none in the group B. In the group B most of the women had only 8 years of education.

Subjective feeling of satisfaction with financial situation was present in 13 women in the group A, whereas most of the women in the group B were dissatisfied with their financials.

There was no statistically significant difference between the two groups comparing the other sociodemographic parameters (Table 2).

Table 1

Average age of women and gestational age

Parameters	Whole sample		Group A		Group B	
	x̄	SD	x̄	SD	x̄	SD
Age of women (years)	28.13	8.151	27.81	7.532	28.47	8.881
Gestational age (weeks)	18.90	2.281	19.19	2.348	18.60	2.207

Group A – pregnant women not satisfying legal criteria of psychiatric indication for second trimester abortion; Group B – pregnant women satisfying legal criteria of psychiatric indication for second trimester abortion.

Table 2

Sociodemographic parameters of the groups

Parameters	Group A		Group B	
	n	%	n	%
Education (years)				
8	9	28.12	20*	66.66
12	19*	59.37	10	33.33
> 12	4*	12.50	0	–
Marriage				
unmarried	13	40.62	11	36.7
parents	19	59.37	19	63.30
Household members				
partner	7	21.87	2	6.66
one family nember	13	40.62	17	56.66
married	12	37.5	11	36.66
Employment				
unemployed	14	43.75	21	70.0
employed	10	31.25	7	23.3
student	8	25.0	2	6.7
Satisfaction with financials				
satisfied	13*	40.62	4	13.30
dissatisfied	19	59.37	26*	86.70
Total	32		30	

χ^2 = 10.912; ss = 2; *p < 0,05; ö = 0.42
Group A – pregnant women not satisfying legal criteria of psychiatric indication for second trimester abortion;
Group B – pregnant women satisfying legal criteria of psychiatric indication for second trimester abortion.

Answers to the question about the reason for late request for abortion were divided into 5 groups according to the answer that was marked as the main. All the reasons were present in both groups. The most frequent reasons in both groups were: interrupted partner relationship during pregnancy and poor financial situation. The other listed reasons were marital conflict, unknown partner and conflict with parents. There was no statistically significant difference in frequencies of these variables between the groups (Table 3).

between the groups on the other dimensions of this questionnaire (Table 4).

The outcome of unwanted pregnancy was almost the same in both groups. In a phone conversation, 6 months after psychiatric evaluation, we received the information that only two women from the group A decided to keep their pregnancy. The rest of the women from the group A had late abortion despite legal restriction.

We used the model of logistic regression including all of the variables. Predictive value of each single parameter for

Table 3

The reasons for requesting late abortion

Reasons for late abortion	Group A		Group B		Group comparison	
	n	%	n	%	χ^2	*p*
Unknown partner	2	6.25	6	20.0	1.9375	0.38
Interrupted relationship	10	31,25	9	30.0		
Marriage conflict	8	25.0	4	13.33		
Conflict with parents	2	6.25	1	3.33		
Dissatisfaction with finances	10	31.25	10	33.33		
Total	32	100	30	100		

χ^2 – chi square test value; *p* – statistical significance.
Group A- pregnant women not satisfying legal criteria of psychiatric indication for second trimester abortion; Group B – pregnant women satisfying legal criteria of psychiatric indication for second trimester abortion.

More than a half of women in the group A had no mental disorder and the rest had distress reaction – 15 women, significantly more than in the group B. Frequencies of mental disorders in the group B, in decreasing order were: major depressive episode, recurrent depression, mental retardation, opioid addiction, schizophrenia and emotional immaturity – age 15 years. On the SCL-90 R questionnaire, elevated anxiety dimension was statistically significantly more frequent in the group A. There were 13 women with clinically significant elevation of anxiety score in the group A and only 3 women in the group B. There was no statistically significant difference

motherhood and abortion was obtained, but with no statistically significant difference between the group. The model based on parameter B, with all variables under the study included, was a significant predictor of unwanted pregnancy outcome.

Abortion in the 2nd trimester, without medical and mental health reasons was predicted with: older age, lower education, unmarried status, life in incomplete parent family, dissatisfaction with financials, hostility, obsessiveness, broken partner relationship or unknown partner, conflictual relationship with parents (Table 5).

Table 4

Distribution of frequencies of elevated psychological dimensions and psychiatric diagnoses with group comparison

SCL-90R (symptom checklist-go)	Group A		Group B		χ^2	*p*	c
	n	%	n	%			
Without clinically significant elevation of dimensions	9	28.1	9	30.0	0.026	> 0.05	0.021
Anxiety	13	40.6	3	10.0	7.585	< 0.05	0.330
Depression	3	9.4	7	23.3	2.230	> 0.05	0.186
Obsessiveness	2	6.3	2	6.7	0.004	> 0.05	0.008
Hostility	5	15.6	5	16.7	0.012	> 0.05	0.014
Psychoticism	0	0.0	2	6.7	2.204	> 0.05	0.185
Paranoid ideation	0	0.0	1	3.3	1.084	> 0.05	0.131
Interpersonal sensitivity	0	0.0	1	3.3	1.084	> 0.05	0.131
M.I.N.I. (psyhiatric diagnosis)							
F 32.2	–	–	14	46.66			
F 33.2	–	–	1	3.33			
F 20	–	–	2	6.66			
F70	–	–	7	23.33			
F43	15	46.87	0	–			
Without psychiatric diagnosis	17	53.12	2	6.66			
F 11	–	–	4	13.33			
Total	32		30				

Group A – pregnant women not satisfying legal criteria of psychiatric indication for second trimester abortion;
Group B – pregnant women satisfying legal criteria of psychiatric indication for second trimester abortion;
MINI – mini-international neuropsychiatric interview.

Table 5
Results of univariant logistic regression with predictive values of the parameters for the outcome of unwanted pregnancy

Step parameters	Parameter B
Socio-demographic variables	
age	-1.580
marital status	-109.292
education	77.675
employment	-42.049
living with parents	-40.887
living with partner	-37.843
dissatisfaction with finances	-8.108
SCL-90 R dimensions	
without elevated SCL dimensions	-108.589
obsessiveness	-98.036
anxiety	15.212
hostility	-69.249
M.I.N.I. diagnosis	
diagnosis F43	9.243
Gestational age of pregnancy (week)	22.034
Stated reasons for demand for late term abortion	
unknown partner	-104.664
broken partner relationship	-211.879
conflict with parents	-210.490
financial difficulties	-59.788

$\chi^2 = 30.885$; df = 17; $p < 0.05$.
SCL – 90R – symptom checklist 90 revised; M.I.N.I. – mini-international neuropsychiatric interview.

Discussion

The women of both groups of our sample had very similar sociodemographic and psychoogical profiles, contrary to our expectancies. Women were in the 3rd decade of life and the average gestational age of pregnancy in both groups was 19 weeks. Unwanted pregnancies occured in both younger and older reproductive ages, in married and single women who never used contraception. In general, they had low or middle level of education, they were not satisfied with their financial situation and less careful in sexual behavior. This might be not only psychological issue, it could be a part of their lifestyle or a consequence of insufficient sexual education. These findings are similar to the results reported by Upson et al. [6]. They showed that almost half of pregnancies in 2006 in USA were unintended the highest rates occured in women younger than 24 years, and in the older women, who did not want any more children. Unwanted pregnancies were associated with no using contraceptives, having no partner and being unmarried. Other findings showed that highly educated women in America have 4–5 times less unwanted pregnancies than poor and uneducated women who deliver unwanted children. According to the National Survey of Family Grow in USA unwanted pregnancy is associated with young age, single status, black race, less education and having one or more children [7].

A statistically significant difference between the two groups in our sample was found in sociodemographic variables: educational level and subjective satisfaction with finances. Women with psychiatric disorder were less educated and more dissatisfied with their financial situation. These could be explained by the fact that psychiatric patients, in general, belong to lower socioeconomic levels, usually their education becomes interrupted because of their disorder, and they have less chances for employment.

We noticed that the two groups of women with unwanted pregnancy did not differ much in psychological dimensions. Elevated anxiety dimension was significantly more frequent in the group A. This finding is understandable having in mind that distress reaction to unwanted pregnancy was much more represented in this group. Evidenced distress reaction in the group A was also characterized with emotional reactions as despair and helplessness. Women were afraid of future life they could not even imagine. Some had nightmares and sleepless nights. Expressed anxiety and other elevated dimensions in the group A were symptoms of their distress reaction, on the other hand, in the group B, they were expressed as the part of the underlying disease. We did not take into account absolute numerical values of every dimension, but only the frequencies of clinically significant elevation. On the other hand, psychological dimensions were not clinically significantly elevated in the group B in comparison with the group A despite the presence of serious mental disorder, because none of the women in this group was actually in exacerbation of her psychiatric disease at the time of psychiatric evaluation.

None of the women knew that psychosocial issues are not sufficient enough for terminating unwanted pregnancy. Psychiatric approval for women in the group B was based upon the diagnosis of mental illness which was present before unwanted pregnancy, and women in the group A were unpleasantly surprised and even more distressed when they found out about the absence of appropriate psychiatric indications for late-term abortion in their cases. Our clients were not aware of legal restrictions and they believed that the stated reasons were sufficient enough for permission for abortion in the 2nd trimester. Distress reaction evidenced in the group A was additionally due to legal obstacles and complicated administrative procedures in the process of obtaining permission for abortion.

Active legislation in Serbia does not recognize emotional distress reactions due to adverse social circumstances, as indication for termination of unwanted pregnancies older than 12 gestational weeks. If mental disorder (psychosis, major depressive disorder) is not present, late-term abortion is not allowed. Our experience and results of this study, quite a contrary, point to the fact that unwanted pregnancy is unique life situation influenced by many personal and social factors which should not be neglected. Mujovic-Zornic [8] in his article also indicates the inadequacy of regulations that force woman to give birth to a child, she does not want.

Our results point to the fact that a change in social circumstances preceded claim for abortion at almost all pregnant women in our sample. Most of the women, even 82.26%, quoted this change as the main reason for demanding termination of their pregnancy, regardless the presence or the absence of psychiatric disorder "appropriate" for late-term abortion.

This begs the question of the importance of psychosocial factors for legal late-term abortion, even in the absence of severe mental illness. Medoff [9] indicates that comprehen-

sive studies with a large number of women in reproductive age, based on self-reported data, could define some important issues in sexual behavior. Possible psychosocial issues could influence decision making and delay of abortion. Providers should predict latter capability for motherhood in unfavorable life circumstances [10–13].

A study in Japan showed that continuation of unwanted pregnancy to the term was associated with health risk for mother and child. The psychological risk is depression, risk health behavior during pregnancy, lack of parental care, low birth weight [14]. Not less important is the fact that abortion in late gestational age is associated with the risk of surgical intervention as well as moral and ethical issues that consider "killing" of a baby, that has right to live.

Notably, little research attention has been paid to explaining why women seek for an abortion in the second trimester instead of the first. A study from England indicates multiple reasons for late abortion: lack of recognizing the signs of pregnancy, denied pregnancy, or rejection of pregnancy after changes in life circumstances [15]. Contributing factors are also difficulties in accessing gynecological services and emotional ambivalence towards parenting [16].

These findings are similar to ours. All the stated reasons for late termination of pregnancy in both groups were equally represented. Our patients based their late request for abortion mainly on changed circumstances in their lives. Late recognizing of pregnancy was always mentioned as additional reason at most of the women, but this was not the main issue for lateness. This occurred in the first two months of pregnancy, but after that period other reasons were the main ones. We expected difference between the groups regarding stated reasons for late request for abortion, but conflict relations and dissatisfaction with financials were equally present regardless psychiatric diagnose in pregnant women. Coleman et al. [17] had very similar results. They concluded that psychiatric disorder was not associated with unwanted pregnancy in all ages.

This could mean that a changed life situation, unfavorable family circumstances, lack of social support, risk sexual behavior, influence on decision making process may contribute to the request for late-term abortion [18]. In some cases, previously wanted pregnancy turned into unwanted. The conflicts within the household or with marriage partner were also present. Some married women had an affair and had difficulty to decide between abortion or deceiving their husbands. Most of them thought this a good reason to terminate pregnancy without any obstacles. They were distressed to find out this not legally possible.

Mental disorder was the main reason for psychiatric approval of late abortion in the group B. Findings in the literature indicate that women with any psychiatric disorder are not more likely to have undesired pregnancy, compared with healthy ones [19, 20]. They experience more often poverty, divorce, partner abuse and negative life events. Psychiatric disorder can affect woman's ability to care for child and her judgment about conception, recognizing pregnancy and ability for motherhood [21–23]. Psychiatric medication and toxic substances used in the first 3 gestational months, can damage the baby.

Our patients with mental disorder did not pay much attention to their amenorrhea, or gave no reason for delaying. Two of them were hospitalized for a long time and had chronic incomplete remission of schizophrenia and no control of their sexual activity. Drug addicts were taking heroin, anti-anxiety medications and analgesics during the whole time of pregnancy and had passive attitude till late in pregnancy. Married psychiatric patients, who had children, did not feel capable to have more children. All of the subjects in the group B were chronically ill, taking antidepressants and neuroleptics for a few previous years and also during their pregnancy. Two minors in our sample, 15 years old, suspected pregnancy but did nothing about it because they were hiding it until it was obvious, or denied it for fearing the reaction of their parents. The same reason was noted in the article of the Coleman [13], explaining that denial of pregnancy in teens might be correlated with their parent's negative response to their pregnancy and cultural attitudes towards unmarried mothers.

After psychiatric evaluation and denied or approved second trimester abortion, all the women were offered counseling. Gynecological counseling for birth control was also recommended. During the follow-up period of 6 months, none of them returned for further psychological help, and we have no data about further use of contraceptives.

The most disturbing result of our study certainly is that, despite being rejected for late-term abortion, almost all (30 out of 32) women from the group A obtained abortion within private gynecology practice or in suspicious circumstances. In the phone interview after six months, the subjects felt uncomfortable to talk about what had happened with their pregnancy. We got only brief answers: women did not want to explain much on how and where they carried out abortion. This observation raises questions about safety and potential health consequences of their late-term abortion.

The other very important question is: which factors have the strongest influence on performing abortion even despite law restrictions, within assumed risky circumstances? In our study logistic regression was used for calculating predictive values of parameters under the study for the outcome of unwanted pregnancy. The statistical model showed that adverse social circumstances and hostility were crucial for late termination of pregnancy.

The limitation of our study is a small sample size, which represents only those women who had some psychological reaction to restriction of abortion. We do not have data about women who accepted their previously unwanted pregnancy, without psychiatric consultation and also those who terminated pregnancy in the second trimester, without coming for psychiatric approval. We also have not got data about psychological and health consequences after late abortion. The question is being raised: could unwanted pregnancy and delivery harm mental health and could it be associated with postpartum psychiatric disorders? Is late termination of unwanted pregnancy less harmful for women's mental health than unwanted parenting? What are the risks for mental health and development of unwanted children?

This aspect of reproductive health requires more exploration, because late abortions are, in fact, carried out despite legal

restrictions. More information about accessibility of abortion service, upper time limits for termination of pregnancy and legal restrictions involved, should be available to women in reproductive age. Educative health prevention programs including education about contraceptive use might reduce this unwanted reproductive event. Valid data on 2nd trimester abortion in Serbia and conditions for its performance would show various aspects of this phenomenon. Medical providers should consider many different issues during the decision making process: consequences for physical and psychological health, contextual social circumstances and law restrictions.

Conclusion

Pregnant women with psychiatric indication for late-term abortion belong to lower socioeconomic and educational level compared to women without this indication. Pregnant women without psychiatric indication for late-term abortion are more often anxious and have distress reaction than women with psychiatric disorder. Social circumstances unfavorable for pregnancy, especially conflict family and partner relationship, as well as poor financial situation are associated with demand for late-term abortion in both groups of women. These factors are predictive for abortion as the outcome of unwanted pregnancy. The second trimester unwanted pregnancy outcomes in abortion despite legal restrictions. It would be important to have more accurate guidelines for mental health indications for legally approved late-term abortion, respecting social circumstances. It would be of great importance to include preventive measures to lower the risk of illegally performed late-term abortions.

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Effect of aqueous solution of stevioside on pharmacological properties of some cardioactive drugs

Uticaj vodenog rastvora steviozida na farmakološka svojstva nekih kardioaktivnih lekova

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Abstract

Background/Aim. Stevioside is a glycoside that supposedly possesses a number of pharmacodynamic effects such as anti-infective, hypoglycemic, along with the beneficial influence on the cardiovascular system. The aim of this study was to determine the effect of rats pretreatment with aqueous solution of stevioside on pharmacological actions of adrenaline, metoprolol and verapamil. **Methods.** Rats were administered (intraperitoneally 200 mg/kg/day) stevioside as aqueous solution or physiological saline in the course of 5 days, then anaesthetized with urethane and the first ECG recording was made. The prepared jugular vein was connected to an infusion pump with adrenaline (0.1 mg/mL), verapamil (2.5 mg/mL) or metoprolol (1 mg/mL). Control animals, pretreated with saline, in addition to the mentioned drugs, were also infused with the solution of stevioside (200 mg/mL) in the course of recording ECG. **Results.** The infusion of stevioside produced no significant changes in ECG, even at a dose exceeding 1,600 mg/kg. In the control group, a dose of adrenaline of 0.07 ± 0.02 mg/kg decreased the heart rate, whereas in the stevioside-pretreated rats this occurred at a significantly higher dose (0.13 ± 0.03 mg/kg). In stevioside-pretreated rats, the amount of verapamil needed to produce the decrease in heart rate was significantly lower compared to the control. The pretreatment with stevioside caused no significant changes in the parameters registered on ECG during infusion of metoprolol. **Conclusion.** The results suggest that pretreatment with stevioside may change the effect of adrenaline and verapamile on the heart rate.

Key words:
stevia; phytotherapy; electrocardiography; epinephrine; metoprolol; verapamil; rats.

Apstrakt

Uvod/Cilj. Steviozid je glikozid za koji se pretpostavlja da poseduje niz farmakodinamskih efekata kao što su antiinfektivni, hipoglikemijski, kao i uticaj na kardiovaskularni sistem. Cilj rada bio je da se utvrdi uticaj pretretmana pacova vodenim rastvorom steviozida na farmakološko dejstvo adrenalina, metoprolola i verapamila. **Metode.** Pacovi su tretirani (intraperitonealno 200 mg/kg/dan) vodenim rastvorom steviozida ili fiziološkim rastvorom pet dana, nakon čega su anestezirani uretanom i urađen im je prvi EKG. U preparisanu venu jugularis infuzionom pumpom potom je primenjivan adrenalin (0,1 mg/mL), verapamil (2,5 mg/mL) ili metoprolol (1 mg/mL). Kontrolne životinje, pretretirane fiziološkim rastvorom, osim navedenih lekova, primale su i rastvor steviozida (200 mg/mL) preko infuzione pumpe. Sve vreme dok su lekovi davani u jugularnu venu, sniman je EKG. **Rezultati.** Infuzija steviozida nije uzrokovala značajne promene EKG-a, čak ni pri dozi koja je prelazila 1 600 mg/kg. U kontrolnoj grupi doza adrenalina od 0,07 ± 0,02 mg/kg smanjila je srčanu aktivnost, dok je kod pacova prethodno tretiranih steviozidom taj efekat postignut pri znatno višoj dozi (0,13 ± 0,03 mg/kg). Kod pacova koji su prethodno tretirani steviozidom doze verapamila potrebne za izazivanje smanjenja srčane aktivnosti bile su značajno niže u odnosu na kontrolu. Prethodni tretman steviozidom nije izazvao značajne promene parametara koji su praćeni na EKG-u tokom infuzije metoprolola. **Zaključak.** Rezultati pokazuju da kod pacova pretretiranih steviozidom dolazi do promena dejstva adrenalina i verapamila na frekvenciju srca.

Ključne reči:
stevia; fitoterapija; elektrokardiografija; adrenalin; metoprolol; verapamil; pacovi.

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Introduction

Stevia leaves have been used by indigenous peoples in Paraguay and Brazil since before recorded history. *Stevia* became more widely known following the 1887, when it was discovered by botanist Antonio Bertoni. Due to its sweetness, *Stevia* has been given many names including honey leaf, sweet leaf of Paraguay, sweet herb and honey *herba* [1]. *Stevia* is used most in the countries of South America, much less in Europe, and since 1970 it has been widely used in Japan as sweetener of various beverages of mass use [2].

The major glycosides found in the leaves of wild *Stevia* plants are stevioside, rebaudioside A, rebaudioside C and dulcosides A and B [3, 4]. Other sweet diterpenoid glycosides which can be isolated include rebaudioside D and E [5].

The sweet taste of *Stevia* tea is due to stevioside, a glycoside that supposedly possesses a number of pharmacodynamic effects such as anti-infective, hypoglycemic, along with the beneficial influence on the cardiovascular system and on seborrheic skin and skin with acne. The importance and actuality of scientific studies on *Stevia* is emphasized in many papers [6-13].

Curi et al. [14] reported that *Stevia* extracts from 5 g of dried leaves administered thrice a day for 3 days to healthy volunteers lowered the plasma glucose levels. However, care should be taken interpreting these results as the plasma glucose level of the *Stevia* treated group was already significantly lower before the administration of the extract [14].

Intravenous administration of stevioside [95% pure, in doses of 50, 100 or 200 mg/kg body weight (bw)] resulted in a significant hypotensive effect in spontaneously hypertensive rats without adverse effects on heart rate or serum catecholamine levels [15]. In a study with humans stevioside (250 mg thrice a day) was administered for 1 year to 60 hypertensive volunteers [16]. After 3 months the systolic and diastolic blood pressure significantly decreased and the effect persisted during the whole year. Blood biochemistry parameters including lipids and glucose showed no significant changes. No significant adverse effect was observed and quality of life assessment showed no deterioration. The authors concluded that stevioside is a well tolerated and effective compound that may be considered as an alternative or supplementary therapy for patients with hypertension. Liu et al. [10] reported that the underlying mechanism of the hypotensive effect of administered stevioside in dogs (200 mg/kg bw) was due to inhibition of Ca^{++} influx from extracellular fluid. Also, Melis and Sainati [6] suggested that stevioside induced in rats a decrease in mean arterial pressure and promoted renal vasodilatation by lowering renal vascular resistance. The vasodilator effect is likely to occur *via* blockage of Ca^{++} channels similarity to verapamil. Stevioside reduces blood pressure by decreasing the vascular resistance *via* inhibition of extracellular Ca^{++} influx and by stimulating the release of a vasodilator prostaglandin. Stevioside also induces diuresis, natriuresis and reduction of Na^{+} reabsorption resulting in the reduction of extracellular fluid volume [17].

Preliminary human studies suggest that stevioside has an influence on the function of cardiovascular system, especially that stevioside can reduce hypertension [18, 19].

The European Food Safety Authority evaluated the safety of steviol glycosides, extracted from the leaves of the *Stevia rebaudiana* Bertoni plant, as sweetener and expressed its opinion on March 10, 2010. The Authority established an acceptable daily intake for steviol glycosides, expressed as steviol equivalents, of 4 mg/kg bw/day. On November 11, 2011, the European Commission allowed the usage of steviol glycosides as food additive, establishing maximum content levels for different types of foods and beverages [20].

The aim of this study was to determine whether stevioside can cause a significant interaction with some cardioactive drugs (adrenaline, metoprolol and verapamil) and modify their effect on the heart rate.

Methods

Stevioside in the form of white powder, purchased in Brazil (Stevita 100% Natural), was a spray-dried commercial formulation containing 95% of stevioside, purified from the *Stevia* leaves; Urethane puriss, (Reanal, Budapest, Hungary); adrenaline, (1 mg/mL, diluted to 0.1 mg/mL), Adrenaline ampoules, Jugoremedija Zrenjanin, Serbia); metoprolol, (5 mg/5 mL), Betaloc® ampoules, Astra Zeneca UK; verapamil (5 mg/2 mL), Isopamil ampoules®, Galenika, Belgrade, Serbia.

The experiments were carried out on adult Wistar rats of both sexes, bw 180-260 g. Before and during the experiment the animals had free access to food and water, with a 12-hour cycle of light and dark periods.

The pretreatment period lasted 5 days, during which the animals were injected intraperitoneally (*ip*) with daily doses of: saline solution (1 mL/kg bw) – the control group (C) or an aqueous solution of stevioside (200 mg/kg bw) – the experimental group (S).

On the 5th day, 2 h after the last injection, the animals were anaesthetized with 25% urethane and connected to the ECG, to take the initial recording. The jugular vein of animals was prepared and animals were connected to the infusion pump.

The animals from the group C were connected to the infusion pump containing one of the investigated cardioactive drugs: adrenaline (0.1 mg/mL), metoprolol (1.0 mg/mL), verapamil (2.5 mg/mL), or aqueous solution of stevioside (200 mg/mL).

The animals from the group S were connected to the infusion pump containing one of the investigated cardioactive drugs: adrenaline (0.1 mg/mL), metoprolol (1.0 mg/mL), or verapamil (2.5 mg/mL).

The infusion rate for verapamil was 0.1 mL/min, ECG was monitored 12 minutes during verapamil infusion got. The infusion rate for the other drugs was 0.2 mL/min, ECG was also monitored during 12 minutes of infusion with other drugs (adrenaline, metoprolol or aqueous solution of stevioside).

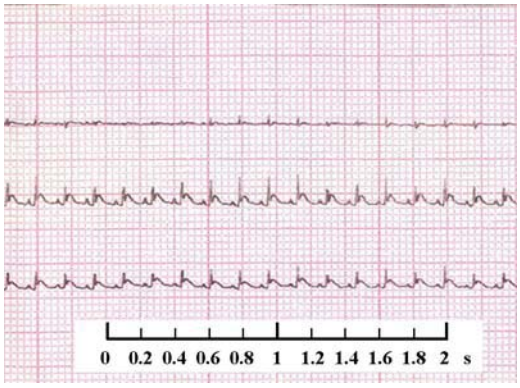
ECG analysis was performed in a single-blinded fashion. In all of animals in the group C and the group S ECG was recorded before application of cardioactive drugs. Also the investigator who conducted the experiment did not interpret ECG. It was interpreted by the investigator who did not know the treatment.

The ECG paper speed was 25 mm/sec, respectively one small block was 40 msec. On the basis of the time interval of infusion duration and the change in the ECG it was possible to calculate the amount of the drug required to produce the observed changes. This amount was correlated with the animal's bw to obtain the specific dose in mg/kg bw.

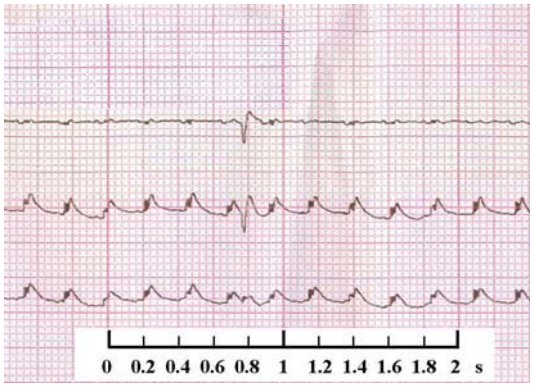
For all animals 5 min before the application of the investigated cardioactive drugs the control ECG was recorded. Therefore, each of the animal was a control for itself (Figure 1a).

The infusion pump and ECG machine started at the same time. During the application of cardioactive drugs changes in the ECG record were monitored.

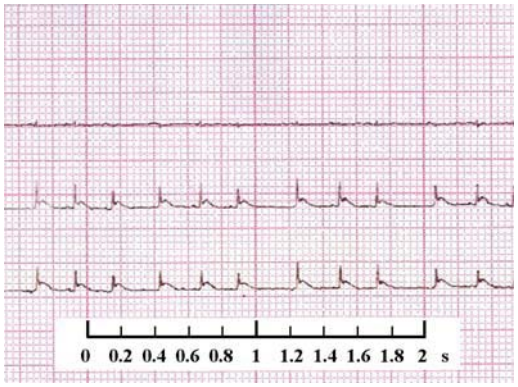
The changes that were observed on the ECG are: 1) the first changes – lonely change in the heart rate that was seen in ECG recording. This changes in the heart rate most frequently were extrasystoles (Figure 1b) or atrioventricular block (Figure 1c); 2) second changes – frequently changes in the heart rate that were seen in the ECG recording (bradycardia was the most frequent change in the heart rate (Figure 1d); 3) the third changes or toxic effect – changes in the heart rate that were seen in the ECG recording in the form of extreme bradycardia (Figure 1e) or asystolia (cardiac arrest) (Figure 1f). Extreme bradycardia was frequency of the heart less than 100 beats *per* minute.



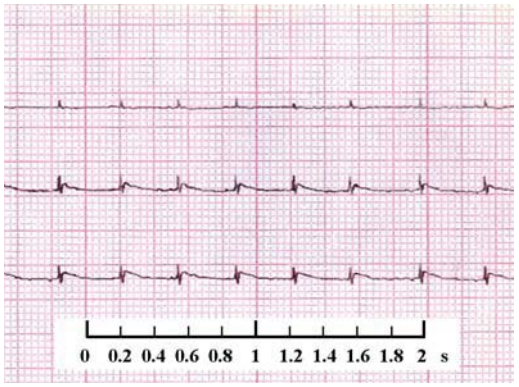
a) ECG record before the infusion of the drugs tested;



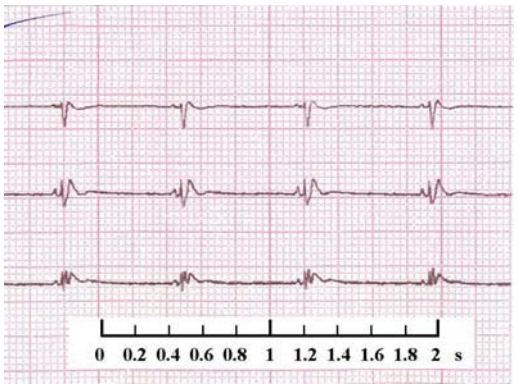
b) The first changes – extrasystoles;



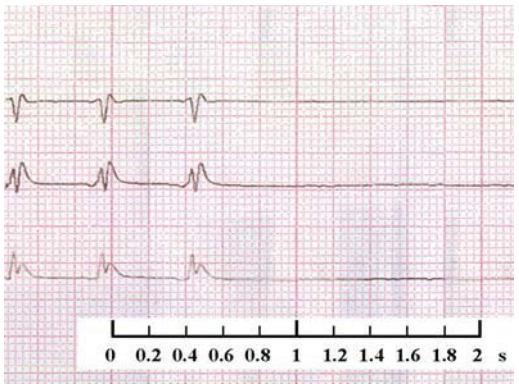
c) The first changes – atrioventricular block;



d) Second changes – bradycardia;



e) The third changes or toxic effect – extreme bradycardia;



f) The third changes or toxic effect – asystolia (cardiac arrest).

Fig. 1 – The changes that were monitored during infusion of the drugs tested.

Statistical analysis was performed by Student's *t*-test for small independent samples. Values $p < 0.05$ were considered statistically significant.

This experiment was carried out in accordance with the ethical principles of working with experimental animals (AEC approval Faculty of Medicine, University of Novi Sad, Novi Sad, Serbia).

Results

No toxic effect was observed in any case of stevioside infusion not even after the dose exceeding 1,600 mg/kg. ECG record of animals in the control group after infusion of stevioside was similar to the ECG record before infusion of stevioside. A decrease of frequency was observed in 2 out of 6 animals, and only after 240 s of infusion, which corresponded to the dose of 640 mg/kg. Further infusion of stevioside produced the normalization of the heart frequency after 180–300 s.

Infusion of adrenaline in all of the animals in the control group decreased the heart rate after the dose of 0.07 ± 0.02 mg/kg. None of the animals died but attenuated amplitudes of the QRS complex were observed in two cases after the dose of adrenaline of 0.56 ± 0.014 mg/kg. During infusion that lasted about 12 min, after the dose of adrenaline of 0.95 mg/kg, the changes in the ECG pattern that could indicate the occurrence of toxicity were not detected.

Pretreatment of rats with stevioside changed the sensitivity of the myocardium to adrenaline (Table 1). Namely, the decrease in the heart rate occurred significantly later compared to the control group of animals, at the doses of 0.13 ± 0.03 mg/kg. In this group of animals, an increased toxic effect of adrenaline was observed. Two out of 6 animals died after infusion of adrenaline of 0.82 mg/kg and 0.92 mg/kg, respectively.

The pretreatment of rats with stevioside decreased the sensitivity of the heart to adrenaline, and increased its toxicity.

The effect of verapamil on ECG of the control group and those treated with stevioside is given in Table 1. The infusion of verapamil in the control group caused the first reaction at the dose of 1.84 ± 0.38 mg/kg, while the second reaction was caused with the dose of 3.78 ± 0.89 mg/kg. Toxic effects during the infusion of verapamil in the control group were registered after the dose of 7.53 ± 1.45 mg/kg. All of the three evaluated parameters (the first, second and the third reaction of the heart to verapamil infusion) in the stevioside-pretreated group, occured significantly earlier comparing with the control group.

The effect of metoprolol on ECG of the control animals and those pretreated with stevioside is given in Table 1.

Metoprolol in the control group caused bradycardia but this change of the heart rate was not significant. It was similar in the group of animals pretreated with stevioside.

Toxic effects of metoprolol were not registered in neither the control nor experimental group.

Discussion

The present data confirm that pretreatment with stevioside play an important role in the increase or decrease of the sensitivity of the myocardium in experimental animals to the studied drugs.

The infusion of aqueous solution of stevioside produced no significant changes in ECG. Only in two cases a temporary decrease in heart rate was observed, but the initial value was restored within 3 to 3.5 min during continuous infusion of stevioside. Similar results were also observed in our first study, when the concentration of stevioside in infusion was 20 mg/mL[18]. Chan et al. [15] found that stevioside applied intravenously in the dose of 200 mg/kg in rats was effective in blood pressure reduction and there was no change in the heart rate. The heart rate in rats were about 350 beats *per* minute. Clinical studies also show that stevioside applied at a dose of 250 mg three times a day orally for one year in people with hypertension exerts antihypertensive effect but no effect on the heart rate [16]. The study confirms our results that stevioside at a high dose of 1,600 mg/kg did not significantly affect the heart rate.

Table 1

Effect of adrenaline, verapamil and metoprolol on the rat heart function monitored *via* ECG changes

Drugs	ECG changes		
	I	II	III
Adrenaline (mg/kg), $\bar{x} \pm SD$			
with S pretreatment	0.13 ± 0.03*	0.62 ± 0.23	0.81 ± 0.08
without S pretreatment	0.07 ± 0.02	0.56 ± 0.14	without toxic effect
Verapamil (mg/kg), $\bar{x} \pm SD$			
with S pretreatment	0.89 ± 0.17*	1.17 ± 0.34*	3.14 ± 0.92*
without S pretreatment	1.84 ± 0.38	3.78 ± 0.89	7.53 ± 1.45
Metoprolol (mg/kg), $\bar{x} \pm SD$			
with S pretreatment	1.89 ± 0.67	5.17 ± 0.42	without toxic effect
without S pretreatment	1.45 ± 0.41	4.84 ± 0.75	without toxic effect

S – stevioside-pretreated rats (200 mg/kg/day, intraperitoneally during 5 days); ECG changes: I – first visible; II – more continuous; III – toxic; $\bar{x}$ – mean value; SD – standard deviation. *$p < 0.05$ (statistically significant difference).

In both cases (with control the and stevioside-pretreated rats) the decreased heart rate was observed during the adrenaline infusion. This result can be explained by the fact that the rats are laboratory animals in which α1 adrenergic receptors are very sensitive to adrenaline, resulting in the significant vasoconstriction and reflex bradycardia during infusion of adrenaline. This fact was first pointed out by Turner in 1965 [21].

In this study, the pretreatment of rats with stevioside reduced the sensitivity of the myocardium to adrenaline (bradycardia occurred later compared to the control).

In our previous study, when the animals were pretreated with a lower dose of stevioside (20 mg/kg bw) the toxic effects (asystolia, cardiac arrest) were not reported [18]. But, the pretreatment with a higher dose of stevioside (200 mg/kg bw) increased thebsensitivity of the myocardium of rats to adrenaline, and cardiac arrest in stevioside–pretreated rats occurred after the administration of adrenaline in a dose of 0.81 ± 0.08 mg/kg bw.

The mechanism of the hypotensive action of verapamil is the blocking of calcium channels. The consequence of calcium channels blocking in the myocardium is the appearance of bradycardia. Stevioside, as reported in several papers is a calcium channels blocker. In stevioside pretreated animals, an increased sensitivity of the myocardium to verapamil was observed and the drug toxicity was significantly increased, too. Thus, a significantly smaller amount of verapamil was required to cause bradycardia in the stevioside–pretreated animals, which needed a significantly smaller amount of verapamil to cause toxic effects (cardiac arrest). In our previous study, when the animals were pretreated with stevioside at a dose of 20 mg/kg bw there was no report on the cardiac arrest after administration of verapamil. This indicates that cardiodepressive effect of stevioside in rats is dose–dependent.

Taking into account these facts it can be concluded that verapamil and stevioside applied together mutually potentiate their effect [6, 10, 15, 16, 18].

Pretreatment with stevioside showed the tendency to reduce the sensitivity of the myocardium to a beta-blocker, but the decrease of the heart rate did not occurr within 12 min of metoprolol infusion. The average decrease of heart rate was 14%, and only with one animal it was 25% of the initial value after 12 min of infusion. No toxic effect was observed, neither in the control nor in the stevioside-pretreated animals. The influence of metoprolol on the heart rate in our previous study, when the animals were pretreated with stevioside at a dose of 20 mg/kg bw was similar [18].

The additional reason for the interaction of stevioside and cardioactive drugs might be the consequence of the influence of stevioside on the regulation of the level of glucose as described in the literature [12]. Namely, treatment with stevioside results in the increase in C-peptide concentrations in healty and diabetic rats. Stevioside influences the function of the endocrine pancreas and stimulates insulin secretion [12]. The results from Jeppesen et al. [9] in the experiments on rats indicate that the treatment with stevioside exhibited hypoglicemic, insulinotropic and glucagonostatic effects, which might influence the sensitivity of the heart to cardioactive drugs actions.

Conclusion

Our results suggest that pretreatment with stevioside might change the effects of adrenaline and verapamil on the heart rate.

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GENERAL REVIEW

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Experimental and clinical use of meshes in urogynecology

Eksperimentalna i klinička upotreba sintetskih hirurških mrežica u uroginekologiji

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Key words:
urinary incontinence, stress; pelvic organ prolapse; surgical mesh; treatment outcome.

Ključne reči:
inkontinencija, urinarna, stres; karlični organi, prolaps; hirurška mrežica; lečenje, ishod.

Introduction

The most common problems in urogynecology, stress urinary incontinence (SUI) and pelvic organ prolapse (POP) are, in other words, problems of pelvic organ support. Extended demand for support in pelvic reconstructive surgery has led to the development of both biomaterials and synthetic grafts. The ideal biomaterial should be physically and chemically inert, mechanically strong, noncarcinogenic, easily fabricated and stabilized, and should induce minimal inflammatory reaction [1].

Experimental studies and mesh behavior

Several studies have analyzed biomaterials derived from dermal, pericardial, dural, and other anatomical sources, and have emphasized their non-uniform graft behavior, variable preparation, and unpredictable quality [2]. Synthetic grafts clearly demonstrated superior durability and long-term success over biomaterials for pelvic floor repair [3].

The most widely accepted method of testing synthetic grafts is through experimental animal studies. In analyzing the literature, it is obvious that rats are the most commonly accepted experimental model. Several study groups have set the standard for methods as well as tissue analysis and tensile strength in experimental studies [4, 5]. Primary repair of full-thickness abdominal wall defect, with respect to the peritoneum, seems like a logical choice. Monofilament polypropylenes were theoretically and practically the first choice for testing, considering the assumption that multifilaments are prone to infection [4]. There is consensus that an average of 90-μm pore size provides the best mechanical anchorage, with peak in-growth reached at around 400–500 μm [1, 2]. Larger pores limit the deposition of collagen to the perifilament region, with central parts being deposited with fat. A solid product, as well as one with smaller pores (< 50 μm), can lead to complete encapsulation or induce an intensive inflammatory reaction. In addition, the intensity of an inflammatory reaction is determined by the amount of synthetic material, therefore suggesting the use of lighter materials [1, 4 6].

It is presumed that multifilaments with interfilamentous spaces smaller than 10 μm are prone to infection, bearing in mind that macrophages are 15 μm in diameter, and bacteria 1–2 μm. Multifilaments were theoretically supposed to have better elasticity, but the same results are achieved with low weight monofilaments [4, 5]. After an initial polypropylene testing, some advances in biocompatibility were to be revealed experimentally on a polypropylene-coated field. Collagen-coated and titanium-coated grafts were to express a less intensive inflammatory reaction, with the same results, on a field of collagen deposition; however, they failed [6]. Arginine administration was also evaluated in experimental models for improved mesh integration, but the experimental data are inconclusive [7]. Some rather interesting data were revealed on initial experimental analysis of semi-reabsorbable (polypropylene-polyglactin) grafts [6]. Later studies determined that the semi-reabsorbable combination proved to be a stable scaffold for collagen deposition, leaving less foreign material after polyglactin reabsorption, with no consequences on tensile strength [5]. Experimental studies have revealed a totally new perspective on the mechanical analysis of incorporated grafts. Tensiometric studies of native graft samples have indicated significant differences in strength among tested samples. When colonized by cells and incorporated with native tissue, explanted samples provided comparable tensiometric strength, regardless of the material used [5].

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Some studies even reported that stronger tensiometric results were achieved with monofilaments than with multifilaments [4].

Experimental studies have suggested a high modality and uniform quality of synthetic meshes, with a rather steady retraction rate of about 20% [1, 4, 5]. Graft retraction seems to correlate with stiffness parameters in the elastic domain of graft deformation [5]. Retraction rates, when summarized, highlight the well-known postulate of tension-free surgery.

Consensus on a cascade Worman's effect is almost absolute. Protein covering of the graft and inflammatory reaction within the first three weeks are the initial steps of foreign body reaction [1]. The reparation process, regardless of the experimental animal model, is characterized by collagen deposition and final stabilization within six weeks [4, 5].

One study even analyzed cell oxidative stress levels in relation to the mesh material used [5]. Conclusions definitely confirmed a positive correlation between cell oxidative stress and the amount of implanted synthetic material. In summary, rat studies have shown good biocompatibility, respectable tensile strength, and fewer complications.

In several studies, rabbits were chosen as an experimental model, but they showed no difference in graft behavior [8]. Pigs are less common, considering that large animals are not as easy to manipulate, and the experimental samples are smaller. The highest level of retraction reported in an experimental study on pigs is 30% [9]. However, this result should be considered cautiously, as retraction was measured with millimeter paper alone. One of the interesting, but rare, experimental studies conducted on very large animals included horses [10]. Fifteen horses were treated with polypropylene meshes for large abdominal hernias and the results were exceptional, except for one fatality.

In summary, future perspectives seem to favor semireabsorbable, low-weight monofilament meshes, with totally inert synthetic grafts being the ultimate theoretic goal.

Mesh in the treatment of stress urinary incontinence

Stress urinary incontinence is a highly prevalent (41%) problem; 72% of patients have moderate to severe symptoms, with only 25% seeking professional help [11]. There is a prognosis that requirements for surgery will increase up to 45% in the future, due to less tolerance of SUI symptoms by patients and the normal aging of female population expected in western countries [12]. Synthetic materials have been generally used in the treatment of SUI after wide acceptance of the Petros and Ulmsten theory of continence [13]. The sling was moved from the bladder neck to the midurethral position and placed in a tension-free manner. The role of the sling is support of the urethra, instead of previous tension sutures placed on the bladder neck or a fascial sling placed on the same position [14, 15].

The sling is placed retropubically, transobturatory, or as a mini (single incision) sling. Tension-free placement has contributed to their non-obstructive behavior, low incidence of urinary retention, no exaggeration of prolapse, and decreased deterioration of the posterior compartment [16, 17]. Postoperative sexual dysfunction is infrequent, due to an unchanged vaginal axis, which is the most frequently recognized flaw of the Burch operation [18, 19]. Retropubic placement of the sling requires cystoscopy as a mandatory part of the procedure, and sling placement was limited in very obese patients and in patients with previous pelvic surgery and suspicious intestinal adhesions. Transobturatory placement performed "inside out" or "outside in" (both ways are almost equal, with "inside out" being slightly superior) [20, 21] became an acceptable alternative that does not require cystoscopy and does not affect the abdominal cavity. Mini slings are the least invasive, placed in a U position or horizontally. There were high expectations and hopes because of the convenient pain profile, but the results were not as encouraging, and their use is performed with caution [22–24]. There are also self-tailored modifications or industrial modifications of the sling, which are applied with similar or equal success rates [25, 26].

However, no one way of sling placement is absolutely complication free [27, 28]. This is especially important to keep in mind, because the surgery is performed with the aim of improving quality of life, and dangerous reported complications, such as bowel perforation and serious bleeding (the majority after retropubic sling surgery), can compromise the procedure seriously.

Success rate (effectiveness) is measured subjectively (interviews, different questionnaires), objectively (e.g. 24-hour pad test, stress test), or as a combined evaluation after surgery. Subjectively evaluated success is always higher than objectively measured one, and varies between 85.7% and 91.6%. The number of satisfied females with significant improvement is higher than the number of completely dry patients [29].

Unfortunately, dryness is not the only outcome of the surgery. Complication rates should not be neglected, but they are usually underreported. The reported complication rate is 4.3–75.1% for retropubic and 10.5–31.3% for transobturatory midurethral slings [30]. The most important predictive factors for sling failure are documented intrinsic sphincter deficiency (ISD) and fixed urethra. It is assumed, in other words, that in patients with a fixed urethra, the main problem is low resistance at the level of the bladder neck, and not urethral support, so the support improvement achieved by the sling is less likely to be curative. Fortunately, the results of tension-free tape (TVT) and adjustable slings in these patients are only a little inferior, or equal, to the results of TVT (66% and 85% cured patients, respectively) [30, 31]. There is a documented follow-up of patients with suprapubic TVT–11.5 years and transobturator tabe (TOT) – 6.5 years, without significant differences between the procedures. Both of these procedures have a stable, long-term success rate (77% and 83%, respectively) [32, 33]. The difference was not spectacular compared with the Burch Tangho operation (70% dry patients) [34].

Recurrent SUI is not an uncommon problem after tension-free procedures. The success rate is lower than after primary treatment (63.5% dry), but it is still acceptable. There is scarce evidence of favorable types of slings for the "redo" surgery, but the retropubic sling seems to be more effective than the transobturatory sling [35, 36].

Postoperative voiding dysfunction and urinary retention after sling procedures are underreported. Frequency of urinary retention (1.8–10%) is highest in patients with the retropubic sling, but it is not absent with the transobturatory or single-incision sling. It is usually temporary, and intermittent catheterization or catheter removal several days later solves the problem. Long-term retention (15 days) usually requires pulling down the sling or a sling section, or removal of the sling. In the majority of cases, it does not result in the recurrence of SUI [37–41].

There was a confusing terminology in previous years regarding the complications-sling exposition and erosion. It is accepted now that sling exposition describes a visible sling in the vagina, uncovered by the vaginal epithelium. Sling erosion is a penetration of the sling into the adjacent viscera. The more frequent surgical complications of the sling is exposition (13%) [41, 42]. Sling exposition is usually expressed as vaginal discharge, pain during intercourse, or sexual discomfort. Sometimes it occurs up to ten years after the surgery [42, 44]. Sling erosion is the consequence of such conditions as migration in the urinary bladder or urethra, or abscess formation. In extreme cases, consequent vesicovaginal or uterovaginal fistulas are possible [45, 46]. Possible explanations for sling exposition are insufficient surgical skill, structure of the sling material, or susceptibility of the hammock to the sling [42]. Sexual function could be improved (29.5% and 32.5% for TVT and TOT, respectively), deteriorated (17.3% and 12.5%), or unchanged after the sling procedures [47]. The vast majority of patients will have improvement in their sexual life, but statements about postoperative sexual function must be included in the informed consent before the surgery [47–49].

Overactive bladder symptoms and mixed urinary incontinence are not contraindications for the sling. Postoperative urgency could start before the surgery and remain after the surgery, or appear as a phenomenon *de novo* [50, 51]. Preoperative urgency is more frequent in patients; mixed urinary incontinence, age, nocturia, maximum cystometric capacity, and choice of sling procedure influence detrusor overactivity and urge urinary incontinence [52]. In the majority of cases, postoperative urgency is sensory and can be controlled with anticholinergics. If urgency persists, removal of the sling is mandatory [53].

Mesh in the treatment of pelvic organ prolapse

Pelvic organ prolapse is a frequent disease among the aging female population. Almost 11% of the female population up to the age of 80 will require some type of surgical correction of POP [54]. Generally, correction of POP is performed with native tissue (NAT repair), mesh adjunct (pure synthetic material, resorbable or not), or rarely, graft (biological material), is used to repair the pelvic floor. Corrections are usually undertaken because of the anatomical disorder (vaginal bulge) and/or coexisting symptoms of prolapse included in symptom/bothersome symptom questionnaires. Vaginal bulge is the most prominent symptom; others include pelvic pressure, associated incontinence or urinary retention and bowel emptying problems, and sexual dysfunction.

Several major influences on POP surgery have been recognized: extensive reports that some degree of prolapse is not obviously symptomatic, changing criteria for performing surgery, and differences in the evaluation of surgery outcome.

Normal anatomic prolapse variations were well recognized in a study of routine clinical examinations of asymptomatic females, and it was confirmed that POP stage I is present in 38%, and stage II in 35% of patients. If it is evaluated as a result of surgery, more than 75% will not meet the criteria for ideal, and 40% for satisfactory outcome [55]. To mitigate a tendency to surgically "overcorrect" the anatomy of asymptomatic females, the evaluation criteria have changed. Acceptable results of successful repair are absent prolapse beyond the hymen, no symptoms, and no need for additional treatment [56]. Therefore, the main advantage of the polypropylene mesh superior anatomical restoration of the female genitalia was lost. In anterior vaginal repair, the efficiency of mesh surgery was confirmed to be superior to that of non-mesh surgery, which has a failure rate of 29% [57]. Ten randomized control studies showed that synthetic absorbable mesh has the highest failure rate (23%), followed by biological graft (18%) and non-absorbable synthetic mesh (5%) [57]. Safety was highest in cases with absorbable mesh (exposure rate, 0.6%), followed by biological grafts (6%) and synthetic non-absorbable mesh (10%) [57]. Posterior repair, with or without mesh, is significantly less frequently reported, and there is no confirmed evidence of a clear superiority of the special method of posterior compartment repair [58]. Clinical experience suggests that mesh will surely keep its place in strict indications (mesh compared to non-mesh surgery: recurrent prolapse/success rate, 90.4% versus 54.8%, respectively; paravaginal defect, 97.6% versus 65.6%; badly damaged pelvic floor, 64% versus 22.9% [59, 60]. Symptoms after mesh correction are based on clinical studies, and use of the mesh was not proved in lower grade prolapse [61]. In other words, restoring anatomy does not mean restoring symptoms at the same time.

Apical defect, although less frequently discussed, is a normal prerequisite for the success of POP surgery. It can be performed with adequate success using a variety of surgical options: vaginal sacrospinous support with mesh (tailored or modified), or colposacral fixation performed by robotics, laparoscopy, or open surgery [62, 63]. Safety of the mesh surgery is lower, according to reported complications, some of them dangerous or life threatening [64, 65].

Sexual dysfunction increases constantly with age. The frequency of sexual dysfunction is up to 50%, and it has a multifactorial origin [66]. Surgeries of any type, as well as concomitant gynecological pathology, could deteriorate sexual function without reliable data to address the source of sexual dysfunction to the mesh directly [67]. However, the Food and Drug Administration (FDA) has recently issued warnings regarding increased complications of mesh surgery and the exaggerated justification for its use in clinical settings [68, 69]. Almost no one could ignore these statements [68].

Finally, all aspects of POP must be evaluated carefully: anatomy, symptoms, quality of life, late complications, and possible repeated surgeries [69]. We have to create a treatment design to meet the criteria of the patients more comprehensively, with native tissue repair whenever it is possible, and with mesh only when it is absolutely necessary.

Conclusion

Synthetic materials have been developed constantly during previous decades, but they have not achieved the standards of ideal foreign material. Their biologic behavior is still unknown, especially after long-term follow-up. Improvements in the treatment of stress urinary incontinence are remarkable, and polypropylene slings have met the criteria for the gold standard of treatment, both for the suprapubic and transobturatory routes of application. It is not the same for mini slings. Meshes in pelvic organ prolapse surgery are superior for the correction of anatomy, but the overall benefit of their use is not remarkable, and complications are more frequent. It is certain that recent experiences will substantially diminish the use of mesh in pelvic organ prolapse surgery, as the restoration of anatomy is not a single goal of surgery. However, its use will be kept for selected patients. Improvement of our knowledge, both fundamental and clinical, is necessary for superior pelvic organ prolapse repair.

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CURRENT TOPIC

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Protein expression, gene amplification, epidermal growth factor receptor mutations and lung carcinoma

Proteinska ekspresija, genska amplifikacija, mutacije receptora za faktor rasta epiderma i karcinom pluća

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Key words:
lung neoplasms; carcinoma, non-small-cell lung; gene amplification; gene expression; mutation; receptor, epidermal growth factor.

Ključne reči:
pluća, neoplazme; pluća, nesitnoćelijski karcinom; geni, amplifikacija; geni, ekspresija; mutacija; receptori, faktor rasta epiderma.

Introduction

Despite significant advances in the detection and treatment of lung cancer it causes the highest number of cancer-related mortality.

Recent advances in the detection of genetic alterations facilitated the development of potent and specific target therapies.

Epidermal growth factor receptor (EGFR)

EGFR belongs to the family of structurally and functionally similar ErbB or HER receptors on cell surfaces, which have intracellular tyrosine kinase activity. What all of these receptors have in common is that they consist of extracellular and membrane components to which ligand and intracellular components are bound *via* the tyrosine kinase activity [1]. Under normal conditions the activity of ErbB receptors is controled by ligands which belong to the group of EGF related growth factors [2].

Protein expression of EGFR and lung carcinoma

EGFR expression was found in normal tissue and lung carcinoma. In normal lung tissue, according to some authors, EGFR expression was localized to the pole with ciliated bronchial cells, whereas EGFR expression was not detected in the bronchial glands [3]. According to others, however, increased protein expression of EGFR in the bronchial epithelium was localized in the basal layer of epithelial cells (Figure 1). Increased EGFR expression is present in 40–60% of primary non-small cell lung carcinomas (NSCLC) and in all histological types, although with squamous cell carcinoma the levels were higher [4]. According to some studies the level of expression is associated with the disease prognosis, as a higher level of expression is found in cancer patients who are in stage III of the disease compared to patients in stages I and II. Moreover, some studies have shown that tumors with high EGFR expression have larger metastatic potential, poorer histological differentiation and higher proliferative potential [5, 6]. EGFR expression is determined by immunohistochemical method, and evaluated by semiquantitative method (Figure 2).

EGFR gene amplification and lung carcinoma

The main mechanism of increased protein expression of EGFR is gene amplification.

Six basic types of EGFR gene amplification in NSCLC have been defined. According to some authors and analyzed by fluorescent *in situ* hybridization (FISH), most often, in about 60% of cases, the classic form occurs in the shape of large clusters with more than 20 gene copies. This type of characteristics is the amplicon with the „homogenously staining region". In about 10–15% of tumors with amplification, there is another form, created by forming small clusters containing 4–10 EGFR gene copies. The third form of gene amplification (15–20% of tumors), EGFR amplicon includes the CEP 7 sequence so that a cluster of EGFR and CEP 7 signals occurs. In about 5% of the tumors, a fourth form of EGFR gene amplification occurs, with atypically large and

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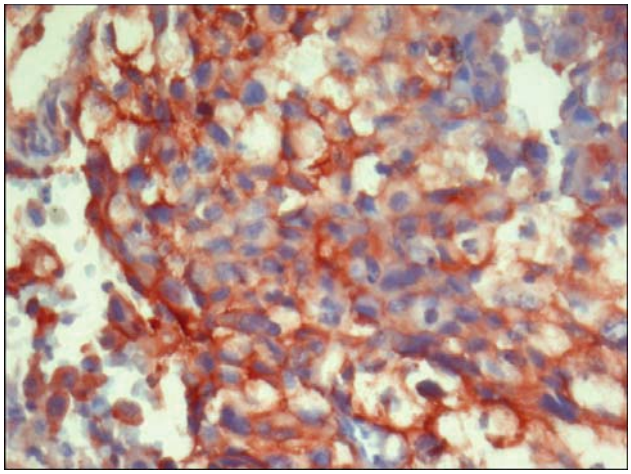

Fig. 1 – Strong, complete membranous staining of tumor cells within lung adenocarcinoma
(anti EGFR antibody, × 200).

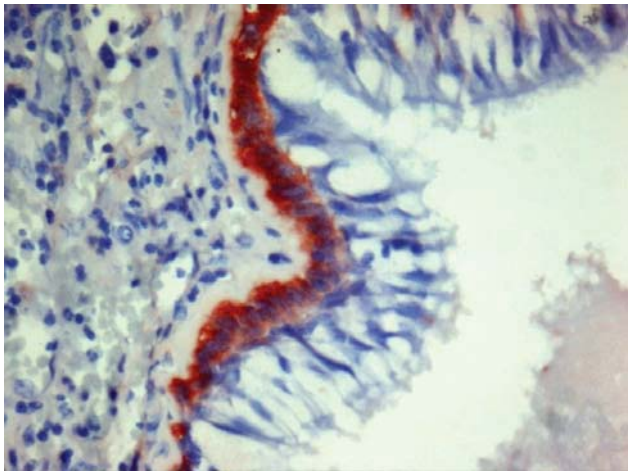

Fig. 2 – EGFR protein expression in the basal cell layer of the bronchial epithelium
(anti EGFR antibody, × 200).

bright signals. In 1% of the tumors, a fifth form of EGFR gene amplification can be found, in the form of fine volatile signals that represent extrachromosomal „double minute". The last, sixth form of EGFR gene amplification occurs in approximately 5% of the tumors as a large number of copies in cells with chromosomal aneusomy [7]. The incidence of the increased number of gene copies, i.e. gene amplification of EGFR in NSCLC, ranges, in published work, between 8% and 10% [8, 9].

Even though FISH is the standard technique for the detection of gene amplification, a disadvantage of this method is that the fluorescence signals are not stable and may fade or disappear within weeks. In addition, it is more difficult to observe the morphology of cells. Some of these issues can be overcome by applying chromogen *in situ* hybridization (CISH). CISH signals do not disappear over time, and it is possible to distinguish stromal and tumor cells [10] (Figures 3 and 4). Gallegos et al. [11] were the first to, in order to analyze the CISH method for the detection of EGFR gene copies in NSCLC, compare the results obtained by FISH and CISH method in the same group of patients and there was concordance of results using these two techniques. It has been confirmed earlier that CISH provides similar results to FISH regarding the detection of HER2/neu gene in breast cancer [10, 12, 13].

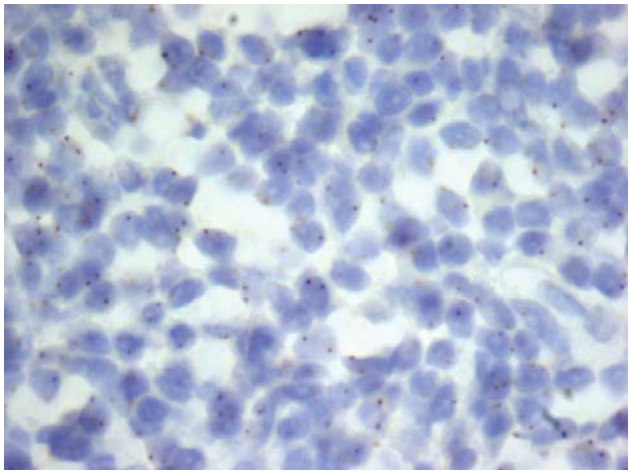

Fig. 3 – Tumor cells of lung adenocarcinoma with normal EGFR gene copy number
(anti EGFR antibody, × 200).

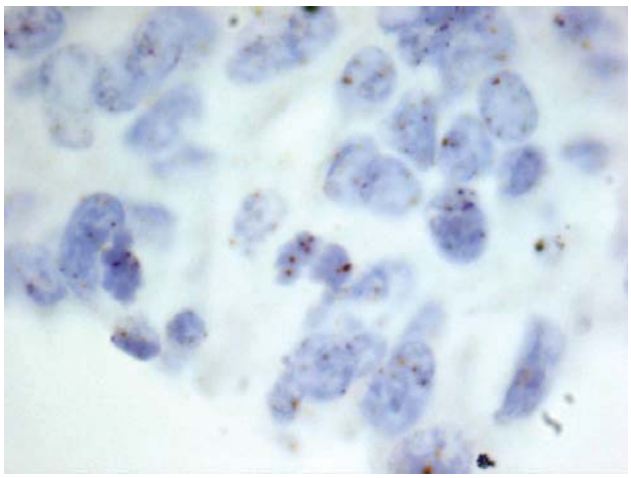

Fig. 4 – EGFR gene amplification in tumor cells of lung adenocarcinoma
(anti EGFR antibody, × 200).

Mutation of the gene for EFGR in lung carcinoma

The EGFR gene is located on the 7p12 chromosome.

Even though a great number of EGFR gene mutations has been documented so far, out of the seven exons encoding the TK domain (exons 18–24), mutations usually occur in the first four exons. A limited number of mutations has been identified in lung adenocarcinomas. These mutations lead to an extension in receptor activation once the ligand is bound. In addition, a consequence of mutations is a change in the TK domain, especially in places near the ATP binding site and the signal activation of pathways that lead to apoptosis resistance [14].

The most commonly used methodology for this purpose has been, and will probably continue to be, direct sequencing of PCR products. The main drawbacks of this method are its

low sensitivity (20–50%) and the significant risk of contamination involved in handling post-PCR products [15–17]. Furthermore, recent advances in molecular techniques have enabled the development of more sensitive methods for detecting mutations with real-time quantitative PCR, using specific probes or amplified refractory mutation system (ARMS™) technology [18]. Most recently, the development of EGFR mutant-specific antibodies for immunohistochemistry (IHC) has presented a new method for consideration [19, 20].

The limit of detection (LOD) of the real-time PCR method is lower than that of direct sequencing while the mutation specific IHC produces excellent specificity. It is necessary to have methods available that give us access to results rapidly and accurately [21].

EGFR gene mutation is more common in nonsmokers than in smokers (51% *vs* 10%), with adenocarcinoma than with other types of cancer (40% *vs* 3%), in patients of Asian descent than those of different descent (30% *vs* 8%) and in women compared with men (42% *vs* 14%). According to some authors EGFR mutation status is not associated with the patient's age, clinical stage of the disease, histologic characteristics and overall survival rate, and was not found in normal lung tissue [22, 23]. According to others, mutations in the tyrosine kinase domain of the epidermal growth factor receptor have prognostic significance, since patients with EGFR-mutant NSCLC have prolonged disease-free survival, compared with those with wild-type disease, regardless of the treatment received [24, 25].

Gene amplification is known to increase the expression of oncoproteins, as is the case with ErbB2 and breast cancer. The combination of gene amplification and mutation in lung carcinoma is relatively rare. However, studies show that EGFR amplification occurs more frequently in tumors with mutations [22, 23]. EGFR gene amplification is detected in some EGFR mutation–positive patients, and it is reported to be associated with disease progression [26]. A subset of lung adenocarcinomas shows activation of EGFR by mutations and/or amplification but the interaction between them is complex and unclear. Amplification can also occur independently, without the mutation, and may explain the increased expression of EGFR in squamous cell carcinomas and other epithelial tumors of the lung [22, 23].

EGFR inhibition in carcinomas

Significant progress has been made in recent decades in the study of specific cellular, molecular and genetic mechanisms that contribute to tumor growth and progression. This progress has instigated development and clinical evaluation of various tumor-specific anticarcinoma therapeutic approaches. A promising antitumor strategy is the inhibition of EGFR signaling. The best studied mode of EGFR inhibition includes monoclonal antibodies (mAbs), which are aimed directly against the extracellular part of EGFR and a small molecule of TK inhibitors (TKI) is directed against the very TK. Monoclonal antibodies bind to the ligand-binding site in the extracellular domain, while the TK inhibitors bind to the cytoplasmic TK receptor domains [27].

Great efforts have been invested into the strategy of choosing the patients for TK inhibitor therapy in those with NSCLC. A history of smoking, performance status, drug-induced itching and molecular biomarkers such as EGFR mutations, EGFR protein expression (immunohistochemically analyzed) and EGFR gene amplification (analyzed by fluorescent *in situ* hybridization) are factors that point to a better response to treatment with TK inhibitors.

The 2 TKI agents currently approved for use in lung cancer, which target lung cancer with EGFR mutations, are gefitinib (2002) and erlotinib (2003). EGFR mutation is a specific target for therapy by TKIs and is a validated biomarker of a treatment response. The clinical utility of this biomarker is supported by prospective clinical trials that have demonstrated a progression-free survival benefit of TKI as first-line therapy in EGFR-mutant patients. Based on current data, predictive biomarker tests for EGFR should involve mutational analysis. EGFR FISH testing is less predictive of TKI response rate than mutation testing in clinical studies, and currently should not be used as a method for EGFR TKI treatment selection [28].

Susceptibility and resistance to EGFR inhibitors

Most frequently detected mutations are those that determine the TK region, i.e. deletion mutations with or without insertion in exon 19 and missense mutation in exon 21. These mutations are more common in patients of Asian descent than in patients of different descent (40% *vs* 19%) and are more common in patients with adenocarcinoma, nonsmokers and women, although they are found in other groups of patients. The response to erlotinib and gefitinib treatment is stronger in patients with mutations than in patients with wild-type EGFR. All EGFR mutations are heterozygous, i.e. affect only one allele and have a dominant oncogenic effect, regardless of the presence the wild-type allele. Mutations are usually located near the ATP binding sites, but also on places where gefitinib binds. It can be assumed that mutations lead to achieving a more stable binding of ATP but also of its competitive inhibitor gefitinib. This could explain the increased activity after receptor ligand binding, as well as the greater susceptibility to the TK inhibitor therapy. Susceptibility to gefitinib has also been demonstrated in patients without EGFR mutations, suggesting that other mechanisms, such as gene amplification can sensitise tumor cells [29–35].

Resistance to TK-targeted therapies rises as a problem. This resistance was first identified in patients with advanced chronic lymphocytic leukemia and an association with point mutation was found or, less commonly, with gene amplification. In gastrointestinal stromal tumor and NSCLC, resistance to TK inhibitor therapy is associated with some types of TK mutations. Research showed that EGFR mutation is connected to resistance to therapy in NSCLC, T790M, and that it causes a blockage in TK inhibitor binding to the ATP site (secondary resistance). In addition, activating mutations GTPase and K-ras also lead to primary resistance [36, 37].

EGFR status and the clinical-pathological parameters of lung cancer with specific reference to adenocarcinoma

According to the current WHO classification, the histological types of adenocarcinomas are: papillary, acinar, solid and the bronchoalveolar. The majority of tumors was built from a combination of these histologic patterns and has thus been classified as a mixed form of adenocarcinoma. However, certain types and subtypes of tumors, i.e. adenocarcinoma of the lung, are associated with the corresponding molecular alterations [38]. Moreover, the subject of numerous studies is the connection between the smoking status, disease stage, tumor differentiation, as well as the patient's gender with the molecular status and especially with the EGFR profile of the tumor.

In their study, Hirsch et al. [39] found no difference when it comes to the respondents' gender, smoking status and histological type of tumor between the groups of patients with and without EGFR gene amplification, demonstraded *via* FISH analysis. In their work they analyzed patients with bronchoalveolar carcinoma in an advanced stage and came to the conclusion that the presence of EGFR gene amplification may be a predictor of better prognosis and better therapy response in patients treated with gefitinib.

Đačić et al. [9] found no correlation between the clinical-pathological parameters and the protein expression of EGFR in the tumor, but have found a greater occurence in the presence of gene amplification in poorly differentiated squamous cell carcinoma.

In patients with lung adenocarcinoma with broncholoalveolar characteristics who were treated with gefitinib and erlotinib the response to the treatment was better [40, 41].

Many studies analyzed K-ras and EGFR alterations in adenocarcinomas of the lung. The frequency rate of both mutations ranges between 10% and 30%. EGFR mutations are more common in the Asian population, non-smokers and nonmucinous tumors whereas K-ras mutation is more common in the non-Asian population, smokers and invasive mucinous adenocarcinoma, and these mutations are mutually exclusive [42].

Under the new proposed classification of lung adenocarcinoma (International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma) [43] certain types of lung adenocarcinomas differ based on molecular alterations, too, and so it is possible to, based on histological appearance of the tumor, predict its molecular profile, with greater or lesser certainty. EGFR mutations are more frequently found in adenocarcinoma *in situ* (AIS), adenocarcinoma with predominantly lepidic growth (LPA), papillary and micropapillary adenocarcinoma, although they may be found in other histologic types. In a large study on 806 NSCLC, EGFR mutations were found more frequently in adenocarcinoma previously classified as BAC or adenocarcinoma with lepidic growth (under new classification AIS, minimally invasive adenocarcinoma – MIA, and LPA). Predominantly solid type of adenocarcinoma is more frequently associated with the presence of K-ras mutations [44].

EGFR gene amplification is more frequently found in primary tumors than in metastases and is more often found in invasive tumors and more poorly differentiated types than in precursor lesions such as atypical adenomatous hyperplasia (AAH) and earlier bronchioalveolar cancer, indicating greater aggressiveness of these tumors in the presence of EGFR gene amplification [45–47].

In our study, performed on 90 patients with lung adenocarcinoma, the presence of EGFR protein expression in tumors was found in 37.78%, while the EGFR gene amplification in tumors, analysed using the CISH method, was present in 11.11% of respondents. Multivariate analyses were conducted on the group of patients with present EGFR protein expression in tumors, and certain characteristics were distinguished: mixed adenocarcinoma with broncholoalveolar characteristics, solid and papillary type, moderately and poorly differentiated tumors, smokers and women; while the analyses conducted on the group of patients with the presence of EGFR gene amplification in tumors distinguished the following characteristics: mixed adenocarcinoma with bronchioalveolar characteristics, papillary and other variants of adenocarcinoma, moderately differentiated tumors, non-smokers and women. There was no difference regarding the presence of protein expression and gene amplification of EGFR in the tumor in relation to T and N status of the patient, recurrence of disease, nor between stages I, II and IIIA of the disease [48].

Conclusion

The classification algorithm, based on histological and molecular characteristics of tumors, is useful in the selection of therapy that targets molecular alterations. In the era of genomics and proteomics, progress is expected in early diagnosis of lung cancer, its classification and selection of therapeutic agents. The profile of the disease can be induvidualised, and with it the choice of therapy for each patient, which will in the future certainly lead to more successful treatment and longer survival rates of patients with lung cancer as well as those with malignant tumors in general.

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CASE REPORT

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Traumatic mesenteric pseudocyst

Traumatska mezenterijalna pseudocista

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Abstract

Introduction. Mesenteric pseudocysts have rarely been described in literature. They belong to a group of mesenteric cysts that are very rare intra-abdominal pathology regardless of the origin. The diagnosis is often difficult to make, because of the diversity of clinical symptoms. The definitive histopathological diagnosis determines the origin and further course of treatment. **Case report.** We reported a patient with post-traumatic mesenteric pseudocyst. It was localised on the mesenteric side, in the direct contact with the small intestine. We surgically removed the pseudocyst along with a part of the small intestine with success. The patient's recovery was eventless, with no complications. **Conclusion.** Only by complete cyst removal, the definitive, accurate histopathological diagnosis and classification can be made.

Key words:
mesentery; cystis; diagnosis, differential; digestive system surgical procedures.

Apstrakt

Uvod. Pseudociste mezenterijuma veoma su retko opisivane u literaturi. One pripadaju grupi cista mezenterijuma koje su, bez obzira na poreklo nastanka, veoma retka intrabdominalna patologija. Dijagnoza je često teška zbog različitosti kliničke simptomatologije. Krajnja patohistološka dijagnoza određuje poreklo i dalji tok lečenja. **Prikaz bolesnika.** Prikazali smo bolesnika sa posttraumatskom mezenterijalnom pseudocistom, lokalizoavanom na mezenterijalnoj strani u neposrednom kontaktu sa tankim crevom. Hirurški smo uspešno odstranili pseudocistu zajedno sa delom tankog creva. Oporavak bolesnika bio je uspešan. **Zaključak.** Definitivnu i tačnu patohistološku dijagnozu moguće je postaviti samo posle potpunog odstranjivanja ciste.

Ključne reči:
mezenterijum; ciste; dijagnoza, diferencijalna; hirurgija digestivnog sistema, procedure.

Introduction

Mesenteric cysts are true rarities of intra-abdominal tumor pathology. They can be found along the entire mesentery of the small intestine, colon and rectum [1]. Fluid-filled cysts are usually solitary, but may be multiple and/or multilocular also [2, 3]. The preoperative diagnosis is often difficult and unclear. Clinically, they are usually presented as tumor mass in the abdomen. The most common symptoms are abdominal pain and disturbance of intestinal passage such as constipation and/or vomiting [4, 5]. The most common complications are ileus and volvulus [6]. Multi-slice computed tomography (MSCT) scan and ultrasonography (US) are proved to be the most successful diagnostic procedures [7]. There are many divisions and classifications of these cysts, but the most important and accurate one is histopathological [8, 9]. A successful method of treatment is surgery, either laparoscopic or open approach [4, 10].

We presented a case with large mesenteric cyst caused by blunt abdominal injury in a car accident.

Case report

A 55-year-old man came to the internist due to a hypertensive crisis, with an arterial blood pressure (BP) value of 230/120 mmHg. The patient know about his arterial hypertension, yet did not go to recall examinations nor took drugs regularly. Since he complained about mild abdominal pain, an abdominal US examination was performed revealing a tumor formation. Physical examinations confirmed the existence of the tumor in the left side of the abdomen, occupying the space of the left hypochondrium and the lateral quadrant of the mesogastrium. It was determined by palpation to be round-shaped, about 15 cm in diameter, slightly sensitive and painful. The laboratory findings including complete

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blood count and clinical biochemistry tests were within normal range, with exception of cholesterol and triglycerides which were elevated. The patient started receiving antihypertensive therapy followed by normalization of BP. Repeated US confirmed tumor formation, which was seen as a hypoechogenic cyst approximately 15 × 12 cm in diameter, with a hyperechoic wall and posterior amplification. MSCT scan showed a hypodense circular formation with hypoechogenous content, which was in direct contact with the jejunal loops, unevenly thin with partially calcified wall, without a change in postcontrast attenuation. In the peritoneum and retroperitoneum no enlarged lymph nodes were found nor the presence of free intraperitoneal fluid (Figure 1). Irrigography excluded the possibility of infiltration of the colon, external compression of the caudal part of the transverse colon was seen, without lumen narrowing. The patient had no previous surgery. The patient stated a traffic accident two years ago, in which he received a blunt impact in the abdomen, without any significant consequences.

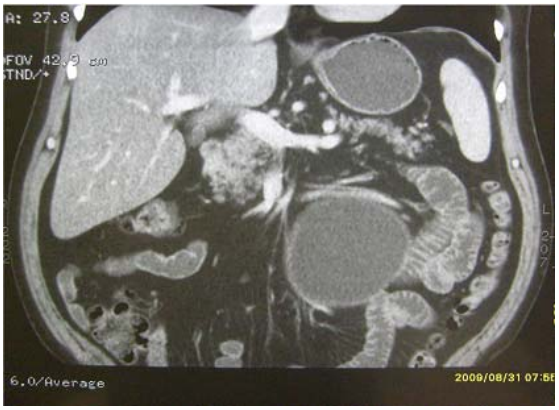

Fig. 1 – Cystic round formation in the close contact with jejunal loops on the frontal sections of multislice computed tomography abdominal scan.

The patient was operated on under general anaesthesia, with an open surgical approach, medial laparotomy. A tense, cystic and oval tumours change was found intraperitoneally, and a slightly tense wall, and direct contact to the proximal part of the jejunal mesentery (Figure 2). The unilocular cyst contained a residual viscous mass similar to milk. An attempt of enucleation was compromised by the vascularization of the jejunum, so partial resection of the jejunum along with the cyst was performed (Figure 3). The continuity of the small intestine was established by end-to-end anastomosis. The postoperative course was eventless. The patient was introduced to peroral food intake on the postoperative day 2 and abdominal drain was removed 4 days after the surgery. The biochemical content of the cysts showed no presence of amylase, while the level of cholesterol and triglycerides was high, and the microbiological culture was sterile.

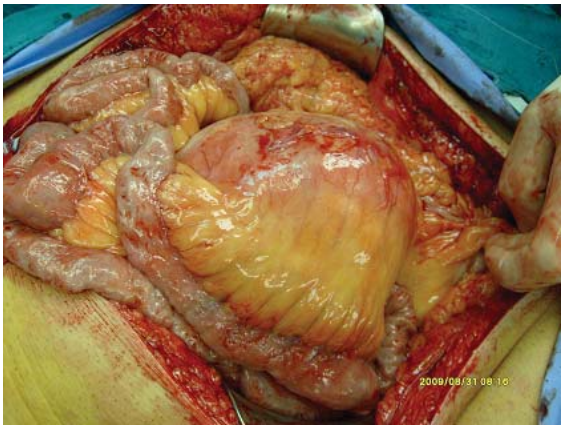

Fig. 2 – Intraoperative findings of a solitary unilocular mesenteric cyst.

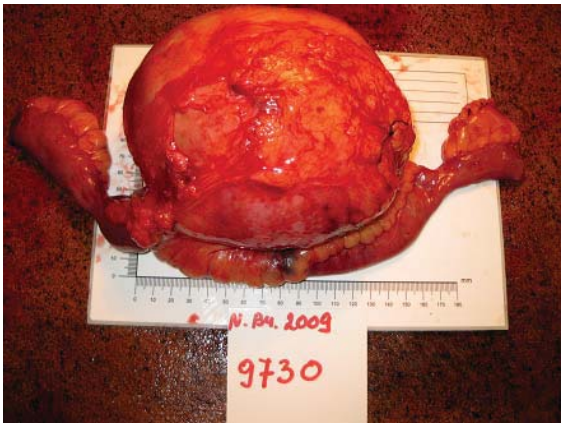

Fig. 3 – A specimen of extirpated unilocular mesenteric cyst with a jejunal loop and thin and smooth wall.

The definitive histopathological examination showed that the wall of macroscopic tumor formation was formed from multiplied acellular connective tissue, and on its outer side an adipose tissue were found. The connective tissue was impregnated with small groups of lymphocytes, as well as foci of calcification. Groups of foamy histiocytes were located on the inner surface. The presence of epithelium was not found (Figure 4). Amorphous eosinophilic material impregnated with small groups of lymphocytes siderophages was located in the lumen. The findings corresponded to the mesenteric pseudocyst.

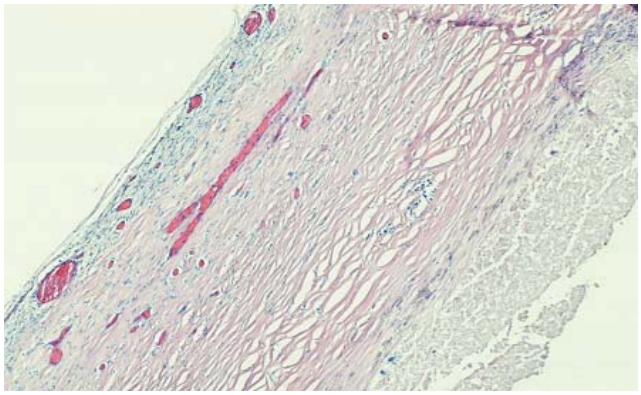

Fig. 4 – Pseudocyst wall consists of multiplied acellular connective tissue, and on its outer side, adipose tissue, while the connective tissue is impregnated with small groups of lymphocytes, as well as foci of calcification. The epithelium was not found (HE, × 5).

Discussion

Benivieni, a Florentine anatomist, first described a mesenteric cyst in 1507, during the autopsy of an 8-year-old boy [11]. The first resection of a cyst was performed in 1880 by Tillaux and Millard [12]. The first successful laparoscopic resection was presented in 1993 by Mackenzie et al [13]. The original classification divides cysts into 4 categories [14]: embryonic, traumatic, neoplastic and infectious. This division clearly indicates the etiology of cyst formation. There are different classifications of cysts, but certainly, the most reliable are histological and immune histopathological classifications, which divide cysts into several groups [8, 9]. In 2000 de Perot et al. [8] proposed a 6-group classification based on histopathology: lymphatic cysts (simple lymphatic cyst and lymphangioma); mesothelial cysts (simple mesothelial cyst, benign cystic mesothelioma, and malignant cystic mesothelioma); enteric cysts (including enteric duplication cyst); urogenital cysts; mature cystic teratoma (dermoid cysts); and pseudocysts (infectious and traumatic cysts).

Several years later a new classification was proposed, based on immunohistochemical findings, as a better prognostic predictor of biological behaviour [9].

Traumatic cysts are relatively rare in intact blunt abdominal traumas, and only several cases are described in the literature [15–17]. They are the result of mesenteric lymphangitis and rupture or hematoma with subsequent resorption and cystic degeneration in most cases [15]. They can be found along the entire mesentery of the small intestine, colon and rectum. They can also be found in the omentum and retroperitoneum. Cysts are usually solitary, but may be multiple and/or multilocular. In any case, whether they are mesenteric, omental or retroperitoneal, the cysts originate from the same embryonic structures [2, 3, 18]. The content of the cyst can be chylous, bloody or purulent [6, 14, 15, 18]. It is clear that only traumatic and infectious pseudocysts do not have epithelium, resemble a pancreatic pseudocyst. The size of cysts may range from a for centimeters up to 10–15 cm [7], as in the presented case, with the literature refers the size of up to 36 cm in diameter [2].

The incidence of mesenteric and omental cysts is low; it is higher in the cases of paediatric hospitalized patients and it is approximately 1/20,000, comparing to the adult population which is about 1/140,000 [2, 19, 20]. In cases of traumatic pseudocysts, considering the incidental etiology, the frequency is unknown. Clinical symptoms are nearly always nonspecific, and presented as intra-abdominal tumor formation which is often incidentally discovered during abdominal US, with exception of uncomplicated cysts. Depending on localisation, they can mimic a pancreatic pseudocyst often, small intestine tumor, or paraovarial cyst [4, 5]. The most common symptoms are abdominal pain and disturbance of intestinal passage, while the most common complications are cyst torsion, rupture and ileus of the small intestine or the colon [6]. In such cases, acute abdominal condition exists, where it is almost impossible to determine the preoperative diagnosis. Unlike adults who can have asymptomatic cysts, children ussualy have symptomatic cysts which are often presented as an acute abdominal condition [21]. US is certainly the most reliable method for preoperative diagnosis [7], but CT scan is very helpful and should be the integral part of preoperative staging. Magnetic resonance imaging can also be helpful in unclear cases [15].

The method of successful treatment is surgical one, which can be open or minimally invasive, such as laparoscopic approach. This kind of surgical method can be applied even in emergency cases when the preoperative diagnosis is accurate [4, 10]. The classical surgical way of cyst solving involves simplex drainage, enucleation, resection or marsupielisation (not suitable because of the possibility of infection and relapse) [2]. Applied surgical method depends on urgency, cyst's localisation, surgeon's experience and the existence of complications such as rupture or volvulus. In our case, we applied the open surgical approach, since the preoperative findings showed that the cyst's wall was in direct contact with the wall of the small intestine. Intraoperatively, we excluded enucleation due to compromising vascularisation of the small intestine. The most favourable surgical procedure for cyst solving is enucleation, but in every third case resection is required. Resection is necessary in adults ussualy, while in children it is required in 50% of cases of mesenteric cysts [2, 18, 22]. However, resection is a method of curative treatment and the laparoscopic approach is the method of choice, as a minimally invasive technique [10, 23]. In most cases, complete resection of cysts usually ends up without intraoperative and postoperative complications. Cyst recurrence is possible in cases with partial resection. The literature describes cases of cyst recurrence after laparoscopic approach [24]. A case of malignant transformations of mesenteric cysts has also been described [2, 25]. In these cases, surgical treatment is undoubtedly needed.

The presented patient informed us about abdominal injury that indicated the traumatic origin of abdominal mass formation. Mesenteric injury probably caused a rupture of small blood vessels with bleeding which spontaneously stopped. After a subsequent resorption of hematoma the pseudocyst remained. Another possible cause of the pseudocyst could be a rupture of lymph vessels with gradual chylous leakage. However, macroscopical findings of viscous fluid similar to milk into pseudocyst, and the presence of siderophages on histopathological examination in the pseudocyst's lumen supported the first pathophysiologic mechanism of pseudocyst evolution. Imaging procedures were helpful, although the definitive diagnosis was made only after cyst extirpation and histological examination.

Conclusion

A traumatic mesenteric cyst is a very rare abdominal pathological finding. Surgical treatment is the method of choice, either open or by the laparoscopic approach. Both techniques are used with equal success. The definite and accurate histopathological diagnosis can be made only by complete cyst removal as in the presented case.

Confilict of interest statement

There was no conflict of interest among the authors.

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CASE REPORT

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Double-hit primary unilateral adrenal lymphoma with good outcome

Double-hit primarni limfom nadbubrežne žlezde sa povoljnim ishodom

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Abstract

Introduction. Primary adrenal non-Hodgkin's lymphoma (NHL) is a rare neoplasm with poor prognosis. On the other side, double-hit lymphomas with BCL2 and MYC translocation are characterized by advanced disease stage, extranodal and central nervous system involvements at presentation or disease progression. **Case report.** We reported a 73-year-old male patient with double-hit primary adrenal lymphoma and preserved adrenal function, showing a favorable clinical course. Computed tomography of abdomen showed a 9 × 7 cm mass of the left adrenal gland. Laparatomy with left adrenalectomy was done and histological examination revealed diagnosis of a diffuse large B-cell NHL (DLBCL), non-GCB subtype. The patient was treated with 6 cycles of R-CHOP chemotherapy with reduced doses of doxorubicin because of the decreased left verticle ejection fraction. The patient was followed up regularly for 20 months with no evidence of tumor recurrence despite the inherently poor prognostic profile and double-hit phenotype of the disease. **Conclusion.** R-CHOP chemotherapy in combination with adrenalectomy can be an effective first-line regimen for primary adrenal DLBCL, despite the inherently poor prognostic profile (non-GCB subtype, bulky disease, elevated lactate dehydrogenase and double-hit phenotype of the disease).

Key words:
lymphoma, non-hodkin; adrenal gland neoplasms; drug therapy; surgical procedures, operative; prognosis.

Apstrakt

Uvod. Primarni adrenalni nehočkinski limfom izuzetno je redak oblik limfoma i odlikuje se lošom prognozom. Sa druge strane, *double-hit* limfomi sa BCL2 i MYC translokacijom karakterišu se uznapredovalom bolešću u vreme postavljanja dijagnoze, često prisutnom ekstranodalnom lokalizacijom bolesti i lošom prognozom. **Prikaz bolesnika.** Prikazali smo bolesnika starog 73 godine sa *double-hit* primarnim limfomom nadbubrega sa očuvanom adrenalnom funkcijom i povoljnim kliničkim tokom. Kompjuterizovanom tomografijom abdomena registrovana je tumorska promena leve adrenalne žlezde, veličine 9 × 7 cm, dok nije utvrđeno prisustvo bolesti na drugim lokalizacijama. Učinjeno je kompletno hirurško uklanjanje tumorske promene i na osnovu histološkog pregleda postavljena je dijagnoza difuznog B krupnoćelijskog limfoma, (DLBCL) non-GCB podtipa. Nakon hirurškog lečenja primenjen je R-CHOP protokol sa redukovanim dozama adriablastina zbog smanjene ejekcione frakcije leve komore. I pored veoma lošeg prognostičkog profila bolesti (non-GCB tip, *bulky* bolesti, visokih vrednosti laktat dehidrogenaze, visokog komorbiditetnog skora i *double-hit* fenotipa) bolesnik je već 20 meseci u kompletnoj remisiji. **Conclusion.** Imunohemioterapija (R-CHOP) u kombinaciji sa hirurškim lečenjem je efikasna prva linija terapije kod bolesnika sa primarnim adrenalnim difuznim B-krupnoćelijskim limfomom i pored nepovoljnog prognostičkog profila i *double-hit* fenotipa bolesti.

Ključne reči:
limfom, nehodžkinov; nadbubrežne žlezde, neoplazme; lečenje lekovima; hirurgija, operativne procedure; prognoza.

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Introduction

The adrenal gland is a rare site of primary extranodal non-Hodgkin lymphoma (NHL), accounting for less than 1% of all NHL cases and only 3% of primary extranodal lymphomas [1]. Compared with nodal diffuse large B-cell NHL (DLBCL), primary adrenal DLBCL is frequently accompanied with many adverse features such as bulky disease, elevated lactate dehydrogenase (LDH), advanced clinical stage and adrenal insufficiency [2]. Prognosis is poor and most patients die due to the progressive disease or its complications within one year after the diagnosis [3, 4]. Double-hit lymphomas with MYC and BCL-2 translocation are rare types of lymphoma (around 2% of NHL) with frequent extranodal disease and extremely poor prognosis [5]. We presented the patient with double-hit primary unilateral adrenal lymphoma, with preserved adrenal function and good outcome.

Case report

A 73-year-old man, with complaints of abdominal pain, fatigue and weight loss for 4 months was admitted to the Hematology Department of the Clinical Hospital Center "Bežanijska kosa", Belgrade, Serbia. The patient had acute myocardial infarction in 2007 and underwent gastrectomy due to bleeding of gastric ulcer 12 years ago. His past medical history was also significant for alcohol abuse (20 years) and cigarette smoking (55 years). Hematological analysis showed mild anemia (hemoglobin level of 103 g/L). Blood biochemistry showed elevated LDH (920 U/L). Ultrasonography and computed tomography (CT) of abdomen demonstrated 9 × 7 cm mass of the left adrenal gland, without splenomegaly or abdominal lymphadenopathy (Figure 1). The patient was referred to the Endocrine Surgery Clinic where left adrenalectomy was done in May 2011.

Histological examination of adrenal tumor revealed diagnosis of DLBCL, non-germinal center B-cell (non-GCB) subtype with following immunephenotype: EMA-, LCA+, PAX5+, inhibin-, synaptophysin-, CD79+, CD20+, CD5-, CD3-, CD10-, CD43-, CD30-, CD38-, CD138-, Mum-1+, BCL2+, BCL6-, ALK-1-, Ki-67 (positive in 60% of lymphoma cells). Fluorescence *in situ* hybridization (FISH) showed MYC(8q24) and BCL2(18q21) rearrangement (Figure 2). In June 2011 the patient was referred back to the Hematology Department. The clinical examination showed pale skin without superficial lymphadenopathy or organomegaly. A serum biochemistry profile showed elevated LDH (506 IU/L) and β2 microglobulin of 4.3 mg/L. Serum sodium, potassium and calcium levels were normal. Endocrinology assessment proved that the patient was euthyroid and eucortisolemic. Mineralocorticoid function and the function of adrenal medulla were also intact. Thoracic CT scan did not show any lymph node enlargement. CT of brain revealed only a few old ischemic lesions. Cerebrospinal fluid sediment did not show any lymphoid cells. Bone marrow trephine biopsy showed no lymphoid infiltration. Echocardiography showed reduced ejection fraction (40%) and aneurysmatic dilatation of the inferior left ventricular wall. Therefore, the patient received immunochemotherapy regimen rituximab–cyclophosphamide/doxorubicin (hydroxydaunomycin)/vincristine (Oncovin®)/prednisolone (R-CHOP) with reduced doses of an antracycline (doxorubicin 25 mg/m^2 i.v. every 3 weeks). After 6 cycles of R-CHOP chemotherapy the patient was in good condition with no evidence of tumor on fludeoxyglucose – positron emission tomography (FDG-PET) scans. The patient was followed regularly in our Outpatient Hematology Clinic. On the

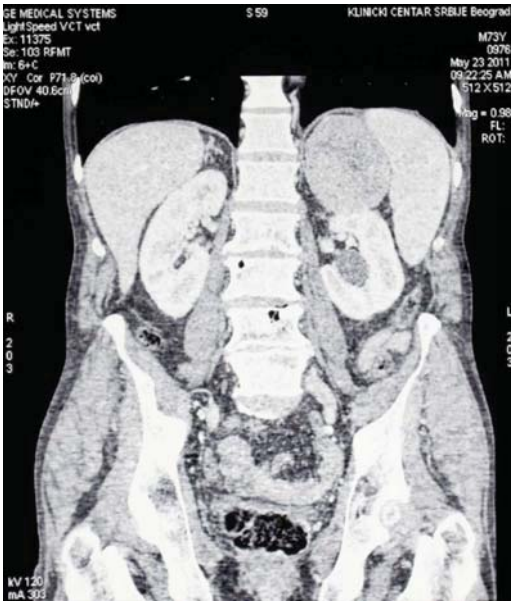

Fig. 1 – Computed tomography scan of abdomen demonstrated 9 × 7 cm mass of the left adrenal gland.

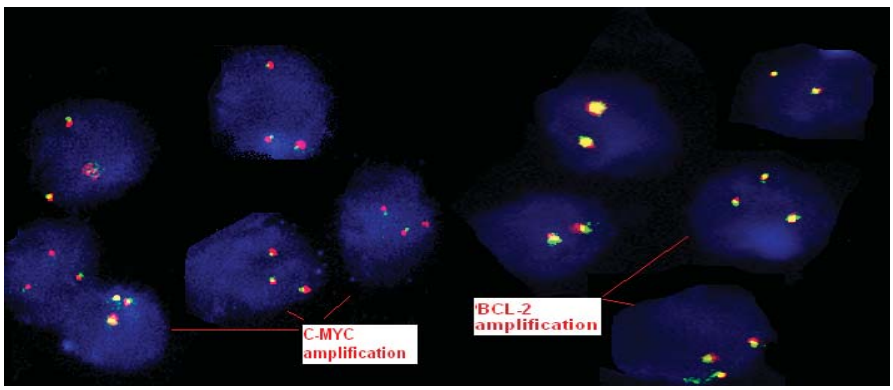

Fig. 2 – MYC (8q24) and BCL2 (18q21) rearrangement (fluorescence *in situ* hybridisation – FISH).

last follow-up (January, 2013), the patient was in good clinical condition, with no evidence of lymphoma recurrence.

Discussion

Primary adrenal non-Hodgkin's lymphoma (PAL) is a rare neoplasm with approximately 120 cases reported worldwide [2]. The disease most frequently affects older men with the mean age of 68 years at diagnosis [4]. The symptoms of the disease and response to treatment vary depending on the type of lymphoma, tumor size, and the presence of adrenal failure. Literature data based mostly on case reports, revealed a high rate of bilateral involvement (60–79% of cases) and adrenal failure (67–69% of cases) [4]. Nodal and extranodal disease in PAL is rare at diagnosis [6]. However, involvement of unusual extranodal organs is often reported at the disease relapse [6]. The most frequent histological subtype of PAL is a diffuse large non-GCB subtype [2, 7]. The presented patient had typical age, histology and symptoms for PAL, but he presented without bilateral involvement and consecutive adrenal failure.

The pathogenesis of PAL is not clear. Immune deregulation may predispose to PAL as well as to certain other lymphomas in some patients [8]. Proposed hypothesis for the occurrence of PAL includes preexisting autoimmune adrenalitis with lymphocyte infiltration, although not conclusively proven due to the rarity of both autoimmune adrenalitis and lymphomas [9]. It is supported by more frequent development of adrenal failure in patients with lymphoma than in patients with bilateral adrenal metastases. Namely, adrenal failure occurs in 1% of patients with adrenal metastasis and only when more than 90% of the adrenal tissue is destroyed [10]. In contrast, a very high frequency of primary adrenal failure (60–70%) has been reported in patients with PAL, including patients with mildly enlarged adrenal glands [9, 10].

The majority of published cases of PAL had a poor prognosis, although there were some with good prognosis [3, 4, 11]. Poor prognosis is related to bilateral disease, high International Prognostic Index score, high LDH, the presence of other localization of disease, non-GCB type and advanced age [2]. Our patient had double-hit phenotype with concurrent expression of MYC and BCL2 which is particularly related to inferior prognosis of DLBCL cases, even in the era of rituximab [5, 12–14]. Despite non-GCB subtype, double-hit phenotype, advanced age, comorbidities and high LDH our patient achieved complete remission and showed no evidence of lymphoma recurrence 20 months from the diagnosis.

Given the rarity of PAL, the optimal therapeutic modality is poorly defined. Available data on outcomes of patients treated by chemotherapy, unilateral or bilateral adrenalectomy, and/or adjuvant radiotherapy gave inconclusive results [2, 15–17]. In earlier trials of CHOP or CHOP-like therapy, the results of PAL treatment have been disappointing; a review of the literature data has shown that most patients died due to the disease or its complications within one year after diagnosis [4, 7, 17]. In contrast, Kim et al. [2] have recently reported that treatment outcomes with R-CHOP for primary adrenal DLBCL were not inferior to those of nodal DLBCL.

Conclusion

This first published case of double-hit primary adrenal lymphoma in Serbia suggests that R-CHOP chemotherapy in combination with adrenalectomy can be an effective first-line regimen for primary adrenal diffuse large B-cell lymphoma, despite the inherently poor prognostic profile and double-hit phenotype of the disease.

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CASE REPORT

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Presurgical orthodontic treatment of patients with complete bilateral cleft lip and palate

Prehirurško ortodontsko lečenje bolesnika sa potpunim bilateralnim rascepom usne i nepca

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Abstract

Introduction. Cleft lips and palates are the most common congenital orofacial anomaly. This type of clefts is the most severe from the orthodontic-surgical therapy aspect. **Case report.** A female newborn with a complete cleft of the primary and the secondary palate was admitted to the clinic, where a multiple-role orthodontic device was specially designed and applied to primarily manage the closure of the existing cleft and help to improve the suckling ability of the baby. Besides the fact that it allows breastfeeding, it has a significant orthodontic effect, too. **Conclusion.** Specificity of this device is the lack of extraoral fixation. What can easily be observed is a progressive reduction of the cleft between the separated segments and the premaxilla retrusion. It, thus, allows the creation of much better conditions for further surgical management of the said defect.

Key words:
cleft lip; cleft palate; infant newborn; palatal obturators.

Apstrakt

Uvod. Rascepi usne i nepca su najčešće kongenitalne orofacijalne anomalije. Ovaj tip rascepa je najteži sa aspekta ortodontsko-hirurške terapije. **Prikaz slučaja.** Žensko novorođenče sa kompletnim rascepom primarnog i sekundarnog palatuma primljeno je na kliniku gde je izrađen višenamenski ortodontski aparat specifične konstrukcije koji omogućava ishranu, ali ima i značajan ortodontski efekt. **Zaključak.** Specifičnost ovog aparata je odsustvo ekstraoralne fiksacije, a zahvaljujući njemu dolazi do progresivne redukcije rascepa između segmenata, kao i retruzije premaksile. Na ovaj način stavaraju se daleko bolji uslovi za hiruršku terapiju koja sledi.

Ključne reči:
usna, rascep; nepce, rascep; novorođenče; opturatori; palatinalni.

Introduction

Cleft lips and palates are among the most common congenital orofacial anomalies. They occur in approximately 700 children born every day somewhere in the world (about 240, 000 newborns a year), and that number is ever increasing [1]. Its etiology has not been completely understood yet despite the fact that the heredity plays a crucial role in its occurrence. Clefts are inherited as a quasi-continuous genetic model with a distinct expressiveness. Cleft palate occurs in about one in 700 live births worldwide.

If we do not take oblique and transversal facial clefts into account, a complete bilateral cleft lip and palate are considered the most severe clinical form.

From the orthodontic-surgical therapy aspect, the clinical picture of this type of cleft is the most complex and severe, because the location of cleft segments is such that they are difficult to be surgically reponed in a way to allow good surgical restitution without the occurrence of postoperative complications such as development of the oronasal fistula. However, the primary problem arising immediately after birth of babies with such a severe deformity is feeding [2–4].

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It is thought that a large gap in children with this type of cleft causes a difficulty in creating an adequate negative pressure within the oral cavity due to the existence of a large oronasal communication. Such a communication prevents normal flow of fluid during breastfeeding and permits the entry of milk or water into the unprotected upper airways, what may result in some very dramatic situation [5]. Nasal regurgitation, an excessive air swallowing and milk aspiration can occur and may be followed by the episodes of choking, coughing, vomiting, difficulty in swallowing and cyanosis [4, 6]. Those babies, even if partially breastfed, are always tired and sleepy. All that can negatively impact the baby's growth [7–11]. The parents' stress when nursing their babies as well as their increased tension and worry because of their babies' insufficient milk intake should not be neglected since it has major effects on the baby's poor weight gain [12]. The mother-child relationship often changes as well [13].

The orthodontic aspect takes a special place in the treatment of cleft newborns. In those children born with bilateral cleft lip and palate, the premaxillary segment is very often protruded forward over the lateral palatinal segments and the lower jaw. The prolabium is very small in size with a hypoplastic muscular layer, and the muscle fibers of the orbicularis oris muscle are inserted near the alar base. A constant retraction not opposed by the lateral lip's muscles makes the diameter of the nose base wider than in the non-cleft cases. Columella is often short, or does not exist, so the nose is flat [14]. The first surgical intervention those patients need is lip adhesion, which could be performed in one or two phases. It depends on the premaxilla position and the quantity of the available tissue. The purpose of early orthodontic treatment is to minimize the need for an additional surgical treatment. Thus, a much better symmetry could be achieved, and visible scars may be minimized. In cases with the premaxilla protruded to a higher degree, one-phase surgical treatment helps the surgeon to reduce the distance between the premaxilla and the cleft lateral segments prior to the surgery.

The families with children born with cleft lip/cleft palate are exposed to stressful life events having social, financial, and mental implications to a much greater extent because clefts palate repair is performed at the time when their children reach the age of two [15].

The aim of this paper was to present the way a stimulator could be fabricated and applied to the baby with severe complete bilateral cleft of the primary and the secondary palate immediately after birth. In the first phase, it would be used as a passive palatal obturator to help the normalization of the baby's suckling ability, and, in the second phase, as an active obturator to allow the initial reposition of the separated segments.

Case report

A duly born female baby with the birth-weight of 2,200 grams and the length of 46 cm was diagnosed with severe and non-syndrome complete bilateral cleft of the primary and the secondary palate (Figure 1).

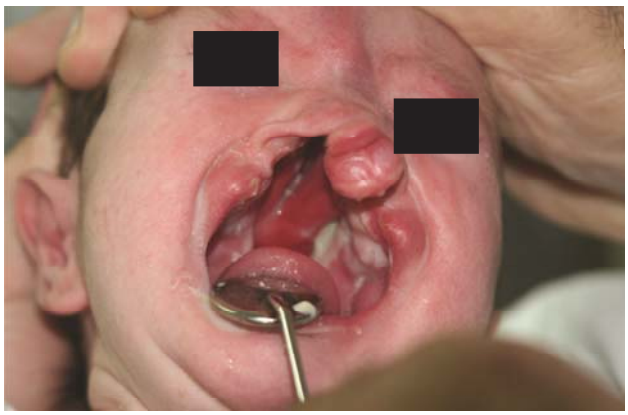

Fig. 1 – Intraoral appearance of the baby with complete bilateral cleft lip and palate.

A couple of hours after birth, the baby was referred to the clinic, and the initial step taken was to manage the problem and allow breastfeeding to baby by fabricating the first RBJ stimulator for closure of the communication between the nasal and the oral cavity.

The first stage in the fabrication of RBJ (Radojičić Božidar and Julija – the authors of the device) obturator is taking an anatomical impression of the upper jaw, which requires an exceptional professional expertise of orthodontists. Premedications given to reduce the stress in infants could provoke prolonged sedation in the postanesthesia period, so they may experience some functional disorders such as eating and sleeping problems even 15 days after the intervention. However, the endotracheal intubation has proved to be difficult in a ceratain number of such cases (4–7% of infants), and endotracheal tube itself covers a considerable space within the oral cavity, which makes taking a proper dental impression extremely complicated and affects both the precision of the obtained impression and the fabrication of an obturator.

The preliminary impression is anatomic, and is taken using an irreversible alginate impression material. The next stage is making an individual tray from this first impression in the dental laboratory, which is required for obtaining the second (corrective, functional) impression. It is very critical for the construction of the obturator because it should accurately show all the details of the baby's alveolar ridges (creases, frenulum). Therefore, impression mass consisting of addition silicones from the Group A featuring a high accuracy, a long-lasting dimensional stability, the hydrocompatibility, thixotropy and hydrophilicity should be used for such a purpose.

When compared to the standard impression technique, the procedure of taking corrective impressions required for the fabrication of a stimulator is specific in the ratio between the quantity of the activator and the quantity of the catalyst of addition silicone. Therefore, a special attention should be paid to that in this phase. The amount of the activator is twice the average amount used in the standard impression procedure, what provides more rapid bonding of the impression mass. The individual tray filled with such prepared impression mass is inserted into the baby's mouth exactly when the process of bonding starts (Figure 2). The baby's body

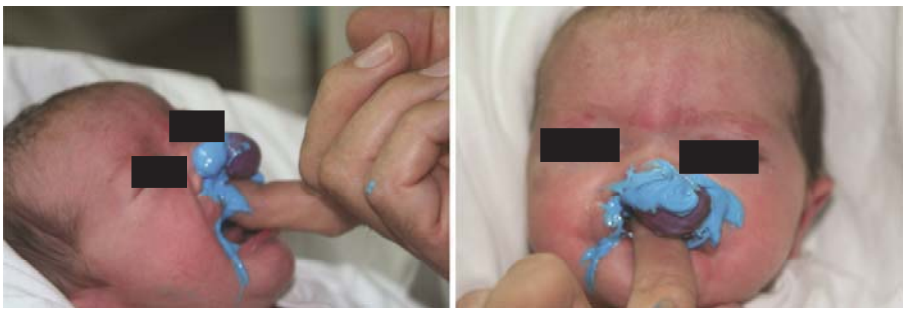

Fig. 2 – A day after birth, the baby's upper jaw impression was taken.

temperature accelerates the process even more, so the entire process takes about 40 seconds to complete, and the tray can be removed from the baby's mouth. However, this should be done with caution since an excessive amount of activator can have the opposite effect, in terms of activator's inability to bind to the catalyst of the impression mass.

When the individual tray with the impression mass is inserted into the baby's mouth, it is necessary to put the left hand on the baby's cheek to manually palpate the cheek muscles, i.e. to pull the cheek and the upper lip downwards in order to precisely outline the working limit between the mobile and immobile mucous membrane (what determines the height of the obturator), and the position of the labial frenulum and other creases. The procedure and obturator making was simmilar to RB obturator made for the baby who had isolated cleft of secondary palate.

After the impression procedure having been completed, a working cast is made in the lab. At this development stage, the baby's alveolar ridges have not fully developed so they are called alveolar margins. The maxillary alveolar ridge is horseshoe-shaped and of a constant configuration. It develops in two parts: the exterior or labio-buccal and the interior or lingual part. The labio-buccal portion is differentiated first and grows more rapidly. That fact should be kept in mind when making obturators, in order to allow the growth of the alveolar ridge.

Accurate impression, regardless of its discontinuity, clearly shows all other anatomic details (surfaces) typical for this stage of the baby's development, and which can serve as orthodontic retainers, or on the contrary, can destabilize the stimulator (Figure 3).

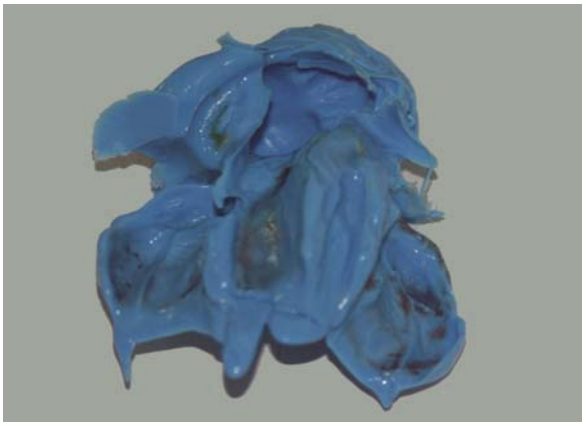

Fig. 3 – The second impression of the upper jaw and the soft palate.

At the next stage, the cleft space delineated on the working cast is filled with wax. That space results from the discontinuity in the fusion between the two palatal shelves. Using the working cast prepared in that manner, the RBJ obturator is made with polymerizing acrylates (Figure 4).

Fig. 4 – The first study model and the first RBJ obturator.

Its construction is almost completed by the polymerization of the acrylates, and the removal of sharp edges. In the end, the obturator is polished for a high shine to prevent a further damage to the mucosa in the baby's oral cavity. The obturator is then tried in the baby's mouth and checked for the accurate fit, while the mother is instructed how to use it when nursing her baby, and how to maintain the hygiene of the orburator (Figure 5).

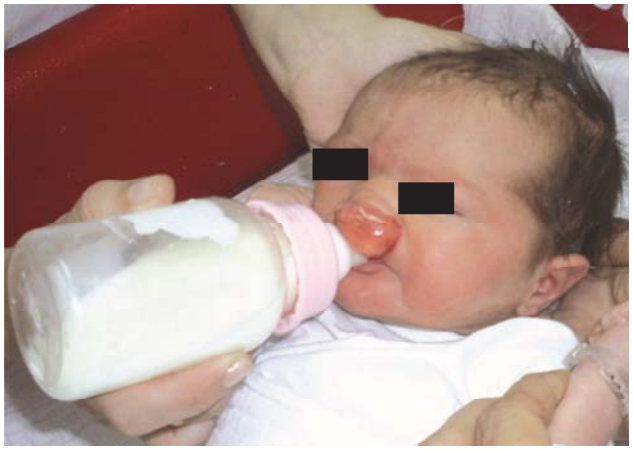

Fig. 5 – Bottle-feeding of the baby.

Over the following treatment period, it is made every week for the first month due to the intensive growth of the baby, but later, it is done every three weeks. The role of the RBJ obturator is to separate the oral from the nasal cavity, what, thus, allow the creation of negative pressure, and enables baby's feeding.

At every two-week follow-up visit, the baby should be checked for the occurrence of lesions involving the soft tissue of the alveolar ridge (decubitus) or lesions on the frenulum that could be caused by the obturator. Then, the existing orburator is adjusted to the growth of the alveolar ridge by removing acrylate from the obturator's inner side (the side that lies on the alveolar ridge from the vestibular side).

In that way, the obturator prevents the cleft from further spreading as the baby grows, and, at the same time, allows positive growth of the alveolar ridges.

Once the baby is accustomed to the regular use of RBJ obturator, a slightly redesigned RBJ stimulator is made. Its purpose is to primarily close the separated segments of the alveolar ridge and palatum, and, thus, direct their further development, decrease the gap, and greatly facilitate the surgical reconstruction of the alveolar ridges and the palatum. This type of RBJ stimulator has an open orthodontic screw (bolt) that allows the active segments to move. By activating the screw, the segments move closer to each other (Figure 6).

Fig. 6 – The second study model and the second RBJ stimulator with a screw.

In order to avoid the discontinuity in the transverse development of the maxilla, acrylates are simultaneously removed from the side of the stimulator which is in contact with the vestibular side of the alveolar ridge. At the same time, this type of RBJ stimulator allows undisturbed feeding of a baby.

The fabrication procedure for this type is almost the same; it is made from the working cast but with a plate cut in two halves attached with an open screw. By its activation, the space between the halves of the plate decreases and the fragments get closer (Figures 4 and 6).

Two months after birth, a visible retroposition of the maxilla could be observed (Figure 7a) on the baby's face, as well as the improved position of the lateral segments when compared to the initial condition (Figure 7b). When necessary, this RBJ stimulator continues to be periodically fabricated.

Four months after the baby's birth, the position of the separated segments progressively improved, what also could be noticed on the baby's face (Figure 8).

Fig. 8 – The fourth RBJ stimulator, four months after birth.

By the time the baby was one year old, the separation distance between the fragments was so small that it could greatly facilitate the surgical treatment, and secure its success to a great extent (Figures 9 and 10).

Another great advantage of the RBJ stimulator is the absence of extraoral fixation. This appliance rests on the ridge of the upper jaw as a complete denture.

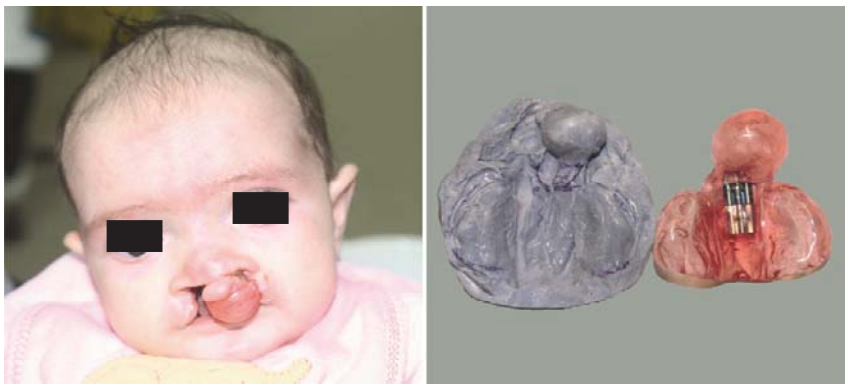

Fig. 7 – The third RBJ stimulator, two months after birth.
a) Visible retroposition of the maxilla; b) Improved position of the lateral segments.

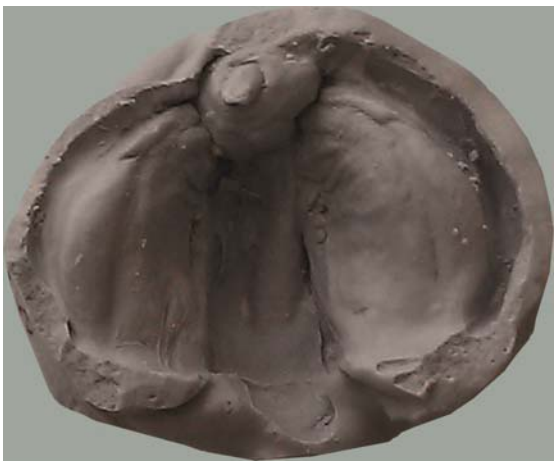

Fig. 9 – A plaster model of the 1-year-old baby's upper jaw.

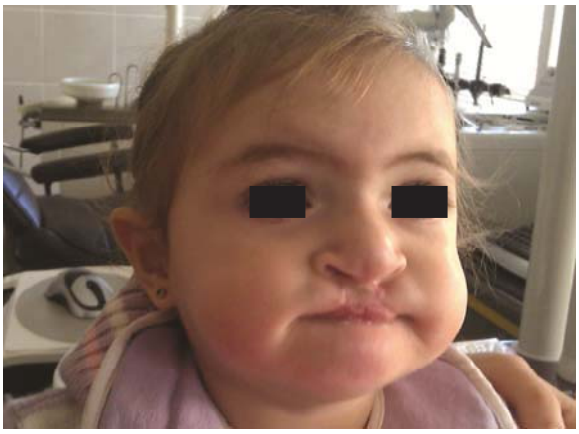

Fig. 10 – The facial appearance of the 1-year-old baby with complete bilateral cleft lip and palate.

Babies are accustomed to its presence in the mouth, so that they always protest when the stimulator is removed from their mouths.

Discussion

In recent years, there have been numerous studies that questioned the validity of early application of orthodontic therapy in babies with cleft, primarily because they allow natural feeding. The most significant are the studies which have proved that the use of palatal obturators could shorten the feeding time, increase the milk intake quantity, and, as a result of all the mentioned above, allow a normal baby's growth and development. Different positions of a baby that could facilitate and ensure successful direct breastfeeding have also been studied.

The second, not less important reason for the application of early orthodontic therapy is early orthodontic treatment of the cleft. The presurgical infant orthopedic surgery as a neonatal therapy for the correction of cleft lip and palate was introduced in the mid-20th century. Some of the problems to which the traditional approach is applied include deformities of the nasal cartilage in both unilateral and bilateral cleft lip and palate cases, and deficient collumela in children with bilateral cleft. Early therapy for cleft lip, alveolus and palate achieves almost normal anatomical relations.

Descriptions of different intra- and extraoral appliances used for that purpose may be found in the literature.

Active intraoral appliances were described by Reisberg et al. [16].

Some authors directed the growth of the premaxilla in the downward and backward direction by application of force on the premaxilla [17–20].

Millard et al. [20] suggest that any application of force could have a restriction impact on the growth of the premaxilla.

Hotz et al. [21] thought that passive intraoral appliances should direct skeletal growth in the desired direction, or even stimulate the growth, what Weil [22] and Nolst et al. [23] confirmed in their studies.

Both active and passive appliances incorporaded to the lateral segments, provide a stabilizing effect on them. That was considered an advantage of those appliances.

Oosterkamp et al. [14] analyzed dimensions of the maxilliary alveolar arch in the patients with bilateral cleft lip and palate. Prior to the surgical closure of the cleft lip, children underwent early orthodontic therapy with intraoral retrusion plates. In 14 children with this anomaly (9 boys and 6 girls) born between 1999 and 2002, the effects of the application of those plates were assessed on plaster models cast from the maxillary impressions. The impressions were taken of the infants' upper jaws, and the passive feeding plates were fabricated. Three weeks later, the impression procedures were repeated, and new retrusion plates were made. These plates contained a microexpansion screw which was positioned in the way to allow movement into two directions: the retrusive movement of the premaxilla, and, if necessary, the realigning movement of the premaxilla relative to the vomer. This position was determined by clinical assessment of the casts. The front plate of the appliance over the premaxilla was connected through the screw with the second plate placed over the lateral segments.

The parents were instructed to turn the screw daily for a quarter of a cycle (0.175 mm/day), producing the retrusion of 2.5 to 3.7 mm in two to three weeks' time. Every three weeks, a new plate was fabricated, and the procedure was repeated until the time of surgical closure of cleft lip, i.e. when the baby reached 4 months of age.

The results indicated that during the therapy there was a significant decrease in the distance between the premaxilla and the lateral segments. That decrease correlated with the increase in the deviation of the premaxilla in relation to the vomer.

For each millimeter decrease in the distance between the premaxilla and the cleft lateral segments, an average increase in deviation of 4.0 degrees was found.

There was also a significant reduction in the width of the left and the right cleft, and a significant increase as well in the premaxilla width, while the transverse dimensions of the premaxilla did not significantly change in the area of lateral segments.

Active presurgical treatment with an oral retrusion plate proved to be very successful in orthodontic treatment of children with bilateral cleft lip and palate.

Abu-Rub et al. [24] studied the effects of an intraoral implant appliance on the reposition of dislocated segments of the upper jaw, as well as the magnitudes of palatine changes dependent on the age of babies born with bilateral cleft lip and palate. By using the 12 plaster study models, the condition of the babies (2–3 weeks after birth) prior to the introduction of the therapy, and their achieved condition at the time the retention (immediately before the cleft lip surgery) was assessed. The intercanine and the intertuberosity width, the palatal length and depth and the intercanine arch length were measured. The authors found an increase in the intercanine and intertuberosity width, and a reduction in the palate and the intercanine arch length. However, the authors did not found any link between these changes and the age of babies.

Spengler et al. [25] estimated the outcomes of presurgical alveolar molding in the therapy of patients with bilateral cleft lip and palate. Their study included the group of eight patients that were treated during the 2002–2004 period. The orthodontic therapy was initiated 34.9 days after birth, and lasted for the average period of 212.5 days. Following the impression procedure (performed under general anesthesia), the appliance was fabricated.

A package for the throat was used to prevent the unwanted entry of alginate materials into the oropharynx. The appliance consisted of acrylate palatal plates and two nasal stents. The stents were added to the appliance only after the intersegmental space had been reduced to less than 6 mm. Repositioning of the premaxilla between the lateral segments was carried by the application of outer force with the aid of sticking plaster and rubber bands, with which the appliance was attached to the child's face. In seven months' time, the columella was lengthened enough, the prolabium was wide enough and rotated inward to the maxillary arch.

The values obtained prior to the initiation of the therapy and after the treatment were compared on the basis of the cast measurements and statistical analysis. The results of the therapy recorded on the upper jaw molds showed that a statistically significant reduction of the premaxillary protrusion and the width of a bigger cleft were achieved. This improvement was comfirmed by the reposition of premaxilla and its alighment with the alveolar segments. However, the width of a smaller segment increased in a half of the studied children as the result of the maxillary repositioning, which was achieved through the width reduction of the bigger cleft.

The authors quantitatively showed that presurgical nasoalveolar molding had significant advantages in bilateral cleft treatment. The shape of the maxillary arch considerably improved after the alignment of the protruded premaxillary segment with the alveolar arches. As a result, the changes coupled with the orthodontic therapy helped reduce the surgical complexity.

Over the 12.5 year period, Garfinkle et al. [26] conducted a trial involving 77 infants born with non-syndromic bilateral cleft lip and palate. The children underwent an early orthodontic therapy with a nasal alveolar denture. Based on the used casts and the measurement results obtained with a digital caliper, the distances between five reference points were measured through the nasal structure. Those measurements were carried out at five different stages. No significant differences between healthy children and children with bilateral clefts were found.

Early presurgical orthodontic therapy has been used in more than 54% cleft palate centers in the world, including our previous case report on the application of palatal RB obturator in babies with isolated palate cleft. Regardless the fact that the results of the above-mentioned studies and this case report proved it to be useful and recommendable, there are, even today, doubts about its viability.

Conclusion

Based on the changes on the working casts monitored through the entire treatment period from the baby's birth up to one year of age, we could conclude that the application of the RBJ stimulator helped in progressive reduction of the cleft between the separated fragments and the premaxillary retrusion as well, in this severe case of complete bilateral cleft lip and palate.

One year after birth, a contact between the premaxilla and the lateral segments was established. That could not be achieved in any other way, but through the use of a stimulator. It further facilitated the surgical procedure to a great extent.

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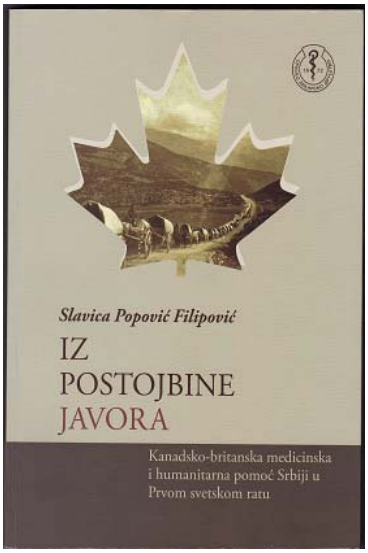
B O O K R E V I E W

Title: From the Homeland of Maple Tree
(The Canadian-British Medical and Humanitarian Help to Serbia in the First World War)
Original title (in Serbian): Iz postojbine javora
(Kanadsko-britanska medicinska i humanitarna pomoć Srbiji u Prvom svetskom ratu)
Author/autor: Slavica Popović Filipović
Publisher/Izdavač: Serbian Medical Society, Belgrade/Srpsko lekarsko društvo, Beograd
Year/Godina izdanja: 2013.



Although Slavica Popović Filipović spent years in researching the topic, this book appeared at the time of the Centenary of the start of the World War One. The author examined the contribution of the Canadian medical and humanitarian assistance to Serbia, during the Great War and afterwards. Since Canada was still a dominion within the British Empire at the time, thus the activities of the Canadian medical staff were described within the context of the British Medical Missions: Dr Howard Green Barrie in the First Unit of the British Red Cross in Skopje; Dr Catherine H. Travis in the First Unit of the Serbian Relief Fund in Skopje, under the leadership of Lady Paget; Dr William Harold Graham Aspland in the Wounded Allies Relief Committee Hospital in Kragujevac. Dr Harriet Cockburn in the Third Unit of the Serbian Relief Fund in Kragujevac; Dr Edna Guest and Dr Honoria Somerville Keer in the Scottish Women Hospitals – The Corsica Unit, in Corsica; Dr Irma LeVasseur, Dr Joseph Henri Albiny Paquette, and Dr Raoul Brault came to Serbia as the members of the Canadian Red Cross, and others. Many Canadian nurses and orderlies were working at various medical units in Serbia or were with the Serbs at different fronts. A separate chapter addresses the establishment of the Canadian Hospitals on the Salonika Front, which were regarded by many as the best allied hospitals. The Author has brought back from oblivion, and given many examples of humane deeds of the Canadian people towards the Serbs via the Serbian Red Cross, the Serbian Relief Fund, the Scottish Women's Hospitals, etc. – all best described by the reports of Jelena Lozanić (later Mrs. Helen Lozanic Frotingham), who was visiting Canada as the representative of the Serbian Red Cross.

The monument to the world renown Serbian inventor Nikola Tesla at the Niagara Falls, the site of his first hydroelectric power station in the world, and the Canadian mountain peak Mt. Putnik, named after Field Marshal Radomir Putnik, the famous Serbian general and strategist, are just examples of historic and cultural links between the two nations, that neither the geographical distance nor the distance in time could obliterate.

The chapters about the organisation and activities of the hospitals are accompanied by numerous eyewitness accounts from both Serbian and foreign writers, that are not just historical records, but valuable recollections about the Serbian people at the time. Above all else, the names of the medical doctors show that these were elite cadres of European, Canadian, American, Australian and New Zealand doctors, who came to help the Serbian people. But who also left the Balkans enriched with medical experiences and knowledge about the Balkans, and that it was not just inhabited by some wild savages, but people with great traditions, and rich historical and cultural heritage.

The book "From the Homeland of Maple Tree" was published under the auspices of the Serbian Medical Society. The book reviews were written by Dr Mile Ignjatović, MD PhD, a surgeon; Prof. Dr. Radoje Čolović, MD PhD, a member of the Serbian Academy of Sciences and Arts, and Assistant Professor Ilija Kajtez, PhD in Political Sciences.

The book contains 352 pages, over 50 illustrations, includes a full index, and an extended list of references. The introduction and numerous first hand eye witness accounts are bilingual – English and Serbian. The translators were Bob Filipovich and Slavica Popović Filipović. The book launch was organised by the State Office for Diaspora at the Belgrade International Book Fair in October 2013.

Historian Momir Ninković wrote a book review, published in the journal "Istorija 20. veka", by the Institute for the Modern History in Belgrade. This book is located in many major libraries in Serbia and around the world, and is regarded as one of the major references in its field.

Col. Mile Ignjatović, MD PhD
Military Medical Academy, Belgrade

In the book is the note of Canadian Dr Harriet Cockburn about the Great Retreat with the Serbian People in 1915:

"I joined the Serbian civilians and military in their retreat through the Albanian wilderness. This was worse than the actual fighting in Serbia, where I found myself at the start of the War, with the group of Canadian physicians. There, on the front, people were killed by a bullet, a bomb, or a projectile; but here, on these icy paths, people were dying because of hunger, cold and exhaustion.

I too was hungry, broken and exhausted. What scarce food was there was given to the Serbian mothers and their children that they carried in their arms, covered in blankets given to them by the soldiers. To me, born in Toronto, in a well to do household, these days of hunger and long walks were particularly hard to bear – I walked for 640 km! I watched those desperate Serbian mothers who were walkingsteadily forward towards to shores of the Adriatic – their safe haven. They stumbled and fell, but rose up again, and kept going. They gave me the strength to continue on this terrible march of death."

U knjizi se nalazi i zapis kanadske lekarke dr Harijet Koburn o povlačenju sa srpskim narodom, 1915:

"Povlačila sam se sa srpskim vojnicima i izbeglicama kroz bespuće Albanije. Ovo je bilo groznije od bojišta u Srbiji, na kojima sam se obrela još u početku rata sa grupom kanadskih lekara. Tamo, na bojištu, padalo se od puščanog zrna, granate, bombe; ovde, na ovim ledenim stazama, od gladi, zime, iznurenosti… I ja sam na tom putu bila gladna, slomljena, iznemogla. Sledovanja hrane, veoma oskudna, davale smo srpskim majkama i njihovoj deci koju su nosile u naručju, uvijenu u šalove koje su im davali vojnici. Meni, rođenoj u Torontu, u izobilju bogatih roditelja, teško su padali i glad i pešačenje – prevalila sam 640 kilometara! Gledala sam nesrećne srpske majke koje su grabile napred, prema obalama Jadrana, prema luci spasenja. One su posustajale, posrtale, padale, ali su se dizale i išle dalje. One su i meni davale snagu da istrajem na ovom surovom maršu smrti…"

(Preveli: Bob Filipović i Slavica Popović Filipović)

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Poziv za reklamiranje u 2014. godini

U prilici smo da vam ponudimo mogućnost oglašavanja i reklamiranja proizvoda i usluga u časopisu „**Vojnosanitetski pregled**“ (VSP). To je sigurno najbolji vid i najzastupljeniji način upoznavanja eventualnih korisnika sa vašim uslugama i proizvodima.

Časopis „**Vojnosanitetski pregled**“, zvanični organ lekara i farmaceuta Vojske Srbije, naučno-stručnog je karaktera i objavljuje radove iz svih oblasti medicine, stomatologije i farmacije. Radove ravnopravno objavljuju stručnjaci iz vojnih i civilnih ustanova i iz inostranstva. Štampa se na srpskom i engleskom jeziku. Časopis izlazi neprekidno od 1944. godine do sada. Jedini je časopis u zemlji koji izlazi mesečno (12 brojeva), na oko 100 strana A4 formata, a povremeno se objavljuju i tematski dodaci (suplementi). Putem razmene ili pretplate VSP se šalje u 23 zemlje sveta. Radove objavljene u VSP-u indeksiraju: *Science Citation Index Expanded (SCIE)*, *Journal Citation Reports/Science Edition*, *Index Medicus (Medline*), *Excerpta Medica (EMBASE), EBSCO* (preko ove baze VSP je dostupan *on line* od 2002. godine u *pdf* formatu) i *Biomedicina Serbica*.

Cene reklama i oglasa u časopisu „Vojnosanitetski pregled“ u 2014. godini su:

1.	Oglas u crno-beloj tehnici A4 formata za jedan broj	20 000,00 dinara
2.	Oglas u c/b tehnici A4 formata za celu godinu (11-12 brojeva)	200 000,00 dinara
3.	Oglas u boji A4 formata za jedan broj	35 000,00 dinara
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5.	Oglas u boji na koricama K3 za jedan broj	50 000,00 dinara
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7.	Oglas u boji na koricama K2 i K4 za jedan broj	55 000,00 dinara
8.	Oglas u boji na koricama K2 i K4 za celu godinu (11-12 brojeva)	530 000,00 dinara

Za sva obaveštenja, uputstva i ponude obratiti se redakciji časopisa „Vojnosanitetski pregled“. Sredstva se uplaćuju na žiro račun kod Uprave javnih plaćanja u Beogradu broj: 840-941621-02 **VMA (za Vojnosanitetski pregled ili za VSP)**, **PIB 102116082**. Uplatnicu (dokaz o uplati) dostaviti lično ili poštom (pismom, faksom, *e-mail*-om) na adresu: Vojnosanitetski pregled, Crnotravska 17, 11000 Beograd; tel/faks: 011 2669 689, e-mail: vsp@vma.mod.gov.rs

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VSP publishes: **editorials, original articles**, **short communications**, **reviews**/**meta-analyses, case reports**, from the **medical history** (general or military), personal views, invited comments, letters to the editor, reports from scientific meetings, book reviews, extensive abstracts of interesting articles from foreign language journals, and other contributions. Original articles, short communications, meta-analyses and case reports are published with abstracts in both English and Serbian.

General review papers will be accepted by the Editorial Board only if the authors prove themselves as the experts in the fields they write on by citing not less than 5 self-citations.

Papers should be written on IBM-compatible PC, using 12 pt font, and double spacing, with at least 4 cm left margin. **Bold** and *italic* letters should be avoided. Observational and experimental articles, reviews and meta-analyses, should not exceed 16 pages (including tables and illustrations); case reports – 6; short communications – 5; letters to the Editor, reports on scientific meetings and book reviews – 2.

All measurements should be reported in the metric system in terms of the International System of Units (SI). Standard, internationally accepted terms should be used.

MS Word for Windows (97, 2000, XP, 2003) is recommended for word processing; other programs are to be used only exceptionally. Illustrations should be made using standard **Windows** programs**.** Avoid the use of colors in graphs.

Papers are reviewed anonymously by at least two editors and/or invited reviewers. Remarks and suggestions are sent to the author for final composition. Galley proofs are sent to the first author for corrections that should be returned within 3 days. Manuscripts accepted for publication are not being returned.

Preparation of manuscript

Parts of the manuscript are: **Title page; Abstract with key words; Text; References**.

1. ***Title page***

a) The title should be concise but informative. Subheadings should be avoided;

b) Full name of each author;

c) Name and place of department(s) and institution(s) of affiliation, clearly marked by standard footnote signs.

2. ***Abstract and key words***

The second page should carry a structured abstract with the title for original articles, metanalyses and case reports. The abstract should state the purposes of the study or investigation, basic procedures (selection of study subjects or laboratory animals; observational and analytical methods), main findings (giving specific data and their statistical significance, if possible), and the principal conclusions. It should emphasize new and important aspects of the study or observations. S t r u c t u r e d abstract should contain typical subtitles: *background/aim, methods, results* and *conclusion*. The abstract for metaanalyses and obrginal papers should have up to 450 words, and up to 150 words for case reports (with subtitles *background, case report, conclusion*). Below the abstract authors should provide, and identify as such, 3–10 key words or short phrases that will assist indexers in cross-indexing the article and will be published with the abstract.

3. ***Text***

The text of original articles is divided into sections with the headings: **Introduction**, **Methods**, **Results**, and **Discussion**. Long articles may need subheadings within some sections to clarify their content.

In the **Introduction** repeat the title of the article, excluding the names of authors. State the purpose of the article and summarize the rationale for the study or observation. Give only strictly pertinent references and do not include data or conclusions from the work being reported.

Methods. Describe your selection of the observational or experimental subjects (patients or experimental animals, including controls) clearly. Identify the methods, apparatus (manufacturer's name and address in parentheses), and procedures in sufficient detail to allow other workers to reproduce the results. Give references to established methods, including statistical methods. Identify precisely all drugs and chemicals used, with generic name(s), dose(s), and route(s) of administration. State the approvement of the Ethnics Committe for the tests in humans and enimals.

Results should be presented in logical sequence in the text, tables and illustrations. Emphasize or summarize only important observations.

Discussion is to emphasize the new and important aspects of the study and the conclusions that result from them. Relate the observations to other relevant studies. Link the conclusions with the goals of the study, but avoid unqualified statements and conclusions not completely supported by your data.

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References should be superscripted and numbered consecutively in the order in which they are first mentioned in the text. **The references must be verified by the author(s) against the original document.** List all authors, but if the number exceeds 6, give 6 followed by et al. Do not use abstracts, secondary publications, oral communications, unpublished papers, official and classified documents. References to papers accepted but not yet published should be designated as ”in press“. Information from manuscripts not yet accepted should be cited in the text as ”unpublished observations“. References are cited according to the ***International Committee of Medical Journal Editors.*** *Uniform Requirements for Manuscripts Submitted to Biomedical Journals. Ann Intern Med 1997; 126: 36–47. Updated October 2001.*

Examples of references:

Jurhar-Pavlova M, Petlichkovski A, TrajkovD, Efinska-Mladenovska O, Arsov T, Strezova A, et al. Influence of the elevated ambient temperature on immunoglobulin G and immunoglobulin G subclasses in sera of Wistar rats. Vojnosanit Pregl 2003; 60(6): 657–612.

DiMaio VJ. Forensic Pathology. 2nd ed. Boca Raton: CRC Press; 2001.

Blinder MA. Anemia and Transfusion Therapy. In: Ahya NS, Flood K, Paranjothi S, editors. The Washington Manual of Medical Therapeutics, 30th edition. Boston: Lippincot, Williams and Wilkins; 2001. p. 413-28.

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

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

Tables

Each table should typed double-spaced on a separate sheet, numbered in the order of their first citation in the text in the upper right corner and supplied with a brief title each. Explanatory notes are printed under a table, using the following symbols, in this sequence: ***, †, ‡, §, ||, ¶, **, ††, ...** Each table has to be mentioned in the text. If you use data from another source, acknowledge fully.

Illustrations

Figures are submitted as photos which should be sharp. Letters, numbers, and symbols should be clear and even throughout and of sufficient size that when reduced for publication, each item will still be legible. Each figure should have a label on its back indicating the number of the figure, author's name, and top of the figure. If a figure has been published, acknowledge the original source.

Legends for illustrations are typed on a separate page, with arabic numerals corresponding to the illustrations. Identify and explain each one clearly in the legend symbols, arrows, numbers, or letters used to identify parts of the illustrations. Explain the method of staining in photomicrographs.

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Rukopis se piše sa proredom 1,5 sa levom marginom od **4 cm**. Koristiti font veličine 12, a načelno izbegavati upotrebu **bold** i *italic* slova, koja su rezervisana za podnaslove. Originalni članci, opšti pregledi i metaanalize ne smeju prelaziti 16 stranica (sa prilozima); aktuelne teme – osam, kazuistika – šest, prethodna saopštenja – pet, a pisma uredniku, izveštaji sa skupova i prikazi knjiga – dve stranice.

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Na drugoj stranici nalazi se strukturisani apstrakt sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **uvod i cilj** rada, osnovne procedure - **metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi - **rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt (**250** reči) ima podnaslove: *uvod/cilj, metode, rezultati* i *zaključak*. Za apstrakte na engleskom dozvoljeno je i do **450** reči. Strukturisani apstrakt je obavezan za metaanalize (istog obima kao i za originalne članke) i kazuistiku (do 150 reči, sa podnaslovima *uvod, prikaz slučaja i zaključak*). Ispod apstrakta, pod podnaslovom „Ključne reči“ predložiti 3–10 ključnih reči ili kratkih izraza koji oslikavaju sadržinu članka.

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Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Zaključak** može da bude posebno poglavlje ili se iznosi u poslednjem pasusu diskusije. U **uvodu** ponovo napisati naslov rada, bez navođenja autora. Navesti hipotezu (ukoliko je ima) i ciljeve rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo strogo relevantne podatke iz literature i ne iznositi opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

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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)

Mladenović T, Kandolf L, Mijušković ŽP. Lasers in dermatology. In: *Karadaglić Đ*, editor. Dermatology. Beograd: Vojnoizdavački zavod & Verzal Press; 2000. p. 1437–49. (Serbian)

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

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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. Za fus-notu koristiti sledeće simbole ovim redosledom: ***, †, ‡, §, ||, ¶, **, ††, ...** Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

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