Vol 59, No 3, September, 2020
UDK 61
ISSN 0365-4478 (Printed)
ISSN 1821-2794 (Online)
www.medfak.ni.ac.rs/amm

ACTA MEDICA MEDIANAE

Naučni časopis
Medicinskog fakulteta Univerziteta u Nišu i
Podružnice Srpskog lekarskog društva u Nišu

AMM

Scientific Journal of the University of Niš Faculty of Medicine
and the Department of the Serbian Medical Society in Niš

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Vol 59, No 3, September, 2020
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http://www.medfak.ni.ac.rs/amm

Acta Medica Medianae (UDK 61; ISSN 0365-4478 štampana verzija; ISSN 1821-2794 elektronska verzija) je zvanični časopis Medicinskog fakulteta Univerziteta u Nišu i Podružnice Srpskog lekarskog društva u Nišu pod pokroviteljstvom Ministarstva za nauku i tehnološki razvoj Republike Srbije. Časopis izlazi četiri puta godišnje od 1962 godine. Izdavač je Medicinski fakultet Univerziteta u Nišu, Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija. Sadržaj i celokupan tekst časopisa dostupan je na sajtu Medicinskog fakulteta http://www.medfak.ni.ac.rs/amm. Godišnja pretplata: za inostranstvo 60 USA dolara, za ustanove 2500 dinara i za pojedince 1500 dinara. Sredstva uplatiti na žiro račun Medicinskog fakulteta u Nišu br. 840-1681666-03, sa naznakom za *Acta Medica Medianae*. Uputstvo autorima se objavljuje u svakom broju, pri čemu je autor dužan da se pridržava navedenih uputstava prilikom predaje rukopisa. Radovi se mogu slati u elektronskom formatu na adresu: acta@medfak.ni.ac.rs. Naknada za štampanje rada iznosi 1000 dinara za autora, a 500 dinara za koautore, za svaki prihvaćeni rad. *Acta Medica Medianae* zadržava pravo dalje distribucije i štampanja radova.
Kontakt adresa: Časopis Acta Medica Medianae, Medicinski fakultet, Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
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Tel+381-18-4533001 lok. 122 fax. +381-18-4534336
Tiraž 200 primeraka. Štampa: "Sven", Niš, Srbija.
Acta Medica Medianae je trenutno indeksirana na *Index Copernicus-u, Srpskom citatnom indeksu, DOAJ i EBSCO*

Acta Medica Medianae (UDK 61; ISSN 0365-4478 printed version; ISSN 1821-2794 online) is the official Journal of the University of Niš Faculty of Medicine and the Department of the Serbian Medical Society in Niš published with the help of the Ministry of Science and Technological Development of the Republic of Serbia. The Journal has been published four times a year since 1962. The publisher is the University of Niš Faculty of Medicine, Institutional address: dr Zoran Đinđić 81, 18000 Niš, Serbia. Table of contents and full texts of articles are available on the Institutional Home Page at http://www.medfak.ni.ac.rs/amm. Prices are subject to change. All subscriptions start with the first issue of the current year. For payment details contact the Secreteriat at acta@medfak.ni.ac.rs. Instructions for authors appear in every issue. Manuscripts accepted for publication are not returned to the author(s). *Acta Medica Medianae* retains the right for further distribution and printing of the articles.
Editorial correspodence: Journal Acta Medica Medianae, Faculty of Medicine, Dr Zoran Đinđić 81, 18000 Niš, Serbia.
Electronic submission of the papers: acta@medfak.ni.ac.rs
Phone: +381-18-4533001 lok. 113 fax. +381-18-4534336
Printed on acid-free paper; 200 issues. Press: "Sven", Niš, Serbia
Acta Medica Medianae is currently indexed in Index Copernicus, Serbian Citation Index, DOAJ and EBSCO

Naučni časopis Medicinskog fakulteta Univerziteta u Nišu i Podružnice Srpskog lekarskog društva u Nišu

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Vol 59, No 3, September, 2020
UDK 61 ISSN 0365-4478 (Printed version)
ISSN 1821-2794 (Online)
http://www.medfak.ni.ac.rs/amm

Autor slike na prednjoj stranici:
Dipl.ing. Jovica Spasić, Medicinski fakultet, Univerzitet u Nišu

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Original article

UDC: 616.617-003.7:613.3
doi:10.5633/amm.2020.0301

THE IMPACT OF BOTTLED "PROLOM WATER" ON LITHOGENESIS OF URINARY TRACT

Dragoslav Bašić[1,3], Bratislav Pejić[1], Svetlana Milosavljević[2], Sanja Jović[2], Gordana Kocić[3], Andrej Veljković[3]

Urolithiasis represents the most common urological condition nowadays, with rising trend of incidence and prevalence rates, according to geographical, climatic, ethnic, dietary and genetic factors. Prophylactic management of urolithiasis in terms of high fluid intake is of great importance in prevention of all types of urolithiasis. Prolom water has been categorized as a sodium hydro carbonic alkaline hypothermal oligomineral water.

The aim of the study was to investigate the effects of bottled Prolom water intake on serum and urinary calcium and magnesium values, as well as on urineary pH and renal microlithiasis.

A multicenter prospective trial included a total of 345 patients who daily consumed 2.5 to 3 liters had underwent of Prolom water intake, in amount of 2.5 to 3 liters/daily, for 14 days, in three follow -up in three periods.

Average values of calcium in serum (mmol/L) at on day zero, 7th and 14th were: 2.24; 2.312 and 2.242, separatelyrespectively. Average values of calcium in urine (mmol/L) at on day zero, 7th and 14th were: 1.046; 1.582 and 1.564, separatelyrespectively. Average values of magnesium in serum (mmol/L) at on day zero, 7th and 14th were: 0.89; 0.82 and 0.81, separatelyrespectively. Average values of magnesium in urine (mmol/L) at on day zero, 7th and 14th were: 1.09; 1.51 and 1.61, separatelyrespectively. Mean urineary pH values were: 6.3 at on day zero; 5.9 at on day 7th; and 6.8 at on day 14th.

Daily intake of 2.5-3 liters of bottled Prolom water has a favorable and antilithogenic effect on calcium oxalate and calcium phosphate urolithiasis.

Acta Medica Medianae 2020;59(3):05-12.

Key words: *bottled Prolom water, lithogenesis, urinary tract*

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Introduction

Urolithiasis represents the most common urological condition nowadays, with reported prevalence rates up to 20%, predominantly higher in industrialized countries (1, 2, 3). Although urolithiasis occurs in all age, sex and racial groups, it is more common in men and older patients, with more than 80% of all stone types presented by calcium oxalate (4). Renal stone disease is associated with high recurrence rates of 50% in 5-10 years and 75% in 20 years, as well as accelerated subsequent relapse course, as reported by Trinchieri and Strauss (5, 6). The etiologic causes of urolithiasis can be classified as infectious (Magnesium ammonium phosphate; Carbonate apatite; Ammonium urate), non-infectious (calcium oxalate; calcium phosphate; uric acid), genetic (cystine; xanthine; 2.8-dihydroxyadenine) and medicamentous (7). The lifetime risk for renal stone disease ranges from 10–25% (8). Epidemiological studies have shown rising trend of incidence and prevalence rates, according to geographical, climatic, ethnic, dietary and genetic factors (7). Of these, lifestyle changes and dietary habits have been considered as the most important causes for this increase (9, 10). Among all dietary habits, fluid intake has been con-sidered as one of the most important.

Prolom water

According to balneological classification, Prolom water has been categorized into the group of sodium-hydro-carbonic-alkaline-hypothermal-oligo-mineral waters. It has been taken from the depth of

200 to 600 meters. The temperature of Prolom water is 20 °C on air temperature of 20 °C, with a specific weight 1.000532 kN/m^3. The pH value is 9.15 which gives an alkaline reaction. Mineralization is 215 mg/L and dry residue at 180 °C is 170 mg/L. The chemical pattern is made of cations with predominance of sodium (Na^+), representing 87.74 mval%, and anions with predominance of hydro carbo-nate (HCO^-3), representing 79.29 mval% (11, 12) (Table 1, 2, 3).

Table 1. Physicochemical characteristics of Prolom water

Water temperature	20 °C	Electrical conductivity	170
Air temperature	20 °C	Mineralization (mg/l)	215
Colour (Pt-Co scale)	0	Dry residue 180°C (mg/l)	170
Fuzziness (NTU)	0	Total hardness (dH)	0.7
pH	9.15	Total ions of alkaline earth metals	
Eh (mV)	-20	(mg/l)	5.0
rH	-	Consumption of $KMnO_4$ (mg/l)	1.0

Table 2. Ionic composition of Prolom water

Kations	mg/l	mmol	mval	mval%	Anions	mg/l	mmol	mval	mval%
Na^+	41.9	1.882	1.882	87.74	HCO_3^-	102.0	1.669	1.669	79.29
K^+	0.2	0.005	0.005	0.24	CO_3^-	6.2	0.20	0.20	9.50
Li^+	0.003	-	-	-	OH^-	< 0.1	-	-	-
$Nh4^+$	< 0.04	-	-	-	Cl^-	6.0	0.17	0.17	8.08
Ca^{++}	4.9	0.123	0.246	11.80	Br^-	< 0.5	-	-	-
Mg^{++}	0.05	0.002	0.004	0.19	J^-	< 0.5	-	-	-
Sr^{++}	0.02	0.0005	0.001	0.02	F^-	< 0.2	-	-	-
Mn^{++}	< 0.01	-	-	-	NO_3^-	1.5	0.024	0.024	1.14
Fe^{++}	< 0.01	-	-	-	HPO_4^{--}	0.04	0.0005	0.001	0.05
Al^{+++}	< 0.04	-	-	-	SO_4^{--}	2.0	0.021	0.042	2.00
Total	47.07	1.952	2.077	100.00	Total	117.75	2.084	2.105	100.00

Table 3. Other substances in Prolom water

Weak electrolytes		Dissolved gases		CO_2	0
H_2SiO_3	48.5	O_2	4.0	H_2S total	0.08
H_3BO_3	0.1	Saturation O_2 %	44.0	H_2S free	0.01
Total solids (mg/l)	213.22	N_2	8.6	HS	0.07

The aim

The aim of the study was to investigate the effects of bottled Prolom water intake on biochemical changes of serum and urinary value of calcium and magnesium cations, as well as on renal microlithiasis.

Materials and methods

The study was a multicenter prospective trial, jointly conducted by Prolom Spa Special Hospital for Rehabilitation, Urological Clinic of Clinical Center Niš and the Institute of Biochemistry of the Faculty of Medicine in Niš, over the period from March 2013 to January 2018. A total of 345 patients (192 male, 153 female), mean age 46.65 years (25-82; SD = 10.69) were included in a multicenter prospective trial through the following inclusion criteria: age > 18 years; the presence of crystalluria in urine sediment (Ca-oxalate); ultrasonography finding of renal microlithiasis. Exclusion criteria encountered renal stone disease, anomalies of renal position, urinary tract infection, active oncological diseases, patients with urinary diversion, patients on renal replacement therapy, pregnancy, non-stabile hypertension.

All patients were informed of the study protocol and gave their consent. Study protocol included:

- extensive medical history,
- laboratory blood and urine analysis (including values of magnesium and calcium) obtained from the first-morning urine and serum specimens on day zero,
- renal ultrasonography on day zero,
- serum and urinary values of magnesium and calcium obtained from the first-morning urine and serum specimens on 7th and14th day,
- renal ultrasonography on 7th and14th day.

According to study design, all patients were treated with daily intake of 2.5-3 liters of bottled Prolom water for 14 days. Laboratory reference ranges are listed in Table 4.

Table 4. Laboratory reference ranges

Reference range (mmol/l)	Ca	Mg
Serum	2.02 - 2.6	0.8 - 1.0
Urine	2.5 - 6.2	0.4 - 4.1

Results

Average values of calcium concentration in serum, within the examined group were: 2.24 mmol/L on day zero (SD = 0.083); 2.312 mmol/L on day 7th (SD = 0.114) and 2.242 mmol/L on day 14th (SD = 0.119) (Table 5).

Average values of calcium concentration in urine in examined patients were: 1.046 mmol/L on day zero (SD = 1.030); 1.582 mmol/L on day 7th (SD = 0.832) and 1.564 mmol/L on day 14th (SD = 1.231) (Table 6).

Table 5. Calcium concentration in serum

Ca serum (mmol/l)	0-day	7th day	14th day
$\bar{x}$	2.24	2.312	2.242
SD	0.083	0.114	0.119

Table 6. Calcium concentration in urine

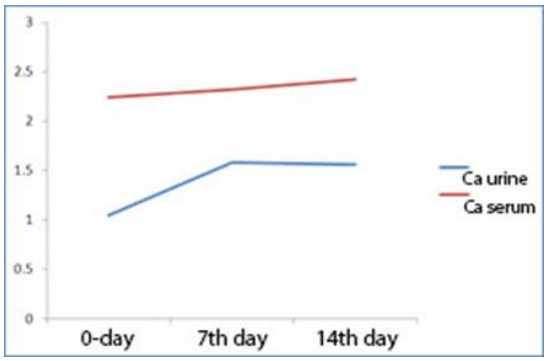
Ca urine (mmol/l)	0-day	7th day	14th day
$\bar{x}$	1.046	1.582	1.564
SD	1.030	0.832	1.231

When analyzing data, it is important to notice that the values of Ca concentration of examined sample were within the reference range, both in urine and serum (Graph 1).

Average values of Mg concentration in serum of all examined patients were: 0.89 mmol/L on day zero (SD = 0.05), 0.82 mmol/L on day 7th (SD = 0.09) and 0.81 mmol/L on day 14th (SD = 0.002) (Table 7).

Average values of Mg concentration in urine in examined patients were: 1.09 mmol/L on day zero (SD = 0.849); 1.51 mmol/L on day 7th (SD = 0.821); 1.61 mmol/L on day 14th (SD = 0.479) (Table 8).

Comparing time 1 to time 3, it is noticeable that there is a relevant growth of magnesium excretion within examined periods, with statistical significance ($p < 0.05$). At the same time, there is a slight decrease in serum values of magnesium, but in lesser extent comparing to urinary excretion increase. However, both serum and urinary magnesium values were within the reference range (Graph 2).



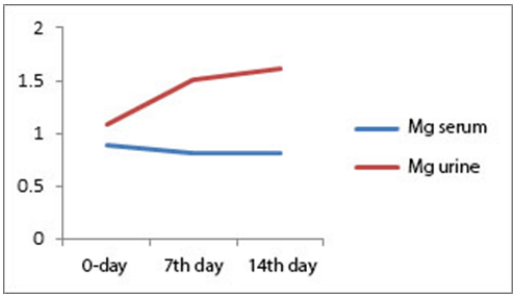
Graph 1. Calcium concentration in urine and serum

Table 7. Mg concentration in serum

Mg serum (mmol/l)	0-day	7th day	14th day
$\bar{x}$	0.89	0.82	0.81
SD	0.05	0.09	0.002

Table 8. Mg concentration in urine

Mg urine (mmol/l)	0-day	7th day	14th day
$\bar{x}$	1.09	1.51	1.61
SD	0.849	0.821	0.479



Graph 2. Magnesium concentration in urine and serum

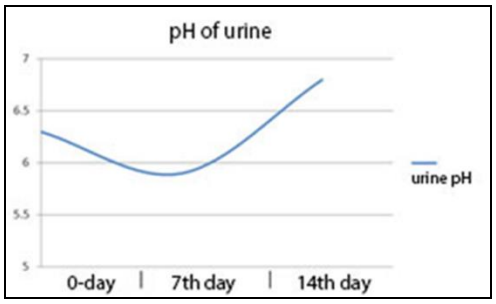
Mean urine pH values within examined group of patients were: 6.3 on day zero (SD = 0.6), 5.9 on day 7th (SD = 0.92) and 6.8 on day 14th (SD = 0.6) (Table 9).

The change in urine pH value during the study period showed slight variations in the range of 0.9. During the first 7 days there was a decreasing trend in its value and moderate acidification of urine, while in the next 7 days it increased to values higher than the initial, with moderate alkalization of the urine to almost neutral value (Graph 3).

From the zero-day onwards, renal ultrasound showed a decreasing trend of diffuse multiple hyperechoic acoustic shadows (microlithiasis), with remarkable regression of microlithiasis at the end of the study (Table 10).

Table 9. Urinary pH

Urinary pH	0-day	7th day	14th day
$\bar{x}$	6.3	5.9	6.8
SD	0.6	0.92	0.6



Graph 3. Urinary pH

Table 10. Renal ultrasonography

Day	Finding
Zero	Normal position of both kidneys, size, shape and structure, preserved corticomedullar border, with signs of diffuse multiple hyperechoic acoustic shadowing (<5mm) on both sides (microlithiasis)
7th	Normal position of both kidneys, size, shape and structure, preserved corticomedullar border, with reduced diffusion of hyperechoic acoustic shadowing (< 5mm) on both sides (microlithiasis)
14th	Normal positions of both kidneys, size, shape and structure, preserved corticomedullar border, with discrete signs of hyperechoic acoustic shadowing (< 5mm) on both sides (microlithiasis)

Discussion

The most common underlying conditions linked to nephrolithiasis have been described with the following prevalence rates: absorptive hypercalciuria (20-40%), renal hypercalciuria (5-8%), resorptive hypercalciuria (3-5%), hyperuricosuric calcium nephrolithiasis (10-40%), hypercitraturic calcium nephrolithiasis (10-50%), hyperoxaluric calcium nephrolithiasis (2-15%), hypomagnesiuric calcium nephrolithiasis (5-10%), gouty diathesis (15-30%), cystinuria (< 1%), infection stones (1-5%), low urine volume (10-50%), miscellaneous (< 3%) (13). The etiologic causes of urolithiasis can be classified as infectious (magnesium ammonium phosphate; carbonate apatite; ammonium urate), non-infectious (calcium oxalate; calcium phosphate; uric acid), genetic (cystine; xanthine; 2.8-dihydroxyadenine) and medicamentous (7).

According to epidemiological data on stone composition, there is a predominance of calcium oxalate which accounts for more than 80% of all stone types (4). However, in terms of pathophysiology and pathogenesis, there are many open-ended questions and ambiguities that are still awaiting answers and clarifications.

Stone formation process encompasses a complex of physicochemical cellular and extracellular events which include: urine saturation, oxidative stress, cell injury and cell membrane rupture, nucleation and crystal growth, aggregation, crystal-cell interaction and retention/adhesion (14). As described by Pearle and Lotan (15), in solutions containing ions, including urine, there is a maximum level of the product of their concentration and at that level, the solution is considered saturated. In this way, the capacity of this solution is completed and the dissolution of additional quantities of crystals is not possible, as their precipitation will occur. However, by changing certain conditions in the solution, such as pH, temperature, or by adding certain substances called crystallization inhibitors, it

is possible to increase the value of the thermodynamic product of solubility, thereby preventing the formation of crystals and their precipitation. The solubility and crystallization states are determined by the thermodynamic solubility product (KsP) and the formation product (Kf). Thus, depending on their values, solutions are classified as undersaturated, metastable and unstable. Of these, the metastable solution represents the most favorable and targeting area for therapeutic action, since the process of additional crystallization is not possible, although the urine has been supersaturated. Crystallization of calcium oxalate occurs after its supersaturation at the point when the concentration product goes beyond the solubility product. Circumstances promoting supersaturation include: increased concentrations of calcium, oxalates, uric acids and phosphates, separately, with a low urinary volume and low concentrations of citrate.

However, there are substances that slow down or inhibit the nucleation, growth, and aggregation of crystals. They accomplish this by acting on the surface of the crystal without affecting the concentration of crystal-forming ions. Nuclei represent precursors of crystals and their persistence in urine depends on the saturation level as well as on the nucleus stability. The last one depends on the impact of promoters and inhibitors of crystallization. In the absence of inhibitors, nucleation extends by adsorption to surrounding structures, such as epithelial cells or preexisting crystals, as described by Alelign et al. and Umekawa et al. (14, 16).

There are organic (citrate, glycosaminoglycans, glycoproteins,lipids) and non organic physiological inhibitors (pyrophosphate, magnesium) for calcium oxalate and calcium phosphate. Among organic inhibitors, citrate, pyrophosphate and magnesium are considered as the most potent. Citrate acts at multiple levels: it inhibits Ca oxalate precipitation, nucleation and crystal aggregation; by competitive binding to Ca, it reduces ionic concentration of calcium and its capacity to form oxalates and phosphates; Anorganic pyrophosphate inhibits calcium phosphate crystallization. Magnesium acts similarly to citrate, by competitive binding to oxalates and thus decreasing their ionic concentration and potential for supersaturation(15, 17).

It has been suggested by several authors that renal urolithiasis promotes the risk to variety of diseases, including chronic kidney diseases (18), diabetes, hypertension (19), and cardiovascular diseases (20). It has also been stated that one of the most important risk factors in urinary stone formation is fluid intake, in reverse proportion (21). Therefore, prophylactic management of urolithiasis in terms of high fluid intake is of great importance in prevention of all types of urolithiasis. It has been reported that an increase intake of water had favorable effects by reducing the reccurrence rates in kidney stone formers. Hence, an increased water intake is advised commonly in all patients with renal stone disease (22-24).

According to our results, bottled Prolom water promotes urinary excretion of magnesium and calcium ions. As an inhibitor of crystallization, magnesium complexes with oxalates forming a soluble compound and therefore prevents further calcium oxalate stone formation. Additionally, by binding itself to calcium ions (70%), magnesium prevents crystallization or inhibits nucleation of calcium oxalate and calcium phosphate. Daily intake of 2.5-3 liters of bottled Prolom water achieves optimal diuresis with a specific weight of urine within the range of 1005-1015. Moreover, it changes overall pH value by decreasing it to 5.8 during the first 7 days, with a significant increase afterwards to 6.8 during the next 7 days. It represents important antilithogenic effect, since low urine pH promotes uric acid and/or calcium stone formation (25).The goal of urine pH change is to be held between 6.5 and 7.2 since these values enable better solubility of urate and cystine in the urine. It has to be emphasized that this value should not exceed 7.2 in order to avoid potential side effect of forming calcium phosphate stones. Results of renal ultrasound showed a decreasing presence of microlithiasis, with remarkable reduction at the end of the study. Although morphological, these findings are consistent with reported changes of Mg and Ca values, supporting results of moderating effects of Prolom water on urinary tract lithogenesis. Prolom water, as an independent factor has high degree of anti-lithogenicity on urolithiasis.

Conclusion

Based on the reported results, it can be concluded that daily intake of 2.5-3 liters of bottled Prolom water has a favorable and antilithogenic effect on calcium oxalate and calcium phosphate urolithiasis. These effects certainly deserve more extensive research, both in terms of pathogenetic mechanisms of action, as well as in terms of laboratory and clinical outcome.

Acknowledgements

This study was supported by the Faculty of Medicine, University of Niš, Internal scientific project number 45.

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Originalni rad

UDC: 616.617-003.7:613.3
doi:10.5633/amm.2020.0301

UTICAJ FLAŠIRANE „PROLOM VODE" NA LITOGENEZU URINARNOG TRAKTA

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Urolitijaza predstavlja najčešće urološko oboljenje danas, sa rastućim trendom incidencije i prevalencije, u zavisnosti od geografskih, klimatskih, etničkih, dijetalnih i genetskih faktora. Profilaktičko lečenje urolitijaze, u smislu visokog unosa tečnosti, od velikog je značaja u prevenciji svih vrsta urolitijaze. Flaširana „Prolom voda" kategorisana je kao natrijum hidrokarbonatna, alkalna, hipotermalna, oligomineralna voda. Cilj studije bio je da se ispitaju efekti unosa „Prolom vode" na serumske i urinarne vrednosti kalcijuma i magnezijuma, kao i na pH urina i bubrežnu mikrolitijazu. Multicentričnom prospektivnom studijom obuhvaćeno je ukupno 345 bolesnika, koji su tokom 14 dana oralno unosili flaširanu „Prolom vodu" u količini od 2,5 litra do 3 litra dnevno, uz praćenje u tri perioda. Prosečne vrednosti kalcijuma u serumu (mmol/l) nultog, sedmog i četrnaestog dana bile su: 2,24; 2,312 i 2,242, ponaosob. Prosečne vrednosti kalcijuma u urinu (mmol/l) nultog, sednom i četrnaestog dana bile su: 1,046; 1,582 i 1,564, ponaosob. Prosečne vrednosti magnezijuma u serumu (mmol/l) nultog, sedmog i četrnaestog dana bile su: 0,89; 0,82 i 0,81, ponaosob. Prosečne vrednosti magnezijuma u urinu (mmol/l) nultog, sedmog i četrnaestog dana bile su: 1,09; 1,51 i 1,61, ponaosob. Srednje vrednosti pH u urinu bile su: 6,3 nultog; 5,9 sedmog i 6,8 četrnaestog dana. Svakodnevni unos 2,5 litra do 3 litra flaširane „Prolom vode" ima povoljan i antitilitogeni uticaj na kalcijum-oksalatnu i kalcijum-fosfatnu urolitijazu.

Acta Medica Medianae 2020;59(3):05-12.

Ključne reči: *flaširana „Prolom voda", litogeneza, urinarni trakt*

Original article

UDC: 616.65-006.6-089.87
doi:10.5633/amm.2020.0302

LAPAROSCOPIC RADICAL PROSTATECTOMY: A SINGLE CENTER EXPERIENCE

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Minimally invasive techniques including robotic-assisted and laparoscopic radical prostatectomy have become the preferred approach for operative treatment of prostate cancer.

The aim of this study was to evaluate and compare results of laparoscopic radical prostatectomy (LRP) and open retropubic radical prostatectomy (ORRP) for localised prostate cancer, in terms of safety, efficacy and oncological outcome.

A total of 123 radical prostatectomies (RPs) for low-risk localised prostate cancer were performed between January 2016 and June 2019 at the University Clinic of Urology Skopje. Of these, 61 (49.6%) were LRP and 62 (50.4%) ORRP, mean patients' age was 54 years (33 to 67). Indications for operative procedure included: pathohistological finding of prostate cancer, age ≤ 70 years, PSA < 10 ng/ml, Gleason score ≤ 7 (3+3 or 3+4), negative bone scintigraphy, stage ≤ T2a, N0, M0. All patients were assessed regarding the demographic data, PSA level, Gleason score, operative time, conversion to open surgery for LRP, blood loss, intra and post operative complications, catheter removal, number blood transfusion, hospital stay and oncological outcomes. LRP proved superior to ORRP, resulting in a shorter operating time, less blood loss ($p < 0.5$), shorter time to resumption of oral intake, shorter postoperative hospital stay ($p < 0.5$), and less analgesic requirements. In terms of oncological outcomes, we observed less positive margins in the LRP group ($p < 0.5$). Our results indicate that although both operative techniques represent safe procedures, offering good quality of operation, in our series, LRP was superior in terms of safety, efficacy and oncological outcomes.

Acta Medica Medianae 2020;59(3):13-19.

Key words: *prostate cancer, laparoscopic radical prostatectomy, open retropubic radical prostatectomy*

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Introduction

Recent epidemiological data show that at the end of 2018, prostate cancer was second in respect to all cancers in the male population, with an incidence that varies by region, from the age-standardized rate of 11.5/100 000 in Asia to 62.1/100 000 in Europe (1, 2).There are several treatment modalities for patients diagnosed with localized prostate cancer. According to EAU Guidelines 2019, it includes deferred treatment (active surveillance/ watchful waiting) and active (curative) treatment. Active treatment encompasses a wide range of options, including radical prostatectomy, external beam radiation therapy, brachytherapy (both high and low dose), hormonal therapy, cryotherapy, high-intensity focused ultrasound, focal therapy (3). With respect to all of these therapeutic options, radical prostatectomy is the therapeutic option of choice for patients with localized prostate cancer whose survival is expected to be longer than 10 years (4). According to a 2014 study by Bill Axelson et al., radical prostatectomy with respect to it significantly reduces CSS, with a relative risk of 0.56 (5).The same authors said in a 2018 study that the survival benefit for patients with localized prostate cancer who underwent radical prostatectomy was at least 2.9 years (6). In regard to lymph node dissection following RP, it has been stated that low-risk disease is rarely associated with nodal involvement (7).

Open radical prostatectomy has long been the only operative technique for the treatment of prostate cancer. It has undergone many modifications

and among them is the anatomical retropubic radical prostatectomy, which he introduced into the clinical practice of Walsh in 1982, and which is characterized by a better knowledge of hemostasis and cavernous nerve preservation (8). Further progress was directed towards reducing the invasiveness of the procedure, and at the end of the 20th century the technique of laparoscopic radical prostatectomy was promoted, with a positive growth trend over the coming years (9, 10). Drawbacks of the laparoscopic technique, which are primarily related to limitations in performing certain manual operations and movements, the absence of a third dimension, as well as a long learning curve, led to the emergence of a new operative technique — robotic assisted radical laparoscopic prostatectomy (RALP), which had been introduced in 2000 by Binder et al. (11). According to the results of recently published studies, as well as the EAU Guidelines, it cannot be stated with certainty that any of the above techniques has clinically significant advantages over the other two in terms of oncological and functional results (12).

The aim of this study was to evaluate and compare results of open retropubic radical prostatectomy (ORRP) and laparoscopic radical prostatectomy (LRP) for localised prostate cancer, in terms of safety, efficacy and oncological outcome.

Patinents and methods

A total of 123 radical prostatectomies (RPs) for low-risk localised prostate cancer were performed between January 2016 and June 2019 at the University Clinic of Urology in Skopje. Of these, 61 (49.6%) were LRP and 62 (50.4%) ORRP, mean patients' age was 54 years (33 to 67). Indications for operative procedure included: pathohistological finding of prostate cancer, age ≤ 70 years, PSA < 10 ng/ml, Gleason score ≤ 7 (3+3 or 3+4), negative bone scintigraphy, stage ≤ T2a, N0, M0. All patients were assessed regarding the demographic data, PSA level, Gleason score, operative time, conversion to open surgery for LRP, blood loss, intra and post operative complications, catheter removal, number of blood transfusions, hospital stay and oncological outcomes. Mean follow-up was 6 months (5 to 36). All patients underwent standardized preoperative procedure of our clinic, including: complete blood count, biochemical analysis, urine, urine culture, PSA, multislice computerized abdomino-pelvic tomography, histopathological result of transrectal ultrasound guided prostate biopsy, cardiac and anesthesia evaluation, urological evaluation. Statistical analysis was estimated by using of Fisher's test and Hi-squared test.

Surgical technique of ORRP was as described by Walsh (8). Surgical technique of laparoscopic prostatectomy was performed by the same surgical team, as follows: an infraumbilical incision was made with approximately 1 cm of length in the midline. Incision of rectus fascia was performed on the linea alba. Creation of preperitonenal space was performed using the fingers and the camera and then insufflation with CO_2 tension of 12 mmHg. We introduced the 0-grade optics through the infraumbilical port and additional four ports were placed under visual control. Two 11 mm trocars were inserted on the pararectal lateral border while two 5 mm were placed half way between the anterior–superior iliac crest and para rectal trocars. During the procedure we used only bipolar tools (clamps and grasps) including the forceps for the operator's left hand. We found this tool comfortable for both dissection and coagulation. After entering into the retropubic space, dissection of the prevesical space of Retzius was performed in a deliberate manner. The superficial dorsal vein, was coagulated with bipolar electrocautery. Subsequently, the endopelvic fascia was cleaned bilaterally. The endopelvic fascia was incised on both sides by bipolar scissors. The fascial incision was carried distally up to the most lateral puboprostatic ligament. The fibers should not be divided close to the prostate in order to avoid injury of large veins that cross on the latero-posterior side of the prostate. Visualization of the prostate apex was the end point of this dissection. The apex of the prostate was defined bilaterally. The deep venous complex of Santorini (DVC) was ligated with a 2-0 vicryl suture. In order to locate the bladder neck, Foley catheter was pulled and inflated with 10-15 ml. The bladder was incised at its junction with the prostate with bipolar forceps. The urethra was dissected at its anterior and lateral aspect and then transversally transected with scissors. The Foley catheter was removed and replaced by a ureteric stent (ch 16), which provided a good visualization of the bladder. This was an important step in order to ensure good preservation of the bladder neck. Next, by pulling the prostate upward in the direction of the pubic symphysis, we were able to uncover vertical fibers of the anterior layer of Denonvilliers' fascia. Its incision showed the retrovesical space in which the vas deferens and seminal vesicals are located. During the preparation of the seminal vesicles, we performed a good hemostasis of the medially situated vessels. The posterior lip of the bladder neck was grasped with forceps and lowered to provide access to the interprostatorectal plane. The vertical fibers of the anterior plane of Denonvillier's fascia covering the seminal vesicles were incised. The ampoule of the right vas deferens was sectioned after coagulation with cold scissors or clipped with a Hem-o-lock clip. A large grip was used to simultaneously coagulate the anterior deferential artery. The seminal vesicle was dissected circumferentially from the base to the apex, taking care to control the vessels. The lateral pedicle of the seminal vesicle was dissected and coagulated following the inferior pedicle dissection and coagulation. We proceeded with the dissection of the lateral surface of the prostate. After sectioning the neurovascular bundle (NVB) and local hemostasis with both bipolar forceps and Hem-o-lock clips, we continued with prostate apex section. Dissection of the apex was started with retraction of the preprostatic tissues using unipolar scissors. The urethra was reached gradually by incising the tissues covering the anterior surface of the urethra. The stent was advanced to make the urethra more

prominent. The posterior surface of the urethra was sectioned at the end. Fibers of the rectourethral muscle were sectioned revealing the plane of the rectum. After freeing prostate we performed the urethrovesical V anastomosis. Prostate gland is placed in catch and extracted trough right pararectus trocar port. Afterward, trocar was taken out and inserted again in the same port beside the endobag.

The urethrovesical anastomosis was performed using a running continuous unidirectional barbed (V-Loc® 180) running sutures. The right tail of the suture started from 5h to 12h position. The left tail of the suture started from 7h to 12h position. Assurance of watertight closure with an intraoperative 150-200 cc saline was performed in all cases. Finally a Foley catheter ch 16 was placed. Once the vesicourethral anastomosis was completed, a 16 F drain was introduced and fixed. The drain was placed in the Retzius space. The endobag was extracted by applying traction and rotation movements throughout the right port followed with incision of rectus fascia and distraction of rectus muscle fiber that ease the extraction.

Results

Basic demographic data and perioperative parameters are listed in Table 1. There were no statistical difference between LRP and ORRP in terms of number of patients, mean age (65.46 ± 3.3 and 65.3 ± 2.5, respectively), clinical stage, preoperative PSA values (6.6 ± 1.8 and 7.7 ± 1.6, respectively), as well as GS values of biopsy and final operative specimen. However, positive surgical margins were statistically different in favor of ORRP group ($p < 0.05$).

Table 1. Perioperative parameters

	LRP	ORRP	p
Number of patients	61	62	
Age	65.46 ± 3.3	65.3 ± 2.5	NS
Prostate volume	68 ± 22	72 ± 41	NS
Clinical stage	≤ T2a	≤ T2a	
Preoperative PSA (ng/ml)	6.6 ± 1.8	7.7 ± 1.6	NS
Gleason score (GS) (biopsy)			
≤ 6	37	42	NS
7 (3+4)	24	20	NS
GS (postoperative)			
≤ 6	29	39	NS
7 (3+4)	32	23	NS

Data on intraopereative and postoperative parameters showed that mean surgical time was similar in both groups, with no statistical difference (126.18 ± 19.5 and 126.66 ± 12.3, respectively). Patients who underwent ORRP had higher blood loss and that difference is statistically significant (355.17 ± 57.75 vs. 275.4 ± 39.79, separately, $p < 0.5$). It was observed that both period of postoperative hospitalization as well as postoperative catheter removal were shorter in LRP group (6.2 ± 0.4 vs. 7.43 ± 0.49, $p < 0.05$; and 6.2 ± 0.4 vs. 7.43 ± 0.49, $p < 0.05$) (Table 2).

Table 2. Intra- and postoperative parameters

	LRP	ORRP	p
Mean surgical time (minutes)	126.18 ± 19.5	126.66 ± 12.3	NS
Blood loss (ml)	275.4 ± 39.79	355.17 ±5 7.75	p < 0.5
Postoperative hospitalization (days)	6.2 ± 0.4	7.43 ± 0.49	p < 0.5
Catheter removal (days)	6.2 ± 0.4	7.43 ± 0.49	p < 0.5

When perioperative complications according to Clavien-Dindo classification have been analyzed, there were 11 grade I events (18%) in the LRP group and 8 (12.9%) in the ORRP group ($p > 0.05$). Grade II that refers to intraoperative blood loss was more frequent in the ORRP (12% vs. 27%, $p < 0.05$). There was 1 (1.6%) LRP event of grade IIIa and 2 (3.2%) ORRP ($p > 0.5$). In all cases, urethral catheter was dropped out, so recatheterization was performed endoscopically. In one case (1.6%) we observed rectal injury during LRP (grade IIIb), so the laparoscopic intervention had been converted into the open. Rectal injury was completely repaired, with no additional complications, and the patient was discharged from the hospital on day 12. Complications of higher grade (IV and V) were not observed. Data on perioperative complications according to Clavien-Dindo classification are summarized in Table 3.

Table 3. Perioperative complications (Clavien-Dindo classification)

Procedure	LRP (n = 61)		ORRP (n = 62)		p
Patients	No	%	No	%	
Grade I	11	18	8	12.9	$p > 0.05$
Grade II	7	12	17	27	$p < 0.05$
Grade IIIa	1	1.6	2	3.2	$p > 0.05$
Grade IIIb	1	1.6	-	-	$p > 0.05$
Grade IVa	-	-	-	-	
Grade IVb	-	-	-	-	
Grade V	-	-	-	-	

Our results indicate that the histopathological finding of the positive surgical margin was significantly more common in the ORRP group (19.6 % vs. 35.5 %) and this difference is statistically significant ($p < 0.5$). Data on postoperative PSA values indicate that it was statistically significantly higher in the ORRP group, after 3 months and after 6 months, respectively (0.0455 ± 0.0524 vs. 0.1708 ± 0.23 and 0.0781 ± 0.0995 vs. 0.115 ± 0.0931) (Table 4).

Table 4. Postoperative PSA and surgical margins

	LRP (n = 61)	ORRP (n = 62)	p
Positive surgical margins (PSM)	12 (19.6%)	22 (35.5%)	$p < 0.5$
After 3 months	0.0455 ± 0.0524	0.1708 ± 0.23	$p < 0.5$
After 6 months	0.0781 ± 0.0995	0.115 ± 0.0931	$p < 0.5$

Discussion

In this study, we presented our initial experience with LRP, with particular focus on results regarding perioperative complications and oncological outcomes, comparing these data with the data from the literature.To the best of our knowledge this is one of the first reports of this procedure in the Balkan region. Radical prostatectomy is a common curative treatment for localized prostate cancer. In this procedure, both oncological and functional outcomes based on health-related quality of life are taken into account. Radical prostatectomy has been developed from an open surgery to a laparoscopic procedure, with improved surgery made possible by magnification of the view of the anatomy around the prostate. Many authors have studied the effects of each of the radical prostatectomy surgery techniques (open, laparoscopic and robotic assisted). Laparoscopic surgery gained great popularity in the early 21st century. The basic motives and reasons for developing this technique are contained in its minimal invasiveness. The effects of laparoscopic technique on tissue have been the subject of study in many studies. Thus, Fornara et al. (13) determined its benefits over open kidney tumor surgery, followed by a decreased inflammatory mediator response. Frakalanca et al. (14) studied the extent of tissue damage using open and laparoscopic radical prostatectomy techniques and found that there were

very small differences in favor of laparoscopic technique. Similar conclusions were reached by Jurczok et al. in their prospective nonrandomized study (15). Open radical retropubic prostatectomy has its qualities, among which stand out especially: availability of performing in smaller centers, short duration of procedure, favorable cost of bone, relatively small invasiveness, possibility of working exclusively in extraperitoneal space, possibility of performing quality lymphadenectomy and relatively fast recovery (16, 17).

There were a total of 123 patients in our study series, 61 LRPs and 62 OORPs. There was no statistically significant difference in the number of subjects, their age and disease stage.

Preoperative PSA levels in both groups were below 10 ng/ml (low risk) and according to the recommendations by the EAU Guidelines, no lymphadenectomy was required. With regard to GS, no statistically significant differences were observed either in the preoperative biopsy appliance or in the definitive histopathological findings (18). Mean surgical time was similar in our two groups, with the period of time consistent with published data which had shown range from 100 to 151 minutes, as reported by Curtoet al. and Stolzenburg et al. (19, 20).

In regard to blood loss, in our series it was higher in ORRP group and it had been statistically significant. The range of blood loss was 235.61 ml to 315.19 ml in LRP and 297.42 ml to 412.92 ml in ORRP group. Average blood loss following LRP is reported to be from 200 ml to 390 ml (Curto, Goeman), and for ORRP 750 ml to 1284 ml (21, 22). Transfusion rates in our series were in 27% of patients after ORRP and in 12% after LRP. The difference is statistically significant and in favor of LRP group. It has been reported by several authors that transfusion rates ranges between 0.9% and 5.3% for LRP (19, 23) and 9.7% and 29% for ORRP (22, 24). We believe that slightly higher transfusion rates as compared to the literature data, with real blood loss consistent with the results of published studies, are primarily the result of a learning curve and increased caution during postoperative recovery in the intensive care unit.

Regarding the duration of hospitalization and removal of the urethral catheter, in both cases the period was shorter in the LRP group and the difference is statistically significant. Several authors, including Bhayani et al. and Reissweileret al. stated that the benefits of minimally invasive radical prostatectomy techniques over open include lower blood loss, lower blood transfusion rates, less need for analgesia, and shorter hospitalization, catheterization and recovery (25, 26). In regard to perioperative complication rates, according to Clavien-Dindo classification, in our series there were higher rate of grade I, grade II, grade IIIa and grade IIIb complications in the ORRP group. However, statistical difference is observed only for grade II (transfusion rates). Other complications were present at a low rate and with no statistical differences between the observed groups, and are consonant with other series.

The oncologic outcome of surgery as seen through positive surgical margins in the definitive histopathologic specimen has been better after LRP because the PSM rate was 19.6%, while in the ORRP group it was 35.5%, and this difference had been statistically significant. The range of PSM varies from 4.7% to 18.3% after LRP, and from 51% to 76.6% after ORRP (24-29). It is evident that the incidence of PSM following ORRP is much higher over LRP, which is in accordance to our results. Finally, PSA values measured at 3 and 6 months postoperatively, although in both groups within the low risk range, were statistically significantly higher after ORRP. This can be explained by a more accurate resection line at LRP as well as a higher rate of PSM at ORRP.

Conclusion

Our results concur with other retrospective reviews comparing laparoscopic and open radical prostatectomy, demonstrating unequivocal advantages of LRP in terms of blood loss, blood transfusions, average rates of Clavien-Dindo complications of grade I to IIIb, duration of hospitalization, catheter removal, positive surgical margins and postoperative values of PSA at 3 and 6 months.

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Originalni rad

UDC: 616.65-006.6-089.87
doi:10.5633/amm.2020.0302

LAPAROSKOPSKA RADIKALNA PROSTATEKTOMIJA: ISKUSTVO JEDNOG CENTRA

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Minimalno invazivne tehnike, uključujući robotski potpomognutu i laparoskopsku radikalnu prostatektomiju, postale su operativne tehnike izbora za operativno lečenje karcinoma prostate.

Cilj studije bio je proceniti i uporediti rezultate laparoskopske radikalne prostatektomije (LRP) i otvorene retropubične radikalne prostatektomije (ORRP), u pogledu bezbednosti, efikasnosti i onkološkog ishoda.

Ukupno 123 radikalne prostatektomije (RP) za nisko rizični lokalizovani karcinom prostate obavljene su u periodu od januara 2016. do juna 2019. godine, na Univerzitetskoj klinici za urologiju u Skoplju. Od toga je 61 radikalna prostatektomija (49,6%) bila LRP, a 62 (50,4%) bile su ORRP, uz prosečnu starost bolesnika 54 godine (od 33 godine do 67 godina). Indikacije za operativni postupak bile su: patohistološki nalaz adenokarcinoma prostate, starost ≤ 70 godina, PSA < 10 ng/ml, Gleson-skor ≤ 7 (3 + 3 ili 3 + 4), negativna scintigrafija kostiju, stadijum ≤ T2a, N0, M0. Svi bolesnici sagledani su kroz demografske podatke, nivoe PSA, Gleason-skor, trajanje operativnog zahvata, konverziju iz LRP u ORRP, gubitak krvi, perioperativne komplikacije, uklanjanje operativnog katetera, transfuziju krvi, boravke u bolnici i onkološki ishod.

LRP se pokazao superiornijim u odnosu na ORRP, što je rezultiralo kraćim operativnim vremenom, manjim gubicima krvi ($p < 0,5$), kraćim vremenom potrebnim za nastavak oralnog unosa hrane i tečnosti, kraćim postoperativnim boravkom u bolnici ($p < 0,5$) i manjim potrebama za analgetskom terapijom. Što se tiče onkološkog ishoda, primetili smo manje pozitivnih resekcionih ivica u grupi LRP ($p < 0,5$). Naši rezultati pokazuju da je, iako obe operativne tehnike predstavljaju bezbedne procedure i pružaju dobar kvalitet operativnog zahvata, LRP pokazala bolje rezultate u pogledu bezbednosti, efikasnosti i onkološkog ishoda.

Acta Medica Medianae 2020;59(3):13-19.

Ključne reči: *karcinom prostate, laparoskopska radikalna prostatektomija, otvorena retropubična radikalna prostatektomija*

Review article **UDC: 615.22:[636.087.7:547.466.64**
doi:10.5633/amm.2020.0303

DILTIAZEM PREVENTS MONOSODIUM GLUTAMATE TOXICITY IN THE RAT TESTES

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Increased exposure to food additives may exhibit harmful effect on the male reproductive system. Neonatal application of high doses of popular taste enhancer monosodium glutamate (MSG) to different kinds of animals cause lesions of the hypothalamic nuclei and the retina. Later in adulthood, animals exhibit a series of neuroendocrine disorders (stunted growth, obesity and decreased fertility).

The mechanism of MSG action is not completely explained yet. We hypothesized that high concentration of MSG could alter permeability of neural membrane for calcium. The objective of our study was to find out whether the pretreatment with diltiazem, a calcium channel blocker, could prevent harmful effect of MSG in the rat testes. Male rat pups were treated with: 0.9% sodium chloride (C group), 4 mg/g BW of MSG (M group), 5 mg/g BW of diltiazem (D group) and diltiazem 5 mg/g BW with MSG (DM group) on 2nd, 4th, 6th, 8th, 10th postnatal day. Animals were sacrificed at the age of six months. MSG treatment resulted in: stunted growth (decreased naso-anal length and tail length; $p < 0.05$), decreased relative testis weight ($p < 0.05$), and increased adipose tissue mass (Lee index; $p < 0.05$), testicular atrophy and decreased histomorphometric parameters (tubular area, tubular perimeter, Feret diameter, tubular diameter, epithelial height; $p < 0.001$). The rats of C, D and DM groups had normal testicular histology and morphometric parameters. Pretreatment with diltiazem has prevented the development of morphological disorders of testes. Our results suggest that calcium overloading may play an important role among mechanisms of MSG testicular toxicity.

Acta Medica Medianae 2020;59(3):20-26.

Key words: *monosodium glutamate, diltiazem, morphometric parameters, testes, toxicity*

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Introduction

The decrease of male fertility has been reported in numerous countries over the past few decades. The meta-analyses data showed a decline in both quality and quantity of human sperm (1-6). A modern life style carries increased exposition to different chemical, biological and physical etiologic factors. The harmful effects on male fertility are the most often attributed to: infections, the negative influence of alcohol, cigarette smoking habit, drug addiction, exposition to heat, excessive exercising, overweight or underweight, exposition to toxic products (lead, cadmium, etc.) and iatrogenic factors (7-10).

A certain risk is hidden in increased use of food additives like monosodium glutamate (MSG). This popular taste enhancer is widely used in commercial and domestic food preparation. MSG is the salt of nonessential glutamic acid. It has a property to enhance the perception that flavors are well blended and full-bodied and disguise unwelcome tastes. This additive is present in almost all food products: dehydrated soups or sauces, canned and frozen foods and meals, fresh sausages, marinated meats, and stuffed or seasoned chicken, bottled soy or oriental sauces, manufactured meats, some hams, flavored tuna, vegetarian burgers and sausages, flavored chips and snacks (11-14).

Numerous studies have shown that application of high doses of MSG (1-4 mg/g BW) especially during the neonatal period may cause lesions of the preoptic nuclei, arcuate nuclei, the circumventricular organs and the retina in different kinds of animals (mice, rats, rabbits, hamsters, dogs, and monkeys)

(13, 15-26). During later life MSG treated animals exhibit a series of neuroendocrine disorders: stunted growth, obesity and decreased fertility (19, 20, 22, 23, 27-30). China Health and Nutrition Survey showed that MSG is responsible for development of obesity in human adults (31).

Negative influence of MSG on testes is documented by: decreased absolute and relative testes weights in treated animals (32-40), testicular atrophy and alterations of testis structure (38, 41-43), significant oligozoospermia and increased abnormal sperm morphology in adose-dependent fashion (44), and testicular hemorrhage, degene-ration and alteration of sperm cell population and morphology (42).

The way of MSG action is not completely explained yet. It is well known that MSG has high excitotoxic potential. We hypothesized that high concentration of MSG could alter permeability of neural membrane for calcium which could be involved in the mechanisms of MSG toxicity.

The objective of the present study was to examine whether the pretreatment with L-calcium channel blocker, diltiazem, may prevent toxic effect of MSG in Wistar rat testes.

Materials and methods

The study was carried out in 24 neonatal male Wistar rats. The pups were injected sub-cutaneously interscapulary on the 2^{nd}, 4^{th}, 6^{th}, 8^{th} and 10^{th} day of life with equal volume of: 0.9% of sodium chloride solution (C group), 4 mg/g BW of MSG (M group), 5 mg/g BW of diltiazem (D group) and 5 mg/g BW of diltiazem and 60 minutes later with 4 mg/g BW of MSG (DM group).The animals were housed in standard cages under controlled laboratory conditions. The room temperature was 23 ± 2 ^{O}C and air humidity 50 ± 5%. The rhythm of light and darkness was established (light phase from 6.00 a.m. to 6.00 p.m.). The pups were weaned at age of 28 days and subsequently had free access to tap water and to standard laboratory chow pellets ("Veterinarski zavod" Zemun). The animals were sacrificed at the age of six months under pentobarbital sodium anesthesia (40 mg/kg BW intraperitoneally). Biometric parameters were measured: body weight, naso-anal length, tail length, absolute and relative testis weight and Lee index. The Lee index was used for assessment of obesity. It was calculated by the formula: cube root of body weight (g) x10^3/naso-anal length (mm) for each animal (45-47).

The testes were carefully removed, cleaned of the surrounding tissue and weighed. The relative testis weight was then calculated as: absolute testis weight (g)/[body weight of rat (g)] x100 for each animal (48). The testis tissue was prepared according to appropriate procedures then stained routinely with periodic acid-Schiff (PAS) and with haematoxylin-eosin (H-E methods), later analyzed and described. Serial sections stained with haematoxylin and eosin were subject of histomorphological examinations. Histomorphometric analysis of the testes was performed on Leica microscope equipped with Leica DC 180 and DC 480 camera using Image J v1.39d. programme. The tubular diameter was measured on at least 30 randomly chosen tubular profiles of round or nearly round shape for each of 3 sections per animal. Area, perimeter and diameter were measured at x20 magnification. The height of the seminal epithelium was measured at x40 magnification at randomly chosen tubular profiles of oval or round shape with at least 6 measurements per tubule, including the highest and the shortest part.

Results of statistical analysis are expressed as means ± standard deviation (SD). Statistical significance was determined with analysis of variance (ANOVA) test. The differences were considered significant at $p < 0.05$ or $p < 0.001$ level. All statistical analyses were performed using the SPSS statistical software (Version 15). All procedures on animals followed Guideline for Work on Experimental Animals approved by the Ethic Committee of Faculty of Medicine, University of Niš.

Results

Statistically significant difference of mean values between analyzed groups was present in all analyzed biometric parameters except in body weight (Table 1). Parameters: naso-anal length, tail length, and Lee index in rats of M group were significantly lower ($p < 0.05$) than in rats of the other groups (C, D and DM). Absolute testis weight was significantly lower in rats of M group than in rats of C and D group. Relative testis weight was sig-nificantly lower in M than in D group of rats.

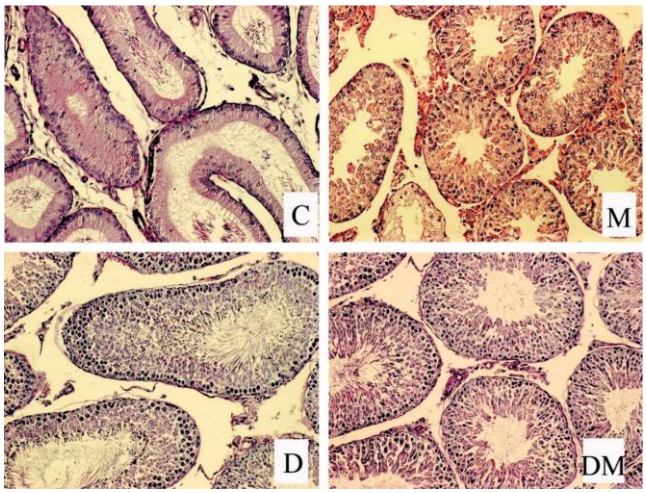
Histological examination revealed testicular atrophy in MSG treated rats. The most prominent histological changes in testes of MSG treated rats were: reduced diameters of the seminiferous tubules with decreased numbers of germ cells and decreased spermatogenesis, edematous interstitium and not prominent Leydig cells (Figure 1. M). Normal testicular histology was found in animals of control group, D and DM group (normal semini-ferous epithelium rich with developing germ cells through continuous spermatogenesis and the semi-niferous tubules containing spermatozoa) (Figure 1. C, D and DM).

Histomorphometric parameters: tubular area, tubular perimeter, Feret's diameter, tubular diameter and epithelial height were determined (Table 2). The lowest values of measured parameters were in group M and they were significantly lower than in groups D, DM and C ($p < 0.001$). The values of parameters in animals of group C were significantly higher than in groups DM and M. The highest values of all measured parameters (tubular area, tubular perimeter, Feret's diameter, tubular diameter and epithelial height) were found in animals of group D. These parameters were significantly higher in group D than in groups DM and M, but not significantly higher in group D than in group C. All parameters in animals of group DM were significantly higher than in group M and lower than in groups D and C.

Table 1. The mean values of biometric parameters in animals of C, M, D and DM group

	Groups			
	C	M	D	DM
Body weight (g)	531.67 ± 27.69	560.00 ± 36.88	528.33 ± 33.71	550.00 ± 41.47
Testis/AW (g)	3.13 ± 0.22	2.60 ± 0.31 *,†	3.16 ± 0.20	3.20 ± 0.27
Testis/RW (g)	0.59 ± 0.05	0.47 ± 0.08 †	0.60 ± 0.07	0.59 ± 0.09
NA length (cm)	26.33 ± 0.41	23.58 ± 0.92 *,†, ‡	26.00 ± 0.63	26.50 ± 1.14
Tail length (cm)	21.28 ± 1.11	19.58 ± 0.80 *,†, ‡	21.17 ± 0.61	22.25 ± 0.42
Lee index	0.31 ± 0.01	0.34 ± 0.02 *,†, ‡	0.31 ± 0.01	0.31 ± 0.01

C – control group treated with 0,9% NaCl;
M – group treated with 4 mg/g of MSG;
D – group treated with 5 mg/g of diltiazem;
DM – group treated with 5 mg/g of diltiazem and 4 mg/g of MSG;
AW – absolute weight; RW – relative weight; NA – naso-anal.
Results are expressed as means ± standard deviation.
Statistically significant difference ($p < 0.05$) between: * M and C; † M and D; ‡ M and DM.



C – control group treated with 0.9% NaCl;
M – group treated with 4 mg/g of MSG;
D – group treated with 5 mg/g of diltiazem;
DM – group treated with 5 mg/g of diltiazem and 4 mg/g of MSG.

Figure 1. Section of testes of rats from C, M, D and DM group; PAS stained; magnification x40.

Table 2. Mean values of testes morphometric parameters in animals of group C, M, D and DM

	Groups			
	C	M	D	DM
Tubular area	77456.9 ± 12231.4	37818.0 ± 9789.6	82248.4 ± 8137.8	57028.0 ± 11014.7
Tubular perimeter	1013.3 ± 81.5	712.2 ± 88.4	1037.9 ± 55.8	874.2 ± 83.1
Feret's diameter	361.5 ± 40.1	258.7 ± 33.5	359.8 ± 34.0	313.7 ± 36.5
Tubule diameter	289.4 ± 25.1	192.1 ± 37.7	291.5 ± 25.9	237.7 ± 27.7
Epithelial height	48.6 ± 9.6	38.5 ± 11.0	48.0 ± 11.3	41.3 ± 9.6

C – control group treated with 0.9% NaCl;
M – group treated with 4 mg/g of MSG;
D – group treated with 5 mg/g of diltiazem;
DM – group treated with 5 mg/g of diltiazem and 4 mg/g of MSG.
Results are expressed as means ± standard deviation. The values of measured parameters in group M were significantly lower than in groups D, DM and C ($p < 0.001$)

Discussion

In recent years glutamate receptors has been given a very important role in pathogenesis of disorders induced by MSG. There are two basic types of glutamate receptors: ionotropic [N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate] and metabotropic (mGluR) (31, 49-51). Glutamate receptors are present in different tissues: hypothalamus, heart, lungs, liver, kidneys, endocrine system, ovaries, uterus, testes, etc. (51-54).

It is referred that administration of high doses of MSG to adult Wistar rats, after two weeks, induced degenerative and atrophic changes in testes (43, 55). Also, increased apoptotic changes in the germinal epithelial cells and decreased germinal epithelial thickness were registered in adult Wistar rats treated with MSG (3 g/kg/BW/) by gavage for 30 days (55).

These findings could be explained by effect of high concentration of MSG on glutamate receptors in peripheral tissues as testes. Activation of glutamate receptors by sustainable high concentration of MSG could alter ionic permeability of neural membrane and induce persistent depolarization (26, 56). Neural death can be induced by such excessive activation of glutamate receptors and overloading with intracellular calcium (38, 57).

The toxic effect of MSG on endocrine (male reproductive system) has been explained for years as the late consequence of hypothalamic nuclei impairment and persisting disturbances of the hypothalamic-pituitary-gonadal axis. Necrosis of hypothalamic structures is the most clearly demonstrated from three to five hours after application of MSG in neonatal animals. It is demonstrated that 24 hours after MSG application, necrotic cells are already phagocytized and signs of edema have disappeared (21, 27).

The presence of the central effect of MSG is well known, but its mechanism is not clear yet. In our experiment the effectiveness of the treatment with MSG is confirmed with the next findings: stunted growth (significantly shorter naso-anal and tail lengths) and obesity ($p < 0.05$). Although the MSG treated rats were not significantly heavier than the controls, they were significantly more obese, what is confirmed with a significantly increased Lee index ($p < 0.05$) (45, 58-62).

In MSG-treated rats, we registered a significant reduction in absolute weight of testes and gonadosomatic index (relative testis weight) compared with these parameters in rats of C, D and DM groups. Our results confirm findings of the studies which registered the decrease of gonadal weights in MSG treated animals (32-38, 40).

Decreased organ weight is a sign of toxic injury. In our study, marked testicular atrophy is shown by light microscopy in rats neonatally treated with MSG. We confirmed alterations in testes structure reported by other authors (38, 41-43, 63, 64).

Normal testicular histology was present in other groups of rats (C, D and DM). These findings are strongly supported by the results of histomorphometric parameters analysis. We recorded highly significantly decreased values of histomorphometric parameters (tubular area, tubular perimeter, Feret's diameter, tubular diameter and epithelial height) ($p < 0.001$) in rats neonatally treated with MSG compared with parameters in rats of C, D and DM group.

Conclusion

The results of our study show that the pretreatment with diltiazem is efficient in prevention of MSG toxicity on testes in Wistar rats. MSG harmful effects: stunted growth, decreased relative testis weight, increased adipose tissue mass, testicular atrophy and decreased histomorphometric parameters are prevented in rats pretreated with diltiazem. We consider that excessive activation of glutamate receptors and overloading with calcium could be responsible for neurotoxic potential of MSG. Subsequent studies should be done to elucidate if pretreatment with slow calcium channel-blocking agents could prevent toxic effect of MSG on testes in adult animals.

Acknowledgment

This research was supported by project 43012 of the Ministry of Science and Technological Development, Republic od Serbia.

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Revijalni rad **UDC: 615.22:[636.087.7:547.466.64**
doi:10.5633/amm.2020.0303

DILTIAZEM SPREČAVA TOKSIČNI UTICAJ MONONATRIJUM GLUTAMATA NA TESTISE PACOVA

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Povećana izloženost aditivima hrani može uticati štetno na muški reproduktivni sistem. Neonatalna aplikacija velikih doza mononatrijum glutamata (MNG), popularnog poboljšivača ukusa, u neonatalnom periodu kod raznih životinjskih vrsta izaziva lezije u jedrima hipotalamusa i mrežnjači. Kasnije, u odraslom periodu, životinje pokazuju niz neuroendokrinih poremećaja: zastoj u rastu, gojaznost i smanjen fertilitet. Mehanizam delovanja MNG-a još nije u potpunosti objašnjen. Pretpostavili smo da velika koncentracija MNG-a može izmeniti propusnost membrane neurona za kalcijum. Cilj naše studije je proučavanje mogućnosti sprečavanja štetnih efekata MNG-a na testise pacova pretretmanom diltiazemom. Mužjaci pacova tretirani su: 0,9% natrijum hloridom (C grupa), 4 mg/g TM MNG-a (M grupa), 5 m/g TM diltiazemom (D grupa) i 5 m/g TM diltiazemom sa MNG-om (DM grupa) drugog, četvrtog, šestog, osmog i desetog postnatalnog dana. Životinje su žrtvovane posle šest meseci. Kod pacova tretiranih MNG-om registrovani su: zastoj u rastu (smanjena nazo-analna dužina i dužina repa; $p < 0,05$), smanjene relativne mase testisa ($p < 0,05$) i povećanje količine masnog tkiva (povećan Lee indeks; $p < 0,05$), atrofija testisa i smanjenje histomorfometrijskih parametara testisa: tubularne aree, tubularnog perimetra, Feret dijametra, tubularnog dijametra i visine epitela ($p < 0,001$). Pacovi C, D i DM grupa imali su normalnu histologiju i morfometrijske parametre. Prethodno tretiranje diltiazemom sprečilo je preopterećenje ćelija kalcijumom i razvoj morfoloških poremećaja testisa. Naši rezultati sugerišu to da preopterećenje ćelija kalcijumom spada u mehanizme toksičnog delovanju MNG-a na testise.

Acta Medica Medianae 2020;59(3):20-26.

Ključne reči: *mononatrijum glutamat, diltiazem, morfometrijski parametri, testisi, toksičnost*

Original article

UDC: 616.12-008.331.1-074:618.3
doi:10.5633/amm.2020.0304

VALUE OF HAEMATOLOGICAL AND SERUM BIOCHEMICAL PARAMETERS IN THE PREDICTION OF PERINATAL OUTCOME IN PREECLAMPSIA

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Preeclampsia is a serious disorder characterized by a generalized maternal inflammatory response associated with diffuse endothelial cell dysfunction. Preeclampsia has a long preclinical phase before it manifests. The possibility of predicting complications in preeclampsia is clinically very significant, as it could contribute to the reduction of maternal and neonatal morbidity and mortality. The aim of this study was to examine whether haematological and serum biochemical parameters may be of use in predicting more severe clinical picture and worse perinatal outcome in preeclampsia.

The prospective observational study included the study group consisted of 30 singleton pregnancies with preeclampsia completed by caesarean section (CS). This study group was divided into two subgroups with respect to severity of preeclampsia (mild and severe). The control group consisted of 20 healthy pregnant women delivered by elective CS. Clinical characteristics of pregnant women, haematological and serum biochemical parameters, as well as perinatal outcome were analyzed. In preeclampsia, the higher values of hematocrit and hemoglobin are noted, and lower platelet count, as well as the higher values of aspartate aminotransferase (AST), alanine aminotransferase, lactate dehydrogenase (LDH), gamma-glutamyl transferase, cholesterol, triglycerides, uric acid, urea and creatinine. Laboratory parameters associated with a severe clinical picture of preeclampsia in our study, as well as with a worse perinatal outcome were thrombocytopenia and increased AST and LDH levels. However, despite being indicators of a poorer outcome, they cannot be used with absolute certainty and in isolation from other indicators to predict poor perinatal outcome in preeclampsia. Deciding the delivery time in relation to an expectative approach should be based on a comprehensive consideration of gestational age, fetal condition, clinical and laboratory maternal indicators.

Acta Medica Medianae 2020;59(3):27-35.

Key words: *biochemical parameters, haematological parameters, perinatal outcome, preeclampsia*

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Introduction

Hypertensive disorders in pregnancy occur in 2-10% of pregnancies worldwide (1). Preeclampsia is a serious disorder, unique in human pregnancy, characterized by a generalized systemic maternal inflammatory response associated with diffuse endothelial cell dysfunction. It is a complex disease in which numerous genetic, immunological and environmental factors interact. It is characterized by widespread systemic vascular endothelial dysfunction and microangiopathy in mothers, but not fetuses. The fetus does not develop any clinical manifestations similar to maternal syndrome, and fetal morbidity and mortality are solely the result of placental insufficiency. Preeclampsia also leads to an increased risk of neonatal morbidity and mortality, especially in relation to neonatal prematurity (2).

The prevailing view is that preeclampsia has its cause in abnormal placentation, which in turn leads to widespread maternal endothelial effects and clinical manifestation of the disease (3). Unusual amounts of placental debris, syncytiotrophoblastic microparticles, products of oxidative damage, pro-inflammatory cytokines and angiogenic factors are assumed to be released into the intervillous space, and then interact with the maternal endothelium and immune cells, causing maternal symptoms (4).

Significant changes in the structure and function of the maternal vascular endothelium lead to altered vascular reactivity, activation of the coagulation cascade, and multisystem damage that occurs in preeclampsia. One of the pathological changes is also in the endothelial cells of the renal glomerular capillaries (glomerular endotheliosis), which results in some clinical manifestations.

Preeclampsia is known to have a long preclinical phase before symptoms and signs of disease become apparent in the second half of pregnancy. The severity of clinical picture in preeclampsia can range from milder to extremely severe forms, and life-threatening pregnancies. The main threats to a fetus are intrauterine growth retardation (IUGR), fetal distress, and even perinatal death of the fetus. On the other hand, while premature termination of pregnancy is always good for the safety of the mother, it can further compromise the fetus due to prematurity. Therefore, it is of utmost importance to adequately assess when the harm of an expectative approach may be greater than the risk of premature termination of pregnancy.

The possibility of predicting complications in preeclampsia is clinically very significant, as it could contribute to the reduction of maternal and neonatal morbidity and mortality.

The aim

The aim of this study was to examine whether haematological and serum biochemical parameters may be of use in predicting more severe clinical picture and worse perinatal outcome in preeclampsia.

Patients and methods

A prospective case-control observational study was conducted at the Clinic of Obstetrics and Gynecology, Clinical Center Niš. The study group consisted of 30 pregnant women whose pregnancies were complicated by preeclampsia, terminated by cesarean section, spontaneously conceived, singletons, with no fetal anomalies and preexisting clinical disorders, as well as without complicating actual pregnancy by diabetes and chorioamnionitis. The criteria for diagnosis of preeclampsia were new-onset arterial hypertension, or diastolic pressures of ≥ 90 mmHg and systolic pressures of ≥ 140 mmHg, measured on two separate occasions within 24h, more than 6 hours apart, and proteinuria of ≥ 300 mg of protein in 24-hour urine samples which were developed after the 20th week of pregnancy in previously normotensive women (5). In the analysis of clinical parameters, the highest recorded values of arterial blood pressure were used. The study group was divided into two subgroups: severe and mild preeclampsia, based on the presence of criterion for severe preeclampsia. The criterion for the diagnosis of severe preeclampsia was the presence of one of the following criteria: systolic blood pressure ≥ 160 mmHg or diastolic ≥ 110 mmHg, proteinuria ≥ 2 g/24h, increased serum creatinine, persistent headache or cerebro-visual disorders, persistent epigastric pain, platelet count < 100,000/mm^3 and/or findings of microangiopathic hemolytic anemia (with increased lactate-dehydrogenase) (6). The neonatal birth weight that was below the tenth percentile for a given gestational age was taken as a criterion for setting the diagnosis of IUGR. The control group consisted of 20 healthy pregnant women with singleton pregnancies, spontaneously conceived, with no fetal anomalies, delivered by elective cesarean section due to obstetric indications that cannot be linked to the etiology of tested disorders (previous cesarean section and breech presentation). The examined and the control group consisted of Caucasian women. The research has been approved by the Ethical Committee of the Medical Faculty, University of Niš and with the informed consent of the involved participants.

The results are systematized, and grouped in the data base. Statistical analysis was performed by using Statistical Package for Social Sciences software (SPSS version 15.0, Inc., Chicago, IL, USA). Continuous variables are presented as mean values, standard deviations and median, while the qualitative variables are presented by their frequency and percentage. Determination of the normality of distribution of continuous variables was performed by Shapiro-Wilk test. If the distribution of continuous data were normal, comparison of arithmetical mean values of two independent samples was performed by Student's t-test for independent samples, and if not, Mann-Whitney U-test was used. Comparison of absolute frequencies of categorical variables was performed by Chi-square test and his variants according to the size of the samples.

Results

Table 1 shows the clinical characteristics of pregnancies complicated by preeclampsia (severe and mild preeclampsia) compared to the control group. Comparisons and establishing the existence of statistical significance of differences were made between the unified study group with preeclampsia and the control group, while the table also shows the values of the parameters in the examined subgroups with severe and mild preeclampsia. The mean age of patients with preeclampsia was 31.3 years and was slightly higher than the control group (29.5 years), but no statistically significant difference in the age of pregnant women was reported. Although patients with severe preeclampsia were on average slightly older, no statistically significant difference in age was reported compared to patients with mild preeclampsia. A striking fact is that in the subgroup with severe preeclampsia, as many as 40.91% of patients were over 35 years old. Parity in pregnant women was significantly higher in the control compared to the study group (1.85 vs 1.40), by $p < 0.01$.

In preeclampsia, the mean gestational age at birth was significantly lower than the control group (36.60 gestational weeks (gw) vs 39.25 (gw) ($p < 0.001$). The proportion of preterm neonates in the

study group was 43.3%, and in 16.7% of the women in the study group the pregnancy had to be terminated before the 34th week of gestation. Proteinuria in severe preeclampsia was 2.81 g/24h with a median as a measure of central tendency of 0.57 g and only a few extremely high values. Severe preeclampsia within the study group was reported in 73.3% of patients, in 63.3% preeclampsia was associated with intrauterine growth retardation, and in 36.67% with oligoamnion.

The mean neonatal birth weight and the weight of the placenta in preeclampsia were significantly lower, the perinatal outcome worse and there were no perinatal deaths.

Table 1. The clinical characteristics of pregnancies complicated by preeclampsia (severe or mild) compared to the control group

Clinical parameters	**Preeclampsia** (n = 30)†				**Control group** (n = 20)†	
	Severe (n = 22)		**Mild** (n = 8)			
Age (years)	31.91 ± 6.55	34.00	29.63 ± 3.54	30.50	29.50 ± 4.54	28.50
Parity	1.32 ± 0.65	1.00	1.63 ± 0.52	2.00	1.85 ± 0.37**	2.00
Gestational age (weeks)	36.32 ± 2.63	36.50	37.50 ± 2.51	38.00	39.25 ± 0.97***	39.00
Systolic blood pressure	172.73 ± 15.69	170.00	147.50 ± 3.53	150.00	106.50 ± 11.93***	110.00
Diastolic blood pressure	110.77 ± 9.17	110.00	96.88 ± 3.34	97.50	65.75 ± 5.91***	70.00
Proteinuria (grams/24h)	2.81 ± 4.01	0.57	0.40 ± 0.10	0.39	0***	
Incidence of IUGR‡	15 (68.18%)		4 (50.00%)		0***	
Incidence of oligoamnion	7 (31.28%)		4 (50.00%)		0**	
Birthweight (grams)	2244.5 ± 773.2	2100.0	2731.2 ± 997.1	2675.0	3425.0 ± 451.4***	3475.0
APGAR score 1 min	7.68 ± 1.17	8.00	7.13 ± 2.42	8.00	8.80 ± 0.41***	9.00
APGAR score 5 min	8.14 ± 0.71	8.00	8.00 ± 1.60	9.00	8.95 ± 0.22***	9.00
Placental weight (grams)	436.3 ± 120.8	395.0	441.2 ± 119.2	485.0	585.5 ± 82.1***	580.0

† Data are presented as mean values ± standard deviation, median, or as incidences and percentages
‡ IUGR – Intrauterine growth retardation
** - $p < 0.01$; *** - $p < 0.001$

Table 2. Haematological parameters in preeclampsia compared to the control group

Haematological parameters	**Preeclampsia**† (n = 30)		**Control group**† (n = 20)	
Total leukocyte count, x 10^9/L	9.85 ± 2.68	9.30	9.29 ± 2.49	8.83
Red blood cells count, x 10^{12}/L	4.17 ± 0.38	4.20	4.01 ± 0.48	3.94
Hemoglobin, g/L	121.40 ± 12.92	126.00	108.75 ± 18.10 **	107.50
HCT - Hematocrit (%)	36.89 ± 3.38	38.00	34.28 ± 5.13 *	33.90
Platelets count, x 10^9/L	201.03 ± 51.52	207.50	253.10 ± 71.87**	238.50
Incidence of thrombocytopenia (< 150 x 10^9/L)	3 (10.00%)		1 (5.00%)	
Neonatal hemoglobin, g/L	152.4 ± 40.30	138.90	116.90 ± 11.50 ***	117.60
Neonatal hematocrit (%)	65.86 ± 6.70	68.00	56.72 ± 3.94 ***	57.00
Neonatal poycytemia (HCT ≥6 5%)	18 (60.00%)		0 (0.00%)***	
Neonatal platelets count, x 10^9/L	168.60 ± 32.25	162.50	189.50 ± 22.02 *	187.50
Incidence of neonatal thrombocytopenia	5 (16.67%)		0 (0.00%)	

† Data are presented as mean values ± standard deviation, median, or as incidences and percentages
* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

The study group reported statistically significant higher hematocrit ($p < 0.05$) and hemoglobin concentrations ($p < 0.01$), as well as lower platelet counts ($p < 0.01$) (Table 2). There were no statistically significant differences in haematological parameters between the two subgroups of the study group with respect to the severity of preeclampsia. However, all cases of thrombocytopenia were in the subgroup with severe preeclampsia.

Neonates in the study group reported significantly higher values of hematocrit and hemoglobin ($p < 0.001$), and significantly lower values of platelet count than the control group ($p < 0.05$). All cases of neonatal thrombocytopenia were in the group with preeclampsia, and were reported in 16.67% of cases. As many as 60% of the newborns in preeclampsia were polycythemic (with hematocrit ≥ 65), while none were reported in the control group ($p < 0.001$).

Table 3 shows the biochemical parameters in pregnant women with preeclampsia compared to the control group. The study group reported statistically significant higher values of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (γ-GT), triglyceride and urea by $p < 0.01$; lactate dehydrogenase (LDH), total cholesterol, creatinine and uric acid by $p < 0.001$ compared to the control group, and lower values of albumin ($p < 0.01$). The neonates in the study group reported statistically significant lower values of neonatal glycemia than the control group ($p < 0.05$).

Table 3. Biochemical parameters in preeclampsia compared to the control group

Biochemical parameters	**Preeclampsia**† (n = 30)		**Control group**† (n = 20)	
AST - aspartate aminotrans. (U/L)	23.63 ± 8.57	21.00	18.26 ± 6.24 **	17.45
ALT - alanine aminotrans. (U/L)	18.51 ± 7.75	16.30	12.28 ± 5.03 **	9.65
LDH - lactate dehydrogenase (U/L)	467.97 ± 229.61	416.30	308.07 ± 80.04 ***	304.50
γ-GT - gamma-glutamyl trans. (U/L)	12.60 ± 5.24	10.45	8.71 ± 3.80 **	7.45
Total bilirubin (μmol/L)	6.34 ± 2.74	5.95	9.69 ± 3.55 ***	8.75
Direct bilirubin (μmol/L)	0.94 ± 0.48	0.80	1.37 ± 0.60 **	1.10
Serum proteins (g/L)	59.67 ± 7.06	59.10	61.39 ± 3.76	61.40
Serum albumin (g/L)	30.32 ± 4.37	29.75	33.3 ± 4.32 **	33.60
Total cholesterol (mmol/L)	8.18 ± 1.75	8.73	6.40 ± 1.82 ***	6.00
Triglycerides (mmol/L)	4.07 ± 1.17	4.00	3.08 ± 0.99 **	2.94
Urea (mmol/L)	3.85 ± 1.68	3.40	2.59 ± 0.80 **	2.45
Creatinine (μmol/L)	71.62 ± 9.81	70.85	62.32 ± 6.80 ***	61.70
Uric acid (μmol/L)	329.38 ± 82.03	327.35	232.02 ± 39.58 ***	225.15
CRP - C-reactive protein (mg/L)	6.59 ± 3.73	6.15	5.20 ± 4.23	3.25
Neonatal glycemia (mmol/L)	2.49 ± 1.13	2.30	3.27 ± 1.07 *	3.40

† Data are presented as mean values ± standard deviation, median, or as incidences and percentages
* - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$

Discussion

The average age of pregnant women in our study group of 31.3 years is approximate to the average age of patients with preeclampsia in many other studies (7, 8). Although there are studies reporting the prevalence of younger pregnant women among those affected by preeclampsia (9, 10), our study showed that patients with severe preeclampsia were on average slightly older than those with mild preeclampsia. It is striking that in the subgroup with severe preeclampsia, as many as 40.9% of patients were of advanced age (35 years and older). Other studies have also found that advanced age is a risk factor for severe preeclampsia (11).

There is a statistically significant difference in the parity of the patients in our study and control group. This information is not surprising and it is predominantly due to the way in which the control group was formed, with the highest frequency of pregnant women with previous birth by caesarean section as the main indication for a repeated caesarean section, aimed at avoiding pathological conditions that could impair the quality of the control group, as well as at excluding the effects of vaginal birth on perinatal outcome. However, within the study group itself, primiparae dominated by 66.7%

in proportion, with an average parity of 1.4, which is in agreement with the findings of other authors (7, 12). This has been explained by the fact that the maternal immune system responds to the genetically strange fetus, based on the hypothesis that the mother's immune system "learns" to adapt to the fetus and that preeclampsia results from failure of maternal tolerance to paternal alloantigens (13). Within the study group, there was a statistically significant difference in the parity of the patients in relation to the severity of preeclampsia. Significantly higher number of primiparae compared to multiparae was in the subgroup with severe preeclampsia compared to those with the mild form of the illness. Thus, primiparae are at greater risk of developing severe preeclampsia.

In the study group, the average gestational age at birth was 36.6 gestational weeks. The research by Aviram et al. reported the approximate average age of pregnancies complicated by preeclampsia, at 36.2 gestational weeks (8), and in the study by Kumari et al. it was 35.5 weeks (10). The proportion of preterm neonates in the study group was 43.3%, which is also in agreement with the findings of other authors (9).

Proteinuria is one of the two essential criteria for defining preeclampsia. It is caused by damage to the endothelium of renal glomeruli as one of the manifestations of generalized endothelial damage that exists in preeclampsia. By the values of proteinuria, our study group is extremely inhomogeneous. Quite divergent results are reported among the existing studies examining whether there is an association between the degree of proteinuria and maternal and fetal perinatal outcomes. While some negate the influence of proteinuria degree on the presence of maternal complications and perinatal outcome (14, 15), others indicate an association between the degree of proteinuria and these complications (10, 16, 17).

Neonates from pregnancies complicated by preeclampsia, and especially preeclampsia associated with IUGR, are known to be at greater risk of various complications (18). The newborn parameters we analyzed, such as birth weight, and Apgar score at the 1^{st} and 5^{th} minutes, were all significantly lower in the study group. Our results are consistent with those of other authors suggesting an association of fetal growth restriction and preeclampsia (8, 19). The existence of cases associated with IUGR and cases with eutrophic growth within preeclampsia indicates at least two etiopathogenetic modalities in relation to the presence of placental dysfunction (20-22).

Of haematological parameters among our patients, the most significant is the statistically lower platelet count in the study group compared to the control group. All cases of thrombocytopenia were from the subgroup with severe preeclampsia. According to the findings of most other authors, low platelet count is one of the most important laboratory indicators associated with poor maternal outcome (23, 24). In pregnant women with a platelet count lower than 50 x 10^9/L, the risk of coagulation disorders is 7.78 times higher, and in those with a platelet count of 50 to 99 x 10^9/L 2.69 times higher than in pregnant women with a platelet count greater than 150 x 10^9/L (24).

In the study group significantly higher are the values of hematocrit and hemoglobin concentration compared to the control group, which is in agreement with the reports of some other authors indicating the association between hemoconcentration and preeclampsia (25). Hemoconcentration leads to the reduction of uterine perfusion. Negative correlation between hemoglobin values and neonatal birth weight was reported in both normotensive women (26) and women with preeclampsia (25, 27).

Of the neonatal haematological parameters, the registered elevated values of hemoglobin, hematocrit, and incidence of polycythemia in neonates in preeclampsia can be explained by chronic hypoxia. Chronic tissue hypoxia induces an increase in plasma erythropoietin levels during fetal life resulting in stimulation of fetal erythropoesis and polycythemia. Polycythemia, due to blood hyperviscosity, further burdens the neonatal hemodynamics and results in impaired cardiopulmonary and metabolic adaptation of the newborn with the deepening of hypoxia. It is often present in preeclamptic newborns, and especially those with impaired growth (28). In our study, the average platelet count in the neonates of the study group was significantly lower compared to the control group, and thrombocytopenia was present in 16.7% of the neonates in the study group, and was not reported in the control group. All of our registered cases of neonatal thrombocytopenia reported mild thrombocytopenia, which is a characteristic of most chronic intrauterine hypoxia - induced neonatal thrombocytopenia (29).

In pregnancies complicated by preeclampsia, thrombocytopenia is usually identified at birth or within the first 72h with resolution within the first 10 days of life in most cases (30). The pathogenesis of neonatal thrombocytopenia in preeclampsia is not completely clarified. One possible mechanism is that chronic hypoxia has a direct depressive effect on megakaryocytic proliferation, which is supported by a study showing that IUGR fetuses have a significant megakaryocytopoetic defect without evidence of increased platelet destruction (31).

The Benoit and Rey study did not determine that decreased plasma albumin levels in pregnant women with preeclampsia could be an independent marker of the severity of preeclampsia (32). The abnormality of any parameter of liver function increases the risk of poor maternal outcome (24), but do some parameters affect more than others?

Laskin et al. indicate a positive correlation of elevated AST, ALT, and LDH levels, decreased albumin levels, and poor maternal outcome (23), whereas von Dadelszen et al. report elevated AST values as one of the major predictors of poor maternal outcome (33). An increase in LDH levels is associated with an increase in the severity of the disease (34). In our study, all AST and LDH values above the reference values were reported in the subgroup with severe preeclampsia, so we considered these two parameters to be the most significant indicators of the severity of preeclampsia among all biochemical parameters.

Changes in lipid status that occur in normal pregnancy are accentuated in preeclampsia (35-37). Our study group, too, reported increased cholesterol and triglyceride levels. There are also data of increased triglyceride levels in preeclampsia with normal cholesterol levels (38). However, numerous studies suggest that there is no difference in the levels of lipid parameters in preeclampsia compared to normal pregnancy (39).

Of the other laboratory-biochemical parameters we analyzed, the values of urea, creatinine and uric acid were significantly higher in the study group. Renal dysfunction is primarily reflected in increased serum uric acid levels, which is the most sensitive laboratory indicator of preeclampsia and its specific marker (40). Elevation of uric acid levels in preeclampsia often precedes hypertension and proteinuria, i.e. precedes the clinical manifestations of this disorder. Although there are studies suggesting that uric acid levels may be a predictor of poor perinatal outcome in preeclampsia (11, 41-43), most have shown that its value in predicting poor maternal and fetal outcome has not yet been confirmed (44, 45). The decrease in its clearance is due to decreased glomerular filtration in preeclampsia, and its elevated values are also a consequence of its increased production under oxidative stress (40). Uric acid is the end product of purine metabolism and the involvement of xanthine oxidase enzyme is important for its synthesis. The oxidative damage to the placenta and the resulting cytokines accelerate the synthesis of this enzyme and thus increase uric acid production. During normal pregnancy, serum concentrations of uric acid drop by 25-30% in early pregnancy due to increased renal clearance resulting from increased glomerular filtration and decreased proximal tubular reabsorption and changes in its production. Later, during pregnancy, the levels of serum uric acid rise, especially due to increasing fetal production and decreased binding to albumin, up until the end of pregnancy when they reach prepregnancy values (40). The most widely accepted explanation for hyperuricemia in preeclampsia is an increase in proximal tubular reabsorption, a decrease in tubular excretion, and a consequence of increased xanthine oxidase activity. The study by Dong et al. reported that serum uric acid levels were approximately equal and not elevated in normal pregnancy and pregnancy with isolated gestational hypertension, and significantly higher in preeclampsia (46), while Williams et al. found that its values were elevated even in pregnancy-induced hypertension without proteinuria (47). Creatinine clearance was decreased in most patients with severe preeclampsia. However, serum creatinine levels were not very helpful because of the wide range of normal values, also shown in our study, with the study group reporting significantly higher urea and creatinine levels, but not beyond the referential ones. Changes in urea clearance are accompanied by changes in creatinine clearance. However, if serum creatinine values rise so much that they fall outside the reference range, a predictor of poor maternal outcome has been confirmed (24, 33).

Regarding the biochemical parameters of new-borns in preeclampsia, dominant is the finding of decreased values of glycemia. The cause of hypoglycaemia in these neonates should be sought in the reduced glycogen reserves (glycogenolysis is the major source of glucose in neonates in the first hours after birth), decreased fetal hepatic gluconeogenesis due to impaired hepatic flow, and decreased maternal glucose transplacental transport. Numerous studies confirm that hypoglycemia is one of the most commonly present biochemical parameters of neonates in preeclampsia (48).

Conclusion

The laboratory parameters in our study, associated with a severe clinical picture of preeclampsia and a worse perinatal outcome, were thrombocytopenia and elevated AST and LDH levels. However, despite being poorer outcome indicators, they cannot be used with absolute certainty and in isolation from other parameters to predict poor perinatal outcome in preeclampsia. Deciding the delivery time in relation to an expectative approach should be based on a comprehensive consideration of gestational age, fetal condition, and clinical and laboratory maternal indicators.

Conflict of Interest

The authors declare that they have no any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations.

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Originalni rad **UDC: 616.12-008.331.1-074:618.3**
doi:10.5633/amm.2020.0304

VREDNOST HEMATOLOŠKIH I SERUMSKIH BIOHEMIJSKIH PARAMETARA U PREDIKCIJI PERINATALNOG ISHODA KOD PREEKLAMPSIJE

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Preeklampsija je ozbiljan poremećaj, koji se karakteriše generalizovanim maternalnim inflamatornim odgovorom, udruženim sa difuznom disfunkcijom endotelnih ćelija. Preeklampsija ima dugu pretkliničku fazu, pre nego postane manifestna. Mogućnost predviđanja komplikacija kod preeklampsije je klinički veoma značajna, jer bi mogla doprineti smanjenju morbiditeta i mortaliteta majki i neonatusa.

Cilj ovog rada je da ispita da li hematološki i serumski biohemijski parametri mogu biti od koristi u predikciji teže kliničke slike i goreg perinatalnog ishoda kod preeklampsije.

Prospektivna opservaciona studija fokusirala se na ispitivanu grupu od 30 jednoplodnih trudnoća sa preeklampsijom završenom carskim rezom. Ova ispitivana grupa podeljena je na dve podgrupe. Ispitanice su podelje u grupe shodno težini preeklampsije (umerena i teška). Kontrolnu grupu činilo je 20 zdravih trudnica, porođenih elektivnim carskim rezom. Analizirane su kliničke karakteristike trudnica, hematološki i serumski biohemijski parametri, kao i perinatalni ishod. Kod preeklampsije, povišene su vrednosti hematokrita i hemoglobina, a umanjen je broj trombocita. Takođe, povišene su vrednosti aspartat aminotransferaze (AST), alanin aminotransferaze, laktat dehidrogenaze (LDH), gama-glutamil transferaze, holesterola, triglicerida, mokraćne kiseline, uree i kreatinina. Laboratorijski parametri, koji su u našem istraživanju bili udruženi sa teškom kliničkom slikom preeklampsije i gorim perinatalnim ishodom, bili su trombocitopenija i povišeni nivoi AST i LDH. Međutim, uprkos tome što su pokazatelji goreg ishoda, ne mogu se sa apsolutnom sigurnošću i izolovano od drugih pokazatelja koristiti u predikciji lošeg perinatalnog ishoda kod preeklampsije. Donošenje odluke o trenutku za porođaj, u odnosu na ekspektativni pristup, trebalo bi da bude bazirano na sveobuhvatnom sagledavanju gestacijske starosti, stanja fetusa, kliničkih i laboratorijskih maternalnih pokazatelja.

Acta Medica Medianae 2020;59(3):27-35.

Ključne reči: *biohemijski parametri, hematološki parametri, perinatalni ishod, preeklampsija*

Original article

UDC: 615.252.349.7:616.379-008.64-08
doi:10.5633/amm.2020.0305

INSULIN THERAPY INITIATION IN A PATIENT WITH TYPE 2 DIABETES IN EVERDAY CLINICAL PRACTICE: IS THERE A DELAY?

Slobodan Antić[1,2], Dragan Zdravković[1,2]

In everyday clinical practice, there is a large number of patients with poorly regulated type 2 diabetes (T2D), which contributes to the development of chronic complications of diabetes. Delayed initiation of insulin therapy in T2D is a particularly significant cause of poor long-term glucoregulation. There are various reasons for this delay, however, in Serbia as well as in Niš, the center of south Serbia, there is no enough data available. The present study was conducted in order to establish whether there was a delay in initiating insulin therapy in Niš, how long it was delayed in comparison to recommendations and experiences of the others, what was glucoregulation like six months prior to initiation of insulin therapy and whether the insulin therapy should have been initiated at that time.

According to the conducted study, at the time of initiation of insulin therapy, HbA1c was 10.51%, which was significantly higher in relation to other comparable studies. The delay can be considered to be at least 6 months, because at that time HbA1c was 9.63%, and all the criteria for initiation of insulin therapy were met.

Acta Medica Medianae 2020;59(3):36-40.

Key words: *type 2 diabetes, insulin therapy initiation, HbA1c, delay*

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Introduction

Diabetes related chronic complications are one of the most important factors that have impact on shortening of life expectancy in people with diabetes, as well as the deterioration of the quality of life (1-4).

The two largest and most significant diabetes studies, UKPDS (5-7) and DCCT (8), have unequivocally shown that good glucoregulation is directly related to the reduction especially of microvascular complications (diabetic retinopathy, diabetic polyneuropathy and diabetic nephropathy), but also to reduction of macrovascular complications and death (8, 9).

In order to have more successful type 2 diabetes management, referent scientific organizations have set HbA1c targets to be below 7% for most of patients, but according to the individual approach, the target can be even lower, between 6.5-8% (9-12).

In everyday clinical practice however, there is a large number of patients with poorly regulated type 2 diabetes, which goes in favor of development of chronic complications of diabetes, i.e. comorbidity. Late start of insulin therapy in type 2 diabetes is a great challenge and a great problem. There are various reasons behind, but basically, it is about insufficient education of both patients and doctors (13-18).

Availability of insulin initiation data in Serbia is quite poor, as well as the answer to the question whether the therapy could have been started earlier.

Research objectives

In accordance with the above mentioned data and performed review of so far available literature, the following research objectives have been set:

1. To examine insulin therapy initiation timeframe in people with type 2 diabetes in relation to glucoregulation.
2. To examine the patient's condition 6 months before insulin therapy introduction, especially in relation to glucoregulation.

Methods and patients

This study was a prospective-retrospective research that included 70 patients with type 2 diabetes treated at the Clinic for Endocrinology, Diabetes and Metabolic Diseases KC Niš in the year 2015.

The study included 40 years old patients and older, with a clinically confirmed diagnosis of type 2 diabetes, at least one year before switching to insulin therapy.

The study included anamnestic and clinical data analysis according to predefined research protocol: at the time of insulin therapy introduction, 6 months before and 6 months after.

All patients were informed in details about the study. Anamnestic data were obtained by a survey questionnaire. Two survey questionnaires (Appendix 10.1 and 10.2) were used throughout the study, and the clinical trial protocol 10.3 was divided into two time intervals:

- Clinical trial protocol 1, which refers to the condition at the time of insulin therapy introduction (Appendix 10.3.1);
- Clinical trial protocol 2, which refers to the patient's condition 6 months before insulin therapy introduction (Appendix 10.3.2).

Statistical data analysis was performed with the SPSS 15.0 software package. Data analysis results are presented in tables and graphs.

Continuous variables are represented by mean values and standard deviations (X ± SD) and medians as a measure of central tendency (Me). Category variables are given as absolute numbers and percentages.

Results

The study included 70 subjects with an average age of 60.97 ± 9.83 years, with a median of 60 years.

Out of the total number of participants, 29 (41.43%) patients were male and 41 (58.57%) were female (Table 1). Patients' age structure is shown in the table.

Female participants were slightly older than the males, but statistically not significant.

The average DM duration was 11.11 ± 6.31 years, and the median duration was 10 years (Table 2).

Table 1. Patients'age/sex characteristics

	Age (years)		
	X ±	SD	(Me)
Male	58,55 ±	9.12	(58.00)
Female	62.68 ±	10.06	(62.00)
Total	60.97 ±	9.83	(6000)

Table 2. DM duration

	DM duration (years)		
	X ±	SD	(Me)
Male	10.34 ±	5.63	(10.00)
Female	11.66 ±	6.77	(10.00)
Total	11.11 ±	6.31	(10.00)

High value of standard deviation indicates the inhomogeneity of this parameter in the examined sample, which is supported by the fact that the minimum of DM duration is one, and the maximum is 38 years. Female subjects have longer DM duration, but not statistically significantly longer vs. male subjects.

Glycosylated hemoglobin (HbA1c) values at the time of transition to insulin therapy were 10.51%, which is statistically significantly higher than 6 months before conversion to insulin (0.88%) (Table 3).

Kohen's d indicates the average effect of drug therapy in the period of 6 months before insulin initiation (Table 4).

Fasting glycemia also increased at the time of insulin initiation vs. 6 months before.

Table 3. HbA1c % at insulin therapy initiation and 6 months before

HbA1c %					
Insulin therapy initiation			6 months before insulin therapy initiation		
10.51 ±	1.49	(10.50)	9.63 ±	1.18	*** (9.65)
Δ (Pr. – 6 months before)			Δ (Pr. – 6 months after)		
0.88 ±	1.16	(0.85)	2.24 ±	1.10	(2.00)

*** - p < 0.001

Table 4. Fasting glycemia (mmol/l) at the time of insulin initiation, and 6 months before insulin initiation

Fasting glycemia (mmol/l)					
Insulin initiation			6 months before		
12.40 ±	2.86	(12,15)	11.81 ±	2.61	(11.50)
Δ (Pr. – 6 months before)			Δ (Pr. – 6 months after)		
0.59 ±	3.39	(0.75)	3.84 ±	2.88	(3.45)

*** - p < 0.001

Discussion

The study included 70 patients - 41 women (59%) and 29 men (41%). The mean age of the patients was 60.97 years. This finding was comparable to similar studies.

Duration of diabetes in our patients was 11.11 years, which is more than in patients in Germany's study INSTIGATE (19), where the insulin therapy is initiated much earlier.

The data about average diabetes duration might be considered with caution since establishing diagnosis of diabetes is often significantly delayed in real life. Evidences of elderly patients with a short known duration of diabetes, duration of around one year, but with marked hyperglycemia and already present complications of diabetes are supporting this claim (20).

Data about glycaemic control and especially in relation to HbA1c, are the most significant finding in the study and they are showing that insulin therapy is initiated with an average hemoglobin of 10.51%. This value of HbA1c is significantly higher than in all comparable studies conducted in Europe and America (19, 21-25). In a similar study (INSTIGATE) (19) in Germany and Spain, insulin therapy was initiated at HbA1c 9.2% and in Greece 9.7%. In the BiAsp/Glargine (21) study conducted in the USA, at the time of insulin administration HbA1c was 9.0%, while in the BIAsp-1556 study in Serbia HbA1c was 10.68%. The BIAsp study was conducted in Serbia and was among the first studies to indicate the late initiation of insulin therapy. (26, 27).

Compared to the period of 6 months before initiation of insulin therapy, there was a significant increase in HbA1c by 0.88%, which unequivocally showed that all patients could be introduced with insulin therapy even 6 months earlier, due to the fact that HbA1c was 9.63 ± 1.18% at that time. Even UKPDS (8, 9) study have shown that decrease in HbA1c values by only 1% leads to a reduced risk of microvascular complications by 33% (5-7). Delay of insulin therapy initiation did not bring any benefit to patients, but on the contrary an increase in HbA1c, which, according to available knowledge, increases the risk of late complications of diabetes (28).

Conclusion

In patients with type 2 diabetes on (sub) maximal therapy with oral hypoglycemics that have been poorly regulated for a long period of time, the initiation of insulin therapy is often delayed. Insulin therapy was initiated to patients in our research at HbA1c of 10.51%, which is significantly higher than in comparable studies in Europe and USA. This delay might be considered to be at least 6 months since the tests performed at that time (HbA1c - 9.63%) are suggesting that even then all the criteria for insulin therapy initiation were present.

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Originalni rad

UDC: 615.252.349.7:616.379-008.64-08
doi:10.5633/amm.2020.0305

DA LI SE KASNI SA UVOĐENJEM INSULINSKE TERAPIJE KOD BOLESNIKA SA DIJABETESOM TIP 2 U KLINIČKOJ PRAKSI?

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U svakodnevnoj kliničkoj praksi, veliki broj bolesnika sa dijabetesom tip 2 (T2D) je loše regulisan, što doprinosi nastanku hroničnih komplikacija dijabetesa. Kasno otpočinjanje insulinske terapije u T2D predstavlja posebno značajan uzrok dugotrajno loše glikoregulacije. Postoje različiti uzroci ovog kašnjenja, ali u Srbiji, kao i u Nišu, kao centru juga Srbije, nema dovoljno raspoloživih podataka. Da bi utvrdili da li kašnjenja u otpočinjanu insulinske terapije ima u Nišu, koliko je kašnjenje u odnosu na preporuke i iskustva drugih, kakva je glikoregulacija bila šest meseci pre otpočinjanja insulinske terapije i da li je tada trebalo otpočeti insulinsku terapiju, sproveli smo navedeno ispitivanje.

Prema sprovedenom ispitivanju, u vreme otpočinjanja insulinske terapije HbA1c bio je 10,51%, što je značajno više u odnosu na druge komaparabilne studije. Može se smatrati da je kašnjenje najmanje 6 meseci, jer je tada HbA1c bio 9,63% i bili su ispunjeni svi kriterijumi za otpočinjanje insulinske terapije.

Acta Medica Medianae 2020;59(3):36-40.

Ključne reči: *dijabetes mellitus tip 2, otpočinjanje insulinske terapije, HbA1c, odlaganje*

Review article

UDC: 616.37-002.1-092-08:618.2
doi:10.5633/amm.2020.0306

ACUTE PANCREATITIS IN PREGNANCY – FROM ETIOPATHOGENESIS TO THERAPY

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Acute pancreatitis (AP) in pregnancy is a relatively rare disease, but at the same time a great challenge for any physician, since it poses a potential threat to both the mother and the fetus. The occurrence of this serious disease requires multidisciplinary efforts in both the diagnosis and treatment, with the involvement of gynecologists, gastroenterologists and surgeons. AP is more frequent in the third trimester and postpartum period. Pregnancy has long been considered as a possible cause of acute pancreatitis. However, more recent studies have shown that in pregnancy the main causes for the development of AP are gallstones or hyperlipidemia, accounting for a higher incidence of this disease in pregnant women. The diagnosis of acute pancreatitis in pregnancy can be a challenge for the clinician, since the clinical manifestations may resemble various pregnancy complications, such as hyperemesis gravidarum, placental abruption, or ruptured ectopic pregnancy. Treatment strategy involves an assessment of both maternal and fetal risks. During severe AP, when there is single or multiple organ failure, emergency childbirth may be necessary. There have been no standardized recommendations for delivery for women with AP in their third trimester in order to reduce maternal and neonatal morbidity and mortality. Larger clinical studies are required for the formulation of recommendations for the diagnosis, follow-up of the clinical course, and treatment of AP in pregnant women.

Acta Medica Medianae 2020;59(3):41-47.

Key words: pancreatitis, pregnancy, diagnosis, therapy

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Introduction

Pregnancy is a specific physiological condition of a women in her reproductive age of life which runs without any complications in over 80% of cases. Acute pancreatitis (AP) in pregnancy is a relatively rare disease, but at the same time a great challenge for any physician, since it poses a potential threat to both the mother and the fetus. The occurrence of this serious disease requires multidisciplinary efforts in both the diagnosis and treatment.

Epidemiology

AP occurs in 0.03-0.09% of pregnancies (1). The incidence of AP in pregnancy has been reported to be as high as 1/1000 (2). AP is more frequent in the third trimester and postpartal period (3). If it occurs during the first trimester, AP is associated with a higher proportion of fetal complications and mortality, and should be taken very seriously in that period of pregnancy (3).

AP in pregnancy may present as a mild, self-limited disease, but more serious forms may develop as well, with local and distant complications, possibly causing the death of both the mother and the fetus (1). Maternal and/or fetal death rates have been markedly reduced thanks to the currently employed diagnostic methods and appropriate and timely therapy (1). Some recent studies have shown that maternal mortality caused by AP is below 1%, but fetal death rates are still rather high – 6.6% (4, 5). In pregnant women with AP preterm births (before gestational weeks 37 or 32) are more common, as well as respiratory distress syndrome and jaundice in their premature infants. Fetal morbidity and mortality are solely the consequence of premature birth (6). In term infants, morbidity and mortality rates have not been reported to be increased.

Ethiopathogenesis

Pregnancy has long been considered as a possible cause of acute pancreatitis. However, more recent studies have shown that in pregnancy the main causes for the development of AP are gallstones or hyperlipidemia, accounting for a higher incidence of this disease in pregnant women (7).

The most common cause of AP in pregnancy are gallstones, in about 70% of cases (4). Other possible etiological factors (Table 1) are alcohol intake, metabolic diseases, medicaments, traumas, medical interventions, infections, vascular disorders (ischemia), genetic factors (trypsinogen gene mutation), systemic diseases, structural anomalies, tumors, insect bites and other, still unrecognized factors (8, 9).

Table 1. Etiology of AP in pregnant women

• Gallstones
• Alcohol
• Hypertriglyceridemia
• Drugs
• Acute fatty liver in pregnancy
• Pre-eclampsia
• Traumas
• Medical interventions
• Infections
• Vascular disorders (ischemia)
• Genetic factors (trypsinogen gene mutation)
• Systemic diseases
• Structural anomalies
• Tumors
• Insect bites
• Unrecognized etiological factors

Gallstones are the most commonly identified etiological factor in the occurrence of AP in pregnancy, similarly as in the rest of (mostly female) population (10). During pregnancy, there are factors predisposing to the development of biliary calculi, such as increased body weight and hormonal changes. Gallbladder volume increases in pregnancy and biliary flow is reduced (10). Cholesterol is increased in the bile in relation to bile acids and phospholipids, which may result in the retention of cholesterol crystals and creation of gallstones. Increased estrogen levels in pregnancy may lead to biliary stasis (10). Increased progesteron levels induce the relaxation of smooth muscles in the gallbladder wall, which increases the pressure at the sphincter of Oddi level, which contributes to biliary stasis.

Hypertriglyceridemia in pregnancy may lead to the development of AP. The level of serum triglycerides exceeding 11 mmol/L may induce an acute pancreatitis attack. In pregnancy, the levels of triglycerides normally rise gradually, especially during the second and third trimester. AP usually occurs in women who had hypertriglyceridemia even before pregnancy (11). Familial hypertriglyceridemia can be further complicated with AP in pregnancy. However, triglyceride values and the severity of clinical picture of AP are not correlated. Hypercholesterolemia is not associated with acute pancreatitis; 10-50% of AP in pregnancy are associated with hypertriglyceridemia (12). AP caused by hypertriglyceridemia in pregnancy tend to be more serious and associated with a higher risk of complications (organ dysfunction, shock, infections) (5, 12, 13). Treatment of hypertriglyceridemia in pregnancy in order to prevent AP is complex and challenging, since it has been found for many lipid-lowering drugs that they cannot be safely used in pregnancy. The patients with hypertriglyceridemia usually also have multiple risk factors, such as diabetes, alcohol consumption, and hypothyroidism, which further complicates their therapeutic management (14).

Hyperparathyroidism and hypercalcemia are relatively infrequent causes of acute pancreatitis. Hyperparathyroidism is rare in pregnancy and is most commonly the consequence of parathyroid adenoma (15). Acute pancreatitis occurs as the consequence of hypercalcemia, which may also cause hyperemesis gravidarum, hypertension, pre-eclampsia, nephrolithiasis, muscle weakness, and even

hypercalcemic crisis. Fetal complications usually include postpartal hypocalcemia, low birth weight, preterm birth and fetal death. A timely diagnosis and adequate therapeutic management may improve the outcome, and in cases of unclear AP etiology it is necessary to take into consideration as well this possible cause of the disease (4).

Acute fatty liver in pregnancy (AFLP) is a rare disorder, described for the first time as a specific clinical entity by Sheehan in 1940. AFLP usually occurs in the third trimester and clinically manifests with nausea, vomiting, moderate enzyme elevation, development of coagulopathy, hyperfibrinogenemia, hypoglycemia and hyperbilirubinemia. Acute pancreatitis develops as a complication of this very serious condition and is life-threatening to the pa-tient. Development of a pseudocyst with secondary infections or hemorrhagic pancreatitis with retroperitoneal bleeding present a special problem in patients with already developed coagulopathy. Serial monitoring of serum lipase and amylase levels several days after the onset of hepatic dysfunction is therefore recommended. Compared to amylase, it appears that serum lipase is more useful in the monitoring of the course of acute pancreatitis. Diagnostic visualization methods (such as magnetic resonance imaging) can be a valuable tool in the diagnosis of pancreatitis and assessment of develop-ment of complications (16).

Pre-eclampsia. A recent study by Haker et al. has demonstrated a significant association of acute pancreatitis in pregnancy with pre-eclampsia (especially severe pre-eclampsia) (6). Pre-eclampsia is associated with microvascular disorders which may involve cerebral, placental, hepatic, renal and splanchnic circulation. Microvascular disorders may lead to the development of pancreatitis (17).

Clinical manifestations

Based on its clinical course and prognosis, according to the Atlanta classification, AP can be mild, moderate (moderately severe) or severe. Mild AP is the most common form of pancreatitis and it lasts for up to a week. It is characterized by the absence of any significant damage to the pancreas and development of local or systemic complications. Moderately severe AP is defined by the presence of transient organ failure (lasting for less than 48 hours) and/or local or systemic complications without any persistent organ failure or aggravation of comorbidities. Severe AP is defined by persistent single or multiple organ failure (MODS), i.e. organ failure lasting for more than 48 hours (Table 2) (18).

Table 2. AP severity according to the Atlanta classification

Mild AP	absence of organ failure and complications
Moderately severe AP	transient organ failure (< 48 h) and/or local or systemic complications
Severe AP	persistent single or multiple organ failure (> 48 h) (MODS)

Acute pancreatitis manifests clinically with unspecific symptoms and signs, which may delay a timely diagnosis. The most common symptom is abdominal pain, occurring suddenly and extending in the form of a "belt" to the back or to the left shoulder and left shoulder blade. The pain is usually accompanied by nausea and vomiting which may represent a reaction to the pain or the consequence of reactive dilation of the stomach due to retroperitoneal spread of the inflammation. The disease is commonly associated with abdominal distension, paralytic ileus and left pleural effusion (19).

Additional complications in pregnant women involve preterm labor, preterm delivery and pregnancy loss (1, 10).

A study by Luo et al. with 121 pregnant women with AP has demonstrated the association between the severity of AP and maternal and fetal morbidity and mortality. The group with severe AP had the highest rate of maternal and fetal mortality. It is assumed that in severe AP systemic inflammatory response can produce generalized endothelial dysfunction, causing further tissue damage (9). A study by Sun et al. reported as well that increased intraabdominal pressure in severe AP was assosiated with a higher risk of fetal death (20).

Diagnosis

The fundamental criteria for the diagnosis of acute inflammation of the pancreas have been for years abdominal pain and elevated serum amylases. Nowadays, new biochemical diagnostic assays and visualization methods are attracting much attention, although clinical picture of the disease and serum amylases are still important indices in identifying individuals with AP.

According to the revised Atlanta classification, the diagnosis of AP is made on the basis of the presence of two out of three criteria presented in Table 3 (18).

Table 3. Diagnosis of AP

1. Abdominal pain (acute attack of persistent, strong epigastric pain, frequently extending to the back).
2. Activity of serum lipase (or amylase) increased at least three times related to upper reference cut-off value
3. Characteristic finding of acute pancreatitis on computerized tomography (MSCT) or, less often, magnetic resonance (MR) imaging or transabdominal ultrasonography.

The diagnosis of acute pancreatitis in pregnancy can be a challenge for the clinician, since the clinical manifestations may resemble various pregnancy complications, such as hyperemesis gravidarum, placental abruption or ruptured ectopic pregnancy (21). Physiologic leukocytosis in pregnancy (< 16,000) should also be taken into account, which can have an impact on the assessment of AP severity. In normal pregnancy, alkaline phosphatase may be three times as high as normal, which can have an impact on the assessment of etiology (21). A study by Tang et al. has reported normal levels of liver enzymes in over 80% of pregnant women with biliary AP (1).

A threefold increase of serum amylase and/or lipase is considered to be an acceptable criterion for the diagnosis of AP in pregnant women. Serum lipase has a higher sensitivity and specificity in the diagnosis of AP compared to amylase (21).

Serum amylase values can be normal in severe forms of AP as the consequence of rapid destruction of acinar pancreatic cells, as well as in an acute exacerbation of chronic alcohol-induced pancreatitis due to deficient exocrine pancreatic secretion. In acute pancreatitis and hypertriglyceridemia, false low serum values of amylases may sometimes be obtained (22).

Echosonography of the abdomen is the first visualization procedure used to verify the diagnosis of AP in pregnant women. Furthermore, it is the initial method used to establish the etiology of AP, since it is highly sensitive in the detection of biliary stones, follow-up of pregnant women with mild disease forms, and monitoring of clearly defined complications of AP (10). Echosonography is usually done on the first day after hospital admission, and repeated as needed. It is the first, fundamental prognostic tool in the assessment of severity of AP (23). The method is capable of visualizing fluid collections, pseudocysts, necrotic collections or incapsulated necrosis. However, echosonography has its inherent limitations. Visualization of the pancreas can be difficult or impossible due to enlarged uterus and presence of excess intestinal gas, especially when a paralytic ileus develops in the first 48 hours (24, 25).

Endoultrasonography represents a combination of endoscopy and sonography and it is better in the detection of cholelithiasis if biliary pancreatitis is suspected. Endoscopic ultrasound is more specific than MRCP in the detection of choledocholithiasis and microlithiasis, and the method can be safely employed during the whole course of pregnancy (25).

Magnetic resonance (MR) is another visualization method which is valuable in the diagnosis of acute pancreatitis. Several clinical studies have shown that MR offers not only an adequate assessment of pancreatic parenchyma in mild AP, but also a reliable detection of pancreatic and peripancreatic necrosis, better discrimination between necrosis and fluid collections compared to CT, obviating the need for radiation exposure and injections of potentially nephrotoxic contrast mediums. Nevertheless, the information is scarce as to the safety of MR in the first trimester of pregnancy because of possible thermal injury to the fetus (25, 26).

MR cholangiopancreatography (MRCP) enables pancreatic parenchyma evaluation and diagnosis of choledocholithiasis with the sensitivity of over 90%, without any maternal and fetal exposure to harmful ionizing radiation. MRCP has limited the use of endoscopic retrograde cholangiopancreatography (ERCP) to exclusively therapeutic purposes (26, 27).

Treatment

Depending on the disease severity and course, the treatment can be conservative or surgical. These two modalities are not mutually exclusive; instead, they supplement each other. Treatment strategy involves an assessment of both maternal and fetal risks. The criteria for the treatment of pregnant women in intensive care units are similar to those for general population, such as the need for permanent fluid replacement, BMI > 30 kg/m^2, pleural effusions, C-reactive protein value of > 150 mg/dL in the first 48 h, necrosis of over 30% of pancreatic tissue and > 3 Ranson's criteria (28). Early ICU admission and adequate treatment reduces maternal morbidity and mortality rates.

During severe AP, when there is single or multiple organ failure, emergency childbirth may be necessary (9, 28). Termination of pregnancy is suggested if a clinical disease exacerbation occurs 24-48 hours after the initiation of active treatment for moderate and severe AP (29).

There have been no standardized recommendations for delivery for women with AP in their third trimester in order to reduce maternal and neonatal morbidity and mortality. The decision depends on the gestation age and severity of AP. Vaginal delivery, if feasible, is safer compared to cesarean section because of more limited chances for infection

(29, 30). In the study by Luo et al., most deliveries have been performed by cesarean section (9).

Conservative treatment involves general therapeutic measures and management of complications.

General therapeutic measures are started by discontinuing food intake by mouth.

In patients with mild AP, oral food intake should be resumed after the cessation of pain (within 24-48 h), i.e. when painful abdominal tenderness has disappeared, but only if bowel peristalsis can be heard (31). Resumption of feeding per os is an important therapeutic measure in AP, providing the integrity of bowel mucosa and preventing the translocation of bowel bacteria and infection of necrotic areas (19, 31,32).

Fluid resuscitation is an essential and emergency measure in AP since it preserves pancreatic microcirculation and prevents necrosis (19). Furthermore, rapid fluid resuscitation is able to prevent hypotension and renal insufficiency. Adequacy of fluid resuscitation should be controlled by way of central venous pressure monitoring; clinically, the values of vital signs should be monitored, such as renal function, electrolytes and HCT. Based on the available data, a conclusion may be drawn that the infusion speed of 5-10 ml/kg is safe if appropriate clinical parameters are monitored (magnitude of diuresis, heart beat rate).

Several studies have investigated the type of fluid to be used for fluid resuscitation. Most recent data has shown that Ringer's lactate solution is superior to physiological solutions in the prevention of SIRS (28, 30).

Analgesia has an important role in the management of pain in AP. A taering pain in severe AP is commonly the main therapeutic problem. It is most important to choose an effective and safe analgesic. Inadequate analgesia may increase patient anxiety and respiratory distress. On the other hand, analgesia may have an impact on the physiological response and immune mechanisms. In the treatment of AP in pregnancy, fentanyl and meperidine can be administered (28).

Antibiotics are not recommended in the prevention of pancreatic necrosis in AP. When their use in AP is justified, a special problem in pregnant women is the choice of antibiotic agents. If cholangitis is present or in infected pancreatic necrosis, the use of an appropriate antibiotic is required. Metronidazole passes the placental barrier, but recent studies have not reported any teratogenic effects of the drug (24). Imipenem belongs to the class of carbapenems and has a broad spectrum of activity. It is currently classified as a fetal risk category C drug. Although certain limited studies on animals have shown some adverse effects on fetus there are no appropriate studies on humans, so that possible benefits could perhaps outweigh potential risks (24). Quinolones too belong to the category C drugs used in pregnancy. Ampicillin/sulbactam and piperacillin/tazobactam are classified as category B (studies on animals demonstrating no risks, but the appropriate controlled studies on human subjects are missing).

Severe forms of AP are often accompanied by acidosis, which should be corrected with bicarbonates.

In pregnant women with severe hypertriglyceridemia heparin and insulin infusions can be used in order to enhance the activity of lipoprotein lipase (3). In some cases, plasmapheresis is recommended (33). However, plasmapheresis can be accompanied by complications such as transfusion and allergic reactions. Again, there are no standardized recommendations for such cases. Pregnancy termination (by labor induction or cesarean section) is considered and decided upon in accordance with the severity of AP. Early therapy in pregnant women with hypertriglyceridemia-related AP can improve the clinical course and outcome of the disease (33).

Low molecular weight heparin can reduce the incidence of pancreatic encephalopathy and mortality rates in severe acute pancreatitis cases in the general population (33).

ERCP should be done within 48-72 hours after the disease onset if AP is complicated by acute cholangitis, when there are clinical or radiographic signs of gallstones present in the common bile duct (dilated bile duct, visible bile duct stone, jaundice, or permanently elevated liver enzyme values). The main problem with the procedure is harmful ionizing radiation. Nevertheless, some studies have shown that the dose of ionizing radiation can be reduced to the values markedly below the allowed values by using adequate lead shields for the pelvis and fetus and by shortening radiation exposure times, which involves adequate prior diagnosis using MRCP and EUS (24). ERCP is also useful in pregnant women in whom surgical intervention is not indicated. Endoscopic sphincterectomy is useful in the prevention of biliary AP recurrence during pregnancy.

Surgical treatment

During the course of AP treatment it is very important to make a timely decision about surgical treatment. It proceeds from the guidelines that in pancreatic necrosis, a surgical intervention is not recommended within 2 weeks of the disease onset, except in severe clinical exacerbations in spite of the measures of intense conservative treatment, in cases with infected pancreatic necrosis, or with multiorgan dysfunction in massive sterile necrosis (34).

Conclusion

Each pregnancy complicated by the development of AP is considered a high risk pregnancy, requiring a special treatment approach. Fortunately, AP in pregnancy is a rare entity. The existing limitations in diagnostic and therapeutic approach to pregnant women with AP require a serious multidisciplinary approach by gastroenterohepatologists, gynecologists and clinical pharmacologists. Larger clinical studies are required for the formulation of recommendations for the diagnosis, follow-up of the clinical course and treatment of AP in pregnant women.

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Revijalni rad

UDC: 616.37-002.1-092-08:618.2
doi:10.5633/amm.2020.0306

AKUTNI PANKREATITIS U TRUDNOĆI – OD ETIOPATOGENEZE DO TERAPIJE

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Akutni pankreatitis (AP) u trudnoći je relativno retka bolest, ali istovremeno i veliki izazov za lekara, jer predstavlja potencijalnu pretnju po majku i plod. Pojava ove ozbiljne bolesti zahteva multidisciplinarni pristup u dijagnostici i lečenju, uz učešće ginekologa, gastroenterologa i hirurga. AP je učestaliji u trećem tromesečju i postpartalnom periodu. Trudnoća se dugo smatrala potencijalnim uzrokom akutnog pankreatitisa. Međutim, novija istraživanja ukazala su na prisustvo drugih etioloških faktora, kao što su kamenci ili hiperlipidemija, čime se objašnjava veća učestalost ove bolesti kod trudnica. Dijagnoza akutnog pankreatitisa u trudnoći može biti izazov za kliničara, jer kliničke manifestacije mogu nalikovati različitim komplikacijama u trudnoći, poput hiperemeze gravidarum, odvajanja posteljice ili rupture vanmaterične trudnoće. Strategija lečenja uključuje procenu rizika za majku i fetus. Tokom teškog AP, kada postoji insuficijencija jednog ili više organa, može biti potreban hitan porođaj. Ne postoje standardizovane preporuke za porođaj žena sa AP u trećem tromesečju, kako bi se umanjili morbiditet i smrtnost majki i novorođenčadi. Za formuliranje preporuka za dijagnozu, praćenje kliničkog toka bolesti i lečenja AP kod trudnica, potrebne su iscrpnije kliničke studije.

Acta Medica Medianae 2020;59(3):41-47.

Ključne reči: *pankreatitis, trudnoća, dijagnoza, terapija*

Original article

UDC: 582.639.21:615.451.1]:612.79.015
doi:10.5633/amm.2020.0307

EFFECT OF APPLICATION OF EMULSIONS WITH STANDARDIZED EXTRACT OF WILD APPLE FRUIT (*MALUS SYLVESTRIS* (L.) MILL., ROSACEAE) ON BIOPHYSICAL SKIN PARAMETERS: AN *IN VIVO* STUDY

Ana Kolarević[1], Dragana Stojiljković[2], Sandra Dinić[1], Ivana Nešić[1]

Wild apple fruit (*Malus sylvestris* (L.) Mill., Rosaceae) represents a valuable source of biologically active compounds, such as polyphenols and fruit acids. These compounds have been found to have a positive effect on human health and also have a beneficial effect on the skin by improving its overall condition and appearance. In the present study, the efficacy of the application of oil-in-water emulsions containing 12% and 15% of wild apple fruit extract was examined on healthy volunteers. During a 28-day long-term study, biophysical skin parameters (electrical capacitance (EC), transepidermal water loss (TEWL), pH, erythema index (EI), and melanin index (MI)) were monitored. As a result, a significant increase in EC and decrease in EI and MI parameters were observed after both 14 and 28 days of application, where the emulsion containing 15% of the wild apple fruit extract was more efficient than the emulsion containing 12% of the wild apple fruit extract. On the other hand, no significant changes in TEWL and pH values were observed. Given their beneficial effects on the skin (increased skin hydration, reduced skin irritation, good skin lightening potential), the tested emulsions might have potential application in the formulation of cosmetic products for the treatment of dry and irritated skin, as well as products intended to reduce skin hyperpigmentation.

Acta Medica Medianae 2020;59(3):48-55.

Key words: *Malus sylvestris, extract, emulsion, biophysical skin parameters, in vivo study*

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Introduction

Wild apple (*Malus sylvestris* (L.) Mill., Rosaceae) is a shrub up to 4 m high or a deciduous tree that grows to a height of 10 m, creating a branched, wide, irregular and dense canopy. It is widespread in Europe. It grows throughout Serbia along forests, on the border between lower and hilly regions. The healing part of the wild apple is the fruit. The fruit is globose, yellow to red, 6-8 cm in diameter in most wild species. It ripens in late summer and in the fall when harvested (1, 2). Wild apple was mainly used as an decorative plant because of its ornamental features, such as the color of leaves and flowers. The fruit is edible, though extremely acidic and bitter. Due to the significant amount of pectin (~3%), wild apples are used for gelling other fruit products, making jams, fruit juices, wine, brandy or syrups (3). Wild apple fruit is also used to make apple cider vinegar, which, in various combinations, usually with water and honey, is a multifaceted drug and a restorative agent for the human body (1).

Wild apple fruit, primarily red varieties, is a valuable source of biologically active compounds, mostly concentrated in the bark of the fruit. In addition to affecting the nutritional values, appearance, taste and texture of the fruit itself, these compounds have a positive effect on human health (4). Polyphenols are considered to be the main bioactive compounds present in wild apple fruit, including flavan-3-ols (30-90%), flavonols (1-13%), dihydrochalcones (1-10%), anthocyanidins (1-7%) and phenolic acids (Figure 1) (4-6). Flavonoids exhibit a wide range of biological activities, with their antioxidant and anti-inflammatory effects being most pronounced (7-10). Further, wild apple fruit contains α-hydroxy acids, such as malic, citric, succinic, tartaric, pyruvic and lactic acid (Figure 2). In addition to affecting the taste of the apple itself, fruit acids exert

numerous positive effects on the skin (accelerate skin desquamation, hydrate the skin, regulate skin pH, lighten hyperpigmented parts of the skin), thereby improving its overall condition and appearance (11-14).

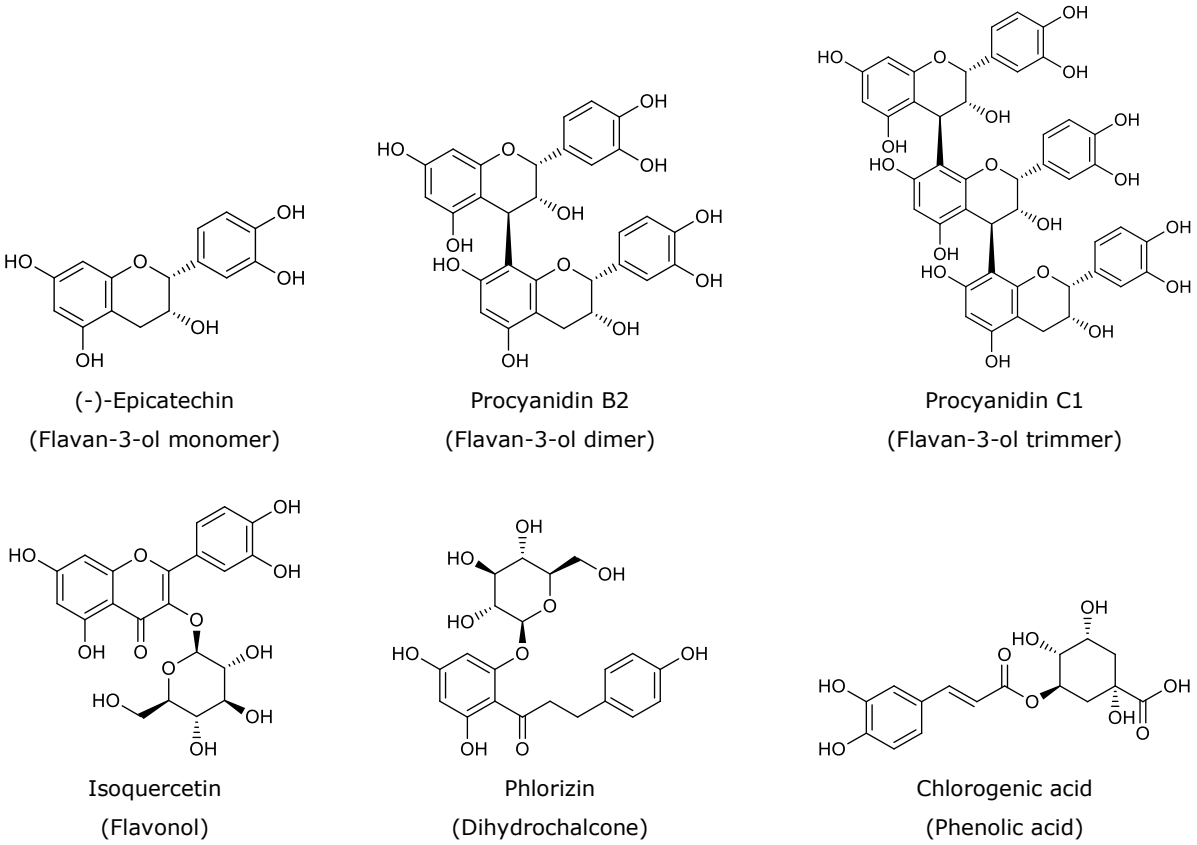


Figure 1. The most common polyphenols in wild apple fruit

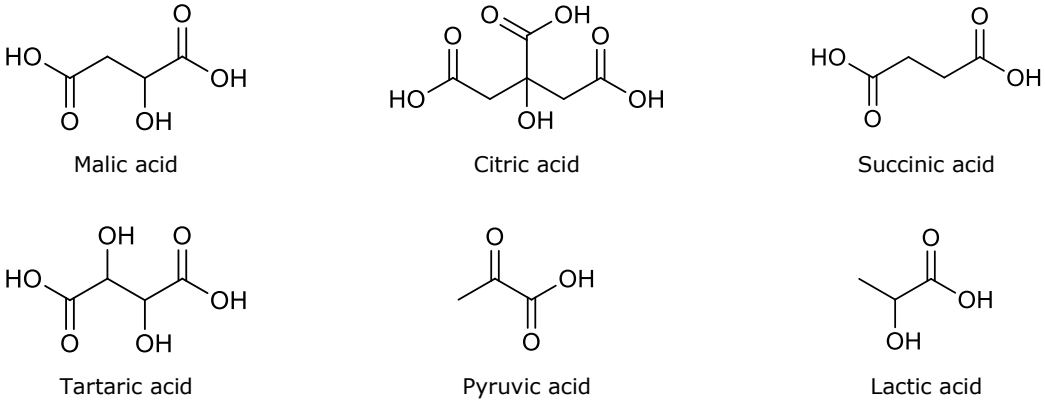


Figure 2. The most common fruit acids in wild apple fruit

Wild apple is also rich in minerals (potassium, calcium, magnesium, iron, phosphorus, zinc), vitamins (A, C and B), plant fibers (cellulose) and sugars (fructose, glucose, galactose and sucrose). Pectin, present in wild apple fruit in large quantities, cleanses the body of heavy metals, prevents the absorption of cholesterol in the small intestine and slows down the absorption of sugar (1).

Extracts rich in bioactive polyphenolic compounds, as well as fruit acids, are often used in the production of phytopreparations for both oral and skin applications. Water as an extraction agent has many advantages over organic extractants, primarily for its safety of application and economy. In addition to polyphenols, aqueous extracts can also contain a large amount of other water-soluble ingredients that can also contribute to better skin appearance (15-17).

Aim of the study

The aim of the study presented in the paper was to evaluate the efficacy of the application of emulsions with aqueous wild apple fruit extract (EWAFE) in an *in vivo* 28-day study on healthy volunteers by monitoring the following biophysical skin parameters:

- ☐ electrical capacitance (EC) - in order to evaluate EWAFE effect on skin humidity;
- ☐ transepidermal water loss (TEWL) - in order to evaluate EWAFE effect on skin barrier function;
- ☐ erythema index (EI) and skin pH - in order to evaluate EWAFE effect on skin irritation and damage;
- ☐ melanin index (MI) - in order to evaluate EWAFE effect on skin color and its lightening.

Materials and methods

Preparation of wild apple fruit extract

The dried and pulverized wild apple fruit was soaked in purified water (as the extractant) in a conical flask and left in an ultrasonic bath for 30 minutes at room temperature. Ultrasonic extraction produced the extract in a drug:extract ratio of 1:5, followed by filtration.

Preparation of O/W emulsions with wild apple fruit extract

The components listed in Table 1 were used to make O/W emulsions containing 12% and 15% of wild apple fruit extract (EWAFE-12 and EWAFE-15, respectively). The fat phase components (coco glucoside and cetearyl alcohol (Montanov™ 82), myristyl alcohol and myristyl glucoside (Montanov™ 14), isopropyl myristate, caprylic-capric triglycerides (Myritol™ 318) and cetearyl alcohol (Lanette 0)) and the aqueous phase components (glycerol, sodium benzoate and purified water) were heated to a temperature of 70 ^{O}C and 72 ^{O}C, respectively. The fat phase was then added to the aqueous phase with stirring (propeller laboratory mixer), first at a speed of 800 rpm for 5 min and then at a speed of 500 rpm for 3 minutes. At the end of the emulsification phase, stirring was continued at a speed of 300 rpm to a temperature of about 40 ^{O}C when wild apple fruit extract was added, followed by cooling to room temperature. The corresponding placebo sample was prepared in the same manner as the test samples, but without wild apple fruit extract (Table 1).

Table 1. Qualitative and quantitative composition of the tested emulsions

Component (INCI name)	Role	EWAFE-12 (g)	EWAFE-15 (g)	Placebo (g)
Coco glucoside and cetearyl alcohol	Emulsifier	7.0	7.0	7.0
Myristyl alcohol and myristyl glucoside	Emulsifier	1.5	1.5	1.5
Isopropyl myristate	Emollient	7.0	7.0	7.0
Caprylic-capric triglycerides	Emollient	7.5	7.5	7.5
Cetearyl alcohol	Emulsifier	1.5	1.5	1.5
Wild apple fruit extract	Cosmetically active compound	12.0	15.0	-
Glycerol	Humectant	2.0	2.0	2.0
Sodium benzoate	Preservative	0.5	0.5	0.5
Purified water	Aqueous phase	61.0	58.0	73.0

In vivo characterization of emulsions tested - biophysical skin parameters determination

In vivo study has been approved by the Ethics Committee of the Faculty of Medicine in Niš, Serbia, No. 12-12123-3, and has been conducted according to previous publications (18-21). The study included 20 healthy volunteers, both sexes, with an average age of 24.1 years, with moderately dry skin, and no history or clinical signs of dermatological disease. The study was conducted in the period January-February 2019. The examinees were instructed not to use any skin care products one week before and throughout the study, but were allowed to wash normally. Volunteers did not take baths, showers or exercise, at least three hours prior to the measurement, and were physically and mentally relaxed.

The measurements of the biophysical skin parameters were carried out in rooms with constant temperature (20-23 ^{O}C) and relative humidity (40-60%), 30 minutes after the acclimatization of the participants. The measuring region (volar parts of the forearmes) was generally hairless or with very little hair among male subjects. The measuring probe was lightly pressed against the skin and placed vertically, and carefully cleaned after each measurement. *In vivo* measurements were performed by only one person using the Multi probe adapter 9 device, Courage & Khazaka Electronic GmbH, Germany, and using the appropriate probes: Corneometer® CM 825 for EC, Tewameter® TM 210 for TEWL, Mexameter® MX 16 for MI and EI, and pH meter® 900 for skin pH measurements.

After the initial measurement of the above mentioned biophysical skin parameters (before the start of the study, basal values), volunteers were instructed to apply samples (EWAFE-12, EWAFE-15 and placebo) at home twice daily, in the morning and evening, after showering, to the skin of the volar side of the forearm for four weeks. Measurements were taken after 14 and 28 days. One part of the volar side of the forearm was left untreated.

Statistical analysis

The results obtained were analyzed by one-factor analysis of variance, followed by Tukey's test, with a statistical significance of $p < 0.05$. Changes in measured parameters at specific time points were checked using the Student t-test.

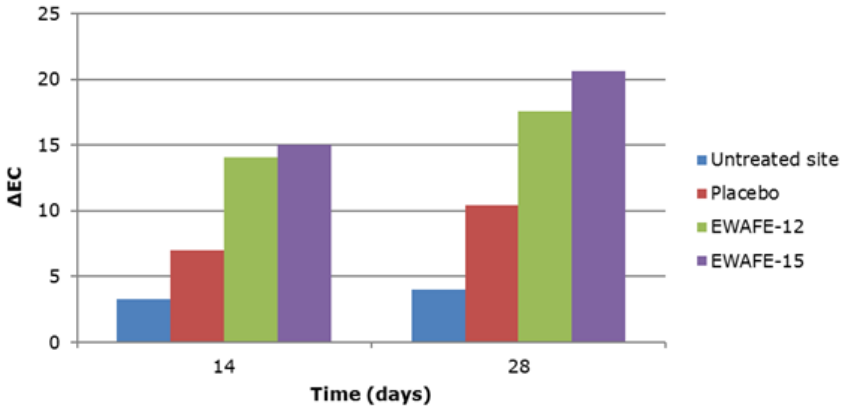
Results and discussion

In vivo efficacy of EWAFE application was examined by measuring the biophysical skin parameters (EC, TEWL, pH, EI, and MI) on healthy volunteers during long-term 28-day study. The results are shown in Graph 1-5.

In vivo testing the effect of EWAFE on EC

According to the results obtained in the present study, the tested emulsions (EWAFE-12 and EWAFE-15) showed, after both 14 and 28 days of application, a statistically significant ($p < 0.05$) increase in EC parameter, that is, skin hydration, compared to the basal values. In addition, both emulsions (EWAFE-12 and EWAFE-15) showed a better moisturizing effect compared to the placebo sample and the untreated site (Figure 3). That is most likely due to the presence of moisturizing compounds in the wild apple fruit extract, such as polyphenolic compounds and fruit acids. The positive effect of emulsions with moisturizing active compounds on the skin humidity has already been shown (18, 22-24). After 14 days of application, both emulsions showed almost the same effect on EC parameter ($\Delta EC_{EWAFE-15}$ = 15.03 ± 7.03, $\Delta EC_{EWAFE-12}$ = 14.1 ± 6.49), while after 28 days EWAFE-15 showed better effect on skin humidity than EWAFE-12 (ΔEC = 20.6 ± 10.51 and 17.6 ± 8.07, respectively) (Graph 1).

The good effect on the skin humidity observed after administration of the placebo sample compared to the untreated site was probably due to the presence of different emollients in the basic formulation (Table 1).



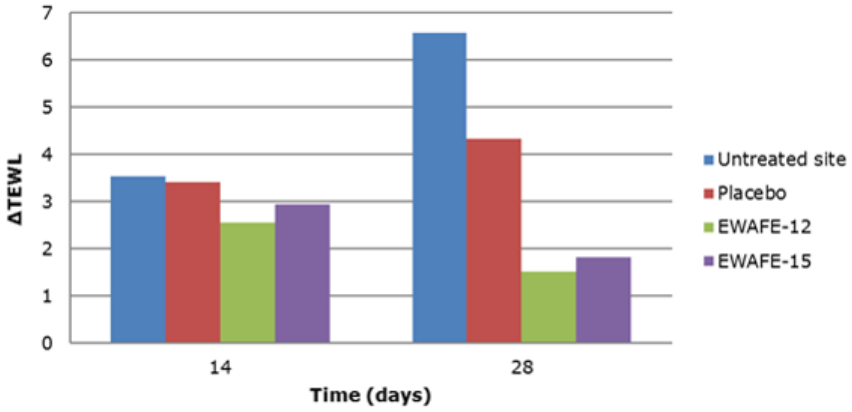
Graph 1. Effect of the tested samples on EC after 14 and 28 days of application

In vivo testing the effect of EWAFE on TEWL

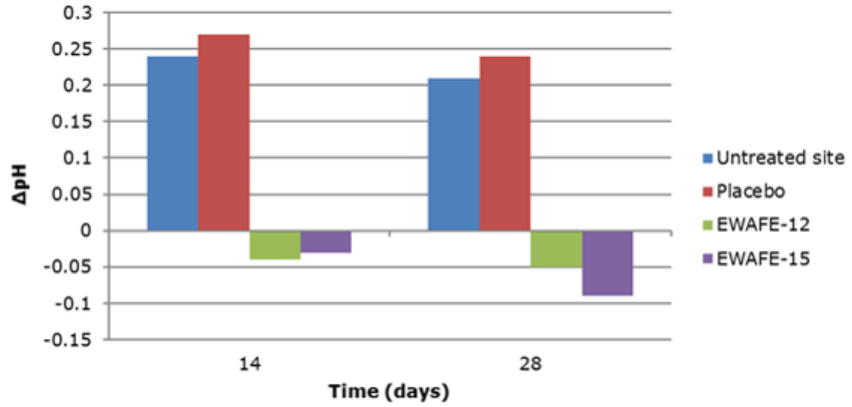
Measurement and monitoring of the TEWL parameter is often used to evaluate changes that occur in the barrier function of the skin after application of the tested samples (19). The obtained results showed that at 14 and 28 days after application of the tested samples, there was no significant increase in TEWL parameter compared to the basal values (Graph 2). Almost unchanged values of the TEWL parameter during the test period indicate that the skin barrier function was not significantly affected by the emulsions tested.

In vivo testing the effect of EWAFE on skin pH

The results obtained by measuring the skin pH of healthy subjects before the application of the tested emulsions, as well as 14 and 28 days after their application, showed that there was no statistically significant change in skin pH (Graph 3). However, a slightly greater decrease in skin pH was observed after EWAFE-15 application than after EWAFE-12 application, which is probably due to the presence of a higher concentration of active ingredients (fruit acids and polyphenolic compounds) from wild apple fruit extract.



Graph 2. Effect of the tested samples on TEWL after 14 and 28 days of application



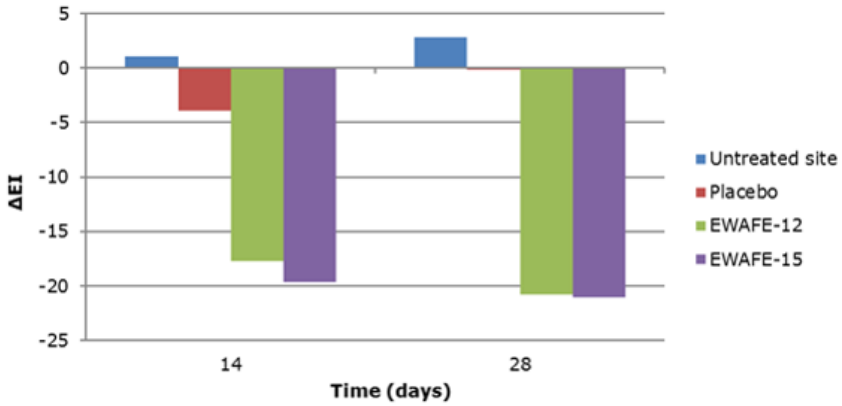
Graph 3. Effect of the tested samples on skin pH after 14 and 28 days of application

In vivo testing the effect of EWAFE on EI

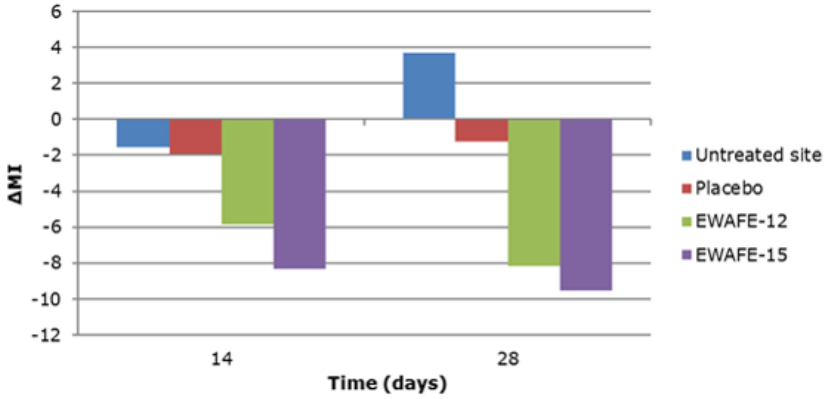
Application of the tested emulsions (EWAFE-12 and EWAFE-15) over a 28-day period showed a statistically significant ($p < 0.05$) decrease in the EI parameter compared to the corresponding controls (untreated site and placebo sample) (Graph 4). Considering that skin irritation is generally accompanied by an increase in the EI parameter, it can be concluded that EWAFEs do not exhibit an irritant effect after prolonged application to the skin. The results showed that slightly better effect was achieved after 28 days than after 14 days of EWAFE application, with EWAFE-15 being slightly more efficient than EWAFE-12 (Graph 4).

In vivo testing the effect of EWAFE on MI

The obtained results indicate the tendency of the tested emulsions to lighten the skin. Namely, at all test sites (untreated site, placebo, and EWAFE sites) there was a decrease in the MI parameter after 14 days of testing. The tested emulsions, including the placebo sample, showed a decrease in the MI parameter also after 28 days of testing, while a slight increase in the MI parameter was observed at the untreated site (Graph 5). During the entire test period, both EWAFE-12 and EWAFE-15 showed a statistically significant decrease in MI parameter ($p < 0.05$), i.e. a slightly better skin lightening effect compared to both controls (untreated site and placebo sample). A slightly better effect was observed after 28 days than after 14 days of the EWAFE application. Additionally, a better effect was observed with the EWAFE-15 than with the EWAFE-12 (Graph 5).



Graph 4. Effect of the tested samples on EI after 14 and 28 days of application



Graph 5. Effect of the tested samples on MI after 14 and 28 days of application

Conclusion

The efficacy of the application of the emulsions containing wild apple fruit extract at concentrations of 12% and 15% was tested *in vivo* on healthy volunteers during 14 and 28 days of application. During the application period, the tested emulsions (EWAFE-12 and EWAFE-15) were shown to lead to increased EC and decreased EI and MI parameters, indicating their positive effect on skin humidity, anti-irritant effect and skin lightening potential, respectively. The best effect on EC, EI and MI parameters was observed after 28 days of EWAFE-15 application. Additionally, the tested emulsions did not significantly affect skin pH and TEWL parameter, i.e. skin barrier function.

The obtained results indicate the potential application of wild apple fruit extract in the formulation of cosmetic products intended for skin hydration, as well as products intended to sanctification of hyperpigmented skin.

Acknowledgment

The financial support of this work by Ministry of Education, Science and Technological Development of the Republic of Serbia (III 45017) and Faculty of Medicine of the University of Niš (Internal project No. 2) is gratefully acknowledged.

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Originalni rad

UDC: 582.639.21:615.451.1]:612.79.015
doi:10.5633/amm.2020.0307

UTICAJ PRIMENE EMULZIJA SA STANDARDIZOVANIM EKSTRAKTOM PLODA DIVLJE JABUKE (*MALUS SYLVESTRIS* (L.) MILL., ROSACEAE) NA BIOFIZIČKE PARAMETRE KOŽE: *IN VIVO* STUDIJA

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Plod divlje jabuke (*Malus sylvestris* (L.) Mill., Rosaceae) predstavlja dragoceni izvor biološki aktivnih jedinjenja, poput polifenola i voćnih kiselina. Utvrđeno je da ova jedinjenja imaju pozitivan uticaj na ljudsko zdravlje, a deluju blagotvorno i na kožu, poboljšavajući njeno opšte stanje i njen izgled. U ovoj studiji, ispitivana je efikasnost primene emulzija „ulje-u-vodi", koje sadrže 12%, odnosno 15% ekstrakta ploda divlje jabuke, na zdravim dobrovoljcima. Tokom dugoročne dvadesetosmodnevne studije, praćeni su biofizički parametri kože (električna kapacitivnost (EK), transepidermalni gubitak vode (TEGV), pH, eritema indeks (EI) i melanin indeks (MI)). Kao rezultat, zabeleženo je značajno povećanje EK i smanjenje EI i MI parametara, kako nakon 14, tako i nakon 28 dana primene, pri čemu je emulzija sa 15% ekstrakta ploda divlje jabuke bila efikasnija od emulzije sa 12% ekstrakta ploda divlje jabuke. Sa druge strane, nisu primećene značajne promene TEGV i pH vrednosti. S obzirom na njihovo blagotvorno dejstvo na kožu (povećana hidratacija kože, smanjena iritacija kože, dobar potencijal posvetljivanja kože), ispitivane emulzije mogu imati potencijalnu primenu u formulaciji kozmetičkih proizvoda namenjenih tretmanu suve i iritirane kože, kao i proizvoda namenjenih posvetljivanju hiperpigmentisane kože.

Acta Medica Medianae 2020;59(3):48-55.

Ključne reči: *Malus sylvestris, ekstrakt, emulzija, biofizički parametri kože, in vivo studija*

Original article

UDC: 615.273:676.164
doi:10.5633/amm.2020.0308

DEVELOPMENT OF BIOACTIVE CELLULOSE SULFATES FOR BIOMEDICAL APPLICATIONS

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Cellulose is the most abundant biomolecule on earth. Chemical derivatives of cellulose have found multitude of uses in industrial and biotechnological applications. Cellulose sulfates (CS) represent a class of water-soluble derivatives that have been employed in industrial application, but not yet in medicine. Here derivatives with different degree of sulfation of anhydroglucose unit (AGU) of cellulose have been studied toward anticoagulant effects and modulating effects of growth factors with heparin-binding domains like fibroblast growth factor 2 (FGF-2). The results show that CS of higher sulfation degree have an anti-coagulant activity comparable to that of heparin with cooperative action to anti-thrombin III that inhibits thrombin and Factor Xa activity making CS interesting for anticoagulant coating of blood-contacting medical devices. Furthermore, the studies show that CS with comparable sulfation degree to heparin have a promoting activity on the mitogenic effect of FGF-2 shown in cell culture studies that indicate their application as coatings of implant materials or component of tissue engineering scaffolds in the area of traumatology and regenerative medicine.

Acta Medica Medianae 2020;59(3):56-67.

Key words: *cellulose sulfates, sulfation degree, anticoagulation, thrombin, growth factors, FGF-2*

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Introduction

Glycans have diverse functions in the organism. They are relevant not only for maintaining the normal functions of cells and tissue, they are also important in wound healing or pathological processes, like bacterial or viral infections, growth and metastasis of tumors (1). An important subgroup of glycans is glycosaminoglycans (GAGs), which comprise specific dimeric repeating units that usually consist of uronic acid connected to a N-acetylglucosamine or N-acetylgalactosamine sugar. Important representatives of GAGs are hyaluronic acid, chondroitin sulfate, heparan sulfate and heparin. It is interesting to note that the degree of sulfation is highest in heparin, followed by heparan sulfate and other sulfated GAGs, while hyaluronic acid is the only non-sulfated GAG (1, 2). Heparan sulfate and heparin are structurally quite similar, but differ mainly in their degree of sulfation, molecular weight and occurrence in the organism (3). Because of its high number of sulfate and carboxyl groups, heparin is the glycosaminoglycan with the highest negative charge density (Figure 1). Heparin is composed of disaccharide-subunits that are either D-glucuronic acid (10 %) bound by a β-(1→4) glycosidic bond or L-iduronic acid (90%) bound by an α-(1→4) glycosidic bond to N-Acetyl-D-glucosamine. The typical structure is usually the trisulfated disaccharide. Sulfate groups can be located at the 2-O position of the iduronic or glucuronic acid, as well as the 3-O and 6-O position of the glucosamine. The amino group of the glucosamine on the other hand can be either substituted by an acetyl or sulfate group. The average molecular weight of a heparin molecule is about 15 kDa (4). By comparison, heparan sulfate has a higher average molecular weight (around 30 kDa) and a lower degree of sulfation. Heparan sulfate chains frequently contain domains with long sequences of either high or low sulfation degree. Heparin shows such inhomogeneities in its degree of sulfation as well, but to a lesser extent in comparison to heparan sulfate (5).The synthesis of heparin hap-

pens mainly in mast cells and basophil granulocytes. Heparin shows a multitude of functions, such as the binding of histamine and the control of the activity of different proteases (6). Heparin that has been released also inhibits the interactions of blood platelets with collagen and binding of von Willebrand factors to them. In addition, it can also bind low density lipoprotein (LPL). Such bound constructs can subsequently be taken up and degraded by macrophages (8). However, the well-known property of heparin is its ability to bind to anti-thrombin III (AT III), which enhances AT III affinity to thrombin and other activated clotting factors of the coagulation cascade, which is the basis of its use as anticoagulant (6).

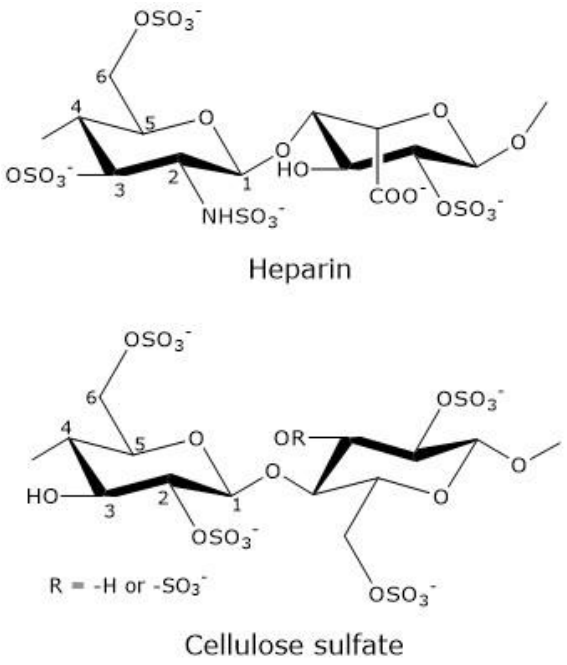

Figure 1. Chemical structure of heparin and cellulose sulfate

On the other hand, heparan sulfate proteoglycans (HSPGs) are localised on the surface of many cells and represent components of the extracellular matrix. The most known representatives of cell membrane localised HSPGs are syndecans and glypicans3. For example, syndecan-1 und -3 carry chondroitin sulfate proximal to the cell membrane, while heparan sulfate (HS) is found at the distal part of proteoglycans. Conversely, syndecan-2 and -4 are decorated exclusively with heparan sulfates.

Because of their cytoplasmic domain, syndecans can transmit signals from the extracellular area to the inner part of the cell. This includes the binding of ligands to the HS chains, followed by oligomerisation of syndecans, which triggers the activation of signalling proteins like kinases in the cytoplasm (1). Many functions of the HSPGs are also related to the regulation of the activity of chemokines, growth factors glycoproteins of the extracellular matrix (ECM) in which they can function as co-receptors. By presenting growth factors as co-receptors for receptor-tyrosine kinases, they can contribute to signal transduction processes (9). The HS chains are directly involved in the formation of the receptor-ligand complex, as described for the fibroblast growth factor FGF-2, and can influence the mitogenic effect of cytokines (10). HSPG can also store growth factors outside the cell as component of ECM and essentially function as a reservoir. The release can be caused either through a change in the degree of sulfation through locally expressed sulfatases (11), through proteolysis of the protein backbone or through the HS chain fission caused by heparanases. The binding of heparin or HS to regulatory proteins occurs through heparin binding sites, which are generally located at the outside of proteins. Those are mostly rift-like domains with a high amount of positively charged amino acids like lysine or arginine (4).

The inhibition of blood coagulation through heparin is an effect that has been used in hospitals for a long time (4). Heparin is also used to make the surface of biomaterials like catheters, tube systems or dialysis membranes more compatible to blood (12). This can reduce the need for a systemic application of heparin, since that can cause side effects like the aggregation of thrombocytes, an increased tendency for bleeding or a retarded healing process

of bone tissue and an increased risk for osteoporosis (13, 14). Besides these well-known risks, there are further disadvantages in the use of natural GAGs since the extraction of them out of animal tissue is time-consuming and carries further risk of infections and immunological reactions during their clinical application. The bioactivity of heparin is additionally very dependent on its biological origin (species, organs), which is related to differences in degree of sulfation and substitution pattern (4). Lastly, the use of natural GAGs in the clinical field opens the possibility to contaminate heparin with highly sulfated chondroitin sulfate falsely claimed with criminal intent as pure heparin, which was fatal for some patients in US (15). Because of these challenges, it would be highly desirable to synthesize biocompatible polymers, which can replace heparin showing less variance in their biological activity with no risk of transmission of diseases and hence better safety.

Cellulose is one of the most abundant polysaccharides that exist in nature. It is a main component of the cell wall of plants, shows a high molecular weight, but is non-soluble in water and most organic solvents. In comparison to other natural polysaccharides like hemicellulose or pectin, cellulose is a non-branched polymer (16). It is composed of D-glucose units, which are connected via β-(1→4) glycosidic bonds. The hydroxyl groups at the C2, C3 and C6 atoms of the anhydroglucose unit (AGU) can be chemically functionalized to synthesize many widespread polymers such as e.g. carboxymethyl cellulose, which are used in the paper industry, food technology and partly in medical applications (17). The sulfation of cellulose has a long tradition and leads to water soluble products with many different application possibilities (18). The main structure of cellulose sulfate (CS) is shown in Figure 1B. It is obvious that sulfation of cellulose leads to derivatives that have similarities to the highly N-acetyl glucosamine unit of heparin. Hence, it seems to be reasonable to assume that CS of higher sulfation degree might be also effective in inhibition of blood coagulation and in supporting the activity of growth factors that possess heparin-binding domains. This article presents the effect of CSs on coagulation and the mitogenic activity of the fibroblast growth factor 2 (FGF-2) showing that sulfation degree has an effect on both phenomena.

Materials and methods

Synthesis of cellulose derivatives

We have already described synthesis and chemical analysis of cellulose derivatives in more detail (28, 29) and therefore will not be described here in detail. The CSs were named later in the result section according to the degree of substitution with sulfate (DS) as CS X.

Analytical methods

The DS of the cellulose derivatives obtained by different sulfation methods was characterized by elemental analysis and ^{13}C-NMR spectroscopy. The substituent distribution within the AGU was assessed from the ^{13}C-NMR spectrum of the cellulose derivatives dissolved in D_2O by integrating the signal areas and comparing those of the substituted position to those of the appropriate non-substituted one.

Study on anticoagulant activity of cellulose sulfates

Collection and preparation of blood

Blood was drawn from healthy human volunteers, who had no medication for at least 10 days. Blood was anticoagulated with sodium citrate (3.8 g/100 ml). The blood was centrifuged at 2000g for 20 min. The supernatant cell free plasma was separated. Plasma samples from 10 different donors were pooled, aliquoted and snap-frozen at -80 °C. For experimental work, plasma was thawed at 37 °C and used within 2 h.

Measurement of clotting times

Thrombin time (TT) was measured using thrombin (Behring Werke, Germany). Partial thromboplastin time (PTT) was estimated using a commercial test kit (Boehringer Mannheim, Germany). Measurements were carried out with a coagulometer KC 4A (Amelung, Germany). Cellulose derivatives were dissolved in TRIS buffer, pH 7.4. 100 µL pooled plasma were mixed with 50 µL cellulose derivative solution and incubated for 1 min (TT) or 3 min (PTT), respectively. TT was measured after the addition of 100 µL thrombin solution (0.3 IU/mL). PTT was estimated after the addition of 100 µL kaolin-cephalic solution, followed by the addition of 100 µL 25 mM $CaCl_2$ solution. After the addition of activator, the time needed for clotting was measured. If samples did not clot within 10 min, it was observed that no clotting occurred afterwards. Therefore, measurements were stopped after 10 min and those samples denoted as non-clottable (n.c.). To still obtain visible data points in the graphs, these values were set at a clotting time of 600 s. However, it should be kept in mind that these data represent conditions under which the plasma did not clot at all.

Inactivation of thrombin and factor Xa

The anticoagulant potential of CSs was tested in addition by their ability to support the inactivation of thrombin (F IIa) and factor Xa (F Xa) in the presence of antithrombin III (AT III). This was possible by the development of amidolytic assays for F IIa and F Xa in separate investigations. Cellulose derivatives or reference substances were dissolved in 50 mM Tris-HCl,175 mM NaCl, 10 mM EDTA, and 0.5 mg/mL human serum albumin (24).

The thrombin assay was carried out mixing 50 µL AT III (activity 0.265 pkat/mL) with 200 µL F IIa (activity 0.53 nkat/mL), and 50 µL of the test substance. After 5 min incubation at 37 °C, 200 µL chromogenic substrate S-2238 (0.22 mM) was added and the mixture was incubated for 2 min. The con-

version of the chromogenic substrate was stopped by the addition of 200 µL acetic acid (20% v/v). The optical density (OD) was measured at 405 nm in 96 well plates with a plate reader (Anthos 2001, Austria). A standard curve was obtained under identical conditions for thrombin activities from 0 up to 1.053 nkat/mL and used for the calculation of residual thrombin activity from the measured OD.

The F Xa assay was performed using 200 µL of F Xa solution (activity 1.06 nkat/mL), 50 µL AT III solution (activity 0.265 pkat/mL), 50 µL test solution and 200 µL chromogenic substrate S-2222 (0.22 mM). The experiment was carried out in the same manner as the thrombin assay. Residual F Xa activities were calculated from a standard curve. Thrombin, factor Xa, AT III, and the chromogenic substrates S-2238, and S-2222 were supplied by Chromogenix, Sweden.

Studies on mitogenic activity of cellulose sulfates

Estimation of binding growth factor FGF-2 to cellulose sulfates

The binding affinity of the synthesized CSs to the growth factor FGF-2 (bFGF) was performed with a competition assay using heparin agarose beads (Fluka, Biochemica). 25 ng of b-FGF obtained from InVitrogen (Karlsruhe, Germany) were mixed with heparin agarose beads and PBS and agitated for 30 min at 200 rpm at RT to allow the binding of growth factors to the beads. The unbound growth factor was removed by washing with PBS twice. For the release of the growth factor from the beads, CSs or heparin (control) were added to the mixture and agitated for 30 min at 200 rpm at RT. After centrifugation, the supernatants with the polysaccharides and the released FGF-2 were applied to a cellulose nitrate membrane in a slot-blot apparatus. A primary antibody against FGF-2 (Sigma, Germany) and a horseradish peroxidase labelled secondary antibody (Dianova, Hamburg, Germany) were applied to the membrane to label bound growth factors. Detection was performed with ECL plus chemiluminescence kit and a CCD camera (Raytest, Diana 2). The quantification of the signals was done by ImageJ.

Cell culture

3T3-L1 fibroblast cells obtained from ATCC (Manassas, USA) were cultured in flasks (75 cm^2, Greiner bio-one, Frickenhausen, Germany) in Dulbecco´s modified Eagle medium (DMEM, Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Biochrom AG) and 1% penicillin-streptomycin-fungizone (PSF, Promocell, Heidelberg, Germany) in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. Cells were harvested by treatment with trypsin/EDTA (Biochrom AG). Trypsinization was stopped by the addition of FBS and cells were washed twice with DMEM.

Investigation of mitogenic effects of cellulose derivatives on 3T3-L1 fibroblasts

3T3-L1 fibroblast cells were seeded at a density of 10.000 cells/well in black 96 well plates (Greiner bio-one) in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin-fungizone and cultured for 24 h. After washing the plates with DMEM only, the cellulose derivatives or heparin were applied to the cells in DMEM without FBS at a concentration range of 1 µg to 1000 µg/ml for 48 h in the presence or absence of 10 ng/ml FGF2. The proliferation was measured based on the DNA content using the Quant -iT^{TM} PicoGreen dsDNA quantification assay (Invitrogen, Karlsruhe, Germany). The fluorescent intensity was measured with an excitation wavelength of 485 nm and an emission wavelength of 520 nm by the plate reader Fluostar Optima. The proliferation was expressed as a ratio to the control wells with 10 ng/ml FGF2. All experiments were carried out with six wells per sample and dilution from which means and standard deviation were calculated.

Results and discussion

Studies on anticoagulant activity of cellulose sulfates

The inhibition of blood coagulation through cellulose sulfates

The sulfation of partially substituted cellulose acetates has been described elsewhere in more detail (19). The total content of sulfur was determined through elemental analysis, while the distribution of the substituents was determined through quantitative ^{13}C-NMR spectroscopy. The synthesized CSs are listed in Table 1 and sorted by degree of sulfation or substitution (DS) and the distribution of the substituents. Figure 1 shows the typical structure of sulfated cellulloses. It is visible that the DS reached from low of about 0.25 to relatively high of 1.35, which is lower than that of heparin. The latter can have two sulfation sites at the N-acetylglucosamine unit with sulfation at C2, C3 and C6 position, while the uronic acid may be substituted at C2 position with a sulfate group (see Figure 1). Hence, the overall DS can have the maximum around 2.0.

The effect of CS on blood coagulation was determined using citrate plasma and commercial test kits for measurements of thrombin time (TT) and partial thromboplastin time (PTT). Figures 2A and B show the results of the TT and PTT coagulation time measurements. It can be seen that an increase in the degree of sulfation of CS leads to an increase in the coagulation times. In addition, the results imply that the inhibition of coagulation increases in the case of TT and PTT, if the degree of sulfation is increased in C2 position, which can be shown by comparing the samples CS1.33 and CS1.35 that have almost the same degree of sulfation, but at different substitution site. (Table 1). This is especially obvious in the case of the determination of TT, since an increase of the DS at C6

and a decrease at C2 position shows a shorter time of coagulation in comparison to a sample that has a similar DS but a higher sulfation in C2 position. At a CS concentration of 25 μg/mL with a DS ≥ 0.95 for TT measurements and a DS ≥ 1.15 for PTT measurements, the blood clotting was completely inhibited, which demonstrates the potential of these CSs to be used as anticoagulant for the modification of blood-contacting materials surfaces like membranes for haemodialysis, blood linings, etc.

Table 1. Degree of sulfation (DStotal) and distribution of the sulfate groups in the derivatives in the determiantion of coagulation inhibition

Cellulose sulfate (CS)	DS_{total} Elemental analysis	DS_{total} ^{13}C-NMR	Substitution pattern of sulfates*		
			C2	C3	C6
CS 0.26	0.35	0.25	0.17	0.08	0
CS 0.95	0.80	0.95	0.55	0.20	0.20
CS 1.14	1.10	1.14	0.74	0.09	0.31
CS 1.33	1.40	1.33	0.76	0.10	0.47
CS 1.35	1.07	1.35	0.67	0.33	0.35

* DS values of the sulfate groups at the C2, C3 and C6 position were determined via 13C-NMR spectroscopy

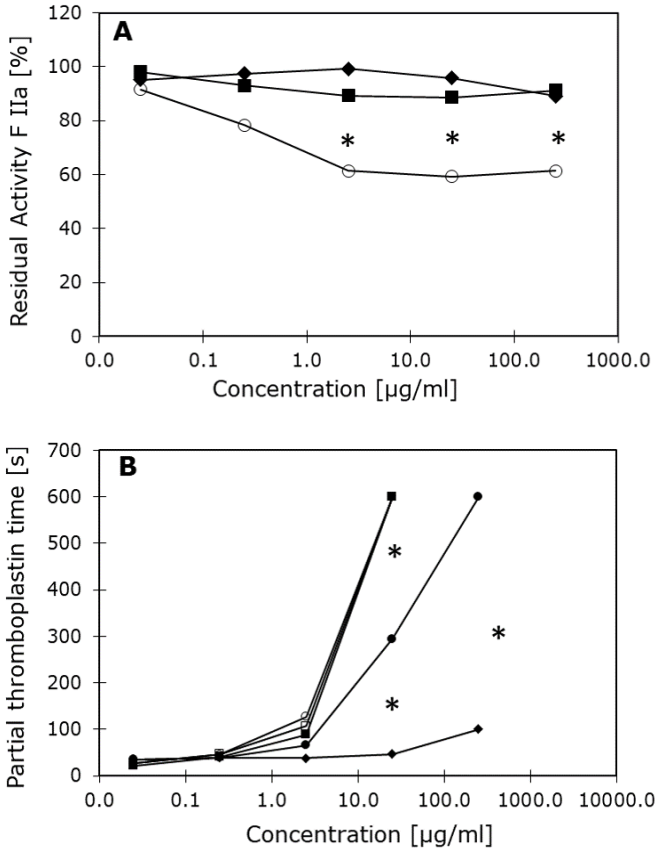

Figure 2. Thrombin time (A) and partial thromboplastin time (B) of citrate in the presence of cellulose sulfates (concentration range: 0.025 μg/mL to 2.5 mg/mL). (♦) – CS 0.26; (●) – CS 0.95; (□) - CS 1.14; (❍) - CS 1.33; (■) - CS 1.35. Asterisks in the figures show significant deviation (p <= 0.05).

Next, we were interested to find out more about the underlying mechanism of inhibition of coagulation regarding the activity of AT III in the presence of CSs toward coagulation F IIa (thrombin) and F Xa inhibition. It is known that for the inhibition of F Xa shorter heparin sequences with specific patterns of sulfation are relevant because of the formation of a binary complex between AT III and heparin that inhibits F Xa. For F IIa inhibition on the other hand, longer heparin sequences play a role because

they form a ternary complex in which heparin forms a quasi-catalytic surface for the interaction of AT III and F IIa (3, 4). In analysing in which way the regioselective derivatisation of cellu-lose with sulfate groups inhibits the blood clotting, some clues were already present that the inhibition of thrombin was stronger, as seen in the TT mea-surements in comparison to PTT measurements. Because of this, tests with F IIa and F Xa were performed in the presence of AT III, selecting CSs with high and low degree of sulfation. For this, single factor tests with chromogenic substances for the specific serine proteinases F IIa and F Xa were de-veloped (19). Figure 3A shows that CS 0.26 showed no inhibiting effect against thrombin, since the re-sidual activity of thrombin was roughly 100% even at CSs concentrations of 2.5 mg/mL. Conversely, sulfated cellulose with a DSs 1.33 (CS 1.33) demon-strated an inhibition of about 40% even at concen-trations as low as 2.5 µg/mL, which points also to the importance of the overall sulfation degree. Also, the sites of sulfation seemed to be important since compared to CS 1.33, the derivative CS1.35 showed an inhibition of about 10%, only. This surprising result can be partially explained with the different substitution pattern of both samples. As already shown in Table 1, the sample CS 1.35 has a higher degree of substitution on position C3, which leads to a lower substitution on position C2 and C6. Although C3 sulfation is critical for an anti-thrombogenic effect (especially for heparin) (20, 21), the case seems different for cellulose sulfates because of their different structure and the β-(1→4) glycosidic bond. Here it seems that a higher sulfation in C2 and C6 position is more beneficial in terms of the inhibition of thrombin. The existence of sulfate groups on the C2 position of the iduronic acid of the heparin is important for the chain conformation and results in a high affinity for anti-thrombin III (20, 21), which might support the anticoagulant activity of the cellulose-2-6 sulfates towards thrombin, too. However, the results of F Xa assay look different (Figure 3B). Here both derivatives with a high DS show a significant inhibition of F Xa at higher concentration ranges starting at 50 µg/mL compared to the low sulfated CS0.26. In summary, these investigation show that CSs with a high DS in position C2 and C6 show an effectiveness analogous to heparin regarding the inhibition of blood coagulation, especially towards the inhibition of thrombin.

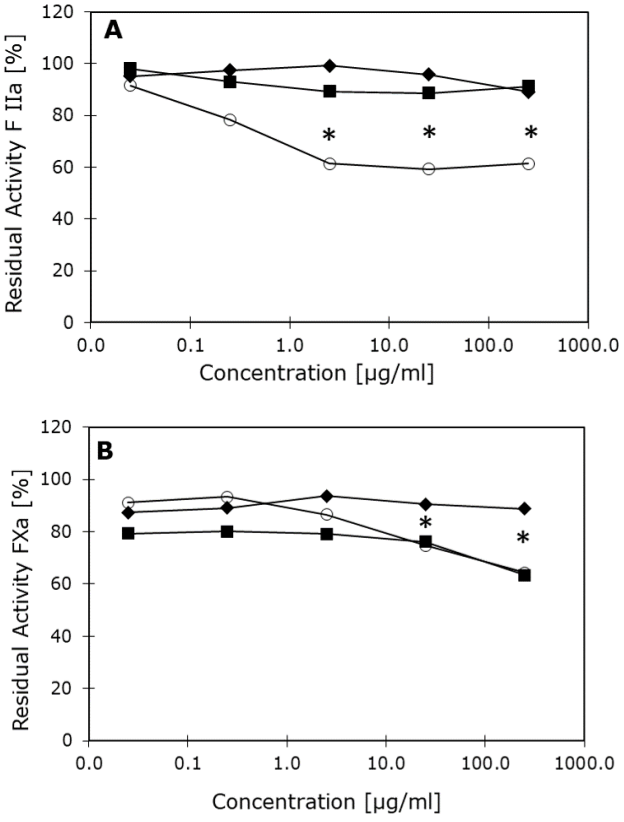

Figure 3 A and 3 B. Residual activity of thrombin (F IIa, A) and factor Xa (F Xa, B) after the addition of chosen cellulose sulfates with different degrees of derivatisation.
(♦) - CS 0.26; (❍) - CS 1.33; (■) - CS 1.35.
Asterisks in the figures show significant deviation (p <= 0.05).

The influence of the degree of derivatisation and regioselectivity of cellulose sulfates on the binding and activity of the fibroblast growth factor FGF-2

Here, a range of CSs were synthesized through acetosulfation or direct sulfation by which a wide range of derivatisation degrees was achieved. Table 2 gives an overview on the synthesized CSs. The distinctive feature of this sulfation procedure is that there was no detectable derivatisation at C3 position of the AGU. The details of this sulfation procedure and characterization of cellulose derivatives can be found in the previous work published by Peschel et al (22).

Table 2. Degree of sulfation (DStotal) and distribution of the sulfate groups in the derivatives in the determiantion of the activity of the growth factor FGF-2

Cellulose sulfate (CS)	DS_S (^{13}C-NMR)*			
	C6	C2	C3	DS_{total}
CS 0.39	0.36	0.03	0	0.39
CS 0.58	0.52	0.06	0	0.58
CS 0.66	0.60	0.06	0	0.66
CS 0.92	0.77	0.15	0	0.92
CS 1.57	1.0	0.57	0	1.57
CS 1.69	1.0	0.69	0	1.69
CS 1.80	1.0	0.80	n.d.	1.80
CS 1.94	1.0	0.94	n.d.	1.94

* DS-values of sulfate groups at the C2- C3 and C6 position were determined 13C-NMR spectroscopy. n.d. – not determined

Binding of the fibroblast growth factor FGF-2 to cellulose sulfates

Growth factors like FGF-2 are presented to their corresponding receptor tyrosine kinases on the cell surface through proteoglycans like syndecan, which is decorated with heparan sulfate side chains followed by the activation of cellular kinases that induce cell proliferation (4, 5, 9). Because of this, the binding affinity of FGF-2 to CSs was determined in a competitive approach. Figure 4 shows the DS of CSs, whereas the light grey and dark grey bars illustrate the derivatization in C2 and C6 position and the observed binding of FGF-2 in comparison to heparin as a control (100%). It can be seen that in comparison to heparin, CSs with a DS ≤ 0.92 allow no significant binding of FGF-2. Only for samples with a DS ≥ 1.57, an increase in the growth factor binding correlating with an increase in DSS up to 60% compared to heparin could be verified. An increase of ca. 40% ($p < 0.05$) was found between the samples of CS 1.94 and CS 1.57.

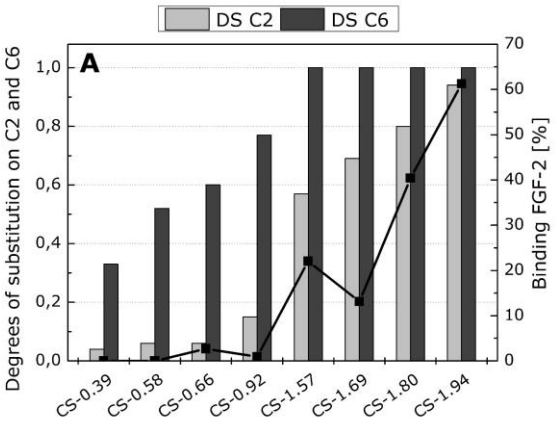

Figure 4. Binding of FGF-2 to sulfated cellulose in dependence of the degree of sulfation in position C2 (light grey columns) and C6 (dark grey columns) compared to the binding to heparin (100%). The binding of FGF-2 is plotted against the degree of sulfation. Values represent the average ± standard deviation (n = 4).

The binding affinity is an important indicator for the biological efficacy of CSs since the binding of growth factors through GAG´s often correlates with the in vitro measured biological activity (23). Here a significant binding of CSs to FGF-2 could only be verified for samples with a DS ≥ 1.00 in C6 position of the AGU, which apparently correlates with an increasing DS in C2 position. Research of other authors could show that none of the hydrogen bonds that develop during the binding of heparin to FGF-2 are realized with the 6-O (C6) sulfate groups, while the sulfation of the 2-O (C2) or the 2-N (C2) position is of some importance for this bond24. This might be the case for samples with a DS > 1.5 that express also stronger binding of FGF-2.

Determination of the effect of cellulose sulfates on FGF-2 induced proliferation of mouse fibroblasts

To determine the influence of FGF-2 induced proliferation through CSs, a cell culture comprised of embryonic mouse fibroblasts (cell line 3T3-L1) was used, analogous to works of other authors (25). For this, fibroblasts were incubated in a culture medium without serum adding 10 ng/mL FGF-2 and CSs for 48 hours. An additional incubation was done with heparin as a control group and cell growth was determined with a Pico-Green DNA quantification assay.

Mitogenic activity of cellulose sulfates at a concentration of 1 mg/mL

The results in Figure 5 show that all samples with a DS ≤ 0.58 inhibited the proliferation of cells. Starting with a DS ≥ 0.66, a stimulation of FGF-2-induced proliferation was observed. With an increasing degree of sulfation, the mitogenic effect of CSs was increased, too. For the derivative with the highest degree of sulfation CS 1.94, a proliferation of 160% was detected, compared with 10 ng/mL FGF-2 used here as a control.

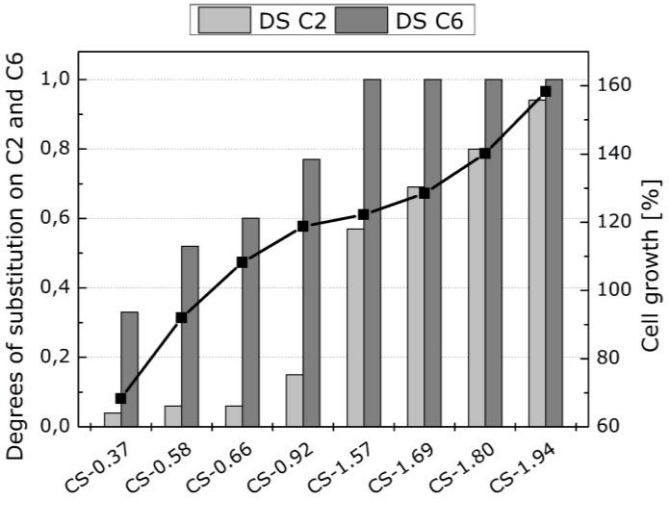

Figure 5. Comparison of fibroblast growth after the addition of FGF-2 (10 ng/mL) and 1 mg/mL cellulose sulfates (CS) with different degrees of sulfation (CS 0.37-CS 1.94).
The proliferation was determined via the content of DNA (average ± standard deviation, n = 5).

In general, the proliferation correlates with the binding affinity of the growth factor, which means that an increase in the sulfation degree of CSs results in a stronger FGF-2 induced mitogenic activity. CSs with a DS ≤ 1.57 that showed no binding of FGF-2 could also lead to an increased proliferation. The reason for this could be based on the high affinity of FGF-2 to heparin, so that those derivatives in the competitive binding assay (Figure 4) could not bind the growth factor. Without the heparin however, the binding of FGF-2 was possible in the cellular assay, which is then visible by the increased FGF-2 activity expressed by cell growth. Similar to these results, Kunou et al. could prove an increase of FGF-1 induced proliferation through dextran sulfate with a DS of about 1.0 (26).

Dependence of mitogenic activity of cellulose sulfates on the concentration in comparison to heparin

In addition to degree of sulfation and position of the sulfate groups, the concentration of heparin-analogous CSs also influences the activity of growth factors. In studies of other authors, concentrations of heparin-analogous substances in a range of less than 1 µg/mL could already increase FGF-2 activity toward cells. Because of this, the analysis of the mitogenic activity of cellulose sulfates was performed in the concentration range from 1 µg/mL to 1 mg/mL. The mitogenic activity was compared with the fibroblast growth in the presence of 10 ng/mL FGF-2 as a control group.

In the following experiments, CSs with a DS of ≥ 0.92 and heparin were used. In Figure 6A and 6B, a strong dependence of the proliferation on the concentration of the samples is visible. At a concentration of 1 mg/mL, with the exception of CS-0.92, all cellulose sulfates showed a proliferation of 3T3 fibroblast cells, that was comparable to that of heparin. In a concentration range of 1 – 500 µg/mL, a stepwise concentration dependent increase in proliferation of 3T3 fibroblasts was visible from low- to high-sulfated derivatives.

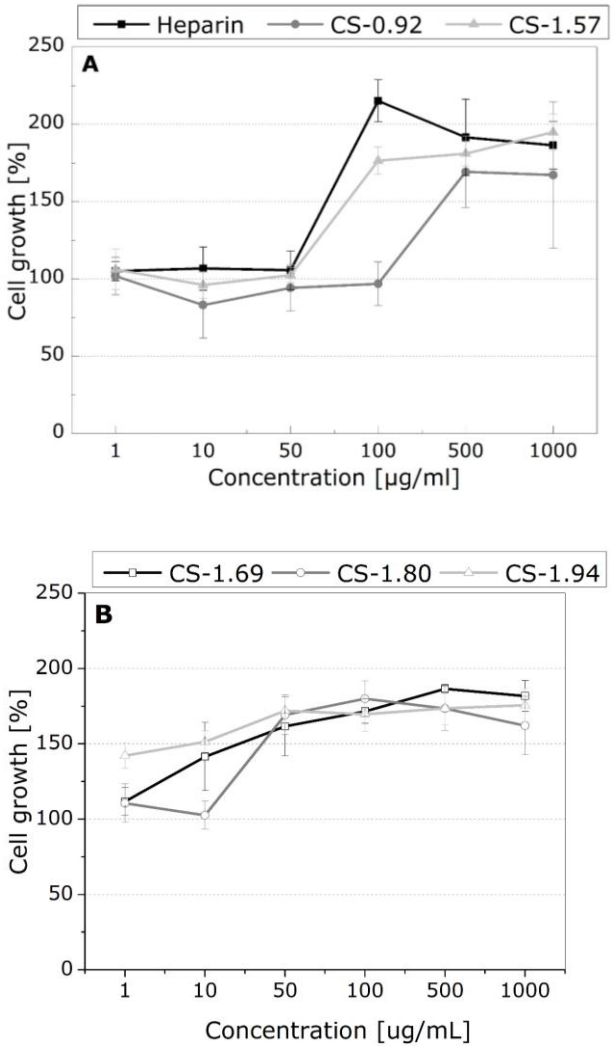

Figure 6. Comparison of proliferation in dependence of the concentration and degree of sulfation of cellulose sulfates (CS) and heparin. 3T3-L1 fibroblasts were incubated with 10 ng/mL FGF-2 and 1 µg/mL bis 1 mg/mL of the derivatives for 48 hours. The proliferation was determined through the DNA content with Pico green.
(A) Heparin and medium sulfated CS, (B) high sulfated CS (average ± standard deviation, n = 5).

The results of these investigations show that with an increasing degree of sulfation, lesser amounts of CS were needed to enhance the FGF-2 induced proliferation. It is important to note that highly sulfated celluloses are needed in significantly lower concentrations than heparin to promote cell proliferation. Since these CSs also show an increased sulfation in C2 position of AGU, this correlates with the increased affinity of FGF-2 to these CSs. Heparin-binding growth factors like FGF-2 show special, often slit-like domains that are rich in basic and partly also hydrophobic amino acids and connect to the charged chains of helically ordered heparin as stretched chains (27). One cause for the comparable or superior activity of these CSs (compared to heparin) could be their high degree of sulfation. The heparin that was used in this experiment possesses a DS of 1.3, which is below the DS of some CS used in these studies (22). Nevertheless, an important role for the interactions between heparin and FGF-2 and the interaction with the FGF-2 receptor on the cell surface is related to the carboxy group in C6 position of the iduronic acid of heparin (20). In the case of a relatively high

sulfation in C2 and C6 position, the β-(1→4) glycosidic bond of AGU in cellulose should lead to a homogeneous charge density on both sides of the chain, which could facilitate the binding of FGF-2 to CSs through Coulomb interactions and hydrogen bonds from remaining hydroxyl group of AGU.

The increase in the mitogenic activity of FGF-2 in combination with CS can be traced back to two causes. Growth factors like FGF-2 have a relatively short half-life period also in vitro because of the rapid proteolytic fission of the protein through proteinases released by cells. *In vitro* experiments have shown that the stability of FGF-2 against proteinases can be increased in the presence of heparin or highly sulfated celluloses because of the interaction between polysaccharide and growth factor (28). On the other hand, the high DS of CSs, which is comparable to heparin, could lead to the formation of a FGF-2 – CS – FGF receptor-complex on the surface of 3T3 fibroblasts, which leads to an activation of the mitogen-activated protein kinases pathway (MAPK/ERK). The research of other authors has shown that especially high sulfation of heparin in C6 position of the glucosamine monomer plays an important role for this effect (29). Because of this and the β-(1→4) glycosidic bond, a higher sulfation in the C6 and C2 position of the AGU of cellulose could be advantageous, which in the end could lead to an enhanced growth of cells in the presence of FGF-2 and higher sulfated celluloses.

Conclusion

In this study CSs were synthesized, which show a bioactivity that rivals or even surpasses that of heparin. It is obvious that a complete sulfation in C6 position and a higher sulfation in C2 position is very important for the biological activity of CS both in anti-coagulation but also promoting mitogenic activity of the heparin-binding growth factor FGF-2. This was evident by the inactivation of thrombin, which is due to a specific interaction with AT III that plays a significant role in the inhibition of blood coagulation. Although the activity of higher sulfated celluloses suggests a medical application, a direct systemic use by intravenous injection to inhibit blood clotting like heparin is not advisable due to reasons of product safety. On the other hand, immobilization of CSs on the surface of medical devices like catheters and tube systems, which have contact to blood could be an interesting alternative to heparin to increase the blood compatibility of biomaterials (30). Aside from the described inhibition of blood coagulation, CS can also bind different growth factors and influence their activity. The here described stimulating effect on the growth factor FGF-2, which has mitogenic and angiogenic effects on cells and tissues, and recent studies showing that CS have modulating effects on other growth factors like the bone morphogenic protein (BMP-2), suggest applications as bioactive coatings on surfaces of implants in the field of tissue engineering (31, 32).

Acknowledgement

I sincerely thank Mr. Wolfgang Wagenknecht as a former colleague from the Fraunhofer Institute for Polymer Science in Potsdam-Golm for the excellent and pleasant cooperation during the regioselective synthesis of cellulose derivatives and the determination of blood compatibility. This work was partially supported by grants from:
Deutsche Forschungsgemeinschaft Gr1290/12-1 and Gr1290/13-1.

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Originalni rad **UDC: 615.273:676.164**
doi:10.5633/amm.2020.0308

RAZVOJ BIOAKTIVNIH SULFATA CELULOZE ZA BIOMEDICINSKE SVRHE

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Celuloza je jedan od najzastupljenijih biomolekula na zemlji. Hemijski derivati celuloze našli su široku primenu i koriste se u industrijske i biotehnološke svrhe. Sulfati celuloze (SC) predstavljaju klasu derivata rastvorljivih u vodi, koji se primenjuju u industriji, ali još uvek ne i u medicini. U ovom radu, ispitivani su derivati različitog stepena sulfatacije anhidroglukozne jedinice celuloze u cilju postizanja antikoagulantnih i modulacionih efekata faktora rasta sa heparin vezujućim domenima, poput faktora rasta fibroblasta 2 (eng. *fibroblast growth factor* 2 – FGF-2). Rezultati su pokazali da SC višeg stepena sulfatacije imaju antikoagulantnu aktivnost, koja se može porediti sa aktivnošću heparina sa udruženim dejstvom na antitrombin III, koji inhibira aktivnost trombina i faktora Xa, što SC čini interesantnim za antikoagulantna oblaganja medicinskih uređaja. Štaviše, studije su pokazale da SC sa stepenom sulfatacije uporedivim heparinu imaju promovišuću aktivnost na mitogeni efekat FGF-2, što je i pokazano u studijama sa ćelijskim kulturama. Ovo ukazuje na njihovu primenu u antikoagulantnom oblaganju materijala za implantiranje ili komponenti skafolda za tkivno inženjerstvo u oblasti traumatologije i regenerativne medicine.

Acta Medica Medianae 2020;59(3):56-67.

Ključne reči: *sulfati celuloze, stepen sulfatacije, antikoagulacija, trombin, faktori rasta, FGF-2*

Original article

UDC: 616.317-006.6-089.844-06
doi:10.5633/amm.2020.0309

SURGICAL MANAGEMENT OF LIP CANCER: A 5 YEAR EXPERIENCE

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Lip cancer is a common malignancy of the oral cavity as it accounts for 25% of them and contributes ~ 12% to all tumors of the head and neck region. The most frequent lip carcinomas are squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and basosquamous carcinoma (BSC). The aim of this study was to describe clinical characteristics of tumors, surgical technique, and outcomes after lip cancer surgery performed at the University Clinical Center Niš. We conducted a single institution retrospective study including patients who consecutively underwent surgery for carcinoma of the lip at the Clinic of Plastic and Reconstructive Surgery, Clinical Center Niš, in the 5-year period. A total of 32 patients with lip cancer were included in the study. Nineteen (59%) patients were male and 13 (41%) were female. There were 20 cases of SCC, 11 with BCC and one with BSC. The mean patient age was 73.44 (SD 9.95) at the time of primary examination. Tumors were excised with a minimum surgical margin of 5 mm. Surgical treatment depended on the size of the tumor and its localization. Different surgical techniques were used for reconstructions of the lip defects after tumor removal. Thirty one percent of patients had postoperative complications., There was no recurrence of tumors or tumor related deaths during the follow-up of patients. In patients who had a wider resection of tissue and a more complex reconstruction technique performed, the possibility of occurrence of early postoperative complications is greater.

Acta Medica Medianae 2020;59(3):68-72.

Key words: *lip cancer, surgical treatment, reconstruction, complications*

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Introduction

Lip cancer is a common malignancy of the oral cavity as it accounts for 25% of them and contributes ~ 12% to all tumors of the head and neck region (1). The most frequent lip carcinomas are squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and basosquamous carcinoma (BSC), but Merkel cell carcinomas, melanomas, malignant adnexal tumors, and dermatofibrosarcoma protuberans have also been reported (2). BCC generally occur in the upper lip and do not usually present lymph node metastases (2, 3, 4). Compared to BCC and BSC, SCC is associated with a significantly increased risk of metastasis, and SCC of the lower lip, ear, and temple seems to be associated with the highest risk of metastasis compared to other locations (5–8).

Surgery is the treatment of choice for most of the tumors of the lip. Surgical resection requires a full-thickness resection of the skin, muscle and underlying mucosa to allow a safe surgical margin. A lot of reconstruction methods after tumor removal have been reported, however, the reconstruction of lip defect remains a challenge (9).

The aim

The aim of this study was to describe clinical characteristics of tumors, surgical technique, and outcomes after lip cancer surgery performed at the University Clinical Center Niš.

Patients and methods

We conducted a single institution retrospective study including patients who consecutively underwent surgery for carcinoma of the lip at the Clinic for Plastic and Reconstructive Surgery, Clinical Center Niš, in the 5-year period July 2011 to June 2016. In our region, invasive cancers of the lips are routinely referred to our department for surgical treatment. Medical histories and a clinical records database of the Clinic for Plastic and Reconstructive

Surgery were used as the sources of data for this study. In addition to regular demographic data (gender and age) all available information was collected regarding: macroscopic features of the primary lesions, location of lesion on the lip, type of surgical treatment, postoperative treatment, pathologic outcome and follow-up.

Analysis of the data was performed using the statistical package software SPSS 13.0 (SPSS Inc., Chicago, IL, USA).

Results

A total of 32 patients with lip cancer were included in the study. Nineteen (59%) patients were male and 13 (41%) were female. There were 20 cases of SCC, 11 with BCC and one with BSC (Table 1). The mean age of all patients was 73.44 years (SD 9.95) at the time of primary examination. Mean age for SCC was 76.90 years (SD 7.00) and for BCC was 66.45 years (SD 11.41).

Chi-square test of independence (whit Yates's correction for continuity), calculated to compare the relationship between gender and type of tumor, did not show statistically significant relationship (BCC and SCC), χ^2 (1, n = 31) = 0.910; p = 0.34.

Independent samples t-test did not show a significant age difference between women (M = 72.42, SD = 10.01) and men (M = 73.68, SD = 10.26); t (29) = -0.340, p = 0.73.

Independent samples t-test showed a significant age difference between BCC and SCC type (M = 66.45, SD = 11.41) and SCC type (M = 76.90, SD = 7.00); t (29) = -3.170, p = 0.04.

Forty one percent of patients had a tumor less than 10 mm in diameter. Twelve patients (37%) had a tumor size between 11 and 20 mm. Six patients (19%) had a tumor size between 21 and 40 mm. Only one patient had a tumor larger than 40 mm. Seventy five percent of the SCCs were located on the lower lip. Five percent of the SCCs were located at the commissures. Contrariwise, 64% of BCCs were located on the upper lip. Nine percent of the BCCs were located at the commissure (Table 2).

Table 1. Patients and tumor distribution, n = 32

Gender	SCC, n (%)	BSC, n (%)	BCC, n (%)	Total, n (%)
female	9 (45)	1	3 (27)	13 (41)
male	11 (55)	0	8 (73)	19 (59)
Total, n (%)	20 (100)	1 (100)	11 (100)	32 (100)

Table 2. Tumor size and tumor localization, n = 32

	SCC, n (%)	BSC, n (%)	BCC, n (%)	Total, n (%)
Tumor size				
0-10	8 (40)	0	5 (46)	13 (41)
11-20	8 (40)	0	4 (36)	12 (37)
21-40	3 (15)	1 (100)	2 (18)	6 (19)
› 40 mm	1 (5)	0	0	1 (3)
Tumor localization I				
Upper lip	4 (20)	0	7 (64)	11 (35)
Lower lip	15 (75)	1 (100)	3 (27)	19 (59)
Commissure	1 (5)	0	1 (9)	2 (6)
Tumor localization II				
Skin	1 (5)	0	2 (18)	3 (9)
Vermilion	14 (70)	0	1 (9)	15 (47)
Both	5 (25)	1 (100)	8 (73)	14 (44)
Total, n (%)	20 (100)	1 (100)	11 (100)	32 (100)

Tumors were excised with a minimum surgical margin of 5 mm or more. Surgical treatment depended on the size of the tumor and its localization. Tumors were most often removed with full thickness excision (94%) including the oral mucosa. Two excisions included only the skin. Four patients did not have a clean surgical margin on the final histopathology after the primary excision.

Fifty percent of all patients were operated on in general anesthesia. Nine patients (28%) were operated on in a local anesthesia and 7 patients (22%) were treated in local anesthesia with sedation.

Different surgical techniques were used for reconstructions of the lip defects after tumor removal. The most used technique for reconstruction was Wedge or "W" shaped excision with direct closure (47%). Karapandzic flap (Figure 1) was used in 10 cases (31%), perialar crescentic melolabial advancement flap (Webster) was used in 3 patients (9%) and 2 patients (6%) underwent reconstructions using the Bernard-Burow advancement flap. In one patient the Pectoralis major musculocutaneous flap was performed for reconstruction.

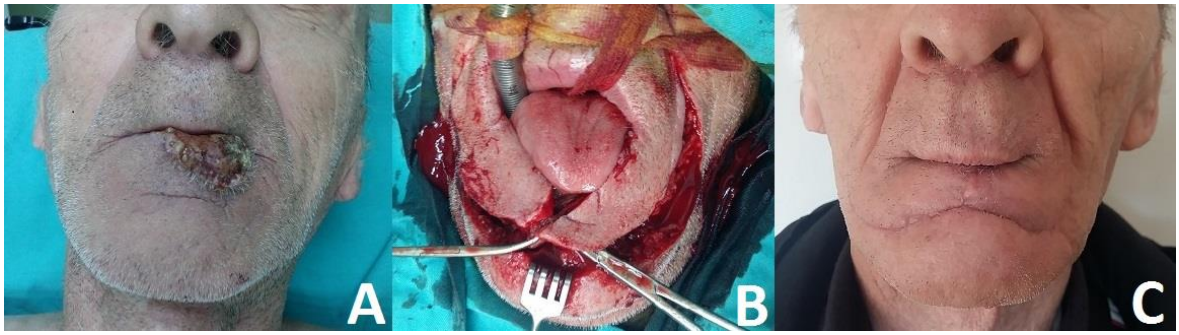

Figure 1. Male patient (79 years old) with SCC of the lower lip. Surgical defect was repaired with Karapandžić flap:
A) preoperative view;
B) intraoperative view;
C) postoperative view after 6 months

Thirty one percent of patients had postoperative complications. Eighteen percent of all patients had postoperative infection. Three patients (9%) had dehiscence of the wound. Only one patient has late complications in the form of microstomia. Other patients had acceptable functional and aesthetic results.

Median follow-up time of the patients was 12 months (min 3, max 24). A difference in patient follow-up time occurred because some patients did not show up at planned control visits after surgery. During the follow-up of patients there was no recurrence of tumors or tumor related deaths.

Discussion

In this article we provided information about lip cancer and described the surgical outcome of a series of 32 patients who underwent surgery for lip cancer and were followed over a 5-year period. The most frequently localization of lip cancer was found on the lower lip, in 59% of the subjects. Also, other authors highlighted same results; Salan A.I. et al. showed that most of the tumors were found in the lower lip (10).

The SCC is most commonly located on the lower lip and the BCC is more common on the upper lip which is similar to reports of other authors (1, 2, 11). We did not find a statistically significant association between gender and tumor type ($p = 0.34$) which corresponds to results of a recent study by Queen D. et al. (12). In our study only 15% of all patients were under the age of 60 and all of them had BCC on final histopathology. The mean age of patients was 73 which is in line with the analysis of other authors (13, 14, 15). The proportion of male and female patients in our sample corresponds to the data of other authors (1, 13, 16, 17).

The unexpected discovery of our study is a large number of early postoperative complications. Kristiensen et al. (18) also reported high frequency of early postoperative wound healing problems which they associated with defect sizes above 20 mm and full thickness excisions. The wider resection margins and the use of full thickness excision require more complex reconstructions and potentially higher risk of complications.

In most international researches, follow-up was about 5 years (1, 13, 19) but some authors (18) also reported problems with follow-up similar to the ones found in our research. Short follow-up led

to the fact that in our study there were no patients with recurrence or deaths caused by disease progression.

Conclusion

We found that a wider resection of tissue and more complex reconstruction techniques that were performed on the patients lead to a higher risk of early postoperative complications. Also, we found no recurrence or tumor related deaths during the follow-up period.

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Originalni rad **UDC: 616.317-006.6-089.844-06**
doi:10.5633/amm.2020.0309

PETOGODIŠNJE ISKUSTVO U HIRURŠKOM LEČENJU KARCINOMA USNE

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Karcinom usne je čest malignitet i čini 25% karcinoma usne duplje i oko 12% svih tumora glave i vrata. Najčešći karcinomi usne su skvamocelularni karcinom (SCC), bazocelularni karcinom (BCC) i bazoskvamocelularni karcinom (BSC). Cilj istraživanja je opisivanje kliničkih karateristika tumora, hirurških tehnika i ishoda nakon operacije karcinoma usne u univerzitetskom kliničkom centru u Nišu. Sproveli smo retrospektivnu studiju, koja je obuhvatila bolesnike hirurški lečene od karcinoma usne na Klinici za plastičnu i rekonstruktivnu hirurgiju Kliničkog centra Niš, u periodu od 5 godina. U istraživanje je uključeno ukupno 32 bolesnika sa karcinomom usne. Devetnaest (59%) bolesnika bilo je muškog pola, a 13 (41%) ženkog. Bilo je 20 bolesnika sa SCC, 11 sa BCC i bio je jedan bolesnik sa BSC. Prosečna starost bolesnika bila je 73,44 godine (SD 9,95) u vreme prvog pregleda. Tumori su ekscidirani sa hirurškom marginom od najmanje 5 mm. Hirurško lečenje zavisilo je od veličine tumora i njegove lokalizacije. Različite hirurške tehnike korišćene su za rekonstrukciju defekata usana nastalih nakon uklanjanja tumora. Trideset jedan posto bolesnika imao je postoperativne komplikacije. Tokom praćenja bolesnika, nije bilo recidiva tumora ili smrti povezanih sa tumorom. Kod bolesnika kod kojih je izvršena šira resekcija tkiva i izvedena složenija tehnika rekonstrukcije, veća je mogućnost pojave ranih postoperativnih komplikacija.

Acta Medica Medianae 2020;59(3):68-72.

Ključne reči: *karcinom usne, hirurško lečenje, rekonsrukicja, komplikacije*

Original article

UDC: 577.11:616.65-006.6-033.2-07
doi:10.5633/amm.2020.0310

PROSTATE SPECIFIC ANTIGEN VERSUS COMBINATION OF PROSTATE SPECIFIC ANTIGEN AND ALKALINE PHOSPHATASE IN PREDICTION OF PROSTATE CARCINOMA BONE METASTASES DETECTED WITH BONE SCINTIGRAPHY

Miloš Stević[1,2], Marina Vlajković[1,2], Marko Kojić[1], Filip Veličković[1], Nina Topić[1], Dragoslav Bašić[2,3]

Prostate cancer (PC) is the second one in morbidity and mortality in a population of men over 50 and has a high affinity for metastasis to the bone system. The aim of this study was to determine predictive value of prostate specific antigen (PSA) versus to combination of PSA and alkaline phosphatase (ALP) for existence of skeletal PC metastases. The study included 620 patients with histologically proven PC with elevated PSA, ALP, or clinical signs that indicated bone metastases. Bone scintigraphy (BS) was performed according to the protocol of the European Association of Nuclear Medicine. Specificity, sensitivity, positive and negative predictive value, and overall accuracy of PSA and the combination of PSA and ALP were evaluated in predicting the existence bone metastases on BS. The PSA showed sensitivity of 91.88%; specificity of 37.5%; positive predictive value of 53.32%; a negative predictive value of 85.62% and an overall accuracy of 61.22% (95% CI). The PSA and ALP combination showed a sensitivity of 99.20%, specificity of 96.88%, positive predictive value of 98.41%, and negative predictive value of 98.41% and an overall accuracy of 98.41% (95% CI). The combination of PSA and ALP showed significantly higher sensitivity, specificity, positive and negative predictive value and overall accuracy than the PSA only. When indicating BS in patients with PC, PSA, ALP, and clinical signs should be evaluated for the early detection of bone metastases and in the aim to avoid unnecessary admission to scintigraphy of patients in whom there is no high suspicion of bone metastases.

Acta Medica Medianae 2020;59(3):73-83.

Key words: *prostate specific antigen, alkaline phosphatase, bone scintigraphy*

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Introduction

Prostate cancer (PC) ranks second in both, morbidity and mortality in a population of men over 50, with a median age of patients of 66 years at the time of initial diagnosis (1). Prostate cancer in its initial stages is often a completely asymptomatic disease. The symptoms that may indicate that there may be changes in the prostate in terms of an incipient prostate cancer are mainly problems associated with urination, such as difficult or frequent urination, or the need for urination that occurs during the night. Sometimes the initial symptoms are pain in the lumbar spine, which is encounter-edusually in advanced stages of the disease, precisely because the axial skeleton is most often affected by metastatic prostate cancer.

Suspicion of the existence of the disease is usually based on elevated serum prostate specific antigen (PSA) values greater than 4 ng/mL. Increased PSA level is also mixed in other conditions associated with prostate disorders such as benign prostatic hyperplasia (BHP). For diagnosis of PC, the basic diagnostic method is biopsy and histological findings.

Prostate cancer metastases mainly occur in the skeleton and represent a very important prognostic indicator, and very important factor in planning of a therapeutic approach in patients diagnosed with PC (2).

Bone scintigraphy is still the most commonly used method for detecting the existence of PC bone metastases. However, BS cannot be used as a screening method for the detection of metastatic disease of any oncological entity, even in the case of PC.

The indication for BS for the detection of PC metastases is mainly related to elevated PSA values. However, elevated PSA alone does not necessarily indicate bone metastases, which would mean that a certain group of patients would needlessly be referred for BS examination.

The clinical picture of patients, especially the presence of the pain in the axial skeleton or other parts of the locomotor system, also does not represent a safe indication for BS examination, considering that the average detection time of PC is 66 years of age, and those kind of clinical signs are usual in this population of men even without PC.

One of the biochemical markers that should be used together with PSA is alkaline phosphatase (ALP). Alkaline phosphatase is released mainly in the liver and skeletal system, and elevated ALP values in patients with PC in the absence of liver disease are largely originated in the skeleton (3).

The aim

The focus of this study was the assessment of accuracy in prediction of bone metastasis in patients with PC using the combination of values of PSA, ALP and the existence of clinical signs, versus the prediction achieved using levels of PSA only as an independent predictor for the existence of skeletal metastases of PC.

Patients and methodology

Six hundred twenty patients, from 51 to 91 years of age, were examined (average 71 years of age SD 7.012). All patients were diagnosed with biopsy and histopathological findings. Laboratory analysis of serum PSA and ALP levels was performed in all patients. All patients with elevated PSA, ALP and with clinical signs such was the presence of the pain predominantly in the axial skeleton or in some other parts of skeleton, underwent BS.

PSA was determined on UniCel Dxl 600 (BECKMAN COULTER) with Hybritec PSA Chemiluminiscent Immunoassay, with normal values in the range from 0.0 to 4.0 ng/mL.

The ALP was determined on UniCel Dxl 600 (BECKMAN COULTER), Chemiluminescent Immunoassay with a reference range from 20 to 140 IU/L.

Bone scintigraphy was performed according to the standard protocol recommended by the European Nuclear Medicine Association (4) on a Siemens dual-head gamma camera, in "whole body" (WBS) modality, or "spot" static scintigrams and/or single photon emission tomography (SPECT) modality when lesions detected on the WBS were suspected but not clear for the existence of secondary deposits. Whole body scintigrams in AP and PA projections were made in one pass, with a recording speed of 12 cm/min in anterior (AP) and posterior (PA) projections with computerized "zipping" of the scintigraphic image, in order to get a full skeleton view in one act. Targeted spot static scintigrams were made in AP and PA projections over the region of interest which was the region where suspected scintigraphic signs of lesions corresponding to secondary deposits were observed on WBS scintigrams. The SPECT method was applied over the suspected regions by rotating the body shape orbit in a 180 degree arc with a step and shoot modality of 30 seconds per projection with a total of 32 positions per detector, respectively, with an arc angle of difference between each projections of 5.6 degrees. The reconstruction of the SPECT tomogram was done along the sagittal, transversal and coronal axes through the region of interest using an iterative method. Radiopharmaceutical that was used was Technetium 99m labeled diphosphonate (99mTc-DPD) in applied dose of 20 mCi (740 MBq) (5). The scintigrams were examined by two independent examiners, both of whom were nuclear medicine specialists with decades of experience in interpreting BS. Positive scintigraphic findings were the existence of one or more foci with significantly enhanced radiopharmaceutical fixation, which corresponded to the existence of a skeletal osteoblastic response to the presence of a secondary deposit of PC in the skeleton. The existence of focal changes with significantly reduced fixation and with the hyperfixation rhyme was considered as the presence of osteolytic metastatic changes, or the existence of a mixed osteoblastic - osteoclastic response to the presence of hematogenous spreading of metastatic disease of PC in the skeleton.

Statistical processing was done with SPSS software, using descriptive testing, with cross tabulation of the obtained results. Normal and pathological value of PSA with the existence and absence of secondary deposits in the skeleton was correlated. In addition, normal and pathological value of ALP was correlated with the existence or absence of secondary deposits, and finally a combination of values of PSA and ALP was correlated with existence or absence of secondary deposits in the skeleton. The pathological values of PSA with the existence of secondary deposits on skeletal scintigraphy were consideredas true positive, the normal values of PSA with normal scintigraphic findings were considered as true negative. The false positive was the pathological value of PSA without proven metastases in the skeleton on the BS, while false negative finding was considered as the normal value of PSA with the presence of metastases on scintigraphy.

The true positive combination of PSA and ALP was the group of patients with elevated PSA and ALP values and skeletal metastases on BS, while the true negative finding was the existence of normal PSA and ALP values without skeletal metastases on BS. False positives were patients with elevated PSA and ALP without metastases on BS, whereas false negative were findings with normal PSA and ALP and with visualized metastases on scintigraphic findings.

Results

Out of 620 patients with histologically proven PC, 349 patients had no signs of skeletal metastases (Figure 1) and 271 patients had scintigraphic signs of secondary deposits in the skeletal system (Figure 2, 3 and 4).

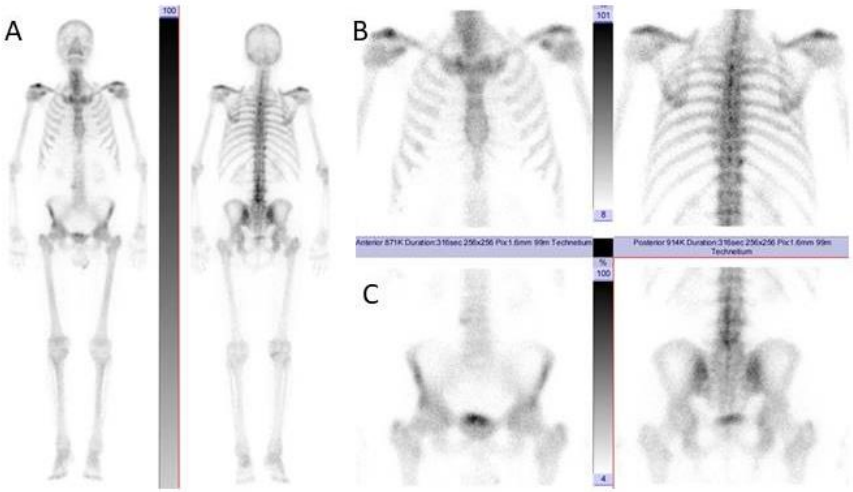


Figure 1. A. Whole body scintigraphy in anterior and posterior projections,
B. Spot scintigrams of thoracic region in anterior and posterior projections,
C. Spot centigrams of pelvis in anterior and posterior projections. Normal finding.

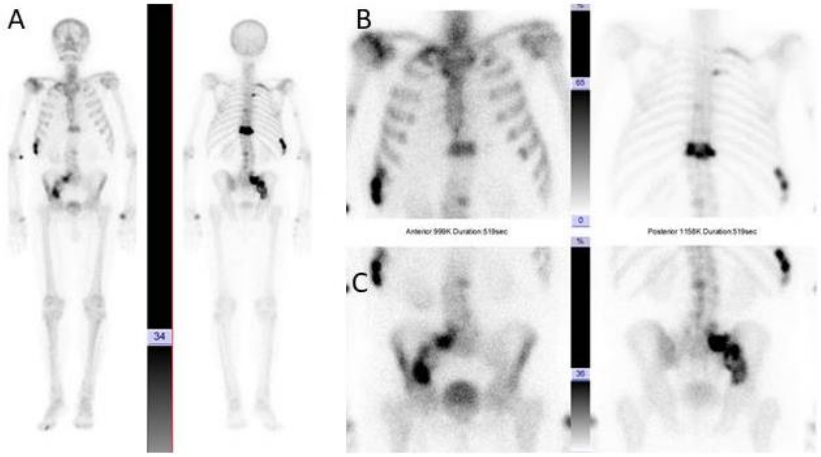


Figure 2. A. Whole body scintigraphy in anterior and posterior projections,
B. Spot scintigrams of thoracic region in anterior and posterior projections,
C. Spot scintgrams of pelvis in anterior and posterior projections.
Presence of metastases in the thoracic spine, posterior aspect of right sided ribs and right sacroiliac joint.

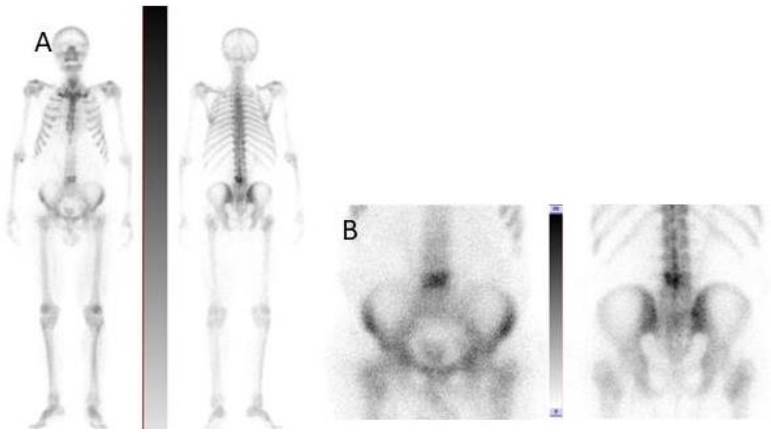


Figure 3. A. Whole body scintigraphy in anterior and posterior projections,
B. Spot scintigrams of pelvis in anterior and posterior projections.
Suspect metastasis in IV lumbar vertebra.

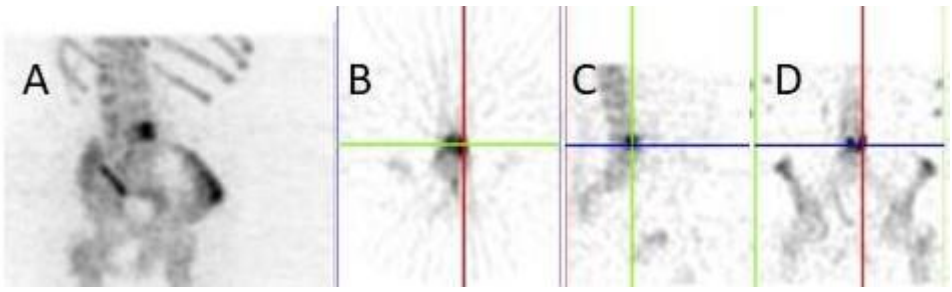
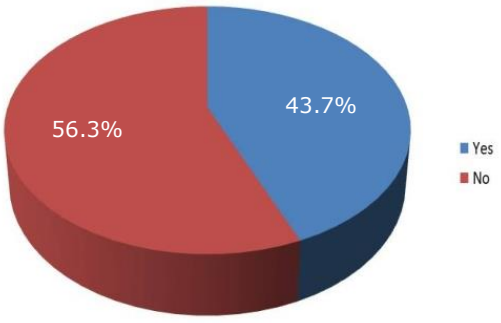


Figure 4. Patient in Figure 3. Solitary metastasis in the body of IV lumbar vertebra SPECT modality study
A. Comprehensive 3D,
B. Transversal tomogram of IV lumbar vertebra,
C. Sagittal tomogram of IV lumbar vertebra,
D. Coronal tomogram of IV lumbar vertebra.



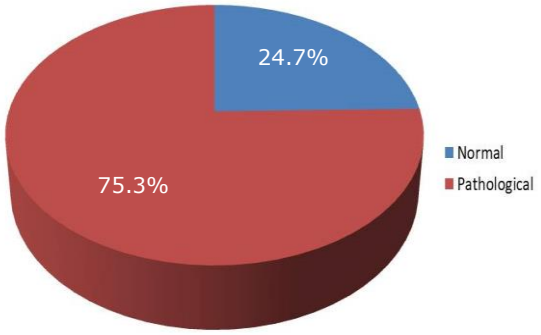
Graph 1. Existance of metastases in skeleton

Expressed in percentages, 56.3% of patients had no signs of skeletal metastases on BS, whereas 43.7% of patients had demonstrated the existence of secondary PC deposits (Graph 1).

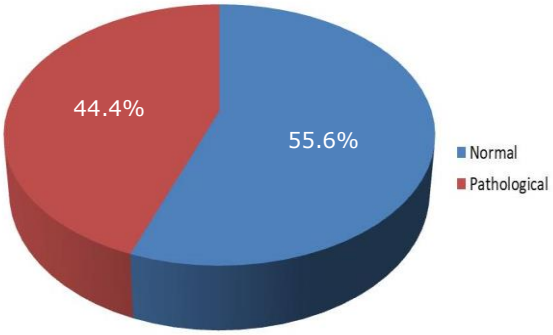
Prostate specific antigen had normal values in 153 (24.7%), while pathological values were found in 467 (75.3%) patients (Graph 2).

Normal ALP values were found in 345 (55.6%) patients and pathological values were found in 275 (44.4%) (Graph 3).

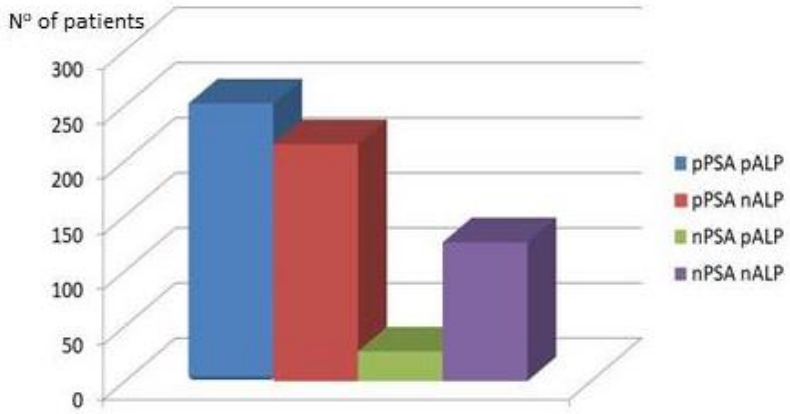
Pathological values of both PSA and ALP were found in 252 (40.6%) patients, pathological PSA with normal ALP values were 215 (34.7%), normal PSA and pathological value of ALP were found in 27 (4.4%) patients, while 126 (20.3%) patients had normal values of both PSA and ALP (Graph 4).



Graph 2. PSA values



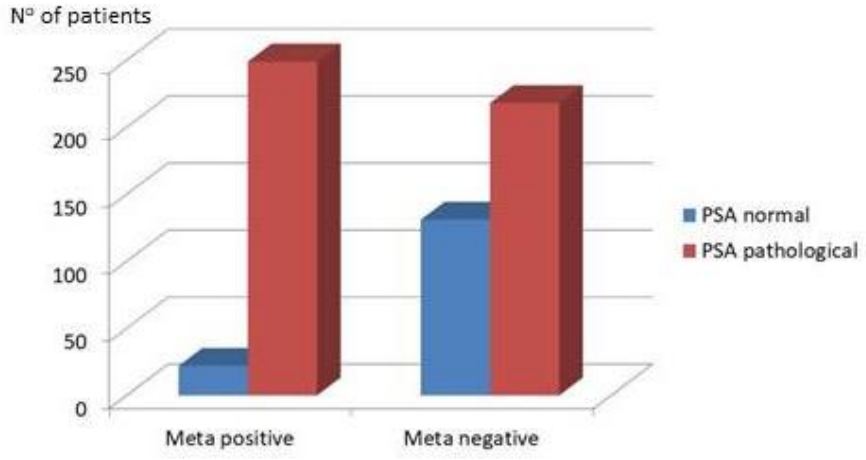
Graph 3. ALP values



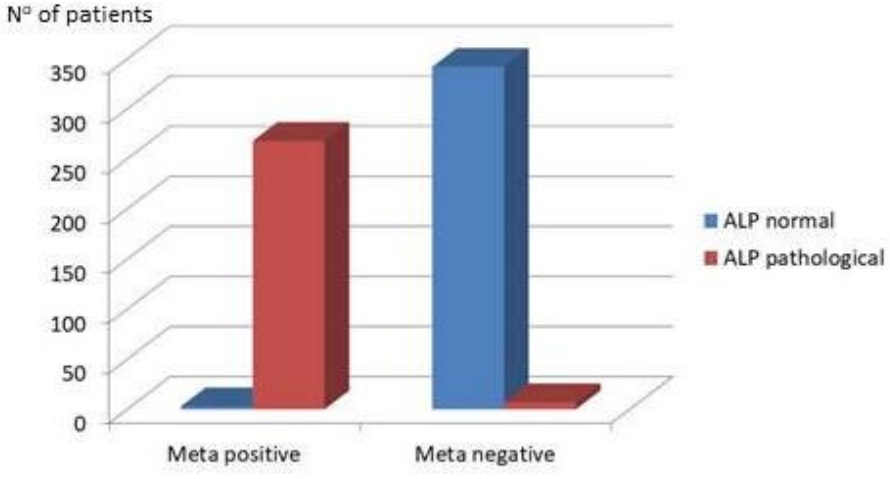
Graph 4. Combined values of PSA and ALP. N – normal values, P – pathological values

Expressed over the number of patients with pathological PSA values, 53.3% of them had a positive scintigraphic finding for the existence of secondary deposits, while (46.7%) had no skeletal metastases, while on the other hand, patients with normal PSA (< 4.0 ng/mL) or low PSA values (< 10 ng/mL) in 14.4% on scintigrams showed the presence of secondary deposits, while 85.6% had no skeletal metastases (Graph 5).

Three hundred forty-two patients with normal ALP values had normal BS, while in three patients with normal ALP, BS showed the existence of secondary deposits of PC. Pathological values of ALP with skeletal metastases on BS were reported in 268 patients, while pathological values of ALP without skeletal metastases were found in seven patients (Graph 6).



Graph 5. Existance of metastases and value of PSA



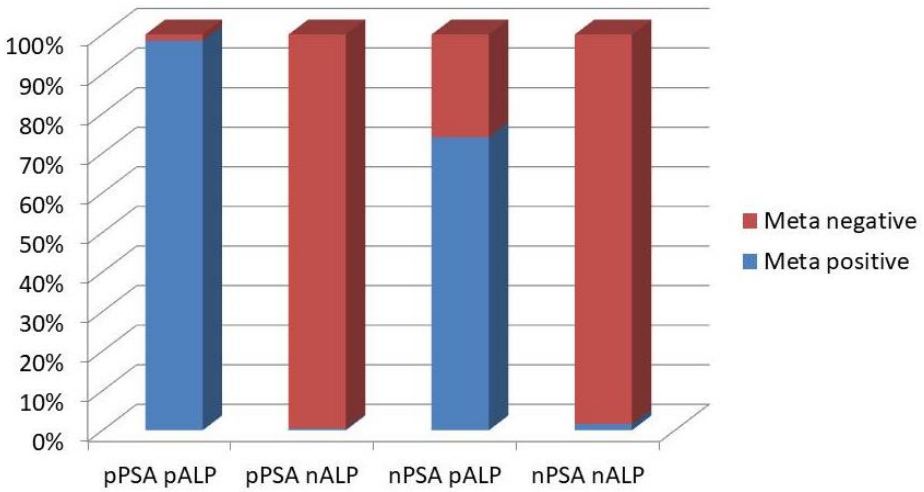
Graph 6. Existance of bone metastases and value of ALP

Pathological values of PSA and ALP were found in 252 patients, of whom 248 had skeletal metastases and 4 did not. Pathological PSA values in combination with normal ALP were observed in 215 patients, one of whom had metastases and 214 had normal BS. Normal PSA and pathological ALP were found in 27 patients, of whom 20 had metastases while 7 had normal BS. Normal PSA and ALP values were found in 126 patients, and only 2 of them had secondary deposits in the skeleton and 124 had

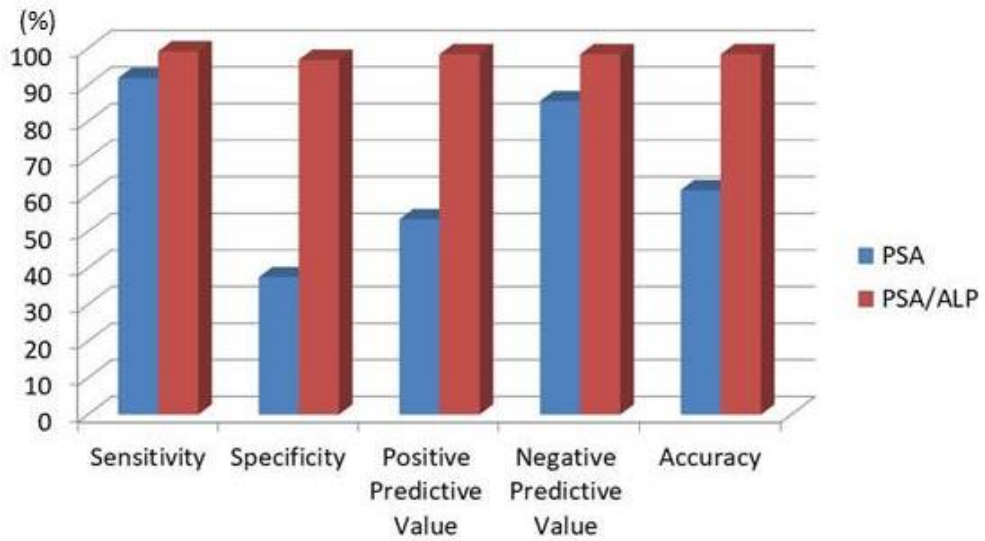
normal BS. A combination of pathological values of both biological markers was considered a positive predictor of skeletal metastases, while a combination of PSA and ALP with values that were within physiological limits was considered a negative predictor (Graph 7).

Statistical processing of the obtained data yielded results that showed that serum PSA values had a sensitivity of 91.88%; specificity of 37.5%; a positive predictive value of 53.32%; a negative predictive value of 85.62% and an overall accuracy of 61.22% (95% CI), for correlation with the existence of PC metastases on BS.

The combination of PSA and ALP was considered positive when both biological markers had pathological values, or negative, when both markers had values within the physiological range. Statistical analysis showed that the combination of PSA and ALP in correlation to the existence of PC metastases in the skeleton had a sensitivity of 99.20%, specificity of 96.88%, positive predictive value of 98.41%, and negative predictive value of 98.41% and overall accuracy of 98.41% (95% CI). Graph 8 shows a comparison of sensitivity, specificity, positive and negative values, and the overall accuracy of PSA as a single biological marker, and the combination of PSA and ALP together in the prediction of the existence of PC metastases in the skeleton found on BS.



Graph 7. Combined values of PSA and ALP as positive and negative predictors for metastases on BS



Graph 8. Comparison of PSA and combination of PSA/ALP as predictors of bone metastases in patients with PC

Discussion

Prostate adenocarcinoma ranks second in morbidity in the male population, and fifth in mortality in the total population. According to GLOBOCAN 2018 (1), there were 1,276,106 new cases of prostate adenocarcinoma reported worldwide during 2018. It has been observed that prostate cancer is more common in African-American populations with the occurrence of more severe forms of cancer, and in members of the male population over 65, regardless of race. No relevant data are yet available to indicate valid measures for the prevention of this type of cancer, and screening tests are recommended in the population over 45 years of age (6). Prostate cancer is very often an asymptomatic disease in the early stages; however, malignant prostate cancer cells migrate, penetrate the blood vessels and have a high affinity for spreading to other organs (7). Hematogenously disseminated prostate carcinoma cells have a significant affinity for expansion into the skeleton, initially engaging the axial skeleton, especially, those places in the skeleton where the red bone marrow is still active (8). The explanation for this spread of prostate cancer is that the red bone marrow represents an almost ideal substrate for the proliferation of prostate cancer cells. In autopsies, it was found that as many as 90.1% of men who died due to hematogenous spread of prostate cancer had skeletal metastases (9). The reaction of the skeleton to the presence of prostate cancer cells is reflected in the existence of osteoblastic or osteoclastic reaction of the bone, or in some cases mixed reactions to the presence of malignant cells. Osteoblastic activity is reflected practically as enhanced process of hydroxyapatite deposition, what results as the formation of a "new bone" at the sites of malignant cell nidation, without previously present bone matrix resorption. Osteolytic lesions are basically represented by the destruction of the bone matrix and this is why they result in the existence of bone softening, pain or even pathological fractures. The mixed response of the skeleton to the presence of malignant secondary deposits is in fact the simultaneous appearance of the two mechanisms described above (10, 11).

Prostate specific antigen is widely accepted as a screening test for early detection of prostate cancer. This approach to the initial detection of prostate cancer is still relevant today, although there are studies showing that its sensitivity to the prediction of prostate cancer is not significantly reliable. Prostate cancer has also been detected in patients who have a PSA level < 4.0 ng/mL which is considered as normal (12). Studies conducted in Australia and accepted by the National Health and Medical Research Council (NHMRC) show that it is appropriate to lower the limit of the physiological range of PSA to < 3.0 ng/mL and that even with such low PSA values, it is possible to have false negative results in the early detection of prostate cancer. Therefore, it is recommended that the testing should be adjusted to the patients' age, and that the lower recommended PSA limits depend on the age of the patients (13).

After prostate cancer is detected, one of the primary goals is to determine the suspicion of metastases in the skeleton, and after establishing the suspicion of the existence of secondary deposits of prostate cancer, to determine their presence. Years, or decades back, the authors have been trying to establish algorithms to predict the existence of hematogenous metastases of prostate cancer in the skeleton. The approaches involve the use of various biologically active markers that may indicate the existence of metastatic spread of prostate carcinoma in the skeleton.

One of the most widely used biologically active marker for screening tests in the initial diagnosis of prostate cancer is PSA, so it was a logical attempt to use the same approach, with measuring the level of PSA in patients with PC to predict the existence of bone metastases.

In a study conducted in 1996, a total of 158 prostate cancer patients reported a high negative predictive value of serum PSA of 98%, its positive predictive value of 74% and an overall accuracy of 92% in the prediction of bone metastases in patients with PC. Based on the results of PSA levels and skeletal scintigraphy, it is concluded that low PSA values almost exclude the existence of metastases (14).

On the other hand, the same author who published a study in 1996 stating that low PSA values almost exclude bone metastases, two years later, published a work performed on 359 patients where the presence of metastases was detected in patients with PSA values < 10 ng/mL, and concludes that PSA provides only limited information regarding the prediction of the existence of secondary deposits of prostate cancer in the skeleton (15).

In our study, the scintigraphic examination included 620 patients, of whom 43.7% had a proven presence of secondary deposits on the scintigraphic examination, while the presence of bone metastases was not scintigraphically observed in 57.3% of patients. Serum PSA levels were normal in 153 (24.7%), whereas pathological PSA values were found in 467 (75.3%) patients. In the group of patients with normal PSA values (< 10 ng/mL), we scintigraphically detected bone metastases in 14.4%. It should be noted that patients in our study were referred for skeletal scintigraphy not only because of high PSA values but also because of a clinical picture that could indicate the presence of bone metastases of prostate cancer. Statistically, we obtained sensitivity of 91.88%; specificity of 37.5%; a positive predictive value of 53.32%; a negative predictive value of 85.62% and an overall accuracy of 61.22% (95% CI) for PSA levels and their relation to the existence of bone metastases. Our results are similar to those obtained in other studies, as stated by the authors in the 2012 paper in which they advocate that symptomatic patients with prostate cancer should undergo skeletal scintigraphy regardless of PSA values (16).

In addition to the clinical picture, that is, the subjective feeling of patients and the value of PSA, there is a need for more objective screening of patients diagnosed with prostate cancer in order to indicate more accurately skeletal scintigraphy for the

purpose of detecting bone metastases. Alkaline phosphatase (ALP) in healthy humans is mainly derived from the skeleton and the liver. Elevated ALP without liver disease in patients diagnosed with prostate cancer comes from the skeleton (17).

The mechanism of occurrence of elevated ALP values in the presence of bone metastases of prostate cancer is related to the activation of osteoblastic activity in the presence of prostate cancer cells. Thus activated osteoblasts produce elevated ALP values (18). Elevated ALP values are directly related to the existence or extensiveness of secondarily depositional skeletal changes in prostate cancer, that is, a decrease in serum ALP levels is directly related to a possible improvement in metastatic disease (19). In our study, we used the opportunity to perform laboratory analysis of serum ALP levels in patients referred for skeletal scintigraphy, either because of elevated PSAs or clinically significant signs that could indicate the presence of bone metastases of prostate cancer. We combined the elevated ALP values with the elevated PSA values and then compared that battery of tests with the existence or absence of bone metastases of prostate cancer.

In our study, correlation of the existence or absence of bone metastases in the two groups of patients, one with low PSA values and ALP values in the physiological limits, and the second one with pathological values of both biological markers, showing significantly lower number of patients with PC bone metastases in the group with low values of PSA and ALP. On the other side, in the group of patients with pathological values of PSA and ALP, BS showed significantly higher number of patients with presence of PC bone metastases.

Conclusion

Prostate specific antigen as an independent biological marker in terms of prediction of bone metastasis in prostate cancer patients showed significantly lower sensitivity, specificity, positive and negative predictive value, and overall accuracy compared to the combination of PSA and ALP.

As part of the evaluation of patients diagnosed with prostate cancer in order to more accurately indicate skeletal scintigraphy for the detection of bone metastases, a combination of serum PSA, ALP, and clinical signs suggesting bone metastasis should be used according to our results.

Such an approach reduces the number of patients who would be unnecessarily referred to skeletal scintigraphy, and on the other side, the number of patients who, due to possibly low or moderately elevated PSA values as a standalone marker, with skeletal metastases, remain with no admission to bone scintigraphy and consequently without detection of existing metastatic changes.

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Originalni rad **UDC: 577.11:616.65-006.6-033.2-07**
doi:10.5633/amm.2020.0310

PROSTATA SPECIFIČNI ANTIGEN U ODNOSU NA KOMBINACIJU PROSTATA SPECIFIČNOG ANTIGENA I ALKALNE FOSFATAZE U PREDIKCIJI SCINTIGRAFSKI DETEKTOVANIH METASTAZA KARCINOMA PROSTATE

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Karcinom prostate (CP) na drugom je mestu po broju obolelih i po mortalitetu u populaciji muškaraca starosti preko 50 godina i ima veliki afinitet prema metastaziranju u koštani sistem. Cilj ovog rada bilo je utvrđivanje prediktivne vrednosti prostata specifičnog antigena (PSA) u odnosu na kombinaciju PSA i alkalne fosfataze (ALP) u predikciji postojanja scintigrafski detektabilnih metastaza CP. U studiju je bilo uključeno 620 bolesnika sa patohistološki dokazanim CP, koji su na scintigrafiju upućivani zbog povišenih vrednosti PSA, ALP ili zbog kliničkih znakova, koji bi mogli da ukazuju na postojanje koštanih metastaza CP. Scintigrafija skeleta (BS) rađena je po ustaljenom protokolu Evropske asocijacije nuklearne medicine (EANM). Statistička obrada podataka podrazumevala je procenu specifičnosti, senzitivnosti, pozitivne i negativne prediktivne vrednosti i ukupne tačnosti PSA i kombinacije PSA i ALP u predikciji postojanja scintigrafski detektabilnih koštanih metastaza CP. U pogledu predikcije postojanja koštanih metastaza na BS, PSA je pokazao senzitivnost u iznosu od 91,88%; specifičnost u iznosu od 37,5%; pozitivnu prediktivnu vrednost u iznosu od 53,32%; negativnu prediktivnu vrednost u iznosu od 85,62% i ukupnu tačnost u iznosu od 61,22% (95% CI). Kombinacija PSA i ALP pokazala je senzitivnost od 99,20%, specifičnost od 96,88%, pozitivnu prediktivnu vrednost od 98,41%, negativnu prediktivnu vrednost od 98,41% i ukupnu tačnost od 98,41% (95% CI). Kombinacija PSA i ALP pokazala je signifikantno veću senzitivnost, specifičnost, pozitivnu i negativnu prediktivnu vrednost, odnosno ukupnu tačnost u odnosu na PSA, kao samostalni biološki marker. Prilikom indikovanja BS, kod bolesnika sa CP treba uzeti u obzir vrednosti PSA, ALP i kliničke znake u cilju rane detekcije koštanih metastaza i izbegavanja nepotrebnog upućivanje na scintigrafiju bolesnika, kod kojih ne postoji visoka sumnja na postojanje koštanih metastaza.

Acta Medica Medianae 2020;59(3):73-83.

Ključne reči: *prostata specifični antigen, alkalna fosfataza, scintigrafija skeleta*

Review article

UDC: 616.155.194:618.2
doi:10.5633/amm.2020.0311

ANEMIA IN GYNECOLOGY AND PERINATOLOGY – NEW ATTITUDES

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Anemia is a global health problem. Among women, it is present in 38% of pregnant women and 29% of non-pregnant women. The most common form is sideropenic anemia. The most common cause of anemia in gynecology is abnormal uterine bleeding that occurs as acute, chronic and intermittent bleeding. In perinatology, there are specific changes at the level of the cardiovascular and hematopoietic systems of a pregnant woman, which impose different criteria for the diagnosis of anemia relative to a non-circulating condition. The basic change is an increase in blood volume that grows more at the expense of plasma versus erythrocyte volume. The erythrocyte volume grows by about 33%, and so does the reticulocyte count. There are two basic approaches to anemia diagnosis - a kinetic approach that seeks to determine the mechanism that led to anemia and a morphological approach that divides anemia relative to the size of the erythrocyte's mean volume and reticulocyte response. The therapy for most common - sideropenic anemia is performed with iron preparations (chemical, divalent and trivalent iron). In pregnancy, it is necessary for all pregnant women, but only in moderate doses. The total antenatal increase should be about 1000 mg. To meet these needs, 4 mg of iron per day is needed in the first half of pregnancy and 6-7 mg in the second half. Due to the poor tolerance of iron, its compliance is estimated at 50%.

Acta Medica Medianae 2020;59(3):84-89.

Key words: *anemia, gynecology, perinatology*

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Introduction

Abnormal uterine bleeding can lead to anemia of gynecological origin.

The FIGO classification of abnormal uterine bleeding (AUB) "PALM-COEIN" system of classification an acute abnormal uterine bleeding, an episode of excessive menstrual blood loss which demands urgent intervention occurs independently or in the context of a chronic abnormal uterine bleeding cases (1).

◇ Chronic abnormal uterine bleeding – abnormal by volume, periods of occurrance and duration, is a disorder which lasts at least six months.

◇ Intermenstrual abnormal uterine bleeding – occuring between clearly defined menstrual cycles.

◇ PALM – objective structural patterns, which can be measured and visualised by diagnostic technics such as ultrasound and/or PH analysis:

1. Polyps
2. Adenomyosis
3. Leiomyomas
4. Malignancy / Hyperplasia

COEIN

1. Coagulopathies
2. Ovulatory disorders
3. Endometrial disorders
4. Iatrogenic patterns
5. Unclassified patterns

Changes of the Hematological system in pregnancy

1. *Growth of the blood volume* – the volume of plasma is higher, the erythrocyte count is less and yet the erythrocyte volume grows by about 33% - 450 ml. The reticulocytes show growth.

2. *Atrial Natriuretic Peptid* (ANP) – There is a growth of renal perfusion and glomerular filtration, less renal secretion, lessens the basal release of aldosterone from the glomerular zone.

3. *Brain Natriuretic Peptid* (BNP) – a very potent vasodilator is being created and is secreted in the amnionic cells (2).

Blood loss in vaginal birth is 500 ml and in sectio Caesarean is 1000 ml.

1. Iron (Fe) needs in a normal pregnancy are enlarged for production 450 ml, Er – 500 mg.

2. Total antepartum increase of Fe is 1000 mg.

3. Day loss of Fe in absense of bleeding is 0.5 – 1 mg.

4. Foetus needs

5. The total Fe needs are:

- For the placenta and the fetus, 300 mg
- For Er growth, 500 mg

6. The amount of Fe needed to fulfill the normal fetus needs and the increase of volume of maternal circulation is 800 mg.

7. The amount of Fe lost during a normal menstrual cycle is 25 mg

8. The absorbtion rate from Fe supplements is 10-20%, i.e., 12 mg a day

9. The amount of Fe in 1ml of Er is 1.1 mg

10. The milk absorbs Fe during lactation about 1 mg a day (3).

Cardiovascular changes during pregnancy

Pulsation rate increases, as does the volume and pulsatory volume, renal angiotensine or aldosterone, while there is a decrease in the arterial pressure, vascular resistance systemic, 21% and pulmonary, 34% (4).

The following tables give the corresponding values of individual blood parameters by trimester of pregnancy (5, 6, 7, 8).

The total amount of iron (Fe) in the organism of an adult human is 3.5-4 g. It is a toxic metal however, the toxic effect is detained by the formation of iron – proteins compound. The proteins which contain iron are divided into proteins that contain heme and heme binding iron, such as hemoglobin, myoglobin, cytochromes and other, and to those which do not contain heme and yet bind iron, such as ferritin, transferrin, and flavoproteins. Referring to these proteins, iron has a key role in transporting oxygen and in the energy metabolism (9).

The digested Fe is absorbed in the organism in a bivalent form in the duodenum and in the upper jejunum with the help of apoferritin protein, which is reversibly bound to Fe. As the amount of apoferritin is limited, resorption of Fe is limited to mucose blockage. The digested trivalent form and the Fe component bound to heme in the form of Fe^{3+} has to be reduced with the help of vitamin C in its bivalent form. About 1 mg of Fe is resorbed daily, which after entering the mucose cells as Fe^{2+} is bound to transportive supstances. Before entering the plasma, oxidation occurs in Fe^{3+} with the help of ceruloplasmin and it sticks to transferrin.

The transport of the Fe ions in the plasma happens via the iron–transferrin complex. As it happens, only two trivalent ions of Fe can be bound to one molecule of transferrin protein. The Fe serum is almost completely tied to transferrin. Transferrin – siderophilin is the transportive protein of iron in the plasma and it is coded with the TF gene. It conducts the transport of iron from the place of absorption and the place of resorption to the place of storage in the bone marrow and partially in the liver. There, iron binds to apoferrin. Transferrin is usually saturated with 30% iron. The amount of iron in the transferrin of plasma is in balance with the iron in these stored forms in the gastrointestinal tract and in the reticuloendothelial (RES) system (10).

There is no ferritin in plasma, but there is apoferritin. In the process of creation of ferritin from apoferritin, first of all, the Fe^{2+} connects to the surface of the inner membrane of the apoferrin and then via oxidation it transforms into Fe^{3+} which binds itself tightly to the ferritin. Reduction releases it from the ferritin in the form of Fe^{2+}.

Iron enters the structure of porphyrin and builds heme – hemoglobin, myoglobin.

Erythrocytopoiesis

Is the process of creation of mature erythrocytes from their stem cells by the process of reproduction, maturity and the loss of nucleus and it almost always happens in the bone marrow in the morphological entity of the erythroblastic island.

In order for this process to occur, many factors are needed: normally built bone marrow with all the necessary elements for division and maturity of the erythrocytic cells – Fe, Cu, Co, proteins, vitamins B12, folic acid, appropriate local temperature and normal endocrine regulation.

The first cell of the erythrocytic strain is the proerythroblast, and in this stage of maturity starts the endocytosis of the Fe^{3+} complex from the extracellular matrix.

With the division of the proerythroblast, the basophilic or early erythroblast is being created. In its ribosomes globin is synthetised, and the synthesis of hem starts in its mitochondrias (11).

Its division creates the polychromatophilic or intermediary erythroblast with visible hemoglobin synthesis.

The acidophilic or orthochromatic erythroblast is the next cell and it contains larger amounts of hemoglobin.

With the ejection of the nucleus from the acidophilic erythroblast, the reticulocyte is created. In this stage, the cells transfer from the bone marrow into the blood stream through the sinusoid capillaries in the process of diapedesis. The endocytosis of iron stops, but synthesis of hemoglobin continues for some time.

A five day period is required for a reticulocyte to get created from the proerythroblast, and another two for it to mature into a full grown erythrocyte.

Erythropoiesis is regulated by the partial pressure of oxygen in the tissues, and the main

factor which influences the increase of erythropoiesis is erythropoietin – 90% from the kidneys and 10% from the liver (12).

The half-life of erythrocytes is 80-120 days after which they disintegrate, mostly in the liver – Kupffer cells and in the spleen, as in the RES macrophage.

Hemoglobin is a protein by nature which can be found in the erythrocytes. It belongs to the class of heteroproteins – apoprotein is a molecule of globin while the prosthetic group is represented by the heme group. Synthesis starts in the proerythroblasts and continues to the reticulocyte and happens in five stages.

In the third stage, the porphyrin IX combines with Fe^{2+} and heme is created, and in the fourth stage, heme reacts with globin which is made of four peptide chains giving a hemoglobin chain and then the four hemoglobin chains get combined to form hemoglobin.

The largest part of hemoglobin in an adult human (95-98%) is made of hemoglobin A or adult hemoglobin, while in a much smaller amount there is hemoglobin A_2 and fetal hemoglobin.

Each molecule of hemoglobin has four atoms of Fe, bound to it are four molecules (8 atoms) of oxygen. The main characteristic of hemoglobin is the ability of reversibility and weak connections with oxygen via coordinative liaisons, in oxyhemoglobin which releases oxygen in the tissues (13).

Iron metabolism regulation

- There is no physiological excretion of Fe from the organism.
- From the reason given above, absorption of Fe must be regulated to suit one's daily needs.
- In the case of Fe shortage, the entire amount of the absorbed Fe is given to the transferrin in circulation, and the stored Iron is being released from the storage.
- In the case of Fe surplus, the process is reversed. It is being stored intensively during the increased synthesis of ferritin, and small amounts are bound almost entirely for the saturated transferrin.
- Iron in the enterocytes bound for the ferritin disappears with its desquamation.
- These processes are regulated systematically and on a cell level (14, 15).

Pulsation increases as does volume and throbbing volume, renal angiotensin and aldosterone. The artery pressure drops, vascular resistance: systemic 21%, pulmonary 34%. A healthy person resorbs 5-10% of iron from food.

Food of animal origin has iron in heme form, and of herbal origin has non-heme form of iron.

The resorption of these two types of iron structures is different. It is being resorbed about 20-30% of heme iron and 2-5% of non-heme iron. With the consumption of vitamin C, the percentage of adopted non-heme iron is up to 50%.

The consumed Fe is resorbed in the organism in its bivalent form in the duodenum and in the upper jejunum with the help of apoferritin protein which in reverse binds itself to the Fe. As the amount of apoferritin is limited, so is the resorption of Fe – mucose blockage. The consumed trivalent form and the Fe component bound to heme in the form of Fe^{3+} must be reduced in its bivalent form with the help of vitamin C. Daily dose of about 1 mg of Fe is being resorbed which after entering the mucose cells as Fe^{2+} binds itself to the transportive substances (16).

Before entering the plasma, oxidation in Fe^{3+} occurs with the help of ceruloplasmin and it ties itself to the transferrin. The Fe serum is almost entirely bound to transferrin. Transferrin-siderophilin is a transporting protein of iron in the plasma and it is coded by the TF gene. It transports iron from the place of absorption to the place of resorption and storage (17).

Two original approaches to anemia diagnostics

Kinetic approach

This approach tends to reveal the mechanisms which lead to the anemia. Anemia can appear due to:

1. Reduced creation of Er

Reduced creation of Er occurs if the bone marrow does not create the necessary amount of Er which would make up for the disintegrated old cells – reduced effective erythropoiesis. It can occur due to: shortage of Fe, vitamin B12 and folic acid; primary disease of the bone marrow; low levels of trophic hormones which stimulate erythropoiesis, such as erythropoietin, the hormones of the thyroid gland and androgens.

2. Accelerated decomposition of Er

Accelerated decomposition of Er – the normal life span of Er is 120 days, so if it is under 100 days, we are talking about hemolytic anemia.

3. Loss of Er

Loss of Er – bleeding clinically clear due to trauma, melee, hematemesis, menometrorrhagia, ocular, iatrogenesis (18).

Morphological approach

The erythrocyte index is an important indicator in anemia.

Mean corpuscular volume (MCV) is a crucial factor which determines the morphological division of anemias.

The values of > 115 fL point to megaloblastic anemia determined by shortage of vitamin B12 or folic acid. MCV < 80 fL speaks of microcytic anemia which appears due to Fe loss most commonly.

The morphological approach divides anemias in regard to the size of mid volume erythrocyte MCV and the reticulocyte ratio (19).

Laboratory analysis

- Amount of iron bound to the transferrin.
- Amount of iron which can be bound to completely saturated transferrin - Total Iron Binding Capacity (TIBC) and total amount of Fe which can be bound to the apotransferrin is unsaturated or latent capacity of iron binding - unsaturated iron binding capacity (UIBC).

In healthy persons, the percentage of transferrin saturation with iron is 20-40%. A level of saturation of 16% leads to a drop of erythropoiesis due to reduced capacity of available Fe in the storage depoes, and a saturation under 10% points to iron deficit in the organism. In case of Fe loss in the storage depoes, its concentration in the plasma also drops while the TIBC value rises, due to the increased synthesis of transferrin.

This parameter is a better indicator of iron deficit than its low concentration.

The sum of serum iron and the UIBC represents the total capacity of iron binding; TIBC is the measure for maximum concentration of Iron which transferrin can bind.

Ferritin level – is a convenient parameter for iron reserves evaluation in the organism and a deficit can be detected in the early stage. Clinically, it is useful to know that the drop in the values under 20 ng/l points to prelatent deficiency of iron, and if the concentration of ferritin drops under 12 ng/l, this points to complete absence of Fe in the storage depoes, although the blood count can morphologically still be normal.

Erythrocytic components – are calculated from the Er count, concentration of Hgb and hematocrit and provide information on the quality of Er.

MCV – average volume of Er, 81-99 fl.

Mean corposcular hemoglobin (MCH) – average amount of Hb in the erithrocyte, 29-32.9 pg.

Mean corposcular hemoglobin concentration (MCHC) – concentration of Hb per liter Er 310-350 g/l.

Red cell distribution width (RDW) – measure variation in size of Er, 11.5-19.5% (20).

Table 1. Red Blood Cell Count (RBC) (whole blood)

Units	Nonpregnant Female	First Trimester	Second Trimester	Third Trimester
X 10^6/mm^3 X 10^6/µl	4 - 5.2	3.42 - 4.55	2.81 - 4.49	2.72 - 4.43
X 10^{12}/L	4 - 5.2	3.42 - 4.55	2.81 - 4.49	2.72 - 4.43

Table 2. Hemoglobin (Hgb) (whole blood)

Units	Nonpregnant Female	First Trimester	Second Trimester	Third Trimester
g/dL	12 -15.8	11.6 - 13.9	9.7 - 14.8	9.5 -15
g/L	120 -158	116 - 139	97 - 148	95 - 150

Table 3. Total iron-binding capacity (TIBC) (serum)

Units	Nonpregnant Adult	First Trimester	Second Trimester	Third Trimester
µg/dL	228 - 428	235 - 408	302 - 519	380 - 597
µmol/L	40.8 - 76.7	42 - 73	54 - 93	68 - 107

Table 4. Iron (Fe) (serum)

Units	Nonpregnant Adult	First Trimester	Second Trimester	Third Trimester
µg/dL	41 -141	72 - 143	44 - 178	30 - 193
µmol/L	7 - 25	13 - 26	8- 32	5 - 35

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Revijalni rad

UDC: 616.155.194:618.2
doi:10.5633/amm.2020.0311

ANEMIJE U GINEKOLOGIJI I PERINATOLOGIJI – NOVI STAVOVI

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Anemija predstavlja globalni zdravstveni problem. Među ženama zastupljena je kod 38% trudnica i 29% negravidnih žena. Najčešći oblik je sideropenijska anemija. Najčeći uzrok anemije u ginekologiji su abnormalna materična krvarenja, koja se javljaju kao akutno, hronično i intermitentno krvarenje. U perinatologiji postoje specifične promene na nivou kardiovaskularnog i hematopoetskog sistema trudnice, koje nameću različite kriterijume za dijagnozu anemije u odnosu na negravidno stanje. Osnovna promena je porast volumena krvi, koji raste više na račun plazme, spram volumena eritrocita. Volumen eritrocita raste za oko 33%, a takođe raste i broj retikulocita. Dva su osnovna pristupa dijagnostici anemije – kinetički pristup, koji nastoji da utvrdi mehanizam koji je do anemije doveo i morfološki pristup, koji deli anemije u odnosu na veličinu srednjeg volumena eritrocita i retikulocitnog odgovora. Terapija najčešće, sideropenijske, anemije vrši se preparatima gvožđa (hemsko, dvovalentno i trovalentno gvoždje). U trudnoći, ono je potrebno svim trudnicama, ali samo u umerenim dozama. Ukupno antenatalno povećanje treba da iznosi oko 1000 mg. Da bi se ove potrebe zadovoljile, u prvoj polovini trudnoće potrebno je 4 mg gvožđa dnevno, a u drugoj polovini od 6 mg do 7 mg. Zbog lošeg podnošenja gvožđa, komplijansa njegove primene procenjuje se na 50%.

Acta Medica Medianae 2020;59(3):84-89.

Ključne reči: *anemije, ginekologija, perinatologija*

Original article

UDC: 616.6-089.819.1-002:615.33. 015.8
doi:10.5633/amm.2020.0312

ANTIBIOTIC RESISTANCE THREATS IN PATIENTS WITH INDWELLING URINARY CATHETERS: BACTERIAL SPECTRUM, INFECTION RATES AND THE EMERGENCE OF MULTIDRUG RESISTANT AND EXTENSIVELY DRUG RESISTANT STRAINS

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The extent of antimicrobial resistance has become a global threat and according to the World Health Organization latest reports on this issue, 21st century could mark the end of the antibiotic era. Catheter-associated urinary tract infections are the leading cause of healthcare-associated bacteremia and a major source of resistant gram-negative organisms. This paper focuses on antibacterial resistance of bacterial species isolated from the urine samples of bacteriuric patients. In this study we examined urine cultures of patients with indwelling urethral catheters hospitalized for operative treatment who are at a higher risk for the emergency due to difficult to eradicate pathogens. We assessed underlying primary health conditions, comorbidities and infection risk factors in an attempt to relate them with rates of resistance. The results of susceptibility testing among positive urine isolates revealed high rates of resistance to β-lactamase inhibitors, third-generation cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole alongside with combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides. Multi-drug resistant strains were isolated regardless of clinically apparent symptoms and signs of the infection with catheterization duration being the dominant factor in comparison to the severity of primary disease and comorbidities. Administration of empirical therapy failed to address resistance patterns of detected pathogens. Catheterization due to strictly defined indications, reduction of catheter presence duration and choice of therapeutic agent in accordance with susceptibility testing are currently best available strategies both for prevention and therapy.

Acta Medica Medianae 2020;59(3):90-97.

Key words: *urinary tract infection, indwelling urethral catheter, health-care acquired infection, multi-drug resistance, extended drug resistance*

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Introduction

Hospital-acquired urinary tract infection is one of the most common healthcare-acquired infections and 70% to 80% of these infections are attributable to the use of an indwelling urinary catheter (1). Duration of catheterization is the most important determinant of bacteriuria. The term catheter-associated urinary tract infection (CA-UTI) refers to UTIs occurring in a person whose urinary tract is currently catheterized or has been catheterized within the past 48 hours. A single insertion of a catheter into the urinary bladder in ambulatory patients results in urinary infection in 1-2% of cases (2, 3).

Indwelling urinary catheters facilitate colonization with uropathogens by providing a surface for the attachment of host cell binding receptors recognized by bacterial adhesions (4). The most common infecting organism is *Escherichia coli, Enterococcus* spp., coagulase-negative *Staphylococcus*, *Pseudomonas aeruginosa* and other non-fermenters (5). Bacterial resistance has major implications for urological practice, particularly in relation to catheter-associated UTIs and infection complications following prostate biopsy or urological surgery (6). Catheter-associated urinary tract infection has been associated with increased morbidity, mortality, hospital costs and length of hospital stay (7).

Patients and methods

A prospective study was performed during a one-year period in the Urology Clinic, Clinical Center Niš. A total of 217 patients admitted for operative

treatment were studied. The inclusion criteria were restricted to patients with indwelling urinary catheter present for more than two days prior to admission. The patients undergoing catheterization for diagnostics or complicated urinary drainage procedures (nephrostomy, urinary stents) were excluded. Duration of catheter presence was considered to be short-term catheterization (STC) if they were in situ for up to 29 days or less and long-term catheterization (LTC) when they were in situ for 30 or more days.

The analysis was performed on the following collected data: urine samples taken on admission, on the 5th day of hospitalization, on the 5th post-operative day and after removal of the catheter. All urine samples were sent to microbiology laboratory and assessed for the presence of pathogens and their antimicrobial susceptibility in accordance to the European Committee on Antimicrobial Susceptibility Testing standards. All positive urine cultures were cross-linked with antibiotics from different classes to obtain a resistance percentage of isolated micro-organisms. Multidrug-resistant (MDR) strains were defined as non-susceptibility to at least one agent in three or more antimicrobial categories and extensively drug-resistant (XDR) as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories. In order to assess general resistance multiple antibiotic resistance (MAR), indexes were calculated by dividing the number of antibiotics isolate is resistant to with a number of antibiotics it is tested against. Empirical therapy was considered adequate if isolates were tested sensitive to administered antibiotics.

Infections were classified as present on admission (POA) and hospital-acquired urinary tract infection (HAUTI). Hospital-acquired urinary tract infection is defined as a microbiologically confirmed or symptomatic UTI with the date of onset. The day one of infection window period (IWP) was the day of admission to the urology clinic regardless of patient transfer from intensive care unit or another ward. Catheter-associated urinary tract infections were defined as positive urine culture with at least one bacterial species isolated at quantitative counts $\geq 10^5$ CFU/ml in patient with indwelling urinary catheter for >2 days prior to infection or symptoms and signs suggestive of UTI (fever > 38 ^{O}C, lower abdominal/flank pain or leukocytosis with no other recognized cause). Catheter-associated asymptomatic bacteriuria (CA-ABU) was defined as at least one bacterial species isolated from urine culture at quantitative counts $< 10^5$ CFU/ml in a patient with an indwelling urinary catheter for > 2 days without symptoms and signs of UTI.

Urinary tract infection risk factors considered were: an indwelling urinary catheter for > 2 days, extended duration of catheterization, previous history of UTI, urinary tract obstruction or reflux, urinary stones, antibiotics or corticosteroids treatment within previous three months and hospitalization within previous six months. These factors were not analyzed separately and were expressed as a total number of factors simultaneously present (minimum one; +1 for LTC). The underlying primary disease was classified with McCabe and Jackson score. Comorbidity severity level was assessed by Carlson Comorbidity Index (CCI). Additional data of interest collected were: baseline characteristics, duration of catheter presence prior to admission, indications for catheters usage, administration of antibiotics, duration of antibiotic therapy and the total length of hospital stay (LOS).

Statistics: Pearson correlation test, Kruskal-Wallis analysis of variance (ANOVA) followed by Man-Whitney U test have been used. SPSS 11 (Chicago, IL, USA). P-values < 0.05 were considered to be statistically significant.

Results

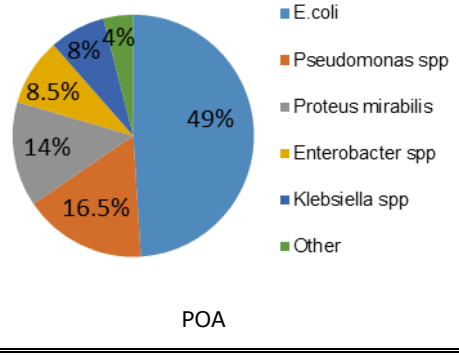
Out of 217 patients included in the study, there were 154 (71%) male patients and 63 (29%) female patients with a median age of 65 years (range 21-84). Long-term catheterization was present in 58 (27%) patients (median catheter time in situ 63.5 days, min 30, max 459) and short-term catheterization in 159 (median 6 days, min 2, max 29). In 126 patients (58%) indication for catheterization was surgery, acute obstruction in 80 (37%) and hematuria or trauma in remaining 11 (5%) patients. The open-drainage system was present in 36 (16%) patients. The most common type (94%) of urinary catheter used was Foley silicone-coated catheter.

Sixty-eight (31%) urine cultures taken on admission were positive for at least one bacterial species. Among these patients six had a fever on admission with accompanying symptoms and signs suggestive of UTI and they were identified as CA-UTI. Of all patients with POA infection, change of causative pathogen occurred in 19 (30%) cases and HAUTI was considered. Of 149 urine samples sterile on admission, 97 (65%) were positive for bacterial presence on the fifth hospital day and stratified as follows: 16 (16.6%) patients had symptoms and were classified as CA-UTI, 25 (25.7%) patients were asymptomatic with bacterial growth $< 10^5$ CFU/mL and were classified as CA-ABU and 56 (57.7%) patients were asymptomatic with bacterial growth > 10^5 CFU/mL and classified as HAUTI. In patients with HAUTI, change of pathogen occurred in 26 (39%) cases on the next sampling. In four of HAUTI patients, infection deteriorated to the level of sepsis. Ratios of organisms isolated from urine samples are presented in Figure 1. Most frequently isolated species present on admission were *E. Coli* (49%), *Pseudomonas* spp. (16.5%) and *Proteus mirabilis* (14%). Among hospital-acquired pathogens most common were *E. Coli* (40%), *E. Faecalis* (16%), *Pseudomonas* spp. and *Klebsiella* spp. (12.5% both). The most common changes of isolated bacteria were in a favor of *Klebsiella* ($p < 0.001$) with consequent reduction of *E. Coli* ($p < 0.05$). The spectrum of causative organisms was virtually identical in LTC and STC patients (higher but not significant presence of *P. Mirabilis* was found in LTC).

Cumulative percentages of resistance toward antibacterial category representatives and additional

data are summarized in Table 1. In 74% of cases antibacterial therapy was empirical with the adequacy of 19%. Twenty-six percent of patients were treated in accordance with antibiogram and in one case treatment was considered non-adequate. Mean duration of antibiotics treatment was 6.5 days (SD = 4.1, max 17). In a total of 351 susceptibility tests performed resistance rates from 68.7% to 100% were found to second-generation cephalosporins, fluoroquinolones and trimethoprim-sulfamethoxazole (TMP-SMX).

Urine culture results	**LTC n = 58**	**STC n = 159**
Pathogen POA, n = 68	33 (57%)	35 (22%)
Sterile on admission	25 (43%)	124 (78%)
Symptomatic on admission	0	6 (3.7%)
10^3-10^5 CFU/ml (POA)	15 (26%)	16 (10%)
≥ 10^5 CFU/ml, ABU (POA)	18 (31%)	19 (11.9%)
Sterile to positive on 2nd sampling	25 (100%)	72 (58%)
Symptomatic HAUTI	5 (20%)	19 (26%)



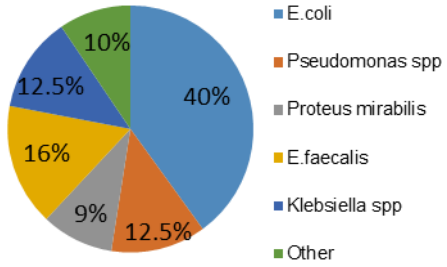
POA

Change of isolated strains:

POA --> 19 (30%)

HAUTI --> 26 (39%)

Change to *Klebsiella spp.* (p < 0.001)



HAUTI

Abbreviations: LTC- long-term catheterization; STC- short-term catheterization; POA- present on admission; ABU-asymptomatic bacteriuria; HAUTI- hospital-acquired urinary tract infection.

Figure 1. Urine culture results and bacterial spectrum of positive isolates

Table 1. Isolated pathogen resistance to commonly used antimicrobials

Antibacterial agent	**Antibiotic resistance percentages (%)**				
	E. Coli n = 152	*Pseudomonas* spp. n = 48	*P. Mirabilis* n = 37	*Klebsiella* spp. n = 42	*Enterobacter* spp. n = 22
Amoxicillin-clavulanate	38.6	73.2	35.7	70.8	84.1
Piperacillin-tazobactam	23.5	43.5	0.0	6.3	9.1
Cefuroxime	47.5	74.1	42.9	82.3	83.8
Ceftriaxone	36.7	65.3	51	59.5	50.9
Cefotaxime	35.3	51	26.7	55.2	56.7
Ceftazidime	33.4	39.8	49	57.8	66.7
Cefepime	14.3	24.5	0.0	32.2	33.6
Ciprofloxacin	68.7	95.7	72.5	88.9	82.4
Gentamicin	45.7	61.3	56.3	89.3	76.5
Amikacin	26.6	38.3	25	57.1	52.9
Trimethoprim/sulfamethoxazole	95.3	100	96.3	100	93.8
Type of resistance					
MDR	21 (13.8%)	9 (18.7%)	5 (13%)	11 (26%)	6 (27%)
XDR	7 (4%)	14 (29.1%)	no	7 (16%)	4 (18%)
POA/HAUTI ratio	0.8	0.91	0.98	0.4	3.0

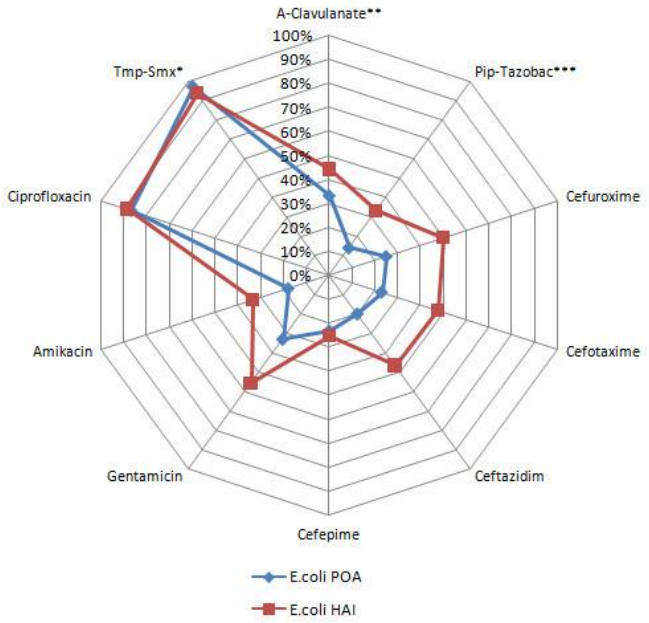
Abbreviations: MDR-Multidrug-resistant. XDR-Extensively drug-resistant. POA-Present on admission. HAUTI-Hospital-acquired urinary tract infection. MAR-Multiple Antibiotic Resistance index.

Sixty percent of POA pathogens and 87.2% of HAUTI were resistant to empirically administered fluoroquinolones, with a higher resistance rate of hospital strains ($p < 0.001$). Resistance rates from 33%-66.7% were recorded to cephalosporins of the third generation along with resistance to cefepime over 30% among *Klebsiella* species and *Enterobacter* species. In regard to cephalosporins, isolated causative pathogens shared similar rates of resistance to the particular antibacterial drug, from moderate 70.3% among *Pseudomonas* and *Proteus* to 97.6% for *E. Coli* and *Klebsiella* spp. None of the isolates were resistant to carbapenem group (imipenem, meropenem). Highest numbers of MDR strains were found among *Klebsiella* (26%) and *Enterobacter* (27%) with *Pseudomonas* spp. being the most prominent pool of XDR pathogens (29.1%). *E. Faecalis* resistance rates are not given in the Table 1 due to data consistency and are presented in the text. Isolated strains of *E. Faecalis* (n = 50) showed the highest resistance to TMP-SMX (98%), ciprofloxacin (90%) and doxycycline (68.4%) along with susceptibility to carbapenems (100%) and aminoglycosides (amikacin 100%, gentamicin 85%).

There was one single vancomycin-resistant isolate of *E. Faecalis*.

Mean MAR index of POA pathogens was 0.62 (SD = 0.25) and in HAUTI mean MAR index was 0.65 (SD = 0.18). In the cases of same species isolated on the next successive sampling, MAR index increased from average 0.42 to 0.57. With the occurrence of pathogen species exchange, mean MAR indexes changed from 0.52 to 0.61 respectively ($p < 0.05$). The most frequently isolated pathogen *E. Coli* change of resistance rates in accordance with the time of isolation is presented in Figure 2.

Based on the duration of catheterization, MAR values were as follows: in LTC mean MAR value was 0.63 (SD = 0.22) and in STC mean MAR value was 0.54 (SD = 0.26) with the signifficant difference between groups $p < 0.05$. In respect to primary disease evaluated by McCabe and Jackson score, patients with LTC had a nonfatal condition in 60% of cases, fatal within 5 years in 30% and life-threatening within 6 months in 10 % of cases. Patients with STC had a nonfatal condition in 52% of cases, fatal within 5 years in 41% and life-threatening within 6 months in 7 % of cases. Significant correlation between severity of primary disease and bacterial resistance was found ($p < 0.05$). Mean Carlson index in LTC was 3.43 (SD = 2.45) and in STC mean Carlson index was 3.48 (SD = 2.42). The relation of pathogen resistance and comorbidity was not significant. A weak positive correlation existed between a number of predisposing factors and occurrence of infection $p = 0.06$. Regarding length of hospital stay (LOS) in LTC patients, mean value was 26.8 days (SD = 20.5) and in STC was 18.9 days (SD = 9.4). The correlation between resistance rates and LOS in both groups of patients was not significant.



Abbreviations: Tmp-Smx*: Trimethoprim-sulfamethoxazole, A-clavulanate**: Amoxicilin-clavulanate, Pip-Tazobac***: Piperacillin-Tazobactam

Figure 2. Resistance pattern between present on admission and hospital-acquired strains of *E. Coli*

Discussion

The urine of patients with indwelling catheters is a source of extended-spectrum beta-lactamase (ESBL) and carbapenem-resistant (CRE) Enterobacteriaceae in both acute and long-term facilities (8, 9). The species isolated in our study demonstrated a high level of resistance to β-lactamase inhibitors, third-generation cephalosporins and combined resistance to cephalosporins, fluoroquinolones, and aminoglycosides. None of these showed resistance to the carbapenem group of antibiotics. European Centre for Disease Prevention and Control (ECDC) report on ABR in 30 countries of EU/EEA warned that the resistance to carbapenems significantly increased from a population-weighted of 6.2% in 2012 to 8.1% in 2015. Resistance to carbapenems was frequently reported in *K. Pneumoniae* invasive isolates from countries in Southern and South-Eastern Europe than other parts of Europe. The vast majority of the resistant isolates had additional resistance to fluoroquinolones, third-generation cephalosporins and aminoglycosides (10). The isolated pathogens we had are showing a similar pattern of combined resistance to that described in ECDC report, suggesting the possibility of ABR expansion to carbapenems. Moreover, observed high correlations of susceptibility/resistance rates among the isolates of different species to particular antibiotic raises the question of the possible cause of this finding.Indwelling catheters with open-drainage systems result in bacteriuria in almost 100% of cases within 3-4 days (3). More than a half of urine cultures of patients with LTC in our study were positive on admission and all of previously sterile become bacteriuric on the day 5 of hospital stay. A large percent of urine cultures obtained from STC patients (58%) became positive during hospitalization. In both groups, symptomatic cases appear with delayed response to therapy and serious complications including sepsis. Part of these findings could be attributed to the presence of open-drainage systems, underlying primary disease and comorbidities potential to alter host defense mechanisms. Another determinant in our case is the unavailability of catheters with additional protective features.

Most hospital-acquired UTIs are associated with catheterization and most occur in patients without signs or symptoms referable to the urinary tract (11, 12). Infections originate from one species of bacteria and as the duration of catheterization lengthens, more bacterial species are usually detected (these tend to be gram-negative bacteria such as *Proteus mirabilis* and *Pseudomonas aeruginosa*) (13).

The risk of infection reduced from 97% with an open drainage system to 8–15% when a sterile closed system was employed. The risk of using antibiotics as a form of prophylaxis is that it may lead to an increase in resistance which, in turn, may reduce the available treatments for patients with clinical infections in the future (14).

Among hospitalized patients with an indwelling catheter 60–80% receive antimicrobials usually for indications other than urinary tract infection (15).

This intense antimicrobial exposure promotes antimicrobial-resistence of pathogens frequently isolated from the urine of catheterized individuals. Bacteria colonizing the drainage bags of catheterized patients have been reported to be a source for outbreaks of resistant organisms in acute care facilities (15).

According to the ECDC reports on high ABR rates in South Eastern Europe, we examined the results of the past and recent studies in geographically close regions. A significant high combined resistance was seen in Bosnian surveillance study of urinary intrahospital infections (16). The setting of this study was confined to the Clinic of Obstetrics and Gynecology and differ both in the choice of the target population and some definitions of standards. Nevertheless, the spectrum of pathogens and high percentages of their resistance to multiple antimicrobial groups alongside with susceptibility to carbapenems and fourth-generation cephalosporines were similar to our findings. A large study of community-acquired UTIs in South Croatia back in 2003 concluded that Enterobacteriaceae had become less susceptible to commonly used antibiotics and that uropathogens were showing a slow but steady increase in resistance (17). The summary of latest reports by ECDC with Croatia as EU member included, confirmed the persistence of aforementioned trends (18).

In a more recent study at the University Clinical Center of the Republic Srpska, the ABR and MDR isolates obtained from different wards were compared. They found that highest percent of MDR isolates from urine samples were comprised of *Pseudomonas* spp. and *Acinetobacter* spp. The leading sources of infections were intensive care units and surgical wards (19). None of the *Acinetobacter* spp. was isolated from samples we obtained at urology ward only.

Microbiological laboratory analysis of the isolates we collected demonstrated highest ABR among hospital-acquired species with *Klebsiella* and *Proteus* being a leading source of MDR strains. Once again duration of catheterization proved to be a decisive factor in the appearance of infection and pathogens harder to eradicate in patients with otherwise similar underlying pathology, comorbidities and risk factors.

The resistance demonstrated to Ciprofloxacin and TMP/SMX resulted in near zero efficiency of these antibiotics among our patients. Furthermore, levels of resistance to amoxicillin-clavulanate, third-generation cephalosporins and gentamicin (ranges from 35% to even 84%) were high above proposed limits for therapeutic efficiency. Infectious Diseases Society of America Guidelines on the treatment of uncomplicated UTIs recommended that the resistance percentage of causative micro-organisms must be < 20% to consider an agent suitable for empirical treatment of a lower UTI and must be < 10% for the treatment of an upper UTI (3, 20). Considering the current resistance percentages of amoxicillin, amoxiclav and trimethoprim/sulfamethoxazole, it can be concluded that these agents are not suitable for the empirical treatment of pyelonephritis in a normal host and therefore also not for treatment of all complicated UTIs. The same applies to ciprofloxacin

and other fluoroquinolones in catheterized urological patients (21). Ciprofloxacin resistance in *E. Coli* isolates is increasing and the use of this antimicrobial agent as empirical therapy for UTI should be reconsidered (22).

In a broader sense, the emergence of ABR strains and their resistance rates are showing a tendency to rise and expand. This is mainly attributed to incorrectly prescribed therapeutics, subtherapeutic antibiotic concentrations and lack of new antibiotics (23). The over-application of antimicrobials usually occurs in extreme cases in hospital-based patients and is relatively controlled. The development and implementation of rapid and accurate diagnostics would alleviate this problem (24).

Conclusion

The non-standard procedure of consecutive urine analysis and susceptibility testing of bacteria found in all catheterized patients regardless of indicative symptomatology gave us the insight into antibacterial resistance profile of often omitted microbes. These bacterial strains demonstrated a high level of antibiotic resistance and combined resistance to third-generation cephalosporins, fluoroquinolones and aminoglycosides. In a management of UTI in catheterized patients, the choice of therapeutic agent should be tailored to the results of susceptibility testing of isolated pathogens since empirical therapies often fail to address the frequent presence of MDR and XDR strains. Asymptomatic bacteriuria should not be treated in catheterized patients. Duration of catheterization is the single independent factor influencing emergence of MDR and XDR strains.

Acknowledgments

This work has been supported by the Serbian Ministry of Education and Science, grant No. 175092 and grant No. III46013.

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Originalni rad **UDC: 616.6-089.819.1-002:615.33. 015.8**
doi:10.5633/amm.2020.0312

OPASNOSTI ANTIBIOTSKE REZISTENCIJE KOD BOLESNIKA SA STALNIM URINARNIM KATETEROM: BAKTERIJSKI SPEKTAR, UČESTALOST INFEKCIJA I POJAVA MULTIREZISTENTNIH I EKSTENZIVNO REZISTENTNIH SOJEVA

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Obimnost antibiotske rezistencije predstavlja globalnu opasnost i prema najnovijim izveštajima Svetske zdravstvene organizacije, 21. vek može da označi i kraj antibiotske ere. Urinarne infekcije povezane sa kateterizacijom vodeći su uzrok nozokomijalnih bakterijemija i glavni su izvor rezistentnih Gram-negativnih organizama. Ovaj rad se fokusira na antibiotsku rezistenciju bakterija izolovanih iz urina bakteriuričnih bolesnika. U ovoj studiji ispitivane su urinokulture bolesnika sa permanentnim urinarnim kateterom, hospitalizovanih zbog operativnog lečenja, kod kojih postoji povećani rizik od pojave rezistentnih patogena. Analizirani su i primarna oboljenja, komorbiditeti i faktori rizika za nastanak infekcija, u nastojanju da se dovedu u vezu sa stopama rezistencije. Testovi osetljivosti izvedeni na uzorcima pozitivnih urinokultura otkrili su visoke stope rezistencije na inhibitore beta-laktamaze, cefalosporine treće generacije, fluorohinolone i trimetoprim-sulfametoksazol, kao i kombinovanu rezistenciju na cefalosporine treće generacije, fluorohinolone i aminoglikozide. Multirezistentni sojevi izolovani su nezavisno od prisustva simptoma i znakova infekcije, pri čemu je trajanje kateterizacije dominantni faktor rizika, u poređenju sa težinom osnovne bolesti i komorbiditetima. Primenjena empirijska terapija pokazala se neadekvatnom u lečenju izolovanih patogena. Kriza antibiotske rezistencije je na vrhuncu i zahteva brz i odlučan odgovor. Kateterizacija prema strogo određenim indikacijama, smanjenje vremena trajanja kateterizacije i izbor antibiotika po antibiogramu, trenutno su najbolje strategije, kako u prevenciji tako i u terapiji.

Acta Medica Medianae 2020;59(3):90-97.

Ključne reči: *urinarne infekcije, uretralni kateter, nozokomijalne infekcije, multirezistentnost, proširena rezistencija*

Review article

UDC: 615.89(497.11)
doi:10.5633/amm.2020.0313

COMPLEMENTARY AND ALTERNATIVE MEDICINE IN SERBIA: A LITERATURE REVIEW

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The aim of this review paper is to present the state of complementary and alternative medicine in the Republic of Serbia and compare it with other developing and developed countries around the world.

In most countries of the world, the legalization and integration of the Complementary and Alternative Medicine (CAM) into the health system went very slowly until the 1970s, when there was an important global change in socioeconomic conditions. WHO estimates that $ 83 billion was spent on traditional medicine in the world market in 2008. Significant variations in financial allocations to CAM across the globe have been observed, however, their direct comparison has been hampered by differences in the definitions and categorization of CAM used, as well as by the use of different currencies in different time periods. The development of CAM in the Balkans, during the 1990s, was hampered by war and transition, and the resolution of CAM was delayed. For the first time, the law regulates the implementation of the CAM in Serbia in 2005 by Article 235 of the Health Care Act.

In the Republic of Serbia, evidence of the extent of use of CAM methods is very modest, although worldwide research shows an accelerated upward trend in the use of CAM. This paper is our contribution to the further development and better recognition of CAM methods by both the Ministry of Health of the Republic of Serbia and the professional public.

Acta Medica Medianae 2020;59(3):98-104.

Key words: *complementary and alternative medicine, CAM, treatment methods, rehabilitation methods*

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Introduction

Back in 1978, the World Health Organization (WHO) at a meeting in Alma Ata, issued a "Health for All" Declaration, recommending that Member States include traditional therapists, i.e. practitioners of the methods of complementary and alternative medicine (CAM) into their health systems (1). The legalization and integration of CAM into health systems in most countries of the world went very slowly until the 1970s, when there was an important global change in socio-economic conditions (1).

WHO estimates that $ 83 billion was spent on traditional medicine in the world market in 2008. Thereafter, "in May 2009, the WHO Assembly adopted resolution 62.13, inviting all Member States and national governments to cooperate and share knowledge while working to strengthen the link between conventional and traditional practitioners" (2).

The aim

The aim of this revision paper is to present the state of CAM in Serbia and compare it with other developing and developed countries around the world.

CAM in Serbia: Legislation and Division of CAM Methods

The development of CAM in the Balkans, during the 1990s, was hampered by war and transition, and the resolution of CAM was delayed (3). At the beginning of the transition in 1989, in our country, all the weaknesses of the current health system

(lack of money, unfavorable contracts between the Health Insurance Fund and health institutions and others) emerged, which made it necessary to move towards changes in the health policy and the health system (4).

For the first time, the law regulates the implementation of the CAM in Serbia in 2005 by Article 235 of the Health Care Act (5). In the following way: "Traditional medicine, within the meaning of this Law, includes those proven professionally unchallenged traditional, complementary and alternative methods and procedures of diagnosis, treatment and rehabilitation that have a beneficial effect or which may have a beneficial effect on a men health or his health status and which are not covered by health services in accordance with applicable medical doctrine" (5). Later on, the methods of diagnosis, treatment and rehabilitation of CAM were defined by the Ordinance on the closer conditions, manner and procedure of performing the methods and procedures of traditional medicine from 2007 (6).

The methods of diagnosis and treatment of traditional medicine were: Ayurveda; acupuncture and related techniques; traditional Chinese medicine; homeopathy; phytotherapy; quantum medicine and related techniques; chiropractic and applied kinesiology; macrobiotics and traditional home medicine (6).The methods of rehabilitation of traditional medicine were classified as: apitherapy; aroma therapy; Chi Gong exercises; spiritual energy medicine; energy therapy, Reiki; detection of harmful radiation; yoga exercises; family schedule and Tai Chi Chi exercise (6). In 2018, a new Ordinance was adopted on the closer conditions, manner and procedure for performing the methods and procedures of traditional medicine (7).

In 2019, the Law on Health Care was amended so that the term traditional medicine was replaced by the term complementary medicine, which within the meaning of this law includes "those traditional and complementary methods and procedures of prevention, diagnostic evaluation, treatment, health care and rehabilitation that have a beneficial effect on human health or medical condition and which, in accordance with applicable medical doctrine, are not covered by conventional medicine methods and procedures" (8).

In January 2020, in accordance with the amendments to the Law on Health Care, the Rulebook on Closer Conditions and Methods of Performing Complementary Medicine Methods and Procedures entered into force, authorizing a total of 12 complementary medicine methods, which are shown in Table 1 (9).

Table 1. Complementary medicine methods approved in Serbia

I. Methods of prevention, diagnostic evaluation, treatment and rehabilitation:
1. Acupuncture
2. Acupuncture microsystem stimulation methods
3. Quantum Medicine
4. Homeopathy
5. Traditional Chinese Medicine
6. Ayurveda - Traditional Indian Medicine
7. Chiropractic
8. Osteopathy
II. Methods of preserving and improving health:
9. Aromatherapy
10. Reiki
11. Anthroposophy medicine
12. Qi gong (dao yin), yoga, that qi chu practice for medical purposes

This Rulebook defines that a healthcare professional may perform complementary medicine methods if he or she has completed the relevant integrated academic studies of the health profession, that is, an appropriate high or high school of health profession, has the approval of the competent chamber of health workers for independent work (license) and has the decision of the minister responsible for health for performing a specific complementary medicine method (license) (9).The permit is issued to a healthcare professional who, in addition to the statutory requirements, must have a certificate of completion of continuing medical education for a specific area of complementary medicine, i.e. adequate higher education for the method of traditional Chinese medicine, Ayurveda-traditional Indian medicine, homeopathy and chiropractic (9). So far, traditional methods and procedures in Serbia

are not funded by the State Health Insurance Fund (1).

Research in the field of CAM in the Republic of Serbia

A number of CAM studies have been conducted in Serbia, and the most important will be presented in this paper. In Vojvodina, being Serbian province, a study was conducted on the population of patients using pharmacy services in this province. The study found that 10.4% of respondents (out of 1,137) used some herbal preparations for the prevention and/or therapy of gastrointestinal and liver disorders, most commonly for constipation (44%) and dyspepsia (23%) (10). Buckthorns-based preparations (16.1%), including Alder buckthorn (8.5%) and dietary fiber preparations (6%) were used by subjects for constipation (10), while preparations with artichoke (11%) and silymarin (9.3%) were most commonly used for liver disorders (10). The decision to choose an herbal preparation was made on the recommendation of a pharmacist (35.6%) or on the patient's own initiative (32.2%), and less frequently on the advice of a physician or other person (10).

A multicenter study conducted as a cross-sectional study of patients in general practitioners' clinics in five Health Centers in Serbia (in Zrenjanin, Pančevo, Zaječar, Zemun and the outpatient department of the Institute for Public Health of the employees of the Ministry of Internal Affairs of Serbia in Belgrade) showed that out of 1,157 respondents, 83.66% of those used traditional medicine methods (11). Phytotherapy (48.8%) and traditional folk medicine (34.7%) were the most commonly used methods of traditional medicine for diagnostics and treatment, and apitherapy (34.7%) were the most used methods (11). Respondents under 65 years of age used acupuncture, Chinese traditional medicine, homeopathy, chiropractic and macrobiotics more often, while respondents over 65 used traditional folk medicine more often (11). Information on traditional medicine methods was most commonly obtained from acquaintances and friends (54.9%) and through the media (39.3%) (11). There was no significant difference in the way information was obtained in relation to gender, and statistically significantly more frequent information via the Internet was obtained by persons younger than 65 years of age (11). Respondents in the city are more likely to receive information about traditional medicine methods online from doctors and pharmacists, and in the countryside from acquaintances and friends (11). The availability of traditional medicine to the urban population was more important, while the price to the rural population was the most important (11).

At the Institute of Oncology of Vojvodina, in the population of patients diagnosed with gastroenterological malignancy, a study was conducted, which showed that 48 (24.9%) patients did not use any of the alternative medicine methods, while at least one alternative therapy used 145 (75.1%) patients (12). About 64% used herbal preparations, most commonly beet juice (about 57%) (12). Mind and body medicine based therapies were used by 16.6% of patients, while spiritual therapy was used by 18.1% of patients and special diets were used by 19.2% of patients (12). Patients were most often informed of alternative therapy by other patients, relatives and neighbors (70.5% of patients) (12).

A study was conducted over total of 300 subjects who underwent chemotherapy at the Clinic for Medical Oncology at the Institute of Oncology and Radiology of Serbia in Belgrade in three time periods: in 1993, 2000 and 2008 (13). The percentage of patients using any of the CAM methods was over 50% in all three observed time periods (13). In 1993 and 2000, about 10% of patients reported that their physicians suggested the use of CAM, while in 2008 this percentage increased to 30% (13).

More educated patients used CAM more often compared to patients who had only primary education (13). The percentage of CAM users among college-educated patients increased from 20% in 1993 to 33% in 2008 (13).

Patients believed that CAM would enhance their immunity (this result was almost identical in all three observed time periods, i.e. approximately 65% of patients) (13). A third of CAM users believed that CAM would cure malignant disease, while most patients expected better effects of standard treatment with CAM (13).

A study conducted in eight Serbian cities among physicians, dentists and pharmacists employed by public and private healthcare institutions, as well as medical, dental and pharmacy students from two state universities found that dental students were better informed about CAM than medical students, pharmacists better than university students professors, while primary care healthcare professionals were more familiar with CAM than pharmacists in public pharmacies (14). Among the students and among employed health professionals, the most commonly used types of CAM were vitamins (71.01% vs. 54.48%) (14).

In a 2019 cross-sectional study conducted in Serbia among consumers of CAM, two-thirds (65.3%) of users take OTC preparations on their own initiative, without a prescription and without a doctor's recommendation (15). It was noted in a study that users of CAM services and consumers of OTC preparations were less frequently hospitalized without the use of sick leave, ambulance, or home treatment in the previous 12 months (15).

Research in the field of CAM in other countries

In the Republic of Serbia, evidence on the extent of the use of CAM methods is very modest, although worldwide research shows an accelerated upward trend in the use of CAM (15). Higher education and high incomes have been significant predictors in most of the studies conducted so far in the world, probably because in most countries CAM is paid out of pocket, so patients with high incomes and usually from professions requiring higher education are able to self-finance the use of CAM (16-19). However, some studies have shown a significant impact of lower education levels (20, 21).

The situation is similar in most countries in the region. Recently, the methods of CAM have been

developing and becoming more popular in Croatia (22, 23). According to the results of a study on the use of CAM in a sample of 228 respondents at the Health Center in Čakovec, 82% of respondents used at least some form of CAM (22). CAM was more commonly used by women, as well as high school graduates, employees and retirees (24). The most commonly used medicinal herbs (87%), bioenergy (29%) and diet therapy (28%) (24). Vitamin and mineral supplements were used by 77% of the respondents (24). CAM was most commonly used to treat diseases of the respiratory tract, urinary tract, musculoskeletal system, as well as to improve the general condition (24). Of the respondents who used CAM, 55% believed it would help (24). Another study conducted in Croatia found that 46% of respondents had used CAM at least once in their lifetime, that the most commonly used methods were herbalism (38%), homeopathy (15.6%) and acupuncture (13.1%), and that the most common beneficiaries were persons between 46 and 55 years of age and of higher education (25). A study conducted on 267 patients with malignancies also showed a high prevalence-60.3%, with naturopathy/folk medicine being the most prevalent, with independent predictors of CAM use being high incomes, divorce, women, and younger life expectancy (26). The use of herbal medicine was specially observed in all parts of Croatia, and traditionally, a large number of herbs were used in the form of teas, tinctures, hydrolysates, fats and oil extracts (27, 28).

A study conducted in Hungary found that 63.9% of surgical patients were interested in using CAM, and 26.8% were using naturopathy (29). According to this study, CAM was more used by women, patients with university degrees, and patients with endocrine diseases (29). A study conducted among anesthetists and surgeons in this country found that they also frequently used CAM methods in their clinical practice such as reflexology, traditional Chinese medicine, herbal medicine and manual therapy (30). A study conducted among breast cancer patients in Hungary found that 52.6% of them used CAM before diagnosis, and 84.4% during treatment, with CAM being more commonly used by more educated women and those living in cities, while during treatment, use was more common in higher-income patients (31).

A comparative study conducted among doctors in Romania and Hungary found that significantly more Hungarian doctors (33.6%) would be more likely to refer patients to CAM practitioners compared to Romanian doctors (12.8%), while the percentage of physicians who once referred a patient to CAM practitioners were approximately similar (57.9% vs. 54.7%) (32). Compared to the countries mentioned above, the situation in Slovenia and Bulgaria is somewhat different. In these countries it has been shown that the prevalence of CAM use in the last 12 months is much lower and is below 10% (33). On the other hand, a survey in the Czech Republic showed that about 76% of the general population had used one or more CAM methods in the previous 30 days in 2011, while in 2014 this percentage was significantly higher and amounted to 87% (34, 35).

In both years, vitamins and minerals, herbal remedies, massage and relaxation techniques were most commonly used (33, 34). A study in Poland showed that the prevalence of using CAM methods in epilepsy patients was 26.8%, with the most commonly used CAM methods being herbal and dietary supplements (32.3%) and energy treatment (31.5%) (36).The use of CAM was more common in younger patients with longer duration of epilepsy who did not experience remission and who had lower levels of education (35).

Significant variations in financial allocations to CAM across the globe have been observed, but their direct comparison is hampered by differences in the definitions and categorization of CAM used, as well as by the use of different currencies in different time periods (16). In the Republic of Serbia a total of RSD329,966,634.66 was spent on herbal and traditional medicines in 2010, i.e. EUR3,104,233.14 (0.43% of total drug turnover) (36), and in 2017 RSD1,012,173,801.44, i.e. EUR8,543,714.03 (0.87% of total drug turnover) (37), while in 2010, a total of RSD30,541,699.02 was spent on homeopathic medicines. EUR287,073.69 (0.04% of total drug turnover) (36), and in 2017, RDS95,585,188.77, i.e. EUR806,830.33 (0.08% of total drug turnover) (37).

A systematic review of studies addressing the prevalence of CAM use in the UK showed that the average cost of using CAM per patient per month was GBP15.99 (range 8.80-28) (38). The estimated total financial allocation in the United Kingdom on an annual basis was GBP1.6 billion in 1999 (39).

In Australia, the total estimated amount spent on CAM products paid out of pocket was AUD621 million in 1993 (40), AUD1.671 million in 2000 (41), AUD1.308 million in 2004 (42) and AUD1.860 million in 2005 (44), with CAM practitioners estimated to have earmarked AUD309 million from citizens' pocket in 1993 (40), AUD616 million in 2000 (41), AUD494 million in 2004 (42) and 1.730 million of Australian dollars in 2005 (43). In Canada, a total of CAD3.8 billion was estimated to have been spent on CAM in 1997 (44) and CAD7.8 billion in CAM (45). In the US, citizens estimated that a total of USD10.3 billion was earmarked in their pocket in 1990 (46), USD34.4 billion in 1997 (47), USD33.9 billion in 2007 (48) and USD30.2 billion in 2012 (49).

Conclusion

This paper is our contribution to the further development and better recognition of CAM methods by both the Ministry of Health of the Republic of Serbia and the professional public. There is a need to conduct research that will allow us to understand both the scope of use of alternative treatment methods and the main characteristics of users in terms of their demographic, socio-economic characteristics and health status, as well as a comparative analysis of the use of health care services and alternative medicine methods. Therefore, there is a need to work together with the Ministry of Health and the

Ministry of Education to form academic studies within the Faculty of Medical Sciences in the form of specialist studies in the field of CAM, in order to provide a high level of training and liaise with universities in the world where this has already been achieved, in order to network and share knowledge. It would also be very good to introduce into the existing practice the methods already approved by the Ministry of Health in the regular practice.

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Revijalni rad **UDC: 615.89(497.11)**
doi:10.5633/amm.2020.0313

KOMPLEMENTARNA I ALTERNATIVNA MEDICINA U SRBIJI: PREGLED LITERATURE

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Cilj ovog revijskog rada je analiza stanja komplementarne i alternativne medicine u Srbiji i poređenje sa stanjima u drugim razvijenim zemljama i zemljama u razvoju širom sveta.

U većini zemalja sveta, legalizacija i integracija komplementarne i alternativne medicine (KAM) u zdravstveni sistem išla je veoma sporo sve do 1970-ih godina, kada je došlo do značajne globalne promene društveno-ekonomskih uslova. Svetska zdravstvena organizacija procenjuje da je na tradicionalnu medicinu, na svetskom nivou, potrošeno 83 milijarde dolara tokom 2008. godine. Primećene su značajne razlike u finansijskim izdvajanjima za KAM širom sveta, međutim njihovo direktno upoređivanje je otežano usled razlika u definicijama i kategorizaciji korištenih KAM, kao i upotrebom različitih valuta u različitim vremenskim periodima. Razvoj KAM na Balkanu, tokom devedesetih godina dvadesetog veka, ometali su rat i proces tranzicije, a donošenje odluka u vezi sa KAM je odloženo. Zakonom je, po prvi put, regulisana primena KAM u Srbiji 2005. godine članom 235. Zakona o zdravstvenoj zaštiti.

U Srbiji su dokazi o obimu primene metoda KAM vrlo skromni, mada svetska istraživanja pokazuju ubrzani trend porasta primene KAM. Ovaj rad je naš doprinos daljem razvoju i boljem prepoznavanju metoda KAM, kako od strane Ministarstva zdravlja, tako i od strane stručne javnosti.

Acta Medica Medianae 2020;59(3):98-104.

Ključne reči: *komplementarna i alternativna medicina, KAM, metode lečenja, metode rehabilitacije*

Case report **UDC: 616.61-089.843:618.19-089.843/.844**
doi:10.5633/amm.2020.0314

KIDNEY GRAFT REJECTION AFTER SILICONE BREAST IMPLANT SURGERY

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In the patient presented in this study, bilateral breast implant surgery was performed by a submuscularly placed silicone gel implant. Unfortunately, a surgical team from another healthcare facility did not consult a nephrologist before the surgery, which was a necessary step in order to adjust the dosage of immunosuppressive therapy. During the early postoperative period, the patient developed febrility and acute mastitis, as well as acute renal transplant rejection. The patient was hospitalized at the Clinic of Nephrology, Clinical Center of Montenegro. After two weeks of treatment and care, her kidney transplant function recovered.
Acta Medica Medianae 2020;59(3):105-107.

Key words: *transplantation, acute renal rejection, silicone implant*

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Introduction

Renal transplantation is a method of choice for treatment of end-stage renal disease. It requires permanent use of immunosuppressive drugs for prevention of acute rejection. Although antigen-dependent immune responses have traditionally been considered critical for induction of both acute and chronic allograft rejection, there is accumulating evidence supporting the observation that antigen-independent injury and subsequent inflammation may trigger allograft rejection. There is no data on the use of silicone breast implants in renal transplant recipients (1, 2).

Discussion

A 49-year-old woman with end-stage renal disease was treated with hemodialysis for three months, after which a cadaveric kidney transplantation was performed. Her posttransplant immunosuppressive protocol included cyclosporin, mycophenolate mofetil and prednisolone. Three years later, she was diagnosed with right-sided breast cancer, treated with right mastectomy and radiotherapy. Throughout this period, the kidney graft function remained satisfactory. Eighteen months after the right mastectomy, the patient was diagnosed with cancer in her left breast, treated with left mastectomy, after which she requested bilateral breast reconstruction for aesthetic reasons. A bilateral breast implant surgery was performed with the submuscularly placed silicone gel implant. Unfortunately, the surgical team that performed the breast implant surgery did not consult a nephrologist prior to the operation, which was a necessary step in order to adjust the dosage of immunosuppressant drugs. During the early postoperative period, she developed fever and acute mastitis (C-reactive protein level of 168 mg/L with pain and serohemorrhagic discharge from breasts), as well as acute kidney graft rejection (creatinine level of 178 μmol/L, blood urea nitrogen level of 7.3, creatinine clearance of 0.36 ml/s and proteinuria of 2.98 g/24h with a diuresis of 2000 ml, and echosonographic signs of acute graft rejection). She exhibited generalized edema with a body mass of 78 kg. Also, drug monitoring showed a cyclosporine level of 71.8 ng/mL. A sudden failure of graft function required pulse corticosteroid therapy with the adjustment of the immunosuppressive regimen, as well as a broad-spectrum antibiotic therapy. After two weeks of treatment, the graft function recovered. On discharge, the creatinine clearance was 0.57 ml/s. Also, there was a marked decrease in proteinuria (0.08 g/24h with a diuresis of 2600 ml), and

her body mass was reduced to 72 kg. Cyclosporine level was 112.1 ng/mL. The inflammation of the breasts also subsided, and her clinical course is uneventful to this day, with a good renal function. Immunosuppressed kidney recipients have a higher incidence of malignant tumors in comparison with the general population, with overall risks ranging from 3.3 to 3.6. (3, 4). However, the relative risk for breast cancer is 0.7, meaning that immunosuppression may not increase the incidence of breast malignancy (3, 5). There are hypotheses considering the role of oncogenic viral infections flaring up during immuno-suppression, as well as the depressed immune reaction to tumor antigens (3). Of 2.139 kidney recipients described in a study by Kwak et al., 11 patients suffered from breast cancer, with a similar prognosis to the general breast cancer population. The authors emphasize the importance of immunosuppressant adjustment and hemodialysis access in posttransplant cancer treatment (4). On the other hand, exposure to silicone potentially results in autoimmune phenomena, due to the fact that silicone is not immunologically inert (6, 7).

Conclusion

In this case, it is plausible that silicone triggered an immune reaction to the kidney graft – a complication of breast augmentation that has not been described before. This report serves to illustrate the need for a nephrologist consultation and the adjustment of immunosuppressants in breast implantation surgery of kidney recipients.

Conflict of interest

The authors declare no conflict of interest.

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Prikaz bolesnika **UDC: 616.61-089.843:618.19-089.843/.844**
doi:10.5633/amm.2020.0314

ODBACIVANJE BUBREŽNOG GRAFTA NAKON UGRADNJE SILIKONSKIH IMPLANTATA U DOJKE

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Kod bolesnice, koju smo prikazali u ovom radu, izvedena je bilateralna operacija implantata dojki submuskularnom primenom implantata silikonskog gela. Nažalost, hirurški tim iz druge zdravstvene ustanove pre operacije nije konsultovao nefrologa, što je bilo neophodno za prilagođavanje doziranja imunosupresivne terapije. Tokom ranog postoperativnog perioda, kod bolesnice se razvila febrilnost, razvio se akutni mastitis i došlo je do akutnog odbacivanja grafta. Bolesnica je hospitalizovana na Klinici za nefrologiju Kliničkog centara Crne Gore. Nakon dve sedmice lečenja i nege, funkcija njenog transplantovanog bubrega se oporavila.

Acta Medica Medianae 2020;59(3):105-107.

Ključne reči: *transplantacija, akutno odbacivanje bubrega, silikonski implantant*

Case report

UDC: 616.26-007.43-053
doi:10.5633/amm.2020.0315

MORGAGNI HERNIA IN THE ADULT PATIENT: A CASE REPORT

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Congenital diaphragmatic hernias (CDHs) occur from a disruption in the muscular formation of the diaphragm, resulting in herniation of abdominal contents into the thoracic cavity (12). First described by Giovanni Batista Morgagni, the anteromedial sternocostal location of diaphragmatic hernia through the defect located between the muscle fibres of the xiphisternum and the costal margin is a rare type of CDH and accounts for only 2% to 3% of cases of all CDHs.

In the neonatal patients, the most common symptoms are pulmonary hypertension and respiratory distress, and in adult patients, these are dyspnea, cough, chest pain and obstruction symptoms.

In this case report, the patient (male, 66 years) reported one month lasting tachycardia, upper abdominal pain and discomfort, claiming certain alleviation of the symptoms in upright position. He had medical history of cardiac disease. The diagnosis was presumed on plain radiogram of the thorax and it was confirmed with CT scan of thorax and abdomen. The patient was treated surgically with primary closure of the diaphragmatic defect.

Acta Medica Medianae 2020;59(3):108-111.

Key words: *Morgagni hernia, surgical treatment, diaphragmatic hernia*

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Introduction

Morgagni hernia consists of a defect in the anterior diaphragm, being more common on the right and allowing herniation of abdominal contents to the thorax (1, 2). Morgagni hernia is the rarest form of congenital hernia which presents in 2 to 5% of all cases (2).

Case report

We report a case of unilateral, right sided Morgagni hernia diagnosed after a previously established suspicion based on a chest radiograph. The patient was immediately diagnosed and successfully rescued by surgical hernia repair.

A 66-year-old male patient presented with one month history of tachycardia, upper abdominal pain, right subcostal discomfort, fatigue, claiming certain alleviation of the symptoms in upright position. He had a medical history of cardiac disease, however, investigations discarded the cardiac origin of the symptoms. The patient reported no previous trauma. Abdominal palpation revealed soft abdominal wall, with no signs of peritonitis, with mild soreness in the epigastrium. The diagnosis was presumed on plain radiogram of the thorax and it was confirmed with CT scan of the thorax and abdomen.

Treatment

Elective surgical treatment with laparotomy approach (upper midline laparotomy) was performed. We revealed 4 cm right diaphragmatic hernia containing almost the entire stomach. Reposition of the stomach into the abdominal cavity and hernia sac excision were undertaken. The diaphragmatic defect was closed using 1/0 Prolene suture. The patient made an uneventful recovery, being discharged on the 3th postoperative day. The patient was scheduled for the first chest radiograph control at the end of the current month.

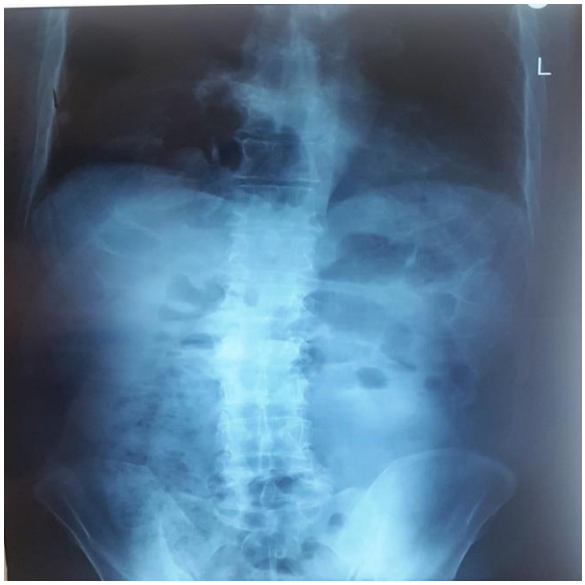

Figure 1. Chest X-Ray in anterior view, right paracardiac opacity (inconclusive, did not exclude diaphragmal hernia, not tumorous formation)

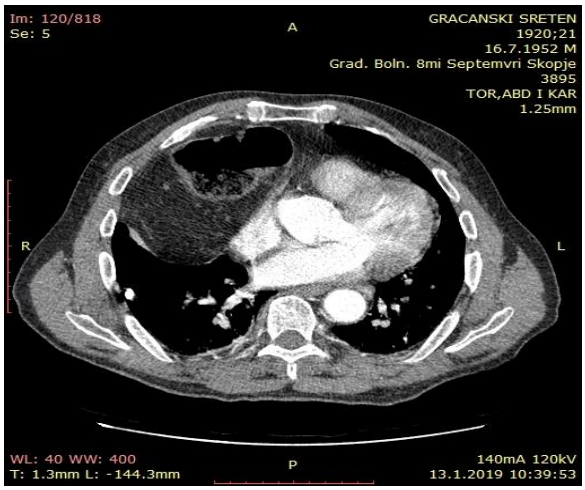

Figure 2. CT scan showing anterior right diaphragmatic Morgagni hernia with stomach content in the right hemithorax

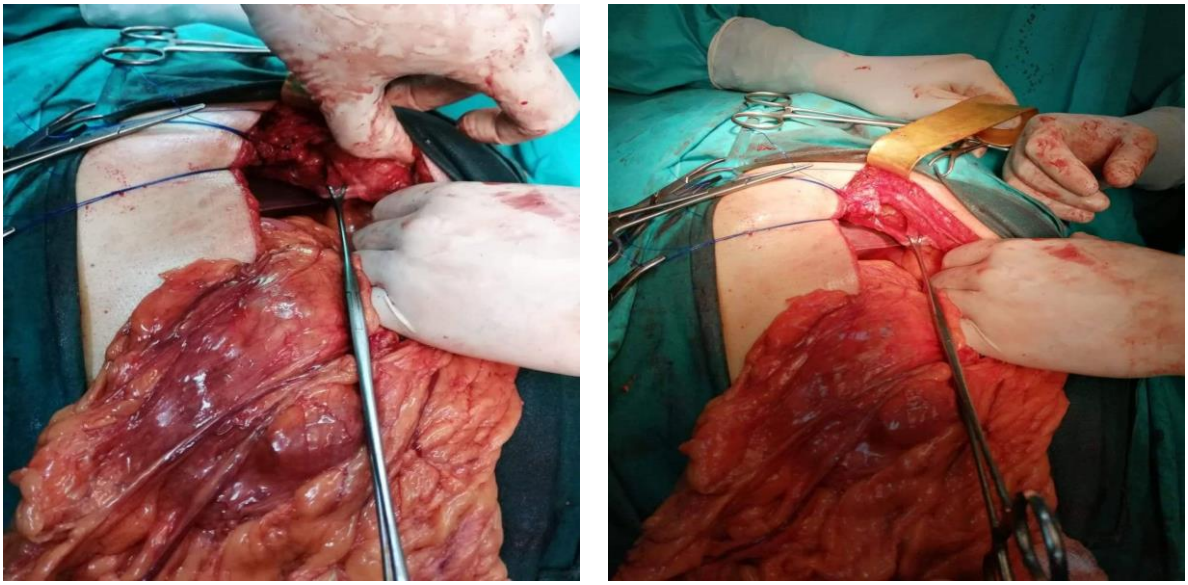

Figure 3. Operative material after reposition of stomach

Discussion

Morgagni hernias develop due to the lack of fusion of the sternal and crural portions of the diaphragm (3). Most of the cases are diagnosed in newborns or in the early childhood. The presence in adulthood is very rare, most remain asymptomatic. Majority of cases are discovered incidentally (chest X-Ray). When symptomatic, it is usually associated with chronic respiratory symptoms or gastrointestinal involvement with occlusive symptoms (4).

Symptomatic adult cases may present with life-threatening complications such as acute obstruction, volvulus, or strangulation due to a delay in diagnosis. CT is considered to be an accurate and non invasive method of diagnosis (5). Surgical repair of the diaphragmatic hernia is recommended in all cases to prevent the emergence of complications. Morgagni hernia can be repaired by a variety of surgical approaches including laparotomy, thoracotomy, laparoscopy, and thoracoscopy (6, 7, 8, 9). The results of surgical repair of foramen of MH are excellent. Operative mortality and morbidity are low,

especially for elective repairs (10, 11, 12). This is the second case of Morgagni hernia in the last ten years in digestive surgery in Skopje.

Conclusion

The unusual presentation highlights the difficulties in diagnosis. A high index of suspicion is required in each symptomatic patient, due to the possibility of life-threatening complications. Early diagnosis and surgery are lifesaving.

Acknowledgement

We are thankful to the patient for allowing us to use his information as well as to the whole team for their assistance during the operation.

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Prikaz bolesnika **UDC: 616.26-007.43-053**
doi:10.5633/amm.2020.0315

MORGAGNI HERNIJA KOD ODRASLOG BOLESNIKA: PRIKAZ SLUČAJA

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Kongenitalne diafragmalne hernije (CDHs) dešavaju se zbog prekida mišićne formacije dijafragme, što dovodi do hernijacije, odnosno prelaska abdominalnog sadržaja u torakalnu šupljinu. Prvi opis dat od strane Giovanija Batista Morgagnija glasi da anteromedijalne sternokostale lokalizacije dijafragmalne hernije, kroz defekt lokalizovan između misićnih vlakana pored sternuma i rebarnih ivica, predstavljaju retki tip CDH i odnose se na samo 2% do 3% svih slucajeva CDH.

Kod neonatalnih bolesnika, najčešći simptomi su plućna hipertenzija i respiratorni distres sindrom, a kod odraslih bolesnika to su dispneja, kašalj, bol u grudima i opstruktivni simptomi.

U ovom prikazu slučaja, bolesnik (muškarac, 66 godina) imao je tahikardiju, bol u gornjem delu trbuha i nelagodnosti, u trajanju od mesec dana, ali sa ublažavanjem svih simptoma u uspravnom položaju. On ima medicinsku anamnezu srčanog oštećenja. Dijagnoza je pretpostavljena na osnovu radiograma toraksa, a potvrđena je putem CT toraksa i abdomena. Bolesnik je tretiran hirurški, sa primarnim zatvranjem dijafragmatičnog defekta.

Acta Medica Medianae 2020;59(3):108-111.

Ključne reči: *Morgagni hernija, hiruški tretman, dijafragmalna hernija*

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General Guidelines

Paper Submission

All research articles published in this journal undergo rigorous peer review, based on initial editor screening and anonymized refereeing by at least two anonymous referees. Remarks and suggestions made by the editors and reviewers are sent to the author for final revision. The papers in English are to be submitted by e-mail: acta@medfak.ni.ac.rs. Manuscripts accepted for publication are not returned to authors.

The first page of a research article must contain: a) article title b) full name of author(s) c) full name(s) of institutions and/or address(es) of department(s) where either reasearch was conducted or research article written d) following signs *, **, ***, #, ##, ### signifying author(s) and institutions e) full address, phone number and e-mail of a corresponding author.

The second page should contain only research article title, abstract and key words without names of author(s) and institution(s). Abstract for research and professional articles, review articles and meta-analyses should have up to 350 words while abstract for all other types of publications should consist of 250 words. Key Words section should have up to 5 key words or phrases related to a submitted article. It is desirable that authors use corresponding descriptors from Medical Subject Heading (MeSH) that can be found on Index Medicus list for key words. The first and the second page should not be numbered.

Body of a Research Article – Research and professional articles, as well as general surveys and meta-analyses should not exceed 11 pages altogether; current topics - 6 pages, casuistics - 6 pages and proceding statements - 5 pages, history of medicine articles - 3 pages while conference reports and book reviews - 2 pages. Research and professional articles should comprise the following mandatory chapters: introduction, aim(s), material and method(s), result(s), discussion and conclusion(s). Result(s) and discussion can be comprized into one chapter. Acknowledgments of any kind in a submitted article should be written at the end of the paper after "Conclusion(s)". It is necessary to clearly mark a place for additions in the text.

References should be written in a separate chapter in the same order of appearance as in a research article. Reference numbers that appear in the text should be written in Arabic numerals and put in brackets. All authors should be listed. If there are more than six authors this should be indicated with *et al*. Use the rules of the Vancouver convention when quoting literature. Pages should be enumerated in Arabic numerals in the bottom right corner.

Additions in form of texts, tables and illustrations (photos, drawings, diagrams, etc) should not be inserted in the reasearch article body but at the end of the text on separate pages which should be marked in the upper left corner as "Table(s), Graphic(s), Illustration(s) etc) with Arabic numeral in the same order of appearance as in the text (for instance, Table 1, Graph 1, etc) with a short title. Short explanations and abbreviations should be stated in footnotes where the following symbols should be used: *, **, ***, #, ##, ###, etc. Table(s), graph(s) and ilustration(s) should be drawn in a Microsoft Office Program. Color should be avoided.

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