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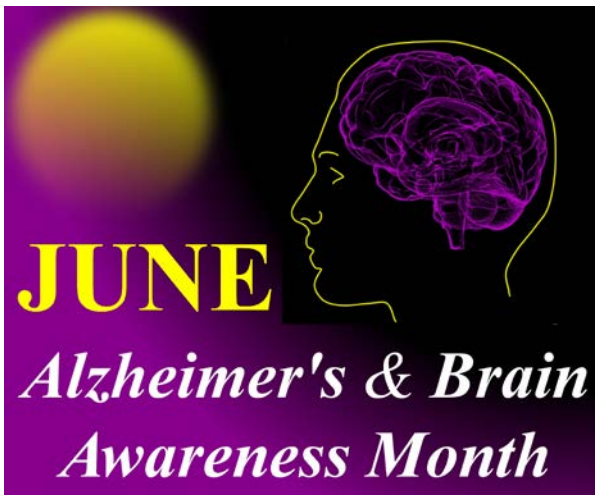
CONTENTS / SADRŽAJ

ORIGINAL ARTICLES / ORIGINALNI RADOVI



Alzheimer's disease, a neurologic condition that causes brain atrophy, is the most frequent cause of dementia which is characterized by progressive loss of cognitive, behavioral and social abilities, ultimately impairing a person's capability to function independently.

June is *Alzheimer's and Brain Awareness Month* used by many organizations as an opportunity for education and fundraising related to the subject. By raising awareness and recognizing the most common symptoms of the disease early on, there is a better chance of a favorable response to therapy.

Alchajmerova bolest je neurološko stanje koje uzrokuje atrofiju mozga i najčešći je uzrok demencije, koju karakteriše progresivni gubitak kognitivnih, bihejvioralnih i socijalnih sposobnosti, što na kraju onemogućava obolelog da živi samostalno.

Jun je *mesec svesti o Alchajmeru i značaju mozga,* koji mnoge organizacije koriste kao priliku za edukaciju i prikupljanje sredstava za rešavanje tog problema. Podizanjem svesti i ranim prepoznavanjem najčešćih simptoma bolesti, veće su šanse za povoljan odgovor na terapiju.

ORIGINAL ARTICLES

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Cardiac injury on admission linked to worse outcomes in hospitalized COVID-19 patients

Povezanost oštećenja srca na prijemu u bolnicu sa lošim ishodom kod obolelih od COVID-19

Yiwu Zhou, Lin Lv, Rong Yao

Sichuan University, West China Hospital, Department of Emergency Medicine, Emergency Medical Laboratory, Chengdu, Sichuan, China

Abstract

Background/Aim. The novel severe acute respiratory syndrome coronavirus 2 (SARS-Cov-2) has triggered a pandemic that causes a disease with complex clinical manifestations (coronavirus disease 2019, COVID-19). Soon it became clear that patients who had some comorbidities had a bigger chance of getting the severe form of COVID-19. The aim of the study was to investigate if there was a link between cardiac injury and COVID-19 severity and mortality in patients. **Methods**. All consecutive patients with laboratory-confirmed COVID-19 were included and followed up until discharge or death from January 30, 2020, to April 5, 2020. **Results**. A total of 261 COVID-19 patients were included, and 29 (11.1%) had cardiac injury on admission. Patients with cardiac injury were older than those without cardiac injury (72.8 *vs* 55.8 years old) and more likely to be male (82.8% *vs* 42.2%). Patients with cardiac injury were also more likely to be smokers (31.0% *vs* 12.5%), more likely to have chronic cardiovascular disease (24.1% *vs* 7.8%), chronic pulmonary disease (17.2% *vs* 3.0%), and chronic kidney disease (10.3% *vs* 2.2%) compared to patients without cardiac injury. Laboratory findings suggested that patients with cardiac injury were more likely to have leukocyte counts > 10×10^9/L, pronounced lymphopenia, direct bilirubin, myohemoglobin, blood urea nitrogen, C-reactive protein, and pro-B-type natriuretic peptide but lower levels of serum total protein and estimated glomerular filtration rates compared to patients without cardiac injury. Patients with cardiac injury experienced more complications (72.4% *vs* 47.8%), including acute respiratory distress syndrome (20.7% *vs* 2.7%), acute kidney injury (10.3 *vs* 0.4%), severe COVID-19 (58.6% *vs* 11.6%) and death (55.2% *vs* 3.9%) compared to patients without cardiac injury. Multivariate analyses showed that cardiac injury was associated with an increased risk of severe COVID-19 [hazard ratio (HR) = 8.71, 95% confidence interval (CI) = 2.37−32.04] and death (HR = 20.84, 95% CI = 1.32−328.22). **Conclusion**. Cardiac injury on admission was associated with a higher risk of disease progression and death in patients with COVID-19.

Key words:
patient admission; cardiovascular diseases; COVID-19; disease progression; mortality; prognosis; risk factors; cardiomyopathies.

Apstrakt

Uvod/Cilj. Novi korona virus *severe acute respiratory syndrome coronavirus 2* (SARS-Cov-2) izazvao je pandemiju bolesti sa složenim kliničkim manifestacijama (*coronavirus disease 2019,* COVID-19). Ubrzo je postalo jasno da su bolesnici sa određenim komorbiditetima imali veću šansu da obole od teškog oblika COVID-19. Cilj rada bio je da se utvrdi povezanost između oštećenja srca (OS) i težine bolesti i mortaliteta kod obolelih od COVID-19. **Metode**. Svi bolesnici sa laboratorijski potvrđenim COVID-19 bili su uključeni i praćeni do otpusta ili smrti u periodu od 30. januara 2020. do 5. aprila 2020. **Rezultati**. Ukupno je bilo obuhvaćeno 261 bolesnika sa COVID-19, od kojih je 29 (11,1%) imalo OS pri prijemu. Bolesnici sa OS bili su starijeg životnog doba od bolesnika bez OS (72,8% *vs* 55,8%) i većina (82,8% *vs* 42,2%) su bili muškarci. Bolesnici sa OS su češće bili pušači (31,0% *vs* 12,5%), imali hroničnu kardiovaskularnu bolest (24,1% *vs* 7,8%), hroničnu bolest pluća (17,2% *vs* 3,0%) kao i hroničnu bolest bubrega (10,3% *vs* 2,2%) u poređenju sa bolesnicima bez OS. Prema laboratorijskim analizama, bolesnici sa OS su značajno češće imali vrednosti leukocita više od 10×10^9/L, izraženiju limfopeniju, više vrednosti direktnog bilirubina, miohemoglobina, uree u krvi kao i C-reaktivnog proteina i pro-B-tipa natriuretičkog peptida, ali niže nivoe ukupnih proteina u serumu i procenjene stope glomerularne filtracije, u poređenju sa bolesnicima bez OS. Bolesnici sa OS imali su više komplikacija (72,4% *vs* 47,8%), uključujući sindrom akutnog respiratornog distresa (20,7% *vs* 2,7%), akutno oštećenje bubrega (10,3% *vs* 0,4%), teški oblik COVID-

Correspondence to: Rong Yao, 37 Guoxue Road, Chengdu, Sichuan, 610041, China. E-mail: yaorong@wchscu.cn

19 (58,6% *vs* 11,6%) kao i veću smrtnost (55,2% *vs* 3,9%) u poređenju sa bolesnicima bez OS. Multivarijantne analize su pokazale da je OS povezano sa povišenim rizikom od obolevanja od teškog oblika COVID-19 (HR = 8,71, 95% CI = 2,37–32,04) i smrti [*hazard ratio* (HR) = 20,84, 95% *confidence interval* (CI) = 1,32−328,22]. **Zaključak**. Oštećenje srca na prijemu u bolnicu kod bolesnika sa COVID-19 bilo je povezano sa višim rizikom od progresije bolesti i smrtnog ishoda.

Ključne reči:
bolesnik, prijem; kardiovaskularne bolesti; COVID-19; bolest, progresija; mortalitet; prognoza; faktori rizika; kardiomiopatije.

Introduction

Since its first outbreak in Wuhan, Hubei, China, in December 2019, coronavirus disease 2019 (COVID-19), which is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has rapidly spread across the whole world. On March 11, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic [1].

The clinical outcome of COVID-19 patients who already have cardiovascular disease (CVD) appears to be worse [2–5]. COVID-19 can cause progressive cardiac injury and worsen the condition in patients who already had cardiac injury through multiple mechanisms, which are closely related to the severity of the disease and the prognosis of death. A small sample size study found that 12% of COVID-19 patients had acute cardiac injury manifesting as an ejection fraction decrease and troponin I (TNI) elevation [6]. Another study involving 416 hospitalized patients suggested that 19.7% of patients had COVID-19-associated cardiac injury and presented with significantly elevated levels of creatine kinase-MB (CK-MB), myohemoglobin (Mb), hypersensitive troponin I (hs-TNI), and N-terminal (NT) pro-B-type natriuretic peptide (NT-proBNP). Notably, COVID-19 patients with cardiac injury had a much higher mortality rate than those without cardiac injury, according to Shi et al. [7]. Another study from Wuhan also confirmed that cardiac injury is significantly associated with fatal outcomes from COVID-19. Cardiac injury was an independent risk factor for mortality from COVID-19 [8].

However, there have been few studies on the characteristics of cardiac injury and its related risks in COVID-19 patients. This study aimed to explore the characteristics of cardiac injury and the prognoses of COVID-19 patients with cardiac injury. Early assessment of myocardial injury in patients with COVID-19 and the development of a targeted cardio protective program can improve the poor prognosis of patients.

Methods

Patients' characteristics

A total of 1,087 patients with COVID-19 were recruited from Hubei and Sichuan Provinces from January 30, 2020, to April 5, 2020. This study was approved by the Ethics Committee of West China Hospital, Sichuan University, and Hubei Red Cross Hospital, Wuhan [2020 (272)]. All of the patients were at least 18 years old. Clinical information was collected, including demographic data, comorbidities, symptoms, laboratory findings, treatment measures, and outcomes. Cardiac biomarkers measured on admission were collected, including hs-TNI, CK-MB, and Mb. Pediatric and asymptomatic patients and those without cardiac biomarkers (hs-TNI) were excluded. Finally, 261patients with complete cardiac markers and complete clinical outcomes were included in this study. All of the patients were followed up until discharge or death.

Cardiac injury was identified when blood levels of hs-TNI were greater than the 99th-percentile upper reference limit, regardless of any new electrocardiographic and echocardiographic abnormalities. All patients were divided according to the presence or absence of cardiac injury into two groups: a group of patients with cardiac injury and a group of patients without cardiac injury. Acute respiratory distress syndrome (ARDS) was defined according to the Berlin definition [9]. Liver dysfunction was diagnosed based on aminotransferase and bilirubin levels greater than the upper reference limits of the local hospital. Acute kidney injury (AKI) was defined according to the definition from Kidney Disease: Improving Global Outcomes [10].

COVID-19 and severity definition

The patients with severe COVID-19 enrolled in this study were diagnosed according to the guidelines for the diagnosis and treatment of COVID-19 and confirmed by RNA detection of SARS-CoV-2 by nasopharyngeal swab. The severity of COVID-19 was defined according to the diagnostic and treatment guidelines for SARS-CoV-2 issued by the Chinese National Health Committee (version 7) [11]. Severe COVID-19 was designated when the patient had one of the following criteria: (1) respiratory distress with respiratory frequency $\geq$ 30/min; (2) surplus pulse oximeter oxygen saturation $\leq$ 93% at rest; (3) oxygenation index (artery partial pressure of oxygen/inspired oxygen fraction, PaO2/FiO2) $\leq$ 300 mmHg; (4) respiratory failure and mechanical ventilation required; (5) shock; or (6) other organ failure requiring intensive care unit monitoring and treatment.

Statistical analyses

Descriptive statistics were obtained for all of the study variables. Categorical variables were summarized as percentages and compared using the χ^2 test or Fisher's exact test (if the expected number was less than five). Continuous variables were expressed as the mean value, standard deviation (SD), or median with interquartile ranges and compared using Student's *t*-test or the Mann-Whitney *U* test, if appropri-

ate. Logistic regression analysis was performed to determine the predictors of cardiac injury and the predictive value of cardiac injury in the disease progression and mortality. Variables with $p < 0.1$ in the univariate analysis were included in the multivariate logistic analysis. Statistical analysis was conducted using SPSS software, version 25.0 (IBM, Chicago, IL, USA). The value of $p < 0.05$ was considered statistically significant.

Results

Patients' characteristics

Out of the 261 patients included, 29 (11.1%) had cardiac injury on admission. Compared with patients without cardiac injury, patients with cardiac injury were older (72.8 ± 13.7 *vs* 55.8 ± 13.9, $p < 0.001$) and more likely to be male (82.8% *vs* 42.2%, $p < 0.001$) and smokers (31% *vs* 12.5%, $p = 0.021$). Comorbidities, including CVD (24.1% *vs* 7.8%, $p = 0.012$), chronic pulmonary disease (17.2% *vs* 3%, $p = 0.005$) and chronic kidney disease (CKD) (10.3% *vs* 2.2%, $p = 0.047$), were more prevalent among the patients with cardiac injury (Table 1).

The laboratory findings are shown in Table 2. Patients with cardiac injury were more likely to have leukocyte counts > 10×10^9/L (21.4% *vs* 6.5%), lymphopenia (58.6% *vs* 24.0%), and lower antithrombin III (90.2% *vs* 81.3%) compared to patients without cardiac injury. They also had a higher international normalized ratio (INR) (1.10 *vs* 1.02), total bilirubin (17.19 *vs* 11.20, μmol/L), direct bilirubin (7.14 *vs* 3.87, μmol/L), aspartate aminotransferase (55.12 *vs* 32.29, IU/L), creatine kinase (469.83 *vs* 85.83, IU/L), Mb (283.9 *vs* 49.9, IU/L), hs-TNI (2.83 *vs* 0.01, ng/mL), blood urea nitrogen (14.18 *vs* 5.31, mmol/L), uric acid

Table 1

Demographic and clinical characteristics of included patients

Characteristics	Cardiac injury (n = 29)	Without cardiac injury (n = 232)	*p*-value
Age (years), mean (SD)	72.76 (13.72)	55.76 (13.94)	< 0.001
Period from first symptoms to hospital admission (days), mean (SD)	4.62 (6.86)	3.78 (4.27)	0.522
Sex, n (%)			
male	24 (82.8)	98 (42.2)	< 0.001
female	5 (17.2)	134 (57.8)	
Drinking, n (%)			
yes	26 (89.7)	204 (87.9)	1
no	3 (10.3)	28 (12.1)	
Smoking, n (%)			
yes	20 (69.0)	203 (87.5)	0.021
no	9 (31.0)	29 (12.5)	
Chronic CVD, n (%)			
yes	7 (24.1)	18 (7.8)	0.012
no	22 (75.9)	214 (92.2)	
CPD, n (%)			
yes	5 (17.2)	7 (3.0)	0.005
no	24 (82.8)	225 (97)	
CKD, n (%)			
yes	3 (10.3)	5 (2.2)	0.047
no	26 (89.7)	227 (97.8)	
CLD, n (%)			
yes	2 (6.9)	10 (4.3)	0.627
no	27 (93.1)	222 (95.7)	
Cancer, n (%)			
yes	3 (10.3)	7 (3)	0.089
no	26 (89.7)	225 (97)	
Diabetes, n (%)			
yes	4 (13.8)	40 (17.2)	0.796
no	25 (86.2)	192 (82.8)	
Hypertension, n (%)			
yes	14 (48.3)	71 (30.6)	0.061
no	15 (51.7)	161 (69.4)	
Temperature on admission (°C), mean (SD)	36.7 (0.6)	36.8 (0.7)	0.567
Heart rate (beats/min), mean (SD)	89 (15)	89 (14)	0.779
Systolic blood pressure (mmHg), mean (SD)	132 (16)	129 (16)	0.347
Diastolic blood pressure (mmHg), mean (SD)	80 (11)	77 (10)	0.374
Respiratory rate (breaths/min), mean (SD)	22 (6)	21 (3)	0.124

CVD – cardiovascular disease; CPD – chronic pulmonary disease; CKD – chronic kidney diseases; CLD – chronic liver disease; SD – standard deviation.

Table 2

Laboratory findings between patients with and without cardiac injury

Parameters	Cardiac injury (n = 29)	Without cardiac injury (n = 232)	*p*-value
WBC (> 10 × 10^9/L), n (%)	6 (21.4)	14 (6.5)	0.016
Lymphopenia (< 1.5 × 10^9/L), n (%)	17 (58.6)	52 (24)	< 0.001
Neutrophils (%), mean (SD)	76.86 (17.98)	64.65 (14.71)	0.001
Eosinophils (%), mean (SD)	0.62 (1.2)	1.2 (1.58)	0.064
Basophils (%), mean (SD)	0.28 (0.22)	0.41 (0.32)	0.006
Monocytes (%), mean (SD)	6.05 (3.63)	8.2 (3.9)	0.007
Hematocrit (%), mean (SD)	0.36 (0.08)	1.17 (5.99)	0.467
D-dimer (ng/mL), mean (SD)	13.47 (34.66)	1.64 (2.99)	0.082
Fibrinogen (FIB) (g/L), mean (SD)	9.5 (22.69)	5.08 (8.74)	0.325
Antithrombin III (ATIII) (%), mean (SD)	81.33 (16.71)	90.22 (12.06)	< 0.001
APTT (S), mean (SD)	29.04 (3.57)	27.94 (3.77)	0.152
PT (S), mean (SD)	14.52 (9.42)	12.25 (4.7)	0.227
INR, mean (SD)	1.1 (0.17)	1.03 (0.12)	0.006
TBIL (μmol/L), mean (SD)	17.19 (10.76)	11.2 (6.06)	0.008
DBIL (μmol/L), mean (SD)	7.14 (4.51)	3.87 (2.24)	0.001
IBIL (μmol/L), mean (SD)	8.04 (3.29)	7 (2.97)	0.204
ALT(U/L), mean (SD)	48.64 (54.61)	35.48 (43.57)	0.255
AST (U/L), mean (SD)	55.12 (38.57)	32.29 (53.07)	0.038
Serum total protein (g/L), mean (SD)	59.39 (5.22)	62.64 (6.52)	0.013
ALB (g/L), mean (SD)	34.49 (4.2)	38.76 (4.6)	< 0.001
GLB (g/L), mean (SD)	25.3 (3.42)	24.14 (4.29)	0.178
TG (mmol/L), mean (SD)	1.58 (0.93)	1.43 (0.83)	0.39
CHOL (mmol/L), mean (SD)	3.81 (0.78)	4.29 (2.84)	0.396
HDL-C (mmol/L), mean (SD)	0.9 (0.35)	1.07 (0.4)	0.038
LDL-C (mmol/L), mean (SD)	2.35 (0.83)	2.56 (0.79)	0.215
CK (U/L), mean (SD)	469.83 (730.67)	85.63 (87.63)	0.019
CK-MB (U/L), mean (SD)	4.73 (6.4)	2.53 (15.44)	0.466
Glucose (mmol/L), mean (SD)	7.1 (3.28)	6.89 (5.28)	0.842
BUN (mmol/L), mean (SD)	14.18 (15.05)	5.31 (6.15)	0.005
Serum creatinine (μmol/L), mean (SD)	158.77 (295.95)	63.17 (28.95)	0.105
eGFR (mL/min/1.73 m^2), mean (SD)	65.49 (31.56)	103.45 (22.61)	< 0.001
Uric acid (μmol/L), mean (SD)	372.63 (233.75)	273.99 (101.08)	0.039
Hypersensitive troponin-I (ng/mL), mean (SD)	2.83 (8.55)	0.01 (0)	0.006
Mb (U/L), mean (SD)	283.9 (300.15)	49.9 (65.08)	< 0.001
CD3 (%), mean (SD)	60.86 (11.85)	67 (10.29)	0.016
CD4 (%), mean (SD)	39.17 (8.01)	41.54 (9.52)	0.31
CD8 (%), mean (SD)	18.71 (9.19)	28.07 (41.51)	0.329
CD3 cell count (cell/μL), mean (SD)	488.79 (255.86)	823.91 (421.2)	< 0.001
CD4 cell count (cell/μL), mean (SD)	312.21 (171.22)	513.56 (287.33)	< 0.001
CD8 cell count (cell/μL), mean (SD)	155.32 (119.38)	279.1 (160.95)	0.001
CD4/CD8, mean (SD)	2.57 (1.33)	2.14 (1.15)	0.124
IgG (g/L), mean (SD)	12.46 (2.7)	12.03 (3.17)	0.578
IgM (mg/L), mean (SD)	0.91 (0.35)	60.75 (278.62)	0.365
IgE (IU/mL), mean (SD)	126.67 (139.63)	165.4 (652.32)	0.802
C3 (mg/L), mean (SD)	1.02 (0.26)	1.06 (0.2)	0.474
C4 (mg/L), mean (SD)	0.25 (0.12)	0.27 (0.1)	0.61
CRP (mg/L), mean (SD)	91.32 (71.06)	34.97 (45.53)	0.002
Procalcitonin (ng/mL), mean (SD)	0.77 (1.17)	0.09 (0.16)	0.008
pro-BNP (pg/mL), mean (SD)	2,469.54 (3,894.11)	265.97 (1,508.47)	0.021

WBC – white blood cell; APTT – activated partial thromboplastin time; PT – prothrombin time; INR – international normalized ratio; TBIL – total bilirubin; DBIL – direct bilirubin; IBIL – indirect bilirubin; ALT – alanine aminotransferase; AST – aspartate transaminase; ALB – albumin; GLB – globulin; TG – triglyceride; CHOL – cholesterol; HDL-C – high-density lipoprotein; LDL-C – low-density lipoprotein; CK – creatine kinase; CK-MB – creatine kinase-MB; BUN – blood urea nitrogen; eGFR – estimated glomerular filtration rate; Mb – myohemoglobin; IgG – immunoglobulin G; IgM – immunoglobulin M; IgE – immunoglobulin E; C3 – complement 3; C4 – complement 4; CRP – C-reactive protein; pro-BNP – pro-B-type natriuretic peptide; SD – standard deviation.

(372.63 *vs* 273.99, μmol/L), C-reactive protein (CRP) (91.32 *vs* 34.97, mg/L), procalcitonin (PCT) (0.77 *vs* 0.09 ng/mL), and pro-BNP (2,469.54 *vs* 265.97, pg/L), but a lower high-density lipoprotein (0.9 *vs* 1.07, mmol/L), serum total protein (59.39 *vs* 62.64, g/L), albumin (34.49 *vs* 38.76, g/L), estimated glomerular filtration rate (eGFR) (65.49 *vs* 103.45, mL/min), $CD3^+$ cells (488.79 *vs* 823.91, cell/μL), $CD4^+$ cells (312.23 *vs* 513.56, cell/μL), $CD8^+$ cells (155.32 *vs* 279.1, cell/μL), with significant difference ($p < 0.05$) compared to patients without cardiac injury. There was no difference in complement and immunoglobulin level.

All patients with cardiac injury underwent electrocardiographic (ECG) examinations after admission, and 22 (91.7%) ECGs were abnormal, with findings compatible with myocardial ischemia, such as T-wave depression and inversion, ST-segment depression, and Q waves.

Treatment and complications

The mean time from symptom onset to admission was similar between the two groups (4.62 days *vs* 3.78 days, $p = 0.522$). Compared with patients without CI, those with CI required more ventilation, including noninvasive (17.2% *vs* 3%) and invasive mechanical (6.9% *vs* 0.4%) approaches, and eventually more intensive care unit admission (13.8% *vs* 1.7%). Most of the patients received antivirals, antibiotic therapy, and traditional Chinese medicine, and no difference was found between the two groups. Notably, patients with cardiac injury received more corticosteroid therapy (44.8% *vs* 24.1%) and nutritional support (34.5% *vs* 10.3%). Overall, patients with cardiac injury experienced more complications (72.4% *vs* 47.8%), including higher ARDS (20.7% *vs* 2.7%), AKI (10.3 *vs* 0.4%), severe COVID-19 (58.6% *vs* 11.6%) and death (55.2% *vs* 3.9%) (Table 3).

Table 3

Treatment and complications in patients with and without cardiac injury

Treatment/complications	Cardiac injury (n = 29)	Without cardiac injury (n = 232)	*p*-value
ICU admission, n (%)			
yes	4 (13.8)	4 (1.7)	0.006
no	25 (86.2)	228 (98.3)	
Noninvasive ventilation, n (%)			
yes	5 (17.2)	7 (3)	0.005
no	24 (82.8)	225 (97)	
Invasive ventilation, n (%)			
yes	2 (6.9)	1 (0.4)	0.033
no	27 (93.1)	231 (99.6)	
Antiviral drugs, n (%)			
yes	29 (100)	212 (91.4)	0.142
no	0 (0)	20 (8.6)	
Antibiotics, n (%)			
yes	22 (75.9)	154 (66.4)	0.304
no	7 (24.1)	78 (33.6)	
Corticoids, n (%)			
yes	13 (44.8)	56 (24.1)	0.017
no	16 (55.2)	176 (75.9)	
Nutritional support, n(%)			
yes	10 (34.5)	24 (10.3)	0.001
no	19 (65.5)	208 (89.7)	
TCM, n(%)			
yes	18 (62.1)	178 (76.7)	0.085
no	11 (37.9)	54 (23.3)	
Total complications, n (%)			
yes	21 (72.4)	111 (47.8)	0.013
no	8 (27.6)	121 (52.2)	
ARDS, n (%)			
yes	6 (20.7)	6 (2.7)	0.001
no	23 (79.3)	220 (97.3)	
Hydrothorax, n (%)			
yes	0 (0)	6 (2.6)	1
no	29 (100)	221 (97.4)	
AKI, n (%)			
yes	3 (10.3)	1 (0.4)	0.005
no	26 (89.7)	226 (99.6)	
Death, n (%)			
yes	13 (44.8)	223 (96.1)	< 0.001
no	16 (55.2)	9 (3.9)	
Severe pneumonia, n (%)			
yes	12 (41.4)	205 (88.4)	< 0.001
no	17 (58.6)	27 (11.6)	

ICU – intensive care unit; TCM – traditional Chinese medicine; ARDS – acute respiratory distress syndrome; AKI – acute kidney injury.

Risk factors for cardiac injury

Univariate analysis indicated that male patients, older age (> 65 years old), smoking status, comorbidities, WBC > 10^9/L, lymphopenia, and PCT were risk factors for cardiac injury. In addition, multivariate analysis showed that male patients [hazard ratio (HR) = 6.13, 95% confidence interval (CI) 1.46–25.69)], older age (> 65 years old) (HR = 4.98, 95% CI 1.25–19.87), CVD (HR = 8.5, 95% CI 1.6–45.18), and PCT (HR = 30.57, 95% CI 3.67–254.98) were independently associated with an increased risk of cardiac injury on admission (Figure 1).

Predictive value of cardiac injury in severe COVID-19 and mortality

More severe COVID-19 was seen among patients with cardiac injury. In univariate analysis, older age (> 65 years old), WBC > 10 × 10^9/L, lymphopenia, eGFR < 60 mL/min, PCT, and cardiac injury were associated with an increased incidence of severe COVID-19. The multivariable-adjusted analysis found that only WBC > 10 × 10^9/L (HR = 4.26, 95% CI 1.09–16.7) and cardiac injury (HR = 8.71, 95% CI 2.37–32.04) were independent risk factors for severe COVID-19 (Figure 2).

Similarly, more deaths were detected in patients with cardiac injury. In univariate analysis, males, older age (> 65 years old), CVD, CKD, hypertension, WBC > 10 × 10^9/L, lymphopenia, eGFR < 60 mL/min, PCT, cardiac injury, and severe COVID-19 were associated with an increased risk of death. In multivariate analysis, only PCT (HR = 3,382.66, 95% CI 3.75–3,051,538.4) and cardiac injury (HR = 20.84, 95% CI 1.32–328.22) were independent risk factors for death (Figure 3).

Discussion

As a rapidly spreading disease with increasing mortality, COVID-19 seriously threatens human life and health, and global economic development. Combating COVID-19 virus

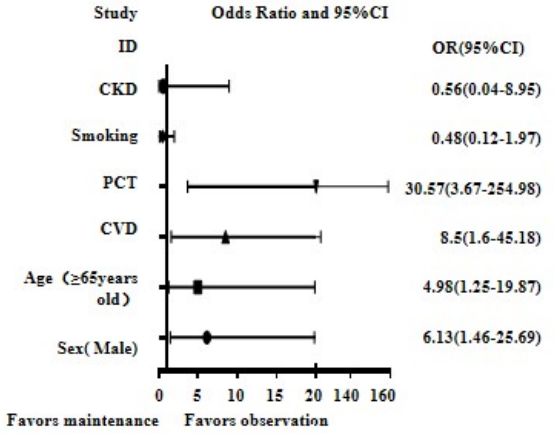

Fig. 1 – Logistic regression analysis of risk factors associated with cardiac injury. CKD – chronic kidney disease; PCT– procalcitonin; CVD – cardiovascular disease; OR – odds ratio; CI – confidence interval.

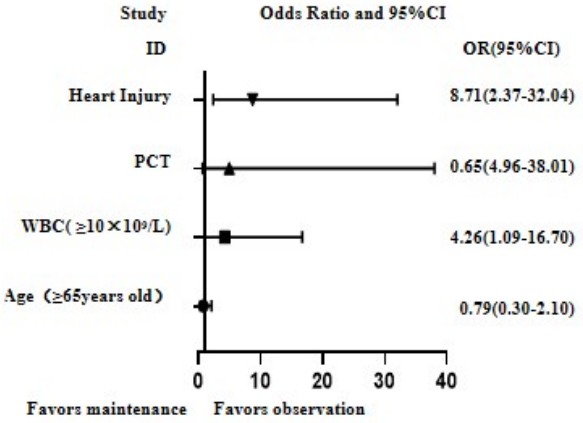

Fig. 2 – Logistic regression analysis of risk factors associated with severe COVID-19. PCT– procalcitonin; WBC – white blood cells; OR – odds ratio; CI – confidence interval.

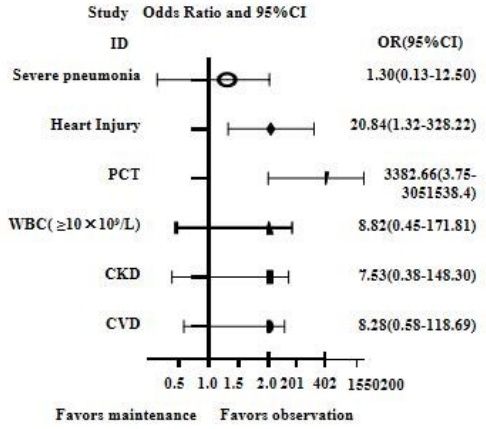

Fig. 3 – Logistic regression analysis of risk factors associated with in-hospital mortality. PCT– procalcitonin; WBC – white blood cells; CKD – chronic kidney disease; CVD – cardiovascular disease; OR – odds ratio; CI – confidence interval.

infection is a global battle in the medical and health industries. The virus mainly invades the lungs; however, it also affects cardiac function through multiple mechanisms [12]. The exact mechanism of cardiac involvement in COVID-19 remains under investigation. A recent study found that angiotensin-converting enzyme 2 (ACE-2) is also highly expressed in the heart [13]. Given that the spike protein can bind to ACE-2, it is plausible that the SARS-CoV-2 virus can infect the human heart, and this speculation was confirmed by a positive real-time polymerase chain reaction (PCR) assay for SARS-CoV-2 in heart tissue [14]. It is known that cyclooxygenase 2 (COX2) is an evolutionary enzyme involved in a variety of physiological and pathological processes and also plays an important role in viral infections [15–17]. Studies have shown that it induces lung developmental damage through the endoplasmic reticulum stress pathway and leads to pulmonary interstitial fibrosis while participating in inflammatory cell infiltration and fibroblast proliferation leading to cardiac injury [18–20]. Chronic basic CVD and risk factors for CVD include the risk of increased COVID-19 and worse clinical outcomes [21]. This study mainly explored the risk factors and clinical characteristics of patients with COVID-19 combined with cardiac injury, examined its risk factors, and identified that early cardiac injury and early intervention for COVID-19 in the progress of the disease improved the prognoses of patients.

This study found that cardiac injury occurred in elderly, male, and smoking patients. These factors are also classic risk factors for CVD [22]. There are vascular lesions and coronary atherosclerotic plaque formation in elderly patients. Long-term smoking can also lead to increased arteriosclerosis, vascular endothelial changes, and atherosclerotic plaque formation in elderly patients and smokers, in addition to direct cardiomyocyte apoptosis induced by inflammatory cell storms [23]. Uncontrolled release of inflammatory cytokines after infection can lead to impaired myocardial endothelial function, a progressive decrease in coronary blood flow, decreased oxygen supply, unstable coronary plaque, and microthrombosis, leading to further cardiac injury [24], consistent with the findings of Wang et al. [25]. This study found that men are more prone to myocardial injury because women secrete estrogen before menopause, which plays an antiatherosclerotic role, while men are more likely to smoke, and smoking accelerates coronary atherosclerotic plaque formation [26, 27]. Therefore, compared with women, coronary artery disease is more serious, and cardiac injury is more likely to occur in men in case of viral invasion and inflammation. For elderly men and COVID-19 patients with bad smoking habits, we should be alert to cardiac injury through early prevention of coronary atherosclerotic plaque formation, and cessation of smoking and other bad habits should be recommended.

In this study, 11.1% of the patients infected with the new coronavirus had cardiac injury. Some studies have shown that 5-38% of COVID-19 patients have cardiac injury [28]. This study found that the WBC counts, CRP, PCT, and other inflammatory indicators in patients with myocardial injury were significantly higher than those in patients without cardiac injury [29, 30]. The same as the results of Shi et al. [7], our study found that the CD3 positive, CD4 positive, and CD8 positive cell counts of patients with CI were significantly lower than those of patients without CI. This may be related to the patient's impaired immune function, indicating that the patient's immune function impairment may also be related to cardiac injury [31]. Moreover, Sandoval et al. [32] found a positive correlation between plasma TNI levels and high-sensitivity CRP levels, supporting the idea that a severe inflammatory response might play an important role in the development of cardiomyocyte injury. Consistent with the PCT-independent risk factor for cardiac injury, a PCT increase of 1 ng/mL will increase the risk of cardiac injury more than 30 times. As a result, COVID-19 patients with high inflammatory responses are more prone to cardiac injury. In addition, 91.7% of patients with cardiac injury had ECG changes, with findings compatible with myocardial ischemia. The main manifestations were T-wave depression and inversion, ST-segment depression, and Q waves. Electrocardiograms can be used as an efficient method for evaluating cardiac injury in COVID-19 patients. Combined with the level of the inflammatory response, early inhibition of inflammatory response, according to the changes in electrocardiography, could guide the treatment decisions for patients with COVID-19 CI.

In this study, the Intensive Care Unit occupancy rate of patients with cardiac injury increased significantly, the support rate of patients with ventilators was higher, and more patients with severe COVID-19 were diagnosed, which was also an independent risk factor for death. Once again, as it is emphasized by Rocco et al. [33], COVID-19 patients with cardiac injury have a worse clinical prognosis, similar to the findings of some other authors [4, 34, 35].

There are several limitations of our study. First, as a retrospective study and with most of our focus on the respiratory system, not all COVID-19 patients had measured cardiac biomarkers, and only 261 patients were included. In addition, other specific information regarding cardiac injury, such as ECG data and echocardiography, was not available. Second, regarding the cytokine storm, we only included parameters CRP and PCT, while inflammatory cytokines, such as interleukin-6 and tumor necrosis factor-alpha, were not presented in the study because the data were incomplete. Third, all cardiac injuries were assumed to be due to SARS-CoV-2 infection. However, it is difficult to differentiate the real cause of cardiac injury, such as medications and other underlying conditions or infections. Therefore, a prospective study with more complete data collection is needed. Finally, we did not evaluate any intervention for cardiac injury, and whether an improved heart condition can result in better clinical outcomes in COVID-19 remains unknown. Thus, future studies which will focus on the effectiveness of treatments specific to cardiac injury are necessary.

Other limitations of our study are: retrospective design, sample size, and in some cases incomplete data on symptoms, laboratory tests, and imaging examinations, given the variation in the structure of electronic databases across different participating hospitals and an urgent data extraction schedule. Besides, we did not collect treatment-related data, which may be critical to the patient's outcome.

Conclusion

Cardiac injury is a common condition in COVID-19 patients on admission, especially in elderly, male, and smoking patients. It is often accompanied by ECG myocardial ischemia and high inflammatory response, which are related to the progression of the disease and fatal outcomes and can not be ignored.

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Conflict of interest

The authors have no conflict of interest to declare.

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Study of the correlation between the expression of nuclear factor kappa B and proliferation regulatory proteins and chronic superficial gastritis

Ispitivanje korelacije između ekspresije nuklearnog faktora kapa B i proliferacije regulatornih proteina i hroničnog superficijalnog gastritisa

Hui Hu*, Zhijian Ma†, Shouzhong Ren†, Yiqiang Xie‡

***Hainan University, Faculty of Chemical Engineering and Technology, Haikou, China; Hainan Medical University, †Faculty of Pharmacy, ‡College of Traditional Chinese Medicine, Haikou, China**

Abstract

Background/Aim. Cell proliferation and the regulation of protein expression play an important role in gastritis, but in chronic superficial gastritis (CSG), they are rarely reported. The aim of this study was to determine the relationship between the expression of nuclear factor kappa B (NF-κB) and regulatory proteins and the rat CSG. **Methods.** The CSG rat model was established artificially, by chemical agents and irregular diet. The expression of epidermal growth factor receptor (EGFR) and proliferating cell nuclear antigen (PCNA) in the gastric mucosa of CSG rats was measured by immunohistochemistry, while mRNA expression levels of NF-κB p65 were detected by *in situ* hybridization. **Results.** There was more obvious infiltration of inflammatory cells in the gastric mucosa of CSG rats than in that of control rats, and the inflammation score was significantly increased. The expression levels of PCNA, EGFR, and NF-κB p65 mRNA in the gastric mucosal cells of CSG model rats increased significantly. Correlation analysis showed that the inflammation score was positively correlated with the expression levels of NF-κB p65 mRNA and EGFR, while it presented no significant correlation with the expression level of PCNA. In addition, there was a significant positive correlation between NF-κB p65 mRNA and EGFR levels. **Conclusion.** High expression of NF-κB and EGFR plays an important role in the occurrence and progression of CSG, and it is significantly positively correlated with the degree of inflammation in the gastric mucosa. Therefore, changes in NF-κB and EGFR expression may be used as important indicators for the assessment of CSG; changes in their expression levels are helpful to assess the degree of gastric mucosal lesions and progression of CSG.

Key words:
antigens, nuclear; cell proliferation; dna-binding proteins; epidermal factor growth; gastritis; immunohistochemistry; rats.

Apstrakt

Uvod/Cilj. Ćelijska proliferacija i regulacija ekspresije proteina igraju važnu ulogu u gastritisu, ali u hroničnom superficijalnom gastritisu (HSG) su nedovoljno ispitane. Cilj rada bio je da se ispita povezanost između ekspresije nuklearnog faktora kapa B (NF-κB) i regulatornih proteina i HSG kod pacova. **Metode.** Kod pacova je HSG bio izazvan veštački, primenom hemijskih agenasa i neadekvatnom ishranom. U mukozi pacova sa HSG imunohistohemijski je određivana ekspresija receptora epidermalnog faktora rasta (EGFR) i nuklearnog antigena proliferišućih ćelija (PCNA) dok su hibridizacijom *in situ* određivani nivoi ekspresije mRNA NF-κB p65. **Rezultati.** Utvrđena je veća infiltracija inflamatornih ćelija i skor inflamacije u sluznici želuca pacova sa HSG, u poređenju sa kontrolnim pacovima. Nivoi ekspresije PCNA, EGFR i NF-κB p65 mRNA u ćelijama sluznice želuca pacova sa HSG bili su značajno povećani. Korelaciona analiza pokazala je da je skor inflamacije bio u pozitivnoj vezi sa nivoima ekspresije mRNA NF-κB p65 i EGFR, ali nije bilo značajne korelacije sa nivoom ekspresije PCNA. Dodatno, nađena je značajna pozitivna korelacija između mRNA NF-κB p65 i nivoa EGFR. **Zaključak.** Visoka ekspresija NF-κB i EGFR ima značajnu ulogu u pojavi i progresiji HSG i u značajnoj je, pozitivnoj korelaciji, sa stepenom inflamacije u sluznici želuca. Dakle, promene u ekspresiji NF-κB i EGFR mogu se koristiti kao važni indikatori za procenu HSG, tj. promene u nivoima njihove ekspresije korisne su za procenu stepena lezije sluznice želuca i progresije HSG.

Ključne reči:
antigeni, nuklearni; ćelija, proliferacija; proteini, dnk vezujući; faktor rasta, epidermalni; gastritis; imunohistohemija; pacovi.

Correspondence to: Shouzhong Ren, Yiqiang Xie, Hainan Medical University, 3 Xueyuan Road, Longhua District, 571199 Haikou, Hainan, China. E-mail: rsz19771029@163.com, mrxieyiqiang@163.com

Introduction

Chronic gastritis is a common disease in human beings. It is estimated that several hundreds of millions of people worldwide may have chronic gastritis in one form or another [1]. Chronic gastritis is also a common disorder in China and is often underestimated in clinical practice and in real life. Chronic superficial gastritis (CSG) is a clinically common and frequently-occurring disease. If the condition is not resolved in the long-term, it may develop into chronic atrophic gastritis (CAG), with a risk of occurrence of malignant transformation of approximately 2.5–5% [2]. Cell proliferation and the regulation of protein expression play an extremely important role during this period. There have been many reports on the proliferation of gastric mucosal cells in CAG and its effect on the disease progression [3], but in CSG, the proliferation of gastric mucosal cells and the regulation of their protein expression are rarely reported. Therefore, it is important to determine the essence, development, and recovery of CSG and drug targets to study the relationship between the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and regulatory proteins related to the proliferation of gastric mucosal cells and chronic superficial gastritis in CSG rats.

Methods

Spraque-Dawley rats (180–220 g), half male and half female, are purchased from Slack Jingda Laboratory Animal Co. Ltd, Changsha, China, [Certificate No: SCXK (Xiang) 2009-0001]. The rats were fed under the condition of controlled temperature (21 ± 2 °C), relative humidity（about 60 ± 10%), 12-h light/dark cycle, and automatic ventilation 8–15 times every hour. All experiments were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals.

Anhydrous ethanol was purchased from Hunan Hui Hong Reagent Co. Ltd (Changsha, China); 28% ammonia was obtained from Hunan Chemical Industry Research Institute (batch No.20100317; Zhuzhou, China). Sodium deoxycholate was a product of Sigma company (St Louis, USA). Mouse polyclonal antibody against epidermal growth factor (EGF) receptor – EGFR, proliferating cell nuclear antigen (PCNA), and NF-κB/p65 mRNA, *in situ* hybridization Kit and SABC kit were purchased from Wuhan Boster company (Wuhan, China).

CSG rat model was established as follows: rats were administered 2 mL of 60% alcohol once every Tuesday and Friday on an empty stomach; 2 mL of 20 mmoL/L sodium deoxycholate orally once daily; 0.05% of ammonia during weeks 1–6 and 0.1% of ammonia during weeks 7–12 in drinking water; an irregular diet involving 2 days of sufficient feeding and 1 day of fasting was performed [4]. The entire experimental period lasted 12 weeks (there were two deaths due to incorrect administration during the modeling process).

The stomachs were rapidly removed from the abdominal cavity, washed with distilled water, fixed with 4% formaldehyde solution, embedded in paraffin, and cut into slices. The slices were stained with hematoxylin-eosin (HE), and morphology was observed with a microscope. To measure the degree of inflammation of the gastric mucosa, a scoring system ranging from a low score of zero to a high score of 5 was used.

After the experiment, the rats were sacrificed, and blood samples were withdrawn from the celiac artery. The blood samples were centrifuged at 3,000 rpm for 10 min to obtain serum samples. Serum levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 were measured by enzyme-linked immunosorbent assay (ELISA) kits.

Analysis of EGFR and PCNA was performed following the operating instructions, and slices were subjected to immunohistochemistry and image analysis. Under an optical microscope at 400x magnification, 5 images were randomly selected, photos were taken of each section, and each picture was scanned on a computer with an HPIAS-1000 pathological image analysis system. The average optical density and the percentage of positive cells (the area of positive cells/total area of statistical field) were determined.

The tissue sections were treated with 3% H_2O_2 at room temperature for 10 min and washed twice in distilled water. Proteinase was added to the sections at 37 °C for 20 min. The sections were then washed three times with 0.5 mol/L phosphate-buffered salines (PBS), five mins each time. The slides were treated with 20 μL of pre-hybridization liquid at 37 °C for 4 hrs, the excess liquid was absorbed, and each slide was treated with 20 μL of NF-κB p65 oligonucleotide probe hybridization liquid and covered with a special slice overnight at 4 °C. Then, the slides were washed twice in 2% saline-sodium citrate (SSC) for five min each and once in 0.5% SSC and in 0.2% SSC for fifteen min each. Afterward, the slides were treated with digoxygenin for antibody visualization with the addition of pre-biotinylated anti-digoxigenin antibody SABC and biotinylated peroxidase successively. The slides were then incubated at 37 °C for twenty min and washed three times in 0.5 mol/L PBS for five min each. The slides were stained with 3,3'-diaminobenzidine (DAB) and hematoxylin and washed in water. Finally, the slides were covered with neutral gum, and after images were taken, the optical density value and rate of the positive area were detected by an HPIAS-1000 pathological image-text analysis system.

All data were expressed as the mean ± standard deviation (SD). Statistical analyses were conducted via ANOVA and Bivariate correlation using SPSS Proprietary Software Release 16.0. Data with p values < 0.05 were considered to be statistically significant.

Results

Under the microscope, in the control group, gastric mucosa epithelial cells were in neat rows, with a few inflammatory cells. In the CSG group, the gastric mucosal injury was obvious. There were visible piles of infiltrated inflammatory cells on the surface of the gastric mucosa. The mucosal inflammation score was significantly higher in the

CSG group than in the control group. The mucosal glands in the gastric antrum in the CSG group were arranged in a disorderly manner and irregular, but the glandular layer was obviously not thinner nor thicker (Figure 1).

After 12 weeks of treatment, the levels of TNF-α and IL-6 in the serum of the CSG group were significantly higher than those in the serum of the control rats (Figure 2).

Expression of PCNA was indicated by brown-yellow granules, which were mainly distributed in the nucleus and occasionally in the cytoplasm. In the control group, PCNA-positive cells were rare in the gastric antral mucosa. Compared with that in the control group, PCNA expression was significantly increased and unevenly distributed in the CSG group, and the optical density value and the number of PCNA-positive cells were significantly increased (Figure 3).

Expression of EGFR was indicated by brown-yellow granules, which were mainly distributed in the cytoplasm and cell membrane. Compared with that in the control group, EGFR expression was significantly increased in the CSG group, and the optical density value and the number of EGFR-positive cells were significantly increased ($p < 0.05$) (Figure 4).

Expression of NF-κB mRNA was mainly observed in the cytoplasm, as determined by *in situ* hybridization. In the control group, its expression was very low. Total expression of NF-κB mRNA was evidently increased and unevenly distributed in the gastric mucosal cells of CSG rats compared to those of control rats, and the optical density value and positive area of NF-κB mRNA were increased compared to those in control rats (Figure 5).

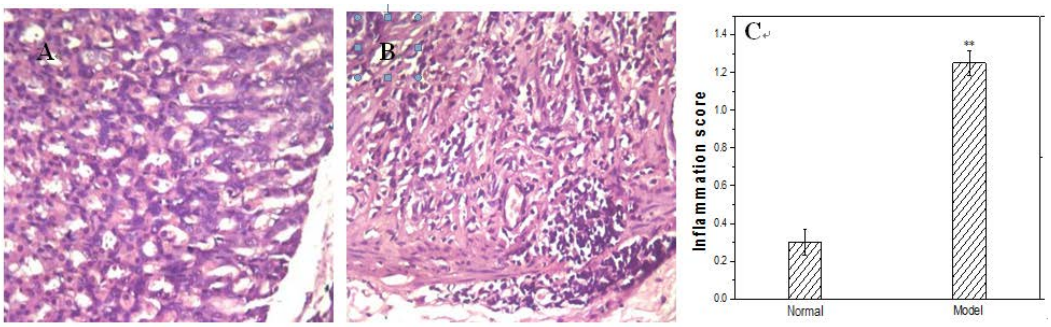

Fig. 1 – Morphologic changes in the gastric mucosa [hematoxylin-eosin staining, ×400]. A) Control group; B) Chronic superficial gastritis (CSG) model group; C) Mucosal inflammation score. [$p < 0.01$, CSG model group *vs* control (normal) group].**

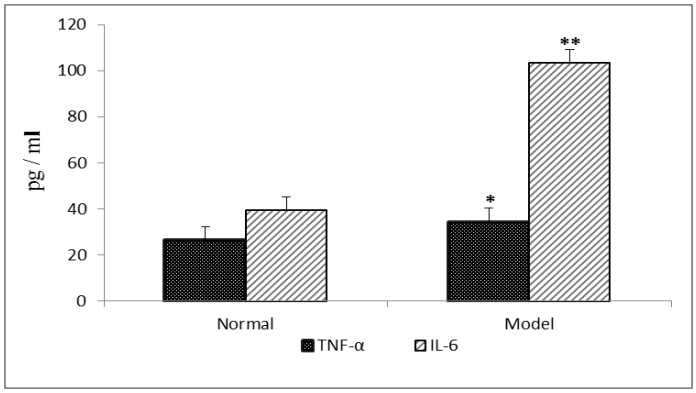

Fig. 2 – The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the serum [*$p < 0.05$, **$p < 0.01$, chronic superficial gastritis (CSG) model group *vs* control (normal) group after 12 weeks of treatment].

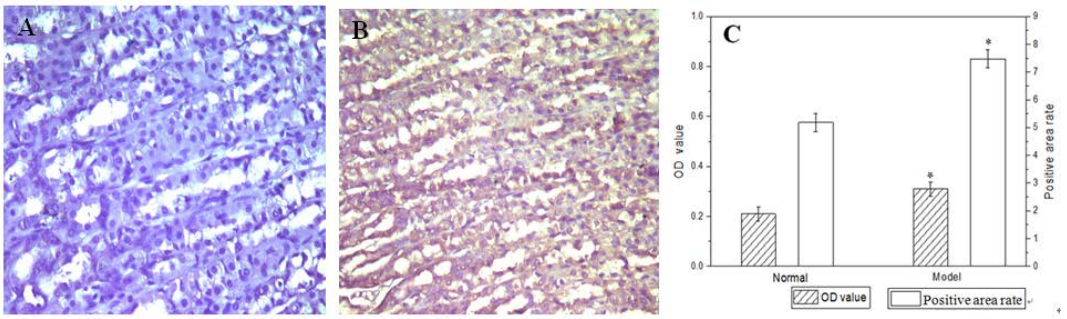

Fig. 3 – Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA) expression in rats [hematoxylin-eosin ×400]: A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area rate (*$p < 0.05$, CSG model group *vs* control group).

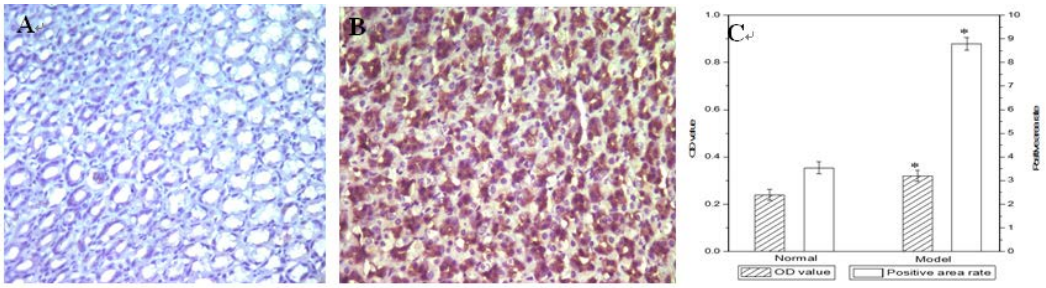

Fig. 4 – Immunohistochemical analysis of epidermal growth factor receptor (EGFR) expression in rats (×400). A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area (*$p < 0.05$, CSG model group *vs* control group).

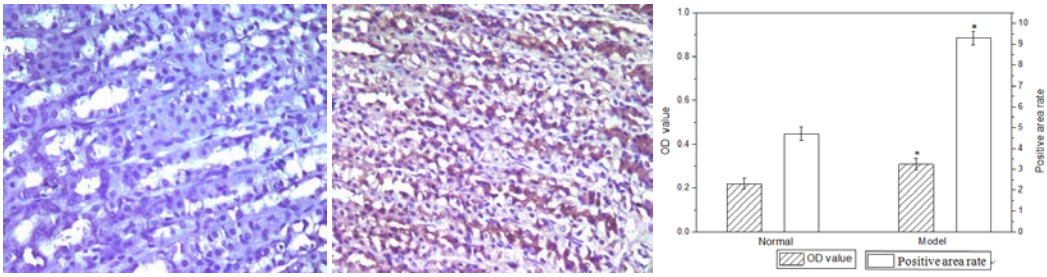

Fig. 5 – *In situ* hybridization analysis of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 mRNA expression (×400): A) Control (normal) group; B) Chronic superficial gastritis (CSG) model group; C) Odds ratio (OD) value and positive area.

Table 1

Correlation between gastric mucosal inflammation score and the expression levels of PCNA, EGFR, and NF-κB p65 in the chronic superficial gastritis group

Inflammation	Inflammation score	Optical density of EGFR	Positive area of EGFR	Optical density of PCNA	Positive area of PCNA	Optical density of NF-κB p65 mRNA	Positive area of NF-κB p65 mRNA
Score							
r	1	0.674	0.200	-0.393	-0.260	0.520	0.606
p		0.033*	0.317	0.167	0.267	0.076	0.042*
Optical density of EGFR							
r			-0.203	-0.334	-0.178	0.666	0.599
p			0.315	0.209	0.337	0.036*	0.058
Positive area of EGFR							
r				-0.619	-0.718	0.148	0.475
p				0.050	0.022*	0.364	0.117
Optical density of PCNA							
r					0.325	-0.325	-0.557
p					0.216	0.216	0.078
Positive area of PCNA							
r						-0.465	-0.578
p						0.123	0.067
Optical density of NF-κB p65 mRNA							
r							0.661
p							0.026*
Positive area of NF-κB p65 mRNA							
r							1
p							

***Confidence (one-sided) is 0.05, the correlation is significant.**

PCNA – proliferating cell nuclear antigen; EGFR – epidermal growth factor receptor; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells.

The statistical results, summarised in Table 1, revealed that in the CSG group, there was a significant correlation between inflammation score and NF-κB p65 mRNA and EGFR levels, but the correlation coefficient between PCNA and inflammation score was very small and presented no statistical significance. Conversely, NF-κB p65 mRNA expression was significantly correlated with EGFR expression, and there was a negative correlation between PCNA and EGFR expression (Table 1).

Discussion

Here, we explored the relationship between the expression of nuclear factor kappa B, EGFR, and PCNA in CSG rats. The pathological results showed that in the control group, the epithelial cells of gastric mucosa were orderly arranged, inflammatory cell infiltration was occasionally found on the surface of the mucosa, and the mucosal glands of the gastric antrum were orderly arranged. In the CSG group, the gastric antrum mucosa was obviously eroded, and there was a large number of infiltrating inflammatory cells, indicating that there was an obvious inflammatory reaction in the gastric mucosa. The inflammation and lesions were mainly located in the antropyloric region of the stomach, and the inflammation scores were higher in the model group than in the normal group and were closely related to inflammatory cell infiltration and damage. The arrangement of gastric antrum mucosal glands was disordered, but there was no obvious thinning or thickening, which was significantly different from CAG and the canceration stage of gastritis [5], indicating that the change in gastric mucosal cells was within the physiological range.

The statistical analysis revealed that in the normal group, the correlation coefficient was very small in the 5% level of significance, and there was no significant difference indicating that there was little change in the expression levels of PCNA, EGFR, and NF-κB p65 and there was no obvious correlation. However, in rats with chronic gastritis, the gastric mucosa was markedly inflamed, and there was a significant positive correlation between inflammation score and the expression of EGFR and NF-κB, with correlation coefficients of 0.674 and 0.606, respectively, and the difference was statistically significant. Meanwhile, there was also a significant positive correlation between the expression of EGFR and NF-κB, with a correlation coefficient of 0.666. The correlation between inflammation score and expression of PCNA was very low and negative, and there was no statistical significance. In other words, based on the changes in the contents of nuclear factors and cell proliferation factors related to CSG and morphological changes, during the development of chronic gastritis, the degree of inflammation of the gastric mucosa was positively correlated with the expression of EGFR and NF-κB p65, and there was also a significant positive correlation between NF-κB p65 and EGFR. The result showed that the expression of EGFR and NF-κB p65 played an important role in the occurrence and development of gastritis.

Inflammation is a crucial factor involved in the pathogenesis of gastric mucosal lesions in CSG. Gastric mucosal damage is accompanied by a substantial increase in the contents of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. The results showed that TNF-α and IL-6 expression levels in the CSG group were significantly higher than those in the control group. In the inflammatory environment, macrophages express a variety of cytokines, such as TNF-α, IL-1β, IL-6, and IL-1. The transgenic expression of inflammatory cytokines in the mouse stomach leads to gastritis and gastric tumor development [6–8], the development of gastritis is also related to cytokine gene polymorphisms [9]. IL-1β and IL-6 are pleiotropic inflammatory cytokines expressed during gastric inflammation, and their overexpression can induce gastric mucosal injury. Proinflammatory cytokines can induce the infiltration of neutrophils, trigger the production of additional inflammatory cytokines and result in an inflammatory response that aggravates gastric tissue damage [10, 11]. Moreover, inflammatory cytokines may trigger oxidative stress pathways and produce reactive oxygen species, which can lead to oxidative damage in gastric mucosal cells. However, the expression of proinflammatory factors is regulated by NF-κB. NF-κB is an important transcription factor expressed in the process of inflammation and immune response. There are functional combination points for NF-κB in many gene promoters and enhancers, which may regulate transcription and expression of many cell factors and inflammatory mediators related to inflammation [12] and mediate both acute and chronic inflammation [13]. After NF-κB is activated, it translocates into the nucleus, where it binds to specific sequences in promoter regions of target genes, further activates target genes, and causes the release of inflammatory factors (IL-1, IL-6, and TNF-α) and inflammatory response by regulating the transcription and expression of genes related to inflammation. Meanwhile, inflammatory factors can also cause further NF-κB activation and induce a cascade response. As a result, inflammation will continue and increase [14]. The experimental results revealed that in control rats, the positive expression of NF-κB p65 in the gastric epithelium was lower than that in CSG rats, and the optical density and the positive area of NF-κB p65 expression were significantly increased, which is consistent with a report by Cui et al. [15], who indicated that NF-κB plays an important role in CSG. Moreover, correlation analysis showed that CSG was closely and positively related to NF-κB p65 expression. Thus, the measurement of its changes could help assess stomach health and the degree of inflammation in the gastric mucosa.

The aberrant activation of NF-κB is invariably associated with inflammation. Activated NF-κB can cause gastritis via the induction of proinflammatory cytokines [16] and reactive oxygen species (ROS), which play an important role in DNA and cell membrane damage in gastric epithelial cells. The inflammation score is an important index used to evaluate the gastric mucosal inflammatory injury. This experiment showed that when the inflammation score was

significantly increased in model rats, high expression of NF-κB was also detected, which was consistent with the changes in the cytokine level and the inflammation score, indicating the degree of gastric mucosal damage. The odd ratio (OD) value of NF-κB was positively correlated with the inflammation score in rats, and there was a correlation between the activity of NF-kB and the inflammatory score, indicating that NF-κB expression correlated well with the severity of gastritis. These results are in agreement with findings by other researchers [17, 18]. The intensity of NF-κB staining correlates with the density of the inflammatory cell infiltrate comprised of neutrophilic and lymphocyte infiltrates but not eosinophilic infiltrates [19], indicating that there were plenty of neutrophilic infiltrates in CSG, which is consistent with the above pathological results. Research has shown that the activation of NF-κB is the primary factor responsible for the initial inflammatory response [18]. The role of NF-κB appears to be predominant in the early stages of the disease when it is responsible for the induction of neutrophilic infiltration. There is no role for NF-κB in the later stages of the disease [19]. Atrophy occurred in the later stages of the disease, and the grade of atrophy had either no or a negative correlation with NF-κB [20]. Atrophic gastritis gradually evolved from superficial gastritis, and superficial gastritis is the early stage of atrophic gastritis. Therefore, high expression of NF-κB also indicates that superficial gastritis at this time is the early stage of gastritis in the rat model.

Chronic inflammation of the gastric mucosa has a positive correlation with EGFR expression. EGFR is the receptor for EGF, and EGF is secreted by the submandibular gland, salivary gland, pancreas, and duodenum [21]. It can restrain the secretion of gastric acid, protect the gastric mucosa, and promote gastric epithelial repair and regeneration [22, 23]. These functions of EGF are achieved when EGF binds to its receptor, and changes in EGFR expression directly affect the function of EGF. The expression of EGFR was rare in the intact gastric mucosa; however, when the gastric mucosal barrier was damaged, its level was elevated. The study showed that there was a low expression of EGFR in the gastric epithelium of control rats and that the expression was higher in model rats, which may be a protective response to inflammatory stimulation in the process of modeling. EGFR expression was high in the mucosal surface layer but low in the deep and muscle layers of the stomach [24], consistent with the pathological location of superficial gastritis. Therefore, the increase in EGFR expression can also imply that there may be inflammation in the gastric mucosa. In addition, the increase in EGFR may also result from overexpression of NF-κB in CSG. Correlation analysis showed that there was a positive correlation between EGFR and NF-κB, with a correlation coefficient of 0.666. NF-κB is a transcription factor that can initiate and regulate the gene transcription and expression of many factors (including EGFR). The appropriate expression of EGFR is beneficial for the repair and regeneration of gastric mucosa [25], but in the case of long-term stimulation, because of repeated inflammatory stimulation, uncontrolled cell proliferation and carcinogenesis will occur [26, 27]. Therefore, it is helpful to assess the degree of mucosal lesions and the progression of CSG to detect changes in EGFR expression, just as PGI and PGII, gastrin-17, and *Helicobacter pylori* antibodies may be used as stomach-specific biomarkers for the noninvasive assessment, diagnosis, and screening of atrophic gastritis [28].

However, increased expression of EGFR has dual effects on gastric mucosal cells; while moderate expression of EGFR can promote mucosal epithelial cell proliferation and is beneficial for mucosal repair, overexpression may be related to canceration [29–31]. The inflammatory microenvironment of the gastric mucosa induces the activation of EGFR signaling. EGFR activation plays a critical role in gastric disease risk. EGFR can combine with EGF and affect the pathway of oncogene expression, disrupting the normal self-regulation of the cell cycle and resulting in the development of gastric cancer. There was high expression of the EGFR protein in the cancer stage of atrophic gastritis in rats, which indicated high trends of carcinogenesis in CAG [32–34]. The expression of EGFR ligands was significantly upregulated in both K19-C2mE mouse gastritis tissues and Gan mouse gastric tumors. These EGFR ligands are induced via a COX-2/PGE2-associated inflammation-dependent mechanism, which leads to the acceleration of tumor cell proliferation. Treatment with an EGFR inhibitor significantly suppressed gastric tumourigenesis [35], and EGFR mutations can modify the responsiveness to EGFR-inhibiting drugs and are associated with acquired resistance to inhibitors [36]. Therefore, the abnormal expression of EGFR is a molecular marker of the malignant growth trend of gastric epithelial cells, which is closely related to the occurrence of carcinogenesis [37].

It has also been shown that the relationship between inflammation of gastric mucosal and expression of PCNA is very small, with no significant difference. PCNA was discovered by Miyachi et al. [38] in the serum of patients with systemic lupus erythematosus in 1978 and was named so because of its presence in proliferative cells (including normal proliferating cells and cancer cells). The expression of PCNA has close relationships with the synthesis of DNA in cells [39], playing an important role in the start of cell proliferation, and is highly involved in cell cycle regulation, replication, repair, and apoptosis [40]. The expression of PCNA occurs mainly in the S and early G2 phases of the cell proliferation cycle, closely reflecting dynamic changes in cell proliferation, and an increase in PCNA expression suggests that cell proliferation is active. Our study found that expression of PCNA was lower in the control group and greater and uneven in the CSG group, as the optical density value and the positive area of PCNA expression increased significantly, indicating that mucosal repair was accelerated. However, the mechanism is not clear and requires further study. Increased expression of PCNA can accelerate mucosal repair and alleviate mucosal inflammation. However, if the stimulating factors exist for a long time, repeated stimulation of the inflammatory microenvironment will lead to its overexpression and then to unregulated mucosal hyperplasia

and the occurrence of gastric carcinoma. There have been many reports [41] regarding the high expression of PCNA in gastric cancer and precancerous lesions.

Conclusion

Our findings show that in the gastric epithelium, the high expression levels of NF-κB and EGFR play important roles in the occurrence and progression of CSG and that there is a strong correlation between NF-kB activation and inflammation score, which indicates the degree of inflammation in the gastric mucosa, suggesting that NF-kB activation is important for neutrophil infiltration and inflammatory factor production. Therefore, changes in NF-κ B and EGFR may be used as important indicators for the assessment of CSG, and the changes in their expression help assess the degree of gastric mucosal lesions and the progression of CSG.

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The relationship between perifoveal capillary ring alterations and visual acuity in diabetic retinopathy

Povezanost promena perifovealnog kapilarnog prstena i oštrine vida u dijabetesnoj retinopatiji

Nenad Petrović*†, Dušan Todorović*†, Sunčica Srećković*†, Tatjana Šarenac Vulović*†, Svetlana Jovanović*†, Svetlana Paunović*, Dejan Vulović‡§, Danijela Randjelović‖

University Clinical Center Kragujevac, *Clinic for Ophthalmology, ‡Center for Plastic Surgery, Kragujevac, Serbia; University of Kragujevac, Faculty of Medical Sciences, †Department of Ophthalmology, §Department of Surgery, Kragujevac, Serbia; ‖Aero Medical Institute, Clinic for Ophthalmology, Zemun, Serbia

Abstract

Background/Aim. The relationship between the foveal avascular zone (FAV) and visual acuity (VA) in retinal diseases remains a matter of discussion. The aim of this study was to determine the impact of diabetic macular ischemia (DMI) on VA through the analysis of the perifoveal capillary network in various stages of diabetic retinopathy - DR (non-proliferative diabetic retinopathy – NPDR and proliferative diabetic retinopathy – PDR). **Methods**. Qualitative and quantitative analysis of 143 angiograms of patients with different stages of DR was performed. The degree of macular ischemia was assessed by the analysis of 2 parameters: perifoveal capillary ring, *ie*, the FAZ outline irregularity, and capillary loss. Finally, a comparison was made between the degree of macular ischemia with the best-corrected VA, depending on macular thickness. **Results.** In the eyes with mild and moderate NPDR, without significant macular thickening, no statistically significant decrease in VA caused by macular ischemia was noticed (p = 0.81). Opposite, in a subgroup with severe NPDR and PDR, without significant macular thickening, a statistically significant difference was presented among eyes with moderate and severe macular ischemia compared to eyes with lower grades of macular ischemia (p = 0.021 and p = 0.018, respectively). In the eyes with moderate NPDR and mild macular ischemia, the increase in macular thickness resulted in a statistically insignificant decrease in VA compared to eyes with a normal macular thickness (p = 0.088). However, in the eyes with severe NPDR, every pathological increase in macular thickness caused a statistically significant decrease in VA, regardless of the degree of macular ischemia (p = 0.018–0.040). A similar relationship was also found in the eyes with PDR (p = 0.017–0.042). In the eyes with a statistically significant decrease in VA, most of the examined eyes (98%) had the FAZ outline irregularity in the nasal perifoveal subfield. **Conclusion.** In the absence of significant macular thickening, the destruction of one-half of the perifoveal capillary network, or greater, is associated with reduced VA. The location of macular ischemic changes in the nasal parts of the perifoveal capillary ring plays a crucial role in its effects on visual function.

Key words:
diabetic retinopathy; fluorescein angiography; ischemia; macula; retina; visual acuity.

Apstrakt

Uvod/Cilj. Povezanost fovealne avaslarne zone (FAZ) i oštrine vida (OV) u bolestima mrežnjače i dalje je predmet debata. Cilj rada bio je da se utvrdi uticaj dijabetesne makularne ishemije (DMI) na OV, analizom perifovealne kapilarne mreže u različitim fazama dijabetesne retinopatije – DR (neproliferativna DR – NPDR i proliferativna DR – PDR). **Metode.** Izvršena je kvalitativna i kvantitativna analiza 143 angiograma bolesnika sa različitim stadijumima DR. Stepen makularne ishemije procenjivan je analizom 2 parametra: perifovealnog kapilarnog prstena, tj. nepravilnostima oboda FAZ i stepena kapilarnog gubitka. Na kraju je izvršeno poređenje između stepena makularne ishemije i najbolje korigovane OV, u zavisnosti od makularne debljine. **Rezultati.** Kod očiju sa blagom i umerenom NPDR i bez značajnog zadebljanja makule, nije uočen statistički značajan pad OV izazvan makularnom ishemijom (p = 0,81). Nasuprot tome, kod očiju sa ozbiljnom NPDR i PDR, bez značajnog zadebljanja makule, utvrđena je statistički značajna razlika u OV kod očiju sa umerenom i ozbiljnom makularnom ishemijom u poređenju

Correspondence to: Dušan Todorović, University Clinical Center Kragujevac, Clinic for Ophthalmology, Zmaj Jovina 30 Street, 34 000 Kragujevac, Serbia. E-mail: drdusantodorovic@yahoo.com

sa očima gde je makularna ishemija bila manjeg stepena (p = 0,021 i p = 0,018, redom). Kod očiju sa ozbiljnom NPDR i blagom makularnom ishemijom, povećanje debljine makule rezultiralo je statistički neznačajnim smanjenjem OV u poređenju sa očima sa normalnom debljinom makule (p = 0,088). Međutim, kod očiju sa ozbiljnom NPDR, svako patološko povećanje debljine makule izazivalo je statistički značajno smanjenje OV, bez obzira na stepen makularne ishemije (p = 0,018–0,040). Slična povezanost je takođe pronađena kod očiju sa PDR (p = 0,017–0,042). Kod očiju sa statistički značajnim smanjenjem OV, većina njih (98%) imala je iregularnost FAZ konture u nazalnom perifovealnom subpolju. **Zaključak.** U odsustvu značajnog zadebljanja makule, destrukcija polovine oboda perifovealne kapilarne mreže ili više, povezana je sa smanjenom OV. Lokalizacija makularnih ishemijskih promena u nazalnim delovima perifovealnog kapilarnog prstena igra presudnu ulogu u njihovom efektu na funkciju vida.

Ključne reči:
dijabetesna retinopatija; angiografija, fluoresceinska; ishemija; žuta mrlja; mrežnjača; vid, oštrina.

Introduction

Diabetic retinopathy (DR) is the leading cause of vision loss in working, active adults. Diabetic macular edema (DME) is defined as the thickening of the macula that occurs due to an abnormal accumulation of edematous fluid in the retinal tissue. This progressively changes the anatomy of the macula and leads to progressive, irreversible photoreceptor degradation and vision loss. The degree of macular thickening is significantly correlated with visual acuity (VA). DME represents the most common cause of vision loss in patients affected by diabetes mellitus (DM), especially in type 2 diabetes [1]. The Early Treatment Diabetic Retinopathy Study defined "clinically significant macular edema"; this definition was introduced to indicate the involvement of the center of the macula and its relationship to visual loss [2].

The macula has one of the highest metabolic intensities per gram of tissue in the body [3]. The outer retinal layers are completely avascular and are dependent on metabolic support by diffusion from the choroidal vascular beds. The inner retina is predominantly supplied by the retinal circulation. The histologic findings have identified three different retinal capillary plexuses in the macular area: the superficial, the deep, and the intermediate capillary plexuses. The vessels in the nerve fiber layer and the ganglion cell layer form the superficial capillary plexus (SCP), while the inner and outer plexiform layers receive blood from the deep capillary plexus (DCP) located in the junction between them [4, 5].

The very center of the macula, the foveola, is mostly avascular and corresponds approximately to the foveal avascular zone (FAZ), which represents the capillary-free zone. The avascular region of the FAZ is surrounded by terminal capillaries forming a perifoveal capillary ring that often has an oval shape with a mean diameter of 362.3 ± 49.7 μm vertically and 410.8 ± 80.7 μm horizontally [4].

The size of the FAZ has been intensively studied both in the healthy eyes and in many retinal disorders. Many studies have shown that the size of the FAZ in normal human eyes can be very variable. In healthy eyes, there are large individual variations in the size of the FAZ, ranging between 0.05 up to 1.98 mm^2 [6–8]. Therefore, the correlation between the size of the FAZ and VA in the normal human eye has not been fully established yet. In healthy eyes, the size of the FAZ does not seem to influence visual function [9].

Macular edema is frequently associated with relative ischemia. Diabetic macular ischemia (DMI) is characterized by the occlusion and loss of the macular capillary network [10]. The health and integrity of the capillaries are essential for ganglion cell survival. The persistent ischemia of both the SCP and DCP may evolve into permanent neurosensory damage [11].

Clinically, macular ischemia (MI) is recognized by two characteristics: enlargement or irregularity of the FAZ and widening of the intercapillary spaces in the perifoveal area due to the capillary dropouts [12, 13]. MI can occur with or without macular edema, but it is very rare to find an isolated case of DMI.

Many studies have observed the increase of the FAZ area in the eyes with DR [7, 14, 15]. Some studies have reported that the relationship between the FAZ size and DMI severity occurs from the earliest stages of DR, non-proliferative DR (NPDR), while others have observed this relationship only in more advanced diseases [7, 15–17]. It is shown that the rate of the FAZ enlargement ranged between 5% and 10% of the baseline FAZ area per year in the eyes with diabetic MI [18].

The relationship between the FAZ size and VA in retinal diseases remains a matter of discussion. The effects of DMI on visual function are ill-defined. Some patients maintain VA near to normal levels in the presence of profound ischemia. Evidence of enlargement of the FAZ greater than 1,000 μm generally indicates visual loss [19].

Historically, since its introduction in the 1960s, conventional fluorescein angiography (FA) has been the gold standard procedure for evaluating the degree of DMI in patients with DR [12]. This technique typically shows enlargement and irregular shape of the FAZ, interruptions of the perifoveal capillary ring, and large areas of retinal hypofluorescence due to the absence of macular capillaries [20].

While previous studies have focused mainly on investigating changes in the size and shape of the FAZ in MI, this study primarily analyzes changes in the perifoveal capillary ring to determine whether its alterations may be a good indicator of the ischemic process.

Methods

The study was conducted at the Clinic for Ophthalmology, University Clinical Center Kragujevac, Serbia. It was designed as a retrospective, cross-sectional study. We performed a qualitative and quantitative analysis of 143 angiograms of patients with different stages of DR. In these pa-

tients, the diagnostic procedure of fluorescein angiography was performed from 2008 to 2019. The study included 123 patients: angiograms of only one eye were analyzed in 103 patients, and angiograms of both eyes in 20 patients.

The main inclusion criterion was the existence of DR. Patients with cataract, high refractive error, corneal leucoma, vitreal hemorrhage, glaucoma, uveitis, previous ocular surgery or trauma, and tractional retinal detachment were not able to participate in the study. Those who received antivascular endothelial growth factor (VEGF) therapy or intravitreal steroids were also excluded.

All angiograms were obtained by performing a standard FA procedure using a digital retinal camera (Carl Zeiss, Meditec, Inc., Dublin, CA) and an intravenous infusion of 5–10 mL of 10% sodium fluorescein. For each angiogram analyzed, there were accompanying fundus color photographs as well as the data on VA, intraocular pressure, and biomicroscopic status of the ocular media.

For all analyzed angiograms, there was an accompanying optical coherence tomography (OCT) image (Stratus Optical Coherence Tomography – OCT3, Carl Zeiss Meditec, Inc., Dublin, CA) with data of mean foveal thickness. For all participants, the best-corrected VA was measured using the Snellen chart.

Initially, the central parts of the angiogram of 3 × 3 mm were excised and then magnified five times. The angiograms processed in this way corresponded to the area of five macular fields according to the Early Treatment Diabetic Retinopathy Study (ETDRS) grid cells scheme: a central foveal ring with 1 mm diameter and an inner macular ring (pericentral) with 3 mm diameter divided into four subfields (nasal, temporal, superior, and inferior) [12]. Fluorescein angiograms were then independently evaluated by the two experienced retinal specialists.

The classification of DR was performed according to the ETDRS grading system [12]. According to these criteria, DR was classified into two basic stages: NPDR and proliferative DR (PDR). NPDR is further divided into mild, moderate, and severe.

The early-phase angiograms (up to 20 sec) were used to observe the superficial capillary plexus, while late-phase angiograms were used to assess leakage intensity, *ie*, blood-retina barrier (BRB) status.

In each of the 4 analyzed subfields (nasal, superior, temporal, and inferior), the irregularity of the perifoveal capillary ring was assessed as follows: Grade 0, normal (no disruption of the FAZ in that subfield); Grade 1, questionable (discrete ring irregularities in that subfield, but the changes are not clearly pathological); Grade 2, mild (outline of the FAZ is destroyed to 25% in that subfield); Grade 3, moderate (outline of the FAZ is destroyed 25% to 50% in that subfield); Grade 4, severe (capillary outline of the FAZ is completely destroyed in that subfield).

The cumulative FAZ outline irregularity was classified as follows: Grade 0, normal (no disruption of the FAZ); Grade 1, questionable (outline not smoothly round or oval, appreciable irregularities seen, but changes are not clearly pathological); Grade 2, mild (outline of the FAZ is destroyed for less than half the original circumference < 180°); Grade 3, moderate (outline of the FAZ is destroyed for greater than half the original circumference > 180°); Grade 4, severe (capillary outline of the FAZ is completely destroyed).

Outline of the FAZ was considered normal when the Grade ranged from 0 to 1, suspiciously abnormal when the Grade was 2, and abnormal when the grade ranged from 3 to 4.

In each of the 4 analyzed subfields (nasal, superior, temporal, and inferior), the capillary loss was assessed as follows: Grade 0, absent (no loss); Grade 1, questionable; Grade 2, minimal (up to 25% loss in the subfield); Grade 3, moderate (> 25% and up to 50% loss in the subfield); Grade 4, severe (> 50% loss in the subfield).

The cumulative capillary loss for all four subfields was graded according to the scale: Grade 0, absent (no loss); Grade 1, questionable; Grade 2, minimal (loss up to 25% of the entire perifoveal capillary network); Grade 3, moderate (> 25% and up to 50% loss of the entire perifoveal capillary network); Grade 4, severe (> 50% loss of the entire perifoveal capillary network).

The capillary loss was considered normal when the Grade ranged from 0 to 1, suspiciously abnormal when the Grade was 2, and abnormal when the Grade ranged from 3 to 4.

Using the fluorescence of the perifoveal vessels as a comparison, the intensity of leakage was classified into 4 grades: Grade 0 corresponded to the absence of leakage; Grade 1 corresponded to the presence of low-intensity leakage (less fluorescent than vessels); Grade 2 corresponded to the presence of mid-intensity leakage (similar fluorescence to the vessels); Grade 3 corresponded to the presence of high-intensity leakage (more fluorescent than the vessels); Grade 4, intensive early diffuse dye leakage that completely blocks the observation of individual blood vessels.

Finally, according to the analyzed parameters in all four subfields, a cumulative diabetic MI was calculated and classified as none (Grade 0), questionable (Grade 1), mild (Grade 2), moderate (Grade 3), and severe (Grade 4). Grades 0 and 1 were considered normal, the Grade 2 was suspected to be pathological, and Grades 3 and 4 were considered pathological.

As a pathological status of increased macular thickness, the OCT 3 definition was used: ≥ 305 μm for males and ≥ 290 μm for females [10]. We compared a cumulative diabetic MI with the best corrected VA depending on the macular thickness.

In analyzing statistical data, SPSS version 22 (IBM Corp., Armonk, NY, USA) was used. Examination of the incidence of the FAZ outline irregularity, capillary loss, and dye leakage was done using the χ^2 test and ANOVA. A value of $p < 0.05$ was considered statistically significant.

Results

The mean age of the participants was 64.27 ± 7.3 years (range 48–72 years). The male to female ratio was almost equal (male 75, female 68). No statistically significant difference was noticed, $p = 0.069$. The mean duration of DM was 15.12 ± 6.8 years. DM type I was presented in 37 pa-

tients, while the other 86 patients had DM type 2. As shown in Table 1, 120 eyes had NPDR (42 eyes mild, 41 eyes moderate, and 37 eyes severe NPDR), while 23 eyes had PDR. Until the moment of FA, 67 eyes had previous focal laser photocoagulation, while in 38 eyes (23 eyes with PDR and 15 eyes with NPDR), the panretinal laser photocoagulation was done. Until the moment of FA, none of the eyes had received intravitreal anti-VEGF therapy. Table 1 shows the distribution of three parameters according to the gradation, as well as the 4 subfields of the perifovea.

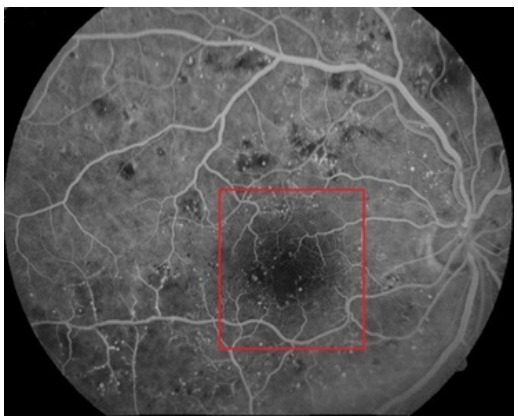
The cumulative DMI compared with the best corrected VA, depending on the macular thickness, is shown in Table 2. In the eyes with mild and moderate NPDR, none of the eyes had cumulative DMI Grades 3 and 4. In the eyes with severe NPDR, moderate and severe (Grades 3 and 4) MI was measured in 6 (16.2%) eyes, while in the eyes with PDR, moderate and severe MI was presented in 12 (52.2%) eyes.

Table 1

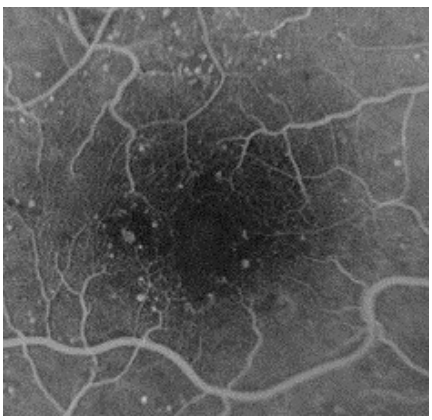
The distribution and gradation of the foveal avascular zone (FAZ) outline, capillary loss, and dye leakage between the groups

Diabetic retinopathy (DR)	FAZ outline subfield				Capillary loss subfield				Dye leakage subfield			
	N	S	T	I	N	S	T	I	N	S	T	I
NPDR												
mild (n = 42)												
0	38	40	41	40	38	39	40	39	36	37	38	37
1	3	1	1	1	3	2	2	2	6	5	4	5
2	1	1	0	1	1	1	0	1	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
moderate (n = 41)												
0	29	32	34	33	31	33	36	35	29	33	35	34
1	7	6	6	5	5	5	4	4	10	8	6	7
2	2	2	1	3	3	3	1	2	2	0	0	0
3	2	1	0	0	2	0	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0	0	0	0	0
severe (n = 37)												
0	18	22	26	22	20	24	26	25	21	24	27	23
1	6	8	5	7	6	8	9	7	9	9	8	10
2	7	4	3	4	9	4	2	4	5	3	2	3
3	3	2	2	2	2	1	0	1	2	1	0	1
4	3	1	1	2	0	0	0	0	0	0	0	0
PDR (n = 23)												
0	2	5	7	4	4	3	4	7	1	2	12	3
1	2	4	5	4	5	6	4	5	7	10	5	10
2	7	6	3	8	9	9	13	8	12	9	6	9
3	7	5	4	4	3	2	2	2	3	2	0	1
4	5	3	4	3	2	1	0	1	1	0	0	0

NPDR – nonproliferative DR; PDR – proliferative DR; N – nasal; S – superior; T – temporal; I – inferior.



a)



b)

Fig. 1 – a) Enlarged foveal avascular zone (FAZ) with the FAZ outline destruction ≤ 180°; b) the same figure magnified ×5.

Table 2
The distribution of cumulative diabetic macular ischemia (DMI) and mean macular thickness (MT), and the best-corrected visual acuity (BCVA) between the groups

Diabetic retinopathy (DR)	Cumulative DMI	Mean MT (μm) and mean corresponding BCVA		*p*
		< 305 (290) / BCVA	> 305 (290) / BCVA	
NPDR				
mild (n = 42)				
0	42	39 / 0.90	3 / 0.85	0.072
1	0	-	-	
2	0	-	-	
3	0	-	-	
4	0	-	-	
moderate (n = 41)				
0	35	29 / 0.90	6 / 0.70	0.051
1	5	4 / 0.85	1 / 0.80	0.088
2	1	1 / 0.90	0 / -	
3	0	-	- / -	
4	0	-	- / -	
severe (n = 37)				
0	14	12 / 0.70	2 / 0.35	0.023*
1	9	7 / 0.75	2 / 0.35	0.021*
2	8	6 / 0.65	2 / 0.35	0.018*
3	3	2 /0.33	1 / 0.20	0.040*
4	3	2 / 0.25	1 / 0.10	0.038*
PDR (n = 23)				
0	0	- / -	- / -	
1	3	3 / 0.65	0 / -	
2	8	7 / 0.70	1 / 0.20	0.023*
3	7	6 / 0.35	1 / 0.10	0.017*
4	5	4 / 0.20	1 / 0.05	0.042*

NPDR – nonproliferative DR; PDR – proliferative DR.
***– statistically significant.**

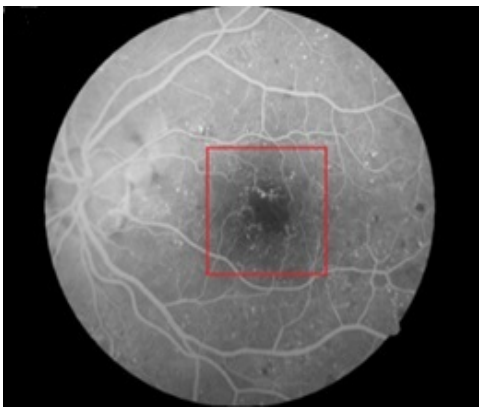
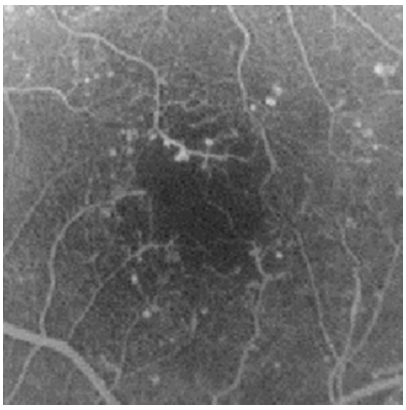

a)

b)

Fig. 2 – a) The cumulative foveal avascular zone (FAZ) outline destruction ≥ 180° with capillary loss ≤ 50%; b) the same figure magnified ×5.

According to the grade of ischemia, analyzing the eyes with mild and severe NPDR, a statistically significant difference was noticed ($p = 0.018$). Comparing severe with moderate NPDR, statistically significant difference was measured as well ($p = 0.037$). In the eyes with PDR, the highest statistically significant difference was found comparing them with the mild ($p = 0.002$), then with the moderate ($p = 0.022$), and finally with the severe NPDR ($p = 0.041$).

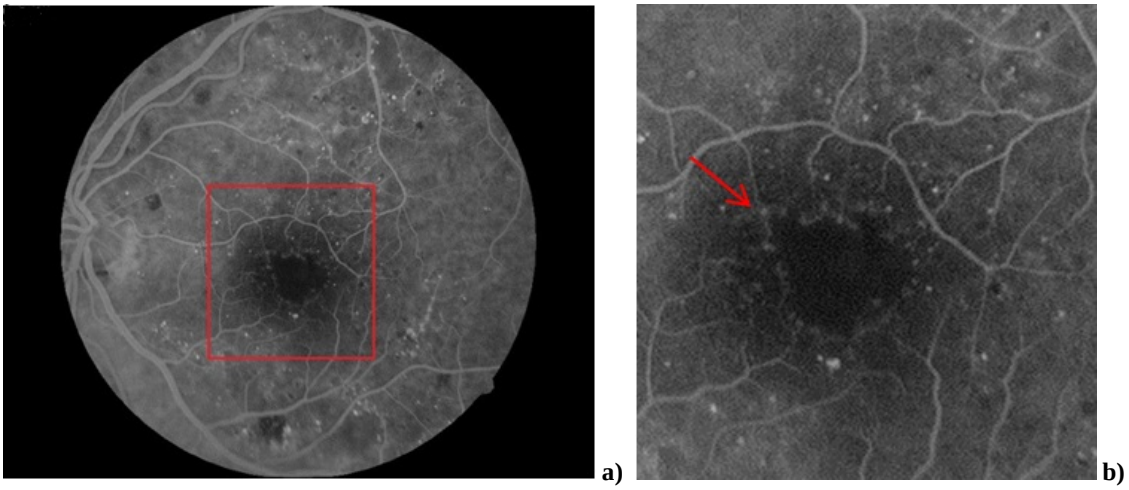
Figures 1a and 1b show the FAZ outline destruction ≤ 180° of the original circumference. Figures 2a and 2b show the FAZ outline destruction ≥ 180° of the original circumference with capillary loss ≤ 50%.

In subgroups with mild and moderate NPDR, without significant macular thickening, no statistically significant decrease in VA caused by MI was noticed ($p = 0.81$). Opposite, in the subgroup with severe NPDR and without significant

macular thickening, a statistically significant difference was presented among eyes with moderate and severe MI (Grades 3 and 4) compared to the eyes with lower Grades of MI (p = 0.021). A similar finding was found in the eyes with PDR (p = 0.018).

In the eyes with a statistically significant decrease of VA, most of the examined eyes (98%) had the FAZ outline destruction in the nasal subfield, while the superior and inferior subfields were destroyed in 71%, and the temporal subfield in 68% of the examined eyes.

As shown in Figures 3a and 3b, the FAZ outline destruction in the nasal subfield leads to a significant reduction in VA.



a) **b)**

Fig. 3 – a) Foveal avascular zone (FAZ) outline destruction in nasal subfield; b) the same figure magnified ×5.

In the eyes with the mild NPDR and without MI, the increased macular thickness caused a statistically insignificant decrease in VA compared to the eyes with a normal macular thickness (p = 0.072). A similar relationship was found in the eyes with the moderate NPDR (p = 0.051). In the eyes with the moderate NPDR and mild MI, the increase in macular thickness resulted in a statistically insignificant decrease in VA compared to the eyes with the normal macular thickness (p = 0.088). However, in the eyes with severe NPDR, every pathological increase in macular thickness caused a statistically significant decrease in VA, regardless of the degree of MI (p = 0.023, p = 0.021, p = 0.018, p = 0.040, p = 0.038). The similar relationship was found in the eyes with PDR (p = 0.023, p = 0.017, p = 0.042).

Discussion

The macula is a unique structural and functional region of the retina, and its nutrition must be well balanced. The outer retinal layers are completely avascular and are dependent on the metabolic support by the diffusion from the choroidal vascular beds. The inner retina is predominantly supplied by the retinal circulation [21–23]. The parafoveal region of the macula is supplied by the dense vasculature with approximately 9 pairs of arterioles and venules [6]. The histologic findings have identified three different retinal capillary plexuses in the macula area: the superficial, the deep, and the intermediate capillary plexuses. The vessels in the nerve fiber layer and the ganglion cell layer form SCP, while the inner and outer plexiform layers receive blood from DCP located in the junction between them. At the level of the SCP, the mean vascular density is 0.28 ± 0.1 mm^2, while at the level of the DCP, the mean vascular density is 0.37 ± 0.12 mm^2 [4, 5].

The very center of the macula, the foveola, is completely avascular and corresponds approximately to the FAZ. The size of the FAZ has been intensively studied both in the healthy eyes as well in many retinal disorders. Many studies have shown that in healthy eyes, there are large individual variations in the size of the FAZ, ranging from 0.05 up to 1.98 mm^2 [7–9, 16, 17, 24–28]. A correlation between the FAZ size and VA in the normal human eyes has not yet been precisely determined. In healthy eyes, the size of the FAZ does not seem to influence visual function [9, 29]. Nevertheless, the relationship between the FAZ size and VA in retinal vascular diseases remains a matter of discussion.

DME represents the most common cause of vision loss in patients affected by DM. The degree of macular thickening is significantly correlated with VA [30–33]. The common pathway that results in DME is disruption of the inner blood-retinal barrier [34, 35].

Macular edema is frequently associated with relative ischemia. Diabetic MI is characterized by the occlusion and loss of the macular capillary network. The capillary dropout may result in larger areas of nonperfusion with a widening of intercapillary spaces in the perifoveal area [10]. Diabetic MI is an important clinical feature of DR. Clinically, DMI is defined by an enlargement of the FAZ and paramacular areas of capillary nonperfusion. The FAZ seems to get larger as the stage of retinopathy advances [12, 13].

DMI can occur with or without macular edema, although it is very rare to find an isolated case of DMI. Thus, each macular edema is the result of two interrelated pathophysiological mechanisms that occur simultaneously: capillary occlusions and disruption of the BRB. Even in the absence of macular edema in diabetic eyes, abnormalities of the

FAZ are often seen, and they include irregular margins and widening of the intercapillary spaces [11, 36]. FA typically shows the large areas of retinal hypofluorescence due to the absence of macular capillaries. In the case of MI, early-phase FA usually can demonstrate enlargement of the FAZ, irregularity of the FAZ outline, a broken foveal capillary ring, and widening of perifoveal capillary spaces [12, 20]. In addition, based on the extent of dye leakage, the FA provides information on the condition of the inner BRB. However, fluorescein leakage does not always correlate with retinal thickness; a simple diffusion of fluorescein without retinal thickening is not included as a part of the definition of macular edema [31, 36, 37].

Optic coherence tomography is another useful clinical tool that can be used for detecting DMI. The ischemic areas of the macula appear thin during OCT investigation. However, the presence of edema usually makes the results of OCT difficult to interpret [38–40].

Many studies have observed the increase of the FAZ area in the eyes with DR [7, 14, 15]. Some studies have reported that the relationship between the FAZ size and DMI severity occurs from the earliest stages of DR [7, 16], while others have observed this relationship only in more advanced diseases [15, 17]. This discrepancy is most likely due to the large intersubject variability of the FAZ.

In our study, we have shown that some degree of MI may exist in the earliest stages of DR (mild and moderate NPDR), but the MI progressively intensifies during the advanced stages of the disease. There is a statistically significant difference in the degree of MI between the severe NPDR in relation to the mild NPDR ($p = 0.0016$) and the moderate NPDR ($p = 0.0022$). This difference is even more pronounced between PDR and the mild NPDR ($p = 0.0011$) and the moderate NPDR ($p = 0.0014$). Furthermore, the degree of MI is statistically slightly higher in PDR compared to the severe NPDR ($p = 0.041$). This finding is consistent with previous studies that have shown that in DR, the FAZ has been enlarged and seems to get larger as the stage of retinopathy advances [41]. It is estimated that the rate of the FAZ enlargement was between 5% and 10% of the baseline FAZ area per year in the eyes with DMI [18].

In this paper, the FAZ outline irregularity has been demonstrated to be a good indicator of MI. The parameters related to the shape of the FAZ may be better parameters for monitoring the FAZ rather than its size. This is in agreement with the results of previous studies that have shown that the circularity and axial ratio are changed significantly more than the size of the FAZ in the eyes with DR [42].

The effects of DMI on visual function are poorly defined. Some patients may have an almost normal level of VA in the presence of profound ischemia. Despite this, numerous studies have demonstrated the link between the presence of DMI and the loss of visual function [13, 19, 43]. Some patients can experience sudden and severe decreases in VA. In these cases, DMI is often responsible for unexplained visual loss, even if the clinical stage of the disease is early or mild [44].

Previous studies have mainly focused on the relationship between the increase in the FAZ size in DR and its effect on VA. It is generally accepted that the doubling of the FAZ size indicates ischemic maculopathy. The enlargement of the FAZ greater than 1,000 μm generally indicates visual loss [19, 45–47]. In their study, Arend et al. [43] showed that in diabetics with decreased VA (0.5 or worse), the FAZ was enlarged by 73% compared with patients whose VA was normal or near to normal (median VA 0.8) [43].

Due to the large variability in the FAZ size and topology in both normal and diseased eyes, we were mainly focused on determining the FAZ outline irregularities and their effect on VA. In our work, in the eyes with the mild and moderate NPDR, without significant macular thickening, MI does not affect visual function ($p = 0.068$, $p = 0.059$). Our study demonstrated that visual function was affected only in those people with moderate to severe MI.

In the eyes with severe NPDR and no significant macular thickening, there was a statistically significant difference in VA ($p = 0.033$) between eyes with the FAZ outline destruction greater than half the original circumference > 180° (Grades 3 and 4) compared to eyes where these alterations are milder (grades 1 and 2). A similar finding was found in the eyes with PDR ($p = 0.025$).

In the eyes with severe NPDR and PDR, in the absence of significant macular thickening, only the destruction of the FAZ outline for greater than half of the original circumference > 180° (Grades 3 and 4) resulted in a significant decrease in VA. Our results confirm, therefore, a definite link between MI and VA.

Our findings indicate that the location of MI changes plays a critical role in its effects on visual function. In our work, the ischemia of the nasal parts of the parafoveal capillary plexus had a particularly strong impact on VA. In almost all eyes with capillary nonperfusion in this part of the FAZ outline, there was a decrease in VA. The capillary network in the nasal parafoveal parts supplies the papillomacular nerve fibers originating from the fovea. We supposed that ischemia in these locations, which contain a high density of axons originating from the macula, may have an association with a reduction in VA. This finding is consistent with the results of other studies that observed a strong significant association between papillomacular ischemia and VA, independent of the FAZ size [44, 48].

The relationship of edema to changes in visual function is complex. In the eyes with the mild NPDR and without MI, the increased macular thickness caused a statistically insignificant decrease in VA compared to the eyes with a normal macular thickness ($p = 0.072$). A similar relationship was also found in the eyes with the moderate NPDR ($p = 0.051$). In the eyes with the moderate NPDR and the mild MI, the increase in macular thickness resulted in a statistically insignificant decrease in VA compared to the eyes with the normal macular thickness ($p = 0.088$). As shown in Table 2, in the eyes with severe NPDR, any pathological increase in macular thickness caused a statistically significant decrease in VA, regardless of the degree of MI. A similar relationship was also found in the eyes with PDR.

Our results suggest that ischemic maculopathy may be compatible with good VA if not accompanied by edema. The

occurrence of macular edema caused by leakage from residual macular capillaries leads to a greater decrease in VA, which is in agreement with the findings of other studies [14, 19]. As mentioned earlier, the fluorescein leakage does not always correlate with retinal thickness. However, in our study, there was a significant agreement on the degree of dye leakage and increased macular thickness in approximately 75% of the eyes.

DMI causes severe irreversible vision loss, and the severity of the disease increases with time [16, 17]. DMI is associated with a poor prognosis of DR. Some studies have linked DMI as a risk factor for progression of DR severity [49]. The one-year risk of developing progressive DR was found to be almost 42% in patients with DMI. The risk for disease progression was significantly lower (by 18%) in diabetics without DMI [50].

Until now, no defined successful treatment method for DMI has been found. The only possible treatment seems to be the management of the risk factors. These include the control of blood sugar levels and optimum blood pressure control. In addition, other risk factors like anemia and nephropathy should also be controlled [51].

Today, the intravitreal application of anti-VEGF drugs is a generally accepted method of treating macular edema, and those drugs can significantly reduce retinal edema. However, several studies have suggested that anti-VEGF therapy could have potential ischemic effects and further compromise the retinal circulation [52–54]. In this regard, a good assessment of the degree of MI is necessary before using these drugs to treat macular edema.

Conclusion

DMI is an important clinical feature of DR, and some degree of MI may exist in the earliest stages of the disease. Results of our study suggest that the assessment of the FAZ outline irregularity may be a good indicator of MI. Ischemic maculopathy may be compatible with good VA if not accompanied by edema. In the absence of significant macular thickening, the destruction of one-half of the perifoveal capillary network or larger is associated with reduced VA. The occurrence of macular edema caused by leakage from residual macular capillaries leads to a greater decrease in VA. The location of MI changes in the nasal parts of the perifoveal capillary ring has a particularly strong impact on VA. Before using anti-VEGF drugs in the treatment of macular edema, a good assessment of the degree of MI is required due to their potentially detrimental effect on the deterioration of macular perfusion.

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Redox status of pregnant women with thrombophilia

Redoks status trudnica sa trombofilijom

Aleksandra S. Dimitrijević*, Jovana V. Bradić†, Vladimir I. Živković‡, Aleksandra B. Dimitrijević§, Mirjana L. Milojević Čorbić‖, Janko M. Djurić§, Dragan V. Vasiljević*¶, Vladimir Lj. Jakovljević‡**

***Institute of Public Health Kragujevac, Kragujevac, Serbia; University of Kragujevac, Faculty of Medical Sciences, †Department of Pharmacy, ‡Department of Physiology, ¶Department of Hygiene, Kragujevac, Serbia; §University Clinical Center Kragujevac, Kragujevac, Serbia; ‖University of Kragujevac, Faculty of Medical Sciences, Kragujevac, Serbia; **University IM Sechenov, 1st Moscow State Medical, Department of Human Pathology, Moscow, Russia**

Abstract

Background/Aim. Since the role of oxidative stress in the pathogenesis of thrombophilia in pregnancy has still not been clarified, the aim of the study was to assess the redox status of pregnant women with thrombophilia. **Methods.** The study involved 120 pregnant women divided into two groups: pregnant women with thrombophilia (n = 60) and women with normal pregnancy (n = 60). Blood samples for biochemical analysis were collected at the end of the first, second, and third trimester of pregnancy. Concentrations of hydrogen peroxide (H_2O_2), nitrites (NO_2^-), and the index of lipid peroxidation measured as thiobarbituric acid reactive substances (TBARS) were measured in plasma. Levels of reduced glutathione (GSH), activities of superoxide dismutase (SOD) and catalase (CAT) were measured in erythrocytes. **Results.** In women with thrombophilia, NO_2^- values were increased in the first and third trimester compared to healthy pregnant women ($p < 0.05$). The higher levels of TBARS and H_2O_2 were noticed in women with thrombophilia in the first trimester compared to healthy pregnant women ($p < 0.05$). The values of SOD and CAT were lower in women with thrombophilia in the third and GSH in the first trimester compared to the control group ($p < 0.05$). **Conclusion.** Our results suggest an increased generation of prooxidants in thrombophilia at the beginning of gestation, which declines as gestation progresses and reaches similar values as in normal pregnancy at the end of pregnancy. Generally viewed, pregnant women with thrombophilia was associated with impaired antioxidant capacity – activities of SOD and CAT were lower in the third and GSH in the first trimester compared to their values in healthy pregnant women.

Key words:
antioxidants; oxidation-reduction; pregnancy; pregnancy trimesters; thrombophilia.

Apstrakt

Uvod/Cilj. Imajući u vidu da uloga oksidacionog stresa u patogenezi trombofilije u trudnoći nije u potpunosti razjašnjena, cilj rada je bio da se proceni redoks status trudnica sa trombofilijom. **Metode.** Studija je obuhvatila 120 trudnica koje su bile podeljene u dve grupe: grupu trudnica sa trombofilijom (n = 60) i grupu sa trudnoćom bez pridruženih bolesti (zdravim) (n = 60). Uzorci krvi za biohemijske analize su sakupljani na kraju prvog, drugog i trećeg trimestra trudnoće. U plazmi su određivane koncentracije vodonik peroksida (H_2O_2), nitrita (NO_2^-) i indeks lipidne peroksidacije meren kao reaktivne supstance tiobarbiturne kiseline (RSTBK). U lizatu eritrocita određivan je nivo redukovanog glutationa (GSH), aktivnosti superoksid dizmutaze (SOD) i katalaze (CAT). **Rezultati.** U grupi žena sa trombofilijom, vrednosti NO_2^- bile su povećane u prvom i trećem trimestru u poređenju sa zdravim trudnicama ($p < 0,05$). Viši nivoi RSTBK i H_2O_2 primećeni su kod žena sa trombofilijom u prvom trimestru, u poređenju sa zdravim trudnicama ($p < 0,05$). Vrednosti SOD i CAT bile su niže kod žena sa trombofilijom u trećem, a GSH u prvom trimestru trudnoće, u odnosu na kontrolnu grupu ($p < 0,05$). **Zaključak.** Naši rezultati ukazuju na to da je kod trudnica sa trombofilijom na početku trudnoće produkcija prooksidacionih parametara povećana, smanjuje se sa progresijom trudnoće i na kraju trudnoće dostiže vrednosti koje se beleže kod zdravih trudnica. Generalno posmatrano, trombofilija je bila povezana sa pogoršanjem antioksidacionog kapaciteta – aktivnosti SOD i CAT su bile niže u trećem, a nivo GSH u prvom trimestru, u poređenju sa njihovim vrednostima kod zdravih trudnica.

Ključne reči:
antioksidansi; oksidoredukcija; trudnoća; trudnoća, tromesečja; trombofilija.

Correspondence to: Vladimir I. Živković, University of Kragujevac, Faculty of Medical Sciences, Department of Physiology, Svetozara Markovica 69, 34 000 Kragujevac, Serbia. E-mail: vladimirziv@gmail.com

Introduction

Thrombophilia may be defined as a disorder of hemostasis characterized by an increased tendency to many thrombotic events [1, 2]. This predisposition to form clots may be both inheritable and acquired, or more commonly, it appears as an interaction between genetic and acquired factors. The most frequent inherited thrombophilic defects include deficiencies of antithrombin, protein C, protein S, mutations in the genes for coagulation factor V (factor V Leiden), and prothrombin G20210A gene polymorphisms. On the other hand, antiphospholipid antibody syndrome is a very often acquired thrombophilia [2, 3].

Epidemiological data have shown that thrombotic events are a significant cause of mortality and morbidity nowadays. There is a growing concern related to thrombophilia in pregnancy since it has been associated with an increased risk not only of pregnancy-related venous thromboembolism but also other complications, including severe preeclampsia/eclampsia, placental abruption, hemolysis, elevated liver enzyme levels, and low platelet levels HELLP syndrome, intrauterine growth restriction, and recurrent miscarriage [4, 5].

Oxidative stress is a condition defined as a disturbed equilibrium between prooxidants/antioxidants in favor of prooxidants [6]. Numerous papers have proven that increased production of prooxidants can be present during a normal pregnancy due to the high energy demands of many body functions [7–9]. The relation between oxidative stress and adverse pregnancy outcome and pregnancy complications such as preeclampsia and diabetes has been established [10]. Literature data indicate that a high oxidative state of the mother corresponds to a high oxidative state of the newborn, thus suggesting the importance of protecting the fetus before the birth process [11].

Furthermore, it has been proposed that thrombophilia during pregnancy induces hemostatic response and microthrombi formation, which leads to the generation of prooxidants. Those events cause further pro-coagulation and consequently further prooxidant generation, which results in many harmful effects on the placenta and placental circulation [12, 13]. Unfortunately, there is a lack of studies referring to the oxidative stress markers and antioxidative defence system during the three trimesters of pregnancy in women with thrombophilia.

Considering the standpoint that the role of oxidative stress in the pathogenesis of thrombophilia in pregnancy has still not been clarified, the aim of our study was to establish the redox status of pregnant women with thrombophilia.

Methods

Study design

This study was designed as a longitudinal study, and it was conducted in 2016. The study protocol was approved by the Medical Ethics Committee of the University Clinical Center "Kragujevac" from March 2, 2016 (01/2862) and was carried out according to the Declaration of Helsinki. All the participants were informed about the research protocol before giving their written consent to participate in the study.

Study population

A total of 120 pregnant women were included in the study. They were divided into two groups: thrombophilia group and normal pregnancy group. The thrombophilia group consisted of 60 pregnant women suffering from thrombophilia, while the normal pregnancy group included 60 physiologically healthy pregnant women. All 120 pregnant women were periodically examined at the Clinic of Hematology at the University Clinical Center "Kragujevac" and had an ultrasonic examination at the Clinic of Gynecology and Obstetrics at the University Clinical Center "Kragujevac" during the year 2016. Among 60 women with thrombophilia, half of them were methylenetetrahydrofolate reductase heterozygous, while the other half included plasminogen activator inhibitor homozygous. They received therapeutic doses of low molecular weight heparin for the treatment of thrombophilia. All participants of the study consumed multivitamin supplements that contained 100 mg of vitamin C and 5 mg of folic acid each day.

Our study involved pregnant women who were physiologically healthy, did not suffer from any chronic disease and have not used any medications, with no current complications, but who have had one or more pregnancies with complications – miscarriages, proven congenital or acquired thrombophilia, single and multiple pregnancies, pregnancy achieved by *in vitro* fertilization. None of the pregnant women had more serious complications during pregnancy and childbirth. All pregnant women gave birth to one child, while one pregnant woman gave birth to twins. Newborns of pregnant women with thrombophilia had a lower body weight at birth compared to healthy women.

Excluding criteria were the following: pregnancies that ended in miscarriage or missed abortion (the cessation of fetal cardiac activity before the 12th week of gestation) and pregnancies that ended with classic miscarriage (pregnancy loss occurring due to bleeding before the 12th week of gestation). Based on these criteria, 17 pregnant women were excluded from the study. Five pregnant women decided to leave the study, two pregnant women had an abortion due to pathologic fetal karyotype, and ten of them had a spontaneous abortion – nine of them had thrombophilia, and one was a physiologically healthy pregnant woman.

Biochemical analysis

Blood samples for biochemical analysis were collected in three specific periods of pregnancy, in each of three trimesters, at the end of the first, second, and third trimester of pregnancy. Blood samples were drawn from an antecubital vein into a Vacutainer test tube containing sodium citrate anticoagulant. Blood was centrifuged to separate plasma and red blood cells (RBCs). In plasma, the following parameters of redox balance were determined:

nitrites (NO_2^-), hydrogen peroxide (H_2O_2), and the index of lipid peroxidation (measured as thiobarbituric acid reactive substances – TBARS). Parameters of antioxidant defence systems such as superoxide dismutase (SOD), catalase (CAT), and reduced glutathione (GSH) were determined in erythrocytes samples. Biochemical parameters were measured spectrophotometrically using UV-1800 Shimadzu UV spectrophotometer, Japan.

H_2O_2 determination

The protocol for measuring H_2O_2 is based on the oxidation of phenol red in the presence of horseradish peroxidase [14]. A 200 μL sample with 800 μL phenol red solution and 10 μL horseradish peroxidase were combined (1 : 20). The level of H_2O_2 was measured at 610 nm. Distilled water was used as a blank control.

NO_2^- determination

Nitric oxide (NO) decomposes rapidly to form stable metabolite nitrite/nitrate products. Nitrites were determined as an index of NO production with Griess reagent [15]. A mixture of 0.1 mL 3 N perchloride acid, 0.4 mL 20 mM ethylenediaminetetraacetic acid (EDTA), and 0.2 mL plasma were put on ice for 15 min, then centrifuged for 15 min at 6,000 rpm. After pouring off the supernatant, 220 μL K_2CO_3 was added. Nitrites were measured at 550 nm. Distilled water was used as a blank probe [16].

The degree of lipid peroxidation in plasma was estimated by measuring TBARS using 1% thiobarbituric acid (TBA) in 0.05 NaOH, incubated with plasma at 100 °C for 15 min, and read at 530 nm. Distilled water was used as a blank probe. TBA extract was obtained by combining 0.8 mL plasma and 0.4 mL trichloro-acetic acid. The samples were then put on ice for 10 min and centrifuged for 15 min at 6,000 rpm. This method was described previously. Distilled water served as a blank probe [15].

Isolated RBCs were washed three times with three volumes of ice-cold 0.9 mmol/L NaCl and hemolysates containing about 50 g Hb/L (prepared according to McCord and Fridovich 1969) were used for the determination of CAT activity [17, 18]. Then 50 μL CAT buffer, 100 μL sample, and 1 mL 10 mM H_2O_2 were added to the samples. Detection was performed at 360 nm. SOD activity was determined by the epinephrine method. A 100 μL lysate and 1 ml carbonate buffer were mixed, and then 100 μL of epinephrine was added. Detection was performed at 470 nm. Distilled water was used as a blank probe [19].

The level of GSH was determined spectrophotometrically, and it is based on GSH oxidation via 5,5-dithiobis-6,2-nitrobenzoic acid. GSH extract was obtained by combining 0.1 mL 0.1 % EDTA, 400 μL hemolysate, and 750 μL precipitation solution (containing 1.67 g metaphosphoric acid, 0.2 g EDTA, 30 g NaCl, and filled with distilled water until 100 mL; the solution is stable for 3 weeks at +4 °C). After mixing in the vortex machine and extraction on ice (15 min), it was centrifuged at 4,000 rpm (10 min). Distilled water was used as a blank probe. Measuring was performed at 420 nm. The concentration is expressed as nanomoles per milliliter of RBCs [20].

Statistical analysis

IBM SPSS Statistics 20.0 for Windows was used for statistical analysis. Descriptive statistics were used to calculate the arithmetic mean with dispersion measures [standard deviation (SD) and standard error (SE)]. Values were expressed as mean ± SE. The distribution of data was checked by Shapiro–Wilk test. Data were analyzed using a one-way analysis of variance (ANOVA) and the *post hoc* Bonferroni test for multiple comparisons. Values of $p < 0.05$ were considered to be statistically significant.

Results

Values of NO_2^- were statistically significantly increased in the second trimester compared to the first and third trimester in the normal pregnancy group ($p < 0.05$). In women with thrombophilia, the level of this parameter was increased when compared to the levels in healthy pregnant women in the first and third trimester ($p < 0.05$) (Figure 1a).

In women with thrombophilia, the release of H_2O_2 was significantly higher in the first trimester compared to the second and third trimester ($p < 0.05$). On the other hand, there was no statistically significant change in H_2O_2 generation during the period of gestation in normal pregnancy. The only difference in the level of this parameter between healthy and thrombophilic mothers was noticed in the first trimester, where the level was higher in thrombophilic women ($p < 0.05$) (Figure 1b).

During the observed pregnancy period, in women with thrombophilia, the level of TBARS was higher in the first trimester compared to the second and third trimester ($p < 0.05$). In addition, the level of TBARS in the third trimester was increased compared to the second trimester ($p < 0.05$). In the normal pregnancy group, an increase in the level of this parameter was found in the third trimester compared to the level in the first trimester ($p < 0.05$). A higher level of TBARS was noticed in women with thrombophilia in the first trimester compared to healthy pregnant women ($p < 0.05$) (Figure 1c).

The activity of SOD in the first and second trimester was similar and higher than in the third trimester in women with thrombophilia ($p < 0.05$). During the followed period, lower activity of this enzyme was found in the first compared to the second trimester in healthy pregnant women ($p < 0.05$). The activity of SOD was lower in pregnancy with thrombophilia compared to normal pregnancy in the third trimester ($p < 0.05$) (Figure 2a).

In a group of pregnant women with thrombophilia, the highest activity of CAT was in the third trimester, significantly higher than in the first and second trimester ($p < 0.05$). The activity of CAT was increased in the first trimester compared to the second and third in normal pregnancy ($p < 0.05$). In addition, the lower activity of CAT

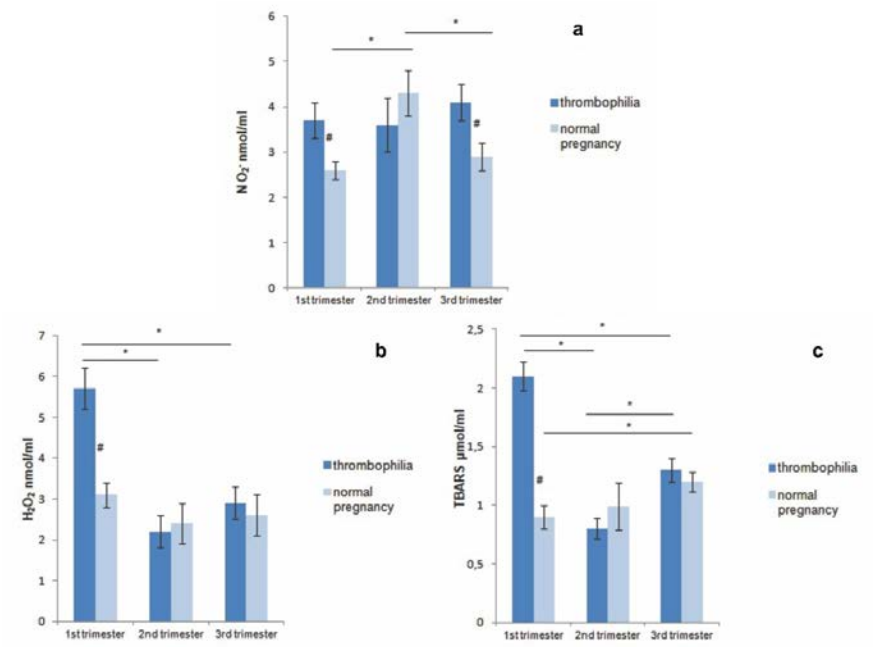

Fig. 1 – Prooxidants in healthy pregnant women and pregnant women with thrombophilia: a) NO_2^-; b) H_2O_2; c) TBARS. * statistical significance at the level of $p < 0.05$, which refers to the comparison of the 1st *vs* 2nd *vs* 3rd trimester; #statistical significance at the level of $p < 0.05$, which refers to the comparison of normal pregnancy *vs* thrombophilia.
NO – nitrites; H_2O_2 – hydrogen peroxide; TBARS – thiobarbituric acid reactive substances.

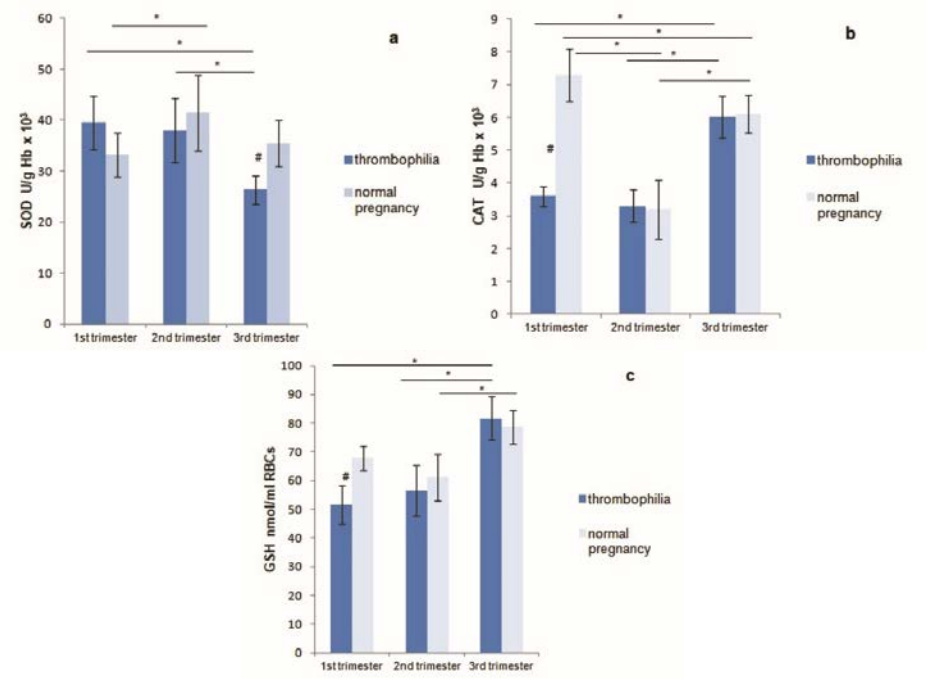

Fig. 2 – Antioxidants in healthy pregnant women and pregnant women with thrombophilia: a) SOD; b) CAT; c) GSH. *statistical significance at the level of $p < 0.05$, which refers to the comparison of the 1st *vs* 2nd *vs* 3rd trimester; #statistical significance at the level of $p < 0.05$, which refers to the comparison of normal pregnancy *vs* thrombophilia.
SOD – superoxide dismutase; CAT – catalase; GSH – reduced glutathione.

was revealed in the second compared to the third trimester in healthy pregnant women ($p < 0.05$). The values of this enzyme were higher in the normal pregnancy group compared to the thrombophilia group in the first trimester ($p < 0.05$) (Figure 2b).

Among women with thrombophilia, the value of GSH was similar in the first and second trimester, and those values were significantly lower compared to the value in the third trimester ($p < 0.05$). During the gestation period in healthy women, there was an increase in the level of GSH in the

third trimester compared to the second trimester ($p < 0.05$). The difference in the level of GSH was noticed in the first trimester between normal pregnancy and pregnancy with thrombophilia, where the level was lower in the thrombophilia group ($p < 0.05$) (Figure 2c).

Discussion

This research was designed to estimate the potential differences in dynamics of production of prooxidative and antioxidative markers during the gestation period in normal pregnancy and pregnancy with thrombophilia. In order to complete the picture of redox status in whole pregnancy, we have chosen to follow up on values of oxidative parameters in three periods, at the end of the first, second, and third trimester.

Our results indicate that in normal pregnancy, the release of H_2O_2 did not differ during the gestation period. In addition, NO_2^- levels raised in the second trimester compared to the first and then in the third returned to similar values from the first trimester. Lipid peroxidation was the lowest in the first trimester, then started increasing until the end of the third trimester. Other authors revealed that in the first trimester of pregnancy, lipid peroxidation was the lowest and then increased during pregnancy [21–23], which is in correlation with our findings. Few studies showed the different dynamics of lipid peroxidation during the gestation period, which may be a consequence of applying a different methodology from ours since they measured conjugated dienes or lipid hydroperoxides as a marker of peroxidation [24, 25].

CAT and GSH values were the lowest, while the value of SOD was the highest in the second trimester of normal pregnancies. On the other hand, the activity of CAT decreased in the third trimester compared to the first. It was previously reported that CAT and SOD activities in placental tissues increased as the pregnancy progressed, while glutathione peroxidase activity (GPx) remained unchanged during the whole pregnancy [22]. In addition, other researchers confirmed unaltered activities of CAT and GPx in maternal erythrocytes during gestation [23]. Antioxidant activity in placental tissues is a significant indicator of the oxidative state of mother and fetus since it has been discovered that a high mother oxidative stress corresponds to an even higher oxidative stress of the newborn [11].

In women with thrombophilia, NO_2^- did not significantly change during the pregnancy period, while TBARS and H_2O_2 levels were the highest in the first trimester, followed by a significant decrease in the second and third trimester. Enhanced activity of CAT, which catalyzes the decomposition of H_2O_2 to water and oxygen at the end of gestation, may explain obtained results for H_2O_2.

The special focus of our investigation was the potential difference in redox status between normal pregnancy and pregnancy with thrombophilia. We noticed higher levels of most of the measured prooxidants in pregnant women with thrombophilia during the first trimester, while during the second trimester, differences in observed values practically did not exist. Furthermore, NO_2^- was increased in the third trimester in the thrombophilia group as well. In addition to these changes, we revealed lower CAT and GSH values in the first trimester and lower activity of SOD in the third trimester in pregnancy with thrombophilia. Generally viewed, the difference between normal pregnancy and pregnancy with thrombophilia in the second trimester was not observed.

Lower antioxidative protection during pregnancy has been reported, but most studies were focused on the redox status of healthy pregnant women or women with diabetes, preeclampsia, etc. However, data referring to the antioxidative defence system during pregnancy with thrombophilia is limited. One research aimed to evaluate the redox status of the placental tissue after Caesarean delivery in pregnancy with thrombophilia and healthy pregnancy, with a focus on the investigation of the origin of oxidative stress by using both fetal blood and amniotic fluid as samples [13]. The obtained findings suggest that higher activities of CAT and GPx are present in the placental tissue of thrombophilic mothers compared to healthy mothers, and the major prooxidant affecting the placental tissue is H_2O_2, probably distributed from the mother's blood and endothelium. This research also showed that antioxidative activity in the fetal blood and oxidative status of amniotic fluid was not changed in thrombophilia, thus suggesting that H_2O_2 is most likely distributed to placental tissue from the mother's blood and endothelium [13].

We noticed an increased level of H_2O_2 in thrombophilia compared to normal pregnancy only in the first trimester. When H_2O_2 passes membranes of placental cells, the placenta tends to defend itself in thrombophilia by antioxidative system activation, particularly CAT, which is indirectly noticed in our study [11, 13]. It is of great importance given the fact that H_2O_2 can cause dysfunction of placental cells [26].

An association between oxidation markers in plasma and antioxidative defence enzymes *antepartum* and *postpartum* in thrombophilia was reported as well. Unlike healthy women, increased oxidative stress in thrombophilic women was noticed after delivery, thus suggesting that the placenta plays a role in antioxidative defence for maternal circulation antepartum [27]. Moreover, a very recent study from the same authors showed that SOD, CAT, and GSH are lower in thrombophilic than in healthy women, which is similar to our findings. Moreover, according to the dynamics of these antioxidative molecules through pregnancy, they highlighted oxidative stress increases with the progress of pregnancy [28].

Conclusion

Our results suggest an increased generation of prooxidants in thrombophilia at the beginning of gestation, which declines as gestation progresses and reaches similar

values as in normal pregnancy at the end of pregnancy. Generally viewed, thrombophilia was associated with impaired antioxidant capacity – SOD and CAT were lower in the third and GSH in the first trimester compared to healthy women. The connection between thrombophilia and oxidative stress suggests the possibility of implementing antioxidant therapy as an adjuvant in the treatment of thrombophilia in pregnant women.

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Conflict of interest

The authors declare no conflict of interest.

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The effects of combined physical procedures on the functional status of patients with diabetic polyneuropathy

Uticaj kombinovanih fizikalnih procedura na funkcionalni status bolesnika sa dijabetesnom polineuropatijom

Vesna Grbović*†, Svetlana Djukić‡§, Srdjan Stefanović‖, Nataša Zdravković-Petrović‡¶, Stefan Simović‡, Aleksandra Jurisić-Škevin*†**

University Clinical Center Kragujevac, *Center for Physical Medicine and Rehabilitation, Clinic for Internal Medicine, §Center for Endocrinology, ¶Center for Gastroenterohepatology, **Clinic for Cardiology, Kragujevac, Serbia; University of Kragujevac, Faculty of Medical Sciences, †Department of Physical Medicine and Rehabilitation, ‡Department of Internal Medicine, ‖Department of Clinical Pharmacy, Kragujevac, Serbia

Abstract

Background/Aim. Diabetic polyneuropathy is a common chronic complication in patients with diabetes mellitus. The aim of this study was to determine the importance of applied physical procedures on the functional status of diabetic polyneuropathy patients compared to the group of respondents treated by alpha-lipoic acid. **Methods.** Sixty subjects were divided into two groups: group A – diabetic polyneuropathy patients treated with physical procedures, and group B – diabetic polyneuropathy patients treated with alpha-lipoic acid. The study has lasted for three diagnostic and therapeutic cycles, each lasting for 16 days with a time between cycles of 6 weeks. **Results.** Manual muscle test, range of motion, Michigan Neuropathy Screening Instrument, and Berg balance scale values showed statistically significant improvement at the end of testing group A respondents, while no improvement was shown in group B respondents. **Conclusion.** The application of the combined physical procedures shows clear benefits for the improvement of muscle strength and mobility of the ankle joint in respondents with diabetic polyneuropathy.

Key words:
ankle joint; diabetic neuropathies; muscle strength; physical therapy modalities; range of motion, articular; thiotic acid.

Apstrakt

Uvod/Cilj. Dijabetesna polineuropatija (DP) je česta hronična komplikacija kod bolesnika sa dijabetesom melitusom. Cilj rada bio je da se utvrdi značaj primenjenih fizikalnih procedura na funkcionalni status bolesnika sa DP u poređenju sa grupom ispitanika lečenih alfa-lipoičnom kiselinom. **Metode.** Ukupno 60 ispitanika je bilo podeljeno u dve grupe: grupa A – bolesnici sa DP lečeni fizikalnim procedurama i grupa B – bolesnici sa DP lečeni alfa-lipoičnom kiselinom. Studija je bila sprovedena tokom tri dijagnostička i terapijska ciklusa, od kojih je svaki trajao 16 dana, sa periodom između ciklusa od 6 nedelja. **Rezultati.** Vrednosti Manuelnog mišićnog testa, obima pokreta, *Michigan Neuropathi Screening Instrument*-a i Bergove skale ravnoteže pokazali su statistički značajno poboljšanje na kraju testiranja kod ispitanika grupe A, dok kod ispitanika grupe B nije ustanovljeno poboljšanje. **Zaključak.** Primena kombinovane fizikalne terapije pozitivno utiče na poboljšanje mišićne snage i pokretljivosti skočnog zgloba kod bolesnika sa DP.

Ključne reči:
skočni zglob; dijabetesne neuropatije; mišići, snaga; fizikalna terapija, metodi; pokretljivost; tioktinska kiselina.

Introduction

The common chronic complication in patients with diabetes mellitus (DM) is diabetic polyneuropathy (DPN) or distal sensorimotor polyneuropathy (DSP) (in more than 50%, it occurs after 25 years of the disease duration) [1]. There are approximately 600,000 people in Serbia suffering from DM, or 8.2% of the population (out of which 95%

Correspondence to: Nataša Zdravković-Petrović, University of Kragujevac, Faculty of Medical Sciences, Department of Internal Medicine, Svetozara Markovića 69, 34 000 Kragujevac, Serbia. E-mail: natasasilvester@gmail.com

account for patients with type 2 DM), and 5.9% have diabetic neuropathy [2]. There is an increased risk of deformity, ulceration, and amputation in these patients [3].

DSP leads to postural balance disorders and increased susceptibility to falls [4]. Deterioration of motor nerve fibers weakens the intensity of stimulation of the muscles, which become hypotrophic. Injuries related to falls in these patients are 15 times more often compared to healthy respondents of the same age [5]. In addition, muscle weakness and limitations of mobility in the ankles and the small joints of the foot occur over time. These disorders result in functional foot impairment, changing pressure points on the foot, and the creation of ulceration [6]. Even 30% of diabetics have limited movements of small or large joints. Limited mobility in the ankle joint and metatarsophalangeal (MTPH) joint is caused by the thickening and shortening of the ligaments and tendons, leading to increased plantar pressure of the forefoot [7]. Muscle weakness mainly dominates in the distal segments of the lower limb, thus threatening gait and other activities in daily life [8, 9].

This study aimed to determine the importance of applied physical procedures on muscle strength and range of motion (ROM) in patients with DPN compared to those treated by alpha-lipoic acid (control group of patients). It also aimed to determine whether there was a statistically significant difference in the values of the Michigan test for examining the neuropathy at the beginning and at the end of the study between the group of respondents who underwent the combined physical therapy and the group of respondents who took alpha-lipoic acid.

Methods

This prospective study included 60 randomized patients older than 18 years at the Center for Physical Medicine and Rehabilitation, University Clinical Center Kragujevac, Serbia and was approved by the local Ethics Committee of the University Clinical Center Kragujevac (no. 01-13598 from 22/12/2011). This study was part of the previously published studies by Grbović et al. [10, 11].

Inclusion criteria were the following: DPN with a timeframe longer than 2 months with DPN signs and symptoms defined as pain, muscular weakness, paresthesia, hyperesthesia to anesthesia, and electromyoneurographic (EMNG) findings; without changes in antidiabetic treatment for at least 6 months; patients who signed consent for participation in the study.

Patients who had the following exclusion criteria were excluded from the study: vitamin B12 deficiency, moderate/severe use of alcohol, chronic kidney disease, dysfunction of the thyroid gland, any state of immunodeficiency, diseases of systemic connective tissues, severe damage of the liver, any kind of cerebrovascular ischemia, decompensation of heart failure, presence of acute coronary syndrome in the previous 6 months, uncontrolled high blood pressure (defined as values of systolic blood pressure higher than 160 mmHg and diastolic blood pressure higher than 80 mmHg), chemotherapy in the last decade, severe polytrauma or state after it, use of peripheral nerves damaging drugs (*eg*, nitrofurantoin, paclitaxel, vincristine, cisplatin, indomethacin, emetine, streptomycin, dapsone, chloroquine, ethionamide, isoniazid, carbamazepine, phenytoin, hydralazine, metronidazole, amiodarone); any sort of contraindication for using any of the arranged physical therapy agents (acute infectious disease, fever, pregnancy, malignancy, any acute vital organ failure, the presence of metal in body); or hypersensitivity to galactose, alpha-lipoic acid, Lapp lactose deficiency, or glucose-galactose malabsorption.

The investigation was organized during 3 cycles of diagnostics and therapeutics, each lasting for 16 days with a timeframe between cycles of 6 ± 1 week (total study duration of six months).

All subjects were divided into two groups, 30 patients in each group, with DM type 2 and DSP, in light of clinical indications and signs, just as the parameters of EMNG discoveries. Utilizing computer randomization, every patient was arbitrarily assigned to one group (therapeutical arms): group A or B.

The group A was treated with combined physical procedures that included a pulsed electromagnetic field, exercise, stable galvanization, and transcutaneous electrical nerve stimulation, while the group B was treated with alpha-lipoic acid as per conditions specified in the marketing license in Serbia. Detailed methods applied in these groups can be found in our previously published study [10].

On admission and after completion of the last diagnostical-therapeutical cycle (after 6 months), the EMNG of the lower extremities, Michigan Neuropathy Screening Instrument (MNSI), evaluation of functionality (manual muscle test – MMT and of range of motion – ROM), and Berg balance scale were done. EMNG examination was made with a Medtronic Keypoint device (Denmark, Skovlunde, www.medtronic.com). MNSI comprises two parts. The first part was a questionnaire that consisted of 15 questions, and the second part included the examination of the patient as follows: inspection of the foot (to determine if there are any changes on the feet, ulcerations, infections, calluses, deformities, etc.), the examination of muscle-tendon reflexes, vibratory sensibility testing and examination of monofilaments. The score ranged from 0 (best result) to 10 (worst result), and the score was the result of marks for both legs. Diagnosis of diabetic peripheral neuropathy with a physical examination score higher than 2.5 was established [12]. The examined were *musculus (m.) triceps surae*, *m. peroneus longus*, *m. tibialis posterior*, *m. tibialis anterior* and *m. peroneus brevis*, based on factors of manual loading and gravity. Gradation of muscle strength was performed according to Kendall, namely: 10 points = grade 5 on MMT; 9 = 4+; 8 = 4; 7 = 4-; 6 = 3+; 5 = 3; 4 = 3-; 3 = 2+; 2 = 2; 1 = 2-; 0 = 0, whereby grade 5 matches the strength of a normal muscle which can make a full ROM against

gravity and a maximum of manual loading; grade 0 means that during the attempt of a movement, a muscle does not show any visible or palpation sensitive contraction [13].

ROM was tested by dorsal flexion, plantar flexion, eversion, and inversion of the foot. ROMs were measured by a manual goniometer. Active dorsal flexion is normally up to 30°, plantar flexion to 45°, inversion to 35°, and eversion up to 10° [14].

Berg balance scale (BBS) examined the balance in elderly people with vestibular disorders, assessing it through specific functional tasks. This is a valid instrument used to evaluate the efficiency of treatment, the quantitative description of the function in the clinical practice, as well as the research. The scale includes 14 functional tasks for assessing the balance in adults in clinical conditions with grades from 0 (the worst result) to 4 (the best result). The full value of 41–56 indicates a low level of the risk of falling; 21–40 = medium level of the risk of falling; 0–20 = high level of the risk of falling. An eight-point difference is enough to show a change in function between the two measurements [15].

The distribution of all continuous variables was determined using the Shapiro-Wilk test, the median value, minimum and maximum values, and standard deviation (SD). Paired *t*-test was used for comparing the mean values of continuous variables within the tested groups with normal distribution or Wilcoxon's test of matched pairs. Independent *t*-test or the Mann-Whitney test for datasets without a normal distribution were used to compare differences between the groups. For comparison of the frequency (incidence) of categorical (dichotomous) variables, the χ^2 test was used. The *p* values less than 0.05 were considered statistically significant. SPSS version 20.0 was used for statistical calculations. The statistics procedures were the same as in our previously published study [10, 11].

Results

The baseline characteristics of respondents in the groups A and B are given in Table 1. Since this study is part of previously published studies by Grbović et al. [10, 11], the detailed baseline characteristics of respondents from the group A and the group B are given in those manuscripts.

MNSI showed statistically significant improvement at the end of testing the group A respondents ($p < 0.001$), while in the group B respondents, no improvement was shown ($p = 0.169$). BBS examination showed that there was a statistically significant improvement at the end of testing the group A respondents ($p = 0.001$), while in the group B there was no significant improvement shown ($p \approx 1.000$).

At the end of the treatment, there was a significant improvement in the dorsal flexion ($p < 0.001$) and plantar flexion ($p = 0.022$) in the group A respondents. At the start of the study, no significant differences were observed in the measures in the range of motion (homogeneous in dorsal flexion, $p = 0.884$; plantar flexion $p = 0.557$; inversion and eversion of the foot $p \approx 1.000$). At the end of the intervention, both the A and B group respondents did not differ significantly in the observed measures in the range of motion (they were homogeneous in dorsal flexion, $p = 0.055$; plantar flexion, $p \approx 1.000$; inversion of the foot, $p = 0.634$ and eversion of the foot, $p \approx 1.000$) (Table 2).

Table 1

Baseline characteristics of the patients with diabetic polyneuropathy (DPN)

Characteristic	Group A (n = 30)	Group B (n = 30)	*p*-value
Sex, n (%)			
male	11 (36.67)	13 (43.33)	0.598[a]
female	19 (63.33)	17 (56.67)	
Heredity for DM, n (%)			
yes	13 (43.33)	13 (43.33)	≈ 1.000[a]
no	17 (56.67)	17 (56.67)	
Age (years), mean ± SD	63.17 ± 7.68	62.77 ± 8.35	0.09[c]
Duration of diabetes (years), mean ± SD	12.22 ± 7.58	11.70 ± 5.75	0.09[c]
HbA1c (%), mean ± SD	7.80 ± 1.87	7.30 ± 1.21	0.403[c]
MNSI questionnaire, mean ± SD	8.57 ± 1.23	7.83 ± 0.79	0.008[c]*
MNSI examination, mean ± SD			
before	6.32 ± 0.23	5.95 ± 0.21	0.008[c]*
after	5.82 ± 0.23	5.87 ± 0.20	0.245[c]
p-value	< 0.001[b]*	0.169[b]	
Berg balance scale, mean ± SD			
before	43.23 ± 1.28	43.60 ± 1.13	0.244[c]
after	43.87 ± 1.28	44.67 ± 1.56	0.34[c]
p-value	0.001[b]*	≈ 1.000[b]	

DM – diabetes mellitus; HbA1c – glycosylated hemoglobin; MNSI – Michigan Neuropathy Screening Instrument; SD – standard deviation.
Statistical test used: [a] – χ^2 test; [b] – Paired sample *t*-test; [c] – Independent sample *t*-test; * significance at *p*-value < 0.05.

Table 2

Range of motion in the ankle joint

Parameter	Group A (n = 30)	Group B (n = 30)	*p*-value
Ankle dorsal flexion			
before	16.33 ± 4.90	16.17 ± 3.87	0.884[b]
after	19.50 ± 1.52	18.50 ± 2.33	0.055[b]
p-value	< 0.001[a]	0.659[a]	
Ankle plantar flexion			
before	40.50 ± 9.50	41.83 ± 7.93	0.557[b]
after	42.17 ± 6.39	42.17 ± 7.39	≈ 1.000[b]
p-value	0.022[a]	≈ 1.000[a]	
Foot inversion			
before	22.67 ± 6.40	22.67 ± 6.12	≈ 1.000[b]
after	23.33 ± 3.56	22.83 ± 4.49	0.634[b]
p-value	0.058[a]	0.083[a]	
Foot eversion			
before	4.33 ± 1.73	4.33 ± 1.73	≈ 1.000[b]
after	4.67 ± 1.27	4.67 ± 1.27	≈ 1.000[b]
p-value	≈ 1.000[a]	≈ 1.000[a]	

All values are given as mean ± standard deviation. The statistical test used: [a] – Paired sample *t*-test; [b] – Independent sample *t*-test.

Table 3 shows the analyzed muscles of the lower extremities. After the intervention, the A group respondents showed significant improvement in muscle strength of *m. tibialis anterior* ($p = 0.002$), *m. tibialis posterior* ($p < 0.001$), *m. triceps surae* ($p = 0.004$), and *m. peronei* ($p < 0.001$). At the beginning of the study, the A and B group respondents were not significantly different in the followed parameters (homogeneous in muscle strength of *m. tibialis anterior*, $p = 0.55$; *m. tibialis posterior*, $p = 0.32$; and *m. triceps surae*, $p = 0.915$), except in muscle strength of *m. peronei* ($p = 0.024$). At the end of the intervention, the A and B group respondents did not differ significantly in muscle strength of observed muscles (*m. tibialis anterior*, $p = 0.276$; *m. tibialis posterior*, $p = 0.457$; *m. triceps surae*, $p = 0.58$; *m. peronei*, $p = 0.098$).

Discussion

Our study shows the clear benefits of the combined application of physical procedures for the improvement of muscle strength and mobility of the ankle joint in respondents with DPN.

The 2014 study indicated the importance of the implementation of exercise in patients with diabetic neuropathy. The testing included 55 respondents with DPN, 26 of which had exercised (2× per week, 12 weeks), while 29 respondents were in the control group. After 12 and 24 weeks, it led to an improvement in foot functionalities in the intervention group [16].

After a 10-week exercise program, Kluding et al. [17] showed a statistically significant improvement in neuropathic symptoms measured by MNSI ($p = 0.01$), while there was no

Table 3

Manual muscle test values of muscles of the lower extremities

Muscle	Group A (n = 30)	Group B (n = 30)	*p*-value
M. triceps surae			
before	4.47 ± 1.14	4.47 ± 0.97	0.276[b]
after	5.00 ± 1.29	5.03 ± 1.13	0.915[b]
p-value	0.004[a]	0.742[a]	
M. tibialis posterior			
before	4.60 ± 0.97	4.80 ± 1.09	0.457[b]
after	5.03 ± 1.00	5.03 ± 1.29	0.32[b]
p-value	0.001[a]	0.321[a]	
M. tibialis anterior			
before	5.20 ± 1.32	5.40 ± 1.43	0.58[b]
after	5.40 ± 1.38	5.63 ± 1.61	0.55[b]
p-value	0.002[a]	0.096[a]	
M. peroneus longus et brevis			
before	5.00 ± 1.39	4.47 ± 1.04	0.098[b]
after	5.73 ± 1.64	4.83 ± 1.34	0.024[b]
p-value	< 0.001[a]	0.419[a]	

All values are given as mean ± standard deviation. The statistical test used: [a] – Paired sample *t*-test; [b] – Independent sample *t*-test; *M. – musculus*.

improvement made in electroneurographic parameters of *n. peroneus*, *n. tibialis* and *n. suralis*. This study was completed by 17 respondents with DPN (8 males/9 females; age 58.4 ± 5.98 years; DM duration of 12.4 ± 12.2 years) or 63.3% of respondents who started the program, which correlates with the results obtained in our study. However, our study also showed improvement not only in the manual muscle test but also in some electroneurographic parameters [10], which could be a result of the combined effect of different physical procedures rather than just exercises used in the study by Kluding et al. [17]. Bosi et al. [18] conducted a study on the effect of pulsed electromagnetic fields in the treatment of diabetic neuropathy, which included 101 respondents (the first group consisting of 50 respondents with the applied pulse magnetic field and the second group consisting of 51 respondents who received placebo). MNSI was being examined and, at the end of the study (3 months later), showed no significant differences between the two groups. Since this study used only the pulse magnetic field and we used multiple physical procedures, again, we could only hypothesize that the improvement in MNSI score in our study was the result of the combined physical procedures.

The 2007 study [19] examined the effect of alpha-lipoic acid in diabetic neuropathy. It included 95 respondents with DPN. The first group included 52 respondents who had been receiving alpha-lipoic acid (600 mg, parenterally) for 14 days, and the second group included 43 respondents with placebo treatment applied. MNSI was examined 7 and 14 days later. There were statistically significant reduced values of the Michigan questionnaire for testing neuropathy in a group of respondents with the applied alpha-lipoic acid ($p < 0.01$) [19]. In our study, we found that multiple physical procedures had a significant effect on MNSI score, but not for patients treated with alpha-lipoic acid. However, patients treated with alpha-lipoic acid had significantly lower MNSI scores before starting the treatment than patients treated with physical procedures.

The study by Song et al. [20] indicated the importance of applying exercise to improve the balance in patients with DPN. After the 8-week exercise implementation (60 min, 2× per week), a statistically significant improvement in balance was made (BBS, $p < 0.05$). As in this study, our study showed that in patients treated with physical procedures, a statistically significant improvement was made in postural equipoise and balance measured by BBS ($p = 0.001$). The group B respondents showed no statistically significant improvements.

Another study indicated the weakness of the foot muscles and limited mobility of the foot joints and ankle joints in patients with DPN, which later became risk factors for foot deformities and ulceration [21]. At the end of our study, the clear effects of the combined application of physical procedures were shown in increasing the range of motion in ankles (dorsal and plantar flexion), while in the second group, such an effect was not achieved.

The study by Andersen et al. [9] also dealt with the examination of muscle strength in patients with DM. The study involved two respondent groups, 36 respondents each (the first group consisted of those suffering from DM, and the second control group consisted of healthy respondents). Muscle strength was determined by using an isokinetic dynamometer. At the end of the study, it was concluded that the first group of respondents had the decreased muscle strength of flexor and extensor of the knee, ankle, wrist, and elbow joint and that it is associated with neuropathy [9]. The importance of the exercise applied in patients with DPN was confirmed in the study by Francia et al. [22]. The study included 26 patients with DM and 17 patients in the control group. After 12 weeks of an exercise program, there has been an increase in muscle strength, mobility, and walking speed, thus preventing the occurrence of disabilities [22].

Similarly to the results of these studies, in our study, at the end of the treatment, we found a significant increase in muscle strength of tested muscles (*m. tibialis anterior*, *m. tibialis posterior*, *m. triceps surae*, and *m. peronei*), while in the group B respondents with the alpha-lipoic acid treatmen, no improvement was made.

Conclusion

The application of the combined physical procedures (pulsed electromagnetic field, exercise, stable galvanization, and transcutaneous electrical nerve stimulation) shows their clear benefit for the improvement of muscle strength and mobility of the ankle joint in respondents with DPN. This is reflected in a significant improvement of neurological symptoms and signs (MNSI), as well as in strengthening muscles and increasing mobility of the respondents, and in improving postural balance.

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Conflict of interest

The authors declare no conflicts of interest.

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Does bronchial asthma influence dental health of the diseased children?

Da li bronhijalna astma utiče na zdravlje zuba obolele dece?

Bojana Davidović*, Mirjana Ivanović†, Dejan Bokonjić‡, Svjetlana Janković*, Jelena Erić*, Jelena Lečić*, Olivera Jovičić†

***University of East Sarajevo, Faculty of Medicine, Foča, Bosnia and Herzegovina; †University of Belgrade, Faculty of Dental Medicine, Department of Pediatric and Preventive Dentistry, Belgrade, Serbia; ‡University Hospital in Foča, Pediatric Clinic, Foča, Bosnia and Herzegovina**

Abstract

Background/Aim. Asthma is a chronic inflammatory lung disorder. The effect of asthma drugs on oral health is still the subject of debate among researchers in dentistry. The aim of this study was to evaluate dental status in asthmatic children and evaluate the possible effect of drugs treating asthma on dental health. **Methods.** Study participants were divided into two groups: the asthma (AG) and the non-asthma (NAG) group. Based on the symptoms of asthma and the possibility for effective control of the disease, the AG group was divided into two subgroups. The oral examination of the teeth was performed using a probe and mouth mirror under artificial light in accordance with the recommendations of the World Health Organization. Saliva analysis was carried out by the GC Saliva-Check Buffer, according to the manufacturer's instructions. **Results.** The study included 136 children aged 6 to 16 years (10.5 ± 3.3). The mean of decayed, missing, and filled teeth (dmft/DMFT) of the children in the AG group (6.0 ± 4.0/3.3 ± 4.4) was higher than in the NAG group (4.8 ± 4.4/2.5 ± 3.4), but significant differences were not observed between the groups. Salivary pH values were found to be similar in both groups, but the quantity and buffering capacity of the stimulated saliva were found to be significantly lower in the AG group ($p < 0.001$ and $p < 0.05$, respectively). **Conclusion.** Although the prevalence of dental caries in the AG group was similar to that of the NAG group in this study, decreased quantity and buffering capacity of the stimulated saliva in the AG group may contribute to higher values of dental caries in asthmatic children in the future.

Key words:
anti-asthmatic agents; asthma; child; dental caries; saliva.

Apstrakt

Uvod/Cilj. Bronhijalna astma je hronično zapaljensko oboljenje disajnih puteva. Uticaj antiastmatskih lekova na oralno zdravlje još uvek je predmet istraživanja u stomatologiji. Cilj rada je bio da se proceni zdravlje zuba dece sa astmom, kao i da se ispita uticaj antiastmatskih lekova na stanje zdravlja zuba. **Metode.** Ispitanici su bili podeljeni na dve grupe: deca sa astmom (AG) i deca bez astme (NAG). Na osnovu prisutnih simptoma astme, kao i mogućnosti njene efikasne kontrolisanosti, grupa AG je podeljena u dve podgrupe. Stomatološki pregled obavljen je pomoću stomatološke sonde i ogledalca pod veštačkim osvetljenjem, u saglasnosti sa preporukama Svetske zdravstvene organizacije. Za analizu pljuvačke je korišćen *GC Saliva-Check Buffer*, prema uputstvu proizvođača. **Rezultati.** Ispitivanje je obuhvatilo 136 dece, uzrasta od 6 do 16 godina (10,5 ± 3,3). Prosečne vrednosti karijesnog, ekstrahovanog, plombiranog (kep/KEP) zuba kod dece u grupi AG (6,0 ± 4,0/3,3 ± 4,4) bile su više u odnosu na iste vrednosti u grupi NAG (4,8 ± 4,4/2,5 ± 3,4), ali nisu utvrđene značajne razlike između ispitivanih grupa. Takođe, pH vrednosti pljuvačke bile su slične u obe grupe, ali su nađene značajno niže vrednosti količine i puferskog kapaciteta stimulisane pljuvačke u grupi AG ($p < 0,001$ i $p < 0,05$ redom). **Zaključak.** Iako je u ovoj studiji prevalenca karijesa u grupi AG bila slična kao i u grupi NAG, smanjene vrednosti količine i puferskog kapaciteta stimulisane pljuvačke u grupi AG mogu doprineti većoj podložnosti karijesu te grupe u budućnosti.

Ključne reči:
antiastmatici; astma; deca; zub, karijes; pljuvačka.

Correspondence to: Bojana Davidović, University of East Sarajevo, Faculty of Medicine, Studentska 5, 73 300 Foča, Bosnia and Herzegovina. E-mail: boki1310@yahoo.com

Introduction

Bronchial asthma is a chronic inflammatory disorder of the airways that causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing (particularly at night or early in the morning) [1]. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. Asthma prevalence has been increasing across all ages and gender worldwide. Asthma is a serious global health problem that usually starts in childhood, and the patients have to take lifelong therapy [2]. The majority of asthmatic patients require long-term medication, which is usually administered using various forms of inhalers. The effect of these drugs on oral health has been the subject of debate among dental practitioners [3].

Systemic diseases like bronchial asthma have a detrimental effect on the oral cavity, such as reduction of salivary secretion and change in salivary composition and pH. The negative effects of dental caries occurrence include pain, dysfunction, poor appearance, and speech problems [4]. However, these oral health problems vary from person to person and depend on the frequency of cariogenic drinks and food consumption, as well as oral hygiene.

Hamid et al. [5] suggested that dental caries and asthma are the most common chronic diseases in childhood. Children with chronic medical disorders mostly require long-term medication, which puts them at high risk of oral diseases in general and dental caries in particular [5].

Caries lesions are formed through a complex interaction between acid-producing bacteria and fermentable carbohydrates. It is also affected by many host factors, including saliva and the inherent nature of the teeth [6]. Saliva plays an important role in oral health as it maintains the integrity of oral hard and soft tissues and protects the oral tissue against bacterial, fungal, and viral infections. Moreover, salivary buffers can reverse the low pH, thus preventing enamel demineralization. Furthermore, an increase in caries prevalence in asthmatic patients was reported to be associated with prolonged use of ß2 agonists that lead to decreased salivary flow, altered saliva composition, and decreased pH, affecting the protective properties of saliva [7]. Higher rates of caries among asthmatics were considered possible due to antiasthmatic medications containing fermentable carbohydrates and sugar [3].

The aim of this study was to compare the prevalence of dental caries in asthmatic and non-asthmatic children, as well as to evaluate the relationship between the types, time of taking, and the duration of asthma medication and dental caries in asthma groups.

Methods

This study was based and designed according to the recommendations for epidemiological surveys (National Oral Health Survey) defined by the World Health Organization (WHO) [8]. This one-year-long study was conducted according to the Declaration of Helsinki of 1975, as revised in 2000. The study was approved by the Ethical Committee of Faculty of Medicine in Foča, University of East Sarajevo (No. 01-8/37).

Study population

The parents and children involved in the study were informed about the objectives and procedure. The parents approved the participation of their children in this study by signing written consent, and children were permitted to leave the process at any time during the study.

The present study was conducted among 136 children aged 6 to 16 years. Participants were divided into two groups. The first group consisted of 68 children with asthma (asthmatic group – AG), without any other systemic disease, treated at the University Hospital or Primary Care Facility in Foča. The second group consisted of children without asthma (non-asthmatic group – NAG) or any other chronic disease who visited the Faculty of Medicine in Foča, Dentistry Program. The AG and NAG were matched by gender and age (± 6 months).

Symptoms were evaluated according to medical history, physical examination, spirometry results, and data obtained after completing the basic disease-related questionnaire. The level of asthma control was determined according to the results of the asthma control test. Based on the symptoms of asthma and the level of asthma control, the AG group was divided into two subgroups. The first subgroup consisted of children with good controlled asthma (GCA), while the second subgroup consisted of children with partly controlled asthma (PCA). Asthma was considered to be under good control if: children had symptoms no more than 2 days a week; these symptoms did not wake them from sleep more than 1 or 2 nights a month; they could perform all usual activities; they took quick-relief medicines no more than 2 days a week; they did not have more than one asthma attack a year that required taking systemic steroids, and their peak flow did not drop below 80 percent of their personal best value. Asthma was considered only partly controlled if: children had symptoms more than 2 days a week; they could not perform normal physical activities and they had nocturnal symptoms; they had more asthma attacks a year that required taking asthma drugs, and their peak flow dropped below 80 percent of their personal best values [1]. Children with uncontrolled asthma were not recorded in this study.

Dental examination

Dental examinations were carried out in a dental chair by using a dental mirror and probe under artificial light, according to the WHO criteria [8]. All participants were examined, and saliva samples were collected at the Department of Pediatric and Preventive Dentistry, Faculty of Medicine in Foča.

Clinical measurements

Caries status was determined by the number of decayed (d/D), missing (m/M), and filled (f/F) primary (dmft) and permanent (DMFT) teeth. No radiographs were taken.

Saliva sampling was conducted in the morning hours. Individuals were instructed not to drink, eat, or use chewing gum for at least 2 h before sampling, not to rinse their mouths, and not to take medicine at least an hour prior to the visit. Patients were placed in an upright, relaxed position with their heads tilted down. Saliva hydration, salivary consistency, resting saliva pH, stimulated saliva flow (quantity), stimulated saliva pH, and saliva buffering capacity were recorded using the GC Saliva-Check Buffer test (GC Corporation, Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis

The results' analysis was carried out using the Statistical Package for Social Sciences (SPSS version 19.0 for Windows, SPSS Inc., Chicago, IL, USA). The χ^2 tests were used to compare differences in categorical variables. An Independent-samples *t*-test was used to compare differences in dental caries status between the AG and NAG, as well as between the subgroups. One-way analysis of variance (ANOVA) was used to test differences in the caries status in relation to the time of taking medication and the type of administered medication. The Mann-Whitney test was used for saliva testing between the AG and NAG, as well as the subgroup with good controlled asthma and partly controlled asthma. A $p < 0.05$ was considered statistically significant.

Results

The study population consisted of 68 (50%) asthmatic and 68 (50%) non-asthmatic children. There were 53 (39%) boys and 15 (11%) girls in the AG ($p < 0.05$) compared to 53 (39%) boys and 15 (11%) girls in the NAG ($p < 0.05$). The mean ages were 10.5 ± 3.3 years and 10.5 ± 3.3 years, respectively, in the two groups. In the NAG, 63.0% of participants were using inhaled corticosteroids (ICS), 13.2% bronchodilators (inhaled long-acting beta-2 agonists), and 23.5% combination of steroid inhalers and bronchodilators. On average, 27.9% of respondents used asthma medications for at least two years, and 57.7% of respondents were taking asthma drugs several times a day.

Characteristics of the participants' salivary samples are presented in Table 1. The resting saliva flow rate was approximately the same between the AG and the NAG. Moreover, there was no statistically significant difference in resting saliva flow between the GCA and PCA subgroups. Sticky saliva was more common in the AG than in the NAG. Lower salivary pH was found in the PCA subgroup. The very low buffering ability of saliva was observed in 27.9% of patients in the AG compared to 13.2% of patients in the NAG (Table 1).

The percentage of children with caries-free primary dentition in the AG and NAG was 16.7% and 25%, respectively, while the percentage of children with caries-free permanent dentition in the AG and NAG was 28.6% and 44.5%, respectively. The results of the present study showed a higher prevalence and severity of dental caries among asthmatic patients compared to the matched healthy children in both primary and permanent dentitions (Tables 2 and 3), but these differences were not statistically significant. Results indicated higher values of mean d/D, F, and DMFT in the AG, group in children who were using the anti-asthmatic drugs in the afternoon (Tables 4 and 5). Moreover, the dmft/ DMFT were higher in the PCA subgroup compared to the GCA, both in primary and permanent dentition, but statistical significance was not observed.

Table 1

Values of saliva testing in the asthma group (AG), non-asthma group (NAG), and subgroups of asthmatic children

Saliva testing (parameters)	AG (n = 68)	NAG (n = 68)	GCA (n = 44)	PCA (n = 24)	*p/q*
Hydration, RS (% ± SD)					
< 30 s	30.9 ± 0.8	38.2 ± 0.7	34.1 ± 0.8	25 ± 0.7	*p = ns; †p = ns
30–60 s	35.5 ± 0.8	45.6 ± 0.7	34.1 ± 0.8	37.5 ± 0.7	
> 60 s	33.8 ± 0.8	16.2 ± 0.7	31.8 ± 0.8	37.5 ± 0.7	
Viscosity, RS (% ± SD)					
watery	26.5 ± 0 .8	45.6 ± 0.7	31.8 ± 0.8	16.7 ± 0.7	*p = 0.001; †p = ns
frothy	33.8 ± 0.8	39.7 ± 0.7	31.8 ± 0.8	37.5 ± 0.7	
sticky	39.7 ± 0.8	1.7 ± 0.7	36.4 ± 0.8	45.8 ± 0.7	
pH, RS (mean ± SD)	6.8 ± 0.5	6.7 ± 0.4	6.9 ± 0.5	6.7 ± 0.5	*q = ns; †q < 0.05
Quantity, SS (% ± SD)					
> 5 mL	29.4 ± 0.8	44.1 ± 0.6	29.5 ± 0.8	29.2 ± 0.8	*p = 0.001; †p = ns
3.5–5 mL	26.5 ± 0.8	42.6 ± 0.6	31.8 ± 0.8	16.7 ± 0.8	
< 3.5 mL	44.1 ± 0.8	13.2 ± 0.6	38.6 ± 0.8	54.2 ± 0.8	
Buffering capacity, SS (% ± SD)					
normal	19.1 ± 0.6	26.5 ± 0.6	22.7 ± 0.6	12.5 ± 0.6	*p < 0.05; †p = ns
low	52.9 ± 0.6	60.3 ± 0.6	54.5 ± 0.6	50 ± 0.6	
very low	27.9 ± 0.6	13.2 ± 0.6	22.7 ± 0.6	47.5 ± 0.6	

GCA – good controlled asthma; PCA – partly controlled asthma; RS – resting saliva; SS – stimulated saliva; n – number of subjects; % – percentage of subjects; *p* – level of statistical significance (Mann-Whitney test); *q* – level of statistical significance (Independent-samples *t*-test); ns – non significant; SD – standard deviation. * – statistical significance between AG and NAG; † – statistical significance between GCA and PCA.

Table 2

Values of dental status in primary dentition

Study group	d ± SD	m ± SD	f ± SD	dmft ± SD
AG (n = 30)	4.6 ± 3.5	1.2 ± 2.2	0.2 ± 0.5	6.0 ± 4.0
NAG (n = 28)	3.4 ± 3.8	0.7 ± 1.5	0.6 ± 1.5	4.8 ± 4.4
GCA (n = 21)	4.4 ± 3.7	0.9 ± 2.1	0.1 ± 0.3	5.5 ± 4.3
PCA (n = 9)	5.2 ± 3.3	1.7 ± 2.5	0.3 ± 0.7	7.3 ± 3.1
p	*ns; †ns	*ns; †ns	*ns; †ns	*ns; †ns

AG – children with asthma; NAG – children without asthma; GCA – good controlled asthma; PCA – partly controlled asthma; n – number of subjects; dmft index – number of decayed (d), missing (m) and filled (f) primary teeth; SD – standard deviation; *p* – level of statistical significance (Independent-samples *t*-test); ns – non significant.
*** – statistical significance between AG and NAG; † – statistical significance between GCA and PCA.**

Table 3

Values of dental status in permanent dentition

Study group	D ± SD	M ± SD	F ± SD	DMFT ± SD
AG (n = 68)	1.5 ± 3.1	0.2 ± 0.5	1.6 ± 2.2	3.3 ± 4.4
NAG (n = 68)	0.6 ± 1.4	0.3 ± 1.2	1.7 ± 2.5	2.5 ± 3.3
GCA (n = 44)	1.0 ± 2.1	0.2 ± 0.5	1.4 ± 1.9	2.6 ± 3.6
PCA (n = 24)	2.4 ± 4.3	0.3 ± 0.6	1.8 ± 2.7	4.5 ± 5.5
p	*< 0.05; †< 0.05	*ns; †ns	*ns; †ns	*ns; †ns

AG – children with asthma; NAG – children without asthma; GCA – good controlled asthma; PCA – partly controlled asthma; n – number of subjects; DMFT index – number of decayed (D), missing (M), and filled (F) permanent teeth; SD – standard deviation; *p* – level of statistical significance (Independent-samples *t*-test); ns – non significant.
*** – statistical significance between AG and NAG; † – statistical significance between GCA and PCA.**

Table 4

Values of dental status (primary dentition) in relation to time of medication administration

Parameters of dental status	Only in the morning	Only in the afternoon	In the morning and evening	Before sleeping	*p*
Decayed teeth ± SD					
AG (n = 30)	3.4 ± 3.6	0.0 ± 0.0	3.5 ± 3.5	7.0 ± 2.5	< 0.05
GCA (n = 21)	3.6 ± 4.3	0.0 ± 0.0	3.7 ± 3.7	6.3 ± 2.8	ns
PCA (n = 9)	3.0 ± 1.4	0.0 ± 0.0	3.0 ± 3.0	8.0 ± 1.8	< 0.05
Missing teeth ± SD					
AG (n = 30)	2.4 ± 3.2	0.0 ± 0.0	1.2 ± 2.3	0.3 ± 0.6	ns
GCA (n = 21)	1.2 ± 2.6	0.0 ± 0.0	1.1 ± 2.5	0.3 ± 0.6	ns
PCA (n = 9)	5.5 ± 2.1	0.0 ± 0.0	1.6 ± 1.5	0.0 ± 0.0	< 0.01
Filled teeth ± SD					
AG (n = 30)	0.3 ± 0.7	0.0 ± 0.0	0.2 ± 0.4	0.3 ± 0.5	ns
GCA (n = 21)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.4	0.0 ± 0.0	ns
PCA (n = 9)	0.3 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.5	ns
dmft ± SD					
AG (n = 30)	6.1 ± 5.2	0.0 ± 0.0	4.9 ± 4.1	7.4 ± 2.7	ns
GCA (n = 21)	4.8 ± 5.7	0.0 ± 0.0	5.0 ± 4.3	6.8 ± 3.4	ns
PCA (n = 9)	9.5 ± 0.7	0.0 ± 0.0	4.6 ± 4.2	8.3 ± 1.5	ns

AG – Children with asthma; GCA – good controlled asthma; PCA – partly controlled asthma; dmft index – number of decayed (d), missing (m) and filled (f) primary teeth; n – number of subjects; SD – standard deviation; *p* – level of statistical significance (One-way ANOVA); ns – non significant.

Table 5

Values of dental status (permanent dentition) in relation to time of medication administration

Parameters of dental status	Only in the morning	Only in the afternoon	In the morning and evening	Before sleeping	*p*
Decayed teeth ± SD					
AG (n = 63)	1.2 ± 2.3	11.5 ± 7.7	1.1 ± 1.8	1.6 ± 3.8	< 0.001
GCA (n = 40)	1.1 ± 2.4	0.0 ± 0.0	1.0 ± 2.2	0.6 ± 0.5	ns
PCA (n = 23)	1.5 ± 2.2	11.5 ± 7.7	1.1 ± 1.3	2.8 ± 5.7	< 0.01
Missing teeth ± SD					
AG (n = 63)	0.0 ± 0.0	0.5 ± 0.7	0.3 ± 0.6	0.1 ± 0.3	ns
GCA (n = 40)	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.6	0.0 ± 0.0	ns
PCA (n = 23)	0.0 ± 0.0	0.5 ± 0.7	0.3 ± 0.6	0.1 ± 0.3	ns
Filled teeth ± SD					
AG (n = 63)	0.9 ± 1.2	2.5 ± 2.1	2.1 ± 2.6	0.3 ± 0.5	< 0.05
GCA (n = 40)	1.0 ± 1.2	0.0 ± 0.0	1.8 ± 2.2	0.3 ± 0.5	ns
PCA (n = 23)	0.5 ± 0.7	2.5 ± 2.1	2.6 ± 3.2	0.0 ± 0.0	ns
DMFT ± SD					
AG (n = 63)	2.1 ± 3.3	14.5 ± 9.2	3.5 ± 3.9	2.0 ± 4.1	< 0.01
GCA (n = 40)	2.1 ± 3.3	0.0 ± 0.0	3.2 ± 4.0	1.2 ± 0.9	ns
PCA (n = 23)	2.0 ± 2.8	14.5 ± 9.2	4.0 ± 3.7	3.0 ± 6.2	< 0.05

AG – children with asthma; GCA – good controlled asthma; PCA – partly controlled asthma; n – number of subjects; DMFT index – number of decayed (D), missing (M), and filled (F) permanent teeth; SD – standard deviation; *p* – level of statistical significance (One-way ANOVA); ns – non significant.

Discussion

Oral health is an important part of overall health. Therefore, the promotion of oral health and the quality of life is an important objective of modern dentistry. Literature data indicate a possible association between systemic diseases, including asthma, and oral health. Systemic diseases can affect the defense mechanisms and patient's motivation and may be considered risk factors for oral diseases [3, 7].

Bronchodilators play a major role in asthma therapy, while corticosteroids are second in line. The more severe forms of asthma require a combination of several antiasthmatic drugs. A recent study showed that inhalation drugs have some negative impact on oral health, depending on their dosage, frequency, and length of use [9]. Factors associated with the severity of the disease and/or medicaments used for treatment may increase the risk for the development of caries due to the reduced secretion of saliva, as well as lower salivary pH in asthmatics [3, 6, 7, 10–15]. Moreover, certain inhalers contain fermentable carbohydrates in the form of lactose, which mask the bitter medication taste and improve patient tolerance but may contribute to an increased caries risk as well [15].

Normal salivary flow is one of the most important protective factors against caries. All changes in the amount or composition of the saliva can alter the oral health status. This study showed that asthmatic children had a smaller amount of unstimulated saliva. Furthermore, the saliva was stickier in the asthmatic children, and the resting salivary flow was below normal as droplets of saliva were formed at the orifices of the minor glands in more than 60 seconds. Sticky and less viscous saliva facilitates accumulation and adhesion of bacteria to the tooth surface, as well as retention of deposits in the mouth, as the capacity of saliva to flush microorganisms and substrates and maintain oral cleanliness may be influenced by its consistency and flow rate [16].

Stimulated salivary flow is important to facilitate flushing away acids originating from the food, dental plaque, or other sources (like gastric reflux). In this study, the amount of stimulated saliva was reduced in the AG. The stimulated flow was lower than 3.5 mL/min in almost half of the children in this group, followed by low and very low buffering capacity. Results of this study also showed that medications used in the asthma treatment did not have any visible impact on the tested saliva parameters with respect to their dosage, frequency, duration of use, as well as the time of medication administration during the day. A significant difference was not observed in saliva pH values between asthmatic and non-asthmatic children in this study which is consistent with the literature data that attributes the absence of the difference to the fact that the measurement of salivary pH was not performed immediately after the use of antiasthmatic drugs [17].

Buffer capacity testing indicates saliva's effectiveness in neutralizing acidity in the mouth. Karova and Christoff [14] pointed out that the use of inhaled antiasthmatic drugs leads to the rapid reduction of the salivary pH. The pH value was recorded to be the lowest in the first five minutes after the drug use and increased during the first 30 min. However, it did not reach the values registered prior to the drug administration. In the other studies, it was reported that 30 min after using beta-2 antagonist, salivary and plaque pH declined to a critical level (pH = 5.5), causing enamel demineralization [3, 15].

The average dmft/DMFT values were higher in primary and/or permanent dentition in asthmatic children in several studies [2, 6, 17–20]. Those values were reported to be higher in children who used inhaler forms of drugs [18], especially salbutamol inhalers (bronchodilators) [19]. In contrast to the above-mentioned studies, other studies do not demonstrate a positive relationship between asthma and dental caries [21–29]. These findings are in accordance with our study. The preva-

lence of dental caries in asthmatic children in this study was higher than in the healthy control group, both in primary and permanent dentition, but it was not statistically significant. However, this finding supports the claim that asthmatic children may be at higher risk for tooth decay. This study showed a higher prevalence, without statistical significance, of dental caries in asthmatic children, particularly in the subgroup of children with partly controlled asthma.

The dmft/DMFT index values in both groups were high. This could be explained by insufficient knowledge, as well as the lack of interest of children and their parents in their oral health, increased consumption of sweet products, inadequate oral hygiene, and insufficient awareness of the importance of regular dental examinations. In addition, the fact that patients involved in this research lived in an area with less than 0.6 ppm F in drinking water might have contributed to the high index values. The devastating fact that, in general, children in this region had higher dmft/DMFT values, as shown by the results of previous studies [30], may have also contributed to the lack of difference in mentioned values between asthmatic and non-asthmatic children.

In this study, non-asthmatic children had higher salivary buffering capacity and a larger amount of stimulated saliva compared to children with asthma. The fact may speak in favor of the influence of asthma medications on composition, pH value, and amount of saliva.

Eloot et al. [23] did not observe any relationship between the severity of asthma, the period of exposure to medication, and the prevalence of caries. In contrast to that, in our study, the average DMFT and the value of component D were higher if the children used asthma medications in the afternoon period. It appeared that poor oral hygiene and dietary habits, usage of antiasthmatic drugs in the afternoon, as well as a decrease in the salivary flow rate and salivary pH, may lead to pronounced caries development in children with asthma.

Vázquez et al. [4] showed that the presence of nocturnal asthma symptoms and usage of antiasthmatic drugs during the night in preschool children could lead to the caries development in primary dentition, although the relationship between caries and asthma was not found. The results of our study showed that the value of component 'd' in primary dentition was higher in patients who consumed asthma medications prior to sleeping.

The difference in dental status between asthmatic and non-asthmatic Brazilian children younger than eleven years of age was not observed, while a larger prevalence of dental caries was recorded in children with asthma older than this age. These differences were followed by a positive correlation between the number of *Streptococcus mutans* and the severity of asthma [31]. Having in mind the fact that caries is a multifactorial disease, other studies [32] reported that lower dmft or DMFT index was found in primary and/or permanent dentition in asthmatic children than in healthy children.

Conclusion

Although children with asthma had a higher average dmft/DMFT values compared to children without asthma, the difference was not statistically significant. Therefore, this study did not confirm a mutual association between asthma and caries.

Further studies are necessary to clarify the possible asthma impact on dental health and thus improve everyday dental practice related to preventive measures planning, as well as dental assessment and treatment of patients with asthma.

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Inflammatory cardiovascular risk markers and silent myocardial ischemia in type 2 diabetic patients

Markeri rizika od kardiovaskularne inflamacije i „tiha" ishemija miokarda kod bolesnika sa dijabetesom melitusom tipa 2

Gabrijela Malešević, Snježana Popović-Pejičić, Aleksandra Marković, Valentina Soldat-Stanković

University Clinical Center of the Republic of Srpska, Department of Endocrinology, Diabetes and Metabolic Diseases, Banja Luka, Bosnia and Herzegovina; University of Banja Luka, Faculty of Medicine, Banja Luka, Bosnia and Herzegovina

Abstract

Background/Aim. A special feature of coronary heart disease (CHD) in patients with type 2 diabetes mellitus (T2DM) is that it is often asymptomatic and occurs as a consequence of cardiovascular autonomic neuropathy. Dysregulation of the autonomic nervous system is associated with elevated values of inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6), which accelerate atherosclerosis and the occurrence of cardiovascular complications in patients with T2DM. The aim of the study was to evaluate the importance of determining inflammatory cardiovascular risk markers IL-6 and hs-CRP in screening for the presence of CHD in asymptomatic patients with T2DM. **Methods.** The study included 169 patients with T2DM without any symptoms and signs of CHD. Ergometric testing proved or ruled out the presence of silent CHD. The levels of hs-CRP and IL-6 were determined by ELISA. **Results.** IL-6 values were significantly higher in patients with a positive ergometric test (6.83 ± 1.99 pg/mL) compared to patients with a negative ergometric test (3.04 ± 1.39 pg/mL) ($p < 0.001$). We also found that hs-CRP values in patients with a positive ergometric test were significantly higher compared to patients with a negative ergometric test (6.37 ± 2.25 *vs* 1.67 ± 1.41 mg/L; $p < 0.001$). Combinations of IL-6 and hs-CRP with age, HbA1c values, and duration of diabetes, presented through three binary logistic regression models, are significant predictors of silent CHD proven by ergometric testing, *ie*, with their increase, the probability of a positive ergometric test also increases ($p < 0.01$). The sensitivity of the associated finding of elevated IL-6 and hs-CRP values in the detection of silent CHD by ergometric testing was 90%, and the specificity was 86%. **Conclusion.** Hs-CRP and IL-6 are significant predictors of silent CHD, and their determination could be recommended for improving cardiovascular risk stratification in asymptomatic patients with T2DM.

Key words:
biomarkers; c-reactive protein; coronary disease; diabetes mellitus, type 2; interleukin-6; prognosis.

Apstrakt

Uvod/Cilj. Posebna osobina koronarne bolesti srca (KBS) kod bolesnika sa dijabetesom melitusom tipa 2 (T2DM) je ta da je često asimptomatska i javlja se kao posledica autonomne neuropatije kardiovaskularnog sistema. Disregulacija autonomnog nervnog sistema povezana je sa povišenim vrednostima markera inflamacije, kao što su visoko senzitivni C-reaktivni protein (hs-CRP) i interleukin-6 (IL-6), koji ubrzavaju aterosklerozu i pojavu kardiovaskularnih komplikacija kod bolesnika sa T2DM. Cilj studije bio je da se proceni značaj određivanja markera rizika od kardiovaskularne inflamacije IL-6 i hs-CRP u „skriningu" na prisustvo KBS kod asimptomatskih bolesnika sa T2DM. **Metode.** Studijom je bilo obuhvaćeno 169 bolesnika sa T2DM, bez simptoma i znakova KBS. Ergometrijskim testiranjem dokazano je ili isključeno prisustvo „tihe" KBS. Nivoi hs-CRP i IL-6 određeni su ELISA metodom. **Rezultati.** Vrednosti IL-6 bile su statistički značajno više kod bolesnika sa pozitivnim ergometrijskim testom u odnosu na bolesnike sa negativnim ergometrijskim testom (6,83 ± 1,99 pg/mL prema 3,04 ± 1,39 pg/mL, redom; $p < 0,001$). Utvrđene su statistički značajno više vrednosti CRP kod bolesnika sa pozitivnim ergometrijskim testom u odnosu na bolesnike sa negativnim ergometrijskim testom (6,37 ± 2,25 prema 1,67 ± 1,41

Correspondence to: Gabrijela Malešević, University Clinical Center of the Republic of Srpska, Department of Endocrinology, Diabetes and Metabolic Diseases, 12 beba bb, 78 000 Banja Luka, Bosnia and Herzegovina. E-mail: gabimalesevic@yahoo.com

mg/L, redom; $p < 0{,}001$). Kombinacije IL-6 i hs-CRP sa godinama starosti, vrednostima HbA1c i trajanjem T2D, predstavljene kroz tri binarna logistička regresiona modela, značajni su prediktori „tihe" KBS dokazane ergometrijskim testom, tj. sa njihovim povećanjem verovatnoća pozitivnog ergometrijskog testa takođe se povećava ($p < 0{,}01$). Senzitivnost udruženog nalaza povišenih vrednosti IL-6 i hs-CRP u detekciji „tihe" KBS ergometrijskim testiranjem bila je 90%, a specifičnost 86%. **Zaključak.** Hs-CRP i IL-6 su značajni prediktori „tihe" KBS i njihovo određivanje bilo bi značajno u poboljšanju stratifikacije kardiovaskularnog rizika kod asimptomatskih bolesnika sa T2DM.

Ključne reči:
biomarkeri; c-reaktivni protein; koronarna bolest; dijabetes melitus, tip 2; interleukin-6; prognoza.

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in three out of four type 2 diabetes mellitus (T2DM) patients. A special feature of CVD in patients with diabetes is that it is often asymptomatic and occurs as a consequence of cardiovascular autonomic neuropathy. The absence of pain during ischemic myocardial episodes and atypical and mild symptoms of acute myocardial infarction delay the start of treatment, causing increased morbidity and mortality in patients. It is believed that autonomic dysfunction leads to the development of silent episodes of ischemia and silent infarction in patients with T2DM [1, 2].

Recent studies have shown that autonomic nervous system dysregulation is associated with elevated inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP) and interleukin-6 (IL-6), and their determination could reflect the severity of atherosclerosis as well as the risk of developing future cardiovascular events in T2DM patients [3, 4].

In order to identify the presence of coronary heart disease (CHD) in asymptomatic patients with diabetes, only an approach based on a detailed assessment of traditional cardiovascular risk factors has been recommended for the time being. Predicting the risk of cardiovascular events occurrence and progression of atherosclerosis and the correlation of inflammatory agents in its progression has increasingly been the focus of research [5, 6]. Traditional risk factors for CVD, such as high low-density lipoprotein (LDL) cholesterol, low high-density lipoprotein (HDL) cholesterol, hypertension, and smoking, explain only a part of cardiovascular risk in T2DM patients [7, 8].

At present, atherosclerosis is considered an inflammatory disease, given the key role of inflammation in all stages of the occurrence and development of the atherosclerotic process, and the inflammatory nature of atherosclerosis is manifested by the correlation of inflammatory marker levels in blood with its occurrence and progression [3, 9]. Vascular complications arise primarily as a consequence of endothelial dysfunction and inflammatory processes that play a role not only in initiation but also in the progression of atherosclerosis. Therefore, it is crucial to determine other risk factors for CVD occurrence, such as progressive inflammatory tissue response to continuous deposition and modification of lipoproteins in the vascular wall [10, 11].

The aim of the study was to evaluate the significance of determining inflammatory markers IL-6 and hs-CRP as atherosclerosis markers during screening for the presence of CHD in asymptomatic patients with T2DM.

Methods

The study was conducted at the University Clinical Center of the Republic of Srpska as a cross-sectional study, and it included 169 T2DM patients, men (n = 71) and women (n = 98). All subjects underwent ergometric testing and based on the obtained results, they were divided into two groups. The first group consisted of 117 T2DM diabetic patients without the presence of CHD, proven by the absence of symptoms and a negative ergometric test. The second group consisted of 52 T2DM patients with silent ischemic heart disease, proven by a positive stress test.

All subjects underwent an anamnestic interview after which they all gave their written consent to participate in the study. Afterward, a physical examination was performed to define the anthropometric measures. Calculation of body mass index (BMI) for the assessment and monitoring of nutritional status was performed according to Quetelet's formula: BMI = body weight in kg/square of body height in meters (kg/m^2). Subjects with T2DM and CHD, with a history of cerebrovascular, peripheral vascular, and malignant diseases were excluded from the study. Moreover, all the subjects who had an acute or chronic infection or who had been receiving corticosteroids or immunosuppressants within their therapy were excluded from our study.

The study was conducted in compliance with the Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. Ethics Committee of the Republic of Srpska University Clinical Center in Banja Luka gave their consent for approval of the research protocol.

CHD diagnosis

Ergometric testing was performed on a general electric treadmill type T-2100. Testing was performed according to the standard Bruce protocol. The test was evaluated as positive in subjects with horizontal or descending ST-segment depression equal to or > 1 mm for 60–80 ms after the J-point, at least in three successive QRS complexes, as well as in patients who experienced ST-segment elevation during the stress test that was characterized as pathological if it occurred with the same characteristics, as well as ST-

segment depression (> 1 mm, lasts longer than 60–80 ms). The test was defined as positive and negative, and the patients in whom it was described as inconclusive were not considered [12].

Laboratory analysis

Biochemical blood tests for laboratory processing were taken in the morning after 12-hour overnight fasting. The total cholesterol, HDL cholesterol, LDL cholesterol, and serum triglycerides were measured directly by homogeneous enzymatic procedure on INTEGRA® 400 plus analyzer manufactured by Roche, and glycosylated haemoglobin (HbA1c) and urine albumin concentration in 24-hour urine by a turbidimetric assay method. Determination of the levels of inflammatory markers hs-CRP and IL-6 was performed using ELISA (R&D Systems, Inc., Minneapolis, USA). It is a quantitative sandwich enzyme-linked immunosorbent assay technique. Blood serum was used for this test. The blood was centrifuged at 3,000 rpm at 4 °C for 15 min and aliquots were stored at -70 °C. A commercial calibrator was used for calibration. Subjects with hs-CRP values above 10 mg/L were excluded from the study because such hs-CRP values indicate the presence of acute inflammatory disease. An hs-CRP value of 1 mg/L indicates low risk for CVD; from 1 to 3 mg/L, moderate risk; from 3 to 10 mg/L, high risk [13]. The lowest level of IL-6 detectability in the serum was 1.5 pg/mL [14]. The coefficients of variation of the test were 5%. Calibrations of the testing instrument were performed as recommended by the manufacturer within the given specifications.

Statistical analysis

The data were analyzed using a commercially available statistical program (SPSS 17.0 for Windows; SPSS, Chicago, IL, USA). Continuous variables are summarized as mean ± standard deviation (SD) or as a percentage of frequency. Categorical variables are expressed as proportions (percentage). Student's *t*-test (for continuous variables) and χ^2 proportion test (for categorical variables) were used. Appropriate descriptive and analytical methods (absolute and relative numbers, *t*-test, Wilcoxon test, Mann-Whitney *U* test) were also used. Multiple logistic regression was applied to predict and evaluate one variable based on the value of the other variable or multiple variables. The significance level was less than 0.05.

Results

A screening test for the presence of CHD was performed in 169 asymptomatic patients with a mean age of 58.71 ± 6.76, ranging from 40 to 70 years, with T2DM without a history of any CVD. The presence of silent CHD was proven in 52 subjects using ergometric testing, while 117 subjects were without CHD. We examined whether there were differences in cardiovascular risk factors between the study groups.

Table 1 shows a comparison of demographic and risk factors between subjects with a positive or negative ergometric test result. The patients with positive ergometric test (silent CHD) were older with a longer duration of diabetes and a higher incidence of smokers compared to the patients with a negative ergometric test result ($p < 0.05$). Prevalence of hypertension, as well as HbA1c values, were statistically significantly higher in subjects with a positive ergometric test compared to the patients with a negative ergometric test ($p < 0.05$). Regarding the lipid parameters, total cholesterol, LDL cholesterol, as well as triglycerides, were significantly higher in the group of subjects with a positive ergometric test ($p < 0.05$), whereas the values of HDL cholesterol did not differ significantly between the study groups. Microalbuminuria was a statistically significantly different characteristic ($p < 0.001$).

Table 1

Demographic and anthropometric characteristics of the type 2 diabetes mellitus patients with positive or negative ergometric test

Characteristics	Patients		*p*
	positive ergometric test (n = 52)	negative ergometric test (n = 117)	
Gender (male/female), n	24/28	47/70	ns
Smoking, n (%)	32 (38.5)	16 (13.7)	< 0.05
Age (years), mean ± SD	58.71 ± 6.76	54.98 ± 6.69	< 0.05
Duration of DM (years), mean ± SD	10.52 ± 4.60	7.08 ± 3.19	< 0.05
BMI (kg/m^2), mean ± SD	27.6 ± 1.58	27.2 ± 1.46	ns
Systolic BP (mm Hg), mean ± SD	139.90 ± 11.7	128.16 ± 10.72	< 0.05
Diastolic BP (mm Hg), mean ± SD	88.56 ± 9.3	81.03 ± 7.27	< 0.05
HbA1c (%), mean ± SD	9.16 ± 1.91	7.43 ± 1.08	< 0.05
Total cholesterol (mmol/L), mean ± SD	6.07 ± 1.33	5.37 ± 1.11	< 0.05
LDL cholesterol (mmol/L), mean ± SD	3.97 ± 1.11	3.33 ± 0.86	< 0.05
HDL cholesterol (mmol/L), mean ± SD	1.17 ± 0.2	1.14 ± 0.37	ns
Triglyceride (mmol/L), mean ± SD	2.35 ± 1.1	2.06 ± 1.37	< 0.05
Microalbuminuria, n (%)	47 (90.4)	20 (17.1)	< 0.001
IL-6 (pg/mL), mean ± SD	6.83 ± 1.99	3.04 ± 1.39	< 0.001
hs-CRP (mg/L), mean ± SD	6.37 ± 2.25	1.67 ±1.41	< 0.001

DM – diabetes mellitus; BMI – body mass index; BP – blood pressure; HbA1c – glycosylated haemoglobin; LDL – low-density lipoprotein; HDL – high-density lipoprotein; IL-6 – interleukin-6; hs-CRP – high-sensitivity C-reactive protein; SD – standard deviation.

When we analyzed inflammatory markers (IL-6 and hs-CRP), we found that IL-6 values were significantly higher in patients with a positive ergometric test ($p < 0.001$) (Table 1). Similarly, we also found that hs-CRP values in patients with a positive ergometric test were significantly higher compared to patients with a negative ergometric test (Table 1).

The results of combinations of IL-6 and hs-CRP with age show the following: increasing the value of IL-6 by one unit increased the possibility of a positive ergometric test by 1.439 times, increasing the value of hs-CRP by one unit increased the possibility of a positive ergometric test by 1.830 times, while increasing the patient's age by one year increased the possibility of a positive ergometric test by 1.160 times (Table 2).

The results of combining IL-6 and hs-CRP with HbA1c in our study showed that an increase in IL-6 by one unit increased the possibility of a positive ergometric test by 1.495 times, increasing the value of hs-CRP by one unit increased the possibility of a positive ergometric test by 1.565 times, while an increase in HbA1c by one unit increased the possibility of a positive test by 1.471 times (Table 2).

The results of combinations of independent variables IL-6 and hs-CRP with T2DM duration show that increasing the value of IL-6 by one unit increased the possibility of a positive ergometric test by 1.581 times, increasing the value of hs-CRP by one unit increases the possibility of a positive ergometric test for 1.663 times while increasing the duration of T2DM by one unit increases the possibility of a positive test 1.293 times (Table 2).

Within all three analyzed binary logistic regression models, combinations of IL-6 and hs-CRP with age, HbA1c values, and duration of diabetes showed that these values, are significant predictors of silent CHD proven by ergometric test, i.e., with their increase, the probability of a positive ergometric test also increases ($p < 0.01$) (Table 2).

A receiver operating characteristic curve (ROC) analysis was used to test the predictive powers of IL-6 with a positive ergometric test. Using the multivariate logistic regression model in ROC analysis, the clinical accuracy of the diagnostic procedure was good with the area under the curve (AUC) of 0.925 confidence interval (CI): 0.882 to 0.969). IL-6 had a sensitivity of 90.4% and a specificity of 82.9% (Figure 1).

Table 2

Binominal logistic regression analysis of risk factors for prediction of ergometric test amongst the study population

Variables	B	S.E.	*p*	OR	95% CI for OR	
					lower	upper
Model 1: IL-6 + hs-CRP + Age						
IL-6	0.364	0.219	0.97	1.439	0.937	2.210
hs-CRP	0.604	0.194	0.002	1.830	1.250	2.678
Age	0.148	0.056	0.008	1.160	1.039	1.294
Constant	-7.454	1.956	0.000	0.001		
Model 2: IL-6 + hs-CRP + HbA1c						
IL-6	0.402	0.209	0.054	1.495	0.993	2.249
hs-CRP	0.448	0.174	0.010	1.565	1.112	2.202
HbA1c	0.386	0.238	0.106	1.471	0.922	2.348
Constant	-7.454	1.95	0.000	0.001		
Model 3: IL-6 + hs-CRP + diabetes duration						
IL-6	0.458	0.219	0.037	1.581	1.029	2.429
hs-CRP	0.509	0.184	0.006	1.663	1.160	2.384
Diabetes duration	0.257	0.081	0.001	1.293	1.105	1.514
Constant	-7.092	1.202	0.000	0.001		

IL-6 – interleukin-6; hs-CRP – high-sensitivity C-reactive protein; HbA1c – glycosylated haemoglobin; OR – odds ratio; CI – confidence interval.

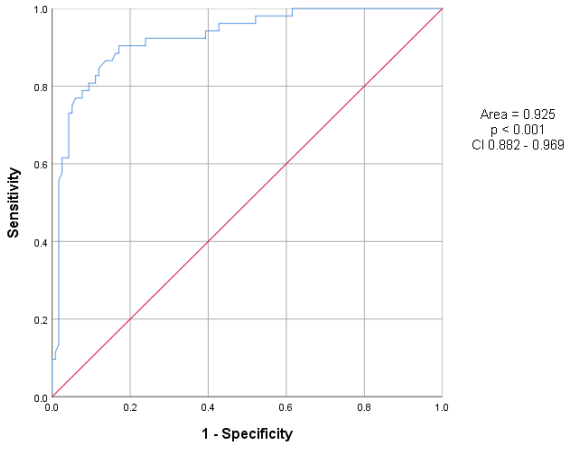

Fig. 1 – A receiver operating characteristic curve (ROC) predictive power of interleukin-6 with positive ergometric test. CI – confidence interval.

ROC analysis was also used in testing the predictive power of hs-CRP with a positive ergometric test. Using the multivariate logistic regression model used in the ROC analysis, the clinical accuracy of the diagnostic procedure was good with the AUC of 0.934 (CI: 0.895 to 0.972). Hs-CRP had a sensitivity of 88.5% and a specificity of 80% (Figure 2).

We examined the significance of the associated risk of increased hs-CRP and IL-6 values in the detection of silent CHD proven by ergometric testing. In patients with T2DM in whom silent CHD was detected, 47 (90%) subjects had a finding of associated risk, while 5 (10%) had no associated risk. In patients with T2D without CHD, there was an associated risk in 16 (14%), while in 101 (86%) subjects, it did not exist. This difference in a frequency distribution is statistically significant ($p < 0.001$) (Figure 3).

Discussion

Our study demonstrated that a large percentage of patients with T2DM have silent CHD and that elevated levels of inflammatory markers (IL-6 and hs-CRP) represent a strong markers for the presence of silent CHD.

Previous research has shown that silent CHD in people with diabetes varied and that there was a need to define the degree of cardiovascular risk in people with silent CHD who could benefit from screening [15]. In detecting ischemia in asymptomatic diabetics, the study was randomly assigned to either stress testing and a 5-year clinical follow-up or to follow-up only. A total of 22% of patients had silent ischemia [16]. The prevalence of silent myocardial ischemia in our study was 29%, which is mostly consistent with previously published literature. Due to all the above, coronary artery disease in patients with diabetes is a diagnostic and therapeutic challenge.

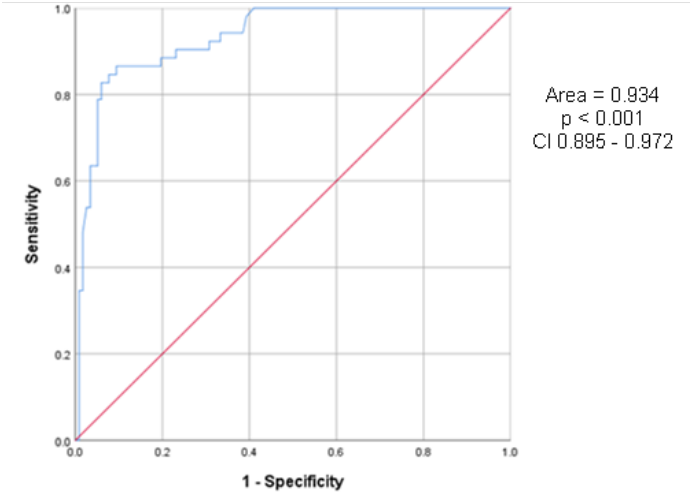

Fig. 2 – A receiver operating characteristic curve predictive power of high sensitivity C-reactive protein with a positive ergometric test.
CI – confidence interval.

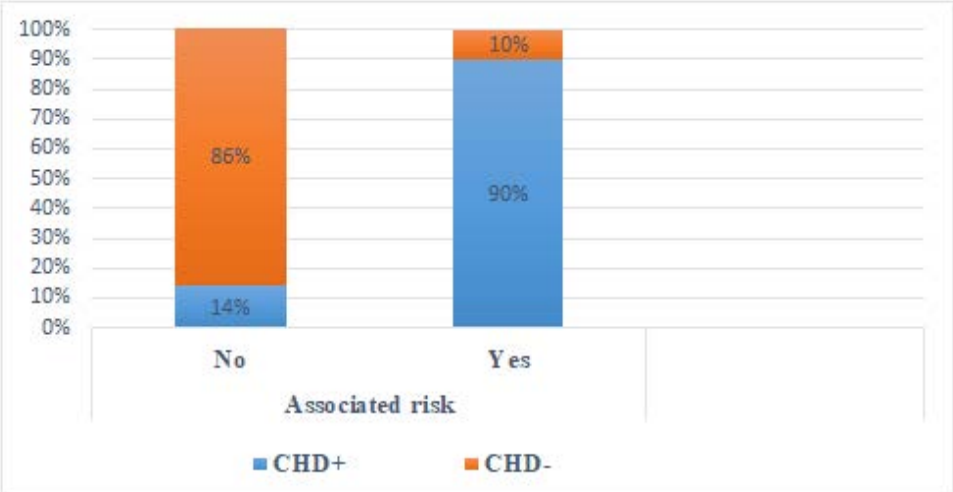

Fig. 3 – Significance of the associated risk of increased hs-CRP and IL-6 values in the detection of silent CHD proven by ergometric testing.
CHD – coronary heart disease.
hs-CRP – high sensitivity C-reactive protein; IL – interleukin.

Although T2DM alone is a large and independent risk factor for the occurrence of CVD, the coexistence of traditional cardiovascular risk factors significantly increases the risk of silent CHD occurring in T2DM patients. The Multiple Risk Factor Intervention Trial showed that multiple risk factors in the same patient significantly increased the overall cardiovascular risk [17]. Gaede et al. [18] reported that an intervention directed at multiple risk factors significantly improves cardiovascular prognosis. This is supported by the results of our study, which showed that the patients with silent CHD were older, with a longer duration of diabetes, higher prevalence of hypertension, poorer glucose regulation, higher values of total cholesterol, LDL cholesterol, and triglycerides as well as a higher prevalence of albuminuria compared to subjects who did not have CHD, while HDL cholesterol levels and BMI had no statistically significant difference between the study groups.

Since the inflammatory process is an integral part of the evolution of atherosclerosis, the use of CRP as a biomarker becomes very useful in combination with the control of classic risk factors such as lipid levels, changes in eating habits, weight loss, regular physical activity, glycemic control, and smoking cessation. The interrelation of these risk factors for CVD are strategies to reduce cardiovascular events in primary and secondary prevention [19]. Due to the relationship between high CRP plasma levels and cardiovascular mortality and morbidity risk, it is important to establish a primary care line to decrease CVD. For this, the evaluation of cardiovascular risk factors is essential for stopping their progression. Several prospective studies having CRP as a central target have shown the benefits of primary prevention. In 1999, the MONICA-Augsburg study, performed on a sample of 936 asymptomatic men, concluded that the increase in hs-CRP leads to a 19% increased risk of fatal and non-fatal coronary events [20]. In the same way, the PREVEND study in 8,139 asymptomatic men and women observed a relationship between hs-CRP and angiographic characteristics and consequently clinical instability of the atherosclerotic plaque [21].

A six-year follow-up study of healthy middle-aged men showed that baseline IL-6 levels greater than 2.28 pg/mL were associated with a 2.3-fold higher risk of future myocardial infarction, which is why IL-6 was also identified as a significant risk predictor of cardiovascular events [22]. Recent research has reported the importance of inflammation in the development and progression of atherosclerosis, as well as the possibility of using inflammatory markers to assess cardiovascular risk. Among the several biomarkers proposed in cardiovascular risk stratification is CRP, which would be used in identifying individuals at risk for developing CVD, but this is not yet recommended in the guidelines [23–25]. Prospective clinical case-control studies, Physicians' Health Study, and Women's Health Study have identified CRP as a strong, independent risk factor for CHD [26, 27]. Previous research has shown that hs-CRP was a predictor of CVD, even after adjusting to traditional risk factors indicating that hs-CRP may provide additional significant prognostic information in cardiovascular risk assessment [28]. Furthermore, elevated levels of IL-6 in serum are correlated with the development of coronary artery disease, which is why IL-6 has become an important cytokine in the assessment of atherosclerosis in people with T2DM, as evidenced by two large genetic studies reporting a correlation between IL-6 receptor signaling and CVD [29–31]. It has been also suggested that elevated IL-6 values were correlated with an increased risk of future myocardial infarction even after adjustment in initial differences in total cholesterol, HDL-cholesterol, BMI, blood pressure, diabetes mellitus, family medical history, alcohol consumption, and doing physical activity [32, 33]. Moreover, elevated levels of IL-6 can play a predictive role in the occurrence of CVD, thus providing a potential prognostic means in the detection of CVD [22].

The results of our research also support these studies. We concluded that the combined finding of increased values of IL-6 and hs-CRP posed a high risk of the presence of silent CHD in T2DM patients.

In this study, we also analyzed the significance of inflammatory cardiovascular risk markers (CRP, IL-6) for the appearance of silent CHD in patients with T2DM. In subjects with silent CHD, there was a direct correlation with IL-6 and hs-CRP values that were significantly higher compared to the subjects without CHD. The results of our study showed that IL-6 and hs-CRP are significant predictors of silent CHD and showed that the association of IL-6 and hs-CRP with age, HbA1c values, and duration of diabetes is significant predictor of silent CHD proven by ergometric testing.

There are some limitations of our study. First, the study was a single-center trial with a relatively small number of subjects. Second, this study was cross-sectional, without appropriate follow-up, so our study could not demonstrate, in the long term, the incidence of silent CHD or the influence of investigated markers on the future appearance of CHD. This could be the main reason to extend the investigation to a larger number of subjects and a longer follow-up in the future in order to get stronger results.

Conclusion

Our study showed that a large percentage of T2DM patients had silent CHD. Elevated levels of inflammatory cardiovascular risk markers, hs-CRP and IL-6, are strong markers of the presence of silent CHD in asymptomatic T2DM patients. Given that traditional risk factors for CVD explain only a part of cardiovascular risk in T2DM patients, and current screening recommendations are based on their use, it would be important to include the determination of inflammatory cardiovascular risk markers in order to improve cardiovascular risk stratification in asymptomatic patients with T2DM. By doing so, we would be able to reduce the incidence of cardiovascular complications occurrence and apply appropriate treatment modalities in a timely manner, whether it was a conservative or invasive treatment.

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Comparison of oncologic outcomes between elective partial and radical nephrectomy in patients with renal cell carcinoma in CT1B stadium

Poređenje onkoloških ishoda između elektivne parcijalne i radikalne nefrektomije kod bolesnika sa karcinomom bubrežnih ćelija CT1B stadijuma

Predrag Marić*, Predrag Aleksić*†, Branko Košević*†, Mirko Jovanović*, Vladimir Bančević*†, Dejan Simić*, Nemanja Rančić†‡

Military Medical Academy, *Clinic for Urology, ‡Center for Clinical Pharmacology, Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Abstract

Background/Aim. In renal cell carcinoma (RCC), the choice of surgical technique, radical (RN) or partial nephrectomy (PN), is still center-dependent because there are still no absolute recommendations for this approach. The aim of this study was to analyze the oncological aspects, time until recurrent disease appears, and cancer-specific survival in patients with RCC in T1bN0M0 stadium depending on the type of surgical procedure, PN or RN technique. **Methods.** In a clinical observational study, data of 154 patients operated at the Clinic for Urology, Military Medical Academy, Belgrade, Serbia with a mean follow-up period of no less than five years were analyzed. Patients were divided into two groups; a group of patients with RN and a group of patients with PN. The inclusion criteria were: renal tumors 4–7 cm, histopathological confirmation of RCC, absence of metastasis, and normal serum creatinine. Exclusion criteria included: the presence of other malignancies, solitary functional kidney or comorbidities that can compromise renal function, bilateral tumors, or unilateral multiple tumors. **Results.** The study analyzed data from 154 patients, 97 (63%) RNs, and 57 (37%) patients that underwent PN. Analyzing cancer-specific survival in four patients with RN, there was a disease advancement that led to a lethal outcome, and one PN patient died as a result of local relapse and distant metastasis. **Conclusion.** Based on our results, PN is a good and safe treatment option for patients with RCC in T1b stadium. PN offers a similar tumor control and better cancer-specific survival.

Key words:
carcinoma, renal cell; kidney neoplasms; neoplasm metastasis; neoplasms staging; nephrectomy; urologic surgical procedures.

Apstrakt

Uvod/Cilj. Kod karcinoma bubrežnih ćelija (KBĆ) izbor hirurške tehnike – radikalne (RN) ili parcijalne nefrektomije (PN) i dalje zavisi od centra gde se resekcija vrši, jer i dalje nema apsolutnih preporuka u vezi sa izborom pristupa. Cilj studije bio je da se analiziraju onkološki aspekti bolesnika sa KBĆ u stadijumu T1bN0M0 u zavisnosti od tipa primenjene hirurške procedure – PN ili RN. **Metode.** Kliničkom opservacionom studijom tipa serije slučajeva analizirani su podaci 154 bolesnika koji su operisani na Klinici za urologiju Vojnomedicinske akademije, Beograd, Srbija, sa prosečnim periodom praćenja ne kraćim od 5 godina. Bolesnici su bili podeljeni u dve grupe: grupu bolesnika sa RN i grupu bolesnika sa PN. Kriterijumi za uključivanje u studiju bili su: tumori bubrega veličine 4–7 cm, patohistološki postavljena dijagnoza KBĆ, odsustvo metastaza i vrednosti serumskog kreatinina u referentnim granicama. Kriterijumi za isključivanje bolesnika iz kliničkog ispitivanja bili su: prisustvo drugih maligniteta, prisustvo drugog funkcionalnog bubrega ili oboljenja koja mogu kompromitovati bubrežnu funkciju, bilateralni tumori i više tumora na jednom bubregu. **Rezultati.** U studiji su analizirani podaci od ukupno 154 bolesnika, 97 (63%) sa RN i 57 (37%) sa PN. Analizirajući preživljavanje bolesnika, u zavisnosti od tumora, utvrđeno je da je kod četiri bolesnika sa RN došlo do smrtnog ishoda zbog napredovanja bolesti, dok je kod jednog bolesnika sa PN smrtni ishod nastupio kao posledica lokalnog recidiva tumora i udaljenih metastaza. **Zaključak.** Na osnovu naših rezultata, PN je dobar i siguran izbor u lečenju bolesnika sa KBĆ u T1b stadijumu. Parcijalne nefrektomije nudi sličnu kontrolu tumora i bolje preživljavanje obolelih od KBĆ.

Ključne reči:
karcinom bubrežnog parenhima; bubreg, neoplazme; neoplazme, metastaze; neoplazme, određivanje stadijuma; nefrektomija; hirurgija, urološka, procedure.

Correspondence to: Nemanja Rančić, Military Medical Academy, Center for Clinical Pharmacology, Crnoravska 17, 11 000 Belgrade, Serbia; E-mail: nece84@hotmail.com

Introduction

Renal cell carcinoma (RCC) presents the third most frequent urological malignancy, 2–3% of all adult malignancies and 80–85% of all primary renal carcinomas [1]. It is the most frequent solid renal tumor whose prevalence increases in Europe and North America [2]. Worldwide, over 350,000 new cases of RCC have been diagnosed annually, with over 140,000 kidney cancer-related deaths (mortality rate around 40%). Therefore, these patients represent a significant health issue [3, 4]. In the European Union, just in 2012, 84,499 new cases of RCC have been diagnosed with 34,700 cancer-related deaths [5].

Surgical treatment is the usual management option for a patient with RCC. Surgical resection is the standard treatment option in patients with localized RCC. Historically [6], radical nephrectomy (RN) has been the benchmark surgical treatment of organ-confined RCC. Partial nephrectomy (PN) has taken primacy in treating RCC up to 4 cm. In selected cases with tumors from 4 to 7 cm, it has proven to be as reliable as RN, even though there are available guidelines recommending that PN be applied even in tumors that exceed 7 cm [7]. So far, a unified and definitive position on the role of PN in clinical-stage T1bN0M0 of RCC has not been proposed when there is no absolute indication for this type of surgery. Most relevant studies specify from the oncological point of view that PN is equally reliable as RN when referring to "cancer-free survival" [8]. In addition, the European Association of Urology (EAU) guideline from 2016 recommends that patients in the T1a clinical stage of RCC should be treated with PN and that PN should be applied whenever possible in patients in T1b clinical stage of the tumor [9].

The aim of this study was to analyze the oncological aspects, such as the time to tumor recurrence and cancer specific survival for patients with RCC in clinical-stage T1bN0M0 depending on the type of surgical treatment, PN or RN.

Methods

The study was adopted as a case series clinical observational study and was conducted on patients that underwent surgical treatment at the Clinic for Urology, Military Medical Academy, Belgrade, Serbia for renal tumors with the histological confirmation of RCC as a result of PN or RN.

Patients were divided into two groups by type of surgical resection: RN group and PN group.

Patients were recruited depending on inclusion and exclusion criteria. The two analyzed groups consisted of patients aged 18–80 years who underwent surgical treatment for renal tumors either by PN or RN from 2006 to 2013. In all patients, RCC was confirmed by histopathology. All significant variables of the disease were registered. The surveillance period was from 2006 to 2018, with a median follow-up period of no less than five years, which depended on the patient's survival following nephrectomy or if there was no lethal outcome.

The inclusion criteria were: renal tumors 4–7 cm, histopathological confirmation of RCC, absence of distant metastasis, and normal serum creatinine level.

The exclusion criteria were: the presence of other malignancies, one functional kidney or other conditions that may compromise renal function in the future, bilateral tumors, and multiple unilateral tumors.

Preoperative diagnostic evaluation in all cases consisted of determining the size of the tumor, absence or presence of metastasis, function, and morphology of the contralateral kidney and was conducted using multi-slice computed tomography (MSCT). This imaging was performed not only in our institution but also in other medical institutions. In some instances, if the MSCT scan was inconclusive, it was repeated in our institution. The contralateral kidney was defined as normal if the serum creatine level and MSCT scan were normal.

Following surgical treatment, the histopathological analysis was performed to determine the tumor grade, vascular or lymphatic invasion, histopathological RCC subtype, and histopathological Tumor-Nodes-Metastasis (TNM) stage.

Postoperative assessment of the patients was performed in an outpatient setting for a month and then six months subsequently following the surgical treatment. All of them included physical examination, laboratory analysis, ultrasound of the abdomen and pelvis minor, chest X-ray, and annual MSCT. Determining the presence of postoperative metastasis and local relapse involved an ultrasound scan, chest X-ray, and MSCT. All of these examinations were performed by a radiologist.

Statistical data analysis was performed by PASW Statistics version 18 statistical software. The χ^2 test was used for statistical analysis between some categories, and the Mann-Whitney *U* test for assessing differences in the continual variables. The value of $p < 0.05$ was considered statistically significant.

The principles of the International Council for Harmonization Good Clinical Practice were strictly followed, and the approval from the Ethics Committee of the Military Medical Academy from December 07, 2016 was obtained.

Results

This study analyzed data from 154 patients, 97 (63%) patients with RN and 57 (37%) patients with PN (Table 1). The male/female ratio was nearly 3/1 (115 vs. 39, respective-

Table 1

Demographic characteristics of patients operated due to renal cell carcinoma

Variable	Nephrectomy		*p*-value
	RN (n = 97)	PN (n = 57)	
Male/female	65 (67.0)/32 (33.0)	50 (87.7)/7 (12.3)	0.008*
Age	61.00 (49.50–68.0)	55.00 (46.50–61.50)	0.027**
Age male	62.00 (54.00–67.50)	56.00 (45.50–63.00)	0.010**
Age female	53.50 (47.00–68.75)	55.00 (48.00–60.00)	0.929**
p-value	0.219**	0.877**	

Results are shown as a number (%) or median (interquartile range).

PN – partial nephrectomy; RN – radical nephrectomy.

***– χ^2 test; **– Mann-Whitney test.**

ly). Furthermore, males were more represented in both groups of patients, but the frequency was statistically more significant in the PN group compared to the RN group (87.7% vs. 67.0%, respectively). When comparing the age of the patients at the time of diagnosing RCC in the analyzed groups, there was a statistically significant difference in the median age in the RN group.

More than 74% of patients in both groups were asymptomatic (Table 2). If present, the most frequent symptom in both groups was pain.

Regarding the histopathological characteristics of the tumors, initially, at the time of surgical treatment, all patients were in the clinical T1bN0M0 stage (Stage I). Table 3 presents the largest diameter of the tumor mass in the PN and RN group; between the groups, there was a statistically significant difference ($p < 0.001$). In the RN group, the mean tumor diameter was 53.00 mm, while in the PN group, it was 43.00 mm. Evaluating the tumor localization, no statistically significant difference in the RN and PN groups was registered. In over 80% of patients in both groups, the confirmed histopathological diagnosis was the clear-cell subtype of RCC.

The ipsilateral adrenal gland in the PN group during tumor resection was left intact, while in the RN group, it was removed in nearly 60% of patients. Once removed, there were nearly no cases of tumor involvement except for two patients in the RN group.

Following surgical tumor resection, a histopathological analysis was performed by defining the tumor grade (Table 4).

Table 2

Symptoms associated with radical and partial nephrectomy

Symptoms	Nephrectomy		*p*-value*
	Radical	Partial	
None	72 (74.2)	47 (82.5)	
Pain	15 (15.5)	8 (14.0)	
Haematuria	8 (8.2)	1 (1.8)	0.323
Hyperkalemia	1 (1.0)	–	
Anemia	1 (1.0)	–	

Results are shown as a number (%).
***– χ^2 test.**

Table 3

Anatomical localization and histopathological characteristics of the tumor

Variable	RN	PN	*p*-value
Diameter (mm)	53.00 (45.00–60.00)	43.00 (40.00–50.00)	< 0.001*
Localization			
upper pole	36 (37.1)	16 (28.1)	0.104**
lower pole	27 (27.8)	25 (43.8)	
interpolar region	34 (35.1)	16 (28.1)	
Histopathological characteristics			
clear-cell	70 (87.5)	43 (82.7)	0.196**
papillary	3 (3.8)	6 (11.5)	
chromophobe	7 (8.8)	3 (5.8)	

Results are shown as a number (%) or median (interquartile range) value.
PN – partial nephrectomy; RN – radical nephrectomy.
***– Mann-Whitney test; **– χ^2 test.**

Table 4

Tumor grade, local invasion and histopathological stage

Variable	RN	PN	*p*-value*
Grade (G)			
G1	2 (2.1)	1 (1.8)	
G2	56 (58.9)	37 (64.9)	0.670
G3	35 (36.8)	19 (33.3)	
G4	2 (2.1)	–	
Lymphatic invasion			
no	23 (29.9)	26 (50.0)	0.034
yes	54 (70.1)	26 (50.0)	
Vascular invasion			
no	19 (25.0)	30 (58.8)	< 0.001
yes	57 (75.0)	21 (41.2)	
T stage			
T1a	11 (11.5)	19 (33.3)	
T1b	43 (44.8)	33 (57.9)	
T2	2 (2.1)	2 (3.5)	< 0.001
T3a	38 (39.6)	3 (5.3)	
T3b	2 (2.1)	–	

Results are shown as a number (%).
PN – partial nephrectomy; RN – radical nephrectomy.
***– χ^2 test.**

Table 5

Clinical progression-free survival of RCC patients subjected to radical or partial nephrectomy

Nephrectomy	Tumor relapse	Clinical progression-free survival (days), mean (95% CI)
Radical	6	1,470.29 (997.56–1,943.01)
Partial	2	1,142.50 (0.00–2,311.64)

RCC – renal cell carcinoma; CI – confidence interval.

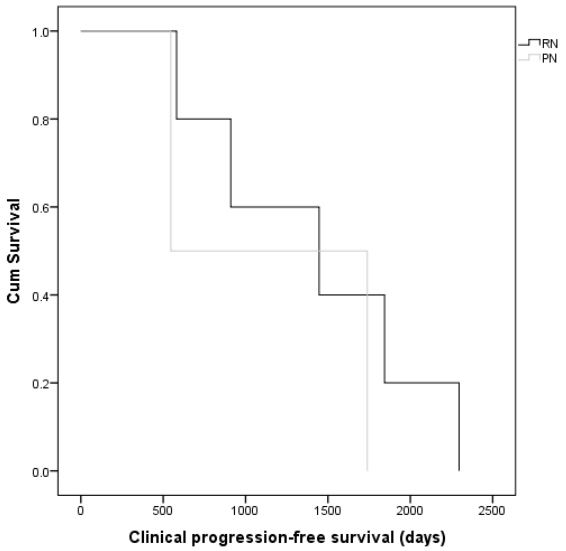

Fig. 1 – Clinical progression-free survival according to the type of surgical resection. PN – partial nephrectomy; RN – radical nephrectomy. Log Rank (Mantel-Cox) test, $p = 0.436$.

No statistically significant difference concerning the tumor grade between the RN and PN group was established ($p = 0.670$). The most frequent tumor grade in both groups was grade 2 and 3 (in over 95%). The lymphatic invasion was statistically significantly more frequent in the RN group in over 70.1% of cases compared to the PN group, where it was present in 50.0% of cases. The same was with vascular invasion in 75% of cases in the RN group, while in the PN group, it was present in 41.2% of cases.

However, considering the histopathological stage of the tumor, a statistically significant difference was established between the RN and PN groups ($p < 0.001$). In the RN group, it was mostly in the T1b and T3a stages, while in the PN group, it was in the T1a and T1b stages (Table 4).

The overall surveillance period in the RN group was 2,343 days (365–4,297 days), while in the PN group, it was 2,175 days (868–4,045 days). The evaluation of the clinical progression-free survival in patients with RCC had shown a low rate of tumor relapse (Table 5). Of the overall number of patients in the RN group, a relapse of the tumor was registered only in 6 patients, while in the PN group, it was registered in two patients. The average time for relapse to occur in the RN group was 1,470 days and 1,142 days in the PN group. This has proven not to be statistically significant [Log Rank (Mantel-Cox) test, $p = 0.436$] (Figure 1).

When analyzing the cause of death in five patients, the occurrence of tumor relapse or the appearance of metastasis were connected to the lethal outcome (Table 6). In all other patients, the leading cause of death was not related to the operated RCC but rather to other comorbidities (cerebrovascular or cardiovascular).

When analyzing the cancer-specific survival, or the mortality from RCC as the single cause of death, we registered that in four patients, the lethal outcome was the result of metastasis, while one patient died because of local relapse and distant metastasis (Table 6, Figure 2).

Table 6

Cancer-specific survival of RCC patients subjected to radical or partial nephrectomy

Nephrectomy	Patients		
	total number	died	censored, number (%)
Radical	95	4	91 (95.8)
Partial	55	1	54 (98.2)

RCC – renal cell carcinoma.

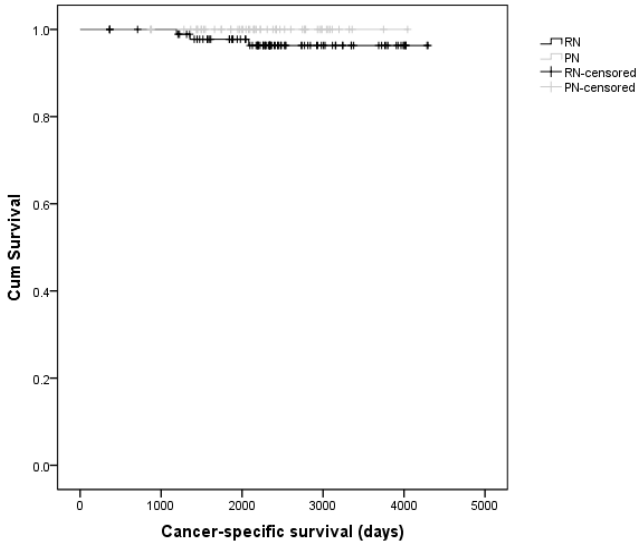

Fig. 2 – Cancer-specific survival according to the type of surgical resection.
PN – partial nephrectomy; RN – radical nephrectomy.
Log Rank (Mantel-Cox) test; p = 0.459.

Discussion

Based on a global assessment of data from 167 countries in 2017, RCC was the seventh most frequent malignancy and represented 3.3% of all newly detected carcinomas [10]. An estimated increase of new cases of RCC was 22% by 2020 [3]. Obesity, smoking, and hypertension are known risk factors for RCC. With the global population aging, there is also an increase in the prevalence of this malignancy [11]. RCC represents around 90–95% of all kidney tumors, and at the time of diagnosis, 25–30% of patients already have metastatic disease [10].

Surgical treatment of RCC is still the gold standard in treating this malignancy [12, 13]. In the early stages of RCC, when the lesions are small and surgical resection is possible, several surgical modalities are available. In the previous decade, an offset occurred from RN towards the necessity of nephron-sparing techniques PN. Preservation surgical techniques have a goal to preserve renal function and, at the same time, have identical oncological results as in RNs [14, 15]. Moreover, when considering the quality of life, renal function, and overall survival, the majority of studies agree that PN has a significant advantage over RN [16–19]. Additionally, an offset from open to laparoscopic and robot-assisted surgery occurred [20]. Now in the leading countries in the world, PN is performed mostly laparoscopically or by robot-assisted techniques.

The classical triad of symptoms (flank pain, macroscopic haematuria, and palpable mass) is present in around 6–10% of cases, but when present, it raises doubt on RCC [21]. In both of our groups, more than 74% of patients were asymptomatic. That is similar to the majority of studies that show that RCC has a devious development, so in most cases, it is incidentally detected [22, 23]. In our study, the most frequent symptom in both groups was abdominal pain (in around 15% of patients). Haematuria was present only in 9 patients. This is explained by the fact that the tumor was low grade and developed pain but not haematuria, anaemia, or other symptoms.

Available literature has shown that tumor size has a major significance and influences patients' survival following tumor resection [24]. The larger the size (especially over 30 mm), the shorter the survival. In tumors smaller than 30 mm, distant metastases are rare. In a study that analyzed 740 patients, Herrlinger et al. [24] reported that distant metastasis was present in only one patient, in whom the tumor size was less than 30 mm. In the PN group, the tumors were, on average, 10 mm smaller than in the RN group (43 mm vs. 53 mm, respectively). At the time of the operation, all patients had the tumo in clinical stage T1bN0M0 (Stage I).

The Corona and Saturn project study [25] has shown that the size of the tumor of 75 mm assessed by computed tomography at the time of diagnosis is the border value and those tumors whose diameter exceeds this value correlate with the appearance of distant bone metastasis. This study also showed that in 1,712 patients, the tumor recurrence of more than 5 years was related to the mean size of the tumor of 60 mm, while the mean tumor diameter of 70 mm was related to the tumor recurrence period less than 5 years following the operation. In tumors 40–70 mm in diameter, there is a probability that 6% of patients have already regional or distant metastasis at the time of diagnosis [26]. Results of these studies can have a role in selecting patients into subgroups as candidates for more aggressive treatments because of the probability of distant metastasis appearance or tumor recurrence following tumor resection.

RN was performed in our patients with tumors mostly localized in the upper pole of the kidney and somewhat lesser in the interpolar region. PN was performed in our patients with tumors mostly localized in the lower pole of the kidney.

An easier anatomical approach to the tumors localized in the lower pole can explain why PN is mostly the opted treatment in these cases and why RN is more frequent in tumors localized in the upper pole or interpolar.

After tumor resection, the protocol is followed by a histopathological examination that determines the tumor tissue differentiation or tumor grade, vascular and lymphatic invasion, tumor histological subtype, and TNM stage. Published studies have shown a direct connection between tumor size and its differentiation, so that increase in tumor diameter increases the volume of patients who have higher tumor grade [26]. The most frequent tumor grade in both of our groups was the grade 2 and 3 in over 95% of cases.

In the RN group, the lymphatic invasion was significantly more frequent than in the PN group (70.1% vs. 50.0%, respectively). It is the same case with vascular invasion, which was more frequent in the RN group (75.0%) than in the PN group (41.2%). The microvascular invasion is defined as the presence of malignant cells that invade the wall of the blood vessel or neoplastic emboluses in the intratumor blood vessels; it is present in 13.6–44.6% of RCC [27]. It is more frequent in higher grades of RCC and larger tumors. This is a significant prognostic factor, but the results in many studies are still controversial [28, 29].

In both analyzed groups, clear-cell RCC (ccRCC) was the most frequent histological subtype of RCC (in over 82% of patients). Similar results have been presented in other published studies [18, 26] because it is a known fact that ccRCC is the most commonly encountered histological subtype of RCC and is present in over 75% of patients, while the others are significantly less common [2, 30].

However, when analyzing the histopathological T stage, a significant difference in patients in the RN and PN groups was established. In the RN group, the T1b and T3a stages were most common, while in the PN group, the T1a and T1b stages dominated. Since one of the inclusion criteria to enter the study was that all of the patients be in the clinical T1b stage, in the RN group, this stage had been confirmed only in 44.8% of cases, while in the PN group, it was more present (57.9%). In the RN group, the rest of the patients had a lower, or more commonly, higher stage. In the PN group, the lower stage was mostly present, and only 5 patients had the higher stage. Data from published studies are similar concerning the difference in pre and postoperative stages [30], with an established difference in T and N stages at around 35%. Most commonly, an error was made in measuring the size of the tumor in 92% of cases and in assessing the local tumor invasion of perirenal fat. The N stage was assessed adequately in 94% of patients. However, the MSCT scan still represents the best method for identifying and assessing the preoperative stage of RCC. The major limitation is assessing the tumor size and local expansion in suspected borderline cases [30].

The ipsilateral adrenal gland was removed only in the RN group – out of nearly 60% of patients, tumor involvement is found in two cases.

Analyzing data in 1,179 patients with RCC, Antonelli et al. [31] showed that preservation of the ipsilateral adrenal gland is recommended only in patients with tumors smaller than 4 cm. They also showed that local expansion and the size of RCC are the best risk predictors of the presence of metastasis in the adrenal gland. Siemer et al. [32] presented similar results and emphasized that the tumor diameter of 4 cm is crucial for deciding whether to perform ipsilateral adrenalectomy or not. The incidence of diagnosing metastasis in the adrenal gland is significantly higher in autopsy studies (6–29%) compared to clinical diagnosis (2–10%) [32–35]. A 19% involvement of the ipsilateral adrenal gland is present in autopsies and 5.5% in urology studies, while even up to 11% of the contralateral adrenal gland involvement is reported [36]. Moreover, it is relevant to take into consideration the possibility of metastasis in other organs, for example, the thyroid gland, lungs, bone metastasis, or other locations, because they are common, especially in higher stages [23]. The EAU Guidelines do not recommend ipsilateral adrenalectomy if there are no clear signs of adrenal gland involvement [37].

When analyzing the cancer-specific survival, our study showed that RCC was the cause of death in 4 patients in the RN group, where metastasis led directly to a lethal outcome, while in the PN group, this was the cause of death in one patient. Our study showed significantly better results than other studies. In a study by Jang et al. [18], a significant difference was not established in the 10-year cancer-specific survival in RCC patients with PN and RNs (85.7% vs. 84.4%, respectively). Similar conclusions were made by other authors who did not prove the advantage of PN to RN based on cancer-specific survival [8, 38, 39]. However, a recent study showed a major difference in the cancer-specific survival rate between laparoscopic PN and RN, where the overall survival, cancer-specific survival, and metastasis-free survival were significantly better in RN patients [40]. Compared with the RN group, patients of the PN group had a 1.9-fold overall survival, 2.9-fold cancer-specific survival, and 2.3-fold metastasis-free survival [40].

In the PN group, both relapses were local. In the first patient, the reoperation was performed 5 years after PN, where the initial tumor was 47 × 45 mm in diameter and localized at the lower pole. The recurrent tumor was 36 mm localized at the site of the previous resection. This was the only PN patient in whom the resection margin was positive. This patient is alive, without tumor recurrence. In the other patient, the tumor at the time of resection was 50 × 45 mm in diameter and localized in the interpolar region of the left kidney in the pT3a stage, with vascular and lymphatic invasion. In less than a year, the patient developed local tumor relapse, and after nephrectomy, also less than a year, developed metastatic disease and shortly after died. In the RN group, no local relapse was detected, but the patients developed distant pulmonary and cerebral metastasis. In a systematic review and meta-analysis of comparative studies, which involved 21 studies and included over 11,000 patients, it was concluded that PN is a sustainable treatment option for large renal tumors because it provides acceptable surgical morbidity, equivalent cancer control, and better preservation of renal function compared to RN with a potential for better overall survival of patients [41].

Conclusion

RNs and PNs are benchmark methods in the treatment of localized RCC. However, PN is a method that preserves the renal parenchyma, hence PN vs. RN provides better postoperative renal function. The results of our study strongly suggest that in patients in clinical-stage T1b of RCC, PN provides the same cancer control as RN. Taking this into consideration, when planning surgical treatment in this clinical stage, elective PN represents the standard treatment method and must be offered to the patient as an effective and safe option.

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Diagnostic accuracy of basal cell carcinoma in dermatology setting in Serbia – a single-center study

Pouzdanost dijagnoze bazocelularnog karcinoma u dermatologiji – monocentrična studija u Srbiji

Milana Ivkov Simić, Branislava Gajić, Dejan Ogorelica, Zorica Gajinov

University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia; University Clinical Center of Vojvodina, Dermatology and Venereology Clinic, Novi Sad, Serbia

Abstract

Background/Aim. The growing incidence of skin tumors requires an accurate diagnosis. Dermoscopy, especially *in vivo*, enhances the diagnosis of basal cell carcinoma (BCC). Total body skin examination (TBSE), a visual inspection of the patient's total body surface, is considered a basic step in the dermatological exam, especially in skin cancer screening. However, TBSE is still a matter of debate regarding its expediency in a real clinical setting. The aim of this study was to analyze the diagnostic accuracy of BCC detected and treated by referred dermatologists. **Methods.** The retrospective analysis included a five-year period of BCC detection during TBSE by visual inspection and dermoscopy. We calculated sensitivity, specificity and positive predictive value for BCC using histopathological results as the correct diagnosis. **Results.** Out of 3,346 biopsied skin tumors, 49.58% were malignant and 50.42% benign. The most common malignant tumor was BCC, accounting for 84.09%. Localization of BCCs was mainly on the trunk (38.92%) and the H-zone of the face (37.63%). Other localizations were face (non-H-zone) (6.67%), neck (3.01%), scalp (3.37%), arms (6.88%) and limbs (3.51%). Of all BCCs, 0.83% were recurrent BCC. The sensitivity for the diagnosis of BCC was 97.71%, and the positive predictive value was 95.08%. **Conclusion.** In the dermatology setting, TBSE and visual inspection with *in vivo* dermoscopy result in a very good diagnostic performance of BCC.

Key words:
dermoscopy; diagnosis; histological techniques; neoplasms, basal cell; sensitivity and specificity; serbia.

Apstrakt

Uvod/Cilj. Zbog povećane incidence tumora kože potrebna je njihova pouzdana dijagnoza. Dermoskopija, posebno *in vivo*, poboljšava dijagnozu bazocelularnog karcinoma (BCK). Pregled kompletne kože tela (PKKT) podrazumeva inspekciju cele površine kože bolesnika i predstavlja osnov dermatološkog pregleda, a posebno pregleda „skrininga" kože na premaligne i maligne lezije. Međutim, svrsishodnost PKKT u svakodnevnoj kliničkoj praksi je i dalje predment debate. Cilj rada bio je da se ispita pouzdanost dijagnostike BCK, detektovanog i lečenog od strane iskusnog dermatologa. **Metode.** Retrospektivna analiza obuhvatila je petogodišnji period detekcije BCK prilikom PKKT vizuelnom ispekcijom i dermoskopskim pregledom. Određivana je senzitivnost, specifičnost i pozitivna prediktivna vrednost dijagnoze BCK korišćenjem histopatoloških rezultata kao tačne dijagnoze. **Rezultati.** Od 3 346 bioptiranih tumora kože, 49,58% su bili maligni a 50,42% benigni. Najčešći maligni tumor bio je BCK (84,09%). Njegova najčešća lokalizacija je bio trup (38,92%) i H-regija lica (37,63%). Ostale lokalizacije bile su: lice (izvan H-regiona) (6,67%), vrat (3,01%), poglavina (3,37%), ruke (6,88%) i noge (3,51%). Od ukupnog broja BCK, 0,83% su bili recidivantni. Senzitivnost dijagnostike BCK iznosila je 97,71%, a pozitivna prediktivna vrednost bila je 95,08%. **Zaključak.** U dermatologiji, PKKT vizuelnom ispekcijom i *in vivo* dermoskopijom omogućava veoma dobru dijagnostiku BCK.

Ključne reči:
dermoskopija; dijagnoza; histološke tehnike; karcinom, bazocelularni; senzitivnost i specifičnost; srbija.

Introduction

The growing incidence of skin tumors is a worldwide problem. In Serbia, the incidence of non-melanoma skin cancer (NMSC) is increased for both genders, with an annual percent change of 2.32%, in the period from January 1999 to December 2015. This continuously increasing incidence rate of NMSC in Serbia urges engagement of all practitioners

Correspondence to: Milana Ivkov Simić, University of Novi Sad, Faculty of Medicine, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia.
E-mail: milana.ivkov-simic@mf.uns.ac.rs

dealing with skin tumors and education about preventive measures in the population, in general, as part of the national preventive strategy [1]. Today, the etiology and pathogenesis of basal cell carcinoma (BCC) are much better understood [2]. BCC is the most common skin cancer in Caucasians, slow-growing, with low metastatic potential. If left untreated, locally invasive BCC can jeopardize the aesthetics and function of the anatomical region and eventually become life-threatening. BCC is still a challenge for dermatologists and other practitioners since its accurate and early diagnosis reduces not only the morbidity rate but also the costs of treatment [3]. For a long time, clinical examination, with naked-eye inspection, was standard for detecting skin tumors, and diagnostic accuracy of BCC with visual inspection alone has been superior to other skin tumors [4–6]. Dermoscopy was introduced into clinical practice about 20 years ago as an additional noninvasive tool for visual inspection; at the beginning, its primary use was for skin tumors [7]. Our knowledge of dermoscopy of BCC has been significantly enriched since dermoscopy was introduced to clinical practice. Variability of dermoscopic structures in BCCs, not only augments the clinical differential diagnosis but also provides additional significant information for guiding type and the management of BCC [8–11]. In recently published meta-analyses, the diagnostic accuracy of skin tumors, BCC of particular interest, with visual inspection and dermoscopy, has been analyzed. Great heterogeneity between studies was found, but it was shown that when dermoscopy is used by specialists, especially in person (*in vivo*), it may be a valuable tool to support visual inspection of a suspicious skin lesion for the diagnosis of BCC [12, 13]. Besides, total body skin examination (TBSE), a visual inspection of the patient's total body surface, is considered a basic step in a dermatological exam, especially in skin cancer screening, but also a matter of debate of its expediency in real clinical setting [14–18]. The diagnostic accuracy of BCC has been studied very rarely in the context of TBSE specified. Papers on BCC diagnostic accuracy in Serbia are lacking.

The main aim of this study was to analyze the diagnostic accuracy of BCC detected and treated by referred dermatologists in the Skin Cancer Unit of the Dermatology and Venereology Clinic, University Clinical Center of Vojvodina, Novi Sad, Serbia with an emphasis on the influence of total body skin examination.

Methods

Clinical setting

This retrospective study included consecutive five-year referrals (from January 1st, 2015, to December 31st, 2019) to the Skin Cancer Unit (SCU) at the Clinic of Dermatology and Venereology, University Clinical Center of Vojvodina in Novi Sad, Serbia (coordinates longitude: 19°51 east, latitude: 45°20 north). The referral Center covers health services for the city of Novi Sad and the Serbian province of Vojvodina, with a population of nearly 2,000,000 inhabitants.

In the SCU, four dermatologists were mainly engaged in the diagnosis and treatment of skin tumors and procedural dermatology. Four clinical specialists were part-time engaged in daily SCU practice. Dermoscopy is incorporated into everyday clinical work. All specialists were practicing dermoscopy, three of them at the level of expert dermoscopy (experience > 10 years and PhD thesis and published papers in the field of dermoscopy), two intermediate (dermoscopy experience 5–10 years), and three with basic knowledge and experience in dermoscopy. Routine protocol in the SCU is held for all dermatologists working in the SCU. Patients were referred to the SCU by general practitioners or dermatologists. Waiting time for a consultant varied from several days to three weeks. For all patients, it was mandatory to perform a TBSE. Every patient was examined by visual inspection and dermoscopy in person. Handheld dermoscope DermLite DL100® was at the disposal of every doctor all the time.

For every lesion planned for biopsy, a digital dermoscopy with photo documentation was performed. Clinical and contact polarized dermoscopic photographs were obtained for each lesion using a Nikon Coolpix 4300® camera attached to a DermLite Foto II Pro®. The dermoscopic diagnosis of BCC was based on pattern analysis: looking for the absence of melanocytic specific criteria (network, aggregated globules, streaks, and homogeneous blue pigment) and by identifying features of BCC: arborizing telangiectasia, large blue/gray ovoid nests, ulceration, multiple blue/gray globules, maple-leaf like areas, spoke-wheel areas, short fine superficial telangiectasia, multiple small erosions, concentric structures, multiple in-focus blue/gray dots. It was mandatory for the dermatologist, who made the decision for surgery, to give a preoperative clinical and dermoscopic diagnosis for any tumor or dermatosis, as a first or as a second – to exclude malignancy and to record it in a database of all biopsies, where diagnoses from histopathology reports have also been collected. When inflammatory dermatosis was considered a referral diagnosis, usually, an incisional biopsy was taken. When a skin tumor was considered a referral diagnosis, usually, an excisional biopsy was taken. The sample was fixated in 4% formalin, and standard paraffin vertical sections, treated with hematoxylin-eosin, were examined by one of three pathologists at the Pathology and Histology Center, University Clinical Center of Vojvodina in Novi Sad. In the SCU, we treat skin tumors less than 20 mm in diameter on all anatomic regions of the skin, while bigger lesions are mainly referred to a surgeon for plastic and reconstructive surgery or maxillofacial surgery. In our SCU, some flat lesions on the trunk or limbs larger than 20 mm, quite suspicious for superficial BCC, are treated with imiquimod or 5-fluorouracil therapy after incisional biopsy and histopathological confirmation. Out of all malignant skin tumors, BCC is the most common lesion treated in the SCU. Before the SCU was established in our Department in 2011, all skin tumors were treated by plastic or maxillofacial surgeons. Still, around 400 malignant tumors a year are treated outside the SCU.

Study design

For this study, we underwent a retrospective analysis of referral and histopathological diagnoses of all consecutive biopsies from the SCU database. We excluded biopsies referred to as inflammatory dermatoses and those suspected of T cell lymphoma. The rest were biopsies suspected of skin tumors. When multiple referral diagnoses were present, we took the first one into account. Lesions were detected following accepted SCU protocol.

The study protocol was approved by the Ethics Committee of the University Clinical Center of Vojvodina from February 27, 2017 (00-15/1222).

Statistical analysis

We calculated sensitivity, specificity, positive and negative predictive value (PPV and NPV, respectively) for BCC using histopathological results as the correct diagnosis. For statistical analysis, we used Statistica® Version 13.5 software.

Results

In the study period, there were 4,033 biopsies. From them, we excluded biopsies with suspicion of inflammatory dermatoses and suspicion of cutaneous T cell lymphoma, 687 (17.03%). The remaining 3,346 biopsies were suspected skin tumors. Of all 3,346 biopsied skin tumors, 49.58% were malignant and 50.42% benign (Table 1).

The most common malignant tumor was BCC, accounting for 1,395 (84.09%) of all malignant tumors. The median age of patients with BCC was 75 years (range 20–95, mean ± standard deviation: 72 ± 12); 48.55% were men and 51.45% were women. Localization of BCCs was mainly on the trunk (38.92%) and the H-zone of the face (37.63%). In all, in the head and neck region, the localization of BCC lesions was 50.68% (Table 2). Referral recurrent BCC was found in 25 cases; histopathology verified 12 of them. Of all BCCs, 0.83% were recurrent BCC.

BCC was a referral diagnosis in 1,459 lesions. Histopathological diagnosis of BCC was made in 1,395 biopsies. In 32 lesions, the referral diagnosis was not BCC,

Table 1

Histopathological diagnosis of excised tumors

Diagnosis	n (%)
Malignant	
basal cell carcinoma	1,395 (84.09)
squamous cell carcinoma	119 (7.17)
Morbus Bowen	78 (4.70)
keratoacanthoma	40 (2.41)
malignant other	27 (1.63)
Total	1,659 (100)
Benign	
actinic keratosis	112 (6.64)
seborrheic keratosis	221 (13.10)
dernatifubrina	78 (4.62)
nevus intradermalis	351 (20.81)
nevus dysplasticus	315 (18.67)
nevus ceruleus	15 (0.89)
haemangioma	78 (4.62)
angiokeratoma	2 (0.12)
fibroma	110 (6.52)
cystis	141 (8.36)
verruca vulgaris	94 (5.57)
benign other	122 (7.23)
no tumor	48 (2.85)
Total	1,687 (100)

Table 2

Localization of basal cell carcinoma lesions

Localization	Lesions, n (%)
Head and neck	
head	
face	
H region	525 (37.63)
non-H region	93 (6.67)
scalp	47 (3.37)
neck	42 (3.01)
Trunk	543 (38.93)
Arms	96 (6.88)
Legs	49 (3.51)
Total	1,395 (100)

but BCC was the histopathological diagnosis. In 96 biopsies, the referral diagnosis was BCC, but it was not proven by histopathology (Table 3). We calculated diagnostic accuracy for BCC as sensitivity, specificity, PPV and NPV (Table 4). Sensitivity was 97.71%, specificity 95.08%, the PPV was 93.42% and the NPV 98.3% (Table 5).

Recently, it was shown that diagnostic accuracy of skin tumors, BCC of particular interest, with visual inspection and dermoscopy, if performed by specialists experienced in dermoscopy, may be a useful tool to help diagnose BCC correctly when compared with visual inspection alone [12, 13].

Our study addressed BCC and investigated the diagnostic accuracy of visual inspection and dermoscopy during TBSE.

Table 3

Diagnostic accuracy of basal cell carcinoma

Diagnoses	FP (unnecessary excisions) n (%)	FN (missed BCC) n (%)
SCC	12 (12.50)	17 (53.14)
Morbus Bowen	3 (3.13)	8 (25.00)
Keratoacanthoma	2 (2.07)	0 (0.00)
Malignant other	1 (1.04)	1 (3.12)
Actinic keratosis	17 (17.71)	3 (9.37)
Other benign	28 (29.17)	3 (9.37)
No tumor	33 (34.38)	0 (0.00)
Total	96 (100)	32 (100)

FP – false positive diagnoses; FN – false negative diagnoses; SCC – squamous cell carcinoma; BCC – basal cell carcinoma.

Table 4

Calculation of diagnostic accuracy for basal cell carcinoma (BCC)

Clinical and dermoscopic diagnosis of BCC*	Histopathological diagnosis of BCC	
	positive	negative
Positive	True positive (n = 1,363)	False positive (n = 96)
Negative	False negative (n = 32)	True negative (n = 1,855)

***Clinico-dermoscopic approach with total body skin examination.**

Table 5

Calculated diagnostic accuracy for basal cell carcinoma as sensitivity, specificity, positive and negative predictive value

Statistical parameters	Value (%)	95% CI (%)
Sensitivity: TP/(TP + FN)	97.71	96.78–98.43
Specificity: TN/(FP + TN)	95.08	94.02–96.00
Positive predictive value: TP/(TP + FP)	93.42	92.11–94.52
Negative predictive value: TN/(FN + TN)	98.3	97.63–98.79

TP – true positive; FP – false positive; FN – false negative; TN – true negative; CI – confidence interval.

Discussion

Seeking the simplest and best diagnostic and therapeutic method for BCC, diagnostic accuracy was investigated from different perspectives. Several previous studies have addressed the diagnostic accuracy of skin cancer based on clinical examination with the naked eye [4–6]. They have considered skin tumors, in general, in different settings, with different profiles of physicians enrolled in the study, such as general practitioners, general practitioners with a special interest in skin cancer, plastic surgeons and general surgeons, and dermatologists. In these studies, the sensitivity of BCC detection was superior to other skin tumors and varied between 63.9–90% [4–6].

Attitude toward TBSE is rarely specified in studies [5]. We have endorsed the concept of TBSE and the clinico-dermoscopic approach as we feel more confident with the diagnosis of skin tumors in general, and we wanted to test it from a perspective of diagnostic accuracy of BCC. Ahnlide and Bjellerup [19] conducted a similar study in Sweden. Both studies had a similar setup and results, with the exception of their study being prospective and our retrospective. TBSE was not specified in the methodology, but given the percentage of BCCs detected on different parts of the skinhead and neck (53.3%), arms (5.9%), legs (10.0%), trunk (30.8%), indicated that a full-body check was performed. Important similarities include the clinical setting of the studies, with only dermatologists as diagnosticians. Anhlide and Bjellerup [19] mentioned for their study that it was "the first

European study with such design". Their study lasted 3 and a half years and included dermatologists familiar with dermoscopy. In their study, 2,953 lesions were analyzed, and 55.1% of the excised lesions were malignant. The most common malignant tumor was BCC, accounting for 72.6% of the malignant tumors. Squamous cell carcinoma (SCC) (including invasive SCC, keratoacanthoma, and SCC *in situ*) accounted for 18% of malignant tumors and 8.4% were melanoma. In our study, there were 49.58% of malignant tumors; of them, 84.09% were BCCs, 14.28% were invasive SCCs, keratoacanthoma, and Morbus Bowen, while other malignant tumors, including melanoma, accounted for 1.63%. In a study by Ahnlide and Bjellerup [19], there were 54 (55.5%) false negative (FN) or misdiagnosed BCCs, of them, 30 (55.5%) were diagnosed as SCCs. In our study, out of 32 FN or missed BCCs, SCCs were diagnosed in 52%, Morbus Bowen in 24%, and actinic keratosis was diagnosed in 9% of cases. Although BCC was missed and incorrectly diagnosed, excision was still justifiable, as there was malignancy in 76% or actinic keratosis in 9% of cases.

Some evidence showed that dermatologists did not routinely perform TBSE while examining patients for skin cancer [14]. Some of them stated that factors influencing such an attitude were the lack of evidence about its efficacy, lack of time, or inadequate reimbursement [16]. Furthermore, in 2009, the US Preventive Services Task Force (PSTF) concluded that for the general adult population, TBSE is questionable as a result for several different reasons. They claimed that current evidence is insufficient to assess the harms and benefits of TBSE for the early detection of skin cancer. They accepted that screening could result in the early detection of skin cancers and pointed out that there was not enough evidence that early detection of skin malignancies was related to morbidity or mortality. Moreover, the US PSTF mentioned potential harms from screening and that their magnitude could not be assessed [14]. Some of the harms they referred to were false positive findings, namely lesions that needed not be biopsied or excised as benign lesions and potential anxiety in screened patients. They also mentioned cancers detected by screening were possibly overtreated because of their slow growth, low metastatic potential, and low mortality rate [14]. In 2012, Argenziano et al. [15] conducted research to give more evidence on TBSE in the light of the US PSTF announcement from 2009. Currently, the US PSTF does not recognize anymore the previous statements for early detection of skin cancer in the view of screening the adult population [20].

Argenziano et al. [15] calculated that in order to find one skin malignancy, 47 patients had to be examined by TBSE, and to detect one melanoma, 400 patients were needed. If TBSE was not performed, the risk of missing one malignancy was 2.17% (95% confidence interval was 1.25–3.74%). A similar prevalence of 2.0% of skin tumors detected by TBSE in dermatology settings was found by other dermatologists [17, 18]. We calculated that 2.17% (n = 30) of 1,395 BCCs found in our study could have been missed. In our study, among 96 FP results (unnecessary excisions), 12 were SCC, and 23 were other malignant tumors and precancerous lesions. Measuring potential harms of TBSE concerning false positive results and 2% of potentially missed tumors, we consider performing TBSE reasonable and justified. We share the opinion of Argenziano et al. [15] that TBSE is a safe procedure that can be easily and rapidly performed by dermatologists who are specifically trained. It has been shown that the median time needed for TBSE performed by well-trained dermatologists was only 70 sec, regardless of whether the patients had few or many lesions [21]. In the study of Ahnlide and Bjellerup [19], the sensitivity for BCC diagnosis was 95.4%, and PPV 85.9%, whereas in the present study, sensitivity was 97.71% and PPV 93.42%. Sensitivity in our study was slightly superior to that from Anhlide and Bjellerup study [19], PPV was also superior, but to a higher extent. It is commonly acknowledged that PPV is influenced by the prevalence of the disease in the population tested/studied. With all the other factors remaining constant, PPV increases with increasing prevalence. The superior result of PPV in our study is very likely the sign of the most important limitation of the present study. Namely, in our SCU, there is a slightly unbalanced frequency among malignant skin tumors, with more BCCs compared to SCCs and melanomas. BCC was found in 84.09% of all malignant tumors. As we stressed earlier, in the SCU, mainly NMSC smaller than 20 mm in diameter are treated, while suspected melanomas and bigger NMSC are referred to the Plastic Surgery Unit, which is equipped with facilities to perform sentinel lymph node biopsy.

Nelson et al. [22] conducted a study to estimate the proportion of BCC lesions that could be referred directly to definitive therapy of BCC escaping incisional biopsy that is usually performed as a part of a treatment plan. In the study based on diagnosing BCC through dermoscopy, it was concluded that clinicians were confident enough to refer about two-thirds of BCCs directly to definitive surgery. With dermoscopy and TBSE, regarding our results and other experiences, we are very confident with the diagnosis of BCC, and we already perform excisional biopsies of BCC upon detection, with very satisfactory results concerning recurrent BCCs.

Another limitation of this study, usually occurring in similar studies, concerns the true sensitivity of the study. The true sensitivity of a referral diagnosis can be determined only if all relevant skin lesions are assessed histologically to give the correct number of FN results. Namely, to assess accuracy and sensitivity completely, each lesion clinically and dermoscopically inspected had to be biopsied. However, this is ethically unacceptable, unnecessary, and reasonably unrealistic in a typical clinical setting.

Conclusion

In the dermatology setting, TBSE and visual inspection with *in vivo* dermoscopy result in a very good diagnostic performance of BCC.

The results of our study appear to be superior in sensitivity and specificity with respect to other referred studies. In our opinion, this can be attributed to TBSE and visual inspection aided with dermoscopy. We also believe that this is a consequence of strengthening the coworking of a small number of experienced specialists engaged in diagnosing and treating BCC in our dermatology setting.

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Patients' perception of the quality of community pharmacy services using the critical incident technique

Percepcija pacijenata o kvalitetu usluga u javnoj apoteci korišćenjem tehnike kritičnih incidenata

Ingrid Kummer*, Jovana Mudrić†, Tamara Čikarić†, Ljiljana Tasić†, Andrijana Milošević Georgiev†, Valentina Marinković†

***Zagreb City Pharmacies, Zagreb, Croatia; †University of Belgrade, Faculty of Pharmacy, Department of Social Pharmacy and Pharmaceutical Legislation, Belgrade, Serbia**

Abstract

Background/Aim. The Critical Incident Technique (CIT) is a qualitative research method for measuring consumer satisfaction by collecting and analyzing information on participants and their activities. This method allows participants to present their detailed experiences related to a particular service in the way they perceive them. The aim of this study was to examine patients' perceptions of an incident that occurred in community pharmacies using CIT and determine recommendations for improving the quality of pharmacy services. **Methods.** A qualitative study using an interview based on the CIT was conducted in three pharmacies in Serbia, on the territory of Kruševac city. The entire course of the interviews was audio-recorded, which provided detailed research. **Results.** A total of 68 critical incidents were collected and divided into two groups: positive (37) and negative (31), depending on the (dis)satisfaction of patients with the services of pharmacists in community pharmacies. The following thematic clusters of pharmacy services were covered: accessibility of community-based pharmaceutical services, pharmacist behavior, patient counseling, dispensing drugs and/or medical devices, compounding, and pharmacy sales/commercial practice. **Conclusion.** The results show that the CIT is a useful tool for evaluating and improving pharmaceutical services. Based on the data collected, various aspects of community pharmacy services can be improved, and further research should be carried out.

Key words:
community pharmacy services; patient satisfaction; pharmacists; pharmacy; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Tehnika kritičnih incidenata (TKI) je kvalitativna metoda istraživanja za merenje zadovoljstva klijenata putem prikupljanja i analiziranja podataka o učesnicima i njihovim aktivnostima. Ta metoda omogućava učesnicima u istraživanju da predstave detalje o svojim iskustvima povezanim sa određenom uslugom, na način na koji ih doživljavaju. Cilj studije bio je da se ispita percepcija pacijenata o incidentu koji se desio u javnim apotekama korišćenjem TKI i da se predlože preporuke za poboljšanje kvaliteta farmaceutskih usluga. **Metode.** Kvalitativna studija pomoću intervjua zasnovanog na TKI sprovedena je u tri apoteke u Srbiji, na teritoriji grada Kruševca. Ceo tok intervjua sniman je pomoću diktafona čime je obezbeđeno detaljno istraživanje. **Rezultati.** Ukupno je prikupljeno 68 kritičnih incidenata i podeljeno u dve grupe: pozitivne (37) i negativne (31), zavisno od (ne)zadovoljstva klijenata uslugama farmaceuta u apotekama. Obuhvaćeni su sledeći aspekti usluga u apoteci: dostupnost farmaceutskih usluga u zajednici, ponašanje farmaceuta, savetovanje pacijenata, izdavanje lekova i/ili medicinskih sredstava, rastvaranje lekova i prodaja/komercijalna praksa farmaceuta. **Zaključak.** Rezultati pokazuju da je TKI korisno sredstvo za procenu i unapređenje farmaceutskih usluga. Različiti aspekti usluga u javnoj apoteci se mogu poboljšati što zahteva dalja istraživanja zadovoljstva pacijenata kvalitetom farmaceutske usluge.

Ključne reči:
apoteka, javna, usluge; bolesnik, zadovoljstvo; farmaceuti; apoteka; ankete i upitnici.

Correspondence to: Ingrid Kummer, Zagreb City Pharmacies, 10 000 Zagreb, Croatia. E-mail: ingrid.kummer2@gmail.com

Introduction

An integral part of pharmacist contribution to healthcare is the improvement of rational and economical prescribing and proper use of drugs. The goal of each part of pharmaceutical care service is relevant to each patient, clearly defined, and comprehensively presented to each healthcare provider involved in the treatment and care of patients [1].

Most of the research conducted in the field of quality of pharmacy services is based on practice; they are focused on the identified problem and seek its clarification, evaluation, and improvement of services [2].

The Critical Incident Technique (CIT) is a well-established qualitative research tool used in many areas of health science, education, as well as management, and marketing. John C. Flanagan was the first to describe the CIT, and the original purpose of this method was applied in organizational psychology [3]. The CIT is used to find the cause of system problems to minimize loss of person, property, money, or data. The technique takes into account the collection, analysis, and interpretation of reports on actions taken by experts in response to their experience. This includes the development of constructs that report critical incidents into defined categories; subsequent analysis allows the researcher to draw conclusions on improving results for future scenarios.

The CIT is a method for measuring consumer satisfaction with services by collecting and analyzing information about participants and their activities. This method allows participants to present their details about experiences regarding a particular service in the way they perceive them instead of asking them questions defined by others.

This approach also allows participants to express their satisfaction or dissatisfaction with a particular part of the service. In this paper, the CIT was used to determine (dis)satisfaction of patients with pharmaceutical care services in community pharmacies.

Studies using the CIT in health care have involved nursing staff, physicians, student-patient relations, and healthcare workers (HCW), as well as their behavior in daily work, the standpoint of HCWs in dealing with patients and their complicated chronic conditions, and specific patient needs [4].

In nursing practice, the CIT as a research tool is used to investigate the experiences of patients suffering from age-related wet macular degeneration, how these patients perceive nursing care, and to what extent they are satisfied [5]. In addition, the CIT tool has been used in patients struggling with advanced chronic obstructive pulmonary disease (COPD) and lung cancer [6], as well as in cancer survivors, to investigate and collect information on complementary and alternative medicine (CAM) [7].

Other studies have examined how HCWs make decisions regarding patients' health and reveal why medication errors occur and how to avoid them [8]. The patients suffering from chronic conditions were investigated concerning their perceptions in interactions with HCWs and their time spent in a hospital setting [9, 10].

When searching for publications from pharmaceutical practice, not enough research regarding the quality of pharmaceutical services exist as well as any specific tools that could help in measuring/assessing patient satisfaction.

In a study by Elvey et al. [11] conducted by the CIT tool, the patient-centered professionalism and behavior of early-career pharmacists working in community and hospital pharmacies were examined. Other research was focused on revealing which factors have an impact on pharmacy students when making decisions about over-the-counter drug recommendations to a patient [12]. A few studies have also dealt with pharmacist-related issues, e.g., how they perceive specific situations in their work, the root of patient aggression, the consequence of this aggression on the pharmacist's work and behavior [13, 14], or why community pharmacists might violate the rules of standardized procedures that should be applied when such a violation could pose a potential threat to patient safety [15].

The aim of this study was to examine patients' perceptions regarding the incident occurring in community pharmacies by using the CIT and determine recommendations for improving the quality of pharmacy services.

Methods

A qualitative CIT-based study [3] using the interview method was conducted following the approval of the Ethics Committe in three community pharmacies located in central Serbia (the territory of Kruševac city).

Participants

The sample of patients was selected based on two criteria: patients who visited the pharmacy to collect chronic therapy (for hypertension, asthma, osteoporosis, or diabetes mellitus) for themselves and patients who had any acute symptoms such as headache, high fever, or rash.

The participants had to meet both criteria, after which the time and place of the interview were subsequently agreed upon. The participants were informed of the purpose and protocol of the study and ensured the confidentiality of the data collected. They also signed informed consent.

Sampling was continued until saturation occurred, i.e., until the addition of new incidents contributed to further information for the analysis. Selecting participants ended once there were no new critical incidents in the respondents' answers.

The research was conducted in three community pharmacies: "Benu", "Anđela", and "Lazarica". One pharmacy is located in the city center, and the other two are located on the outskirts of the city (not on the same side). The pharmacies were selected so that one pharmacy is in public ownership ("Lazarica" Pharmacy), and the other two pharmacies are in private ownership, in the pharmacy chain. One of the pharmacies operates only in the local municipality ("Anđela" Pharmacy), while the other operates throughout Serbia ("Benu" Pharmacy).

Ethical approval and consent for participation were applied and waived by the Ethics Committee of the Faculty of Pharmacy, University of Belgrade, No. 430/2. Twenty patients were examined; half of them were in the age group from 30 to 50 years and were predominantly female (90%).

The procedure, analysis, and rigor of data collection

An open-ended interview was designed (Appendix 1); the interviewer (TC) was trained on how to approach participants during the interview. Each interview was audio-recorded and conducted at a time and place convenient for each patient. The interviews lasted 5 to 20 min (10 min on average). All interviews were transcribed verbatim in written format. Each episode was analyzed to gain an in-depth understanding of the significance of the previous participant in a given context [16].

An inductive analysis [16] was performed by identifying the mechanism on which each episode was based and comparing all mechanisms to identify differences and similarities between the studied events. A descriptive list of elements was made and subsequently revised to remove redundancies. Then a list of descriptive elements was organized into a cluster of topics. The expert group (VM, LJT, IK) reached a consensus on the final list of the thematic cluster of pharmacy services and the relationship between the topics. For each example, citations were identified and included anonymously (patient statements only concerning a particular incident).

Results

A total of 20 respondents (90% of women and 10% of men) were included in this study. Table 1 shows data on the sociodemographic characteristics of 20 interviewed respondents, including their age, gender, employment, and the pharmacies they visit.

All critical incidents were sorted according to patients' satisfaction and experience with particular pharmacy services and pharmacist behavior. After classifying the critical incidents, all elements were classified into six thematic clusters (three categories of the structure and three of the procedures), as shown in Table 2.

A total of 68 critical incidents were collected and divided into two groups: positive (37) and negative (31), depending on patients' satisfaction/dissatisfaction with community pharmacy services. The same critical incident was assessed by both participants positively and negatively based on a number of dependent variables: age, gender, prior experience with community pharmacists and pharmacy services, emotional state, and the impact of other patients' experiences (Figure 1).

Table 1

Demographic characteristics of the respondents (n = 20) included in the survey

Characteristics	Respondents n (%)
Age (years)	
< 30	2 (10)
30–50	10 (50)
50–70	6 (30)
> 70	2 (10)
Gender	
female	18 (90)
male	2 (10)
Employment status	
unemployed	3 (15)
employed	6 (30)
retired	11 (55)
Pharmacies included in the survey	
"Anđela"	9 (45)
"Benu"	6 (30)
"Lazarica"	5 (25)

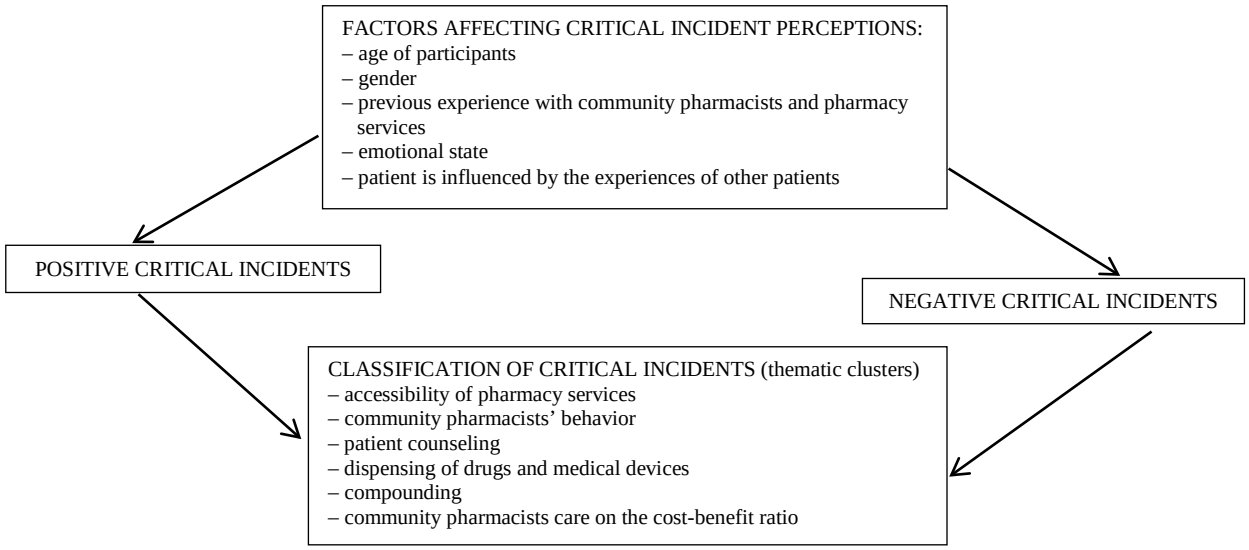

Fig. 1 – Classification of critical incidents (thematic clusters) as perceived by patients.

Table 2

Thematic clusters of pharmacy service and descriptors and a list of quality characteristics leading to the satisfaction/dissatisfaction of patients

Thematic clusters of pharmacy service descriptors	Positive critical incidents (satisfaction of patients)	Negative critical incidents (dissatisfaction of patients)
Accessibility of pharmacy services in community pharmacies	Pharmacy opening hours (working day, weekend)	Pharmacy opening hours (working day/weekend) – working hours too short, especially on Sundays
	Waiting time for pharmacy service	Waiting time for pharmacy service
	Pharmacy access (parking zone for customers, access for people with special needs)	Pharmacy access (parking zone for customers, access for people with special needs)
	Proximity to complementary services (e.g., medical center and laboratory)	
	Pharmacist available at a community pharmacy by phone or mail	
Dispensing of drugs and medical devices	Community pharmacy equipment (wide assortment of prescription drugs, medical devices, dietary supplements, etc.)	Partial (incomplete) information on the proper use of medical devices
	Legible and clear dosing instructions on drugs' packaging	Illegible and confusing writing of the dosing instructions on the drug packaging
	Giving complete information on drugs (name, interval, dosing, route of administration)	Not giving complete information about the drug (name, interval, dosing, route of administration)
	Informing the patient on possible adverse drug/medical device reactions and clinically significant interactions and measures to avoid or alleviate them	Emphasizing the possibility of adverse drug/medical device reaction – the patient is afraid of using the drug prescribed to him/her
	Recognition and reporting of adverse reactions to drugs/medical devices	
	Careful reading of prescriptions	
	Appropriate expiration date of drugs	
	Ordering availability (for products that are not currently available in the pharmacies)	
	Organizing work in the pharmacy	
Pharmacist behavior in a community pharmacy	Politeness and appropriate facial expression of the pharmacist	Community pharmacist using a higher tone of voice
	Responsibility of the pharmacist	Lack of pharmacist responsibility
	Understanding the patient's emotional state	Lack of empathy
	Providing support (assistance) and readiness to help the patient	Community pharmacist who behaves inappropriately
	Careful listening to the patient	Lack of communication with patients
	The patience of the pharmacist in contact with the patient	
Patient counseling	Giving correct and complete evidence-based information during counseling	Providing redundant and incomprehensible information during counseling
	Using appropriate language that patients can understand (without technical and foreign terms)	Dispensing the prescription drugs without counseling (e.g., without giving correct information on how to use the drug)
	Adjusting the tone of voice when addressing patients with hearing difficulty	
	Using capital letters on written materials if the patient has vision problems	
Compounding	Proper labeling of the compound	Illegible writing on the outer compound package
	Control of the expiration date of the compound	Expired date of the compound
	Control of the compound inner package	Lack of detailed instructions
Pharmacist behavior in sale/commercial aspects	Notification on the discounts	Non-reporting on drugs discounts
	Informing the patient about the benefit/cost ratio of the treatment	Short discount periods for dietary supplements and cosmetic products
	Accurate drug billing (participation, total drug costs)	Dispensing more expensive drugs (not covered by insurance)
		Dispensing financially inaccessible drug

Table 3

Thematic clusters of pharmacy service and patients' narratives of positive and negative experiences

Thematic clusters of pharmacy service	Patients' statements (positive critical incidents)	Patients' statements (negative critical incidents)
Pharmacy services accessibility in the community (n = 9)	"The phone line at the pharmacy is never busy, so I can get information on my prescribed drugs from very kind pharmacists."	"I can never find free parking in front of the pharmacy. I take my prescription drugs, and I have no time to ask for any detailed advice (explanation for drugs), as I am in a hurry so that my car does not get towed." "In many community pharmacies, the entrance either has stairs or is so narrow that a wheelchair cannot pass through it."
Dispensing drugs and medical devices (n = 20)	"Although my eyesight is very poor, the pharmacist writes the instructions for taking the drug in capital letters. I try to use it regularly so that it can help me."	"The pharmacist wrote on the drug packaging how to use it for high blood pressure as 0+0+1, so I take it every third day. My blood pressure varies, so I will ask my physician to prescribe me some stronger drug."
Pharmacist behavior (n = 13)		"I was arguing with pharmacists in a community pharmacy for half an hour, trying to explain that it was not the same drug I had been taking for years. He assured me that the manufacturer had changed the packaging and the medicine was the same, and the price was the same. Finally, I took that drug with new packaging when I saw that other pharmacies also no longer had the drug in old packaging." "I went to the pharmacy to get my prescription for diabetes, but also to find out what should I modify in my diet to avoid using insulin so often. The pharmacist was very unpleasant, and she did not want to dispense the prescribed drug to me and emphasized that she was not an endocrinologist." "Once I went to the pharmacy to get my prescribed drugs… they were completely unorganized - piles of scattered papers, prescriptions, etc. The pharmacist dealt more with the papers than listening to me, and she gave me a urinary tract tea instead of the respiratory tract tea that I requested, as I had a dry cough."
Patient counseling (n = 13)	"Lately, my son has been having a harsh, strange, and mutated voice. It was very strange since he is not yet in puberty, but the pharmacist explained that it could be due to the inhaler he was using, so she suggested he should use the Volumatic chamber. She also advised him to rinse his mouth with water so that the drug particles would not deposit in his throat."	"I take this drug for my hypertension only when my blood pressure is high. Why should I take it every day if I do not have high blood pressure every day? Nobody, neither the physician nor the pharmacist, told me that it was important to take it regularly." "The pharmacist scared me when he told me that my son could get an anaphylactic shock if I gave this antibiotic to him. He has never taken this drug before, so I'm not sure if he is allergic or not."
Compounding (n = 6)		"After waiting a long time to perform the OGTT test, I failed because the glucose I bought at the pharmacy did not dissolve in water. When I returned, I went to the pharmacy and informed the pharmacist to check the glucose because I did not want other patients to have the same problem during testing. However, the pharmacist was not interested (he did not want to hear my objections), so I turned and left."
Pharmacist behavior in sale/commercial aspects	"The drug for osteoporosis that my physician prescribed was expensive. The pharmacist offered me a more affordable drug at a discount."	"Ever since I quit smoking, I've been anxious and started eating more, and I've gained 10 pounds. The pharmacist tricked me into selling me the most expensive weight loss formulation that didn't help me lose weight."

Community pharmacy services comprise different inter-related segments, and the underdevelopment of one can cause the weakening of other segments, resulting in health/clinical, economic, and legal consequences. The most interesting patient statements, i.e., descriptions of situations, both positive and negative, are shown in Tables 2 and 3.

Discussion

Patient-centered care describes the partnership between the patient and HCW, where patients' expectations and experiences with their disease, as well as experiences with drugs, determine their willingness to adhere to prescribed drugs [17].

In recent years, the pharmaceutical practice has expanded significantly, with the increase mainly related to person-centered care and patient needs [18].

The context in which health care services are provided by different HCWs, such as physicians, nurses, or pharmacists, is different, but the expectations of patients in these systems are somewhat similar [6].

Behavioral research involving patients exists, but research in pharmaceutical practice from the patients' point of view is rare, so the research instrument used in this study is interesting because it has been used in other disciplines before (e.g., nursing, medical practice).

In this study, the number of actual critical incidents (37) exceeded the number of negative critical incidents (31), indicating that participants were generally satisfied with pharmacy services in community pharmacies. Positive experiences with patients mean that in cooperation with a pharmacist, patients could achieve positive clinical outcomes, while negative critical incidents indicate that pharmacy services require improvement to reduce errors in providing pharmacy services in community pharmacies. A significant cluster of pharmacy services is the process of counseling at the time of dispensing drugs that should be processed with sufficient privacy.

Our results are consistent with the research by Emsfors et al. [5]. In that research, when patients were perceived to have been treated with respect and when they were involved in the process, it created confidence and trust among the patients and a willingness to cooperate. The most common reasons why participants said they were discouraged from asking for advice and help from a pharmacist were: lack of time, lack of privacy, insufficient number of qualified pharmacists in the pharmacy, pharmacists dealing with administrative matters, and inadequate pharmaceutical education. Lack of perceived privacy is an obstacle to patients as well as in the study conducted among cancer survivors, where privacy concerns were a restrictive factor for participants, the same as for patients in our study [7]. It should be noted that patients in different health care institutions (e.g., community pharmacies, hospitals) have almost similar problems when receiving care. Lack of attention and continuity in care, poor communication [6], and insufficient information have reduced the patients' ability to participate in deciding about their care if they were not considered "co-partners" [5]. Therefore, this is considered poor health care.

Of the total number of critical incidents collected, participants had the most positive experiences with the service in a community pharmacy dispensing drugs and medical devices since this service is the most common reason for visiting a community pharmacy and the main and traditional service in pharmacy practice.

Pharmacists' behavior in a community pharmacy can impair communication with patients if the pharmacists do not listen to what their patients say, do not try to explain things in a way the patients can understand, have a negative attitude (insecure, preoccupied, pessimistic), express disinterest in patients' problems, ignore patients' emotional state or patients' fears, or if the community pharmacists are in a constant rush due to various assignments.

Patients should be informed about the drugs they use and how they should be used to achieve the best possible outcome. The same problems arose in nursing practice research conducted by Bailey et al. [6] when patients were unaware of what to expect from the treatment, what would precede their recovery, or when patients felt that their emotional support was gone [7.] As a solution, pharmacists should strengthen the sense of trust with patients, providing them with clear and unambiguous explanations about the use of drugs and possible adverse drug reactions.

Another very important question for patients is how HCW perceives them. It is vital that they are considered equal partners in deciding their treatment with their HCW; they recognize themselves as persons and receive sufficient information regarding their health condition. In a community pharmacy, when patients were not satisfied with the proposed solution or explanation given by the pharmacist, they felt that the quality of service was declining. Our findings are well related to the results of Emsfors et al. [5], who studied patients suffering from age-related wet macular degeneration, and similar nursing behavior resulted in patients' satisfaction and the perception that they received the treatment they needed.

Recommendations on how to overcome barriers in communication between community pharmacists and patients [19] include: avoiding technical language when addressing patients, using written instructions instead of verbal ones where necessary, checking that the patients understand instructions, actively listening to the patients, patiently answering patients' questions, paying attention to patients' emotional and social needs, and communicating with confidence and empathy. Empathy in communication in health care implies humanity, care, altruism, and sharing emotions. It has been noticed that it has a positive effect on the patients' well-being and their perception of the therapy that is received [20].

The results of this study show that the CIT could be a useful tool for improving the quality of pharmacy services in the community by increasing patient satisfaction. Based on the results obtained, various aspects of community pharmacy services can be improved. Further research is needed using the CIT in more community pharmacies and more countries to allow the comparison of results.

Limitations

This study describes the experiences of patients from three different pharmacies in a smaller city in Serbia (Kruševac, about 60,000 citizens). Therefore, the small size of the sample does not allow a conclusion to be drawn about the wider population of patients.

Conclusion

The CIT recognized six descriptors/thematic clusters of incidents in community pharmacy practice as a foundation for a quality improvement recommendation. Patients' perception of pharmacists' behavior was considered a very important descriptor. It was identified especially in three basic pharmacy services (dispensing, canceling, and compounding drugs) to meet the patients' needs and achieve positive therapeutic results. Good communication between a patient and a pharmacist increased patient satisfaction. Moreover, it had an impact on the quality use of non-prescribed drugs (self-medication). Based on the data collected, various aspects of public pharmacy services can be improved, and further research should be carried out.

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Appendix 1

Open-ended questions that led the interview*:*

1) Introductory statement

“We are conducting a study that aims to examine your experiences with community pharmacy services and community pharmacists in any of your previous visits to community pharmacies”;
“May I interview you?”;
“May I record your voice during the interview?” (Signed consent form)

2) Remembering and contextualizing the episode

“Do you remember any good or bad experiences you had with community pharmacists while providing pharmacy services in the community pharmacy?”

3) Describing the dynamics of the event

“Please, describe the event in detail and how it happened.”;

“Please, tell me the details of the episode (e.g., what was the pharmacist doing, how did they behave?)”;

“Have you ever experienced any benefit or consequence as a result of the pharmacist's behavior in the community pharmacy?”

MINI REVIEW

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Clinical trials of resveratrol efficacy and safety

Klinička ispitivanja efikasnosti i bezbednosti resveratrola

Veljko Ćućuz*, Jelena Cvejić*, Ljiljana Gojković-Bukarica†

***University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Novi Sad, Serbia; †University of Belgrade, Faculty of Medicine, Department of Pharmacology, Toxicology and Clinical Pharmacology, Belgrade, Serbia**

Key words:
antioxidants; phytotherapy; protective agents; resveratrol; review.

Ključne reči:
antioksidansi; fitoterapija; protektivni agensi; resveratrol; pregled.

Introduction

Trans-resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a phytoalexin class of stilbene, phenolic compound from non-flavonoid group. Plants produce it in response to fungal infections (*Botrytis cinerea, Plasmopara viticola*, etc.) and other stress factors such as ultraviolet (UV) radiation, ozone, heavy metal ions, mechanical injury to plant tissues, or frost [1]. It was first detected in 1940 from the root of white *Veratrum grandiflorum*, and since then until today, its derivatives (glycosides and oligomers) have been isolated and identified in over 70 plant species [2]. The well-known source of this compound in the human diet is certainly wine [3–5]. In traditional Asian medicine, white *Veratrum* root has been used for many purposes, such as for treating atherosclerosis, cough, asthma, hypertension, and cancer [6]. Numerous *in vitro* and preclinical animal studies have demonstrated the ability of trans-resveratrol to exhibit a wide range of potential benefits for human health, such as antioxidant, anti-inflammatory, cardioprotective, neuroprotective, antidiabetic, and anticancer activity [7–14].

We presented the available data on the most significant clinical trials of resveratrol's biological effects and evaluated its efficacy in humans. Resveratrol studies were found via a search of the PubMed, Web of Science, and SCOPUS databases using "resveratrol" and "clinical trial" as keywords. The search was limited to studies published between 2010–2019 reporting on cardioprotective activity, circulatory function, metabolism, and anticancer activity of resveratrol. These conditions were selected due to the fact that most of the clinical trials were conducted in these treatment areas. Unregistered clinical trials, trials without clear and specific end-point outcomes, and clinical trials focusing solely on general pharmacokinetics of resveratrol were excluded.

Studies related to cardioprotective activity

In their study, Bo et al. [15] examined the beneficial effects of resveratrol on markers of inflammation and oxidative stress in smokers (Table 1). A study of 50 healthy smokers who received 500 mg of resveratrol daily for 30 days was randomized, double-blind, and crossover. The results showed that resveratrol significantly decreased C-reactive protein (CRP) and triacylglycerol concentrations while the total antioxidant status was increased by 74.2 μmol/L. Concentrations of uric acid, glucose, insulin, cholesterol, and liver enzymes, as well as weight and blood pressure values, did not change significantly. Due to the demonstrated anti-inflammatory, antioxidant, and hypotriglyceridemic effects, resveratrol supplementation may be beneficial for reducing cardiovascular risk in healthy smokers.

In a much longer study of one year, Tome-Carneiro et al. [16] examined the effect of resveratrol-enriched grape supplementation on the inflammatory and fibrinolytic status of high-risk cardiovascular subjects receiving statins for primary prevention. A randomized, triple-blind, parallel, placebo-controlled study included 75 subjects divided into 3 groups. The resveratrol group received 8 mg of resveratrol for the first 6 months and twice the dose for the next 6 months. In the resveratrol-enriched grape supplement group, a highly-sensitive CRP (-26%), tumor necrosis factor (TNF) α (-19.8%), plasminogen activator inhibitor type 1 (-16.8%),

Correspondence to: Jelena Cvejić, University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia. E-mail: jelena.cvejic@mf.uns.ac.rs

Table 1

Clinical trials of resveratrol

Subjects	Daily resveratrol dosage/length of trial	Biomarker changes	Effect	Ref.
50 healthy smokers	500 mg /30 days	↓ CRP and triacylglycerol concentrations; ↑ the total antioxidant status	Beneficial	15
75 high-risk cardiovascular subjects receiving statins for primary prevention	8 mg/first 6 months 16 mg/next 6 months	↓ highly-sensitive CRP, TNF-α, PAI-1, IL-6/IL-10; ↑ anti-inflammatory IL-10	Beneficial	16
166 patients with stable angina pectoris	Group I: 20 mg, Group II: 20 mg + 112 mg of calcium fructoborate, Group III: 112 mg of calcium fructoborate; 60 days	↓ highly-sensitive CRP, N-terminal prohormone of brain natriuretic peptide	Beneficial	17
40 patients who have suffered a heart attack	10 mg/3 months	Improved left ventricular diastolic function and endothelial function; ↓LDL cholesterol	Beneficial	18
75 subjects receiving statins for primary prevention of cardiovascular disease	8 mg/6 months	↓LDL cholesterol, ApoB, and oxidized LDL cholesterol; no changes in hepatic, renal, and thyroid function	Beneficial	19
71 patients with dyslipidemia	100 mg/2 months	↓Total cholesterol and triacylglycerol concentrations; no significant differences in HDL- and LDL-cholesterol concentrations	Beneficial	20
28 obese subjects	75 mg/6 weeks	↑ Flow-mediated dilation; unchanged blood pressure and arterial compliance	Beneficial	25
22 healthy subjects	250 or 500 mg/single doses	Dose-dependent increase in cerebral blood flow (measured via total hemoglobin concentration); no significant change in cognitive function	Beneficial	26
66 patients with type 2 diabetes	1,000 mg/45 days	↓ Systolic blood pressure, blood glucose, hemoglobin A1c, insulin and insulin resistance; ↑ HDL cholesterol; unchanged markers of liver and renal function	Beneficial	27
24 obese men	1,500 mg/4 weeks	No effect on blood pressure, ectopic or visceral lipid content, inflammatory and metabolic biomarkers	None	30
29 postmenopausal women with normal glucose tolerance	75 mg/4 weeks	Unchanged plasma lipids, inflammation markers, and insulin sensitivity of the liver, skeletal muscle, and adipose tissue	None	31
28 obese men (11 Caucasians and 17 non-Caucasians)	2,000 mg/30 days	Significant improvement in insulin resistance and glucose homeostasis only in Caucasians	Beneficial	32
39 adult women with increased risk of breast cancer	50 mg/12 weeks	↓ Methylation of tumor suppressor gene RASSF-1α	Beneficial	33
9 patients with colorectal cancer and hepatic metastases	5,000 mg of micronized resveratrol SRT501/2 weeks prior to surgery	↑ Cleaved caspase-3, a marker of apoptosis, in malignant hepatic tissue	Beneficial, well-tolerated	34
24 patients with relapsed and/or refractory multiple myeloma	5,000 mg of micronized resveratrol SRT501 with or without bortezomib/~4 months	Not available	Severe adverse events: Nephro-toxicity to renal failure	35

interleukin (IL)-6/IL-10 (-24%) were significantly decreased, while anti-inflammatory IL-10 (+19.8%) was significantly increased compared to placebo and the resveratrol-free supplementation group. Adiponectin was increased by 6.5%, while soluble intercellular adhesion molecule-1 decreased by 5.7%. No adverse effects were reported. The results of the study indicated that one year of resveratrol-enriched grape supplementation could improve the anti-inflammatory and

fibrinolytic status of patients receiving statins for primary prevention of cardiovascular disease and thus be used together for better effect.

By enrolling a larger number of subjects, Militaru et al. [17] conducted a randomized, double-blind, controlled, parallel study of 166 patients with stable angina pectoris for 60 days, divided into three groups. The group I received resveratrol (20 mg/day), the group II was a combination of resveratrol and calcium fructoborate, and the group III only had calcium fructoborate (112 mg/day). Biomarkers of inflammation, markers of left ventricular function, and lipid markers were measured. The results showed that there was a significant decrease in a highly-sensitive CRP in all three groups, but the largest decrease was in the group III (39.7%). On the other hand, the marker of left ventricular function (N-terminal prohormone of brain natriuretic peptide) was decreased by 59.7% (the group I) and 52.6% (the group III), while the combination of resveratrol and calcium fructoborate (the group II) was the most effective (65.5%). This combination significantly reduced the weekly frequency of angina attacks and, by that, improved the quality of life of the respondents. Lipid markers changed only slightly from baseline values.

Through a randomized, double-blind placebo-controlled study, Magyar et al. [18] investigated the cardioprotective effects of resveratrol in patients who have suffered a heart attack. The subjects (n = 40) were divided into two groups, where one group received 10 mg of resveratrol for three months and the other one placebo. The results demonstrated that resveratrol improved left ventricular diastolic function as well as endothelial function, lowered low density lipoprotein (LDL) cholesterol, and protected patients with coronary artery disease from adverse hemorheological changes.

In a randomized, triple-blind, placebo-controlled study, Tome-Carneiro et al. [19] included 75 subjects receiving statins for primary prevention of cardiovascular disease. Their aim was to investigate a 6-month-effect of grape supplementation containing 8 mg of resveratrol on oxidized LDL cholesterol, Apolipoprotein B (ApoB), and serum lipids. Compared to the placebo group, LDL cholesterol (-4.5%), ApoB (-9.8%), and oxidized LDL cholesterol (-20%) decreased significantly. No changes in hepatic, renal, or thyroid function were observed. No adverse effects were reported in any of the subjects. Thus, the resveratrol-enriched grape extract may have the effect of reducing atherogenic markers and exerting cardioprotective activity [19].

In a randomized, double-blind, placebo-controlled clinical trial from 2019, Simental-Mendia and Guerrero-Romero [20] examined the effect of resveratrol on the lipid status of men and women (n = 71) with dyslipidemia at a dose of 100 mg daily for two months. As an outcome, resveratrol supplementation significantly reduced total cholesterol (-19.2) and triacylglycerol (-33.3) levels compared to the placebo group, whereas there were no significant differences for high density lipoprotein (HDL) and LDL cholesterol.

In 2013, Sahebkar [21] conducted a systematic review and meta-analysis of seven randomized, controlled studies in order to investigate the effects of resveratrol supplementation on plasma lipids. This meta-analysis included 282 subjects (141 in each group). The results demonstrated that resveratrol supplementation had no significant effects on any of the lipid parameters: total cholesterol (-8.70%), LDL cholesterol (-3.22%), HDL cholesterol (-0.26%), and triacylglycerols (-4.30%). The obtained results were robust against the sensitivity of the analysis and did not depend on the dose of resveratrol, the time of supplementation, or the cardiovascular risk of the study population. The results indicated that other mechanisms, other than hypolipidemic, are responsible for the cardioprotective properties of resveratrol. A more recent systematic review and meta-analysis from 2018 included twenty-one randomized clinical trials and provided the same results, with the only difference that a statistically significant difference occurred for triacylglycerol levels. However, after eliminating only one study from the meta-analysis, this significance was also lost [22].

In a new systematic review and meta-analysis of 17 randomized, controlled clinical trials from 2019, Fogacci et al. [23] compared the impact of resveratrol administration on human blood pressure. The results showed that resveratrol supplementation did not significantly affect systolic or diastolic blood pressure. However, administration of higher doses of resveratrol ($\geq$ 300 mg daily) significantly reduced systolic blood pressure in diabetic patients and thus exhibited cardioprotective activity.

Studies related to circulatory function

Wong et al. [24] demonstrated earlier, acute, dose-dependent, flow-mediated dilation (FMD) of the brachial artery after the administration of resveratrol in mildly hypertensive, obese subjects. Resveratrol supplementation also showed an acute increase in cerebral blood flow without affecting cognition. This time, the study was conducted to evaluate the effects of chronic resveratrol supplementation on flow-mediated dilatation and cognitive performance (Table 1). Obese but otherwise healthy subjects (n = 28) were randomized into two groups. In a double-blind, crossover study, one group received 75 mg per day of encapsulated resveratrol and the other one placebo for 6 weeks. The results showed that resveratrol supplementation for 6 weeks was well-tolerated and resulted in a 23% increase in flow-mediated dilation compared with the placebo group. A single dose of resveratrol (75 mg) followed by chronic resveratrol supplementation resulted in a 35% stronger acute FMD response than placebo supplementation. On the other hand, blood pressure and arterial compliance remained unchanged. In conclusion, chronic resveratrol supplementation has the potential to maintain the healthy circulatory function of obese subjects [25].

Through a randomized, double-blind, crossover investigation, Kennedy et al. [26] evaluated the impact of resveratrol on cognitive performance and localized cerebral blood flow. Healthy volunteers (n = 22) received a placebo or two different single doses of resveratrol (250 or 500 mg).

Administration of resveratrol led to a dose-dependent increase in cerebral blood flow, which was measured via total hemoglobin concentration. After the administration of both doses of resveratrol, there was also an increase in deoxyhemoglobin. However, the cognitive function of subjects did not change significantly.

Studies on metabolism

Through a randomized placebo-controlled double-blind, parallel study, Movahed et al. [27] examined the efficacy of resveratrol on lowering blood glucose levels in the presence of standard antidiabetic drugs (Table 1). The study included 66 patients with type 2 diabetes who received resveratrol supplementation (1 g/day) for 45 days and the placebo (control) group. The results showed that resveratrol treatment significantly reduced systolic blood pressure, blood glucose, hemoglobin A1c, insulin, and insulin resistance, while HDL cholesterol was significantly increased compared with the placebo group. Markers of liver and renal function remained unchanged. No significant changes in body weight and body composition occurred. This study showed that resveratrol supplementation could exert potent antidiabetic activity in patients with type 2 diabetes, unlike previous reports that showed only mild effects on hyperglycemia and hyperinsulinemia [28, 29].

However, Poulsen et al. [30] obtained different results after conducting a randomized, double-blind, placebo-controlled study involving 24 obese but otherwise healthy men. Subjects were given 500 mg of resveratrol three times a day for four weeks. The results demonstrated that endogenous glucose production, turnover, and oxidation remained unchanged, whereas insulin sensitivity slightly decreased in both groups. Supplementation with resveratrol had no effect on blood pressure, ectopic or visceral lipid content, as well as inflammatory and metabolic biomarkers.

Another study that also did not have positive results was conducted by Yoshino et al. [31]. In a randomized, double-blind, placebo-controlled study, 29 postmenopausal women with normal glucose tolerance received 75 mg of resveratrol a day for four weeks. Although resveratrol supplementation led to an increase in resveratrol concentration in plasma, plasma lipids and inflammation markers remained unchanged. There was also no increase in insulin sensitivity of the liver, skeletal muscle, and adipose tissue. Therefore, resveratrol supplementation in this study did not exhibit beneficial metabolic effects in postmenopausal women with normal glucose tolerance.

An interesting pilot study from 2019 was conducted by Walker et al. [32], and it included 28 obese men with metabolic syndrome. The subjects (11 Caucasians and 17 non-Caucasians) received orally 2 g of resveratrol/day (in two daily doses) or a placebo over 30 days. The results showed that resveratrol supplementation led to a significant improvement in insulin resistance and glucose homeostasis, but only in Caucasians. These different reactions between members of different races are due to their differences in the gut microflora, where resveratrol in the case of Caucasians reduced the diversity of gut microflora and increased the number of microbe *Akkermansia muciniphila*, which has been shown to have beneficial effects on obesity and diabetes in experimental animals. As this was a pilot trial, more people should be included before reaching conclusions.

Studies on anti-cancer activity

Zhu et al. [33] conducted a randomized, double-blind study of 39 adult women with an increased risk of breast cancer. For 12 weeks, one group received a placebo, and other two groups received resveratrol. One of the two groups got 5 mg and the other 50 mg of resveratrol. The obtained results provided new insights into the effects of resveratrol, which included a decrease in the methylation of tumor suppressor gene RASSF-1α with an increase in serum resveratrol levels.

In 2011, Howells et al. [34] conducted the first phase of a randomized, double-blind study with the micronized resveratrol SRT501, whose micronization improved the absorption and thus the bioavailability of resveratrol. SRT501 was given to patients with colorectal cancer and hepatic metastases at a dose of 5 g daily for 14 days. The aim of the study was to evaluate the safety, pharmacokinetics, and pharmacodynamics of this resveratrol formulation. The obtained results led to the following conclusions: daily use of SRT501 for 14 was well-tolerated in patients with colorectal cancer; C_{max} for SRT501 was significantly higher compared to equivalent doses of non-micronized resveratrol; ingestion provided measurable concentrations in tissue distant from the gastrointestinal tract (specifically in the liver), which led to a significant pharmacological effect (significant increase in cleaved caspase-3, a marker of apoptosis, in malignant hepatic tissue).

A year later, in the second phase of the clinical study, the same form of micronized resveratrol SRT501 was given to patients with relapsed and/or refractory multiple myeloma (n = 24), where a severe adverse event for SRT501 was observed – nephrotoxicity to renal failure. Since this adverse event was not recorded in the first phase of the clinical study in patients with colorectal cancer, this is considered an adverse event only for patients with multiple myeloma [35]. New large-scale studies are required to assess the use and safety of resveratrol as a chemoprotective or chemotherapeutic agent.

Evaluation of resveratrol efficacy in humans

The results of clinical efficacy of resveratrol indicate that resveratrol may have beneficial cardioprotective effects in smokers [15], persons with stable angina pectoris [17], persons who have suffered from a heart attack [18], and persons who already receive statins for the primary prevention of cardiovascular disease [16, 19]. In addition, resveratrol can improve circulatory function [24–26] and glucose metabolism [27], while anticancer activity in humans remains poorly investigated [33, 34]. These studies were not patient-oriented but

mainly focused on changes in some biochemical parameters that are indicators of the existence or severity of diseases.

Some studies gave conflicting results, such as whether resveratrol supplementation changes inflammatory or metabolic biomarkers. Reasons for obtaining inconsistent results may be differences in the characteristics of the involved patients, the dose of resveratrol, as well as the duration of supplementation [36]. One of the biggest challenges in evaluating resveratrol efficacy in clinical studies is the fact that regarding its very low bioavailability, there is a wide range of used doses (from 5 mg to 5 g). Furthermore, some supplements contain additional components with a presumed synergistic effect where synergism is reflected by increasing the bioavailability or bioactivity of resveratrol, such as the resveratrol/calcium fructoborate combination [17]. These synergistic components may also influence the results, and their relative individual contribution is still unknown [37].

What undoubtedly encourages future clinical trials of resveratrol is that its administration at a dose of 5 g/day for one month is safe and well-tolerated [38].

Conclusion

The results of clinical trials suggest that resveratrol may exert some beneficial effects on human health. However, important questions such as the dose and length of a treatment that would make the most of resveratrol potential remain unsolved. It is expected that future extensive and better-designed clinical trials will give answers to these challenges.

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Tacrolimus-induced optic neuropathy – a case report

Optička neuropatija izazvana takrolimusom

Lepša Žorić*†, Aleksandra Ilić†‡, Emina Čolak§, Miloš Mirković*, Jelica Pantelić†‡, Dijana Mirić‖, Bojana Kisić‖

University of Priština, Faculty of Medicine, *Ophthalmology Department, ‖Institute of Medical Biochemistry, Kosovska Mitrovica, Serbia; University Clinical Center of Serbia, †Institute of Eye Diseases, §Institute of Medical Biochemistry, Belgrade, Serbia; ‡University of Belgrade, Faculty of Medicine, Belgrade, Serbia

Abstract

Introduction. Tacrolimus (fujimycin or FK506) is a potent immunosuppressive drug with growing usage. It is usually used in the prevention of transplanted organ rejection. Its use is highly valuable, but like other immunosuppressants, it has adverse effects. One of them is optic neuropathy. **Case report.** A 47-year-old male patient, who had received tacrolimus therapy for nine years after kidney transplantation, developed a subacute, painless vision loss in both eyes. He was thoroughly examined on different possible optic neuropathies and other causes of vision loss. After exclusion of other possible causes, the diagnosis of toxic optic neuropathy was established. The patient's therapy was converted to cyclosporine by his nephrologist, but his vision had improved only slightly. **Conclusion.** Toxic optic neuropathies are presented in everyday ophthalmological practice, but they are underestimated. Diagnosis can be demanding, especially when it comes to drugs and substances whose possible toxic effect on the optic nerve is not widely known. Unlike other adverse effects of tacrolimus therapy on the nervous system, optic neuropathy can cause great and permanent functional impairment.

Key words:
drug-related side effects and adverse reactions; tacrolimus; toxic optic neuropathy; treatment outcome.

Apstrakt

Uvod. Takrolimus (fujimycin, FK506) je potentan imunosupresivni lek čija upotreba je u porastu. Obično se koristi u prevenciji odbacivanja transplantiranih organa. Njegova primena je dragocena, iako, poput drugih imunosupresivnih lekova, ima i neželjena dejstva. Jedno od takvih dejstava je optička neuropatija. **Prikaz bolesnika.** Bolesnik muškog pola, star 47 godina, koji je zbog transplantiranog bubrega primao takrolimus devet godina, razvio je bezbolni gubitak vida na oba oka, subakutnog toka. On je detaljno ispitan na moguće uzroke optičkih neuropatija i druge moguće uzroke gubitka vida. Nakon isključenja drugih mogućih uzroka, postavljena mu je dijagnoza toksične optičke neuropatije. Nadležni nefrolog je izmenio terapiju i uveo ciklosporin, ali vid se samo diskretno poboljšao. **Zaključak.** Toksične optičke neuropatije se javljaju u svakodnevnoj oftalmološkoj praksi, ali se na njih retko posumnja. Postavljanje dijagnoze može biti zahtevno, posebno u slučaju lekova i suspstanci čije moguće toksično dejstvo na očni nerv nije šire poznato. Za razliku od ostalih neželjenih dejstava takrolimusa na nervni sistem, toksična optička neuropatija može izazvati značajan i trajan gubitak vida.

Ključne reči:
lekovi, neželjeni efekti i neželjene reakcije; takrolimus; neuropatija, optička, toksična; lečenje, ishod.

Introduction

Tacrolimus (fujimycin or FK506) is an immunosuppressant used mainly after allogeneic organ and bone marrow transplantation to prevent transplanted organ rejection and graft versus host disease (GVHD). This macrolide was isolated from a strain of *Streptomyces*. The mechanism of action is similar to cyclosporine, but it is more potent and has less serious adverse effects [1]. Tacrolimus acts by the calcineurin phosphatase inhibition and so intervenes on interleukin (IL)-2 transcription and T lymphocyte signal transduction. In recent years it has been increasingly used, even in ophthalmology, for some unwanted or excessive topical or systemic immune responses inhibition [2].

Immunosuppressive drugs have revolutionized transplant medicine. However, they have numerous adverse effects on almost every organ system [3]. Calcineurin inhibitors are known for their neurotoxicity, both central and peripher-

Correspondence to: Lepša Žorić, University Clinical Centre of Serbia, Institute of Eye Diseases, Pasterova 2, 11 000 Belgrade, Serbia. E-mail: zoriclepsa@gmail.com

al [4]. One of the most frequent toxic effects is posterior reversible encephalopathy syndrome (PRES). Side effects related to a visual deficit in this syndrome occur in nearly 40% of patients [5], but they are usually reversible after the therapy modification. Peripheral toxic neuropathies are also described, and they develop after weeks or months of therapy [4].

Toxic optic neuropathies (TONs) are usually bilateral, more or less symmetric, painless, and progressive, but otherwise they have characteristics similar to some other optic neuropathies (diminution of vision, dyschromatopsia, normal or edematous optic disc, visual field scotomas, a disorder of pupillary response to light, and later, some degree of optic nerve atrophy) [6]. Although they are not uncommon in ophthalmic practice, elucidating TONs demands a serious and demanding approach. The diagnosis is made based on exhaustive anamnesis, the disease features, and course and exclusion of other possible causes. The most widely known causes of TONs are antituberculosis drugs (isoniazid, ethambutol, streptomycin), some antibiotics (chloramphenicol, linezolid, sulfonamides), antimalarials (chloroquine, quinine), antiarrhythmics (amiodarone, digitalis), anticancer agents (vincristine, methotrexate, cyclosporin), alcohols (methanol, ethylene glycol), heavy metals (mercury, lead, thallium), and other (carbon monoxide, tobacco) [7], and inhibitors of phosphodiesterase 5 (sildenafil). If they are caused by drugs, the majority of them recover after the therapy cessation or conversion, but in others, such as optic neuropathy induced by tacrolimus, the favorable outcome may be lacking.

Case report

A 47-year-old male patient was first seen after he received intravenous pulse methylprednisolone therapy with prednisone tapering because his condition was diagnosed as bilateral inflammatory retrobulbar optic neuropathy. As there was no improvement on the subsequent checkups, he was directed to the Ophthalmology Department for further examinations.

The onset of the disease manifested with the patient's vision deterioration bilaterally, gradually, for eight to ten days before visiting an ophthalmologist. At first, the patient had noticed visual disturbances for distance and shortly after for near vision. Visual loss was painless, with slight daily variations and without other neurological symptoms. As his life quality decreased rapidly and seriously, he decided to visit an ophthalmologist.

The patient had a blunt trauma of his right eye some 20 years ago with residual light visual decline and posttraumatic mydriasis. Secondary glaucoma and incipient cataract developed years after that accident and was recorded during this hospitalization. Occupied with other health and family issues, he has not been controlled ophthalmologically for years. He started wearing a hearing aid twelve years ago because of bilateral sensorineural hearing loss. He had kidney transplantation in 2010, and since then, he has been on tacrolimus therapy (3 mg prolonged-release capsules), together with mycophenolic acid 540 mg twice daily, with regular checkups and without any adverse effects. At the time of this hospitalization, his therapy was also enalapril and amlodipine. He had stopped smoking cigarettes and consuming alcohol more than ten years ago.

On admission, visual acuity on his right eye was counting fingers on 30 cm, and on his left eye, on 1m. He had an incipient cataract on both eyes, more prominent on the right but not dense enough to explain vision loss. Both optic nerve heads were somewhat paler, and the right one had shallow excavation (Figure 1). Foveal reflex was absent on the right and decreased on the left eye. Blood vessels were thin. His pupils reacted weakly and sluggishly. Intraocular pressure was 24 on the right and 20 mmHg on the left eye. His visual field showed serious defects, without response on his right eye, and significant scotomas on his left eye (Figure 2).

In order to examine the possible origin of optic neuropathy, a series of analyzes and exams were performed. The serum level of tacrolimus (7.56 ng/mL) was within therapeutic concentration range (target range 5–20 ng/mL). Inflammatory markers (erythrocyte sedimentation rate, C-reactive protein, and fibrinogen) were within normal ranges. Antinuclear antibody (ANA), antineutrophil cytoplasmic antibody (ANCA), and rheumatoid factor (RF) tests were negative.

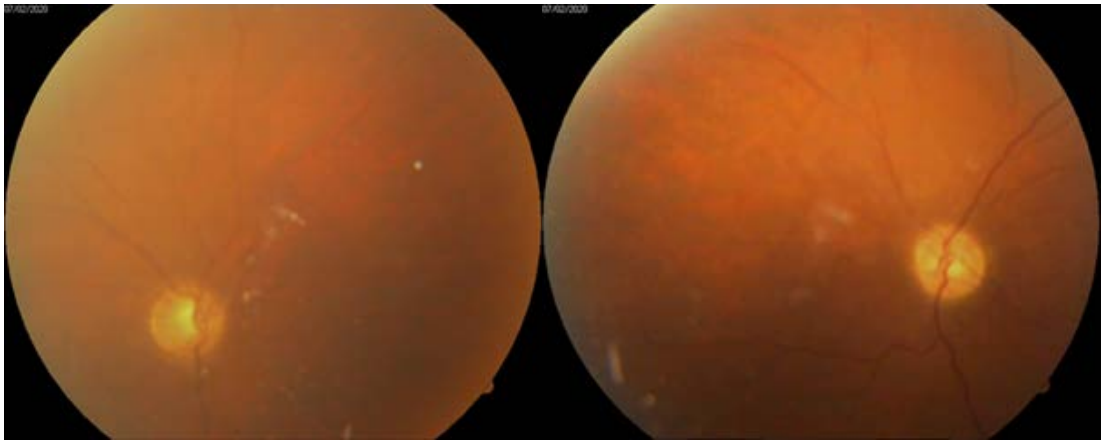

Fig. 1 – Photo fundus of the right and the left fundus: optic nerve heads are paler and arterial blood vessels are thinner; on the right eye is visible excavation (glaucomatous); details are less visible due to incipient cataract.

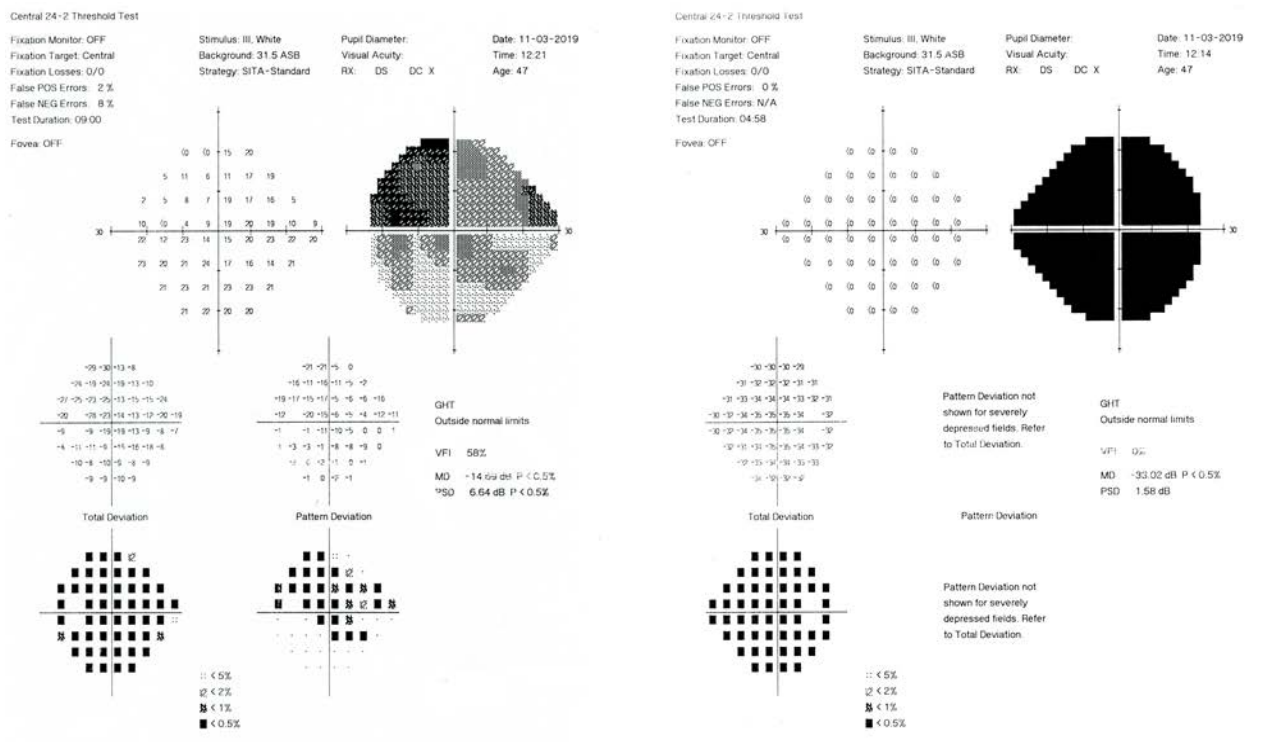

Fig. 2 – On admission, visual field deficits on the left eye of the patient were irregular and covered the central and upper, more temporal region; sensitivity is low; visual field on the right eye shows perimetrically blind field.

Biochemical analyses showed only high triglyceride level (7.07 mmol/L), while antibodies to viruses such as herpes simplex (HSV1), varicella-zoster (VZV), cytomegalovirus (CMV), hepatitis B and C, human immunodeficiency virus (HIV), and *Treponema pallidum* (TPA) were negative. Quantiferon gold tuberculosis (TB) test was also negative, as well as aquaporin 4 antibodies. Angiotensin-converting enzyme (ACE) was within normal limits (23.85 U/L), as well as homocysteine (7 μmol/L) and coagulation factors levels. The laboratory results also did not point to thrombophilia. Vitamin B12 concentration was high (1,131.0 pg mL^{-1}, normal range: 239–931 pg/mL), probably because the patient was taking supplements for weeks since the disease started, and folate (5.50 ng/mL) and vitamin D concentration (26.3 nmo/L) were normal. Arterial pressure was normal all the time.

Lungs, core, and sinuses radiography revealed maxillar sinusitis on his right side. Postcontrast magnetic resonance imaging (MRI) showed infra and supratentorial cortical reductive changes and vasculopathic changes in subcortical frontoparietal regions and a slightly reduced diameter of the left optic nerve at the level of orbital apex.

Although our suspicion was directed to toxic neuropathy from the beginning, as his cousin lost vision in her thirties for no clear reasons, we performed a genetic analysis for Leber hereditary optic neuropathy.

Another reason was that nephrologists were reasonably satisfied with the patient's therapy, and they did not meet such side effects in their numerous patients for almost two decades of usage. However, their first step was a conversion from an extended-release formulation that is to be taken every 24 hours, which he had used in a 3 mg dose, to the immediate-release formulation to be taken every 12 hours, 1.5 mg twice a day. The rest of the therapy remained the same, except that atorvastatin was introduced. However, further decline in visual acuity in the next two weeks (VOD L+ P+/-, VOS L+P+) convinced them to convert therapy to cyclosporine A 125 mg twice daily, while the rest of the therapy remained the same. A few weeks after being dismissed from the hospital, we received results for Leber mitochondrial base-pair mutations G11778A, T14484C, and G3460A, and they were negative.

After a month of cyclosporine therapy, his visual acuity was L+P+ on his right eye and his fingers counting on 50 cm on his left eye. In the further course, it improved a little on his right eye and now is stabile on counting fingers on 50 cm on each eye, with a discrete improvement of the visual field on the right eye.

Pattern visual evoked potentials showed low amplitudes, lower on his right eye, while the latencies were within normal range. Pattern electroretinogram had low values of N95 amplitudes, better on the left side, while P50 were just below normal values. His optic nerves were pale. Optic coherence tomography revealed retinal nerve fiber layer (RNFL) thinning in all sectors of his right eye and partial on his left eye (Figure 3), as well as ganglion cell layer (GCL). All this confirms consequent bilateral atrophy of the optic nerve after neuropathy.

The patient tolerated cyclosporine therapy well, without the appearance of possible side effects for a year and a half.

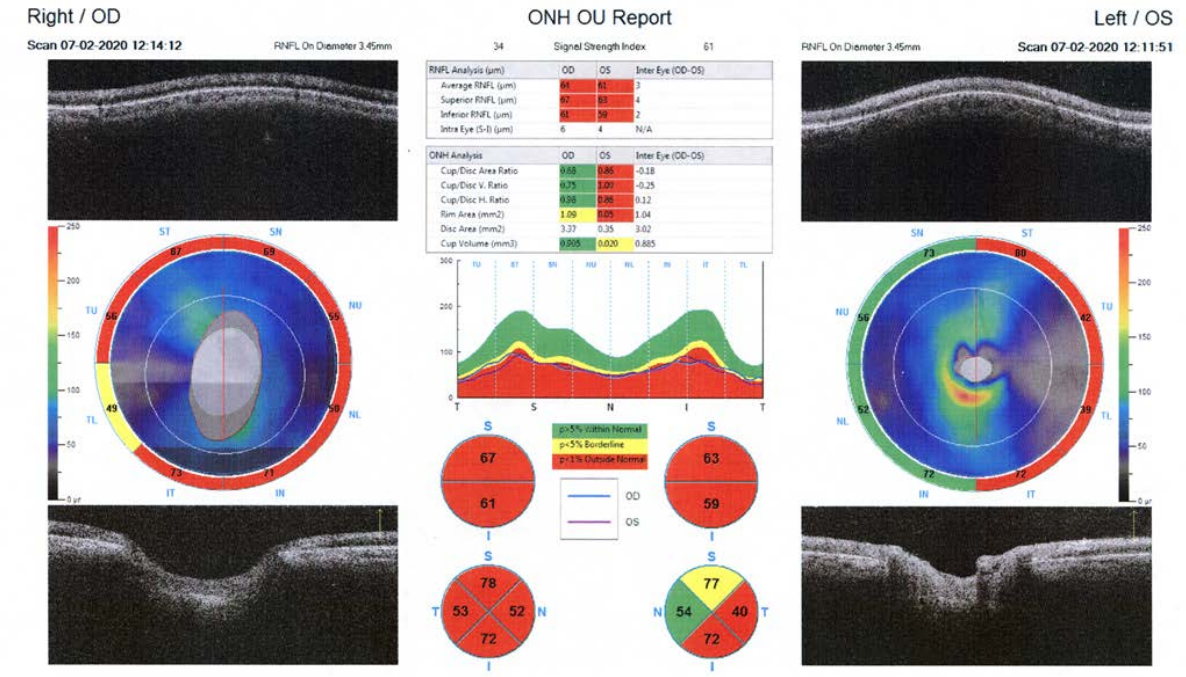

Fig. 3 – Retinal nerve fiber layer (RNFL) analysis shows thinning on both eyes, slightly more prominent on his right eye; poor fixation resulted in optic nerve interpapillar parameters differences, which do not otherwise exist (see Figure 1) (note: a better, control shot was used).

Moreover, he was on latanoprost topical therapy for glaucoma, which was recently converted to dorzolamide/timolol for better control of intraocular pressure.

This presentation was made with the patient's written consent to use the data and photographs describing his case.

Discussion

Tacrolimus is a valuable immunosuppressant, but like other similar drugs, its use is associated with some serious side effects. Almost one-third of patients on this or similar therapies have neurological complications [4]. PRES, which predominantly affects the parietooccipital lobes, is the most common tacrolimus toxic effect of the central nervous system. Besides other significant neurological effects, significant visual loss may occur, but with a favorable outcome after the therapy modifications [5, 6]. Peripheral nerves could be affected as demyelinating or axonal forms. Possible mechanisms of toxicity and risk factors are numerous [7–9].

Since tacrolimus optic neuropathy was recognized some twenty years ago [8], there has been a small but permanent increase of reports of this toxic effect on the optic nerve. It appears sporadically after liver, kidney, multivisceral, or bone marrow transplantation [9]. This complication is rare and usually occurs after several months to a few years of immunosuppressant therapy, rarely after longer usage [10]. However, both PRES and optic neuropathy complications may manifest in the same patient [11]. On the other hand, even unilateral tacrolimus TON was described [12].

It is important that all case reports find appearing of TON independently of tacrolimus blood concentration. Possible mechanisms of toxic tacrolimus influence on the nervous system and optic nerve are not fully understood, and there are few possible explanations. The most cited are direct neurotoxicity on oligodendrocytes, whose damage can lead to demyelination, vascular complications where neurotoxicity may be caused by vasoconstriction in cerebral microvasculature (like probably in PRES) [4], or genetic variations in tacrolimus elimination mechanism from the central nervous system [13]. The male gender and type and duration of the disease which preceded transplantation or TON may play a role [9, 14]. There is a relatively high incidence of neurotoxicity after liver transplantation, which may be due to changes in tacrolimus metabolism, leading to cumulative toxicity. Unusually, tacrolimus optic neuropathy was described even in the patient who was on this drug therapy for nephrotic syndrome and not in GVHD [15].

Recovery of visual acuity is described occasionally, mainly in cases that have been significantly shorter on tacrolimus therapy, after the therapy conversion, and/or in those where an inflammatory component exists that provides a good response to anti-inflammatory therapy [9].

Our patient developed toxic neuropathy after nine years of excellent enduring tacrolimus. The only possible side effects, until then, were high lipid levels and arterial hypertension, which nephrologists expected in such patients [16]. Both conditions were regulated by the listed therapy (amlodipine, enalapril, atorvastatin). They are risk factors for ischemic optic neuropathy, as well.

Visual loss and other findings on his right eye are, without doubt, to some extent connected with previous trauma and consequent glaucoma, but the visual decline and subsequent optic atrophy are bilateral now. Because of the course of his visual loss and optic atrophy, the absence of

pulse corticosteroid therapy answer and the length of tacrolimus therapy, as well as slight improvement after the therapy conversion, the most likely mechanisms of tacrolimus action was a toxic accumulation of the drug. Previous illness and vasculopathic changes on MRI may contribute another assumption to the vascular, ischemic causes. However, posterior ischemic optic neuropathies are very rare, especially as bilateral simultaneous occurrences [17].

According to the clinical aspect, the diagnosis of toxic optic neuropathy is of the exclusion type. Diagnosis of tacrolimus-induced optic neuropathy is even more difficult, as it is described in literature exclusively as case presentations with a great amount of variability of clinical features and, as it appears, independently of the blood drug concentration. For these reasons, it is a reasonable restraint of the other specialists, but from a neuro-ophthalmic aspect, after eliminating demyelinating and non-demyelinating inflammatory, compressive, infiltrative, traumatic, nutritive, to great extent ischemic and paraneoplastic, and even some hereditary neuropathies, our patient's diagnosis is toxic neuropathy. In less than two weeks, from a man who was reading, watching TV, and hanging out with people, the patient became a person who does not recognize faces and moves precariously while touching objects around him. The exclusion of all possible causes is methodologically and temporally very difficult and is neither rational nor necessary. Monitoring a patient who has not taken good care of his eyes until profound bilateral visual acuity loss sets an additional aggravating circumstance in establishing a conclusion.

According to the pharmacovigilance, the likelihood that optic neuropathy was induced by tacrolimus is probable (score 7) as stated by the Naranjo Adverse Drug Reaction Probability Scale (APS) [18]. On the World Health Organization – Upsala Monitoring Center (WHO UMC) scale, our case is somewhere between probable ("reasonable") and certain [19]. In this and similar cases, it is impossible to meet all the requirements set in the scales (therapeutic rechallenge, use of placebo, dose increasing). There is no ideal scaling system or diagnostic procedure.

Conclusion

Vision disorders can be caused by many substances and drugs, and early recognition may be important for treatment and prognosis. A thoughtful approach to all patients with optic neuropathies is essential and, as a first step, a detailed medical history and similar consequent examination are crucial in establishing the diagnosis. TONs are underestimated in ophthalmology practice, and unfortunately, on some occasions, they could be diagnosed when vision has already been severely damaged.

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CASE REPORT

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Prophylactic heme arginate therapy in acute intermittent hepatic porphyria – a case report

Profilaktička terapija hem arginatom u akutnoj intermitentnoj hepatičkoj porfiriji

Žarko Krnetić*, Tatjana Pešić*, Željka Savić*†, Tatiana Jocić*, Olgica Latinović Bošnjak*, Vladimir Vračarić*, Dimitrije Damjanov*†

***University Clinical Center of Vojvodina, Clinic for Gastroenterology and Hepatology, Novi Sad, Serbia; †University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia**

Abstract

Introduction. Among the acute hepatic porphyrias, a small percentage of patients, predominantly female, present with recurrent cyclic attacks of acute intermittent porphyria that occur more than three times a year and sometimes at intervals of less than a month. In women, the attacks are typically related to the menstrual cycle, requiring several days of hospitalization and administration of heme arginate. For these patients, prophylactic heme arginate therapy may be the optimal treatment modality. **Case report.** We presented a 40-year-old female patient who has been suffering from porphyria for seventeen years. The first attack occurred in 2003, presenting with severe neurological symptoms, requiring the use of heme arginate (Normosang®, Orphan Europe), which resulted in a favorable therapeutic response. In 2004 and 2007, gonadorelin analogue goserelin (Zoladex®) was used, but without beneficial effects on the course of the disease. In 2008, a preventive administration of heme arginate was initiated. The patient received heme arginate in the early phase of symptoms, every month in the premenstrual phase of the cycle, which resulted in milder symptoms, full recovery within 24 hours, lower doses of Normosang® (1-2 ampoules), and fewer hospital days (1-2 days) per month. This regimen has significantly improved the patient's quality of life and reduced the risk of potential adverse effects. **Conclusion.** Preventive use of Normosang® is the optimal therapeutic modality in patients with frequent, recurrent severe attacks that are unresponsive to other therapeutic regimens. As a result, patients have a better quality of life due to an effective, short-term, targeted treatment regimen.

Key words:
hem arginate; porphyrias; tertiary prevention; treatment outcome.

Apstrakt

Uvod. Među bolesnicima sa akutnim hepatičkim porfirijama niži procenat obolelih, uglavnom ženskog pola, ispoljava rekurentne ciklične napade akutne intermitentne porfirije, koji se javljaju više od tri puta godišnje, a ponekad i u intervalima kraćim od mesec dana. Kod žena su napadi uglavnom povezani sa menstrualnim ciklusom, zahtevaju po nekoliko dana hospitalizacije i terapiju hem arginatom. Za te bolesnike, preventivni režim davanja hem arginata može biti optimalan terapijski modalitet. **Prikaz bolesnika.** Prikazana je 40-godišnja bolesnica koja je 17 godina bolovala od porfirije. Prvi napad bolesti desio se 2003. godine i prezentovao se teškim neurološkim simptomima, što je zahtevalo primenu hem arginata (Normosang®, Orphan Europe) sa povoljnim terapijskim odgovorom. Tokom 2004. i 2007. godine sprovedena je terapija analogom gonadorelina, goserelinom (Zoladex®, Astrazeneca), ali bez povoljnog odgovora na tok bolesti. Tokom 2008. godine započeto je preventivno davanje hem arginata. Lek je davan u ranoj fazi simptoma, svakog meseca u premenstrualnoj fazi ciklusa, što je dovelo do ublažavanja simptoma bolesti, punog oporavka u toku 24 časa, uz smanjenje doza leka Normosang® (1–2 ampule) i kraćeg trajanja hospitalizacije (1–2 dana) mesečno. Tim režimom je značajno poboljšan kvalitet života bolesnice i snižen rizik od eventualnih neželjenih efekata leka. **Zaključak**. Preventivno davanje hem arginata je optimalan terapijski modalitet kod bolesnika sa čestim, rekurentnim, teškim napadima koji ne reaguju na druge terapijske mere. Kao rezultat, zahvaljujući kratkom i ciljanom terapijskom režimu, bolesnici imaju bolji kvalitet života.

Ključne reči:
hem arginat; porfirija; prevencija, tercijarna; lečenje, ishod.

Correspondence to: Dimitrije Damjanov, University of Novi Sad, Faculty of Medicine, Novi Sad, Hajduk Veljkova 3, 21 000 Novi Sad, Serbia. E-mail: dimitrije.damjanov@mf.uns.ac.rs

Introduction

One of the precipitating factors of an attack in acute intermittent hepatic porphyria may be the physiological oscillation of female sex hormones during the premenstrual or luteal phase of the menstrual cycle [1]. A small percentage of women have acute cyclical attacks occurring every month on average a few days before menstruation [2–4]. These are commonly severe forms of attacks that may be life-threatening and require hospitalization, intensive monitoring, and intravenous heme arginate therapy (Normosang®, Orphan Europe). In such patients, preventive administration of heme arginate may be the optimal therapeutic modality [5, 6].

Case report

We presented a 40-year-old female patient who has been suffering from acute intermittent hepatic porphyria since 2003. The disease initially presented with severe acute seizure and neurological symptoms (grand mal epileptic seizures, status epilepticus, and coma). The patient's positive family history (mother had porphyria) indicated a qualitative urine test for porphobilinogen (Watson-Schwartz), and it was positive. A urinary porphyrin test was also done, showing elevated levels (15–25 times) of delta-aminolevulinic acid, coproporphyrin, uroporphyrin, and porphobilinogen (Table 1).

Genetic testing of HMBS and PEPT2 genes is unavailable in Serbia. It can be done only abroad, and it is extremely expensive; for that reason, and given the clear clinical picture, it was not done.

The patient was treated in the Intensive Care Unit with all supportive measures. Specific treatment with intravenous heme arginate (Normosang®) was initiated, which resulted in a favorable therapeutic response and complete recovery. After discharge, the patient had recurrent attacks in the premenstrual phase of the cycle, including myalgia and abdominal pain, requiring hospitalization. Since the problems continued despite administration of hypertonic glucose infusions and symptomatic replacement therapy, heme arginate treatment was continued. In the further course of the disease, heme arginate appeared as the only effective treatment. The average duration of an acute attack was 5–7 hospital days per month, with the administration of 2–4 doses of heme arginate (one per day). Since the seizures were related to the luteal phase of the menstrual cycle, the use of gonadorelin analogue goserelin (Zoladex®, Astrazeneca) was attempted in order to induce amenorrhea but without beneficial effects on the course of the disease (the patient received a subcutaneous Zoladex® implant at a dose of 3.6 mg, once per month for 6 months, on two occasions in 2004 and 2007).

After five years of heme arginate treatment, a preventive application of the drug was initiated in 2008, every month in the premenstrual phase of the cycle and in the early phase of symptoms. This therapeutic regimen resulted in milder symptoms, lower urinary porphyrin levels (Tables 1 and 2), full recovery within 24 hours, and lower doses of Normosang® (1–2 ampoules). The patient received a dose of 250 mg of heme arginate, according to the manufacturer's instructions, through a Port-A-Cath system implanted in the jugular vein. Heme arginate was not given with albumin, nor was the heme/albumin molar ratio calculated. There were no complications such as phlebosclerosis or thrombophlebitis. This therapeutic response allowed a hospital stay of 1-day, with a lower dose of the drug. In this way, the patient's quality of life was significantly improved in the last ten years and reduced the risk of potential adverse effects.

All parameters of potential drug toxicity are documented and presented in annual reports to the manufacturer (Orphan Europe) (Tables 3 and 4) without complications related to the application of the drug, such as thrombophlebitis and phlebosclerosis.

The preventive regimen has proven to be more effective in several aspects. In the first five-year period of preventive use of heme arginate (2009–2013), the patient had 49 seizures that required 202 hospital days of treatment and 117 ampoules of Normosang®. In the second five-year period of preventive regimen (2014–2018), the patient had 52 seizures which required 227 days of hospitalization and 130 ampoules of Normosang®, as opposed to the five-year period (2004–

Table 1

Urinary prophyrin levels in the presented patient during the first attack and attack in the heme arginate treatment regimen

Porphyrins	First atack	Attack in treatment	Reference value
Coproporphyrin, moL/d	958	643	20–274
Delta aminolevulinic acid, moL/d	1,354	931	11.4–57.2
Uroporphyrin, mmoL/d	107.8	483	4.8–60
Porphobilinogen	253	113.4	< 9

d – daily diuresis (24 h urine).

Table 2

Urinary porphyrin levels during the attack in the heme arginate preventive treatment regimen

Porphyrins	Prior to heme arginate use	After the first heme arginate dose
Coproporphyrin, (moL/d)	266	192.1
Delta aminolevulinic acid, (moL/d)	331	90.8
Uroporphyrin, (nmoL/d)	186.5	148
Porphobilinogen, (nmoL/d)	88.6	42.9

For porphyrins reference values see in Table 1.

Table 3

Biological parameters in the serum during preventive application of heme arginate

Parameter	Average value	Reference values
Ferritin, µg/L	64.8	10–120
C-reactive protein, mg/L	0.3	0–5
Transferrine saturation, g/L	2.46	2–3.6
Iron, mmol/L	18.1	12.5–32.2
Gamma-glutamyltransferase, IU/L	10	1–55
Alanine aminotransferase, IU/L	16	5–40
Creatinine, mmol/L	91	30–127

Table 4

Cumulative dose and side effects of heme arginate given preventively

Parameter	Values
Dose *per* infusion, mg	250
Number of courses	27
Total number of ampoules *per* year	28
Adverse drug reaction	No
Iron overload	No

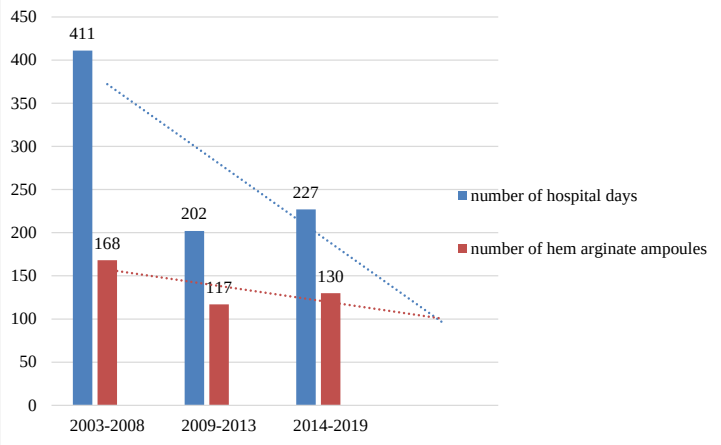

Fig. 1 – Total number of hospital days and heme arginate dosage during the three five-year periods (therapeutic and preventive regimens).

2008) when heme arginate was the treatment regimen, which resulted in 56 seizures and 411 hospital days during which the patient received 168 ampoules of Normosang® (Figure 1).

Discussion

Cyclical attacks of acute intermittent hepatic porphyria associated with the menstrual cycle affect a small number of patients. Therefore, there is a lack of literature on the long-term treatment of such patients. Inducing amenorrhea in order to prevent seizures is one of the therapeutic modalities that may be applied [4, 7, 8]. In the case of our patient, this therapy did not give favorable results in preventing seizures. In patients with cyclic forms of the disease who require intravenous administration of heme arginate during the attacks, there is a possibility of applying a preventive heme arginate regimen immediately before the expected period of discomfort [2–4], which can give satisfactory therapeutic results in terms of reducing the intensity of attacks, shortening the hospital stay, and decreasing the total dose of heme arginate [9]. This regimen significantly reduces the risks of adverse drug reactions.

Conclusion

The presented case leads to the conclusion that preventive heme arginate therapy may be the optimal therapeutic modality in patients with cyclic attacks of acute intermittent porphyria precipitated by physiological hormonal oscillations during the premenstrual phase of the cycle, as well as in case of negative therapeutic response to other treatments. This therapeutic approach significantly reduces the intensity of seizures and improves the quality of life of patients due to shorter hospital stay and reduced risk of potential side effects of the drug.

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Epidemic typhus in the Lithuanian army from 1919 to 1923

Epidemijski pegavac u litvanskoj vojsci od 1919. do 1923.

Viktorija Šimkutė, Vilma Gudienė

Lithuanian University of Health Sciences, Department of Drug Technology and Social Pharmacy, Kaunas, Lithuania

Key words:
disinfection; history, 20th century; history of medicine; lithuania; military personnel; typhus, epidemic louse-borne.

Ključne reči:
dezinfekcija; istorija, xx vek; istorija medicine; litvanija; vojni kolektiv; tifus, pegavi.

Introduction

Population movements, particularly those connected with war, spawn infectious diseases. These were noted in the Peloponnesian War between Sparta and Athens in 430 BC. In "Rats, Lice, and History", Hans Zinsser emphasized: "And typhus, with his brothers and sisters – plague, cholera, typhoid, dysentery – has decided more campaigns than Caesar, Hannibal, Napoleon, and all the inspector generals of history" [1]. Biblical writers depicted these diseases as one of the four apocalyptic horsemen that resulted in more soldiers being killed than by weapons. The First World War was the first military conflict in which this rule no longer applied [2]. However, despite the progress in military medicine, during the Great War, the armies in Eastern Europe faced major epidemics of typhus.

Typhus, in particular, was a tremendous scourge. Also known as "war typhus", the disease appears to have first surfaced in Serbia in 1914. Because of malnourishment, unsanitary conditions, and disruption of supplies, armies were decimated by epidemic typhus. The migration of soldiers and refugees accelerated the contagion. Death rates ranged from 20% to 80%, with the highest percentage occurring in 1918 [3]. Typhus exploded in Russia during the Civil War (1917–1922). The Red Army alone lost 100,000 men from 1918 to 1920 [4]. Typhus also felled armies in neighboring Poland, Ukraine, Romania, and the Baltic States. These countries tried to take preventive measures but found it difficult to stop the disease.

The newly created Lithuanian army was plagued by epidemic typhus from the very first days of its formation at the end of 1918. Troops suffered from typhus throughout the Lithuanian War for Independence (1918–1920)* with the highest incidence rates, compared to all other infectious diseases, occurring in 1919 [5]. Because of severe shortages in the Lithuanian army, it seemed impossible to combat typhus. Everything was missing, from clean clothes to disinfectants. However, the Sanitation Department of the Lithuanian Ministry of National Defense used all, albeit minimal, resources to carry out the prevention of epidemic typhus, and gradually the situation improved.

The history of epidemic typhus

Epidemic, or exanthematic typhus, is an infectious disease caused by a small bacteria *Rickettsia prowazekii* which spreads through the body louse *Pediculus humanus humanus*. The name "typhus" (derived from the Greek word "typhus", meaning smoke or haze) was first introduced by Hippocrate, who used it to describe the state of a sick person's stupor rather than a specific disease [6]. The first record of the disease was *Cronica cavense* – an Italian manuscript about "severe fever with peticuli" composed in the monastery near Salerno in 1083 [1]. Due to the similarity of symptoms, epidemic typhus was confused with other diseases for a very long time. It was separated from the plague by Girolamo Fracastoro in 1546 and from typhoid – by John Huxham in 1739. These perceptions were confirmed by Francois Boissier de la Croix

* Lithuanian Wars of Independence – the fights against Bolshevik, Bermontians, and Poland in order to defend the restored independence of Lithuania. The battles began in the late 1918 and ended in the late 1920s. However, sometimes the end of these battles is considered to be the year 1923 when the Klaipėda region was regained. As the activities of the Sanitation Department narrowed down in the same year, 1923 will be considered the end of the Wars of Independence in this article.

Correspondence to: Viktorija Šimkutė, Lithuanian University of Health Sciences, Department of Drug Technology and Social Pharmacy, Sukilėlių av. 17, LT-50161, Kaunas, Lithuania. E-mail: v.simkute.vi@gmail.com

de Sauvage, who introduced the term "exanthematic typhus" in 1760 and applied it to the disease, which we still call by the same name today [7]. Although, at that moment, it seemed that the disease had become quite well known, two essential things were still unclear: the pathogen and the transmission pathways. This was clarified in the twentieth century. The louse as the vector of epidemic typhus was discovered by Charles Nicolle in 1909. Soon after, the causative agent *Rickettsia prowazekii* was revealed by H. T. Ricketts and J. M. Prowazek. It was one of the most important discoveries in history as it fostered the development of preventive measures against the pathways of the disease. The main prevention measure – vaccine, was discovered by prof. Rudolf Stefan Weigl in the 1930s [8].

Relationship between epidemic typhus and military

Historically, epidemic typhus was one of the most terrible diseases connected with military conflicts. It followed the soldiers during the Granada War (1482–1491), the Thirty Years War (1618–1648), and the War of the Austrian Succession (1741) [9]. The classical example of the relationship between epidemic typhus and the army is the effect on Napoleon's Grande Armée during the invasion of Russia in 1812. Since the etiology of typhus was not well-known at that time, soldiers did not take any preventive measures to defeat these parasites. As a result, 80,000 French soldiers had died or contracted epidemic typhus by July 1812. Even the best sanitation in the world at the time could not help stop the rise in these numbers [10].

Nicolle's discovery in 1909 was a real revolution in the history of epidemic typhus. From the start of the First World War, armies knew that the disease could be overcome by fighting lice. Delousing stations were installed on both the Eastern and the Western fronts. Disinfection with steam, dry heat, benzene fumes, sulfur and carbolic acid, or hydrogen cyanide played an important role [4]. The soldiers also used a wide variety of insecticides: N.C.I. (naphthalene 96%, creosote 2%, iodoform 2%), Vermijelli, Crude Oil Ointment, Mercury Ointment, White Mercury Powder, etc. [11]. Much attention was paid to general sanitary conditions, particularly the cleanliness of the body and clothes. Baths were built near the barracks. Education of soldiers about the disease was also a particularly important preventive measure.

On the Western Front, since multiple preventive measures were taken, curtailing epidemic typhus was fairly successful [1]. The situation on the Eastern Front was much worse. After the end of the First World War, local wars continued. Revolutions, famine, and other communicable diseases exacerbated the spread of lice. Most of the soldiers were uneducated and tired after four years of war. During the Russian Civil War, not only were disinfection devices lacking, but clean clothes, warm water, and soap were scarce as well [4]. Hans Zinsser wrote: "As everyone who has really been to war knows, let the water supply fail, or soap become scarce, or a change of clothing be delayed, it takes no time at all before the louse comes back to its own" [1]. The situation was similar in all the neighboring states that continued their struggles for independence, including Lithuania.

Epidemic typhus in the Lithuanian Armed Forces

The Lithuanian Army was formed on November 23 in 1918 – nine months after the proclamation of Lithuania's independence. The first two years were particularly difficult. This period was referred to as the "period of extreme illness and severe loss" in the Lithuanian Armed Forces [5]. As noted, epidemic typhus had the highest incidence rate in 1919 – the first year of the army's existence. In that year alone, 1,559 soldiers fell ill with the disease. Inpatient morbidity was more than 15% and accounted for 12% of the overall morbidity (Figures 1 and 2) [5]. Mortality rates were also very high (Figure 3).

The main cause was poor sanitation. German troops had occupied the western Russian provinces and Lithuania, in particular, during the Great War. Thus, there was a lack of clean barracks, clothing, footwear, disinfection chambers, saunas, and disinfectants available to the new Lithuanian army. The first Lithuanian military units settled in the barns, abandoned houses, or barracks that were taken over by the German occupying army. Usually, these were simple wooden buildings damaged or left in ruins by the retreating German soldiers. There was a shortage of mattresses. On August 13, 1919, during the sanitary inspection of the Kaunas

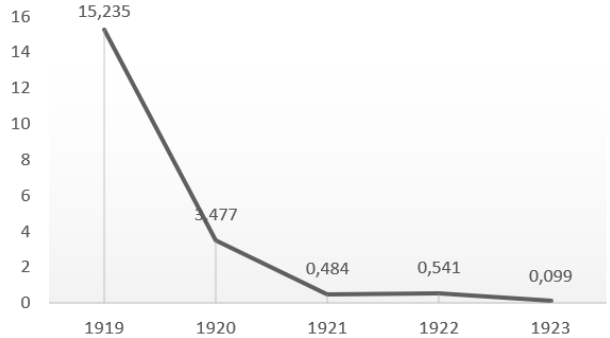

Fig. 1 – Morbidity (%) of epidemic typhus (numbers of sick per every 100 soldiers). (Made based on "Morbidity and Mortality in the Lithuanian army" by S. Barkauskas [5]).

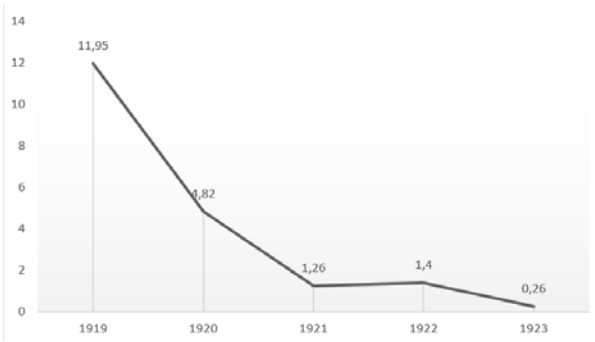

Fig. 2 – Proportion (%) of epidemic typhus in whole inpatient morbidity. (Made based on "Morbidity and Mortality in the Lithuanian army" by S. Barkauskas [5]).

Fig. 3 – Mortality (%) of epidemic typhus (deaths per every 100 sick soldiers). (Made based on "Morbidity and Mortality in the Lithuanian army" by S. Barkauskas [5]).

Battalion, it was found that 25% of soldiers did not have them. In addition, 20% of soldiers did not have blankets [12]. Basic clothing was lacking. In July and August 1919, sanitary inspections revealed that most battalion soldiers had only one pair of clothes (although it was required to have at least two), and pants and shoes particularly were in poor condition. For example, in the Panevėžys battalion, 30% of the soldiers did not have shoes or were badly worn. In the Marijampolė Battalion, 90% of soldiers had only one set of clothes [12].

The shortage of disinfection chambers was a huge problem. This was especially felt in hospitals. At the beginning of 1919, when the epidemic of typhus was raging in Telšiai, the military doctors working here faced a serious problem – there was not a single disinfection chamber in the whole district [13]. Moreover, the Lithuanian army lacked specialists called "disinfectors" who knew how to use disinfecting equipment. In July 1919, there was only one such qualified person capable of disinfecting, and 6,934 soldiers in the Lithuanian army [14]. In addition to these shortages, the soldiers' poor understanding of hygiene helped spread typhus. The first soldiers came mainly from the villages and were not highly literate. They had a poor understanding of cleanliness and its importance in the prevention of epidemic typhus. One military doctor I. M. noted in the newspaper "Military word": "There is no clear understanding of this in the vast crowds of our army. If the soldiers talk among themselves about the danger to them, it is about the danger of a bayonet or a bullet. But it is forgotten that while in the army, they are also at risk from invisible enemies – carriers of various infectious diseases" [15]. Sanitary inspections revealed numerous examples of poor hygiene. For example, during the sanitary inspection of the Panevėžys Battalion on July 25–26 in 1919, it was observed that even when baths were available, soldiers visited them irregularly or infrequently [12].

Prevention of epidemic typhus in the Lithuanian Army

Despite the shortages and intense fighting in 1919, the Sanitation Department of the Ministry of National Defence used all, albeit minimal, resources to improve the situation ever since. This body, in collaboration with the Supply Division, struggled to increase preventive. The head of the Sanitation Department, General Vladas Nagevičius, played a major role in this effort. In the almanac "Shield", the general was described as follows: "With General Nagevičius at the forefront, everything began to grow and develop rapidly. His tremendous efforts, inexhaustible energy, rigor, extraordinary talent of the organizer, ability to orientate quickly..., ability to choose doctors for responsible positions quickly rectified the military sanitation" (Figure 4) [16].

Fig. 4 – Vladas Nagevičius, the head of military sanitation, inspects sanitary units (1921) [16]. (The archive of the Museum of the History of Lithuanian Medicine and Pharmacy).

Nagevičius was an indefatigable warrior in the fight against infectious diseases in the Lithuanian army. By establishing many contacts with foreign countries, the international Red Cross, and promoting contributions from Lithuanian civilians to the well-being of the army, he succeeded in increasing the most important preventive measures for the troops. His work enabled the Sanitation Department to control epidemic typhus. The main focus was on living conditions, disinfection, cleanliness, and education.

Living conditions

The Sanitation Department carried out regular sanitary inspections in the army and reported the deficiencies to the Supply Department. These sanitary inspections covered five broad areas: condition of barracks, food and water, clothing, shoes, and saunas [14]. Usually, the Sanitation Department asked the Supply Department to repair the barracks and temporary apartments. The Sanitation Department emphasized that soldiers could not sleep on the ground and requested straw, mattresses, and bedding. Linen mattresses that could be washed were to replace the paper mattresses formerly used [17]. The sanitation staff asked for laundries and driers to wash clothing. They emphasized the need for clean water, sufficient food, and building as many baths as possible. They made efforts to ensure that soldiers did not live in cramped conditions. Each soldier had to have at least 16 cubic meters of air space in the barracks; distances needed to be maintained between the beds [18]. Although these goals were not achieved in all units, general living conditions in the army began to improve significantly.

Disinfection

For the disinfection process, the Lithuanian Armed Forces used disinfection chambers. These were metal or wooden boxes containing steam (Figure 5).

Some disinfection chambers were stationary, but most were portable. The latter were more practical because, in the event of outbreaks of epidemic typhus, they could be easily transported from one place to another. Disinfection chambers were used to disinfect clothes, bedding, and upholstered furniture. This was a demanding process. People performing it had to be well prepared, strong, and healthy. Individuals with ulcers or sores on their hands could not use chemicals for disinfection. Those performing disinfection also needed special clothing and good knowledge of the process [19]. Professionals, called "disinfectors", had to undergo special training conducted by disinfection instructors. Those without the special education required to work with disinfection chambers were generally not accepted. For example, civilian Juozas Jaras knew how to use a disinfection camera because he took part in the fight against epidemic typhus in the vicinity of Skuodas. He was drafted into the army for the same purpose. However, when it became clear that he had not completed any special disinfection courses, Juozas Jaras was not accepted because he did not meet the disinfector qualifications [20, 21]. As a result of these strict requirements, there were relatively few disinfectors. To fill this gap, doctor's assistants were sent to be trained in the process.

Because of the scarcity of both disinfection chambers and professionals who could use them, other disinfection methods were also used. In addition to heat, disinfection with chemicals was performed. Soap and carbolic acid solution, purified carbolic acid, sulfur and carbolic acid solution, sublimate, lime milk, chlorinated lime, tree tar, formalin, and sulfur gas were used [19]. The choice of type of disinfection depended on the object that would be disinfected (Table 1).

These disinfection techniques were applied by trained military doctors and sanitary personnel. The textbook "Igiena" described detailed disinfection processes for sanitary lieutenants. Military periodicals also described some disinfecting methods that soldiers themselves could apply.

Cleanliness

Maintaining cleanliness was one of the most important ways to fight epidemic typhus. The Lithuanian army tried to install as many saunas as possible and provide soldiers with soap and clean clothes. In the Congress of Military Doctors on January 21–25, 1921, further increases in soap rates,

Fig. 5 – Staff of the disinfection service of the First Separate Infirmary (1923) [19]. (The archive of the Museum of the History of Lithuanian Medicine and Pharmacy).

Table 1

Methods of disinfecting certain objects
(Made based on "Handbook for Sanitary Lieutenants, Hygiene" by Baranov, 1921 [19])

Object to be disinfected	Type of disinfection
Body	1% / 2% carbolic acid solution or 0.1% sublimate.
Premises	Soap and carbolic acid solution, 2% carbolic acid solution, 1% soda solution with water, sublimate solution, and 20% lime milk.
Doors, windows, and wooden furniture	Sublimate solution, soda, and green soap solution.
Upholstered furniture	Water vapor, formalin vapor, carbolic acid solution, soap and carbolic acid solution.
Metal and leather goods	5% solution of carbolic acid or soap and carbolic acid. Fur – formiline or sulfur gas.
Blankets, mattresses, and clothing	Water vapor, formalin vapor, boiling water, straw-burned.
Clothes	Water vapor, soap and carbolic acid solution, boiling water and soda solution, 0.1% sublimate solution.

amount of baths, and washrooms were required [17]. Various orders and statutes defined the importance of cleanliness in the military. The Statute of the Internal Service stated that it was the duty of every soldier to be clean and negligent. Soldiers were required to wash their faces, necks, and hands every morning. Visiting the baths was the primary mean of keeping the body clean overall, and the first step for new recruits was a visit to the sauna. Service soldiers were required to visit the sauna as often as possible, at least twice a month. It was obligatory to go to the sauna in groups with sanitation staff. Before going to the sauna, the soldiers had to leave all of their clothes in the disinfection chambers. The same statute specified hygienic measures in the barracks. Clean clothes were to be kept separate from dirty clothes. It was recommended that dirty clothing be kept in separate rooms where soldiers did not live or in warehouses [18]. Failure to maintain the cleanliness of clothing was punishable. For example, the Statute of Discipline reported that one soldier was arrested for three days because he did not clean his clothes for the next shift [22].

Education

The statutes and orders specified strict requirements for soldiers. However, soldiers also needed concrete examples of good hygiene. Here periodicals played a major role. The journal "Soldier" wrote that "the most important law of health science is cleanliness, which is achieved with water and soap" [23]. In the journal "Word of Soldiers" soldiers were taught: "Do not be lazy to go to the sauna and wash yourself; the more often you go to the sauna – the healthier your body will be, the spirit will be stronger and more alert, the more useful you will be to your homeland". Newspapers stressed the importance of using soap on a daily basis, washing hands and feet and cutting nails frequently, and using only one's own towels [24]. Educating soldiers about hygiene was a very important part of prevention because most of them were from poor families and had little understanding of the importance of cleanliness. With a limited amount of supplies, the formation of soldiers' knowledge of proper use of those available was important.

Treatment of epidemic typhus

Soldiers with epidemic typhus were mostly treated in the First Separate Infirmary (Figure 6).

Fig. 6 – Outpatient Clinic of the First Separate Infirmary in Šančiai, Kaunas (1923). (The archive of the Museum of the History of Lithuanian Medicine and Pharmacy).

The treatment consisted of good nursing to strengthen the patients and relieve symptoms. The main treatment was directed to the cardiovascular system. Soldiers' medical histories show that treatment began with an application of camphor. In patients whose blood pressure was low and who were in a weakened condition, camphor stimulated heart and blood circulation. Camphor was administered in the form of tablets or injections. Caffeine and red wine were given for the same purposes. In addition, potassium chloride, digoxin, and strophanthus tincture were used to regulate heart function in case of arrhythmias. Antipyretics and analgesics (e.g., codeine phosphate, antipyrine, aspirin) were used for

high temperatures (up to 40 degrees Celsius) and for pain relief. Sodium chloride infusions, usually 500 mL in volume, were used to restore body fluids. Castor oil, calomel, or enema were prescribed for constipation [25].

Besides treatment with drugs, good nursing was particularly important. The room where the patient was being treated was well ventilated, and bedding and clothes were disinfected and frequently changed. Patients were washed frequently (Figure 7).

the retreating German army only: the first medicines and sanitation measures were supplied by the sanitary depot of the German army in Šančiai [27]. Lithuanian industry was just beginning to develop, so foreign aid was necessary. First foreign help was received from Germany, France, Great Britain, and the United States. In 1919, a consignment was received from France that provided Lithuanian soldiers with medicine and clothing for as many as 40,000 soldiers [28]. A lot of medicines, disinfection chambers, and disinfectant

Fig. 7 – Patient bathing in Kaunas Military Hospital (1921) [26].
(The archive of the Museum of the History of Lithuanian Medicine and Pharmacy).

For patients with a high temperature, the body was cooled by immersing them in cool baths and placing a piece of ice on their heads. Sometimes they were wrapped in wet sheets. Doctors examined the patients' mouths and tongues regularly, washing them with water or lubricating them with glycerin. Patients were given only drinking water and easily digestible food [26]. In general, caring for a patient with epidemic typhus was difficult because the symptoms were very severe, and the disease lasted for a long time without effective treatment.

The sources of materials

The Supply Department and the pharmaceutical part of the Sanitation Department were two main institutions that took care of the entire supply of sanitary property to the Lithuanian army. In the early days, the help was sought from

fluids were obtained from Germany. American Red Cross provided the largest consignments of clothes, bedding, mattresses, footwear, "American soap", and medicines to the Lithuanian army [29]. On each month of January, February, and March 1921, this organization donated 240 kg of cresol for disinfection [30]. Significant help from American Red Cross came because of Vladas Nagevičius's connections with Edward Ryan, the head of the American Red Cross headquarters in Riga. Moreover, small organizations in Lithuania had been established thanks to the head of the Sanitation Department.

Over time, the Lithuanian industry also recovered. The first factories of slippers, leather, and footwear opened. Soap factories in Kaunas were of very great importance. The Lithuanian army received soaps from Vislickis, "Florance", and other soap factories [31]. The "Florance" soap was especially advertised in military periodicals (Figure 8).

Fig. 8 – An advertisement for soap "Florance 503" in the military periodical "Soldier". The advertisement says soap eliminates pimples and makes skin whiter and softer [31].

In 1921, the army took over the soap workshop in Aleksotas from the Lithuanian Ministry of Supply and Nutrition, and soap production was introduced in the army itself. Four soldiers worked in this workshop [31]. This recovery in production in Lithuania meant that fewer resources had to be sought abroad. In addition, the requisitions that tortured the Lithuanian people could be reduced.

Conclusion

The epidemic of typhus affected not only the Lithuanian army but also armies in neighboring countries. Fortunately, the disease was controlled quickly enough in the Lithuanian army: in the second year of the army's existence, the incidence of epidemic typhus was almost five times lower than in the first year (Figures 1 and 2). The successful fight against epidemic typhus depended solely on proper prevention. It was not possible to take other measures at that time. In the pre-antibiotic era, treatment was more harmful than beneficial. Soldiers sick with typhus needed many different drugs. Many of these, in turn, were poisonous and thus did not reduce mortality (Figure 3). This prevented proper disease management and did not let reduce the mortality. Mortality rates in the Lithuanian army were high throughout the whole period from 1919–1923. As a result, it seemed better to prevent the spread of the disease rather than treat the sick soldiers with ineffective measures.

The measures against epidemic typhus used in the Lithuanian army were not as diverse as in the Western countries, but they were innovative and effective. The main focus was on disinfection, cleanliness of person and living conditions, and education. Every effort was made to control the disease as much as possible within the army and prevent the admission of sick people from the outside. Strict requirements defined in statutes and other documents forced soldiers to follow all instructions. Military doctors greatly contributed to the eradication of typhus. Treating diseases was no longer their only task. They became disinfection specialists, providers of sanitation norms, and teachers.

The achievements in preventing contagious diseases, especially typhus, were due to unstinting activities of the Sanitation Department and, in particular, an increased supply of preventive materials. Increased production and acquisition of food, clothing, sanitation equipment, and medicines, as well as armaments, was crucial to the success of the Lithuanian army. More than half the territory of Lithuania was occupied in 1919. This impacted the number of materials available and disrupted supplies from abroad. When the strongest battles ended at the close of 1920, the situation was more stable, and both local people and foreign countries were able to deliver more supplies. Moreover, when the military conflict subsided, officers and doctors could shift their focus from fighting armed enemies and treating war wounds to fighting disease. As a result, the incidence of epidemic typhus and other communicable diseases greatly decreased. Having carried out its main work, the Sanitation Department reduced its activities to the minimum in 1923, leaving only a few facilities for treating soldiers.

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