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POSSIBLE ROLE OF TGF B PATHWAYS IN SCHIZOPHRENIA

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MOGUĆA ULOGA TGF B SIGNALNIH PUTEVA U SHIZOFRENIJI

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ABSTRACT

e phenomenological uniqueness of each patient with schizophrenia is determined by complex symptomatology, particularly the overlapping of symptoms and their prominence in certain phases of this mental disorder. Establishing biological markers is an important step in the further objectivisation and quantification of schizophrenia. Identifying the cytokine profiles that precede a psychotic episode could direct the strategies for relapse prevention and be useful in predicting disease progression and treatment response. In the context of inflammation, TGF-β exerts potent anti-inflammatory and immunosuppressive functions by inhibiting pro-inflammatory cytokine synthesis, but it can also have pro-inflammatory functions through its stimulatory e ects on inflammatory 17 cells. It has been shown that the T helper cell type-1 and type-17 responses are reduced and type-2 response is increased in patients with schizophrenia. Both data from the literature and our results also indicate the presence of an anti-inflammatory response through production of the TGF-β regulatory cytokine. A meta-analysis of plasma cytokine alterations suggested that TGF-β is the state marker for acute exacerbation of schizophrenia, and we showed that TGF-β can also be a valuable marker for psychosis. Hyperactivity of TGF-β signalling pathways in schizophrenia may be both a neuroprotective mechanism and a possible therapeutic target.

Keywords: *schizophrenia, biomarkers, TGF-β, neuroplasticity*

SAŽETAK

Konstelacija simptoma opservirana kod svakog pacijenta sa shizofrenijom je jedinstvena i može se menjati progresijom bolesti. Aktuelni pokušaji objektivizacije i kvantifikacije u shizofreniji obuhvatili su i istraživanja bioloških markera ovog poremećaja. Određivanje specifičnih citokinskih profila u prodromalnoj fazi poremećaja može usmeriti nove strategije prevencije relapsa i biti od koristi u predviđanju toka bolesti i odgovora na terapiju. U kontekstu inflamacije, TGF-β ispoljava snažnu antiinflamatornu i imunosupresivnu aktivnost sprečavanjem sinteze proinflamatornih citokina, ali može imati i proinflamatornu ulogu stimulacijom inflamatornih 17 ćelija. Pokazano je da su imunski odgovori tipa-1 i tipa-17 oslabljeni i tip-2 odgovor pojačan kod pacijenata sa shizofrenijom. Podaci iz literature i naši rezultati ukazuju i na antiinflamatorni odgovor u shizofreniji sekrecijom regulatornog citokina TGF-β. Meta- analiza studija, koje su određivale plazmatske nivoe citokina pacijenata sa shizofrenijom, ukazala je na TGF-β kao marker pogoršanja shizofrenije, a naši rezultati takođe pokazuju da TGF-β može biti koristan marker psihoze. Hiperaktivnost TGF-β signalnih puteva u shizofreniji može biti neuroprotektivni mehanizam i potencijalni terapijski cilj.

Ključne reči: *Shizofrenija, biomarkeri, TGF-β, neuroplastičnost*

INTRODUCTION

The phenomenological uniqueness of each patient with schizophrenia is determined by complex symptomatology, particularly the overlapping of symptoms and their prominence in certain phases of this mental disorder (1). Different approaches to evaluating and characterising schizophrenia can lead to misunderstandings among clinicians and researchers (2).

To address this problem, reliable diagnostic criteria have been defined (3, 4). Additionally, clinical assessment scales are used to evaluate the severity of illness and the degree of treatment response. New diagnostic criteria should account for the knowledge gained over the past 20 years, especially in the field of neurobiology of mental disorders (5). Establishing biological markers is an important step in

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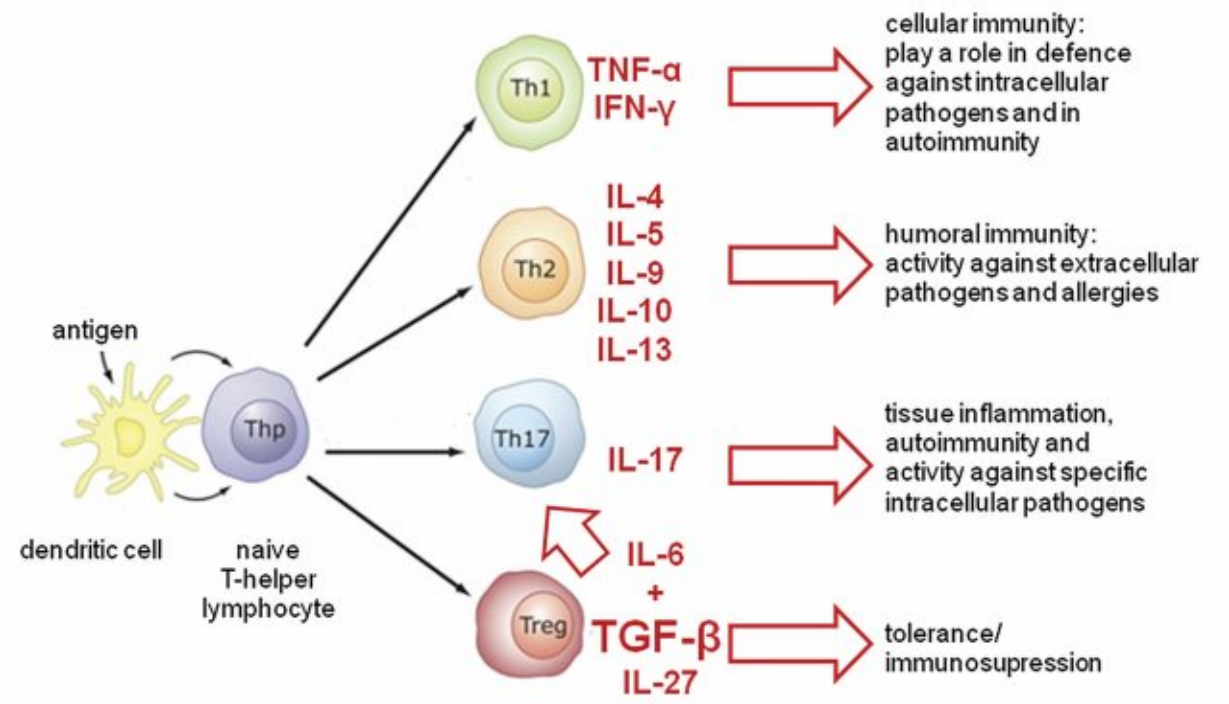

Figure 1. Types of immune responses and role of TGF-β

the further objectivisation and quantification of symptoms of schizophrenia.

Studies have shown that the molecular basis of schizophrenia changes from early stage of the disease to the chronic form, thus providing proof of the progressive nature of the schizophrenic disease process (6). Short-term schizophrenia is linked to transcription, metal binding, expression of ribonucleic acid and vesicular transport, whereas the long-term, chronic form of the illness has been linked to inflammation, response to stimuli and immune functions (7).

Cytokines as biomarkers in schizophrenia

Studies of the role of the immune system in the emergence and development of schizophrenia have shown its important role in the wide range of possible factors affecting schizophrenia (8- 10). Th lymphocytes direct the function of other immune cells by secretion of cytokines. Activated Th cells can be divided into Th1, Th2, Th17 and regulatory T cells (Tregs), based on the cytokines secreted (11) (Figure 1). Macrophages, natural killer, natural killer T cells and dendritic cells may also secrete the same cytokines. Thus, it is more appropriate to categorise immune responses based on their specific cytokine profile, i.e., as type-1, type-2 or type -17.

The type-1 cytokines, such as IFN-γ and TNF-α, activate macrophages and play a role in both the defence against intracellular pathogens and autoimmunity (12). IL-4, IL-5, IL-9, IL-10 and IL-13 are mainly secreted in the type-2 immune response (13). Type-2 cytokines have a role in humoral immunity via activity against extracellular pathogens and in allergies (14). The representative cytokine of the type-17 immune response is IL-17, which is a potent mediator of inflammatory response in autoimmune disorders (15, 16). Regulatory T-cells also have an important role in controlling immune response, as a decrease in regulatory T-cell activation leads to autoimmunity (17). IL-23 was required during the restimulation of TGF-β in addition to IL-6-stimulated cells to maintain their IL-17 production (18).

Identifying the cytokine profiles that precede a psychotic episode could direct strategies for relapse prevention and be useful in prediction of disease progression and treatment response (19, 20). It has been shown that the type-1 response is blunted and that the type-2 response is heightened in schizophrenia (14, 21), and our results indicate reduced type-17 and anti-inflammatory response through production of the TGF-β regulatory cytokine (22- 24).

TGF-β is a pleiotropic cytokine that is secreted by immune and non-immune cells. TGF-β plays a role in immune regulation, but it is also important for embryonic development, cellular differentiation and wound healing (25, 26). The over-expression of TGF-β has been linked to impaired effector T cell responses to viral infections (27).

In the context of inflammation, TGF-β exerts potent anti-inflammatory and immunosuppressive effects by inhibiting pro-inflammatory cytokine synthesis and by dampening natural killer cell activity and growth of T- and

B-cells. However, TGF-β also has pro-inflammatory functions through its stimulatory effects on inflammatory Th17 cells (28) (Table 1). Stimulation of inflammatory Th17 cell genesis by TGF-β occurs primarily in the presence of IL-6 (18, 29- 30) (Figure 1).

TGF-β in schizophrenia

TGF-β family members bind to and activate transmembrane serine/threonine receptors (31). During canonical signalling, type I receptors phosphorylate receptor-activated Smads, which then associate with Smad4, which in turn accumulates in the nucleus and modulates transcription of target genes (32). Signalling through the canonical pathway is involved in multiple aspects of neurodevelopment, adult neurogenesis and neuroprotection (reviewed in 33). There is evidence that TGF signalling contributes to neurodegeneration in Alzheimer's disease (34, 35) and influences cognitive abilities and the level of cognitive decline between male and female schizophrenia patients (36, 37); further, it is altered in the hippocampus in schizophrenia and bipolar disorder (38) and in anxiety and depression (39- 41).

Elevated total TGF-β has been reported in the cerebrospinal fluid of patients with malignancies in the central nervous system, AIDS dementia complex, and neuropathologic disorders, including communicating hydrocephalus, Alzheimer's disease and glioblastoma (42- 44), and in patients with schizophrenia (45).

It has been observed that patients with schizophrenia have a higher percentage of anti-inflammatory regulatory T cells and IL-4-producing lymphocytes in peripheral blood (46). Schizophrenia has been associated with the enhanced peripheral release of TGF-β (47) and increased lymphocytic expression of TGF-β receptors (48, 49). Data from the literature also indicate that the serum levels of TGF-β are significantly increased in patients with schizophrenia in relapse and first-episode psychosis compared with a control group (21, 49). A meta-analysis of plasma cytokine alterations suggested that TGF-β is the state marker for acute exacerbation of schizophrenia (21) and that it can be a valuable marker for psychosis (22). Recent studies have questioned these findings and did not find a difference in the TGF-β serum levels between patients with schizophrenia and controls (50).

In our studies, we found that the IL-17 levels were decreased and that the IL-17/TGF-β ratio was significantly lower in drug naïve patients who were having their first psychotic episode. We also found that the levels of TGF-β and IL-23 were increased in all psychotic patients and that the IL-6 serum levels decreased only after antipsychotic treatment (22- 24). At the onset of illness, TGF-β most likely plays an immunosuppressive role, and IL-23 may have pathogenic effects unrelated to IL-17. In contrast to our results and those of others (51), several reports have found an increase in serum IL17 in patients whose schizophrenia is in relapse and an increased activation of Th17 cells in the first episode of schizophrenia, with a decrease in Th17 cells after risperidone treatment (50, 52). These differences may be due to the significantly shorter duration of illness in our study population, the possible predominance of IL-6 signalling pathways in later stages of schizophrenia (14), and the diverse effects of different antipsychotic drugs (21). The meta-analysis by Tourjman et al. (53) confirms our findings that TGF-β is unaffected by antipsychotic treatment (23).

Data from a pathway analysis of genome wide association study suggested that TGF-β signaling is associated with schizophrenia (54). The TGFB1+869T>C gene polymorphism is associated with schizophrenia, especially in females in the context of TGF-β and estradiol interaction (55).

TGF-β pathways and neuroplasticity in schizophrenia

TGF-β signalling is a crucial factor in neural stem cell maintenance and differentiation and determines the growth and size of the developing brain (56). Activated microglia seems to have a positive effect on the secretion of the anti-inflammatory cytokine TGF-β (57). Hyperactivity of TGF-β signalling pathways in schizophrenia is considered a neuroprotective mechanism (58). In animal models, TGF-β promotes the survival of midbrain dopaminergic neurons (59), and TGF-β over-expression increases neurogenesis in the subventricular zone (60). Additionally, TGF-β expression is induced following a variety of types of

Table 1. TGF-β role and its concentrations in schizophrenia

parameter	role	serum concentration	cerebrospinal fluid concentration	cytokines ratios	antipsychotic treatment
TGF-β	- inhibition of pro-inflammatory cytokine synthesis - inhibition of natural killer cell activity and growth of T- and B- cells - stimulation of 17 cells in the presence of IL-6	↑ [21, 49] \ [50]	↑ [45]	IL-17/TGF-β ↓ [22]	\ [53]

↑- increased; ↓- decreased; \ - unchanged

brain tissue injury, which attenuates brain damage through anti-inflammatory, apoptotic, and excitotoxic actions and promotes angiogenesis and neuroregeneration (61).

CONCLUSION

Early interventions in schizophrenia have great importance in preserving cognitive abilities, possibly due to the neuroprotective effects of TGF-β signalling. The biological markers measured during the prodromal phase could have clinical importance in determining diagnosis, further treatment strategies, and prognosis. Targeting the TGF-β signalling pathways with new psychoactive drugs may allow the re-establishment of synaptic transmission in many neuropsychiatric disorders, including schizophrenia.

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HEPATITIS C THERAPY RELATED HAEMATOLOGICAL SIDE EFFECTS ARE ASSOCIATED WITH TREATMENT OUTCOME

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HEMATOLOŠKA NEŽELJENA DEJSTVA TERAPIJE HEPATITISA C SU POVEZANA SA ISHODOM LEČENJA

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ABSTRACT

Treatment of patients su ering from chronic hepatitis C with standard pegylated interferon alpha 2a plus ribavirin has limited e cacy. erapy outcome is dependent on several factors of both the host and virus, including age, sex, stage of fibrosis, viral genotype, viral load, and occurrence of haematological adverse events during chronic hepatitis C treatment. e aim of this study was to determine the relationship between the viral and host factors and the haematological side e ects of therapy with sustained virological response.

Fifty-four patients were treated with combined pegylated interferon alpha 2a plus ribavirin therapy. Hepatitis C virus genotyping, viral load, histopathological liver changes and biochemical parameters were evaluated for each patient before beginning treatment. Each patient's blood count was analysed during each clinical visit.

Sustained virological response was achieved in 75,9% of patients. Baseline AST and ALT levels were significantly higher in patients with a poor response to therapy ($p<0,05$). Other clinical and laboratory parameters did not reach statistical significance. Both responders and non-responders developed anaemia. A decrease in thrombocytes, neutrophils and white blood cells was significantly associated with a sustained response to therapy ($p<0,05$, $p<0,05$ and $p<0,001$, respectively).

Sustained virological response was associated with lower baseline AST and ALT values and thrombocytopenia, leucopenia and neutropenia at the end of the treatment. All treated patients developed anaemia.

Keywords: *chronic hepatitis C, pegylated interferon alpha 2a, ribavirin, anaemia, thrombocytopenia, leucopoenia, neutropenia*

SAŽETAK

Standardna terapija hronične HCV infekcije, primenom pegiliranog interferona alfa 2a i ribavirina, ima ograničenu efikasnost. Na ishod terapije utiču brojni faktori domaćina i virusa: starost, pol, stadijum fibroze, genotip virusa, nivo bazalne viremije, ali i pojava neželjenih efekata terapije. Cilj ovog istraživanja je bio da se utvrdi povezanost neželjenih hematoloških efekata terapije i trajnog virusološkog odgovora kod pacijenata sa hroničnom HCV infekcijom.

Ispitivanjem je bilo obuhvaćeno 54 bolesnika sa hroničnim HCV hepatitisom. Lečenje je sprovedeno tokom 24/48 nedelja, u zavisnosti od genotipa virusa. Svim bolesnicima su pre početka terapije određivani bazalni nivo viremije, genotip virusa, stepen histopatoloških promena u jetri i biohemijski parametri, dok je kompletna krvna slika je određivana prema standardnom protokolu tokom i nakon završetka terapije.

Trajni virološki odgovor postignut je u 75,9% pacijenata. Bazalni nivo AST-a i ALT-a je bio značajno veći kod pacijenata sa slabim odgovorom na terapiju ($r <0,05$). Drugi klinički i laboratorijski parametri nisu dostigli statističku značajnost. Anemija se razvila u obe grupe ispitanika, i u grupi respondera ali i u grupi non-respondera. Smanjenje broja trombocita, neutrofila i leukocita je bilo značajno povezano sa dobrim odgovor na terapiju ($r <0,05$, $r <0,05$ i $r <0,001$).

Utvrđeno je da je trajni virusološki odgovor povezan sa nižim vrednostima AST i ALT, trombocitopenijom, leukocitopenijom i neutropenijom na kraju tretmana. Kod svih lečenih pacijenta javila se anemija.

Ključne reči: *hronični hepatitis C, pegilovani interferon alfa 2a, ribavirin, anemija, trombocitopenija, leukopenija, neutropenija.*

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INTRODUCTION

Hepatitis C infection is a leading cause of chronic liver disease, fibrosis and liver cirrhosis, which leads to hepatocellular carcinoma in 1-5% of cases (1, 2). In approximately 75% of patients, acute hepatitis C evolves to chronic disease (3). The current standard treatment of hepatitis C is pegylated interferon alpha 2a (PEG-IFNα-2a) and ribavirin (RBV) (4). However, this therapy has limited efficacy and is successful in only 55-80% of patients. Several studies have been conducted to identify factors that influence therapy outcome. Previous studies have been conflicting, as the size and composition of the studied groups varied. Achieving sustained virological response (SVR) depends on many factors, such as HCV genotype, viral load, age, gender, genetics and patient ethnicity, degree of histopathological liver changes and infection duration (5, 6). However, combined PEG-IFNα-2a+RBV therapy has diverse side effects that are similar to flu-like symptoms, including fever, headache, cough, nausea, and haematological disorders (7). Some of these adverse effects can be severe and require modification of the therapy dosage or withdrawal from therapy. However, a previous study by Pawlowska et al. showed that alterations in haematological parameters are associated with response to therapy (8).

Therefore, the aim of our investigation was to determine the relationship between various viral and host factors and haematological side effects in response to PEG-IFNα-2a+RBV therapy in chronic HCV patients.

PATIENTS AND METHODS

A retrospective study was conducted of 76 patients with chronic hepatitis C infection who completed combined PEG-IFNα-2a+RBV therapy in the Clinic for Infectious Diseases, Clinical Centre of Kragujevac, from January 2007 to December 2010. Of the 76 patients who started therapy, 22 failed to return for follow-up evaluation of blood count; therefore, a total of 54 patients with complete blood count follow-up were reviewed. The study was approved by the local Ethics Committee, and written informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Anamnesis, laboratory data, liver biopsy, serum HCV viral load and genotyping were obtained for each patient prior to therapy. The degree of histopathological changes in the liver was determined by Haematoxylin-Eosin staining of biopsy specimens. Blood count and haemoglobin were assessed during each clinical visit.

Patients infected with HCV genotypes 1 and 4 were treated with PEG-IFNα-2a (180 μg/week) and ribavirin (800-1200 mg/day) for 48 weeks, whereas patients infected with HCV genotypes 2 or 3 completed the same treatment for 24 weeks. Sustained virological response (SVR) was defined as undetectable HCV RNA 24 weeks after therapy. If HCV RNA was steadily detectable during and at the end of therapy, patients were defined as non-responsive to therapy (NR), whereas the reappearance of viral RNA in patients whose serum HCV RNA had been undetectable was categorized as a relapse (relapse responders [RR]).

Clinically relevant cut-offs were defined as follows: leucopoenia (WBC less than $4x10^9$ cells/L) neutropenia (neutrophils less than $1.5x10^9$ cells/L) thrombocytopenia (platelets less than $135x10^9$ cells/L) and anaemia (haemoglobin less than 120 g/L for women and less than 130 g/L for men; erythrocytes less than $3.86x10^{12}$ cells/L for women and $4.34x10^{12}$ cells/L for men). The PEG-IFNα-2a dose was reduced for patients with neutrophil counts $<0.75x10^9$

Table 1. Baseline demographic and clinical characteristics and laboratory data of the study population

Characteristics	*Total (n=54)*
Male gender, n (%)	36 (66,7)
Age, years, mean±SD	41.1±13.4
Baseline HCV RNA ($x10^6$ IU/ml±SD)	8.1±12.9
HCV genotypes, n (%)	
G1	31 (57.4)
G2	1 (1.9)
G3	21 (38.9)
G4	1 (1.9)
Liver biopsy results (fibrosis), n (%)	
F0	6 (11.1)
F1	26 (48.1)
F2	12 (22.2)
F3	6 (11.1)
F4	4 (7.4)
Risk factor for acquiring HCV, n (%)	
IDU	22 (40.7)
Blood transfusion	12 (22.2)
Perinatal Infection	1 (1.9)
Sexual partners with HCV	1 (1.9)
Unknown factors	18 (33.3)
Treatment response rate	
SVR/ETR	41 (75.9)
NR/RR	13 (24.1)
Biochemical parameters, mean±SD	
AST (IU/l)	115.5±52.0
ALT (IU/l)	149.4±58.4
AF (IU/l)	66.1±17.9
AFP1 (ng/ml)	3.9±3.1
Bilirubin unconjugated (μmol/l)	10.8±4.0
Bilirubin conjugated (μmol/l)	2.7±1.4
Blood Proteins (g/l)	73.0±5.7
Albumins (g/l)	45.7±3.9
Globulins (g/l)	27.6±6.9
INR (s)	1.0±0.1
Triglycerides (mmol/l)	1.3±0.7
Cholesterol (mmol/l)	4.3±1.1

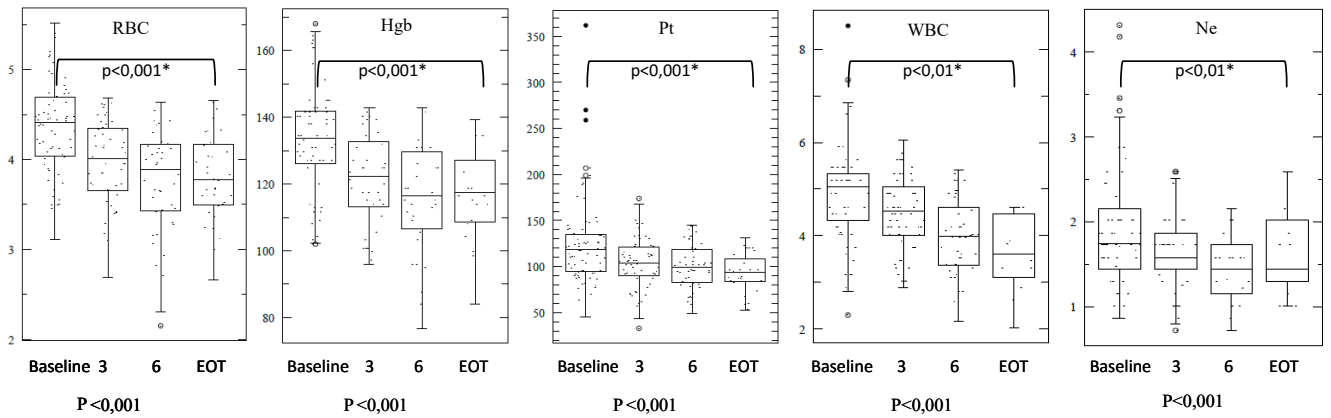

Figure 1. Complete blood count determined before treatment (baseline), 3 and 6 months after beginning treatment and at the end of therapy (EOT). P values were determined using ANOVA for di erences in values during treatment. *Statistical di erences between the baseline and EOT time points were determined using a Mann-Whitney U-test.

cells/L and platelet counts <50x10^9 cells/L and was withheld for patients with absolute neutrophil counts <0.5x10^9 cells/L and platelet counts <25x10^9 cells/L. Ribavirin doses were modified for patients with a haemoglobin concentration <100 g/L and was withheld for patients with a haemoglobin concentration <85 g/L.

All statistical analyses were conducted using commercial SPSS software (version 19.0, SPSS Inc., Chicago, IL). Collected data were stratified by subgroups of patients of interest and analysed using central tendency, variability and frequency. Contingency Tables were used to analyse the relationship between two or more variables. The distributions of data were evaluated for normality using the Kolmogorov-Smirnov test. Quantitative parametric data were compared between two study groups using an unpaired t-test. A Mann-Whitney U-test and Kruskal-Wallis test were used for comparative analysis between groups of non-parametric data. For analysis of the association between haematological parameters and therapy response, logistic regression and multivariate analysis were performed. A p-value of <0.05 was considered statistically significant.

RESULTS

The majority of the patients were male (67%), and the average patient age was 41,1±13,4 years (Table 1). The median HCV-RNA viral load was 8,1±12,9x10^6 IU/ml. HCV genotype 1 was dominant (57,4%), while genotype 3 was present in 38,9% of patients and genotypes 1 and 4 were present in 1,9% of patients each. The major route of infection was injection drug use (40,7%), followed by blood transfusion (22,2%); in 33,3% patients, the virus transmission route was unknown. Liver fibrosis was present in 88,9% of patients, primarily at the F1 (48,1%) or F2 stage (22,2%). The majority of patients achieved sustained virological response (SVR) (75,9%), and 24,1% patients showed poor response to therapy (RR and NR). Laboratory data of patients are presented in Table 1. Haematological parameters during follow-up visits showed a significant decrease in haemoglobin (Hgb) levels and in the count of red blood cells (RBC), platelets (Pt), white blood cells (WBC) and neutrophils (Ne) (p<0,001) during therapy (Figure 1).

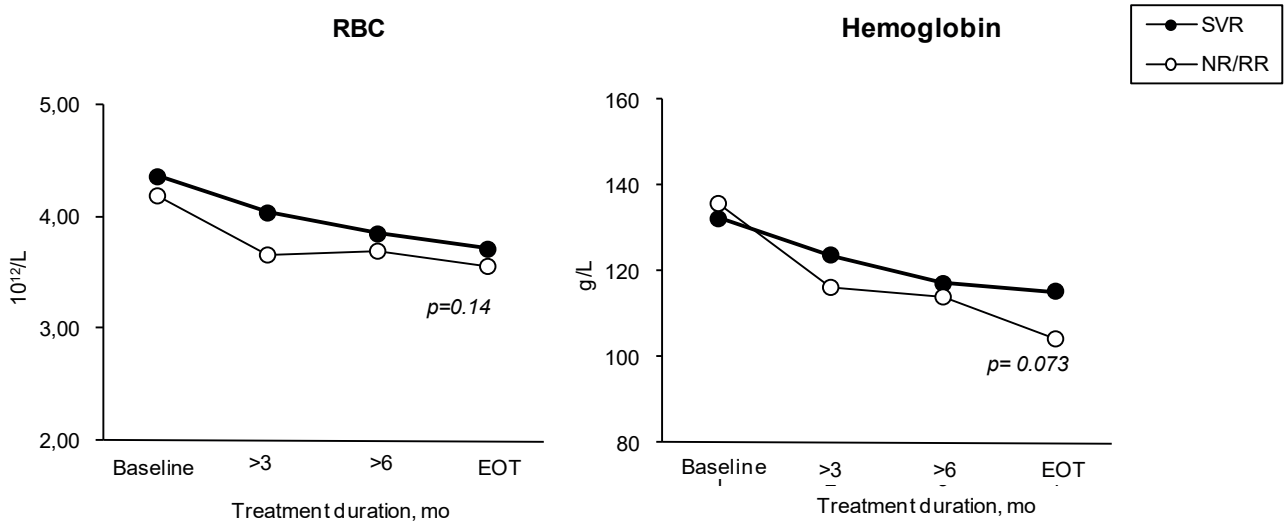

Figure 2. Median erythrocyte count and haemoglobin levels in the SVR and NR/RR groups before treatment (baseline), 3 and 6 months after beginning treatment and at the end of therapy (EOT);
*Di erences between SVR and NR EOT time points were determined using a Mann-Whitney U-test.

Table 2. Comparison of demographic and clinical characteristics and laboratory data between patients with or without sustained virological response. * p<0.05

Characteristics	*NR/RR (n=13)*	*SVR (n=41)*
Male gender, n (%)	10 (76.9)	25 (61.0)
Age, years, mean±SD	42.1 ± 12.3	40.8±14.5
Baseline HCV RNA (x10^6 IU/ml±SD)	6.2±8.9	8.6±13.9
HCV genotypes, n (%)		
G1	9 (69.2)	22 (53.7)
G2	1 (7.7)	0 (0)
G3	3 (23.1)	18 (43.9)
G4	0 (0)	1 (2.4)
Liver biopsy results (fibrosis), n (%)		
F0	2 (15.4)	4 (9.8)
F1	5 (38.5)	21 (51.2)
F2	2 (15.4)	10 (24.4)
F3	3 (23.1)	3 (7.3)
F4	1 (7.7)	3 (7.3)
Risk factor for acquiring HCV, n (%)		
IDU	4 (30.8)	18 (43.9)
Blood transfusion	3 (23.1)	9 (22.0)
Perinatal Infection	0 (0)	1 (2.4)
Sexual partners with HCV	1 (7.7)	0 (0)
Unknown factors	5 (38.5)	13 (31.7)
Biochemical parameters, mean±SD		
AST (IU/l)	**142.7±40.7**	**106.8±52.3***
ALT (IU/l)	**180.5±52.0**	**139.5±57.3***
AF (IU/l)	72.1±16.7	64.4±18.1
AFP (ng/ml)	4.7±2.1	3.5±3.3
Bilirubin unconjugated (μmol/l)	12.3±4.2	10.4±3.9
Bilirubin conjugated (μmol/l)	3.3±1.7	2.5±1.2
Blood Proteins (g/l)	73.2±3.9	73.0±6.2
Albumins (g/l)	45.7±4.2	45.7±3.8
Globulins (g/l)	28.3±6.3	27.3±7.1
INR (s)	1.0±.1	1.0±0.1
Triglycerides (mmol/l)	1.5±0.8	1.3±0.6
Cholesterol (mmol /l)	4.0±1.5	4.3±1.0
Laboratory data, median (range)		
RBC baseline (x10^{12} cells/L)	4.19 (2.69-4.68)	4.36 (3.11-5.52)
RBC EOT (x10^{12} cells/L)	3.57 (2.15-4.64)	3.72 (2.66-4.66)
Hgb baseline (g/L)	136 (102-168)	132 (96-164)
Hgb EOT (g/L)	104 (76-142)	115 (84-139)
Platelet baseline (x10^9 cells/L)	142 (54-324)	142 (59-311)
Platelet EOT (x10^9 cells/L)	**136 (59-248)**	**110 (39-174)***
WBC baseline (x10^9 cells/L)	4.32 (2.30-6.05)	5.18 (2.88-8.50)
WBC EOT (x10^9 cells/L)	**5.04 (2.16-8.93)**	**3.65 1.93-5.62)***
Neutrophil baseline (x10^9 cells/L)	2.06 (0.86-3.02)	2.02 (0.86-3.17)
Neutrophil EOT (x10^9 cells/L)	**1.85 (1.01-2.45)**	**1.49 (0.72-2.59)***

We compared all of the data between the patients with and without sustained virological response. As shown in Table 2, there was no statistically significant difference between groups in relation to gender, age, basal viral load, virus genotype, infection route or fibrosis stage. Among the biochemical parameters tested, only AST and ALT serum levels were significantly higher in patients with poor response to therapy (p<0,05). For haematological parameters, RBC count and haemoglobin levels decreased in both groups during treatment, but there was no significant difference between the two groups at the end of therapy (Figure 2). Platelet and white blood cell counts were within

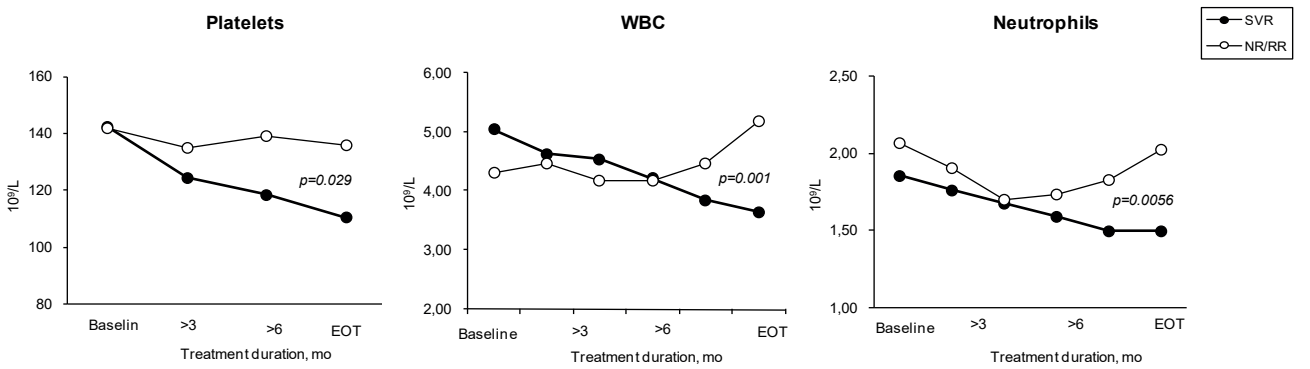

Figure 3. Median platelet, WBC and neutrophil counts in SVR and NR/RR groups before treatment (baseline), 3 and 6 months after beginning treatment and at the end of therapy (EOT); *Di erences between SVR and NR EOT time points were determined using a Mann-Whitney U-test.*Di erences between SVR and NR EOT time points were determined using a Mann-Whitney U-test.

the normal range in NR/RR patients during therapy, but decreased in the SVR group. At the end of therapy, there was a statistically significant difference in these values between the two groups (Pt: p<0,05 and WBC: p<0,001). The neutrophil count decreased during the first 3 months of treatment in both groups. Neutrophil counts continued to decrease in the SVR group, but increased back to baseline in the NR/RR group. The neutrophil count at the end of treatment was statistically lower in responders than in patients with poor response (p=0,005) (Figure 3).

To identify clinically relevant associations between the analysed parameters and the treatment outcome, we determined independent predictors for sustained virological response using logistic regression and multivariate analysis. Of the parameters analysed in the univariate model, we found a negative association between therapy response and platelet, WBC and neutrophil counts. The results showed that therapeutic response depends on platelet count (Odds ratio=0.869; 95% CI=0.772–0.979; p=0.020), as an increase in platelet count reduces the chances for a good therapeutic response by 13.1%. Similarly, treatment response depends on WBC (Odds ratio=0.018; 95% CI=0.001–0.0.396; p=0.011) and neutrophil counts (Odds ratio=0.003; 95% CI=0.000–0.182; p=0.006) such that an increase in WBC or neutrophil count reduces the chances for a good therapeutic response by 82% and 97%, respectively. However, the multivariate model showed that the most significant predictors of treatment response were WBC (Odds ratio=0.013; 95% CI=0.000–0.656; p=0.030) and neutrophil count (Odds ratio=0.000; 95% CI=0.000–0.273; *p*=0.018).

DISCUSSION

Considering the insufficient efficacy (55-60%), cost and diverse side effects of PEG-IFNα-2a+RBV therapy (7, 9) it is important to determine the factors that influence or are associated with response. The major predictors for achieving sustained virological response were viral genotype and basal viral load. HCV genotype 1 infection and viral load >600.000 IU/ml are related to poor therapy response (10, 11). Among other factors, male sex, age >40 years and advanced fibrosis have been shown to predict a lower response rate (12, 13). However, in our study, gender, age, basal viral load, HCV genotype, histopathology and risk factors for acquiring HCV infection were not associated with therapy response (p>0,05). These discrepancies may be related to the size of the study group, as our previously published data with a larger group of 121 patients showed that treatment outcome was associated with baseline HCV RNA, HCV genotype, infection route and the degree of histopathological liver changes (14).

Clinical data regarding the relevance of transaminase levels and therapy outcome are conflicting. While Zeuzem et al. showed that patients with elevated transaminases respond better to therapy (15), Gordon et al. found that reaching SVR is independent of the ALT baseline level (16). Our results are inconsistent with those findings, as we found that patients with a poor response to therapy had significantly higher baseline AST and ALT levels (p<0,05).

Common side effects of PEG-IFNα-2a+RBV therapy include anaemia, leucopoenia and thrombocytopenia (17-19). Ribavirin has haemolytic effects and often causes anaemia, whereas IFNα has been shown to suppress haematopoietic progenitor cells in the bone marrow to induce leucopoenia and thrombocytopenia (20-22). Likewise, our study showed a statistically significant decrease in Hgb levels and RBC, Pt, WBC and Ne counts (p<0,001).

Recent studies have shown that the degree of haemoglobin alleviation during therapy is associated with a higher response rate (23, 24). We did not observe such a relationship, but found a similar decrease in Hgb levels and RBC counts in both the SVR and the NR/RR groups. However, the Pt, WBC and Ne counts decreased in the SVR group, and they were significantly lower at the end of treatment in the SVR group compared with the NR/RR group. Our results are consistent with previous findings that leucopoenia and thrombocytopenia are significantly more pronounced in patients who achieved sustained virological response (8, 25). We suggest that this difference is related to individual variations in pharmacokinetics and biochemical and physiological effects of drugs.

In conclusion, our study showed that treatment failure was significantly associated with higher baseline AST and ALT levels. All treated patients developed anaemia, irrespective of therapy response. Sustained virological response occurred more often in patients who developed thrombocytopenia, leucopoenia and neutropenia.

ACKNOWLEDGMENTS

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THE EFFECTS OF CISPLATIN AND ITS PT II ANALOGUE ON OXIDATIVE STRESS OF ISOLATED RAT HEART

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EFEKTI CISPLATINE I PT II ANALOGA CISPLATINE NA OKSIDACIONI STRES IZOLOVANOG SRCA PACOVA

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ABSTRACT

To date, numerous platinum (II) complexes have been successfully used in the treatment of di erent types of cancer. erapeutic platinum complexes are di erent in terms of their structure, chemical reactivity, solubility, pharmacokinetics and toxicity. e aim of our research was the evaluation of cardiotoxicity of dichloro-(ethylendiamine) platinum (II) in a model of isolated rat heart using the Langedor technique. Oxidative stress was assessed by determination of superoxide anion radical, hydrogen peroxide, iobarbituric Acid Reactive Substances and nitric oxide levels from coronary venous e uent. All reagents were perfused at increasing concentrations from 10^{-8} to 10^{-4} M for 30 minutes. In this paper, we report that substances administered at higher doses did not induce dose-dependent e ects on oxidative stress markers. e results of this research may be of great interest for future studies in this area. ere are many novel platinum compounds that had previously demonstrated antitumour activity, and these types of experiments in our study can assist in the examination of their cardiotoxicity. ese results could be helpful for understanding dose-dependent side e ects of existing and novel platinum compounds.

Keywords: *isolated rat heart, oxidative stress, novel-etilendiamin complex of platinum, cisplatin*

SAŽETAK

Danas se veliki broj kompleksa Pt(II) sa velikim uspehom koristi u terapiji različitih vrsta tumora. Kompleksi platine koji se koriste u terapijske svrhe se razlikuju u pogledu strukture, hemijske reaktivnosti, rastvorljivosti, farmakokinetičkih osobina i toksičnosti. Cilj ovog istraživanja bio je da se utvrdi kardiotoksičnost kompleksa dihloro-etilendiamin-platina(II) na modelu izolovanog srca pacova metodom po Langedor -u. Oksidativni stres je određivan merenjem superoksid anjon radikala, vodonik peroksida, indeksa lipidne peroksidacije i azot-monoksida u koronarnom venskom efluentu. Sve supstance su aplikovane u rastućim dozama od 10^{-8} do 10^{-4}M tokom 30 minuta. U ovom radu smo naveli da ispitivane supstance pri višim dozama nisu pokazale dozno zavisni efekat na parametre oksidativnog stresa. Rezultati ovog istraživawa mogu biti od velikog značaja za neka nova istraživawa u ovoj oblasti. Postoji veliki broj novih kompleksa platine koji su pokazali antitumorsku aktivnost i ovakav vid eksperimenata bi mogao da posluži za ispitivawe njihovih kardiotoksičnih efekata. Ovi rezultati bi mogli biti od koristi za razumevanje dozno zavisnih neželjenih efekata postojećih i novih komplesa platine.

Ključne reči: *izolovano srce pacova, oksidativni stres, novi etilendiaminski kompleksi platine, cisplatina*

ABBREVIATIONS

DNA - deoxyribonucleic acid
EN – ethylenediamine
GSH - tripeptide gluthathione
H_2O_2 - hydrogen peroxide
$K_2[PtCl_4]$ - Potassium-tetra-chloroplatinum
NHCP - platinum-N-heterocyclic carbene complex
NO - nitric oxide
$O_2^{\cdot-}$ - superoxide anion radical
$Pt^{(II)}ENCl_2$ - cis-$[Pt(en)Cl_2]$ – dichloro-(ethylendiamine) platinum(II)
RNA - ribonucleic acid
ROS - reactive oxidative species
TBARS - Thiobarbituric Acid Reactive Substances

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INTRODUCTION

Synthesis of Peyron`s chloride (cisplatin) was the first step in research on biologically active platinum complexes; the biological activity of cisplatin was discovered many years later (1). To date, numerous platinum (II) complexes have been successfully used for the treatment of different types of cancer (2). Therapeutic platinum complexes differ in terms of their structure, chemical reactivity, solubility, pharmacokinetics and toxicity. Cisplatin affects tumour cells and prevents synthesis and repair of DNA (3). Nucleophiles such as DNA, RNA and proteins interact with cisplatin and cause several side effects (4, 5). Cisplatin has a central role in chemotherapy, but severe toxicity (e.g., nephrotoxicity, peripheral neuropathy, ototoxicity, neutropenia, thrombocytopenia, embryotoxicity, mutagenicity and cardiotoxicity) and cross-resistance have limited its therapeutic use and initiated development of new analogues (2, 6, 7).

Cisplatin-induced nephrotoxicity, ototoxicity and neurotoxicity have been elucidated in detail, but less is known about cardiotoxicity (8-11). The toxic effects of cisplatin may be caused by inhibition of protein synthesis, DNA damage, peroxidation of the cell membrane and mitochondrial dysfunction (12). One serious side effect of cisplatin treatment is acute, cumulative cardiotoxicity, which can be a limiting factor for its use in therapy. Cardiotoxicity resulting from the use of cisplatin is caused by the formation of reactive oxidative species (ROS) and the induction of immunogenic reactions by the presence of antigen presenting cells in the heart tissue (13). A few cases of acute myocardial infarction after treatment with cisplatin have been described previously (14, 15). Studies have shown that cisplatin stimulates the production of ROS such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), Thiobarbituric Acid Reactive Substances (TBARS), and nitric oxide (NO) (16).

The aim of our research was to evaluate cardiotoxicity by direct perfusion of isolated rat heart with cis-$[Pt(NH_3)_2Cl]$ and cis-$[Pt(en)Cl_2]$ ($Pt^{(II)}ENCl_2$). Oxidative stress was assessed by the measurement of $O_2^{\cdot-}$, H_2O_2, TBARS and NO levels in coronary venous effluent.

MATERIALS AND METHODS

Isolated rat heart preparation

Male Wistar-albino rats (body weight 180-200 g) were anaesthetized with diethyl ether and were killed by cervical dislocation according to Schedule 1 of the Animals (Scientific procedures) Act 1986, UK. After urgent thoracotomy and rapid arrest of the beating heart by superfusion with ice-cold isotonic saline, the hearts were isolated and perfused according to the Langendorff technique. The composition of the Krebs-Henseleit buffer (perfusion medium) was as follows (in mmol/l): NaCl (118); KCl (4.7); $CaCl_2 \times 2H_2O$ (2.5); $MgSO_4 \times 7H_2O$ (1.7); $NaHCO_3$ (25); KH_2PO_4 (1.2); glucose (5.5). The buffer was equilibrated with a gas mixture (5% CO_2-95% O_2) at 37°C (pH 7.4). Constant left ventricular draining through the dissected mitral valve was performed, and the sensor (*transducer BS4 73-0184, Experimetria Ltd, Budapest, Hungary*) was inserted into the left ventricular cavity via the left atrium for continuous recording of the functional cardiac parameters (17).

Perfusion of isolated rat heart

After 30 minutes of stabilization, myocardial perfusion was established at a constant coronary perfusion pressure of 70 cm H_2O. Once flow was stabilized, hearts were perfused for 30 minutes with Krebs-Henseleit buffer without (control group) or with the following test compounds at different concentrations from 10^{-4} to 10^{-8} M: $Pt^{(II)}ENCl_2$, cisplatin, potassium-tetra-chloroplatinum ($K_2[PtCl_4]$), ethylenediamine (EN) and Krebs-Henseleit buffer.

Biochemical assays

Samples of coronary venous effluent were collected at the end of each period of perfusion (30, 60, 90, 120 minutes).

Index of lipid peroxidation
(Thiobarbituric Acid Reactive Substances – TBARS)

The degree of lipid peroxidation in coronary venous effluent was estimated by measuring thiobarbituric acid reactive substances (TBARS). The measurement was taken using 1 % thiobarbituric acid (TBA) in 0.05 sodium hydroxide (NaOH) and was incubated with coronary effluent at 100 °C for 15 minutes. The results were read at 530 nm. Krebs-Hensenleit solution was used as a blank probe (18).

Nitrite determination

Nitric oxide was assessed as nitrite and quantified by the spectrophometric method using the *Griess* reagent. 0.5 ml of perfusate was precipitated with 200 µl of 30% sulfosalicylic acid, vortexed for 30 min and centrifuged at 3000 x g. Equal volumes of the supernatant and Griess reagent, containing 1% sulphanilamide in 5% phosphoric acid and 0.1% naphthalene ethylenediamine-dihydrochloride, were added and incubated for 10 min in the dark. The results were read at 543 nmol/l. Nitrite levels were calculated using sodium nitrite as a standard (19).

Superoxide determination

The level of superoxide anion radical ($O_2^{\cdot-}$) in coronary venous effluent was measured using a Nitro Blue Tetrazolium (NBT) reaction in TRIS-buffer. The results were read at 530 nm. Krebs-Hensenleit solution was used as a blank probe (20).

Hydrogen peroxide determination

The level of hydrogen peroxide (H_2O_2) in coronary venous effluent was determined by measuring phenol red oxidation of H_2O_2 in the presence of horse-radish peroxidase. The results were read at 610 nm (21).

Reagents

$Pt^{(II)}ENCl_2$ was synthesized according to Keppler et al (22). Cisplatin, $K_2[PtCl_4]$, and EN as well as substances

necessary for the preparation of Krebs-Henseleit buffer were purchased from Sigma-Aldrich GmbH, Germany.

Statistical Analysis

The concentration-response relationship was determined by linear regression on logarithmically transformed data calculated according to the least squares method. The effect of each concentration of tested substances was expressed as a percentage of the maximal response. Significance of the linear regression was tested by analysis of variance, with a p-value of less than 0.05 considered significant. For each substance, the concentration that gave 50% of the maximum response (EC_{50}) was calculated.

RESULTS

Superoxide anion radical ($O_2^{\cdot-}$)

Superoxide anion radical ($O_2^{\cdot-}$) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=1.72, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=1.21, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.73, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=1.02, df_1=4, df_2=25, p>0.05).

Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric Acid Reactive Substances (TBARS) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.14, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=0.53, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.15, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=1.06, df_1=4, df_2=25, p>0.05).

Nitric oxide (NO)

Nitric oxide (NO) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.97, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=1.13, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=1.19, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=0.75, df_1=4, df_2=25, p>0.05).

Hydrogen peroxide (H_2O_2)

Hydrogen peroxide (H_2O_2) levels were not significantly affected by treatment with $Pt^{(II)}ENCl_2$ (from 10^{-4} M to 10^{-8} M; F=0.14, df_1=4, df_2=25, p>0.05), EN (from 10^{-4} M to 10^{-8} M; F=0.26, df_1=4, df_2=25, p>0.05), $K_2[PtCl_4]$ (from 10^{-4} M to 10^{-8} M; F=0.82, df_1=4, df_2=25, p>0.05) and cisplatin (from 10^{-4} M to 10^{-8} M; F=0.81, df_1=4, df_2=25, p>0.05).

DISCUSSION

Cisplatin use leads to acute and cumulative cardiovascular complications such as arrhythmias, myocarditis, cardiomyopathy and electrocardiographic changes (23). These complications have led to the reduction of cisplatin doses or the discontinued use of cisplatin for chemotherapy (24). Drug-induced oxidative stress is generally one of the key features involved in the mechanism of toxicity in the cardiovascular system. It is well known that most anticancer drugs are associated with toxic side effects and that the formation of ROS plays a crucial role in the mechanism of anticancer drug-induced cardiotoxicity. There is no clear evidence for the cellular and molecular mechanisms involved in cisplatin cardiotoxicity, but some experimental and clinical studies support the idea that an increase in oxidative stress may lead to cardiotoxicity (24-28).

Cisplatin is a potent chemotherapeutic agent that exhibits multiorgan toxicity. *In vitro* studies have shown that ROS such as $O_2^{\cdot-}$, H_2O_2 and ·OH are involved in the cytotoxicity induced by cisplatin. The role of oxidative stress in the pathophysiology of cisplatin-induced toxicity was investigated by using different antioxidants and superoxide dismutase mimetics and it was shown that the use of ROS scavengers prevents or reduces cisplatin-induced cytotoxicity. These results provide additional evidence that ROS have important roles in the pathogenesis of cytotoxicity induced by cisplatin (12, 27, 29). There is evidence that acute administration of cisplatin leads to a significant increase in the biochemical markers of oxidative stress in postmitochondrial and mitochondrial fractions in cardiac tissues in rats (30).

Lower doses of cisplatin induce apoptosis mediated by superoxide and hydroxyl radicals, and higher doses of cisplatin induce necrosis mediated by superoxide and hydrogen peroxide in renal tubular epithelial cells (29, 31-33). In our research, we applied two different platinum complexes at increasing concentrations (range 10^{-8}-10^{-4}) to isolated rat heart. As seen in Tables 1 and 2 , when the hearts were exposed to lower concentrations of the complexes (range 10^{-8}-10^{-7}), the production of oxygen free radicals was higher than when the hearts were exposed to higher concentrations. This phenomenon has been previously observed as the mechanism of cisplatin cytotoxicity that is responsible for cell death in culture (12).

In addition to the production of oxygen free radicals, there is evidence that cisplatin induces lipid peroxidation and decreases the activities of antioxidant enzymes in rat kidneys (34). Cisplatin especially decreases the activity of the tripeptide gluthathione (GSH), which represents its most important non-DNA target (35). GSH is known to protect mitochondria against oxidative stress, inhibiting free radical mediated injury by eliminating hydrogen peroxides. Oxidation of GSH by cisplatin changes the intramitochondrial redox status, and contributes to the establishment of a prooxidative state that favours generation of hydroxyl radicals and oxidative damage to macromolecules such as mitochondrial proteins and lipids. The depletion of GSH seems to be one of the most important factors for lipid peroxidation (36). In our research, we measured TBARS as an index of lipid peroxidation (Table 3), and we noticed that cisplatin induced higher lipid peroxidation than $Pt^{(II)}ENCl_2$ (concentration range 10^{-8}-10^{-6}); However, both substances caused a decrease in TBARS levels at the

Table 1. e e ects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on H_2O_2

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	23.54 ± 13.26	13.61 ± 4.33	30.72 ± 8.00	7.84 ± 5.22	2.97 ± 2.74
10^{-7}	18.58 ± 5.43	11.90 ± 6.50	26.27 ± 15.93	7.77 ± 8.28	1.40 ± 1.25
10^{-6}	19.67 ± 7.48	9.28 ± 5.03	18.43 ± 11.57	8.39 ± 6.82	1.08 ± 0.53
10^{-5}	17.30 ± 10.94	8.95 ± 5.30	15.48 ± 9.96	3.68 ± 2.56	0.55 ± 0.51
10^{-4}	20.01 ± 14.97	4.47 ± 2.96	14.10 ± 8.13	0.53 ± 0.51	0.26 ± 0.20

Table 2. e e ects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on $O_2^{\cdot-}$

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	55.72 ± 18.09	23.80 ± 14.61	32.73 ± 24.29	26.06 ± 12.31	36.40 ± 22.90
10^{-7}	73.52 ± 40.52	43.96 ± 22.35	35.19 ± 29.48	27.57 ± 11.16	29.16 ± 18.95
10^{-6}	45.04 ± 26.20	27.93 ± 26.95	12.99 ± 5.93	18.80 ± 12.46	52.90 ± 47.37
10^{-5}	70.17 ± 42.31	14.36 ± 8.84	28.21 ± 18.51	30.85 ± 18.85	21.47 ± 15.83
10^{-4}	49.87 ± 15.36	13.14 ± 12.36	19.98 ± 7.67	2.20 ± 1.48	8.95 ± 5.16

Table 3. e e ects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on TBARS

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	14.72 ± 9.91	28.68 ± 23.76	15.57 ± 4.84	12.51 ± 6.02	40.04 ± 18.38
10^{-7}	11.73 ± 10.26	20.65 ± 16.24	13.81 ± 8.57	10.20 ± 7.33	48.84 ± 18.59
10^{-6}	14.95 ± 10.50	24.56 ± 11.41	12.76 ± 9.37	16.52 ± 11.83	37.10 ± 22.36
10^{-5}	12.58 ± 9.23	15.97 ± 10.15	10.41 ± 9.73	12.10 ± 4.09	18.35 ± 6.52
10^{-4}	13.56 ± 8.10	8.49 ± 4.60	5.23 ± 5.23	6.95 ± 3.43	7.03 ± 4.39

Table 4. e e ects of cisplatin, $Pt^{(II)}ENCl_2$, EN, K_2PtCl_4 on NO

	X±SD (nmol/l)				
	Control	Cisplatin	$Pt^{(II)}ENCl_2$	EN	K_2PtCl_4
10^{-8}	5.20 ± 4.39	10.24 ± 4.79	15.13 ± 6.88	3.62 ± 1.80	12.19 ± 3.96
10^{-7}	3.72 ± 2.40	10.60 ± 4.20	6.16 ± 6.98	2.60 ± 1.53	8.90 ± 3.90
10^{-6}	2.35 ± 1.94	8.99 ± 2.22	3.71 ± 4.65	3.36 ± 1.36	7.58 ± 1.12
10^{-5}	2.80 ± 1.33	6.29 ± 4.16	4.67 ± 4.16	3.21 ± 1.80	3.81 ± 0.62
10^{-4}	2.05 ± 1.46	3.12 ± 0.85	1.56 ± 1.12	0.24 ± 0.21	1.60 ± 0.82

higher concentration range (10^{-5}-10^{-4}), possibly because of myocardium necrosis.

Administration of cisplatin causes overproduction of nitric oxide in the heart and kidney. Overproduction of NO may increase cellular injury by decreasing intracellular GSH levels and production of peroxynitrite anion, which causes protein nitration and tissue injury (31). In our research, we measured NO as a parameter of nitrosative stress (Table 4), and it can be observed that production of NO is higher at lower doses of each complex (concentration range 10^{-8}-10^{-7}) and is lower at higher doses (concentration range 10^{-6}-10^{-4}).

Many scientists have studied the connection between cisplatin use and imbalance of production and removal of

ROS. They observed the effects of joint use of cisplatin and different antioxidants on production of ROS in various tissues such as liver, heart and kidney. They showed that acute administration of cisplatin to rats (a single dose of 5-30 mg/kg) leads to overproduction of oxidative stress and reduction of antioxidant defences. When cisplatin was administered to rats together with certain antioxidants, the production of ROS was lower in liver, heart and kidney tissues (29-31, 37-38).

In accordance with the potential of cisplatin to cause damage to various tissues because of oxidative stress, there have been attempts to overcome these side effects. One such attempt was the formulation of new generations of platinum complexes (1). Ciftci et al. tried to prove that novel platinum agents, such as the platinum-N-heterocyclic carbene complex (NHCP), are less toxic than cisplatin (39). This study had the same design as earlier studies (30, 32, 36). The researchers showed that NHCP produced the same oxidative stress level as cisplatin at the lower dose of 5 $mgkg^{-1}$, but at the higher dose of 10 $mgkg^{-1}$, NHCP was more toxic than cisplatin.

In our study, we investigated the influence of acute administration of $Pt^{(II)}ENCl_2$ (concentration range: 10^{-8}-10^{-4}) to isolated rat heart compared to treatment with cisplatin (same concentration range) on oxidative stress parameters (Tables 1-4). We also tried to prove that $Pt^{(II)}ENCl_2$ is less toxic than cisplatin. However, our results show that, regardless of the dose, neither platinum complex induced statistically significant changes in redox status.

The results of this research may be of great interest for future studies in this area. There are many novel platinum compounds that had previously been shown to exhibit antitumour activity, and the types of experiments in our study could assist in the examination of their cardiotoxicity. Our results could be helpful for understanding dose-dependent side effects of existing and novel platinum compounds.

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EFFICACY AND TOLERABILITY OF A FIXED COMBINATION OF PERINDOPRIL/AMLODIPINE/INDAPAMIDE IN PATIENTS WITH ESSENTIAL HYPERTENSION: PILOT STUDY

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EFIKASNOST I TOLERABILNOST FIKSNE KOMBINACIJE PERINDOPRIL/AMLODIPIN/INDAPIMIDA KOD PACIJENATA SA ESENCIJALNOM HIPERTENZIJOM: PILOT STUDIJA

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ABSTRACT

Hypertension is the major risk factor in Serbia and worldwide for the morbidity and mortality from cardiovascular and cerebrovascular diseases. A majority of patients need two or more antihypertensive drugs to adequately control blood pressure.

Our study group consisted of 12 patients with uncontrolled essential hypertension, without comorbidities, divided in two groups and followed for 12 weeks. The first group was treated with a single-pill of fixed-combination Perindopril 5 mg/Indapamide 1.25 mg and an additional tablet of Amlodipine 5 mg. The second group received a single-pill fixed-combination of Perindopril 5 mg/Indapamide 1.25 mg/Amlodipine 5 mg. Our research showed significant decreases in systolic (p=0,05) and diastolic (p<0,05) blood pressure in both groups after 12 weeks of treatment. The study also showed a higher percentage of patients who achieved the targeted blood pressure (< 140/90 mmHg) on the single-pill triple combination drug (69.7%) in comparison with the other group (50%). No adverse effects were recorded in both groups.

Our results revealed significant efficacy and tolerability of a single-pill triple-fixed combination Perindopril/Amlodipine/Indapamide in patients with uncontrolled essential hypertension without comorbidities.

Keywords: *hypertension, pharmacological therapy, fixed combination*

SAŽETAK

Hipertenzija je glavni faktor rizika u Srbiji i širom sveta u pogledu morbiditeta i mortaliteta od kardiovaskularnih i cerebrovaskularnih bolesti. Većini pacijenata je potrebno dva ili više lekova za sniženje pritiska kako bi adekvatno kontrolisali krvni pritisak. Studijska grupa se sastojala od 12 pacijenata sa nekontrolisanom esencijalnom hipertenzijom bez komorbiditeta, podeljenih u dve grupe koje su praćene 12 nedelja. Prva grupa je tretirana jednom fiksnom kombinacijom perindopril 5 mg/indapamid 1.25 mg u piluli i dodatno tabletom amlodipina 5 mg. Druga grupa je dobijala jednu tabletu fiksne kombinacije perindopril 5mg/indapamid 1.25mg/amlodipin 5 mg.

Naše istraživanje je pokazalo značajan pad sistolnog (p = 0,05) i dijastolnog (p <0,05) krvnog pritiska u obe grupe posle 12 nedelja tretmana. Studija je takođe pokazala veći procenat pacijenata sa postignutim ciljanim krvnim pritiskom (<140/90 mmHg) u grupi bolesnika na tretmanu trostrukom fiksnom kombinacijom leka (69,7%) u odnosu na drugu grupu (50%). Nema zabeleženih neželjenih efekata ni u jednoj grupi. Naši rezultati ukazuju na značajnu efikasnost i podnošljivost trostruke fiksne kombinacije perindropil/amlodipin/indapamid kod pacijenata sa nekontrolisanom esencijalnom hipertenzijom bez komorbiditeta.

Ključne reči: *hipertenzija, farmakološka terapija, fiksna kombinacija*

INTRODUCTION

Hypertension is the leading risk factor worldwide, and specifically in Serbia, for morbidity and mortality from cardiovascular and cerebrovascular diseases (1). The prevalence of hypertension in Serbia has increased throughout the first decade of the 21st century, and almost every second resident of Serbia has high blood pressure. In addition, it has been shown that a majority of patients with primary hypertension worldwide do not have adequately controlled blood pressure (2). The recommendations of the European Society of Hypertension/Cardiology from 2013 proposed a combination of antihypertensive drugs for use when monotherapy is not sufficient (3). As indicated in a large

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epidemiological study, most patients require two or more antihypertensive drugs to adequately control their blood pressure (4). A number of studies have demonstrated the efficiency of a fixed combination (in a single pill) of different classes of antihypertensive agents. These combinations have proven to be safe and efficient because drugs act via different mechanisms, and the side effects of individual drugs were significantly reduced. Additionally, the fixed combination enabled early and effective attainment of targeted blood pressure levels, a significant increase in treatment compliance and adherence to antihypertensive therapy, a reduction of adverse effects and a reduction of the cardiovascular and cerebrovascular mortality (5). The most commonly used combinations of two anti-hypertensive drugs have been combinations of ACE inhibitors and diuretics and combinations of ACE inhibitors and calcium antagonists. In recent years, research has focused on a triple combination of antihypertensive drugs, with a single tablet encompassing all the above-mentioned advantages of fixed combinations. Several large studies have identified a significant percentage of patients who required three or more antihypertensive drugs to adequately control their blood pressure (ALLHAT-23% of patients; ACCOMPLISH-32% of patients) (6, 7). In terms of efficiency, rationality and safety, a fixed three-in-one combination of an ACE inhibitor, calcium antagonist and diuretic is emerging as an important link in the chain of continued approaches to the current treatment of high blood pressure.

PATIENTS AND METHODOLOGY

Our research is a part of an international, multicentre, randomized prospective study. The objective of the study was to evaluate the clinical efficacy and safety of a fixed-dose combination of Perindopril 5 mg/Indapamide 1.25 mg/Amlodipine 5 mg in a single pill compared to a fixed combination of Perindopril 5 mg/Indapamide 1.25 with Amlodipine 5 mg given as a separate drug over a period of 12 weeks.

The study included adult patients of both sexes with poorly regulated hypertension who were previously on maximum doses of monotherapy or dual therapy for hypertension (without using Perindopril, Amlodipine and Indapamide in the past two months). The definition of poorly regulated hypertension included systolic blood pressure ≥ 140 mmHg and <160 mmHg and diastolic blood pressure ≥ 90 mmHg and <100 mmHg measured at the two different visits. The consumption of grapefruit juice during the study was not allowed due to its interaction with Amlodipine.

Exclusion criteria included the following: minors, pregnant women (positive β-hCG test), breastfeeding women, women of childbearing age with inadequate contraception and the possibility of conception, patients with orthostatic hypotension, obese with a body mass index >32 kg/m^2, renal impairment, anaemia (haemoglobin <100 g/L), electrolyte imbalance, a history of heart disease (myocardial infarction, heart failure, coronary revascularization, severe aortic or mitral valve stenosis or hypertrophic obstructive myocardiopathy, unstable angina pectoris), heart rhythm disorders, transaminase values that were 1.5 times greater than the reference value (known complicated liver disease: chronic hepatitis, cirrhosis), microalbuminuria > 300 mg/24 h, severe gastro-intestinal tract disorders, diabetes mellitus type 1 or 2 under treatment, endocrine diseases (Chusing's syndrome, acromegalia, hyperparathyroidia), chronic pancreatitis, and history of a severe mental or psychiatric disorder.

Table 1. Demographic characteristics of examined patients and related factors

Drug		Number of patients	Percentage
	Sex		
Drug 1	1 Male	4	66,7
	2 Female	2	33,3
Drug 2	1 Male	6	100,0
	2 Female	0	,0
	Family history		
Drug 1	1 Positive	4	66,7
	2 Negative	2	33,3
Drug 2	1 Positive	3	50
	2 Negative	3	50
	HLP		
Drug 1	1 Elevated	6	100
	2 Normal	0	0
Drug 2	1 Elevated	5	83,3
	2 Normal	1	16,7
	Smoking		
Drug 1	1 Active	1	16,7
	2 Passive	3	50,0
	3 Former	2	33,3
Drug 2	1 Active	3	50,0
	2 Passive	3	50,0
	3 Former	0	0

In addition, patients whose blood pressure was unregulated and exceeded 160/100 mmHg on two measurements with an interval of 15 days were excluded from the study.

Each patient underwent a physical examination at the beginning of the study that included body height and weight measurement, calculated body mass index, 12-lead ECG, heart rate, and screening for microalbuminuria with dipstick strips (if the result was positive, proteinuria in 24-hour urine was required). After 12-hours fasting, blood samples were taken for haematology (haemoglobin, haematocrit, erythrocytes, platelets, leukocytes and leukocyte counts) and biochemical tests (sodium, potassium, chloride, calcium, uric acid, glucose, total protein, triglycerides, cholesterol, ALT, AST, GGT) in the morning.

Patients were divided into two groups. One group received a fixed-dose combination of Perindopril 5 mg/Indapamide 1.25 mg/Amlodipine 5 mg ("drug 2" group), and a second group received two drugs: a combination of Perindopril 5 mg/Indapamide 1.25 mg as one pill and Amlodipine 5 mg given as a separate drug ("drug 1" group).

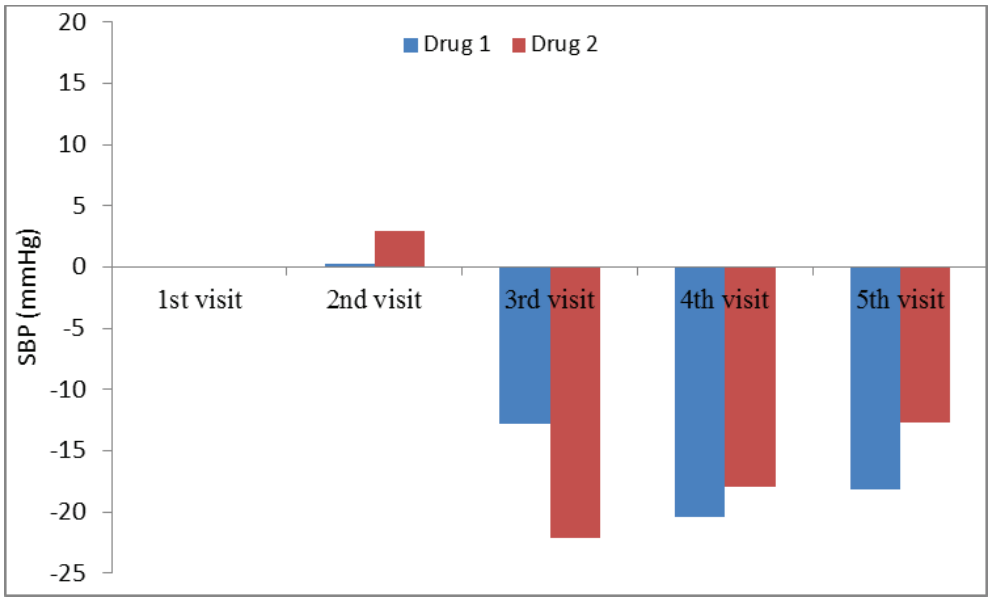

Figure 1. - Changes in the average SBP per visit and drug (mmHg)

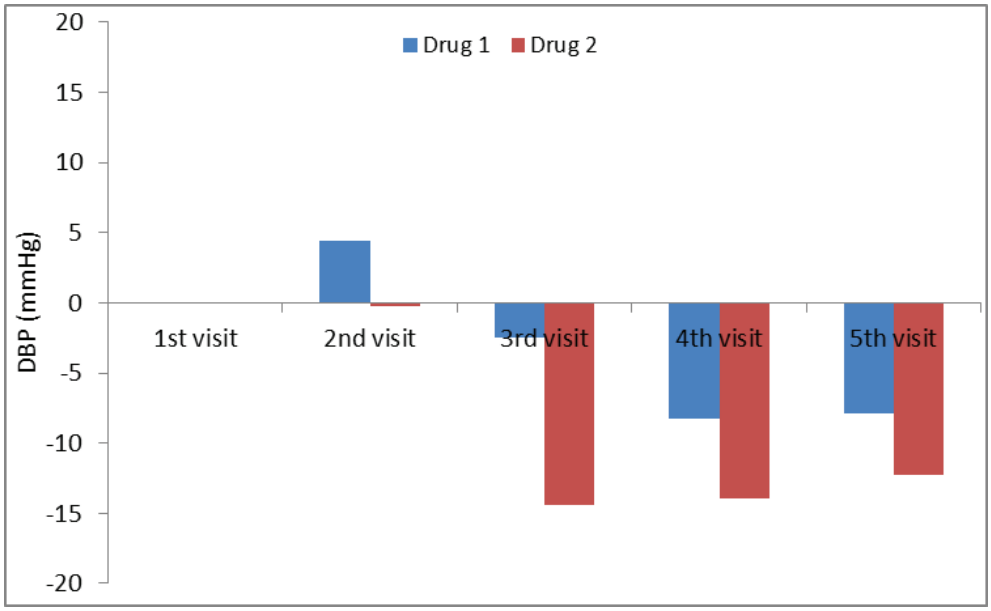

Figure 2. - Changes in the average DBP per visit and drug (mmHg)

Upon enrolment in the study, follow-up visits were performed after 4, 8 and 12 weeks. At every visit, blood pressure was measured both in the sitting and standing position, and heart rate was calculated. Blood pressure was measured using a sphygmomanometer cuff that covered 2/3 arm and included at least 80% of its volume. The measurement was performed three times in both hands with one minute breaks in between measurement; the average value was then calculated.

Before inclusion in the study, all patients provided signed informed consent. The local institution's Committee of Ethics approved the study. Data were described using descriptive statistical methods and analysed via ANOVA repeated measures. Descriptive statistical methods included measures of central tendency (mean, median), indicators of structure (expressed in percentage) and measures of variability (standard deviation, minimum and maximum value).

RESULTS

The study included 12 patients, each of whom was randomly assigned to one of two groups. The first group included 6 patients (four men and two women) who received "drug 1" (fixed-combination drug Perindopril 5 mg/Indapamide 1.25 mg and Amlodipine 5 mg as a separate drug). The second group also consisted of 6 patients (6 men) who received "drug 2" (triple fixed combination of Perindopril 5 mg/Indapamide 1.25 mg/Amlodipine 5 mg). The average age of the patients was 40.00 ± 9.49 years in the "drug 1" group and 40.67 ± 10.82 years in the "drug 2" group. The average body mass index (BMI) for patients on "drug 1" was 29.17 ± 2.64 (min 24, max 31), and for patients with "drug 2" the average was 27.67 ± 3.39 (min 22, max 32). There was no statistically significant between-group

difference between patients taking "drug 1" and "drug 2" in the prevalence of other risk factors for cardiovascular disease (family history, hypercholesterolemia, smoking) (Table 1).

The study showed a significant decline in the average systolic blood pressure (SBP) in both groups from the first to the fifth visit (F = 3.99, p = 0.05).

At the first visit, the average SBP in patients who were taking "drug 1" was 153 ± 6.57 mmHg; a decrease was noted through the fifth visit at which time the average SBP was 132.5 ± 25.38 mmHg. In patients who taking "drug 2," the average SBP (measured at the first visit) was 149.17 ± 24.06 mmHg. This value significantly decreased to SBP 140.5 ± 8:02 mmHg by the fifth visit (Figure 1). The results also showed a significant reduction in mean diastolic blood pressure (DBP) from the first to fifth visit (F = 20.16, p <0.05). At the first visit, the average DBP for patients who taking "drug 1 " was 94.83 ± 2.32 mmHg. After a slight increase noted at visit two, a decrease in DBP was recorded through the fifth, visit, at which time the average DBP was 84.50 ± 10.29 mmHg. The average value of DBP for patients taking "drug 2" was 94.00 ± 3.22 mmHg at the first visit. This average DBP value significantly decreased to 83.83 ± 5.98 mmHg by the fifth visit (Figure 2). Our results showed no statistically significant difference between "drug 1" and "drug 2" in their effects on the average values of the SBP (F = 0.24, p> 0.05) and DBP (F = 1.29, p> 0.05) between the first and fifth visit.

In our study, a higher percentage of patients achieved target blood pressure (<140/90 mmHg) in the group with triple fixed dose combination drugs (69.7%) compared to patients taking a combination of three drugs in two tablets (50%). There was no statistically significant difference between patients in the "drug 1" and "drug 2" groups in terms of average heart rate frequency between visit 1 and visit 5. No adverse effects from the drug therapy or any signs and symptoms of other diseases were reported during the study period in all patients.

DISCUSSION

Our study showed that the use of a fixed combination in one pill (Perindopril 5 mg/Indapamide 1.25 mg/Amlodipine 5 mg) as well as a combination of two pills (fixed combination of Perindopril 5 mg/Indapamide 1.25 and Amlodipine 5 mg) during the 12 weeks of therapy produced significant decreases in both systolic and diastolic blood pressure in patients with unregulated essential hypertension without comorbidity. The fixed combination of three drugs at the end of the monitoring period had lower DBP and slightly higher SBP compared to patients who used two drugs, although this difference was not statistically significant.

The effective treatment of hypertension is based on the regular use of medication. Numerous studies have shown that the attainment of the target blood pressure (<140/90 mmHg) requires the use two or more drugs (8, 9). According to the JNC 7 report (Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure), polypharmacy is marked as one of the major obstacles in achieving target blood pressure values due to poor compliance with the medication regimen (10). Therefore, the report recommended a fixed combination of drugs. The first use of triple-fixed drug combinations was described in 1966. It introduced the use of a combination of reserpine, hydralazine, and hydrochlorothiazide, which was quickly abandoned due to the drugs' adverse effects (11). Since the 1980s, several fixed drug combinations have been introduced. The European and American Guidelines (2003) for the treatment of hypertension recommended an initial treatment of hypertension with fixed-combination drugs (12). These recommendations were based on a number of studies which have shown that the use of fixed combinations is associated with better compliance, fewer side effects and faster achievement of target blood pressure values (13). A meta-analysis that included 11.925 respondents from nine studies, of which three were subjects with hypertension, came to the conclusion that the use of fixed combinations of drugs reduced noncompliance by 24-26% (14).

Today, triple fixed-dose combinations consist of different drug groups. Calcium antagonists and diuretics are the most common, combined with an ACE inhibitor, receptor antagonist ATII or aliskiren as a third drug. Calhoun and colleagues showed that the fixed combination of Aliskiren/Valsartan/Hydrochlorothiazide (HTZ) showed greater reduction in blood pressure and a higher percentage of patients successfully reaching the target blood pressure compared to a control group that received a fixed combination of Aliskiren/Valsartan and Valsartan/HTZ (15). Oparil and colleagues in the TRINITY study yielded similar results, in which the use of a fixed combination of Olmesartan/Amlodipine/HTZ was superior in terms of efficacy and tolerability compared to the control group with a dual-fixed combination of these drugs (16).

The combination of Perindopril/Indapamide/Amlodipine has a special therapeutic significance as one of the possible fixed combinations of antihypertensive drugs. In this combination, drugs act by different mechanisms in lowering blood pressure, and Perindopril and Amlodipine have been shown to have strong antiatherosclerotic effects. The calcium antagonist Amlodipine has a renin-independent mechanism of lowering blood pressure by blocking the L-calcium channels in the cell-smooth muscle arteriolar, which causes its vasodilatation. The diuretic Indapamide affects natriuresis, peripheral vascular resistance, and indirect stimulation of the renin activity. The ACE inhibitor Perindopril inhibits the system RAAS (renin-angiotensin-aldosterone system). The combination of the renin-dependent and renin-independent mechanisms leading establishing a balance between renin and sodium, reduced blood pressure and reduced adverse effects of individual components of the drug. Several studies have demonstrated a significant antihypertensive and antiatherosclerotic effect of the combination of these drugs. The ASCOT

study showed a strong effect of the combination of Perindopril/Amlodipine in reducing not only blood pressure but also cardiovascular and cerebrovascular target events (17). The EFFICIENT study has shown great efficacy and tolerability of a fixed combination of Amlodipine/Indapamide in patients with uncontrolled primary hypertension (18).

The effect of the triple combination of these drugs has been confirmed by the results of the PIANIST study (19). The study included 4,731 patients with high or very high cardiovascular risk with hypertension that was not well controlled despite antihypertensive therapy. Patients were followed for 4 months. After switching to a fixed combination of Perindopril/Indapamide/Amlodipine, a decrease was observed in the mean arterial pressure of 28.3/13.8 mmHg and blood pressure was regulated at 72%, which is similar to the results of our research. Despite the fact that patients did not have regulated blood pressure first, significant results were observed after only a month of switching to a fixed combination of drugs. Additionally, this study recorded a significant decrease of blood pressure variability.

Finally, it should be noted that it is shown that fixed triple drug combinations additionally enhance compliance and adherence to therapy and have a significant positive effect on the cost of antihypertensive therapy (20).

CONCLUSIONS

This study demonstrated significant efficiency and safety of a fixed combination of three drugs (Perindopril, Indapamide and Amlodipine) in one tablet in patients with uncontrolled essential hypertension without associated comorbidity. The application of this drug is characterized by a rapid achievement of target blood pressure, excellent adherence to the compliance and the absence of side effects. A limitation of this study was its relatively small number of subjects and the short follow-up period.

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Conflict Of Interest

The authors declare that they have no conflicts of interest.

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VOLAR LOCKING PLATING TREATMENT RESULTS IN FRAGMENTED INTRAARTICULAR DISTAL RADIUS FRACTURES

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REZULTATI TRETMANA METODOM "ZAKLJUČAVANJA" RUČJA KOD VIŠESTRUKTIH DISTALNIH INTRAARTIKULARNIH PRELOMA RADIJUSA

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ABSTRACT

Volar locking plating treatment results in fragmented intra-articular distal radius fractures in adults.

Twenty-five patients (14 men, 11 women; mean age, 44.32±12.12 years) who were treated with volar locking plates due to distal radius fractures were prospectively studied. Among the fractures, 44% were C2 and 56% were C3, according to the AO classification system. Clinical analyses were performed using the Gartland and Werley scale, the short version of the Arm, Shoulder, and Hand Questionnaire (QuickDASH), and the visual analogue scale (VAS); radiological evaluations were performed using the Stewart evaluation criteria.

e mean patient follow-up time was 20.36±7.62 months. According to the Gartland and Werley assessment scale, 68.0% of the patients (n = 17) had excellent results, 28.0% (n =7) had good results, and 4.0% (n =1) had poor results. e mean VAS score was 0.84±1.07, and the quick-average DASH subjective question score was 7.07±12.95. According to the Stewart score, the percentages of patients with perfect and good results were 92% and 8%, respectively. Among the patients, the mean grip strength was 77.3% when it was evaluated on the healthy side. One patient was diagnosed with complex regional pain syndrome, while another patient was diagnosed with flexor tenosynovitis and paraesthesia in the distribution of the median nerve; two patients were diagnosed with flexor tenosynovitis.

Volar locking plating is an e ective surgical method for treating distal radius fractures. e plate should be properly placed during surgery, and post-operative movement should begin early.

Keywords: *volar locking plate, distal radius fracture, surgical treatment*

SAŽETAK

Rezultati tretmana "zaključavanja" ručja kod odraslih pacijenata sa višestrukim distalnim intrartikulranim prelomima radijusa. U studiju je uključeno i prospektivno praćeno 25 pacijenata (14 muškaraca, 11 zena; starosti 44,32±12,12 godina) koji su podvrgnuti tretmanu "zaključavanja" ručja usled disatlnog preloma radijusa. Svi prelomi su podeljeni prema AO klasifikacionom sitemu, od kojih je 44% bilo C2 i 56% C3 klase. Za kliničke analize su korišćene Gartland i Werley skala, skraćena verzija Ruka, Rame i Šaka Upitnika (QuickDASH) i vizuelna analogna skala (VAS); radiološka procena je sprovedena upotrebom Stewart kriterijuma za procenu. Prosecan period pracenja pacijenata je bio 20,36±7,62 meseci. Na osnovu rezultata Gartland i Werley skale 68% pacijenata (n=17) je imalo odličan rezultat, 28% (n=7) je imalo dobar rezultat i 4% (n=1) je imalo loš rezultat. Izmerena srednja vrednost VAS skora je bila 0,84±1,07 a QuickDASH skora je bila 7,07±12,95. Na osnovu dobijenih rezultata korišćenjem Stewart skale 92% pacijanata je imalo odličan rezultat, a preostalih 8% je imalo dobar rezultat. Dobijena srednja vrednost jačine stiska je bila 77,3% na zdravoj strani. Jednom od pacijenata je dijagnostikovan sindrom kompleksnog regionalnog bola, dok je kod jednog pacijenta dijagnostikovan tenosinovitis fleksora sa paresteziјama nerva medijanusa; kod dva pacijenta je dijagnostikovan tenosinovitis fleksora. "Zaključavanje" ručja predstavlja uspešnu hirušku metodu za lečenje distalnog preloma radijusa. Graft treba pravilno postaviti u toku operacije, a sa pokretima šake treba početi tokom postoperativnog oporavka što je moguće ranije.

Ključne reči: *"zaključavanje ručja", distalni prelom radijusa, hiruški tretman*

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INTRODUCTION

Fractures in the hand and wrist area, which are frequently subject to trauma, are the primary pathologies treated by orthopaedic clinics. The distal radius is one of the most commonly fractured bones in the body (i.e., 8 – 15% of all broken bones) (1). Distal radius fractures comprise approximately 1/6 of all fractures treated in the emergency room (2). In total, 75-80% of distal radius fractures are extra-articular stable fractures, which are conservatively treated in the emergency department (3).

When examining the distribution of age-related broken bones, the following two age groups stood out: physically active children 5 to 10 years of age and older adults 60-69 years of age (approximately 80% were women with less active lives) (4).

It is important to diagnose and treat these fractures because they are common, and they are closely related to an individual's daily functioning. Together with the type of fracture, the patient's age, general condition, physical condition, mental capacity, additional diseases, adherence to treatment and personal expectations should be considered in the treatment plan.

The treatment goal is to ensure and protect normal anatomy and to provide a suitable, functional wrist at the end of the treatment. The type of fracture, degree of displacement, and the stability of the fracture help to determine whether surgical treatment is required. Successful results are often obtained with closed reduction and plaster casts in low energy, extra-articular and stable fractures.

In terms of treatment, problematic fractures are those with high energy and multiple parts, as well as unstable and intra-articular fractures. Although several surgical methods and detection materials have been identified for unstable fracture treatment, a standard treatment method has not yet been determined.

Surgical treatment alternatives include percutaneous pinning or external fixation after closed reduction; nailing after limited open or open reduction, internal fixation, or some combination of these treatments (and additional grafting); and arthroscopic assisted reduction and stabilization (3).

Regardless of which method is chosen, the basic requirement is that the radial length, radial inclination and palmar inclination are at the most appropriate levels and that the distal radial articular surface is anatomically repaired (5,6,7).

In this study, we evaluated the anatomic, radiographic and clinical treatment results of the patients with intra-articular comminuted distal radius fractures who underwent treatment at our clinic using open reduction and locking plates, and we examined the impact of the treatment results on the patients' daily work and social lives.

MATERIALS AND METHODS

In this study, 25 adult patients (with 25 distal radius fractures) who underwent open reduction and volar locking plates due to distal radius fracture were prospectively evaluated at Hamidiye Şişli Etfal Education and Research Hospital Orthopedics and Traumatology clinic between April 2010 and April 2013. Patients who were followed for at least 6 months were included in this study.

Of the 25 patients, 44% (n = 11) were female and 56% (n= 14) were male. They ranged in age from 24 to 68 years (average 44.32±12.12 years).

A total of 64% (n = 16) of our patients had left-side fractures, and 36% (n =9) had fractures on their right sides; in 40% of the patients (n= 10), the affected extremities were dominant.

Patients who presented at our hospital with wrist injuries were examined. After physical and radiological assessments, they were diagnosed with wrist fractures. The patients were first treated for more serious issues, as needed. Patients with extra-articular distal radius fractures were not included in this study.

For the patients with distal radius fractures related to their joints, control radiographs were taken, following administration of a short-arm splint and closed reduction under sedoanalgesia.

Patients without good reduction (i.e., the dorsal angulation was more than 10°, volar angulation was more than 20°, radial shortening was more than 5 mm and joint surface separation was greater than 2 mm) and unstable comminuted fractures who consented to surgery and a sufficient follow-up time were admitted to the study group.

All patients underwent surgery under general anaesthesia, and a pneumatic tourniquet was applied. Following anaesthesia, 1 g of cefazolin sodium antibiotic I.V. was administered for prophylactic purposes.

Patient complaints were recorded at their final examinations. Forearm rotation and wrist movements were examined during the clinical examination.

Hand grip strength was comparatively measured with a dynamometer (Jamar, Baseline hydraulic hand dynamometer, Irvington, NY, USA) at 90°, with the elbow, forearm and wrist in a neutral position. The measured values were calculated as a percentage, based on the healthy side. Posterior-anterior and lateral radiographs of the wrist were taken for comparison.

Clinical measurements were performed using the Gartland and Werley (8) and QuickDASH (9) clinical assessments. Angular evaluations were performed, according to the Stewart et al. (10) radiological assessment. The clinical evaluations were evaluated in comparison with the VAS (Visual Analog Scale) (11).

RESULTS

Patient demographic information and a classification of the fractures (based on the AO system) are shown in Table 1. Our study included 25 patients (44.0% [n = 11] female and 56% [n= 14] male). The patients ranged in age from 24 to 68 years (average 44.32±12.12 years).

The following mechanisms of trauma were recorded: 36% (n = 9) of the fractures were caused by falls from

Table 1.

Demographic Information		Min-Max	Mean ± SD
Age (years)		24-68	44.32±12.12
		n	**%**
Gender	**Female**	11	44.0
	Male	14	56.0
Trauma mechanism	**Fall from height**	9	36.0
	Falling while walking	8	32.0
	Falling down stairs	6	24.0
	NVTA	1	4.0
CTA		1	4.0
Time until surgery		2-13	5.56±3.11
Length of stay in hospital		3-13	8.08±2.29
Follow-up duration (months)		6.60-41.17	20.36±7.62
Side	**Right**	9	36.0
	Left	16	64.0
Dominant hand	**Right**	22	88.0
	Left	3	12.0
Open/Closed	**Closed**	23	92.0
	Open (Type 1)	2	8.0
AO	**C2**	11	44.0
	C3	14	56.0

height; 32% (n = 8) were caused by falling while walking; 24% (n = 6) were caused by falling from the stairs; 4% (n = 1) were caused by non-vehicle traffic accidents (NVTA); and 4% (n = 1) were caused by car traffic accidents (CTA). Among the patients, the time until surgery ranged from 2 to 13 days, while the average was 5.56±3.11 days. The length of stay in the hospital ranged between 3 and 13 days, with an average of 8.08±2.29 days. The follow-up period ranged from 6.60 to 41.17 months, with an average ranging between months and 20.36±7.62 months. For 88% of the patients (n = 22), the right hand was dominant, while for 12% (n = 3), the left hand was dominant. For 64% of the patients (n = 16), the fractures were on the left side, and for 36% (n = 9), the fractures were on the right side. In 92% of cases (n = 23), the fractures were closed fractures, and in 8% (n = 2) of cases, the fractures were open (type 1) fracture. In 28% of patients (n = 7), additional pathologies were detected due to trauma.

According to the Gartland and Werley assessment scale, 68% of the patients (n = 17) obtained excellent results, 28% (n = 7) were good, and 4% (n = 1) were poor. The mean VAS score was 0.84±1.07, and the quick-average DASH subjective question score was 7.07±12.95. According to Stewart, 92% of the patients had perfect scores, and in 8% of cases, good results were obtained. The mean grip strength of the patients was 77.3% when it was evaluated based on the healthy side.

At the final follow-up examination, the average radial length in the patient group was 11.82±1.52; the radial inclination was 20.68±4.89, and the volar tilt was 9.04±6.91. The wrist range of motion of the patients at the final examination was as follows: the average volar flexion was 68.80±12.44, dorsal flexion was 65.60±14.17, ulnar deviation was 30.80±7.59, radial deviation was 21.00±5.00, pronation was 84.40±6.18, and supination was 81.60±8.26 (Table-2, Patient samples Fig 1, 2).

Table 2. ROM value distribution

ROM	Robust side		Broken side		*p*
	Min / Max	Mean ± SD	Min / Max	Mean ± SD	
Dorsal flexion	50/90	72.60±9.14	20/85	65.60±14.17	***0.002*****
Volar flexion	60/85	76.40±6.21	20/80	68.80±12.44	***0.002*****
Ulnar deviation	30/40	37.40±2.93	5/40	30.80±7.59	***0.001*****
Radial deviation	20/30	24.60±3.51	10/30	21.00±5.00	***0.001*****
Pronation	80/90	86.60±4.26	65/90	84.40±6.18	***0.009*****
Supination	80/90	85.20±4.89	60/90	81.60±8.26	***0.002*****

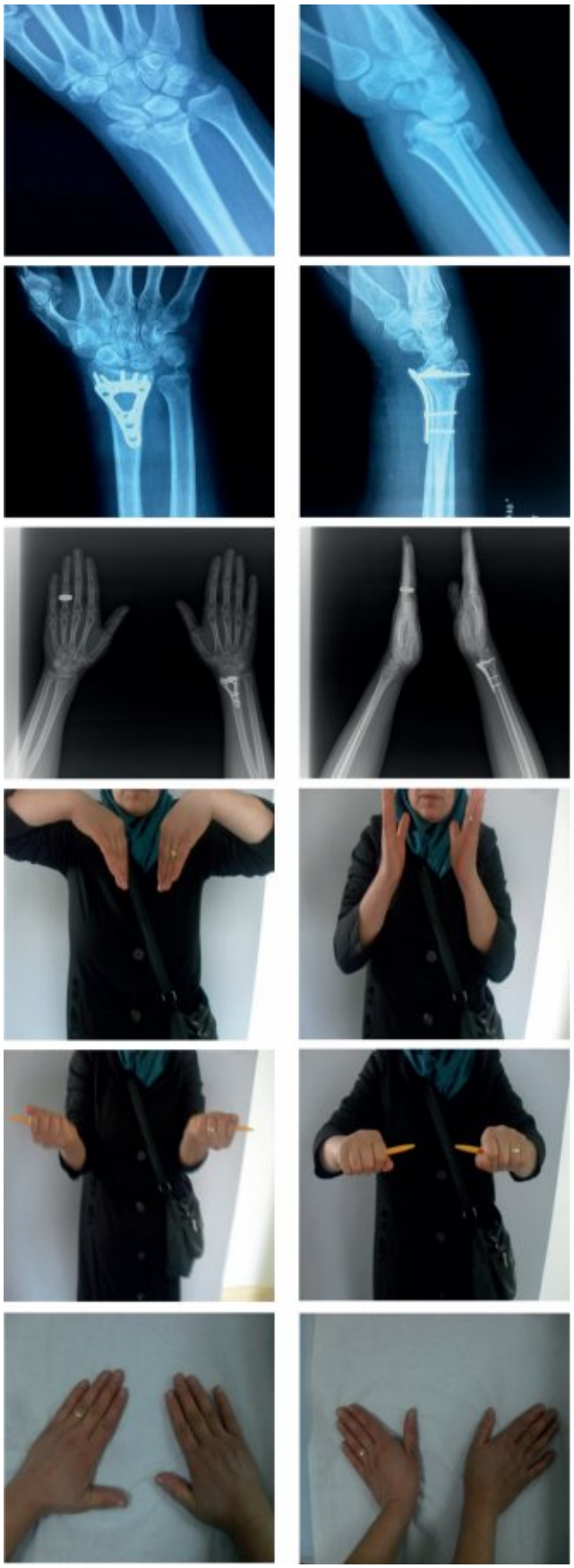

Figure 1: Case No: 14, ZC, age 44, woman, AO Type C3.2, before and after surgery, demonstrating the range of motion, with the final control radiography.

Complex regional pain syndrome was detected in one patient; flexor tenosynovitis and paraesthesia in the distribution of the median nerve were detected in another patient, and flexor tenosynovitis was detected in 2 other patients.

DISCUSSION

Distal radius fractures are most often the result of simple falls that occur in normal life. However, the increased life expectancy, more common use of motor vehicles, industrialization, and increase in the number of people playing sports have resulted in a higher incidence of these fractures as well as more complex fractures. Today, while unstable fractures and joint related fractures are the preferred surgical treatment, simple and stable distal radius fractures are still treated with conservative methods.

Discussions regarding the treatment of distal radius fractures are still ongoing. Although the type of fracture is the most important factor when considering a treatment choice, the patient's age, occupation, functional status, general health status should also be taken into account. Closed reduction and plaster, percutaneous pinning and plaster, percutaneous pinning with limited or open reduction and external fixation, and open reduction internal fixation are the current treatment options for distal radius fracture (12,13).

Each fracture should be evaluated individually, and an appropriate treatment should be selected. The possibility of arthritis increases if the step-off on the joint surface is more than 2 mm, the radial shortening is more than 5 mm, and the dorsal angulation is more than 20 degrees (14).

Intra-articular, dissociated, metaphyseal comminuted fractures caused by high-energy trauma have become more common recently, and some of the challenges experienced in providing sufficient stability by conventional plates has led to the search for new detection methods. For this purpose, appropriate anatomic plates are designed to be suitable for the distal radius volar surface tilt (13). Therefore, the use of volar fixed-angle plate application treatment for fractures with dorsal angulation has become more common. There is no need to align the bone exactly, as in conventional fixation plates, because locking plates are based on the internal fixation principle. Plate and locked screws, which act as a single unit to keep bone fragments together, create the angular stability. Therefore, the volar fixed-angle plates do not need to fit in perfectly with the cortical surface. Thus, the plating technique simplifies the blood flow, which is necessary to ensure fracture recovery (13,15,16).

Demirbaş et al (17) treated 34 displaced fractures of the distal radius with volar fixed-angle locking plates, and they observed excellent results in 10 patients, good results in 19 patients, and bad results in 1 patient, according to the Gartland and Werley scoring system. They found excellent results in 15 patients, good results in 18

patients and moderate results in 1 patient, according to the Stewart radiologic evaluation system. Rozental and Blazar (18) treated 41 distal radius fractures with a volar angle plate, and they reported that the average movement of the wrist flexion 52°, extension was 53°, pronation was 73° and supination was 71°. Excellent results were obtained in 27 patients, and good results were obtained in 14 patients, according to the Gartland and Werley scoring system. In their series of 30 patients who were treated with volar fixed-angle plate, Wong et al. obtained excellent results in 24 patients, good results in 5 patients and moderate results in 1 patient; they obtained 22 excellent results and 8 good results according to the radiological evaluation. The grip strength ratio was 68% compared to the opposite side. Figl et al. (19) found that the average wrist flexion was 52°, and the extension was 54° in the 80 patients they treated. The grip strength ratio was 65% compared to the opposite side. Kostanitinidis et al. (20) treated 40 distal radius cases using the volar approach; when examining the patients' clinical outcomes, they observed excellent results in 26 patients, and good results were reported for in 11 patients. Arora et al. (21) treated 114 patients who were treated with volar fixed-angle locked plates and followed them for an average of 15 months. They observed a mean wrist flexion of 54° and 46° extension. The grip strength ratio was 70% compared to the intact side. According to the Green and O'Brien's assessment system, they observed excellent results in 31 patients, good results in 54 patients, moderate results in 23 patients, poor results in 6 patients. Among their patients who were treated with locked plates, Gallacher et al (22) observed the following functional outcomes: 56,5º mean flexion, 57º extension, 81º as pronation and 83,3º supination. In their study, Knight et al. (23) observed among their patients a post-operative mean wrist movement flexion of 67º, extension 74º and complete forearm rotations; the grip strength was 81%. The wrist motion values, scoring results and grip strength results of our 25 patients who were treated with volar locked plates were consistent with the literature.

In the literature, the complication rate for unlocked volar plates ranges from 14 to 40.5%. The complication rate for locked plates is between 34% and 3% (24).

We detected a 16% (4 patients) complication rate in our patients. In one patient, complex regional pain syndrome was detected; in another patient, flexor tenosynovitis and paraesthesia in the distribution of the median nerve were detected, and in 2 patients, flexor tenosynovitis was detected. In only one case, the plate had to be removed. The complication rates and types of complications were similar to those reported in the literature.

Johnson et al (25) reported a 9.7% (20 patients) complication rate in their 204 series of disease. They observed 7 (3.4%) tendon problems, 4 (1.9%) cases of complex regional pain syndrome, 4 (1.9%) plate irritation problems and 3 reduction problems. In their study of 50

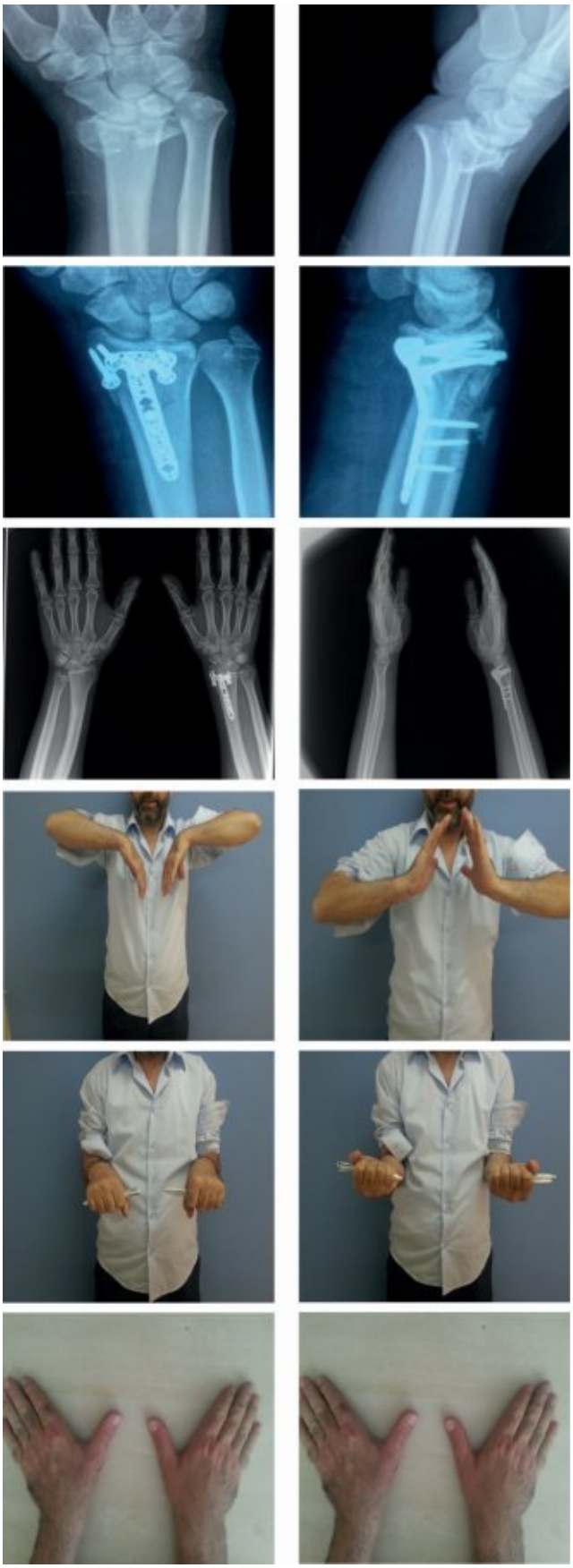

Figure 2: Case No: 6, İ.E.Ş, age 42, male, AO Type C3.2, before and after surgery, demonstrating the range of motion, with the final control radiography.

distal radius fractures, Drobetz and Kutscha-Lissberg (26) detected 6 (12%) patients with flexor pollicis longus tendon ruptures, 3 (6%) patients with complex regional pain syndrome, one (2%) with carpal tunnel syndrome, one (2%) with screw loosening and one (2%) with extensor tendon rupture.

Although open reduction and plating is a classic method, it is still debateable which patients are candidates and which approach and which implant should be applied. According to data from our study, the volar approach allows patients to achieve the appropriate reduction. The new generation of locked plates has allowed physicians to act earlier to reduce the complication rate and to achieve more successful final results in vital patients. Despite the early rehabilitation and preserved reduction, no loss of fixation was observed. Suitable reduction criteria obtained in the majority of patients aligned with the good functional results obtained. The complication rate aligned with that in the literature, and complications specific to the implant are rare.

In the light of these findings, the locked plates application with the volar approach is a safe treatment alternative for distal radius fractures, and in terms of patient functional outcomes, it can provide a reliable cure rate.

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DIABETES MELLITUS DIRECTS NKT CELLS TOWARD TYPE 2 AND REGULATORY PHENOTYPE

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DIABETES MELITUS USMERAVA DIFERENCIJACIJU NKT ĆELIJA U PRAVCU TIP 2 I REGULATORNOG FENOTIPA

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ABSTRACT

Diabetes mellitus is chronic disorder characterized by hyperglycaemia. Hyperglycaemia induces mitochondrial dysfunction, enhances oxidative stress and thus promotes reactive oxygen species (ROS) production. Earlier studies suggested that reactive oxygen species (ROS) are involved in the pathogenesis of many diseases. Previous studies have revealed that hyperglycaemia changes the functional phenotype of monocytes, macrophages, neutrophils, NK cells and CD8⁺ T cells. e aim of this study was to investigate whether diabetes a ects the functional phenotype of NKT cells.

Diabetes mellitus was induced in BALB/c mice by intraperitoneal injection of streptozotocin at a single dose of 170 mg/kg body weight. e number and functional phenotype of splenic NKT cells was assessed by flow cytometry, 28 days after diabetes induction.

e diabetic condition facilitated the production of antioxidant enzymes, including catalase ($p<0.05$) and superoxide dismutase. Hyperglycaemia enhanced oxidative stress and thus decreased the number of splenic NKT cells but did not change the percentage of splenic CD3⁺CD49⁺ NKT cells that express the activatory receptor NKP46 or produce IFN-γ. However, hyperglycaemia increased the frequency of splenic NKT cells that express KLRG-1 and produce TGF-β, IL-4, and IL-5, and it decreased the frequency of IL-17⁺ NKT cells.

Our study indicates that diabetes mellitus induces oxidative stress and switches the functional phenotype of NKT cells towards type 2 (IL-4 and IL-5 producing NKTs) and regulatory (TGF-β producing NKTs) phenotypes. ese findings are correlated with the clinical observation in humans that diabetic patients are more prone to infections and tumours.

Keywords: *diabetes, hyperglycaemia, oxidative stress, NKT cells*

SAŽETAK

Dijabetes melitus je hronično oboljenje koje se karakteriše hiperglikemijom. Hiperglikemija utiče na funkciju mitohondrija, pojačava oksidativni stres i time podstiče produkciju kiseoničnih slobodnih radikala. Ranije studije su pokazale da kiseonični slobodni radikali igraju važnu ulogu u razvoju mnogih bolesti. Hiperglikemija utiče na funcionalni fenotip monocita, makrofaga, NK ćelija i CD8⁺T limfocita. Cilj istraživanja je bio ispitati da li hiperglikemija utiče na funcionalni fenotip NKT ćelija.

Dijabetes melitus je indukovan BALB/C miševima jednom dozom streptozotocina intraperitonealno u dozi od 170 mg/kg. Broj i funkcionalni fenotip NKT ćelija je analiziran protočnom citometrijom 28. dana nakon indukcije dijabetesa.

Dijabetes je povećao produkciju antioksidantnih enzima, katalaze i superoksid dizmutaze. Dijabetes i pojačan oksidativni stres su smanjili ukupan broj NKT ćelija u slezini hiperglikemičnih miševa, dok se procenat NKp46⁺NKT ćelija i NKT ćelija koje produkuju IFN-γ u slezini nije značajno razlikovao u poređenju sa normoglikemičnim miševima. Međutim, hiperglikemični miševi su imali veću procentualnu zastupljenost NKT ćelija koje eksprimiraju KLRG-1 i produkuju TGF-β, IL-4, and IL-5, dok je učestalost IL-17⁺ NKT ćelija bila značajno manja u poređenju sa normoglikemičnim miševima.

Rezultati ukazuju da dijabetes melitus pojačava oksidativni stres i usmerava polarizaciju NKT ćelija ka tipu 2 i regulatornom fenotipu, što je u skladu sa kliničkim studijama koje potvrđuju da su osobe sa dijabetesom sklone razvoju infekcija i tumora.

Ključne reči: *dijabetes, hiperglikemija, oksidativni stres, NKT ćelije*

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INTRODUCTION

Diabetes mellitus, one of the most common chronic diseases, increases the susceptibility to obesity and many other diseases (1). The major characteristic of diabetes mellitus is hyperglycaemia (2). Hyperglycaemia is usually caused by low insulin levels or insulin resistance and is associated with the damage of many tissues and organs, especially nerves, blood vessels, kidneys and eyes (2). One of the major effects of hyperglycaemia is increased production of reactive oxidative species (3, 4). Excess glucose in cells is involved in glucose oxidation and the nonenzymatic glycation of proteins (5). The final products of these pathways are reactive oxidative species (5). Increases of reactive oxidative species harm cellular organelles and increase lipid peroxidation (6).

Many studies have shown that immune system function is impaired in individuals with diabetes mellitus (7). The immune system can be subdivided to innate and acquired immunity (8). The innate immune system is characterized by rapid responses to pathogens and is mediated mainly by macrophages, dendritic cells, granulocytes, natural killer (NK) cells and natural killer T (NKT) cells, while the acquired immune system is composed of T and B lymphocytes (8). Earlier studies have shown that hyperglycaemia changes effector functions of innate immune cells. The decreased expression of MHC class II on circulating monocytes, reduced response of macrophages to multiple TLR ligands, reduced neutrophil degranulation, impaired $\gamma\delta$ T cell proliferation (9), significant decrease in the expression of activating receptors NKG2D, NKp30, and NKp46, and interferon-γ (IFNγ) and perforin production in NK cells (10, 11) are all described as phenomena that accompany diabetes.

Natural killer T (NKT) cells constitute a subset of T cells that serve as a bridge between innate and adaptive immunity (12-14). NKT cells recognize exogenous and endogenous lipid antigens presented in the context of the MHC class I-like molecule CD1d (15). One of the main functions of NKT cells is cytokine production (13). Thus, NKT cells play an important modulatory role in the induction or prevention of many pathogenic conditions (12-14). NKT cells can be subdivided according to transcriptional factor expression and subsequent cytokine production, such as IFNγ, interleukin IL-4, IL-10, IL-13, IL-17, and IL-22; tumour necrosis factor-α (TNFα); and granulocyte-macrophage colony-stimulating factor (GM-CSF), which modulate the innate and adaptive immune response (13, 16).

In the available literature, there is no evidence of the effect of diabetes on NKT cells. The aim of our study is to investigate the effect of the diabetic condition on the functional phenotype of NKT cells in mice.

MATERIAL AND METHODS

Animals

BALB/C mice (female, 6-8 weeks old) were used in all experiments. Animals were maintained under standard laboratory conditions. The protocols for animal experiments were approved by the Animal Ethics Board of the Faculty of Medical Sciences, University of Kragujevac, Serbia.

Induction of diabetes

Mice were divided randomly into two groups: the experimental and control group. Diabetes was induced in the experimental group by the intraperitoneal injection of streptozotocin dissolved in a sodium citrate buffer (pH=4.5) at a single dose of 170 mg/kg body weight, while the control group was given a sodium citrate buffer (pH=4.5). Blood samples were obtained from the lateral tail vein after four hours of starvation. Blood glucose levels were determined twice a week with *Accu-Chek Performa*, *Roche*.

Determination of antioxidant enzymes

Isolated RBCs were washed three times with 3 volumes of ice-cold 0.9 mmol/l NaCl and haemolysates containing approximately 50 g of Hb/l (17), which were used for the determination of catalase (CAT) and superoxide dismutase (SOD) activity by spectrophotometry. According to Beutler, for the determination of CAT activity, lysates were diluted with distilled water (1:7 v/v) and treated with chloroform-ethanol (0.6:1 v/v) to remove haemoglobin. Then, 100 µl of a sample and 1 ml of 10 mM H_2O_2 were added to a 50 µl catalase buffer (18). Detection was performed at 360 nm. According to the methods of Misra and Fridovich, superoxide dismutase (SOD) activity was determined using epinephrine. Approximately 100 µl of lysate and 1 ml of carbonate buffer were mixed, and then 100 µl of epinephrine was added (19). Detection was performed at 470 nm. The activities of SOD and CAT in red blood cells (RBCs) are presented in units per gram of haemoglobin x 10^3 (U/g Hb x 10^3).

Cell preparation

Mice were sacrificed on day 28 after diabetes induction, and their spleens were isolated. Single-cell suspensions were obtained from the spleens by mechanical dispersion through a cell strainer (BD Pharmingen, USA) in a complete growth medium (Dulbecco's-Modified Eagles Medium supplemented with 10% foetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L penicillin–streptomycin, 1 mmol/L mixed nonessential amino acids (Sigma, USA)). Erythrocytes were removed from the splenocyte cell suspension by a lysing solution (BD Pharmingen), and cells were resuspended in complete growth medium. The number of viable cells was determined by trypan blue staining, and only cell suspensions with > 90% viable cells were used.

Flow cytometry

Single-cell suspensions from spleens were incubated with mAbs that were specific for mouse CD3, CD49, NKp46, IFNγ, KLRG1, IL-4, IL-5, IL-17 and TGF-β or isotype-matched controls (BD Pharmingen/BioLegend); they were then analysed using a FACSCalibur flow cytometer

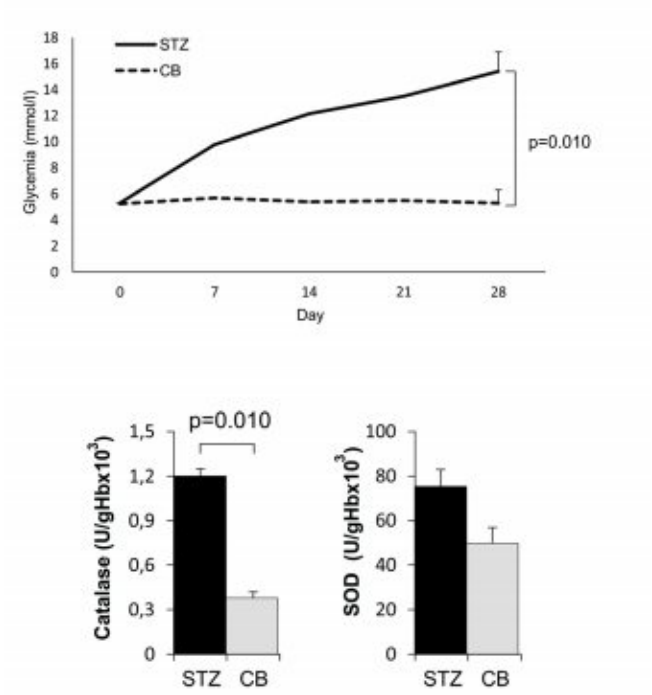

Figure 1. STZ application increases glycaemia and oxidative stress. Experimental diabetes was induced in BALB/c mice by intraperitoneal injection of streptozotocin dissolved in sodium citrate at a single dose of 170 mg/kg body weight. Hyperglycaemia was measured twice a week. Mice were sacrificed on the 28th day after streptozotocin application. e activity of antioxidative enzymes was measured in the isolated RBCs. Data are presented as the mean+SEM from two experiments. Statistical significance was tested by Mann–Whitney rank-sum test or Student's unpaired t-test where appropriate (p<0,05).

(BD). Dead cells were excluded from the analysis by positive propidium-iodide staining. The gate used for FACS analysis was the mononuclear cell region in FSC/SSC plots (20000 events were acquired). Data were analysed using CELLQUEST (BD) and FlowJo (Tristar) software.

Intracellular cytokine staining

For the analysis of IFNγ, IL-4, IL-5, IL-17 and TGF-β expression, splenocytes were stimulated with phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (500 ng/ml, Sigma) with GolgiStop (BD Pharmingen) and incubated for 4 h at 37ºC, 5% CO_2. After fixation and permeabilisation, intracellular staining was performed using anti-IFN-γ, anti-IL-4, anti-IL-5, anti-IL-17 and anti-TGF-β anti mAb (BD Pharmingen) and analysed by flow cytometry (20).

Statistical analysis

The data were analysed using the statistical package SPSS version 20. The normality of the distribution was tested by the Kolmogorov–Smirnov test. The two-tailed Student's t-test or the nonparametric Mann–Whitney U test were used. The results were considered significantly different when p <0.05.

RESULTS

Diabetes increases the production of catalase and superoxide dismutase

Glycaemia was measured twice a week during all experiments. As shown in figure 1, the blood glucose level was significantly increased in mice treated with streptozotocin compared to CB-treated mice at day 28. Twenty-eight days after diabetes induction, we measured antioxidant enzyme activity in erythrocytes. The activity of antioxidant enzymes did not differ among the experimental and control groups on day 0 (data not shown). Hyperglycaemic mice had significantly increased activity of catalase compared to normoglycaemic mice (p=0,03, Figure 1). The activity of superoxide dismutase was also measured; our data showed that hyperglycaemic mice have increased activity of superoxide dismutase, but the difference did not reach statistical significance (Figure 1).

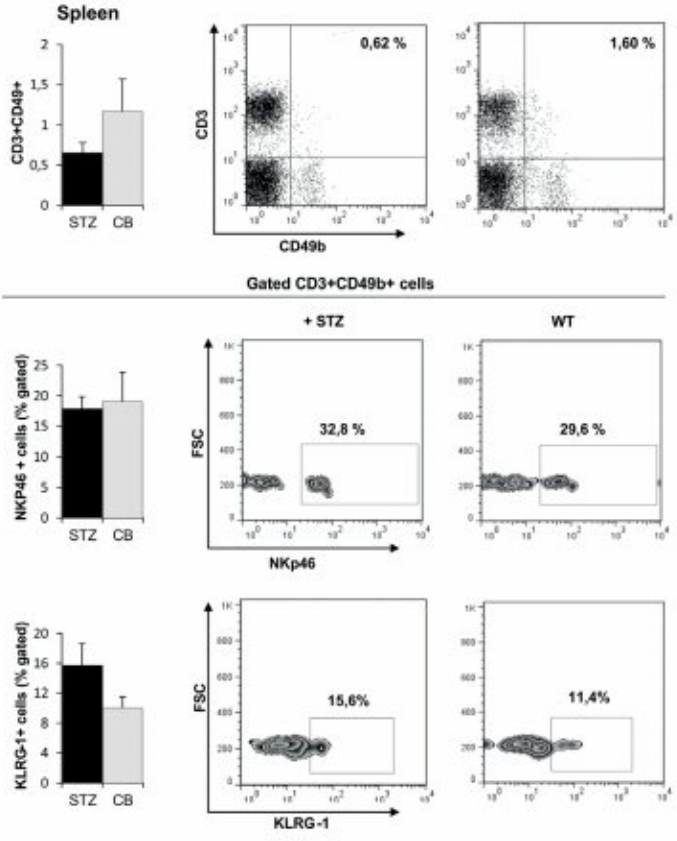

Figure 2. Diabetes decreases the total number of splenic NKT cells and increases the number of KLRG1$^+$NKT cells in the spleen. Mononuclear cells were isolated from spleens of streptozotocin-injected mice, and non-treated mice were determined on day 28 of the experiment. Mononuclear cells were labelled with fluorochrome-conjugated anti-mouse antibodies and analysed by flow cytometry. Data are presented as the mean+SEM of two separate experiments, and each was carried out with seven mice per group. Statistical significance was tested by the Student's unpaired t-test (p<0,05).

Diabetes decreases the total number of spleen NKT cells and increases the number of KLRG-1$^+$ NKT cells

We assessed the frequency and functional phenotype of NKT cells in the spleens of hyperglycaemic and normoglycaemic mice at the 28th day after streptozotocin induction. Our results showed that the frequency of CD3$^+$CD49$^+$ NKT cells was decreased in spleens of hyperglycaemic mice compared to normoglycaemic mice, but the difference did not reach statistical significance (figure 2). Further, we analysed the expression of activatory and inhibitory receptors on NKT cells. Diabetes increased the incidence of CD3$^+$CD49$^+$ NKT cells expressing KLRG1$^+$ (which did not reach statistical significance), but it did not affect the percentage of NKP46$^+$ CD3$^+$CD49$^+$ NKT cells (Figure 2).

Diabetes increases IL-4$^+$, IL-5$^+$ and TGF- β$^+$ NKT cells and decreases IL-17$^+$NKT cells

To further determinate the functional phenotype of NKT cells, we analysed cytokine production. As shown in figure 3, hyperglycaemia increased the frequency of splenic IL-4$^+$ and IL-5$^+$ NKT cells (which did not reach statistical significance, respectively) and NKT cells producing TGF-β$^+$ (p=0.004). Diabetic conditions also decreased the percentage of IL-17$^+$-producing NKT cells (p=0.004), while it did not affect the number of IFN-γ$^+$NKT cells (Figure 3).

DISCUSSION

The aim of this study was to investigate whether diabetic conditions changed the functional phenotype of NKT cells. For this purpose, hyperglycaemia was induced in one group of mice by intraperitoneal injection of streptozotocin, while the other group served as healthy controls. Streptozotocin-treated mice exhibited significantly higher levels of glycaemia in comparison to CB-treated mice on the 28th day of the experiment (Figure 1). We also reported that hyperglycaemic mice had increased systemic levels of catalase (p=0,03) and superoxide dismutase (level of superoxide dismutase was not statistically significant) in

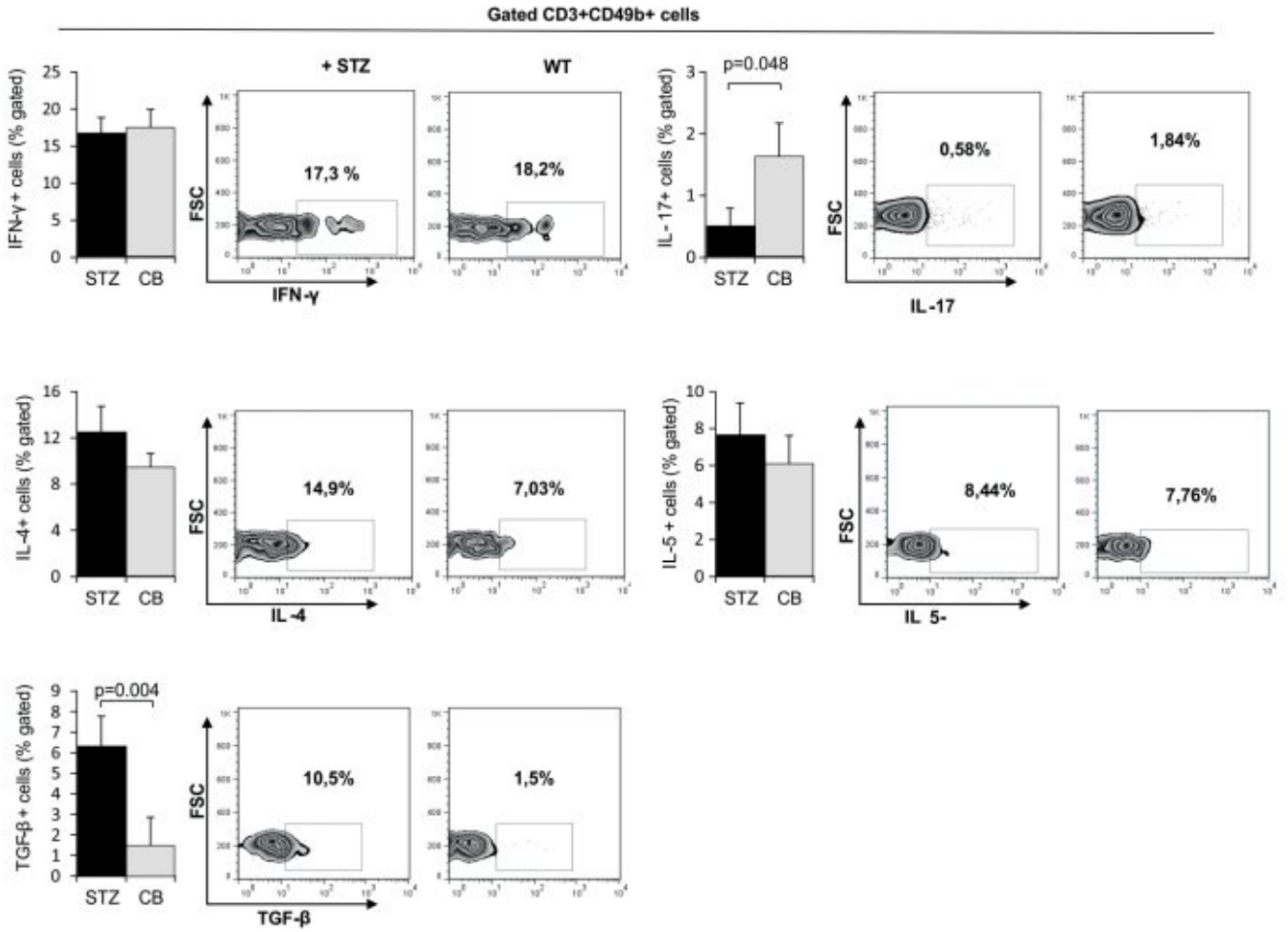

Figure 3. Diabetes increases IL-4$^+$ and IL-5$^+$ and TGF-β$^+$NKT cells and decreases IL-17+NKT cells in the spleen. Mononuclear cells isolated from spleens of streptozotocin-injected mice (7 mice per group) and non-treated mice (7 mice per group) were determined on day 28 of the experiment using fluorochrome-labelled Abs and analysed on a FACS Aria. Mononuclear cells were gated by size and granularity on FSC/SSC. Results are presented as the mean+SEM of two separate experiments. Statistical significance was tested by the Student's unpaired t-test (p<0,05).

comparison to normoglycaemic mice (Figure 1). The frequency of $CD3^+CD49^+$ NKT cells was decreased, while the incidence of NKT subpopulations that express KLRG-1 or produce IL-4, IL-5 and TGF-β were higher in the spleens of hyperglycaemic mice (Figure 2 and 3). Furthermore, the percentage of IL-17-producing NKT cells was lower in mice injected with streptozotocin (Figure 3).

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia (5). One of the major phenomena caused by hyperglycaemia is the production of reactive oxidative species (21). Excess glucose in cells activates the polyol pathway, the hexosamine pathway, protein kinase C (PKC) activation, and the formation of advanced glycation end products; it thus accelerates the production of reactive oxygen species, hydroxyl radicals, superoxide anion, hydrogen peroxide and nitric oxide (21). Oxidative stress occurs when the production of free radicals exceeds the antioxidant defence mechanism (6). If cellular antioxidants do not remove free radicals, abnormally high levels of ROS harm DNA, lipids, and proteins, which leads to the accumulation of damaged molecules (6, 22). Additionally, ROS are known as inducers of cell apoptosis and regulators of gene expression (23). Although hyperglycaemia increases the production of free radicals, it also aggravates the endogenous antioxidant system (5). Antioxidants, such as the enzymes superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase, as well as vitamins A, C, and E, work in synergy against different free radicals (24). The focus of our investigation was on two important antioxidant enzymes: catalase and glutathione reductase. Catalase enzyme, present in the peroxisome, converts hydrogen peroxide to water and oxygen and thus neutralises its toxic effects (25). Superoxide dismutase converts superoxide anion radicals to hydrogen peroxide, which is further detoxified to water (H_2O) by catalase or glutathione peroxidase (26). In this study, we reported the increased activity of antioxidant enzymes, catalase and superoxide dismutase, which was derived from erythrocytes of streptozotoin-treated mice on the 28^{th} day of the experiment in comparison to CB-treated mice (Figure 1). Our results are in line with other studies that confirmed that hyperglycaemia increases the production of reactive oxidative species and thus increases the production of antioxidative enzymes (27).

Natural killer T (NKT) cells lie at the interface between innate and adaptive immunity and are important mediators of immune responses and tumour immunosurveillance (28). Two major subsets of NKT cells can be distinguished based on their TCR repertoire and lipid reactivity (12). Type I or invariant NKT (iNKT) cells express an invariant TCRα paired with a restricted repertoire of Vβ chains (12), while type II NKT cells express a more variable TCR repertoire (29, 30) and can modulate immune responses, suppress autoimmunity and inhibit tumour rejection (16). Additionally, functionally heterogeneous NKT cells (28) can be subdivided according to the expression of transcription factors and subsequent cytokine production (31). T-bet^{high} NKT1 cells are capable of producing large amounts of IFN-γ, while alternatively polarized NKT2 cells express a GATA3 transcription factor and produce IL-4, IL-5 and IL13 (31, 32). Recent studies have revealed a new type NKT17 cell that, like $CD4^+$Th17 cells, constitutively expresses the RORγ-t transcription factor and IL-23R and produces high levels of IL-17 (33). The other studies defined Foxp3-type INKT cells that, similarly to Tregs, suppress the proliferation of $CD4^+$ T cells (28). To evaluate the influence of diabetic conditions, we analysed the expression of NKp46 and KLRG-1 receptors on NKT cells. NKp46, a transmembrane type I glycoprotein, is a major activating receptor that is important in the elimination of virally infected cells and tumour cells (34, 35). NKp46 triggers lysis by recognizing membrane ligands on infected and tumour cells (34, 35). KLRG1 is an inhibitory lectin-like receptor, predominantly expressed on NK cells that produce lower levels of IFN-γ (36). Our results show that hyperglycaemia decreased the percentage of total $CD3^+CD49^+$ NKT. Further, hyperglycaemia did not affect the percentage of splenic $CD3^+CD49^+$ NKT cells that express activatory receptor NKP46, while it increased the frequency of NKT cells that express inhibitory receptor KLRG1 (Figure 2). Shimizu et al. showed that $KLRG1^+$ iNKT cells coexpress CD49d and granzyme A live longer than conventional NKT cells and have the potential to be involved in a second immune response on the same antigen (37). In our study, we focused on all subpopulations of NKT cells, not only iNKT cells. Thus, our data suggest that hyperglycaemic mice have a lower percentage of highly active and functional NKT cells capable of dealing with infections or tumour cells.

In further analyses of the functional phenotype of NKT cells, we investigated the production of cytokines IFN-γ, IL-4, IL-5, IL-17 and TGF-β by NKT cells. There was no statistically significant difference in the percentage of IFN-γ-producing NKT cells between hyperglycaemic and normoglycaemic mice on the 28^{th} day of the experiment (Figure 3). We also measured the production of cytokines that are markers of type 2 immune responses. Hyperglycaemic mice had higher frequencies of splenic $CD3^+CD49^+$NKT cells that produce IL-4 and IL-5 compared to normoglycaemic mice (Figure 3). Finally, we analysed the production of IL-17 and TGF-β by NKT cells. Diabetic conditions significantly decreased the percentage of IL-17-producing NKT cells in the spleen, while it significantly increased the percentage of TGF-β-producing NKT cells in comparison to normoglycaemic animals.

Earlier studies have shown that immune deviation towards a type 1 response and the production of IFN-γ promotes tumour rejection, while a type 2 immune response prevents tumour rejection (38, 39). Additionally, IFN- γ is an important cytokine in combating intracellular pathogens (8). IL-4 can be marked as the most critical cytokine in the induction of type 2 immune responses (38, 39). The development of a type 2 immune response is followed by GATA3 expression, and GATA3 inhibits type 1 immune responses by the down-regulation of the STAT 4 tran-

scriptional factor (40). Thus, our data suggest that diabetic conditions facilitate the development of type 2 NKT cells, which suppress the type 1 immune response and make these mice more *prone* to developing *cancer and more susceptible to infections with intracellular microorganisms.*

IL-17 plays a vital role in protecting the host from infection, primarily extracellular bacterial infections and fungal infections, but it is also important for protection against intracellular bacteria and some viruses (41). IL-17 has potent pro-inflammatory functions, including the induction of IL-6 and TNF-a, that increase the recruitment of neutrophils and regulate the production of anti-microbial peptides, which contribute to the host defence (28, 41, 42). Our data indicate that hyperglycaemic mice with a significantly lower percentage of type 17 NKT can be highly susceptible to infection by extracellular pathogens.

Earlier studies showed that NKT cells produce TGF-β and thus suppress anti-tumour immunity (43). $NK1.1^+$ T cells in TIL show immunosuppressive activity in the anti-tumour immune response through the production of TGF-beta and the preferential cytolysis of B7-expressing cells (44). Earlier studies have shown that TGF-β plays an important role in tumour escape from immune surveillance via the down-regulation of $CD8^+$CTL and the suppression of antitumour cell activity, which results in the uncontrolled growth of tumour cells (45). TGF-β also affects myeloid cells, which modulate host immune surveillance and the tumour microenvironment and thus facilitate tumour growth and metastasis (46). TGF-β also inhibits the proliferation and effector functions of macrophages, neutrophils and T lymphocytes and thus supresses the innate and adaptive immune responses (8).

In line with these studies, our results revealed that diabetic mice had higher percentages of TGF-β-producing NKT cells, which can make them more susceptible to developing cancer and infections in comparison to normoglycaemic mice.

Conclusion

Collectively, diabetes mellitus can modulate NKT cells' functional phenotype in at least two ways: through enhanced expression of the inhibitory receptor KLRG1 and direction toward type 2 and regulatory phenotypes. These findings are in line with data that show that diabetic patients are more prone to infections and tumours.

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Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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QUALITY OF ROOT CANAL FILLINGS IN A BOSNIAN ADULT POPULATION TREATED IN PUBLIC AND PRIVATE DENTAL CLINICS

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KVALITET PUNJENJA KANALA KORENA KOD ODRASLOG STANOVNIŠTVA U BOSNI I HERCEGOVINI LEČENIH U DRŽAVNIM I PRIVATNIM STOMATOLOŠKIM KLINIKAMA

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ABSTRACT

e aim of this study is to examine the technical quality of root canal fillings in an adult population in the Republic of Srpska, Bosnia and Herzegovina, who were treated in public dental institutions and private dental clinics.

Radiographs of faculty patients, aged 25-60 years old, who came for the first time were examined for the technical quality of root canal fillings. Patients with endodontically-treated teeth were invited for an interview to reveal the providers of the endodontic treatment.

e percentages of teeth with adequate length ($p<0.01$) and taper ($p<0.01$) in institutions of public health cases were significantly greater than those in private clinic cases. In addition, the frequency of teeth with adequate root canal fillings in patients treated in public dental institutions was significantly greater than those treated in private dental clinics ($p<0.01$).

A significantly higher percentage of adequate canal fillings is present in teeth where endodontic treatment was performed in public dental institutions compared to private dental clinics. is result indicates the need to improve the quality of endodontic treatment through more intensive training for dentists in private practice.

Keywords: *endodontic treatment, quality of root canal fillings, radiographic evaluation, adults*

SAŽETAK

Cilj ove studije je da se ispita kvalitet punjenja kanala korena endodontski lečenih zuba kod odraslog stanovništva u Republici Srpskoj, Bosna i Hercegovina, koji su lečeni u državnim stomatološkim ustanovama i privatnim stomatološkim klinikama.

Na radiografskim snimcima pacijenata koji su se prvi put javili na fakultet, starosti 25-60 godina, analiziran je kvalitet punjenja kanala korena. Pacijenti sa endodontski lečenim zubima su pozvani na intervju kako bi se utvrdilo mesto sprovođenja endodontskog tretmana.

Procenat zuba sa adekvatnom dužinom ($p<0.01$) i konič-nošću ($p<0.01$) kanalnog punjenja bio je znatno veći kod zuba koji su lečeni u državnim u poređenju sa privatnim stomatološkim klinikama. Pored toga, učestalost zuba sa adekvatnim kanalnim punjenjem kod pacijenata lečenih u državnim ustanovama bila je znatno veća, od onih koji su lečeni u privatnim stomatološkim klinikama ($p<0.01$)

Znatno veći procenat adekvatnog kanalnog punjenja je prisutan kod zuba čiji je endodontski tretman sproveden u državnim stomatološkim ustanovama u poređenju sa privatnim. Ovakav rezultat ukazuje na potrebu za poboljšanjem kvaliteta endodontskog lečenja kroz intenzivnije obuke stomatologa u privatnoj praksi.

Ključne reči: *endodontsko lečenje, kvalitet kanalnog punjenja, radiografska procena, odrasli*

INTRODUCTION

The goal of endodontic treatment is to eliminate, or at least significantly reduce, bacterial population from the root canal space to levels that are compatible with periradicular tissue healing (1).

Numerous studies have shown that the success of endodontic treatment of teeth without periapical changes is very high, up to 95% (2-4). Results of these studies are most often related to the treatment outcome performed in university-based or teaching hospital-based settings. A markedly lower success rate (65 to 75%) was recorded in general dental practices (5, 6). This discrepancy in success rate may reflect the possible difference in the quality of the endodontic treatment.

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It is expected that the root canal filling provide a hermetic seal of the canal system and prevent reinfection. Epidemiological studies revealed that technically satisfactory endodontic treatments were performed in 14 to 65% of cases (7-13). Data on the technical quality of root canal treatments are needed to evaluate the endodontic status of the population, since it is known that the prognosis of endodontic treatment depends on the quality of root canal filling (7, 9, 11-13).

The large majority of cross-sectional studies evaluating the quality of root canal fillings have been performed in Europe and the USA (7-12, 14-17). However, for Bosnia and Herzegovina, only limited information is available (18).

The aim of this study is to examine the technical quality of root canal fillings in an adult population in the Republic of Srpska, Bosnia and Herzegovina, who were treated in public dental institutions and private dental clinics.

MATERIALS AND METHODS

The sample for this cross-sectional study included 275 adult patients who consecutively presented for the first time seeking routine dental care (not emergency care) at the Faculty of Medicine, University of East Sarajevo, Republic of Srpska, Bosnia and Herzegovina, between 2013 and 2014. In order to be enrolled in the study, the patients had to be older than 18 years, have more than nine natural teeth and possess a panoramic radiograph or full-mouth series of periapical radiographs. When only a panoramic radiograph was present, supplemental periapical radiographs were taken of all endodontically-treated teeth. All patients were contacted by telephone and were invited to an interview. Since most of the patients had been enrolled as active patients at the faculty clinic, they were interviewed during a dental visit. Other patients were contacted by telephone and invited to an interview. General information and data about the place where the endodontic treatment of teeth was completed were collected during the interview: public dental institutions (clinics, hospitals and faculties) or private dental clinics. All patients were informed about the survey, and they gave written informed consent.

Two endodontic specialist examiners evaluated all the radiographs. Calibration of the examiners was carried out on 30 periapical radiographs (not related to the study samples) representing teeth with root canal fillings. Inter- and intra-examiner agreement for length, density, taper and adequacy of the root canal filling were measured by Cohen's kappa coefficient. The value of the coefficient for all examined canal filling parameters was greater than 0.77 and 0.81 for inter- and intra-examiner agreement, respectively. Then, the examiners independently analysed periapical radiographs of endodontically-treated teeth, utilizing a magnifying lens and an X-ray viewer.

The quality of root canal fillings was assessed according to the length, density and taper (19).

Length of the canal filling was assessed as follows:

- "Adequate" – root filling ending ≤2 mm short of the radiographic apex;
- "Underfilled" – root filling ending >2 mm short of the radiographic apex;
- "Overfilled" – root filling ending beyond the radiographic apex.

Density of the root canal filling was estimated as:

- "Acceptable" – uniform density of the root canal filling without voids and the canal space is not visible;
- "Poor" – non-uniform density of the root canal filling with a clear presence of voids and the canal space is visible.

Taper of the canal filling was assessed as follows:

- "Acceptable"- consistent taper from the coronal to the apical part of the filling, with good reflection of canal shape,
- "Poor"- inconsistent taper from the coronal to the apical part of the filling.

The quality of endodontic treatment was estimated as:

- "Adequate" – adequate length, density and taper of the canal filling;• "Inadequate" – underfilled and/or overfilled and/or poor density and/or poor taper.

In multi-rooted teeth, the root with the worst treatment quality was used.

SPSS 19.0 for Windows (IBM Corp., Armonk, NY, USA) was used for data processing and statistical analysis. The $\chi 2$ test was applied to look for differences in the treatment quality between the two groups. A probability level of 0.05 was used as the criterion for statistical significance.

RESULTS

A total of 275 patients (103 women) were examined, with a mean age 35.68 ± 5.13 years. Out of the 502 endodontically-treated teeth, 218 were from private dental clinics and 284 were from public dental institutions.

Adequate canal filling lengths were found in 286 out of a total of 502 teeth (57%). A significantly higher percentage of teeth with adequate filling length (65.5%) was registered in a group where the treatment was performed in public compared to private dental clinics (45.9%) ($p<0.01$) (Table 1).

An acceptable density of root canal fillings was found in 397 teeth; 232 of them were treated in public institutions and 165 were treated in private dental clinics (Table 2).

An acceptable taper of root canal fillings was present in 71.1% of treated teeth, and a significantly greater percentage of teeth with acceptable taper was registered in cases coming from public institutions (77.8%) in comparison to private clinical cases (62.4%) ($p<0.01$) (Table 3).

Adequate root fillings, defined as having adequate filling length, acceptable density and acceptable taper, were

Table 1. Length of the root canal filling in relation to the facility where endodontic treatment was performed

Endodontic treatment	Number of teeth	Length of root canal filling		
		Adequate	Underfilled	Overfilled
Public dental institutions	284	186 (65.5%)	76 (26.8%)	22 (7.7%)
Private dental clinics	218	100 (45.9%)	100 (45.9%)	18 (8.3%)
Total	502	286 (57.0%)	176 (35.1%)	40 (8.0%)

p<0.01

Table 2. Density of root canal filling in relation to the facility where endodontic treatment was performed

Endodontic treatment	Number of teeth	Density of root canal filling	
		Acceptable	Poor
Public dental institutions	284	232 (81.7%)	52 (18.3%)
Private dental clinics	218	165 (75.7%)	53 (24.3%)
Total	502	397 (79.1%)	105 (20.9%)

seen in more than half of the teeth (51.2%). The percentage of root canals with adequate filling in public cases (59.9%) was significantly greater than that in private clinic cases (39.9%) (p<0.01) (Figure 1).

DISCUSSION

This study investigated the technical quality of root canal fillings in an adult population who were treated in public institutions and private dental clinics in the Republic of Srpska, Bosnia and Herzegovina. The results of this study indicate that there were more root canal fillings with adequate length, density and taper in patients coming from public institutions.

Periapical radiographs were used in this study, as in many other similar studies (12, 18, 15). The criteria for scoring endodontic quality vary among studies. Some of them adopted length as the only criterion for evaluation of endodontic treatment technical quality (9, 20, 21), some used both the length and the density (22, 23), or, in addition to these two factors, taper can be used as well (19, 24, 25). This study used all three parameters for radiological assessment (19).

In this study, an adequate filling length was found in 57.0% of 502 treated teeth, which is similar to findings from Chueh et al. (61.7%) (15). The percentage of canal fillings with an adequate length is higher in comparison to the results of Lupi-Pegurier et al. (38.7%) (26), as well as the study of Boltacz-Rzepkowski and Pawlicka (48.9%) (27), but was slightly lower compared to the results of Adebayo et al. (71%) (14). Our results show that a significantly higher percentage of adequate length was found in teeth treated in public dental institutions (65.5%) in comparison to private clinics (45.9%). A similar difference was observed in the study from Chueh et al. (15) where the percentage of adequate length was found to be longer in public clinics (66.9%) compared to private clinics (57.9%).

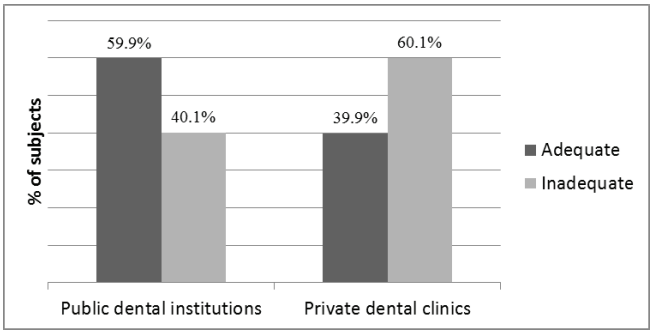
Density of the root canal filling is an important factor for assessing the outcome of endodontic therapy (23, 28, 29). In our study, 79.1% of canal fillings were assessed as having an acceptable density, which is in accordance with the results of Elemam et al. (75.8%) (30), and was much higher in comparison to Adebayo et al. (58.1%) (14) and Chueh et al. (38%) (15). In this study, an acceptable density of the filling was present in 81.7% of the public cases and 75.5% of cases from private clinics. Similarly, in an adult population in Taiwan, a smaller percentage of acceptable fillings was recorded in private clinics (31.6%) compared to public institutions (46.8%). However, this percentage is lower compared with our research (15). The differences can be attributed to the fact that, according to the Bureau of National Health Insurance regulations, greater importance is attributed to filling length than obturation density regarding root canal treatment payments (15).

Table 3. Taper of root canal filling in relation to the facility where endodontic treatment was performed

Endodontic treatment	Number of teeth	Taper of root canal filling	
		Acceptable	Poor
Public dental institutions	284	221 (77.8%)	63 (22.2%)
Private dental clinics	218	136 (62.4%)	82 (37.6%)
Total	502	357 (71.1%)	145 (28.9%)

p<0.01

Figure 1. Quality of root canal filling in relation to the facility where endodontic treatment was performed



p<0.01

Acceptable tapering is seen in 71.1% of cases of this study; this corroborates with the findings of Román-Richon et al. (71%) (31) and Fonseca et al. (82%) (32). A high percentage of acceptable tapers is presented in both samples of our research (77.8% in public and 62.4% in private dental clinics).

More than half (51.2%) of endodontically-treated teeth met the criteria for an adequate canal filling. Kulic et al. (18) found adequate canal fillings in 51.6% of teeth treated by dental students in the Republic of Srpska. In the general population in Serbia, adequate root canal fillings were found in 44.1% of teeth (13). In other studies, adequate root canal fillings were recorded in 14-65% of cases (7-13). The discrepancy in results can be attributed to the use of different parameters for evaluation of root canal filling quality. The length of the root filling (9, 20, 21) or length and density (22, 23) were used as parameters for the assessment of root canal filling quality in most of the studies. However, some also included the taper of the root canal filling (19, 24, 25). Moreover, different criteria have been used for assessing the length, density and taper (15, 30). Regardless of the parameters used, the quality of canal filling that was analysed in our research can be considered to be poor.

In our study, the quality of canal fillings performed in public institutions (59.9%) was significantly better than those performed in private clinics (39.9%). In the study by Chueh et al. (15), more adequate root canal fillings were also recorded in public clinics (38.1%) in comparison to private ones (24.3%). Epidemiological studies have shown that there was a significant difference in the outcome of endodontic treatment carried out by endodontic specialists and general dentists (5, 6, 33). In our study, public institutions included clinics, faculties and hospitals where endodontic therapy is usually carried out by endodontic specialists or dentists under the mentorship of endodontic specialists. However, according to the study conducted by Bjorndal et al. (34), general dentists are more focused on the appearance of clinical symptoms and factors determining appearance during root canal filling, than on the microbiological status of the teeth and the technical quality of canal filling. Moreover, studies also emphasize the importance of continuous training of general dentists in order to improve the quality of endodontic treatment (35). This can partly explain a difference of almost 20% in the quality of root canal filling between those from public dental institutions and from private dental clinics, as indicated in our research. The obtained results also indicate the need for improving the knowledge and skills related to endodontic procedures in order to increase the quality of endodontic treatment. This is primarily related to the knowledge and skills of clinicians practicing in private dental clinics.

CONCLUSION

In this study, the quality of endodontic treatment was evaluated as adequate in more than half of the endodontically-treated teeth. A significantly higher percentage of adequate canal filling was present in endodontically-treated teeth in public dental institutions compared to private dental clinics. This result suggests the necessity of an improvement in the quality of endodontic treatment in private practice.

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A COMPARATIVE ANALYSIS OF LAPAROSCOPIC APPENDECTOMY IN RELATION TO THE OPEN APPENDECTOMY IN CHILDREN

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UPOREDNA ANALIZA LAPAROSKOPSKE APENDEKTOMIJE U ODNOSU NA OTVORENU APENDEKTOMIJU KOD DECE

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ABSTRACT

Acute inflammation of the appendix in childhood usually requires an appendectomy. Surgical methods are open appendectomy (OA) and laparoscopic appendectomy (LA). Both have the same goal of removing the appendix.

Data collected from the medical records of children who underwent hospitalization and operation for acute appendicitis have been retrospectively analysed and statistically processed. e patients underwent surgery in 2010 at University Children's Hospital in Belgrade, and the methods that were used were open appendectomy (OA) and laparoscopic appendectomy (LA). e analysed data refer to gender, age, length of hospital stay, surgery duration, use of pain management therapy, and antibiotic therapy, complications during surgery, complications after surgery, re-hospitalizations, and reoperations.

A total of 218 children underwent an appendectomy operation, of which 158 (72.5%) underwent OA and 60 (27.5%) underwent LA. e average age of patients who had OA was 11.44 years, and 10.87 years for those who underwent LA. e surgery duration was significantly longer for LA (45.3 vs. 42.1 minutes, p = 0.003). e total number of postoperative complications was lower in LA (1 vs. 12), but there was no statistically significant di erence. e number of hospital stay days was significantly lower in LA (3.48 vs. 5.45 days), with a high statistical di erence, p = 0.00.

e advantages of LA compared with OA are shorter hospital stay, lower total number of intraoperative and postoperative complications, and fewer reoperations. e advantage of OA compared with LA is shorter surgery duration.

Keywords: *appendicitis, appendectomy, laparoscopy, length of hospital stay, complications.*

SAŽETAK

Akutna upala crvuljka u dečjem uzrastu najčešće zahteva izvođenje hirurške intervencije-apendektomije. Hirurške metode koje se koriste su otvorena apendektomija (OA) i laparoskopska apendektomija (LA), i obe imaju isti cilj, odstranjenje apendiksa.

Retrospektivno su analizirani i statistički obrađeni podaci prikupljeni iz istorija bolesti hospitalizovane i operisane dece zbog akutne upale crvuljka. Bolesnici su operisani 2010. godine u Univerzitetskoj dečjoj klinici u Beogradu, metodama otvorene apendektomije (OA) i laparoskopske apendektomije (LA). Analizirani podaci se odnose na pol, godine starosti, dužinu bolničkog lečenja, trajanje hirurške intervencije, primenu terapije bola i antibiotika, komplikacije tokom operacije, komplikacije posle operacije, ponovne hospitalizacije i ponovne operacije.

Ukupno je operisano 218 dece od kojih je 158 (72,5%) operisano OA a 60 (27,5%) LA. Prosečna starost bolesnika operisanih OA je bila 11.44 godine, odnosno 10.87 godina onih kod kojih je primenjena LA. Vreme trajanje hirurške intervencije je značajno duže kod LA (45.3 vs. 42.1 minuta, p=0.003). Ukupan broj postoperativnih komplikacija je manji kod LA (1 vs. 12), ali ne postoji statistička značajna razlika. Broj dana bolničkog lečenja je znatno kraći kod LA (3.48 vs. 5.45 dana) i postoji visoka statistička razlika p=0.00.

Prednost LA u odnosu na OA je kraća dužina bolničkog lečenja, manji ukupan broj intraoperativnih i postoperativnih komplikacija i manje reoperacija. Prednost OA u odnosu na LA je kraće vreme trajanje operacije.

Ključne reči: *apendicitis, apendektomija, laparoskopija, dužina bolničkog lečenja, komplikacije*

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INTRODUCTION

Acute inflammation of the appendix (*appendicitis*) is considered to be the most common disease in surgery that requires performing an emergency operation. The incidence of acute appendicitis is 1.1/1000 inhabitants per year in developed countries, and the risk that the population has the disease is 7.5% during a lifetime. It is equally common in both sexes, with the highest incidence between 10 and 19 years of age (1). It is diagnosed in emergency children's clinics in approximately 8% of children who present with acute abdominal pain (2, 3). In 1894, McBurney was the first to apply the open appendectomy (OA) surgical method, which is currently successfully used (4). As a basic surgical technique, it has become an integral part of the catalogue of mastering surgical skills (5). In 1973, Gans successfully introduced laparoscopy in the paediatric population (6), which was an introduction to further development of this method. The first laparoscopic appendectomy (LA) was described by Kurt Semm, a German gynaecologist in 1983 (7). In contrast to the open method, LA became a new alternative in the late 1980s.

The aim of the present study was a comparison of LA with OA by length of hospital stay, surgery duration, intraoperative and postoperative complications, and re-hospitalizations.

MATERIALS AND METHODS

A retrospective comparative study was made. Data were gathered from 218 medical records of children under the age of 18 who were hospitalized for acute appendicitis and were surgically treated with OA and LA methods in 2010 in the abdominal and laparoscopic surgery ward of the Center for Pediatric Surgery at the University Children's Hospital in Belgrade.

The following variables have been analysed: surgery type, gender, age of the child, hospital stay length, surgery duration, complications during the operation, postoperative complications, preoperative leukocyte count, body temperature, pain therapy, antibiotic use, re-hospitalization and reoperations.

Children who underwent an appendectomy in some other surgical interventions and patients who underwent an operation in other health institutions in Serbia and then were moved to the University Children's Hospital in Belgrade because of postoperative complications have been excluded from the analysis.

Surgical techniques

An open appendectomy involves opening the abdominal cavity by cutting the lower right quadrant of the abdomen and removing the appendix. The additional use of retractors is necessary to make the operative field wider in order for the appendix to be accessed.

Laparoscopy involves visual inspection of the abdominal cavity by using optical instruments and is facilitated by insufflation of CO_2 medical gas. The instruments are brought into the abdominal cavity through three small holes on the stomach. The LA method allows full visualization of the operative field by looking at the screen.

Progress in the development of instruments and components, especially lights, optics, fibre optic transmission, gas insufflation, video equipment, in addition to the development of anaesthesia monitoring, have contributed to safe and more frequent applications of LA. Access to the abdominal cavity with minimal trauma makes LA a minimally invasive surgical technique (8). The clinical advantage of LA compared with OA has been shown in many studies (9, 10). From the beginning of its implementation, the method of laparoscopic appendectomy, as a minimally invasive technique, has been a topic among the professional public and has been a constant object of scrutiny, regarding surgery duration, complications, and length of hospital stay compared with the traditional open method of surgery. A large number of assays, studies of individual health care institutions, meta-analyses, randomized studies, and even national studies have given different opinions regarding the comparison of advantages and disadvantages of these two methods (9-17). Both methods are still used in children and adults. The surgery method is determined by the surgeon's personal choice, equipment availability and clinical experience. The proponents of LA justify this method by stating that it reduces postoperative pain, shortens hospital treatment, and provides a faster return to daily activities. However, surgeons who prefer OA explain that the classical method is more justified because the duration of the operation is significantly shorter, the percentage of postoperative complications is lower, and the operation costs are lower.

Statistical analysis

The data obtained in the research have been analysed by descriptive and analytical statistics. Continuous data were expressed as the mean ± SD, and categorical data were expressed as percentages. Normality of the data distributions were tested with Kolmogorov Smirnov[a] tests and graphical depictions (histogram, QQ plot, and a detrended QQ plot). Continuous variables were compared using Student's t-test (normal distribution) or the Mann–Whitney U-test (non-normal distribution). A chi square test was appropriate for categorical variables. P-values ≤ 0.05 were considered to be statistically significant. The results are presented in tables and graphs. All statistical analyses were performed using SPSS® statistical software, version 15.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The research includes a total of 218 children who underwent acute appendicitis surgery at the University Chil-

dren's Hospital. The OA method was used in 158 (72.5%) cases, and LA was used in 60 (27.5%). The number of male children who underwent the OA method was 86 (54.4%), and the number of female children was 72 (45.6%); the number of male children who underwent the LA method was 23 (38.3%) compared with 37 (61.7%) females. The ratio of distribution according to gender and the applied surgery methods of OA and LA shows that there is no significant difference between gender (x^2 = 3.886, df = 1, p = 0.49) and that LA was more frequently performed in females. The age range of children in the OA group was from 4-17 years old, with an average age of 11.2 ± 3.5, while in the LA group, the age ranged from 5-18 years, with an average age of 12.1 ± 3.5 years; there was no statistically significant difference (t = 0.488, df = 2, p = 0.61) (Table 1).

According to the surgeons' findings, the following types of inflammation were present during the operation: catarrhal, phlegmonous, gangrenous, perforated, and peritonitis. A total of 158 children underwent the OA method, of whom 45 children (28.5%) had the catarrhal type of inflammation, 49 (31%) had phlegmonous, 54 (34%) gangrenous, 7 (4.4%) perforated, and 3 (4.4%) peritonitis. From a total of 60 children who underwent the LA method, 38 (68.3%) had the catarrhal type of inflammation, 16 (26.7%) had phlegmonous, 3 (5%) had gangrenous; there was no perforate peritonitis or conversion (Table 2).

The average OA surgery duration was 42.12 ± 8.47 minutes, while in the group of children who underwent the LA method, the average surgery duration was 45.25 ± 6.20 minutes. The OA surgery duration is shorter than LA, and there is a statistically significant difference (t-test = -2.990, df = 1, p = 0.03) (Table 3).

The average duration of hospitalization was 5.45 and 3.48 days for OA and LA, respectively; there is a statistically significant difference, p = 0.00 (Table 3).

Bleeding and injuries of hollow organs were the intraoperative complications that were analysed. Bleeding during surgery occurred only in children who underwent OA, and it was observed in 3 of 158 children, i.e., 1.9%. From a total of 60 children who underwent the LA method, bleeding during surgery did not occur. There were no hollow organ injuries in either applied method. There is no statistically significant difference in the occurrence of intraoperative complications in relation to the type of operation (x^2 = 0.180, df = 1, p = 0.672) (Table 3).

The postoperative complications during hospitalization were: increased body temperature (BT) that lasted over 48 hours, abscess, ileus, bleeding, wound infection, peritonitis, and reoperation. In OA, 2 children, or 1.26%, had increased body temperature as a reactive response to tissue trauma, while one child had increased BT in LA, i.e., 1.6% (1/60). A total of 10 children who underwent OA surgery had increased body temperature associated with other postoperative complications (abscess, wound infection, and reoperation): 3 abscesses, i.e., 1.89% (3/158), 6 wound infections, i.e., 3.7% (6/158), and one reoperation, i.e., 0.63% (1/158). In LA, there were no postoperative complications (0/60). There was no statistically significant difference in relation to postoperative complications and surgery method (x^2 = 7.150, df = 4, p = 0.128). The frequency of re-hospitalization in the first 30 days after surgery did not differ significantly in children who underwent the OA method in comparison to LA (x^2 = 0.000, df = 1,

Table 1. Comparison of gender and age with the method of operation

Characteristics	OA *n*=158	LA *n*=60	*p*
Gender			
Male	86(54.4%)	23 (38.3%)	0.049
Female	72(45.6%)	37 (61.7%)	
*Age, years (min/max)	11.2±3.5 (4-17)	12.1±3.5 (5-18)	0.061

* mean ± (SD).

Table 2. Intraoperative findings

	OA *n*=158	LA *n*=60	*p*
Catarrhal	4(28.5%)	38 (63.3%)	p<0.05
Phlegmonous	49 (31%)	16 (26.7%)	p>0.05
Gangrenous	54 (34%)	3 (5.0%)	p< 0.05
Perforated	7 (4.4%)	0 (0%)	p>0.05
Peritonitis	3 (1.9%)	0 (0%)	p>0.05

Table 3. Surgery duration, length of hospital stay, intraoperative and postoperative complications and re-hospitalization

	OA *n*=158	LA *n*=60	*p*
*Surgery duration (min)	42.1±8.5	45.3±6.2	0.03
*Length of hospital stay (days)	5.45±2.94	3.48±1.37	0.00
[1]Intraoperative complications	3 (1.9%)	0 (0%)	0.67
[1]Postoperative complications	12 (6.9%)	1 (1.7%)	0.12
[2]Re-hospitalization	4 (2.5%)	1 (1.7%)	1.00

* mean ± (SD); [1] total; [2] up to 30 days due to complications

Table 4. Leukocyte count, antibiotic treatment and pain reduction therapy

	OA *n*=158	LA *n*=60	*p*
*Le (x 109/L)	13.4±8.56	11.6±4.7	0.02
min/max	(4-27.9)	(4.9-28.9)	
Antibiotics	158 (100%)	60 (100%)	
Pain reduction therapy	100%	100%	

* Le-leukocyte, mean ± (SD);

p = 1.00). There were four children (2.5%) who were re-hospitalized in the OA group and one child (1.7%) who was re-hospitalized in the LA group (Table 3).

The average leukocyte (Le) count in the children who underwent the OA method was 13.47 ± 8.56 (x 10^9/L) compared with the LA group, 11.6 ± 4.7 (x 10^9/L), indicating that there is a statistically significant difference (Mann-Whitney test, df = 1, p = 0.022). Antibiotics were included in all operated patients, regardless of the type of surgical intervention. Additionally, all children were treated for postoperative pain reduction (Table 4).

DISCUSSION

The results of our study related to the distribution by gender and age indicated no statistically significant difference, but the more common method used for appendicitis operations in girls was LA. There was also no statistical significance between surgery type and age, p<0.0061. A retrospective multicentre cohort study in southern California (18), which included 12 regional hospitals and 7,000 children who underwent LA and OA (where children were analysed by gender, age, and complications) showed no statistically significant differences by gender, but there was a significant difference regarding their age, p<0.001. The same study showed that in the ten-year period from 1997-2007, the number of LA operations increased on average in comparison to OA (22% vs. 70%). The percentage of postoperative complications (wound infections and intra-abdominal abscesses) was reduced, and duration of hospital stay was also reduced. The conclusion of this study indicates that the LA method for acute appendicitis treatment in children is always associated with a lower risk of postoperative complications and shorter hospital stay (18). The results of our study are consistent with this study. Postoperative complications such as soft tissue infections and ileus and intra-abdominal abscesses were less common in children who underwent LA in comparison to OA; it is worth noting that the use of LA in children reduces complications. Our study shows no statistically significant differences between the two applied methods in relation to the incidence of postoperative complications regardless of whether the complications were observed individually or collectively. An explanation for this finding can be the fact that a smaller number of children underwent the operation. No similar published data can be found in Serbian literature. The results of a meta-analysis (19), which included 23 comparative studies (retrospective, prospective, and randomized) and a total of 6,477 children who underwent surgery in the period from 1992-2004, showed that LA surgery is shorter, but there were no significant differences. A meta-analysis (20) of all 44 randomized control clinical studies published in English in the period from 1990-2009, which included both children and adults who underwent an operation with one of the mentioned methods (OA and LA), concluded that LA has the following advantages: shorter hospital stay, lower incidence of postoperative pain, better postoperative recovery, and lower complication rate. In this analysis, the average duration of surgery was 12.35 minutes longer for LA compared with OA, and the length of hospital stay was 0.6 days shorter. In summary, increasing use of LA is related to a better therapeutic effect, which recommends LA as a routine method of choice where there is professional staff and technical equipment availability. In our study, the LA operation duration was 3.5 minutes longer and hospitalization was two days shorter compared with OA (Table 3). The only study found in literature (21) that analyses the factors that influence the choice of operative technique draws a conclusion that the appendicitis severity, gender, operation duration, and surgeon experience are significant factors that affect the method choice and that complication rates and hospital stay length are significantly lower in LA. Both techniques have the same value, suggesting that a better selection of patients will provide better treatment. A conclusion of a large study (22) carried out by the University Department of Surgery in Munich that included 1,400 LA during the period from 1991 to 2005 suggests that LA can be introduced to university centres, especially for surgeons who perform fewer operations and where there is a liberal learning policy. Considering the fact that the percentage of complications and reoperations is low (2-4%), the LA method is an ideal laparoscopic training procedure for young surgeons (22). An increased leukocyte count is an integral part of clinical diagnosis confirmation in terms of inflammatory markers (leukocytes, C-reactive protein) as predictors of surgical procedure; they are the most obvious in phlegmonous and perforated appendicitis (23). In our study, the average number of Le in patients who underwent OA was 13.47 ± 8.56 (x 10^9/L) compared with patients who underwent LA, 11.6 ± 4.7 (x 10^9/L), which indicates that there is a statistically significant difference (p = 0.022). One American study showed that for LA, the length of hospital stay was only 0.9 ± 0.5 days (24); in contrast, our study had an average length of treatment of 3.48 ± 1.37 days in children who underwent LA. Summarizing the results, we concluded that our study concurs with the results of some other studies.

STUDY LIMITATIONS

This study included patients who underwent an operation in one paediatric hospital in Serbia. The clinical knowledge of surgeons (training for LA application) had a significant impact on the choice of surgical treatment method and its results. More frequent use of OA is associated with a lack of conditions regarding the equipment needed for LA, which does not allow for equal application of these methods based on the surgeon's choice.

CONCLUSION

Laparoscopic appendectomy is nearly three times less frequent than open appendectomy in University Children's Hospital in Belgrade. There is no significant difference in the applica-

tion of these two methods in relation to gender and age of the patients. The average length of hospitalization of children who underwent laparoscopic appendectomy was shorter compared with open appendectomy. There are no differences in the incidence of intraoperative and postoperative complications, or in the frequency of re-hospitalization. A disadvantage of LA is a longer surgery duration. Both methods are applied concurrently in surgery, while surgeons' clinical experience is crucial for deciding which method will be applied, with restrictions regarding the availability of equipment and educated staff in the abdominal and laparoscopic surgery ward at the Center for Pediatric Surgery of the University Children's Hospital in Belgrade.

Conflict of interest:

The authors report no conflicts of interest.

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ZINC AND GOLD COMPLEXES IN THE TREATMENT OF BREAST CANCER

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ABSTRACT

Metals are essential components in indispensable biochemical processes for living organisms. is review article highlights the metals zinc and gold in the development and treatment of breast cancer. Metal compounds o er many advantages as therapeutics due to their ability to coordinate ligands in a three-dimensional configuration. In aqueous solution, they form positively charged ions that can bind to negatively charged biological molecules. Metal complexes that contain metal ions such as zinc(II) and gold have received considerable attention as potential anticancer agents. Zinc is an essential trace element that plays a critical role in a wide range of cellular processes that include structural, signalling, catalytic and regulatory functions. Zinc acts as a key structural component in many proteins and enzymes, including transcription factors, cellular signalling proteins, and DNA repair enzymes, and perturbed levels of zinc in tissues may play a role in cancer aetiology and outcome. Unlike zinc, gold is feasible as a component of compounds for e ective anticancer therapy. Some progress in anticancer therapy may include interactions between zinc and gold.

Keywords: *Zinc, gold complexes, breast cancer, anticancer therapy*

SAŽETAK

Metali su ključne komponente u svim važnim biohemijskim procesima u živim organizmima. Ovaj revijski članak opisuje najvažnije karakteristike određenih metala, cinka i zlata i njihovu ulogu u razvoju i lečenju karcinoma dojke. Kompleksi metala imaju značajne prednosti u odnosu na ostale terapeutike zbog sposobnosti da vezuju ligande i formiraju trodimenzionalne strukture. U vodenim rastvorima su u formi pozitivno naelektrisanih jona i tako mogu da vezuju negativno naelektrisane biomolekule. Metalni kompleksi koji sadrže jone metala kao što su cink(II) i zlato se intenzivno ispituju kao potencijalni antitumorski lekovi. Cink je esencijalni element koji ima ključnu ulogu u različitim procesima u ćeliji kojima se kontrolišu strukturne, signalne, katalitičke i regulatorne funkcije. Cink je sastavni deo mnogih proteina i enzima, uključujući transkripcione faktore, signalne proteine ćelije, enzime koji učestvuju u popravci DNA, a poremećena koncentracija cinka u tkivima može da ima ulogu u razvoju i ishodu tumora. Zlato se nalazi u sastavu različitih kompleksa koji pokazuju efikasan anititumorski odgovor. Neki od protokola koji se koriste za lečenje tumora uključuju kombinovanu primenu preparata koji sadrže cink i zlato.

Ključne reči: *Cink, kompleksi zlata, tumor dojke, antitumorski lekovi*

INTRODUCTION

According to the World Health Organization, breast cancer accounts for approximately 16% of all types of cancer deaths globally. It is the most frequently diagnosed solid tumour in women, and its incidence increases with age. Both molecular and genetic factors have been documented to play roles in the initiation and promotion of breast tissue oncogenesis (1). Among genetic factors, the most common cause is inherited mutation in the BRCA1 or BRCA2 genes (2). Deregulation of mechanisms that contribute to increased oxidative stress and the consequent genomic instability contribute to breast cancer development (3). The underlying mechanism may also rely on the ability of oestrogen and oestrogen metabolites to generate reactive oxygen species (ROS), which induce DNA synthesis, increased phosphorylation of kinases, and activation of transcription factors responsive to either oxidants (e.g., toxins,

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including metal compounds) or oestrogen. Environmental factors such as nutrition (obesity and alcohol consumption), smoking, and exposure to carcinogens (e.g., metal compounds) also play a decisive role in breast carcinogenesis. Deficiencies in key micronutrients may contribute to increased cellular stress and associated DNA damage (4-6). In addition, multiple reports show that metallic compounds can function as oestrogen disruptors (7), while other studies emphasize the connection between exposure to metals or metal compounds and breast cancer risk (8,9).

The present review discusses the association of specific metals, zinc and gold, with regard to their effects in contributing to breast cancer oncogenesis and to the beneficial effects of these metals in treating the same cancer.

ZINC TRANSPORTERS IN BREAST CANCER AND ZINC CYTOTOXICITY

Metals and metal compounds have been implicated in breast cancer biology in a few ways. They can be a possible risk factor for development of breast cancer, while on the other hand, their ability to induce cytotoxicity and apoptosis in breast cancer cells can be used for anticancer therapy, or they can be used as diagnostic markers. Zinc is involved in many aspects of cellular metabolism. It is required for the catalytic activity of enzymes, it plays roles in immune function, protein synthesis, wound healing, DNA synthesis, and cell division, and it is critical for the functioning of greater than 3000 transcription factors (10-12). The role of zinc in cell growth and division as well as basal homeostasis is of key importance. Zinc is needed for the stabilization of the nucleic acids DNA and RNA (13). In fact, all RNA polymerases (I, II, and III) are zinc metalloenzymes. Substitution of zinc has been shown to advantage DNA synthesis, while deficiency in this mineral inhibits DNA synthesis (14).

The participation of zinc in a number of physiological processes requires strict control of cellular zinc levels (15). Zinc cannot passively diffuse through the cell membrane and requires transporters for its passage (16,17). In addition, the intracellular distribution of zinc is tightly regulated by a family of proteins that control the uptake, efflux, and compartmentalization of zinc. There are three known families of zinc transporters, the Zrt-Irt-like proteins (ZIP family), the Cation Diffusion Facilitator family, often called the ZnT family, and the zinc-sensitizing MTs (18). The MTs play an important regulatory role in zinc uptake, storage, distribution, and release (19). Transporters of the ZIP family are responsible for the uptake of zinc from outside the cell into the cytoplasm and also contribute to zinc efflux from subcellular organelles into the cytoplasm. Members of the ZnT family of transporters, however, perform the opposite role, functioning in the efflux of zinc from the cytoplasm out of the cell, as well as in the translocation of zinc from the cytoplasm into organelles, effectively decreasing the cytosolic zinc concentration (20). The expression and cellular distribution of ZIPs and ZnTs are predominantly (but not always) regulated by changes in extracellular and intracellular zinc concentrations. The cellular distribution of zinc into organelles is precisely managed to provide the zinc concentration required by each cell compartment. There is evidence of ZnT and ZIP genetic polymorphisms, which could influence dietary zinc requirements and zinc metabolism (21).

Alterations of both cellular and serum zinc content have been shown in patients with breast cancer (22, 23). There is a 72% increase in zinc concentration in breast cancer tissue in comparison with normal tissue. This evidence regarding breast cancer is coupled with an observed reduction in serum zinc levels (24). In addition to the observed difference in zinc concentration within individual patients, cancerous breast cells tend to accumulate more zinc than ancillary non-cancerous breast cells (25). Significantly higher concentrations of zinc in breast cancer tissues compared to healthy breast tissue and lower levels in serum of patients with breast cancer seems to be due to altered expression of zinc transporters in breast cancer tissue (26-28).

The zinc transporter LIV-1 has been observed to be significant for breast cancer (29, 30). LIV-1 and ZIP10, both from the ZIP family, are associated with breast cancer metastasis to lymph nodes, and they may play a causal role in this process (31). The zinc transporter ZIP10 has been implicated in the migration and metastasis of breast cancer cells, and this invasive behaviour could be inhibited by the knockdown of ZIP10 expression (31). This study was consistent with findings from clinical samples showing that breast cancers with lymph node metastases expressed significantly higher levels of ZIP10 than those without lymph node metastases. Comparable results have been demonstrated with LIV-1 in HeLa cells (32).

ZIP6 is normally localized in the plasma membrane of mammary epithelial cells, where it imports zinc into the cytoplasm (33, 34). High levels of ZIP6 are found in metastatic breast cancer cells (35) and are positively correlated with lymph node metastasis (36), suggesting the possibility that it plays a role in tumour progression. A unique characteristic of ZIP6 is the highly conserved putative metalloprotease motif that resembles the active site found in matrix metalloproteinases (MMPs) (37). Increased expression of certain MMPs is associated with tumour growth, invasion, metastasis and angiogenesis and correlates with poor prognosis (38).

ZnT2 is abundantly expressed in the mammary gland and is over-expressed in ER+T47D cells (39). Hyperaccumulation of Zn in the malignant T47D breast tumour cells is correlated with ZnT2 overexpression and increased vesicular zinc pools. Further, attenuation of ZnT2 expression in malignant cells protects the metallothionein-null breast tumour cells from zinc-induced cytotoxicity by redirecting zinc into vesicular compartments (39). Because malignant breast cancer cells accumulate zinc, and exposure

to high levels of zinc activates apoptosis (40), mechanisms have evolved to protect cells against zinc-modulated cell death. Two genetic variants of ZnT2 have been characterized that may further implicate ZnT2 dysfunction in breast disease (41).

The broad involvement of zinc in biological processes indicates that aberrations in zinc status may play a significant role in cellular dysfunction, including the development and/or progression of cancer. Zinc is known to be essential for cell proliferation (42, 43) and may play a role in tumour growth (22-24). The effects of growth factors on proliferation are accompanied by an increase in the concentrations of labile zinc, whereas in the absence of zinc, cells are arrested in the S-phase, with cell proliferation being attenuated (10).

Opposing effects of exposure to zinc on cell survival have been described. Effects of high concentration of zinc in cells appear to be cell-type dependent. It has been reported that zinc induces apoptosis in different cells, including epithelial cells of prostate, ovaries, oesophagus, neurons, glial cells, and hepatoma cells. On the other hand, zinc has anti-apoptotic effects on breast and lung epithelial cells, renal cells, macrophages, lymphocytes, thymocytes, and pancreatic acinar cells (44). Further, it has been shown that exposure to low zinc levels induces apoptosis, whereas exposure to high zinc levels inhibits apoptosis (45). There is still no explanation for these apparently opposite actions of zinc. However, because of the critical role that zinc plays in biological systems and its unique properties, zinc has become a potential anticancer agent.

GOLD METALLOPROTEINS IN ANTICANCER THERAPY

Gold in solution exists as Au+ and Au3+. It has intriguing properties because gold does not react with water, air, oxygen, ozone, nitrogen, fluorine, hydrogen, sulfur, iodine or hydrogen sulfide under normal conditions.

An important issue, both theoretically and experimentally, is the interaction between gold and DNA. Recent experimental studies have shown that DNA bases, adenine (A), thymine (T), guanine (G), and cytosine (C), interact with Au surfaces in a specific and sequence-dependent manner. The relative binding affinities of these nucleobases for adsorption on polycrystalline Au films obey the following order: A > C ≥ G > T. Two key bonding ingredients underlie the base-gold and base pair-gold hybridizations: the anchoring, either of the Au-N or Au-O type, and the nonconventional N-H Au hydrogen bonding. The former is the leading bonding factor and results in stronger binding and coplanar coordination when the ring nitrogen atoms of the nucleobases are involved (46).

One of the most commonly proposed mechanisms for gold (III) compound-induced cytotoxicity is the induction of apoptosis by mitochondrial death pathways related to reactive oxygen species (ROS) (47). Gold(III) tetraphenylporphyrin downregulates the expression of genes involved in angiogenesis and inhibits formation of microvessels by epithelial cells. Further, gold(III) tetraphenylporphyrin has been shown to inhibit migration and invasion of nasopharyngeal carcinoma cells (48).

Several gold(I) and gold(III) complexes have shown *in vitro* anticancer properties against human cancer cell lines, including cell lines resistant to cisplatin. Cysteine-containing proteins appear to be likely targets for gold complexes due to the thiophilicity of gold. Among these proteins, Cys4 zinc finger domains have attracted significant attention because gold(I) and gold(III) complexes have been shown to inhibit poly(adenosine diphosphate ribose) polymerase-1, an essential protein involved in DNA repair and cancer resistance to chemotherapies (49).

Gold(III) complexes have shown promising results as anticancer agents due to their high cytotoxic effects on tumour cells both *in vitro* in tumour cell lines and *in vivo*, but they show reduced or even absent systemic or renal toxicity (50). In the presence of aurothioglucose, A549 human lung cancer cells exhibited a marked reduction in growth kinetics.

GOLD NANOPARTICLES IN THERAPY FOR BREAST CANCER

Ultrasmall gold nanoparticles (GNPs) consisting of a few to roughly one hundred gold atoms are promising candidates for delivery vehicles for anticancer drugs (51). Colloidal gold nanoparticles have great potential to overcome delivery limitations because of their biocompatibility, low toxicity, small size, and tuneable surface functionalities. If they are exposed to the biologicals in fluid, a protein adsorption layer forms around them. Gold nanoparticles have been coated with different biological agents including tumour necrosis factor, paclitaxel, and docetaxel (52-55). It was shown that compared with free or liposomal doxorubicin, doxorubicin-conjugated hollow gold nanoshells (HAuNSs) stimulated with an NIR laser enhanced eradication of tumours in vivo and were less cardiotoxic, most likely because the conjugated form was associated with less free doxorubicin in the blood (56).

A number of studies have shown that gold nanoparticles conjugated with antibodies are efficient in targeting and destroying cancerous tissue (57). A 4-component antibody-phthalocyanine-polyethylene glycol-gold nanoparticle conjugate is described as a potential drug for targeted photodynamic therapy of breast cancer. Gold nanoparticles, stabilized with a self-assembled layer of a zinc–phthalocyanine derivative (photosensitizer) under irradiation with visible red light efficiently produced cytotoxic singlet oxygen. It was shown that these gold nanoparticles, when conjugated with anti-HER2 monoclonal antibody, could be

effective photodynamic therapy agents for breast cancer cells that overexpress HER2 (58).

Another form of nanoparticles, Au-Fe3O4 conjugated with anti-HER2 monoclonal antibody and cisplatin, allowed target-specific delivery of platinum compounds to HER2-positive cells. Cisplatin conjugated to nanoparticles is released in endosomes after uptake of the conjugates, and it is considered that intracellular release of cisplatin is stimulated by the lower pH in endosomes (59). The higher release of cisplatin is followed by higher cytotoxicity (60).

CONCLUSION

Metals and metal compounds interfere with breast cancer in several means. Under specific conditions, they can represent possible risk factors for development of breast cancer, but their cytotoxicity might also have beneficial effects in inducing apoptosis and cytotoxicity in breast cancer cells. These include zinc- and gold-containing complexes, which have allowed significant progress in the pursuit of developing novel anticancer drugs. Advantages of metal-containing compounds are based on their ability to coordinate ligands in three-dimensional configurations, thus allowing functionalization of groups that can be tailored to defined molecular targets.

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CANNABIS AS A POSSIBLE TREATMENT FOR SPASTICITY IN MULTIPLE SCLEROSIS

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KANABIS KAO MOGUĆI TRETMAN U LEČENJU SPASTIČNOSTI KOD MULTIPLE SKLEROZE

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ABSTRACT

e therapeutic potential of cannabis has been known for centuries. Cannabinoids express their e ects through two types of receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). Present studies indicate that cannabis-based drugs can make a positive impact in the treatment of di erent diseases. For many years, multiple sclerosis patients have self-medicated with illegal street cannabis to alleviate spasticity, a common and debilitating symptom that impairs quality of life.

Nabiximols is the cannabis-based medicine approved in many countries as an add-on therapy for symptom improvement in patients with spasticity who have not responded adequately to other medications. Adverse events such as dizziness, diarrhoea, fatigue, nausea, headache and somnolence occur quite frequently with nabiximols, but they are generally of mild-to-moderate intensity and their incidence can be markedly reduced by gradual uptitration. e prerequisite for the therapeutic use of cannabis in Serbia arerequires legal clarification for the use of the drug in a clinical environment.

Keywords: *cannabis, multiple sclerosis, spasticity, nabiximols*

SAŽETAK

Vekovima unazad je poznato da kanabis ima terapijski potencijal. Kanabinoidi ispoljavaju svoje efekte vezujući se za dve vrste receptora, kanabinoid receptor 1 (CB1) i kanabinoid receptor 2 (CB2). Dosadašnje studije su pokazale da lekovi bazirani na kanabisu mogu imati značajnu ulogu u lečenju mnogih bolesti. Godinama unazad, oboleli od multiple skleroze samoinicijativno, ilegalno koriste kanabis za ublažavanje spastičnosti. Spasticitet je jedan od najčešćih simptoma bolesti koji dovodi do nastanka invaliditeta i može značajno uticati na kvalitet života.

Nabiximol je lek od kanabinoida biljnog porekla koji je danas u mnogim zemljama, zvanično odobren za lečenje spastičnosti kod multiple skleroze. Indikovan je kod pacijenata kod kojih druge terapijske opcije nisu dale zadovoljavajuće rezultate.

Neželjeni efekti nabiximola kao što su vrtoglavica, dijareja, umor, mučnina, glavobolja i pospanost su česti, ali blagog do umerenog intenziteta, a njihova učestalost se smanjuje postepenom titracijom doze leka. Osnovni preduslov za terapijsku upotrebu kanabisa u našoj zemlji je promena postojećih zakonskih okvira.

Ključne reči: *kanabis, multipla skleroza, spasticitet, nabiximol.*

ABBREVIATIONS

CB1-Cannabinoid receptor 1
CB2-Cannabinoid receptor 2
CBD-Cannabidiol
FDA-Food and Drug Administration
HIV-Human immunodeficiency virus
THC-Tetrahydrocannabinol

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Cannabis consumption throughout the centuries

Medications derived from cannabis have been used for therapeutic purposes for centuries. In ancient Greece, Rome, China and India, cannabis was used to ameliorate muscle spasms, cramps and pain (1). At the end of the 19th century in Europe, cannabis was used for the treatment of pain, asthma, sleep disorders, depression, and loss of appetite (2). UntilBefore 1964, scientists were unable to establish the chemical structure of the essential ingredient of the cannabis plant, but after the principal active ingredient of cannabis was defined as trans-delta-9-tetrahydrocannabinol (THC) (3), an Currentlyextensive literature on the effects of cannabis-based compounds in clinical studies and animal models has been developed.

Cannabinoid receptors and endocannabinoids

Cannabis-based medications have been the subject of intense research since the endogenous cannabinoid system was discovered more than twenty years ago (4, 5). Two principal types of receptors have been found to be part of the endocannabinoid system. Cannabinoid receptor 1 (CB1), predominantly located in the terminals of nerve cells (central and peripheral neurons and glial cells), the reproductive system, some glandular systems and the microcirculation, was identified in 1990 (4). Three years later, cannabinoid receptor 2 (CB2) was found initially in multiple lymphoid organs with the highest expression detected in B lymphocytes, moderate expression in monocytes and polymorph nuclear neutrophils and the lowest expression in T lymphocytes; subsequent studies have identified them in microglial cells (5, 6). The highest distribution of cannabinoid receptors was found in the hippocampus, the basal ganglia, and the cerebellum, areas associated predominantly with memory and motor coordination (7). Cannabinoid receptors also occur in high density in many areas related to pain, such as the periaqueductal grey mass, the rostral ventrolateral medulla, and superficial layers of the spinal dorsal horn. They are also found in the dorsal root ganglion, from which they are transported to both central and peripheral terminals of primary afferent neurons (8, 9, 10). These discoveries consequently lead to the identification of natural ligands for the CB1 receptor: arachidonoylethanolamide, named anandamide, and 2- arachidonoylglycerol (11).

As reviewed by Wegener and Koch in 2009 (12), CB1 receptors are expressed in the presynaptic membrane of neurons, and they utilize a negative feedback mechanism to regulate transmitter release at GABAergic, glutamatergic and dopaminergic synapses. Cellular activity of the postsynaptic neuron promotes continuous synthesis and release of the endocannabinoids from phospholipids of the cell membrane, which then bind to the CB1 receptors in the presynaptic membrane. Stimulation of the CB1 receptors blocks the activity of the presynaptic neurons through activation of A-type potassium channels and potassium inward rectifiers and through the inhibition of voltage-dependent calcium channels and adenylate cyclase. The outcomes are reduced transmitter release in the presynaptic terminals and reduced cellular excitability.

The endocannabinoid effect is regulated through the transport of endocannabinoids into the cell via specific transporters and subsequent degradation through the membrane-bound enzyme fatty acid hydrolase. (13).

erapeutic potential of cannabis

Cannabis preparations exerts numerous therapeutic effects, and the augmentation of cannabinergic tone is therapeutically beneficial in the treatment of multiple disease states (14). Numerous studies, most of them carried out in the 1970s and 1980s, have demonstrated that cannabinoids are equally or more effective in treating chemotherapy-related nausea and vomiting as the standard antiemetics (15, 16, 17). Some studies have shown that cannabis is effective in the treatment of anorexia and cachexia in patients with human immunodeficiency virus (HIV) (18), tumours (19) and Alzheimer's disease (20), as well as in the treatment of chronic neuropathic pain and multiple sclerosis (21,22). However, cannabis may be ineffective in treating patients with acute pain (23). Small randomized controlled trials have shown a positive effect of cannabis preparations in the treatment of tics in Tourette syndrome (24) and levodopa-induced dyskinesia in Parkinson's disease (25).

Cannabis treatment of spasticity in multiple sclerosis

Spasticity is a consequence of a damaged corticospinal tract, which plays a role in controlling voluntary movements (26). Therefore, it is characterized by sudden involuntary movements, muscle stiffness, or muscle spasm sufficient to cause pain, particularly in the lower back and legs. Spasticity ,and itcan result in difficulty moving the limb at all. This condition is often associated with spinal cord injury, amyotrophic lateral sclerosis, cerebral palsy and brain damage (27).

Cannabis preparation has been discussed as a promising agent in multiple sclerosis treatment, particularly for spasticity. Spasticity is the most commonly reported symptom, occurring in 90% of patients, causing significant disability and quality of life impairment. Spasticity may also contribute, directly or indirectly, to hroughother symptoms of multiple sclerosis, such as bladder or bowel dysfunction (28), and it can be associated with pain, weakness, clonus, sleep disturbance, fatigue and loss of dexterity (29). At worst, severe spasticity can lead to complete immobility (30). Prolonged immobility may lead to pressure sores and thromboembolism (31).

Standard drugs used to treat spasticity include centrally acting agents, such as benzodiazepines, baclofen, tizanidine and gabapentin, and peripherally acting agents such as dantrolene (29, 32). There is a very limited evidence

base for these drugs, and they provide only moderate relief from spasticity (33). Because many patients are refractory to treatment with existing oral drugs (29, 32, 33), there is a clear need for new treatments for spasticity in multiple sclerosis.

Demographic evidence has shown that many people with multiple sclerosis use cannabis for self-medication (34). The antispastic effect of cannabis has been supported through a demonstration of the inhibitory properties in exogenous agonists for cannabis receptors found in the central nervous system (35). Stimulation of CB1 receptors by cannabis-based drugs have the potential to regulate aberrant levels of glutamatergic excitability during spasticity (36). The endocannabinoid system is upregulated in lesioned areas to provide further control of aberrant neurotransmission, suggestings that further enhancement of endocannabinoid tone by stimulating endocannabinoid synthesis or blockade of endocannabinoid degradation may exhibit anti-spastic activity. CB1 receptors in the basal ganglia are probably the target for cannabinoid control of tremor, spasticity, and painful muscle spasms in multiple sclerosis. CB1 is densely expressed in the output neurons of the substantia nigra, pars reticulata and globus pallidus (37). Activation of CB1 within these neurons can suppress excessive motor output and consequent muscle spasm (38). Cannabinoids can reduce chronic pain, one symptom of multiple sclerosis, but in this case, it is clinically important that cannabinoids can also reduce pain which is a consequence of muscle spasm (38).

Available cannabinoid-based medication for spasticity

For patients with multiple sclerosis, nabiximols is the first cannabis-based medicine to be licensed for the treatment of symptoms (39). The drug is a pharmaceutical product standardised in composition, formulation and dose. Nabiximols differs from all other pharmaceutically produced cannabinoids currently available because it is a mixture of compounds. Its principal active cannabinoid components are the cannabinoids tetrahydrocannabinol (THC) and cannabidiol (CBD) (Figure 1).

Although there is considerable structural overlap between THC and CBD, their conformational structures differ significantly. As a result of this, CBD does not bind to or activate the CB1 receptor, which leads to a complete lack of psychoactivity by CBD, unlike THC, which is the psychoactive principle of cannabis. However, CBD shows significant pharmacological activity, such as anti-inflammatory and immunomodulatory effects, that appear to be mediated by the adenosine A2A receptors and could possibly influence the progression of the illness (40). Finally, it has been suggested that the combination of CBD and THC shows a better therapeutic profile than each cannabinoid component alone, with a lower predominance of unwanted, adverse side effects (41). Smoking cannabis achieves the fastest absorption, and the effects are manifested in a few minutes. Inhalation of 8 mg of THC results in a 24 times faster achievement of peak blood concentrations than 0 msublingual administration of 10 mg of THC. When inhaledAdditionally,This peak is achieved in 17 minutes when inhaled, and in 263 minutes when ingested sublingual (37).

Nabiximols is formulated as an oromuscular spray which is administered by spraying into the mouth. Each spray delivers a near 1:1 ratio of CBD to THC, with fixed a dose of 2.7 mg THC and 2.5 mg CBD (42). An essential part of the therapeutic use of the drug is that it is patient-directed and dose-optimized through self-titration. The titration period may take up to 2 weeks to find the optimal dose. On the first day of the titration period, one spray in the morning and one spray in the afternoon/evening should be administered. This dosage should be increased by one spray each day depending on efficacy and adverse effects. The average effective dose is 8-9 sprays per day, up to a maximum of 24 sprays per day (43).

Among all tested analogues, such as oral formulations Dronabinol and Nabilone, it has been concluded that only sublingual spray nabiximols hasve a sufficient evidence base to justify its theiruse in the treatment of spasticity to improve patient quality of life, particularly in patients who are refractory to current treatments (44).

Indications and contraindications for use of nabiximols

Nabiximols is registered for the relief of spasticity, tremor and pain in patients with multiple sclerosis who have not responded sufficiently to standard medication and who show a worthwhile degree of improvement during a 4-week trial period (44). It is contraindicated in patients with known or suspected allergies to cannabinoids or any of the other ingredients and for patients with severe psychiatric disorders other than illness-associated depression. Use is not advised for nursing mothers, they are adolescents or children under 18 or elderly patients (44).

Adverse e ects of nabiximols

Because cannabis is a restricted drug, the use of cannabis for medical purposes inevitably raises a number of problems such as possible intoxication and neurotoxicity, the development of tolerance and dependence, as well as legal and ethical dilemmas. Using cannabis for medicinal purposes and the use of marijuana and hashish, which are derivatives of cannabis and are used as forbidden “soft” drugs, should not be equated. Many psychological effects of cannabis and THC are biphasic and bidirectional, depending upon the modality of administration, dosage, individual variability, the degree of tolerance, as well as other environmental factors (45). Acute effects can range from euphoria, relaxation, excitation, sharpened perception and increased motor activity to sedation, distortions of perception, ataxia, and loss of coordination (6). In larger doses,

cannabis can cause dysphoric reactions, anxiety, panic and hysteria (46). All of these central effects occur only after the activation of CB1 receptors (4).

The optimal doses of CB1 receptor agonism in motor control centres would invariably be associated with stimulation of CB1 receptors in cognitive centres, which could also be associated with some unwanted side effects. Acute exposure could also produce a full range of transient psychotomimetic symptoms that last only during the period of intoxication but also and acute psychosis that lasts longer (47). THC produces the full range of transient, positive psychotomimetic symptoms, negative symptoms and cognitive deficits observed in schizophrenia, while CBD has been shown to have anxiolytic properties and even to inhibit the psychotomimetic effects of THC. Cannabis also produces transient, dose-related cognitive impairments, especially in the domains of verbal learning, short-term memory, working memory, executive function, abstract ability, decision-making and attention. Variable duration to full recovery (absence of persistent neuropsychiatry deficits) has been demonstrated to last from a week to an average of 2 years of abstinence (47).

The safety profile of nabiximols, in randomized studies, conducted on patients suffering from multiple sclerosis, indicates that the drug is well tolerated and the most common side effects are dizziness, fatigue, nausea, diarrhoea, drowsiness, headache and somnolence (48).

There is no evidence that nabiximols causes intoxication, cognitive impairments or any of the other central side effects commonly associated with recreational use. T, he reason for this may beis the presence of cannabidiol in nabiximols, which is not psychotropic and may reduce THC levels in the brain and attenuate its psychotropic side effects (49, 50). All cannabinoids in current therapeutic use have a therapeutic index that is relatively narrow for most uses, with adverse effects limiting dose titration. Under everyday clinical practice conditions, nabiximols at a mean daily dose of <7 sprays in ehas been shown to relieve spasticity in approximately 70% of patients previously resistant to treatment. In large observational studies, >80% of patients reported no adverse events. Subjectivity of the spasticity assessment and coexistence of other symptoms in patients with multiple sclerosis must be taken into account as a serious obstacle when evaluations of drug efficacy are conducted. (51). Further limitations in designing adequate comparative studies that cannot be ignored are the legal aspects of psychotropic drug use and the possible influence of pharmacological companies.

Regulation of cannabis use in di erent countries

There is no unified position regarding the use of cannabis and its analogues, from full legalization and free traffic, through legislation or controlled traffic to a complete ban. In June 2010, the Medicines and Healthcare products Regulatory Agency of the United Kingdom licensed nabiximols as a prescription-only medicine for the treatment of spasticity due to multiple sclerosis (52). This regulatory authorization represents the world's first full regulatory approval for the medicine. Currently, the drug is available for medical use in the United Kingdom, Spain, the Czech Republic, Germany, Denmark, the Netherlands, Sweden, Italy, Austria, Norway, Iceland, Poland, Finland, Switzerland, France, and in some countries in Asia and in Israel (52). In the USA, nabiximols is not officially approved by the FDA for the treatment of spasticity in multiple sclerosis (53, 54). In countries where the medical use of cannabis is approved, there are legally defined conditions for the cultivation of cannabis. For example, in England and the Netherlands, cannabis is grown with the permission of the Government under strictly controlled conditions (55).

If it is in use, there are strictly defined indication areas in which it can be used, and cannabis can be obtained for medical use only by physician prescription. In Serbia, according to the existing legislation, possession, production and trade of marijuana is illegal (56). Laws provide for the strict distribution of cannabis for therapeutic use. C theannabis is availableonly issued by prescription andto doctors and specialists and only after a clear indication of how it will be used. Also it is necessary to define who can grow cannabis and under which conditions it can be produced and distributed. The eventual formation of a user registry is a priority. Regardless of the dilemmas that exist regarding the legislation of marijuana, patients have a right to all beneficial treatments and to deny them access to treatments violates their basic human rights. Therefore, it is necessary to harmonize the scientific, clinical knowledge and national legislative opinions to provide proper use of this psychotropic substance. Considering all of the positive findings, regulatory bodies and the Ministry of Health in Serbia are trying to initiate discussion and establish a legal environment for the use of this compound.

Concluding remarks

Cannabis preparations have been used to relieve nausea, improve appetite and reduce pain for thousands of years. The development of synthetic drugs in the 20th century supplanted these and herbal remedies, but in the past several decades there has been a resurgence of interest in using cannabis and cannabinoid preparations for medical purposes. There is evidence from controlled trials that cannabinoids are effective in relieving nausea and vomiting, alleviating acute pain and improving appetite in people with HIV-related disorders. The potential role of cannabinoids in the treatment of spasticity in multiple sclerosis was highly controversial following the publication of initial studies. Most of the clinical trials conducted in recent years have shown that nabiximols is a useful treatment option for its approved indication, treating spasticity in multiple sclerosis. Apart from reducing spasticity, nabiximols also offers moderate relief of pain associated with muscle spasm and also with centrally generated neuropathic pain. Quality of life, particularly with respect to

sleep, is improved for patients taking the drug, and urinary incontinence is moderately reduced (57). The pharmacoeconomical aspects also favour this therapeutic option because a lower severity of spasticity can lead to reduce resource consumption such as psychotherapy and medication. (58) All of the aspects of cannabis use as a treatment of spasticity in multiple sclerosis must be thoroughly discussed in Serbia.

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OVARIAN DYSGERMINOMA

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DISGERMINOM JAJNIKA

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ABSTRACT

Ovarian dysgerminoma (OD) is one of the most common malignant tumours of the ovarian germ cells, comprising 1-2% of all malignant ovarian tumours. It most commonly occurs between the ages of 10 and 30. e treatment is primarily surgical; however, in the advanced stages of the disease, surgical treatment is followed by chemotherapy.

We report a case of a 19-year-old female patient who was diagnosed with bilateral ovarian dysgerminoma. e patient was first operated for dysgerminoma on the left ovary and an ovariectomy was performed. A month later, she underwent surgery again because of the appearance of a residual tumour in the lodge of the removed ovary; the rest of the tumour was removed at that time.

e histological finding was dysgerminoma ovarii, FIGO stage 1c. As agreed on by the consulting team, the patient returned for a follow-up ultrasound examination every three months. Nevertheless, nine months after the operation, she was diagnosed with dysgerminoma of the right ovary. Surgical treatment was performed again, and the tumour was removed. e histological finding on the second occasion was dysgerminoma ovarii, FIGO stage IIa, according to the TNM classification T2aNxMx. To preserve the reproductive capacity of the patient, the consulting team agreed to treat the patient with chemotherapy, i.e., 3 cycles of BEP [Bleomycin, Etoposide, Cisplatin (Platinum)]. Magnetic resonance imaging, ultrasound and clinical examination after the therapy were normal. e patient is monitored in regular intervals, feels good and has no signs of the disease after five years.

Keywords: *dysgerminoma, malignant ovarian tumour, chemotherapy, surgical intervention*

SAŽETAK

Disgerminom jajnika je jedan od najčešćih malignih tumora germinativnih ćelija jajnika. On čini 1-2% svih malignih tumora jajnika. Najčešće se javlja između 10. i 30. godine života. Lečenje je primarno hiruško. Kod uznapredovalih stadijuma bolesti nakon hiruškog lečenja primenjuje se i hemioterapija.

Prikazujemo pacijentkinju staru 19 godina koja je operisana zbog disgerminoma na oba jajnika. Pacijentkinja je prvi put operisana zbog disgerminoma na levom jajniku. Urađena je ovariektomija. Mesec dana nakon operacije zbog pojave rest tumora u loži odstranjenog jajnika urađena je ponovo operacija i odstranjen je rest tumor. HP nalaz – Dysgerminoma ovarii, stadijum FIGO 1c. Nakon toga, po odluci Konzilijuma pacijentkinja je kontrolisana ultrazvučnim pregledima na 3 meseca. Devet meseci posle ove operacije dijagnostikovan je disgerminom na desnom jajniku. Urađena je operacija i odstranjen je tumor jajnika. HP nalaz - Dysgeminoma ovarii. Stadijum bolesti FIGO IIa. Prema TNM klasifikaciji T2aNxMx. Zbog očuvanja reproduktivne sposobnosti pacijentkinje, a po odluci Konzilijuma, sprovedeno je lečenje hemioterapijom,3 ciklusa po protokolu BEP [(Bleomycin, Etoposide, Cisplatin (Platinum)]. Kontrolni pregled magnetne rezonance,ultrazvuka i klinički pregled nakon sprovedene terapije bili su uredni. Pacijentkinja je redovno kontrolisana i pet godina nakon operacije dobro se oseća i nema znakova recidiva bolesti.

Ključne reči: *disgerminom, maligni tumor jajnika, hemioterapija, hiruški zahvat*

ABBREVIATIONS

OD – Ovarian Dysgerminoma

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INTRODUCTION

Ovarian dysgerminoma (OD) is one of the most common malignant tumours of ovarian germ cells. It belongs to the group of germ tumours of the ovary and arises by the malignant alteration of the primordial germ cells. In some cases, the tumour may occur on the anomalies of the genital tract, such as gonad dysplasia or testicular feminization [1]. It usually occurs in patients aged 20 to 30 years. Dysgerminomas comprise 1-2% of all malignant ovarian tumours [2] and occur bilaterally in 10-15% of all cases. In approximately 5% of patients with dysgerminomas stage Ia, microscopic metastasis can be found in the other ovary. Macroscopically, the tumours are solid with lobular structure and may reach a size of approximately 15 cm. The most common clinical symptoms are abdominal pain and the presence of a tumour mass in the abdomen [3]. The tumours usually spread through the lymphatic system into the paraaortic lymph nodes; haematogenous spread may occur in the advanced stages of the disease. Conservative surgery is the treatment of choice for unilateral encapsulated unruptured tumours, with negative retroperitoneal lymph nodes and negative swabs from the pelvic and paracolic area in order to preserve the patient's reproductive function. In advanced stages of the tumour, radical surgical procedure is followed by chemotherapy. The five-year survival rate of the disease in stage I is 80-90% [4,5].

CASE REPORT

A 19-year-old patient, nulliparous, nulligravida, menarche at the age of 14, with regular menstrual cycles at 28 days lasting for 4-5 days was admitted to the Gynaecology and Obstetrics Clinic "Narodni Front" in Belgrade for surgery because of a residual tumour in the left ovary lodge. A month prior to this admission, the patient had a laparoscopic ovariectomy performed at another hospital due to the presence of a tumour in the left ovary, which was 9x8 cm large and of predominantly solid lobular structure. The tumour was encapsulated, and the capsule was intact. The histological finding of that tumour was dysgerminoma ovarii. A month after the aforementioned laparoscopic surgery, the patient underwent a regular follow-up ultrasound examination when another cystic formation measuring 8x6 mm was diagnosed in the lodge where the left ovary was previously extracted. Magnetic resonance imaging of the left ovarian lodge revealed a tumour of a lobular structure, 8x6 mm in diameter. The uterus had normal size and structure. The right ovary was normal in size, with microcystic structure. LDH, AFP, hCG and CA-125 values were within normal limits. The paraaortic and pelvic lymph nodes were not enlarged. The patient was presented to the consulting team for malignant diseases, and the team decided that it was best to perform an exploratory laparotomy and to act upon the findings. The patient was operated with salpingectomy on the left side and had a tumour formation of 8x6 mm in diameter with finely cystic structure removed. A biopsy was performed on the right ovary, which had a microcystic structure and a normal size. The right fallopian tube was normal. A partial resection of the omentum was performed. During the operation, no pathological findings were reported on the liver, stomach, small and large intestine, omentum and parietal peritoneum. Douglas's pouch contained approximately 10 ml of fluid. The fluid was collected for cytological analysis; swabs from the left and right subdiaphragmatic region and from the left and right paracolic region were also taken. The histological finding of the removed tumour was dysgerminoma ovarii, FIGO stage 1c. A histopathological biopsy of the right ovary and the omentum showed the tissue of ovaries and omentum without pathological changes. The cytological findings on malignant cells were negative. The patient was presented to the consulting team once again after surgery, and the team decided to have her followed up every three months, including conducting magnetic resonance imaging of the abdomen and pelvis. The follow-up results after 3 months were normal. The patient was regularly monitored by ultrasound every 3 months. Nine months after surgery, however, a follow-up ultrasound scan revealed a tumour in the upper pole of the right ovary, predominantly of a solid material with dimensions of 3x3 cm, with a unilocular cystic formation measuring 3x4 cm with transonic media. LDH values were slightly increased, while AFP, HCG and CA-125 values remained within normal limits. Magnetic resonance imaging revealed a tumour formation in the upper pole of the right ovary with a diameter 3x3 cm, multilocular, predominantly cystic structure with some solid tissue and thin partitions, as well as another unilocular cystic formation 3x4 cm that probably corresponded to follicular cysts. The patient's karyotype was normal, 46XX. Suspecting another dysgerminoma, we decided to perform the surgery once again. The patient underwent a laparotomy with the resection of ovarian tumours in total, a punction of the cyst and a suture of the ovary. The tumour was encapsulated. The removed tumour was sent for histological analysis, and the content of the cyst was sent for cytological analysis. A peritoneal washing was collected for cytological analysis. A partial resection of the omentum was performed. No macroscopically visible secondary deposits were visible on the liver, stomach, small and large intestine, urinary bladder, omentum or parietal peritoneum. Once again, the histological finding was dysgerminoma ovarii **(Figures 1 and 2).**

However, the tumour was focally infiltrating the ovarian capsule. A lymphatic and vascular infiltration was not found. The disease was at FIGO IIa stage. According to the TNM classification, it was T2aNxMx. The cytological finding of the cyst and of the peritoneal washing for malignant cells was negative. The consulting team decided to carry out the adjuvant chemotherapy treatment in order to preserve the reproductive capacity of the patient. The patient received 3 cycles of chemotherapy with BEP [(Bleomycin, Etoposide, Cisplatin (Platinum)], according

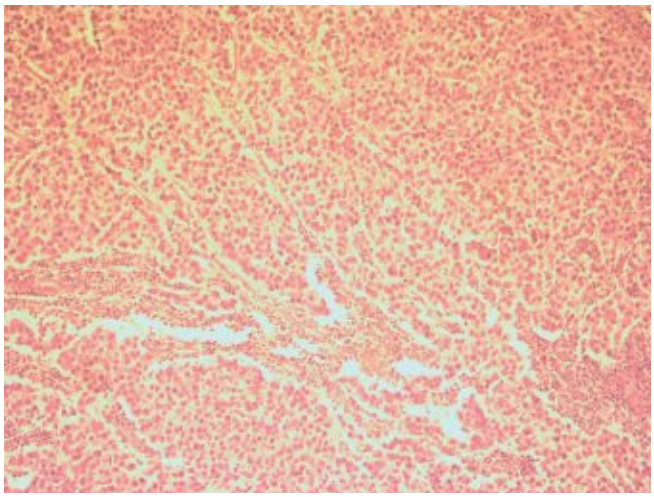

Figure 1. Dysgerminoma, 10x, haematoxylin and eosin stain

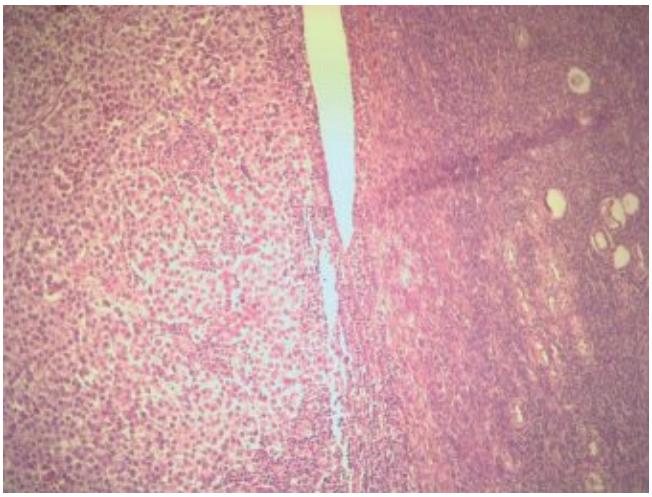

Figure 2. Dysgerminoma with ovarian tissue, 10x, haematoxylin and eosin stain

to protocol. Afterwards, she underwent a check-up magnetic resonance imaging, ultrasound and clinical examination. All findings were normal. The patient was presented to the consulting team once again, and the team decided to have her monitored every three months with magnetic resonance imaging of the pelvis and abdomen. The first follow-up imaging was uneventful, and the team decided to monitor the patient every 3 months. During the following five-year period, the patient was regularly monitored by ultrasound examination, and once a year she had magnetic resonance imaging of the abdomen and pelvis. Five years after surgery, the patient was feeling well and there was no evidence of the disease.

DISCUSSION

Dysgerminoma is the most common type of malignant germ cell tumours, comprising 1-2% of all malignant ovarian tumours. In most cases, it occurs in women between the ages of 20 and 30 [2]. Our patient was 19 years old. In approximately 5% of cases, dysgerminoma may even occur before puberty. Dysgerminoma cells originate from the primordial sexually undifferentiated embryonic gonads. This disease may be associated with pregnancy in 17% of cases; if that is the case, the tumour can be removed, and the pregnancy can be preserved[6,7]. In approximately 2% of non-pregnant women, a pregnancy test can be positive due to the creation of hCG in the isolated syncytiotrophoblast cells. The tumour most often occurs unilaterally, which makes up approximately 80-85% of all cases. However, it occurs bilaterally in 10-15% of cases, as was the case in our patient. Dysgerminomas tend to spread by the perirectal lymphatic system to lymph nodes near the aorta [8]. It can disseminate haematogenously to the lungs, liver and bones at advanced stages of the disease. The largest part of the dysgerminoma is associated with elevated levels of serum lactate dehydrogenase (LDH), which is sometimes used as a tumour marker in monitoring the progression of the disease [9]. In our patient, the values of the tumour markers AFP, HCG and CA-125 were within normal limits, while LDH values were slightly increased. The main clinical feature of this tumour is its rapid growth. Symptoms usually persist from one month to six months before the tumour is diagnosed. The first symptoms are abdominal pain and vaginal bleeding, followed by abdominal distension and the presence of a mass in the abdomen[10]. Abdominal effusion and rupture of the tumour occur in 25% of cases. It is usually spread through the lymphatic paraaortalne in the lymph nodes, and haematogenous spread takes place in the advanced stages of the disease [8]. In our patient, there were no pathological findings on the liver, stomach, small and large intestine, omentum or parietal peritoneum. Douglas's pouch contained approximately 10 ml of a free fluid. The cytological finding of this fluid and the swabs taken from the left and right subdiaphragmatic region and from the left and right paracolic region were negative. Dysgerminoma is composed of aggregates and large islands of uniform cells surrounded by varying amounts of connective tissue containing a small number of lymphocytes. The cells are round or oval with eosinophilic or light cytoplasm. The nucleus is circular with clear boundaries, finely granular chromatin and one or two nucleoli [10].

Considering the fact that 85% of women with ovarian dysgerminoma are younger than 30 years old, a conservative surgical treatment is highly recommended in order to preserve fertility. Conservative surgery is the treatment of choice for unilateral encapsulated unruptured tumours in stage Ia, with negative retroperitoneal lymph nodes and negative swabs from the pelvic and paracolic area and without dysgenetic gonads, in order to preserve reproductive function[3]. In more advanced stages of the disease, such as Ib and above, a radical surgical approach is advised. This includes hysterectomy with bilateral salpingo-oophorectomy, lymphadenectomy and omentectomy[4].

Commonly, in women with advanced-stage disease who insist on having children, a conservative surgical treatment may be applied with a certain risk. Our case was specific to the development of dysgerminoma on the right ovary following the ovariectomy of the left ovary due to the same disease. The second dysgerminoma was of stage IIa, according to FIGO classification. Despite the fact that it was diagnosed at an advanced stage, this patient underwent a conservative surgical procedure of the right ovary in order to remove the tumour only and to preserve childbearing capacity at the same time. Chemotherapy consisting of 3 cycles of BEP was applied after the surgical treatment. Dysgerminomas are sensitive to radiation; adjuvant chemotherapy is highly recommended whenever possible to preserve fertility. The so-called BEP protocol [(Bleomycin, Etoposide, Cisplatinum (Platinum)] is considered a gold standard and is widely accepted around the world[11,12]. The number of chemotherapy cycles is limited to 6. A concurrent chemotherapy and radiation therapy are reserved for women with stage III disease. The five-year survival rate for stage I disease is 90% and approximately 74% for stage III disease[5,13].

According to the recommendations of the European Society for Medical Oncology, the clinical follow-up of patients with ovarian tumours is performed every 3 months during the first 2 years, every 4 months in the third year, and every 6 months in years 4 and 5[14]. Our patient was monitored regularly for five years after surgery and showed no evidence of the development of the disease.

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ENCEPHALOPATHY DURING H1N1 INFLUENZA A VIRUS INFECTION

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ENCEFALOPATIJA KOD INFEKCIJE VIRUSOM INFLUENCE A PODTIP H1N1

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ABSTRACT

Influenza virus type A is known for its capacity to transform its antigenic structure and create new viral subtypes. e clinical picture varies from non-febrile, mild upper respiratory tract infection to severe or fatal pneumonia. Neurological complications include encephalitis, encephalopathy, Reye's syndrome and other neurological diseases. Patients with encephalopathy exhibit a disturbed state of consciousness lasting more than 24 hours, and patients with encephalitis exhibit high temperature, focal neurological signs and pathological CSF results in addition to disturbed state of consciousness.

A 54-year old, previously healthy male farmer was hospitalized at the Clinic for Infectious Diseases of the Clinical Centre Kragujevac on the fifth day of disease. In addition to general symptoms of the disease, the clinical picture was dominated by a disturbed state of consciousness (Glasgow Coma Scale score <8). e aetiological agent was an H1N1 influenza A virus, which was isolated from nasopharyngeal secretions. No other causes of infection were demonstrated from both serum and cerebrospinal fluid specimens. Interstitial pneumonia was detected by radiographic examination of the chest. ere were also some changes present in the EEG. e patient was cured without consequences.

Because our country is in a whirlwind of pandemic H1N1 virus activity, we should think of all the possible complications that this virus can produce regardless of the epidemiological data and the clinical picture.

Keywords: *H1N1 virus, neurological complications, diagnosis, disturbed state of consciousness.*

SAŽETAK

Influenca virus tip A je poznat po sposobnosti menjanja antigene strukture i stvaranja novih podtipova virusa. Klinička slika varira od nefebrilnih, blagih infekcija gornjih disajnih puteva pa sve do teške ili fatalne pneumonije. Neurološke komplikacije uključuju nastanak encefalitisa, encefalopatije, Rejovog sindroma i drugih neuroloških bolesti. Kod bolesnika sa encefalopatijom, prisutan je poremećaj stanja svesti u trajanju dužem od 24 h, a kod bolesnika sa encefalitisom pored poremećenog stanja svesti prisutna je i povišena temperatura, fokalni neurološki znaci i patološki likvorski nalaz.

Bolesnik T.J. muškog pola, star 54 godine, prethodno zdrav, poljoprivrednik, hospitalizovan u Infektivnu kliniku KC Kragujevac petog dana bolesti. Pored opštih simptoma, kliničkom slikom dominira poremećaj stanja svesti (Glasgow Coma Scale score <8). Etiološki uzročnik je bio virus Influence A, H1N1 koji je izolovan iz nazofaringealnog sekreta. I iz seruma i iz likvora nisu izolovani neki drugi uzročnici. Radiografskim pregledom grudnog koša nađena je intesticijalna pneumonija. Prisutne su i promene u elektroencefalogramu. Bolesnik je izlečen bez sekvela.

S obzirom da se i naša zemlja nalazi u pandemijskom vrtlogu infekcije virusom H1N1 treba misliti na sve moguće komplikacije koje ovaj virus može dati bez obzira na epidemiološke podatke i kliničku sliku.

Ključne reči: *H1N1 virus, neurološke komplikacije, dijagnoza, poremećaj stanja svesti.*

ABBREVIATIONS

CSF - cerebrospinal fluid
EEG - electroencephalography
RT-PCR - reverse transcription polymerase chain reaction
AST - aspart aminotransferase
ALT - alanine aminotransferase
a PTT - Activated Partial Thromboplastin Time
PT - prothrombin time

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INTRODUCTION

The H1N1 influenza A virus is an orthomyxovirus and has a segmented RNA genome (8 segments) (1). Orthomyxiviruses are very sensitive to disinfectants and antiseptics, which is very important from an epidemiological point of view. At a temperature of 54-56°C, H1N1 influenza A deteriorates for half an hour (2). Influenza virus type A is known for its capacity to transform its antigenic structure and create new viral subtypes. The most common subtype is H1N1. Swine flu is the name for the respiratory infection caused by influenza A virus subtype H1N1. This subtype represents the genetic combination of avian, human and swine flu (3). In contrast to the typical swine flu, this form of the virus is transmitted from human to human. It is spread by sneezing, coughing or touching your nose and mouth with unwashed hands. There is no evidence of the transmission of swine flu by eating pork.

The clinical picture of H1N1 infection ranges from nonfebrile, mild upper respiratory tract infection to severe or fatal pneumonia. The greatest number of cases typically present with an uncomplicated seasonal flu that resolves spontaneously. Cough, fever, sore throat, fatigue and headache are registered in most of the cases (4). In a large number of patients, gastrointestinal disturbances (nausea, vomiting, and diarrhoea) can occur as well. In the majority of patients, laboratory diagnosis detects moderate leukopenia, accelerated sedimentation, increased transaminase activity and increased creatine phosphokinase (CK). C-reactive protein (CRP) and fibrinogen are within normal values, except in cases of bacterial superinfection. Characteristic radiographic changes of lung parenchyma can also occur and may not correlate with the clinical results.

A large number of authors have described the neurological complications of infection with influenza viruses A and B, but not with infection by influenza A H1N1 (5). However, a number of authors in America and especially in Japan have described neurological complications in patients who are infected with H1N1 influenza. Neurological complications include encephalitis, encephalopathy, Reye's syndrome and other neurological diseases (6). Patients with acute neurological complications had serological confirmation of a new virus and a clinical picture that was dominated by encephalopathy or encephalitis within five days of disease onset (7). Patients with encephalopathy exhibit a disturbed state of consciousness lasting more than 24 hours and patients with encephalitis exhibit a high temperature (over 38°C), focal neurological signs and pathological CSF result in addition to a disturbed state of consciousness (8). Electroencephalographic changes may be indicative of encephalitis and a pathological neuroimaging result is often present as well.

CASE REPORT

A 54-year old, previously healthy male farmer was hospitalized at the Clinic for Infectious Diseases of the Clinical Centre Kragujevac on the fifth day of disease. The patient had a high temperature (over 39°C) at home for three days, accompanied by sore throat, muscle pain, extreme exhaustion and occasional dizziness. On the fourth day of the disease, his vertigo intensified and he complained of a headache at times, walked with assistance of others and developed a disturbed state of consciousness in the evening hours. The patient became confused, aggressive and uncommunicative, at which point he was referred to the neurologist of the General Hospital in Jagodina. Because his state of consciousness deepened towards the shallower state of somnolence, he was sent to the Clinic for Infectious Diseases in Kragujevac on suspicion of viral encephalitis.

On admission to the Clinic for Infectious Diseases, the patient was febrile (38.6°C), awake and disoriented in time and space and toward persons. He reacted to external stimuli but verbal communication was not established. His Glasgow Coma Scale score was 11, which indicates a moderate disturbance of the state of consciousness. The patient had tachypnea (respiratory rate 30/min.), cardiocirculatory stability, TA of 130/70 mmHg, and oxygen saturation of 94%. His pharynx was lightly hyperaemic and rare, bilateral inspiratory crackles were present in the lungs. Apart from a disturbed state of consciousness, other neurological as well as somatic results were within normal limits.

The following laboratory analyses were conducted on admission (reference values are given in parentheses): Er 5.46 x 10/l (4.00 - 6.00), Le 9.4 x 10/l (4.5-10.5), haemoglobin 148 g/l (110-180), Tr 240 x 10/1 (150-450), SE 18 / urine b.o., fibrinogen 3.281 g/l (2.0 to 5.0), CRP 18, 0 mg/1 (0.0-5.0), AST 128 IU/l (0-40), ALT 79 IU/l (0-40), CK 201 U/l (1-171), urea 3, 2 mmol/l (3.0-8.0), creatinine 79 µmoll /l (49-106), glycaemia mmol/l 5.9 / 1 (3.6-6.1), ionogram within the reference values, total protein 67 g/l (64-83), albumin 42 g/l (35-52), procalcitonin <0.05 ng/ml (0.5-2.0), Pt Rec. 13.9 s (10.0-14.09), ARTT SP 27.9 s (25.0 to 35.0), D-dimer 2651 ng/ml (0-250).

Endocranial examination was conducted by magnetic resonance, and no pathological changes were observed except for reductive changes. The patient was diagnosed with viral encephalitis and a lumbar puncture was conducted. Cerebrospinal fluid (CSF) was clear with increased pressure. CSF analysis results were as follows: no cellular elements, protein 1.3 mmol/l and glucose 3.6 mmol/l. Glycaemia of 5.9 mmol/l was observed in the serum. CSF was also taken for virological diagnosis.

In the afternoon, the patient experienced cramps in certain muscle groups, entered into a deepened stage of somnolence and had a Glasgow Coma Scale score of <8, which indicated severe disturbance of the state of consciousness. He was tachypneic (respiratory rate 40/min.) and his oxygen saturation dropped to 89% in spite of continuous oxygen therapy. An anaesthesiologist was consulted and the patient was transferred to the intensive care unit due to imminent respiratory failure. Although the

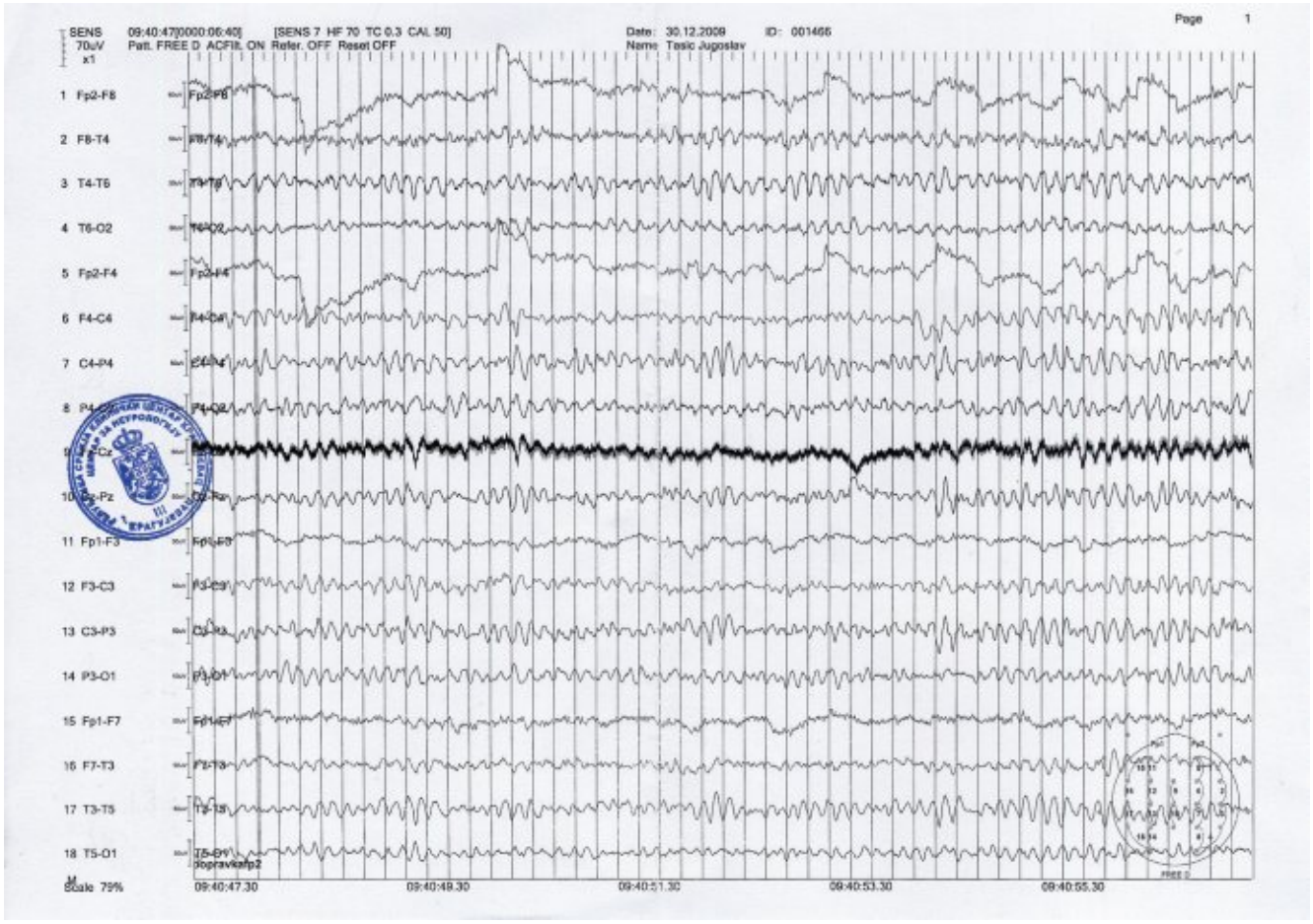

Figure 1. Electroecephalogram showed a di use slowing down of waves, particularly over the right hemisphere

clinical picture was not dominated by respiratory symptomatology and epidemiological data had not linked the patient to a case of H1N1, a swab of nasopharyngeal secretions on antigen A H1N1 was taken. With the reaction binding a complement with the antigen of the influenza A H1N1 virus, a positive titre of 1/32 was obtained. RT-PCR in the serum was performed on the same virus and a positive result was obtained. An electroencephalogram was performed and it showed a diffuse slowing down of waves, particularly over the right hemisphere (Figure 1). Oseltamivir, at a dose of 150 mg / 24 h, was immediately added to the current therapy (clarithromycin 500 mg 2x 1, ciprofloxacin amp. 200 mg / 12 h, amoxiclav amp. 1,2 gr / 8 h, durofilin caps. 125 mg / 12 h, verapamil 80 mg 2x1/2, antipyretics, and, v. re-hydration). Subsequently, there was a rapid stabilization of vital functions. The patient was afebrile and his cardiocirculatory status was stable with an oxygen saturation of 98% on 21 / min. of oxygen with a mask and a TA of 150/90 mmHg. However, the patient maintained a disturbed state of mind (verbal contact was not established, and the patient was anxious, turned his head and opened his eyes when called, executed orders, and reacted violently to external stimuli and manipulation). On both sides of the lungs, bronchial breath sounds were present with basilar crackles on both sides. With the exception of qualitative disturbances of consciousness, no other neurological disturbances were observed. CSF taken for viral diagnosis was negative for herpes simplex virus, parainfluenza virus, influenza B virus and Mycoplasma pneumoniae.

The patient responded favourably to treatment with gradual stabilization of the state of consciousness. On the eighth day of hospitalization, the patient was conscious and oriented to time and space and towards personalities. Additionally, verbal communication was adequate. The patient was discharged from the hospital on the fifteenth day of illness in good general condition and with a satisfactory state of consciousness, laboratory results within the reference values and proper chest radiography results. A repeat electroencephalogram was completed and the result was within normal values.

DISCUSSION

Respiratory infections caused by influenza A and B viruses can cause neurological manifestations of influenza disease. However, neurological manifestations caused by influenza A H1N1 virus are very rare (9). The pandemic wave that swept through a large number of countries noted a wide variety of clinical manifestations of H1N1 infection, which is the result of its antigenic instability. The

highest percentage of patients had respiratory symptoms that ranged from mild upper respiratory tract infection to severe viral interstitial pneumonia. However, the patient presented in this report had no respiratory symptoms. In this case, the overall clinical presentation was dominated by neurological symptoms, leading to suspicion of encephalitis caused by another aetiologic agent. Epidemiological data (absence of contact) did not indicate that infection with H1N1 virus was in question. However, laboratory analysis, chest radiography and CSF results indicated that this was a generalized viral infection. Serodiagnosis confirmed that infection with H1N1 virus was in question, but the presence of the virus was not detected in the cerebrospinal fluid. Serological responses in the cerebrospinal fluid were negative for herpes virus, influenza virus B, parainfluenza virus and Mycoplasma pneumoniae. The results of electroencephalograms and magnetic resonance examination of the endocranium also provided evidence of a generalized viral infection. The patient responded well to treatment, resulting in an improvement and stabilization of the state of consciousness on the eighth day of hospitalization. He was discharged from hospital without any consequences.

Our results correlated with data from the literature. Since the beginning of the pandemic wave caused by influenza A H1N1 virus, a rather small number of works which describe neurological manifestations caused by this virus have been published. A group of researchers from a national centre for diagnosing, monitoring and controlling infectious diseases in Texas reported seven cases of H1N1 infection associated with neurological complications (10, 11). The virus was isolated from nasopharyngeal secretions but not from CSF. This finding correlates with our data because the presence of H1N1 virus was not established in our patient's CSF. All diseased patients had severe symptoms of respiratory tract infection that were not observed in our patient. CSF results suggested a generalized viral infection that our patient exhibited. In all seven cases, the clinical picture was dominated by a disturbed state of consciousness (from confusion to somnolence) with the absence of focal neurologic disorders. These clinical manifestations were present in our patient as well. In all seven patients, the electroencephalogram result was obtained and showed a diffuse pathological slowing down, and magnetic resonance examination of the endocranium was within normal limits. All patients were treated with oseltamivir, and all were healed without consequences.

A group of Japanese authors described the neurological manifestations caused by influenza A H1N1 virus in children (12). Children are more often infected with the H1N1 virus than adults (13). All affected children were previously healthy and had flu-like symptoms. Shortly after the appearance of respiratory symptoms, focal neurologic disorders occurred accompanied by disturbances of the state of consciousness. These neurological manifestations were commonly associated with the second or third day of disease and were correlated with the general difficult condition of the disease. Such a course of the disease in children in Japan left a number of neurological consequences and often led to death (14). It was described that in Japan, approximately 20% of children under 5 years of age who were infected with influenza A H1N1 also developed encephalopathy (13). Typical neurological signs developed 1-2 days after the onset of symptoms of influenza. Manifestations included psychotic behaviour, irritability, loss of consciousness and epilepsy seizures. In these children a complete recovery occurred in 50% of cases, while in 20% of cases the outcome was fatal.

CONCLUSION

Studies originating from the United States, Japan and some other countries provide data on the neurological manifestations of infection with influenza A H1N1 virus, while there is a very small number of data published in our country. Because our country is in a whirlwind of pandemic H1N1 virus activity, we should think of every possible manifestation that this virus can produce regardless of the epidemiological data and the clinical picture.

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An abstract of less than 250 words should concisely state the objective, findings, and conclusions of the studies described in the manuscript. The abstract does not contain abbreviations, footnotes or references.

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The introduction is concise, and states the reason and specific purpose of the study.

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ACKNOWLEDGMENTS

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Both letters concerning and those not concerning the articles that have been published in Serbian Journal of Experimental and Clinical Research will be considered for publication. They may contain one table or figure and up to five references.

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All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.

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