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TOXIC EFFECTS OF METALLOPHARMACEUTICALS

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TOKSIČNI EFEKTI METALOFARMACEUTIKA

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ABSTRACT

Discovery of the metallopharmaceutical cisplatin and its use in antitumour therapy has initiated the rational design and screening of metal-based anticancer agents as potential chemotherapeutics. In addition to the achievements of cisplatin and its therapeutic analogues, there are significant drawbacks to its use: resistance and toxicity. Over the past four decades, numerous transition metal complexes have been synthesized and investigated in vitro and in vivo. The most studied metals among these complexes are platinum and ruthenium. The key features of these investigations is to find novel metal complexes that could potentially exert less toxicity and equal or higher antitumour potency and to overcome other pharmacological deficiencies. Ru complexes have a different mode of action than cisplatin does, some of which are under clinical trials for treating metastatic or cisplatin-resistant tumours. This review consists of the current knowledge, published and unpublished, related to the toxicity of metallopharmaceuticals, and special attention is given to platinum [Pt(II) and Pt(IV)] and ruthenium [Ru(II) and Ru(III)] complexes.

Key words: *antitumor therapy, metallopharmaceuticals, platinum complexes, ruthenium complexes, toxicity*

SAŽETAK

Otkrićem cisplatine, metalofarmaceutika kojime se postigao ogroman uspeh u terapiji tumora započeo je process racionalnog dizajna i ispitivanja agenasa na bazi metala kao potencijalnih citostatika za hemijoterapiju. Pored navedenog uspeha cisplatina i njeni terapijski analozi ispoljili su značajne nedostatke: pojava rezistentnosti i toksičnost. U poslednjih četiri decenije veliki broj kompleksa prelaznih metala je sintetisan i ispitivan in vitro i in vivo. Najviše proučavani prelazni metali su platina i rutenijum. Ključni cilj ovih istraživanja ogleda se u izvođenju novog metalofarmaceutika sa smanjenom toksičnošću, istim ili jačim antitumorskim dejstvom i prevaziđenim ostalim farmakološkim nedostacima. Kompleksi rutenijuma poseduju drugačiji mehanizam dejstva u poređenju sa cisplatinom a neki se ispituju u kliničkim studijama za lečenje metastaza tumora koji su rezistentni prema cisplatini. Ova revija opisuje trenutna (publikovana i ne publikovana) saznanja koja se odnose na toksičnost metalofarmaceutika, pri čemu je posebna pažnja posvećena kompleksima platine [Pt(II) i Pt(IV] i rutenijuma [Ru(II) i Ru(III)].

Ključne reči: : *antitumorska terapija, metalofarmaceutici, kompleksi platine, kompleksi rutenijuma, toksičnost*

The wide use of metallopharmaceuticals in contemporary oncology dates to the discovery of cisplatin by Rosenberg and coworkers in 1965 (1). This discovery opened the gate to the unexplored world of metal-based chemotherapeutic agents, which have different pharmacokinetic, pharmacodynamic and pharmacological mechanisms of action than do conventional organic drugs (2). Today, there are many successful metallopharmaceuticals that are primarily used in clinical trials not just to treat cancer but to fight a range of diseases, including parasitic and bacterial infections (3). Over the past several decades, several cisplatin analogues have been screened as potential antitumour agents, but of these, only two (carboplatin and oxaliplatin) have entered worldwide clinical use (4). The clinical use of

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these agents is severely limited by their toxic side effects. In addition to platinum, special attention over the past several decades was paid to many ruthenium complexes because of their potential low toxicity. Numerous ruthenium complexes have been evaluated for clinical applications, particularly in the treatment of cancer due, in part, to the fact that Ru(II) and Ru(III) complexes exhibit a similar spectrum of kinetics for their ligand substitution reactions as Pt(II) complexes do (5). The representative group of cytotoxic Ru compounds are Ru(II) arene complexes, which were developed primarily by Dyson and coauthors (5) and Sadler and coauthors (6), although none of these compounds has yet entered clinical trials.

Toxicities

There are growing interests in designing new metallopharmaceuticals that are capable of overcoming the problems of clinically used drugs while maintaining their efficacy. The main goal is to reduce systemic toxicity and increasing the spectrum of activity. The toxicities associated with metallopharmaceuticals such as platinum and ruthenium complexes range from mild to severe. The most common and serious toxicities of these complexes are nephrotoxicity, neurotoxicity, ototoxicity and vascular toxicity (7,8).

Nephrotoxicity

Nephrotoxicity is associated with cisplatin treatment but is rare with the later-generation analogues carboplatin or oxaliplatin (9,10). Because cisplatin nephrotoxicity is stereospecific to the cis and not the trans isomer, the platinum atom is not the proximate nephrotoxicant. It is likely that a metabolite of cisplatin, possibly an aquated and/or hydroxylated complex, mediates the nephrotoxicity of cisplatin (11). The nephrotoxic effect of cisplatin appears to be related to its preferential uptake by the proximal tubular cells of the inner cortex and outer medulla, especially in the S3 segment. Other segments of the renal tubule also accumulate cisplatin, although to a lesser extent, and their damage may contribute to clinical nephrotoxicity (12). The persistent reduction (20% to 30%) in glomerular filtration found in long-term follow studies indicates that these cisplatin-induced changes are irreversible (13,14). Some investigators have reported that the severity of persistent renal impairment is correlated with the dose of cisplatin applied (14,15).

Based on current research, it is known that ruthenium complexes also show toxic effect on kidneys. However, a study by Kersten and coworkers suggested that compared to cisplatin, proteinuria was significantly lower after the administration of any of three ruthenium coordination complexes (KP418, KP692, KP1019) in rats (16).

Peripheral neuropathy (neurotoxicity)

The peripheral neuropathy was observed in patients with testicular cancer, and this is mainly attributed to cisplatin. The primary target of cisplatin-induced damage in the central nervous system is the dorsal root ganglion of the spinal cord (17). The most frequent clinical signs of neurotoxicity are paraesthesia, dysesthesia, disturbances of position, vibratory sensations and relative sparing of motor units, which disappear in most cases after chemotherapy (18). Carboplatin is significantly less neurotoxic than cisplatin in conventional doses, but high doses of carboplatin are associated with sensory ataxia soon after treatment (19). In contrast, oxaliplatin neuropathy has a wide spectrum, ranging from an acute sensory neuropathy immediately following treatment to a chronic, dose-limiting neuropathy that usually takes several weeks of treatment to appear (20). Motor dysfunctions were associated with low serum levels of magnesium and can be managed by treatment with calcium gluconate or magnesium sulfate before and following treatment (21). Additionally, vitamin E has been shown to be decrease sensory neuropathy in patients treated with cisplatin (22). Because there are almost no previous studies, to the best of our knowledge, that investigate the neurotoxicity of ruthenium complexes, it is important that future experimental research provide information about this type of toxicity.

Ototoxicity

The incidence of ototoxicity established by audiometric techniques is approximately 20% to 40% (17,22,23). Higher bolus doses of metallopharmaceuticals, especially cisplatin, have been shown to be more ototoxic and nephrotoxic than repeated infusion at lower doses in adults. Conversely, prolonged infusions in children are less nephrotoxic than bolus doses are (24,25). Cisplatin-induced ototoxicity depends on more than the dose, as there are marked interindividual variations in toxicity in patients receiving similar cumulative doses of this agent. Other factors are considered important, and it has been hypothesized that genetic variation may be a key component in determining a patient's susceptibility to the effects of cisplatin (23). Ototoxicity is probably caused by cisplatin damage to the secretory mechanism of the organ of corti and manifests as high-frequency hearing loss and tinnitus (26). Ototoxicity observed with platinum complexes may be acute or delayed and irreversible, and no preventive treatments are available (27). In the literature, there is no clear evidence about the ototoxicity of ruthenium complexes, which was expected because as mentioned above, these complexes are not yet in clinical use.

Vascular toxicity

Vascular toxicity occurs in approximately 3% to 49% of patients, and one of the most common manifestations after treatment with metallopharmaceuticals is Raynaud's syndrome, a clinical consequence of small-vessel disease (28,29). Studies that used provocative testing suggested that even asymptomatic patients might exhibit a

vasospastic response to cold stimuli (29). Literature data suggest that it is a possible delayed onset, with a median time of 10 months after chemotherapy (30). Ruthenium complexes are still not approved for clinical use, so there are no reports about vascular toxicity to these metallopharmaceuticals.

The toxicities of metallopharmaceuticals are probably a result of the increased production of reactive oxygen species. In the literature data, there is evidence to support a role of metallopharmaceutical induced oxidative stress in each of these adverse effects (31,32). Both *in vitro* and *in vivo*, cisplatin has been shown to increase oxidative stress by increasing the levels of different free radicals (31,33). Additionally, some of the examined ruthenium complexes lead to increased cellular oxidative stress and promote cell death *via* apoptosis (34).

CONCLUSION

A vast number of metallopharmaceuticals has been evaluated as antitumour agents, but only a very small fraction has shown sufficient promise during preclinical evaluation to enter human clinical trials. It is believed that ruthenium complexes will demonstrate significant clinical advantages over the current platinum complexes (36). Considering the toxic potential of metallopharmaceuticals, further experimental studies and careful clinical monitoring during treatment are necessary to overcome this problem. Thus, efforts should be focused on designing more selective metallopharmaceuticals that possess the ability to overcome resistance and toxic side effects.

Oxidative stress is probably one of the molecular mechanisms in the development of toxicity induced by the administration of platinum or ruthenium complexes. Understanding the individual differences of metallopharmaceuticals and the potential for redox effects to manifest as toxicities is increasingly valuable, not only for existing therapies but also for tailoring clinical metal complex development. In addition to the design and screening of new metallopharmaceuticals, extensive efforts should be directed towards investigating their molecular mechanisms of action.

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SYNTHESIS AND CHARACTERIZATION OF PLATINUM(IV)-COMPLEXES WITH S-ALKYL DERIVATIVES OF THIOSALICYLIC ACID AND THE CRYSTAL STRUCTURE OF THE S-BUTYL DERIVATIVE OF THIOSALICYLIC ACID

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SINTEZA I KARAKTERIZACIJA PLATINA(IV)-KOMPLEKSA SA S-ALKIL DERIVATIMA TIOSALICILNE KISELINE. KRISTALNA STRUKTURA S-BUTIL DERIVATA TIOSALICILNE KISELINE

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ABSTRACT

*New platinum(IV)-complexes with S-alkyl derivatives of thiosalicylic acid (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) have been synthesized and characterized by microanalysis, infrared spectroscopy, and ^{1}H and ^{13}C NMR spectroscopy. The bidentate S,O ligand precursor, the S-butyl derivative of thiosalicylic acid (S-bu-thiosal), was prepared, and its crystal structure was determined. Single crystals suitable for X-ray measurements were obtained by slow crystallization from a DMSO-water system. S-bu-thiosal crystallized in a $P2_1/c$ space group of a monoclinic crystal system with a = 8.0732 (3) Å, b = 19.6769 (4) Å, c = 8.2291 (3) Å and Z = 4. S-bu-thiosal also has a coplanar geometry.*

Keywords: *S-alkyl derivative of thiosalicylic acid, platinum(IV)-complexes, IR and NMR spectroscopy, crystal structure*

SAŽETAK

*Novi platina(IV)-kompleksi sa S-alkil derivatima tiosalicilne kiseline (alkil = benzil-(**L1**), metil-(**L2**), etil-(**L3**), propil-(**L4**), butil-(**L5**)) su sintetisani i okarakterisani na osnovu rezultata mikroanalize, infracrvene i ^{1}H i ^{13}C NMR spektroskopije. Sintetisan je bidentatni S, O ligand prekursor, S-butil derivat tiosalicilne kiseline, (S-bu-thiosal), i ispitivana je njegova kristalna struktura. Kristali nagrađenog jedinjenja pogodni za rendgensku strukturnu analizu dobijeni su sporom kristalizacijom iz sistema DMSO-voda. Navedeni ligand kristališe u prostornoj grupi $P2_1/c$ monokliničkog kristalnog sistema sa dimenzijama jedinične ćelije a = 8,0732 (3) Å, b = 19,6769 (4) Å, c = 8,2291 (3) Å i Z = 4. Molekul navedenog jedinjenja poseduje koplanarnu strukturu.*

Ključne reči: *S-alkil derivati tiosalicilne kiseline, platina(IV)-kompleksi, IR i NMR spektroskopija, kristalna struktura*

ABBREVIATIONS

DNA - deoxyribonucleic acid
DMSO-d_6 - deuterated dimethyl sulfoxide
IR - infrared
K_2PtCl_6 - potassium-hexachloroplatinate(IV)
LiOH - lithium hydroxide
NMR - nuclear magnetic resonance
Pt - platinum
Ras - rat sarcoma proteins
TMS - tetramethylsilane

INTRODUCTION

Recent studies have shown important progress towards the use of transition metal complexes as drugs for the treatment of various human disorders. In the past, platinum-based drugs, mainly cisplatin and carboplatin, have dominated the treatment of various types of cancers by chemical agents because of their pharmacological properties. Relationships between structure and activity for a class of platinum coordination compounds confirmed that only those compounds having *cis* geometry block cell growth. The most active complex, cisplatin, was found to exhibit antitumor activity, while its *trans* isomer showed no such activity. Many derivatives of cisplatin also inhibit

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tumor cell growth, and these compounds have at least one N-H group that is responsible for important hydrogen-bond donor properties. However, the clinical utilization of cisplatin has often been limited by its severe side effects. Furthermore, platinum(II)-based drugs are associated with high reactivity and thus, poor biological stability (1-3).

After the discovery and use of platinum(II)-complexes, research has been directed towards complexes of platinum(IV), primarily due to the adverse effects of platinum(II)-complexes. The platinum(IV)-complexes display potential advantages due to their greater stability and bioreductive activation, thereby allowing for a greater proportion of the drug to arrive at the target intact. Currently, attention is focused on platinum(IV)-complexes with bioactive ligands because of lower toxicity, the possibility of oral administration, and the fact that they can coordinate to DNA (4). From the studies of platinum complexes in different cancer cell lines and DNA binding studies, some important structure activity rules have previously been summarized. The three important factors in designing platinum drugs appear to be chain length and flexibility, hydrogen bonding capacity, and charge of linking chain and the geometry of the chloro ligands to the linking chain (5). Two compounds of Pt(IV), iproplatin and ormaplatin, have undergone clinical trials. However, these compounds were abandoned due to severe neurotoxicity in the case of ormaplatin and the lack of superior performance in the case of iproplatin (6). Furthermore, numerous new complexes based on the platinum(IV)-ion have been synthesized, and their antitumor activities have been documented (7-9).

Thiosalicylic acid and its derivatives are used in cosmetics, for reducing hair growth, and for treatment of inflammatory, allergic and respiratory diseases. Farnesyl thiosalicylic acid, a novel Ras inhibitor, dislodges Ras proteins from the cell membrane, leading to inhibition of cell transformation and tumor growth. Ethyl mercury covalently linked to thiosalicylate, known as thimerosal, has been extensively used as a preservative in vaccines (10-13).

Our investigations presented in this paper focus on the synthesis and characterization of the corresponding Pt(IV)-complexes with S-alkyl derivatives of thiosalicylic acid. The preparation and spectral characterization of S-alkyl derivatives of thiosalicylic acid have been previously published (14,15). The structures of the isolated complexes are proposed based on elemental microanalysis, and infrared and nuclear magnetic resonance spectra. The bidentate S,O ligand precursor, S-butyl derivative of thiosalicylic acid (S-bu-thiosal), was prepared, and its crystal structure was determined and presented in this paper.

MATERIALS AND METHODS

Materials and measurements

All chemicals were obtained commercially and used without further purification. Elemental microanalyses were performed on a Vario III CHNOS Elemental Analyser, Elemental Analysensysteme GmbH. For the infrared spectra, a Perkin-Elmer Spectrum One FT-IR spectrometer was employed. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 NMR spectrometer using TMS in DMSO-d_6 as an internal reference at 22°C and with 10 mM solutions of the complexes.

Syntheses

General procedure for the synthesis of S-alkyl derivatives of thiosalicylic acid (L1)-(L5)

S-alkyl derivatives of thiosalicylic **L1-L5** (benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) were prepared (16) via alkylation of thiosalicylic acid by adding the alkyl halogenides to an alkaline water-ethanol solution. A crystal of the S-butyl derivative of thiosalicylic acid suitable for X-ray analysis was obtained after slow crystalization from a DMSO-water system.

Preparation of [$PtCl_2$(S-bz-thiosal)$_2$] (C1), a platinum(IV)-complex with the S-benzyl derivative of thiosalicylic acid

K_2PtCl_6 (0.1000 g, 0.2056 mmol) was dissolved in 10 mL of water in a steam bath, and the S-benzyl derivative of thiosalicylic acid (0.1005 g, 0.4112 mmol) was added to the solution. The resulting mixture was stirred for 2 h, and during this time, an aqueous solution LiOH (0.0099 g, 0.4112 mmol in 10 mL of water) was introduced. The complex [$PtCl_2$(S-bz-thiosal)$_2$] (C1) formed a yellow precipitate and was filtered, washed with water and air-dried, with a yield of 0.15 g (58.80%). Anal. Calc. for [$PtCl_2$(S-bz-thiosal)$_2$]=$PtC_{28}H_{22}O_4S_2Cl_2$ (Mr=752.59): C, 44.68; H, 2.95; S, 8.52. Found: C, 44.26; H, 2.88; S, 8.60. IR (KBr, cm^{-1}): 3437, 3062, 3028, 2924, 1629, 1561, 1493, 1463, 1412, 1318, 1254, 1142, 1046, 868, 799, 750, 697, 667, 652, 552. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 4.01 (s, 4H, CH_2), 7.23-8.24 (m, 18H, Ar и bz). ^{13}C NMR (50 MHz, DMSO-d_6,δ ppm): 17 (CH_2), 124; 125.5; 127.1; 127.4; 127.7; 133.1; 133.9; 136.2 (Ar и bz); 169.1 (COO^-).

Preparation of [$PtCl_2$(S-met-thiosal)$_2$] (C2), a platinum(IV)-complex with the S-methyl derivative of thiosalicylic acid

The complex [$PtCl_2$(S-met-thiosal)$_2$] (**C2**) was prepared as described using the S-methyl derivative of thiosalicylic acid (0.0692 g, 0.4112 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.12 g (56.40%). Anal. Calc. for [$PtCl_2$(S-met-thiosal)$_2$] = $PtC_{16}H_{14}O_4S_2Cl_2$ (Mr=600.40): C, 32.00; H, 2.35; S, 10.68. Found: C, 31.54; H, 2.59; S, 10.22.IR (KBr, cm^{-1}): 3436, 2923, 2794, 2439, 1634, 1581, 1552, 1469, 1423, 1361, 1290, 1274, 1149, 1116, 1056, 970, 858, 798, 754, 697, 653, 568. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.47 (s, 6H, CH_3), 7.41-8.30 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 15.6 (CH_3), 125; 125.5; 126.4; 129.9; 134.1; 137.2 (Ar), 169.3 (COO^-).

Preparation of [$PtCl_2$(S-et-thiosal)$_2$] (C3), a platinum(IV)-complex with the S-ethyl derivative of thiosalicylic acid

The complex [$PtCl_2$(S-et-thiosal)$_2$] (**C3**) was prepared as described using the S-ethyl derivative of thiosalicylic acid (0.0749 g, 0.4112 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.13 g (58.10%). Anal. Calc. for [$PtCl_2$(S-et-thiosal)$_2$] = $PtC_{18}H_{18}O_4S_2Cl_2$ (Mr=828.46): C, 34.40; H, 2.89; S, 10.20. Found: C, 34.14; H, 2.71; S, 10.11. IR (KBr, cm^{-1}): 3436, 2521, 1692, 1634, 1563, 1437, 1404, 1274, 1143, 1122, 1050, 997, 872, 794, 749, 693, 643, 568. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 1.27 (t, 6H, CH_3), 2.81 (q, 4H, CH_2), 7.42-8.28 (m, 8H, Ar).^{13}C NMR (50 MHz, DMSO-d_6,δ ppm):12.9 (CH_3), 13.8 (CH_2), 125.1; 126.4; 126.6; 133.3; 133.8; 137.1 (Ar), 169.2 (COO^-).

Preparation of [$PtCl_2$(S-pr-thiosal)$_2$] (C4), a platinum(IV)-complex with the S-propyl derivative of thiosalicylic acid

The complex [$PtCl_2$(S-pr-thiosal)$_2$] (**C4**) was prepared as described using the S-propyl derivative of thiosalicylic acid (0.0807 g, 0.4112 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.13 g (56.90%). Anal. Calc. for [$PtCl_2$(S-pr-thiosal)$_2$] = $PtC_{20}H_{22}O_4S_2Cl_2$ (Mr=656.51): C, 36.59; H, 3.38; S, 9.77. Found: C, 36.17; H, 3.30; S, 9.61. IR (KBr, cm^{-1}): 3444, 3061, 2963, 2930, 2873, 2600, 1706, 1639, 1586, 1562, 1461, 1436, 1416, 1293, 1253, 1138, 1091, 1052, 863, 798, 753, 691, 652. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 0.90 (t, 6H, CH_3), 1.34 (m, 4H, CH_2), 2.75 (t, 4H, CH_2), 7.40-8.31 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 13.0 (CH_3), 23,2 (CH_2), 12 (CH_2), 125.3; 126.1; 126.4; 133.2; 133.9; 136.9 (Ar), 169.1 (COO^-).

Preparation of [$PtCl_2$(S-bu-thiosal)$_2$] (C5), a platinum(IV)-complex with the S-butyl derivative of thiosalicylic acid

The complex [$PtCl_2$(S-bu-thiosal)$_2$] (**C5**) was prepared as described using the S-butyl derivative of thiosalicylic acid (0.0865 g, 0.4112 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.14 g (59.30%). Anal. Calc. for [$PtCl_2$(S-bu-thiosal)$_2$] = $PtC_{22}H_{26}O_4S_2Cl_2$ (Mr=684.56): C, 38.60; H, 3.83; S, 9.37. Found: C, 38.35; H, 3.71; S, 9.28. IR (KBr, cm^{-1}): 3437, 3054, 2956, 2931, 2869, 2629, 1673, 1644, 1635, 1583, 1561, 1462, 1433, 1410, 1318, 1286, 1250, 1137, 1100, 1060, 1049, 916, 863, 754, 738, 698, 652, 551. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 0.89 (t, 6H, CH_3), 1.43 (m, 4H, CH_2), 1.60 (m, 4H, CH_2), 2.73 (t, 4H, CH_2), 7.41-8.28 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 13.4 (CH_3), 21.5 (CH_2), 31(CH_2), 10 (CH_2), 124.9; 125.9; 126.5; 133.1; 134.2; 136.9 (Ar), 168.9 (COO^-).

X-ray crystal structure determination

A single crystal of S-butyl thiosalicylic acid was selected and mounted on a glass fibre. Diffraction data were collected using the Oxford Diffraction Gemini S four-circle goniometer equipped with a Sapphire CCD detector. The crystal to detector distance was 45.0 mm, and graphite monochromated CuKα (λ = 1.5418 Å) radiation was used for the experiments. The data were reduced using the program CrysAlisPRO (17). A semi-empirical absorption-correction, based upon the intensities of equivalent reflections, was applied, and the data were corrected for Lorentz, polarization, and background effects (17). The structure was solved by direct methods using the Sir 97 program (18) and refined by full-matrix least-squares procedures on F^2 using SHELXL-97 programs (19) as implemented in the WinGX program suite (20). The non-H atoms were refined anisotropically. The positions of hydrogen atoms were found from the inspection of the difference Fourier maps. Crystallographic data and refinement parameters are listed in Table 1. The figures representing molecular structure were created using the ORTEP-3 (21) and PLATON (22) programs.

RESULTS AND DISCUSSION

Synthesis and chemical characterization

S-alkyl (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) derivatives of thiosalicylic acid were prepared (16) via alkylation of thiosalicylic acid by addition of the corresponding alkyl halogenides to an alkaline water-ethanol solution (Scheme 1).

Platinum(IV)-complex with S-alkyl derivatives of thiosalicylic acid, [$PtCl_2$(S-alkyl-thiosal)$_2$], were obtained via the direct reaction of K_2PtCl_6 with the S-alkyl derivatives of thiosalicylic acid (in a molar ratio of 1:2) in water (Scheme 2).

Bidentate coordination (S-O) of S-alkyl derivatives of thiosalicylic acid to the platinum(IV)-ion is expected. In the infrared spectrum of isolated ligands (15), we observed valencione asymmetric vibrations of the carboxyl groups at lower values than expected (from 1700 to 1750 cm^{-1}) (23-25), which could be explained by the presence of large R-S groups in the *ortho* position. The positions of these bands in the corresponding complexes (C1-C5) are located in the expected region (1600 to 1650 cm^{-1}), which confirms their deprotonation and coordination to the metal ion (Table 2).

The chemical shifts of hydrogen and carbon atoms of the obtained S-alkyl derivatives of thiosalicylic acid and the corresponding platinum(IV)-complexes were found to be almost the same as the expected chemical shifts. We observed only slight differences in the chemical shifts of the carbon atoms of the carboxyl group of the S-alkyl derivative of thiosalicylic acid and the corresponding platinum(IV)-complexes. These differences in the chemical shifts of the carboxyl group may be explained by the coordination of the ligands over the oxygen atom of the carboxyl group to the platinum(IV)-ion.

Based on the IR and NMR spectra of the ligands and the corresponding Pt(IV)-complexes, we concluded that the ligands are bidentately coordinated to the platinum(IV)-ion. However, based on the mentioned spectroscopic results,

Table 1. Experimental details: Crystallographic data and refinement parameters.

Crystal data	
Chemical formula	$C_{11}H_{14}O_2S$
M_r	210.28
Crystal system, space group	Monoclinic, $P2_1/c$
Temperature (K)	293
a, *b*, *c* (Å)	8.0732 (3), 19.6769 (4), 8.2291 (3)
b (°)	119.084 (5)
V (Å^3)	1142.40 (7)
Z	4
Radiation type	Cu *K*a
No. of reflections for cell measurement	4178
q range (°) for cell measurement	4.5–72.2
m (mm^{-1})	2.30
Crystal size (mm)	0.33 × 0.28 × 0.21
Data collection	
Diffractometer	Xcalibur-Gemini S diffractometer
Absorption correction	Multi-scan *CrysAlis PRO*, Agilent Technologies, Version 1.171.36.24 (release 03-12-2012 CrysAlis171 .NET) (compiled Dec 3 2012,18:21:49) Empirical absorption correction using spherical harmonics, implemented in SCALE3 ABSPACK scaling algorithm.
T_{min}, T_{max}	0.557, 1.000
No. of measured, independent and observed [$I > 2s(I)$] reflections	6832, 2038, 1898
R_{int}	0.020
$(\sin q/l)_{max}$ (Å^{-1})	0.597
Refinement	
$R[F^2 > 2s(F^2)]$, $wR(F^2)$, *S*	0.035, 0.100, 1.06
No. of reflections	2038
No. of parameters	183
No. of restraints	0
H-atom treatment	H atoms treated by independent refinement
ρ_{max}, ρ_{min} (e Å^{-3})	0.20, -0.16

Computer programs: *CrysAlis PRO*, Agilent Technologies, Version 1.171.36.24 (release 03-12-2012 CrysAlis171 .NET) (compiled Dec 3 2012,18:21:49), *SIR* 97 (Altomare *et al.* (1999) J. Appl. Cryst. 32, 115-119), *SHELXL97* (Sheldrick, 1997).

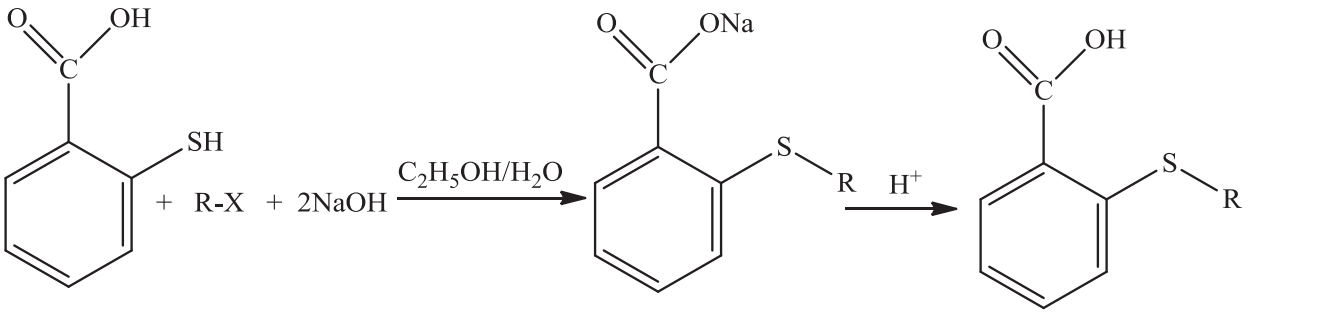
we could not conclude anything about the complex geometry.

In a previously published study of palladium(II)-complexes with S-alkyl derivatives of thiosalicylic acid (15), we have confirmed a square-planar structure with *cis*-O *cis*-S geometry of two bidentate ligands in a coordinated sphere of the metal ion. Based on these results, we can expect that octahedral platinum(IV)-complexes also contain two molecules of S-alkyl derivatives of thiosalicylic acid in the equatorial plane with *cis*-O *cis*-S geometry and two axial monodentate anionic ligands.

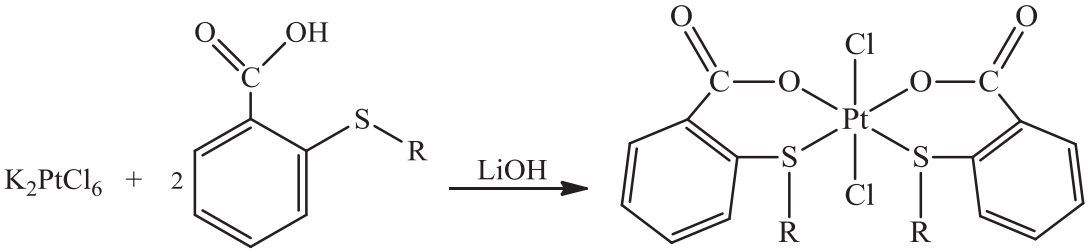
Crystal structure of the S-butyl derivative of thiosalicylic acid

S-butyl-thiosalicylic acid was prepared by the alkylation of thiosalicylic acid using the corresponding alkyl halogenide in an alkaline water-ethanol solution. The lack of S-H stretching absorption bands in the range of 2600-2550 cm^{-1} (2556 cm^{-1}) suggests the deprotonation of the S-H group in thiosalicylic acid and its alkylation with a butyl group (26). The carboxylate asymmetric stretching band from S-butyl-thiosalicylic acid (1674 cm^{-1}) is located at a lower energy range than expected (1700–1750 cm^{-1}) (26, 27). This fact could be explained by the presence of a large S-butyl group in the *ortho* position with a -COOH group. Chemical shifts arising from carbon and hydrogen atoms of this type of thioether were found at the expected positions.

The perspective view of the molecular structure of the title compound ($C_{11}H_{14}O_2S$) is shown in Figure 1. The arrangement of the molecules in the unit cell is shown in Figure 2, where broken lines represent hydrogen bonds that connect the molecules of each dimer.



Scheme 1. The preparation of the S-alkyl derivatives of thiosalicylic acid; R= benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**).



Scheme 2. The preparation of the platinum(IV)-complexes with S-alkyl derivatives of thiosalicylic acid ; R= benzyl-(**C1**), methyl-(**C2**), ethyl-(**C3**), propyl-(**C4**), butyl-(**C5**).

As expected, S-butyl thiosalicylic acid crystalizes in the same monoclinic crystal system and $P2_1/c$ space group as was found for the crystal form of benzoic acid (28). The bond lengths and angles in the title compound are within the expected ranges (Table 3). The differences between C-C bonds in the benzene ring are in the range of 0-0.035 Å, but displacement of the benzene carbon atoms is not significant, suggesting that the ring can be assumed as strictly planar. The dihedral angle between the thiosalicylic and butyl groups is -179.50(11)°, indicating a co-planar molecular geometry.

Due to the intermolecular hydrogen bonds between carboxylic groups, a dimeric form of this structure is also expected. The hydrogen bond distance of 2.634(3) in the crystal form of S-butyl thiosalicylic acid is very similar to the same distances in, for example, benzoic acid (2.64 Å)

Table 2. The most important infrared bands (cm^{-1}) of the investigated compounds.

Compound	-COO- (as)
$[PtCl_2(S\text{-bz-thiosal})_2]$ (**C1**)	1629
$[PtCl_2(S\text{-met-thiosal})_2]$ (**C2**)	1634
$[PtCl_2(S\text{-et-thiosal})_2]$ (**C3**)	1634
$[PtCl_2(S\text{-pr-thiosal})_2]$ (**C4**)	1639
$[PtCl_2(S\text{-bu-thiosal})_2]$ (**C5**)	1644,1635

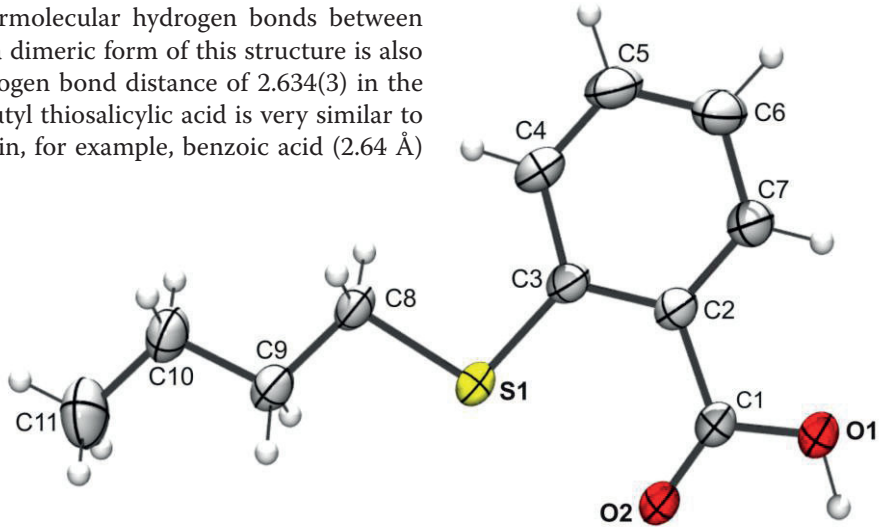


Figure 1. Molecular structure of compound $C_{11}H_{14}O_2S$ with the non-H atom numbering scheme with thermal ellipsoids at 30% probability level.

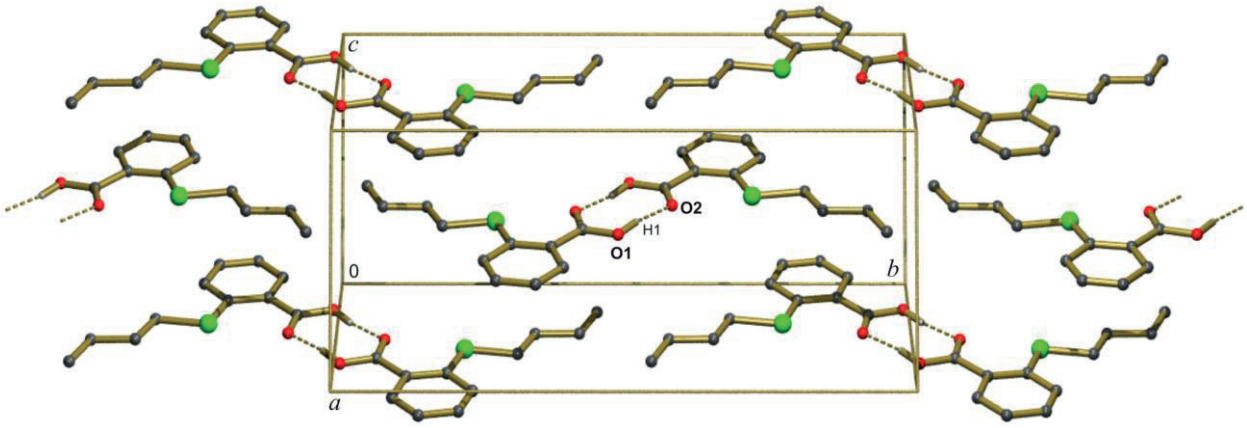


Figure 2. PLATON drawing showing crystal packing for $C_{11}H_{14}O_2S$. Intermolecular hydrogen bonds O1-H1....O2 are shown as dashed line. H atoms not involved in hydrogen bonds are not shown for clarity.

Table 3. Selected geometrical parameters for S-butyl derivative of thiosalicylic acid.

Bond lengths [Å]		Bond angles [°]	
S1—C3	1.7593 (18)	C3—S1—C8	103.82 (7)
S1—C8	1.8169 (15)	O2—C1—O1	122.10 (13)
O2—C1	1.216 (2)	O2—C1—C2	123.63 (13)
O1—C1	1.3088 (18)	O1—C1—C2	114.26 (13)
C1—C2	1.479 (2)	C7—C2—C3	120.33 (13)
C2—C7	1.399 (2)	C7—C2—C1	117.66 (14)
C2—C3	1.411 (2)	C3—C2—C1	122.01 (13)
C3—C4	1.404 (2)	C9—C8—S1	107.70 (10)
C4—C5	1.370 (3)	C6—C7—C2	121.00 (17)
C6—C5	1.364 (3)	C11—C10—C9	113.4 (2)
C8—C9	1.513 (3)	C4—C3—C2	116.58 (15)
C7—C6	1.376 (2)	C4—C3—S1	121.52 (13)
C10—C9	1.515 (2)	C2—C3—S1	121.89 (10)
C10—C11	1.507 (4)	C8—C9—C10	112.42 (14)
		C5—C6—C7	118.81 (18)
		C5—C4—C3	121.56 (17)
		C6—C5—C4	121.68 (16)

Torsion angles [°]	
O2—C1—C2—C7	-179.10 (16)
O1—C1—C2—C7	0.8 (2)
O2—C1—C2—C3	0.8 (2)
O1—C1—C2—C3	-179.33 (14)
C3—S1—C8—C9	-179.50 (11)
C3—C2—C7—C6	1.0 (3)
C1—C2—C7—C6	-179.14 (17)
C7—C2—C3—C4	-1.8 (2)
C1—C2—C3—C4	178.29 (14)
C7—C2—C3—S1	177.09 (12)
C1—C2—C3—S1	-2.8 (2)
C8—S1—C3—C4	-0.56 (15)
C8—S1—C3—C2	-179.45 (12)
S1—C8—C9—C10	176.41 (13)
C11—C10—C9—C8	-177.3 (2)
C2—C7—C6—C5	0.4 (3)
C2—C3—C4—C5	1.4 (3)
S1—C3—C4—C5	-177.57 (15)
C7—C6—C5—C4	-1.0 (3)
C3—C4—C5—C6	0.0 (3)

(28), acetic acid (2.62(2) Å) (29), nicotinic acid (2.66 Å) (30) and *o*-phtalic acid (2.67(0.05) Å) (31). The differences between the two C-O bonds are almost 0.093 Å higher than in benzoic acid (0.046 Å), but are similar to the differences in salicylic acid (0.1 Å) (32). This observation could be explained by the greater similarities of the title compound with salicylic acid than with benzoic acid.

The pair of O1—H1...O2 interactions connects inversion-related molecules into dimers (Table 4, Figure 2).

CONCLUSION

Platinum(IV)-complexes with S-alkyl derivatives of thiosalicylic acid (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) have been synthesized and characterized by microanalysis, infrared spectroscopy, and ^{1}H and ^{13}C NMR spectroscopy. The S-butyl derivative of thiosalicylic acid was crystalized in a $P2_1/c$ space group of a monoclinic crystal system. The crystal form and crystal packing are determined by intermolecular hydrogen bonds O1-H1...O2. The S-butyl derivative of thiosalicylic acid also has a co-planar geometry.

Acknowledgement

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Table 4. Hydrogen bonding geometry for $C_{11}H_{14}O_2S$.

D-H....A	*D-H (Å)*	*H....A (Å)*	*D ....A (Å)*	*q (°)*
O1-H1....O2[a]	0.90(3)	1.75(3)	2.634(3)	172(2)

[a] 1-x,1-y,1-z

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THE IMPACT OF POSITIVE ACCELERATION (+GZ) ON ANTIOXIDANT CAPACITY AND HISTOPATHOLOGICAL ALTERATIONS IN DIFFERENT ORGANS AND TISSUES IN RATS

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UTICAJ POZITIVNOG UBRZANJA (+GZ) NA ANTIOKSIDATIVNI KAPACITET I HISTOPATOLOŠKE PROMENE NA RAZLIČITIM ORGANIMA I TKIVIMA PACOVA

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ABSTRACT

Since the early 1940s, a significant amount of research has been conducted to describe the impact of the high-G acceleration on the cardiovascular system. The objective of the present study was to examine the role of the antioxidant enzyme system under biodynamic stress in the liver, heart and gastric mucosa in response to high-magnitude +Gz exposure in a rat model. Twenty adult male Wistar albino rats (10 rats per group; 9-11 weeks old, 200-250 g b.w.) were divided into the following two groups: control and G (exposed to a biodynamic stress model under positive (+7 Gz) acceleration for 40 s). The influence of acute biodynamic stress on pro-oxidative parameters in the rat liver (xanthine oxidase (XOD), catalase (CAT), peroxidase (Px), glutathione peroxidase (GSH-Px), total content of glutathione (GSH), lipid peroxidation (LPx)) and on histopathological alterations in the liver, cardiac muscle and gastric mucosa was examined. Biodynamic stress resulting from positive (+7 Gz) acceleration resulted in a highly statistically significant increase of CAT GSH-Px activity compared to the control group. The LPx levels were significantly decreased, but the GSH contents and the activities of other enzymes were not significantly changed. Significant microscopic changes in the liver, heart and gastric mucosa were observed in the G group. These results clearly indicate that +Gz acceleration alters biochemical systems. These alterations in cellular processes may be mediated by influences of hypoxia or ischaemia via changes in the antioxidant capacity.

Keywords: *+Gz acceleration, antioxidant enzyme, liver, heart, gastric mucosa, rats*

SAŽETAK

Od ranih 1940-ih, značajan broj istraživanja je sproveden u pokušaju da se opiše uticaj visokog G ubrzanja na kardiovaskularni sistem. Cilj naše studije je bio da ispita ulogu sistema antioksidativne zaštite u stanju biodinamičkog stresa u jetri, srcu i gastričnoj mukozi, prouzrokovanog izlaganjem visokim vrednostima +Gzna modelu pacova. Koristili smo 20 odraslih Wistar albino pacova muškog pola, (10 po grupi; starosti 9-11 nedelja, telesne mase 200-250g) podeljenih u kontrolnu grupu i G grupu (izloženi modelu biodinamičkog stresa pozitivnom (+7Gz)ubrzanju tokom 40s). Ispitivali smo uticaj akutnog biodinamičkog stresa na prooksidativne parametre u jetri pacova (oksidaza (ksantin XOD), katalaza (CAT), peroksidaza (Px), glutation peroksidaza (GSH-Px), ukupan sadržaj glutationa (GSH), lipidna peroksidacija (LPx)) i na histopatološke promene u jetri, srčanom mišiću i gastričnoj mukozi. Biodinamički stres uzrokovan pozitivnim ubrzanjem (+7Gz) je izazvao visoko statistički značajno povećanje aktivnosti CAT u poređenju sa kontrolnom grupom, kao i aktivnosti GSH-Px. Nivo LPx se značajno smanjio, ali sadržaj GSH i aktivnost ostalih enzima se nije značajno promenio. U G grupi otkrili smo značajne mikroskopske promene u jetri, srcu i gastričnoj mukozi. Naši rezultati jasno ukazuju na to da +Gz ubrzanje ima sposobnost da promeni biohemijske sisteme. Ove promene u ćelijskim procesima mogu biti posredovane uticajima hipoksije ili ishemije, menjajući antioksidativni kapacitet.

Ključne reči: *+Gz ubrzanje, antioksidativni enzimi, jetra, srce, gastrična mukoza, pacovi*

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ABBREVIATIONS

G force - ratio given acceleration/due to gravity
+Gz - inertial force that acts from head to feet (positive)
CAT - catalase
GSH - reduced glutathione
GSH-Px - glutathione peroxidase
GSSG - oxidized glutathione
XOD - xanthine oxidase
Px - peroxidase
LPx - lipid peroxidation
EDTA - ethylenediaminetetraacetic acid
TRIS - Tris(hydroxymethyl)aminomethane
MDA – malondialdehyde
ROS – reactive oxygen species

INTRODUCTION

Reflecting improvements in the aviation industry, highly manoeuvrable aircrafts have been introduced to military aviation worldwide. Since the early 1940s, several research studies been conducted to describe how acute high-G acceleration exposure affects cerebral perfusion, regulatory cardiovascular mechanisms and consciousness in high-performance aircraft pilots during aerial combat manoeuvres (1).

The pilots of these aircraft are exposed to acceleration forces (particularly +Gz) at higher magnitudes and for longer durations. "G" is a measure of the force experienced by a person due to acceleration, expressed in terms of multiples of the Earth's gravitational acceleration. +Gz is a description of the G vector in which the vertical (z) axis is parallel to the long spinal axis of the body, and the direction (+) is from head to foot. The G capability of a modern aircraft is high, but the G tolerance of a human organism is limited. The human body acclimates to conditions of high acceleration forces through physiological compensation mechanisms (2).

Repeated exposure to high +Gz can induce significant physiological adaptation reactions associated with blood pressure regulation (blood volume reduction and decreased cardiac output) (3). In addition, cardiomyocytes and muscle fibres become damaged as a result of mechanical force and severe haemodynamic changes. The exposure of the human body to +Gz acceleration produces dramatic effects on the cardiovascular system (4). For example, during +Gz stress, the heart rate has been reported to increase in excess of 200 b /min^{-1}, and the left ventricular pressure has been estimated to reach 300 mm Hg. Several reviews concerning the overall effects of +Gz stress on the cardiovascular system have been published (5). Acceleration on the +Gz axis increases pressure in the cardiac chambers. Some authors have confirmed that the effects of high +Gz were similar to the haemodynamic changes and redox imbalance observed in ischaemia reperfusion (I/R) (6).

Other consequences of +Gz positive acceleration have also been described. Several studies have demonstrated that +Gz affects many organs, such as the lungs, liver, bones and kidneys, and even consciousness and acute gastric mucosal injury have also been observed. However, the effect of the exposure of pre-existing gastric mucosa injuries to +Gz conditions has not been extensively examined (4-8).

The normal weight, hydrostatic pressure and physiological ventilation/perfusion gradients in the lungs are exaggerated under high +Gz forces, resulting in increased pulmonary arterio-venous shunting (5-7), which leads to the impairment of circulatory oxygenation and may also result in acceleration-induced atelectasis. Moreover, the effects of +Gz acceleration forces on brain damage as a result of ischaemia and hypoxia and the limitations of the cervical musculature have been described. The liver is the largest internal organ and plays an important role in metabolism. In experimental studies, repeated +Gz exposure can transiently cause liver dysfunction and trigger pathological changes (5-8).

Furthermore, +Gz exposure likely induces accumulative stress damage in the body, inducing organ dysfunction and triggering pathological changes and the activation of the biological defence system, including antioxidant and immune functions (7, 8). Oxidative stress is defined as an imbalance between the production and removal of reactive oxygen species (ROS), which could lead to cell damage and cell death (9). However, the activation of antioxidative processes induced by ROS likely inhibits several types of oxidative damage (10, 11).

However, the mechanisms underlying injuries to the heart and other organs under high +Gz stress have not been systematically studied. The objective of the present study was to investigate the role of the antioxidative enzyme system in the liver, heart and gastric mucosa under biodynamic stress induced through high-magnitude +Gz exposure in a rat model.

MATERIALS AND METHODS

Experimental protocol

The present study used 20 adult male *Wistar albino* rats, 9-11 weeks old, weighing 200-250 grams, which were specially bred in a vivarium at The Center for Scientific Research ICN Galenika, Belgrade. Before seeing, rats (4-6 animals) have stayed in standard Plexiglas cages, in a room with controlled temperature of 22±1°C, relative humidity

(65-70%) and day/night cycle (12:12 brightness / darkness). The rats were fed with standard food dedicated for laboratory rats. All research procedures were performed in accordance with the European Directive for the Welfare of Laboratory Animals (No 86/609/EEC) and the principles of Good Laboratory Practice (GLP).

All animals were divided into 2 groups (10 rats per group): C group (healthy animals without exposure to +Gz acceleration) and G group (animals exposed to positive (+7 Gz) acceleration).

In the present study, we examined the influence of acute biodynamic stress (+7 Gz) on the dynamics of the parameters of oxidative stress in the liver, such as the activities of xanthine oxidase (XOD), catalase (CAT), peroxidase (Px), and glutathione peroxidase (GSH-Px), the total contents of glutathione (GSH) and lipid peroxidation (LPx) and the histopathological alterations in the liver, cardiac muscle and the gastric mucosa.

+Gz stress exposure

Biodynamic stress through positive (+Gz) acceleration in gravity-altitude laboratories (*CFC-35 centrifuge*) was used to induce acute stress. The experimental animals were subjected to growing linear acceleration tests at increments from 0.1 G/s to + 7Gz in the gravity-altitude laboratories (centrifuge) of the Institute of Aviation Medicine, Military Academy in Belgrade, Serbia.

Prior to the growing linear acceleration tests, all animals were fasted (without food) for 24 hours in individual metabolic cages to prevent coprophagia. The centrifuge type-CFC-35 comprises two branches with cabins on both sides (for both humans and animals). These arms, which are attached to the pivot, around which rotation is made, are 9 metres in size. Positive (+Gz) acceleration was produced by the position of weights attached below the vertical axis of the cabin. A centrifuge has a maximum acceleration of 35 G (x, y, z) with an acceleration weight gain of 0.1 G/s. A programmer managed the centrifuge either manually or automatically. After fixation of the cage in the centrifuge cabin intended for animal experiments, the animals were exposed to positive linear acceleration (+7 Gz) for 40 s.

Thirty minutes after the centrifuge test, all rats were euthanized by decapitation. The chest and stomach were quickly opened, and the hearts, livers and gastric samples were harvested, repeatedly flushed with 0.9% saline, and stored at -80°C.

Tissue preparation for the determination of enzyme activity in the liver (GSH, GSH-Px, CAT, Px, XOD, and LPx)

Whole livers were isolated from the rats, and the tissues were homogenized in cold phosphate buffer (pH 8.0). The final tissue concentration in the tissue homogenate was 20 mg of tissue per ml of buffer (12). The liver tissue homogenates were used to determine the levels of enzymes, such as GSH, GSH-Px, CAT, Px, XOD and LPx.

Determination of reduced glutathione (GSH) content

The GSH content in the homogenates was determined as the amount of non-protein residues using -SH Ellman's reagent according to *Benzie et al* (13). To determine the GSH content in the homogenate, 1 ml of the homogenate was centrifuged in 2 mL of sulfosalicylic acid (4%) for 10 min at 3000 bmp/min. Subsequently, 0.05 ml of the supernatant was mixed with 2 ml of Ellman's reagent, and the absorbance was measured at 412 nm. The GSH content was calculated from the molar ratio and converted to per mg of protein over total protein.

Determination of glutathione peroxidase (GPx) activity

The activity of glutathione peroxidase was determined using cumene hydroperoxide according to *Flohe et al* (14, 15). To this end, 0.05 ml of the homogenate was mixed with 0.75 ml of 50 mmol/dm^3 Tris-HCl buffer, pH 7.6 (Buffer 1), and controlled using a thermostat for 10 min at 37°C. Subsequently, 0.1 ml GSH (0.006 g in 10 ml of Buffer 1) was added to the control sample, and 0.1 ml GSH and 0.1 ml of cumyl hydroperoxide (0.05 ml in 10 ml MeOH) was added to the experimental sample, and both samples were incubated at 37°C for 5 minutes. Subsequently, the probe was added to 1 ml TCA (20%) for both samples, and 0.1 ml of cumyl hydroperoxide was added to the control. The cooled solution was centrifuged at 3000 bmp/min for 10 min. A total of 2 ml of a 0.4 M Tris-HCl buffer, pH 8.9 (Buffer 2) and 0.1 ml DTNB (0.02 g in 5 ml of Buffer 2) was added to 1-ml samples of the supernatant of each probe, and the absorbance was measured at 412 nm. The results are expressed in nmol/mg protein/min (ε= 1.36x10^4 dm^3 mol^{-1}cm^{-1}).

Determination of catalase (CAT) activity

Catalase activity was determined using hydrogen peroxide as a substrate according to *Viviez-Bauza et al* (16). A total of 3 ml of substrate (50 mM phosphate buffer, *p*H 7, 0.075 ml of 30% H_2O_2 per 50 ml of KPi) was added to 20 µl of homogenate. The absorbance was measured at 240 nm, and the results are expressed in nmol/mg protein/min (ε=4.36x10^4 dm^3 mol^{-1}cm^{-1}).

Determination of peroxidase (Px) activity

The peroxidase activity was determined using hydrogen peroxide as a substrate according to *Bergmayer et al* (17, 18). To this end, 3 ml of phosphate buffer (0.1 mol dm^{-3}, pH 7), 50 µl of guaiacol (250 g in 100 ml of H_2O) and 40 µl of hydrogen peroxide (140 µl of 30% H_2O_2 in 100 ml of H_2O) were added to 50 µl of homogenate, and the absorbance of the reaction product was measured at 436 nm. The results are expressed in nmol/mg protein/min (ε= 2.3x10^4 dm^3 mol^{-1}cm^{-1}).

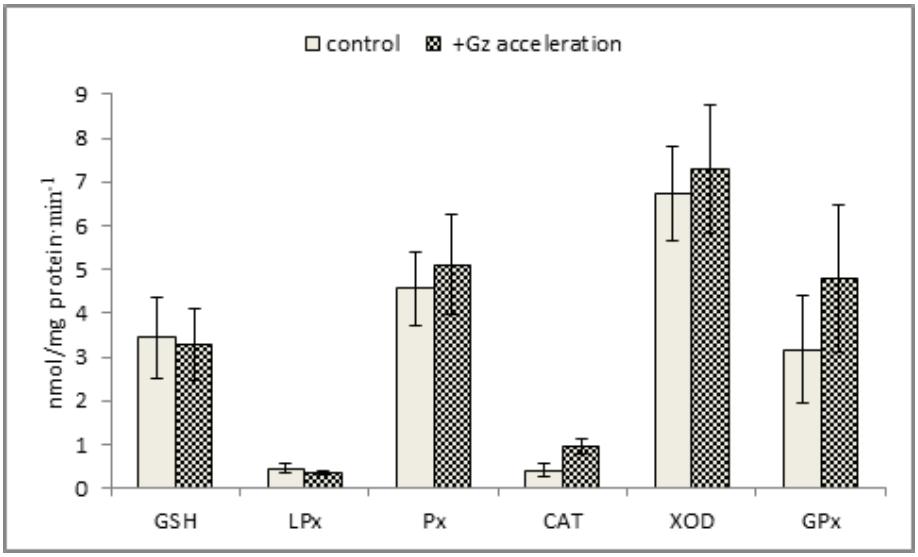

Fig. 1 - Changes in the parameters of oxidative stress in the livers of animals in the G group compared to the control group exhibited a positive (+7 Gz) acceleration. CAT activity, Px and GSH-Px are expressed in nmol/mg protein · min^{-1}; XOD activity is expressed in mmol/mg protein · min^{-1}; LPx intensity expressed as nmol of malondialdehyde/mg protein; GSH content is expressed as GSH nmol/mg protein. The values are presented as the means±SD (*=p <0.05; ***=p<0.01).

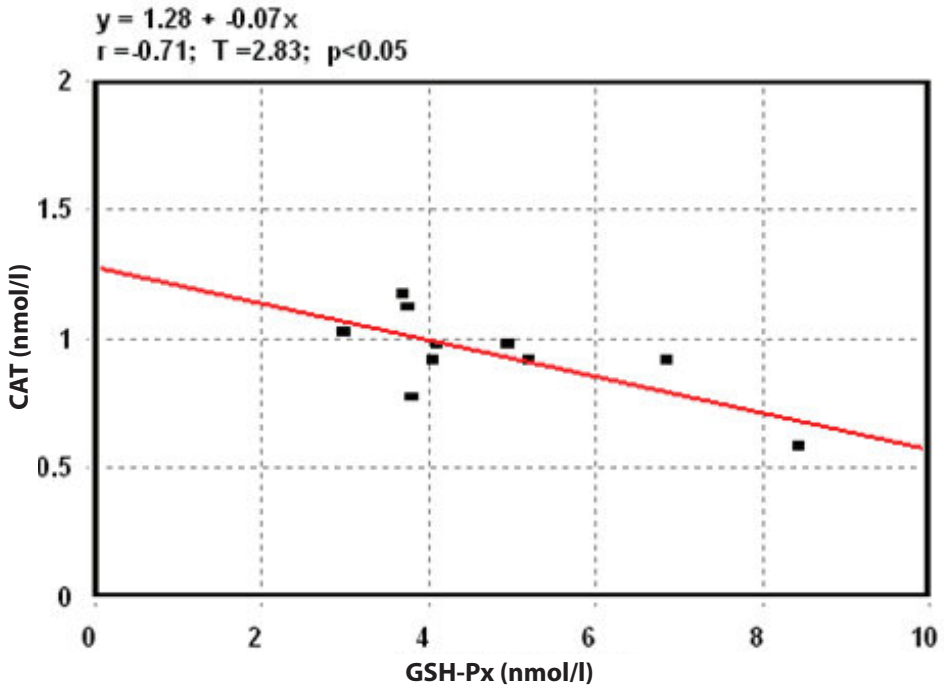

Fig. 2 - Correlation between CAT and GSH-Px activity in the experimental group (G group) (r= -0.71)

Determination of xanthine oxidase (XOD) activity

The activity of XOD was spectrophotometrically determined by varying the optical density at 293 nm after passing hypoxanthine to uric acid, according to *Bergmayer et al* (18, 19). A total of 3 cm^3 of 0.05 mol/dm^3 potassium phosphate with *p*H=7.5, containing the EDTA and hypoxanthine, at a concentration of 1 mmol/dm^3, was added to 30 µl of liver homogenate or 100 µl of haemolysate. The solution was centrifuged at 3000 bmp/min for 10 min. Finally, the absorbance of the reaction product was measured at 293 nm, and the results are expressed in µmol/g of liver or µmol min/mg protein/min (ε= $1.2x10^4$ dm^3 mol / cm).

Index of lipid peroxidation measured as the Malondialdehyde (MDA) concentration

The malondialdehyde (MDA) concentration was determined using the thiobarbituric acid method according to *Halliwell et al* (20), wherein lipid oxidation is measured by

the cell membrane via the lipid-peroxide reaction products formed in a reaction system using thiobarbituric acid. A total of 0.5 ml of homogenate was heated for 15 minutes in a water bath and subsequently added to a solution of 3 mL of thiobarbituric acid 0.375% to 15% TCA (3.75 g TBA+15+20.72 g of CCl_3COOH mL of 37% HCl + 1-2 drops of α-tocopherol per 1 dm^3 solution). The solution was subsequently centrifuged for 10 minutes at 3000 bmp/min, and the absorbance of the resulting reaction product was measured at 535 nm. The results are expressed as nmol of malondialdehyde/mg of protein/min ($\varepsilon = 1.56 \times 10^5\ dm^3\ mol^{-1}cm^{-1}$).

H&E staining of paraffin-embedded tissues and detection of microscopic changes

Macroscopic and microscopic changes in the heart, liver and gastric tissues were observed in control and experimental groups. For histological processing, heart, liver and gastric mucosa samples were prepared using standard techniques (21, 22). The specimens were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned on a sliding microtome at 4-6 microns thick, and subsequently stained with haematoxylin-eosin (H&E). Histological samples were analysed using a *Leica DML type S2* light microscope, harbouring a specific change in the photodocumented type of *Canon Power Shot S70* digital camera (21, 22).

In the experimental group, we observed and recorded every alteration of the gastric mucosa using a magnifying glass (3x zoom, *Luxo Magnifier*). The sizes of all changes were expressed in mm and documented using an *Olympus C350* type digital photo camera.

Ulcer index of gastric mucosa

The ulcer index was recorded and calculated using *Guth's* method. Ulcer length ≤ 1 mm (including erosion foci) was scored as 1; 1 mm < ulcer length < 2 mm was scored as 2; 2 mm < ulcer length ≤ 3 mm was scored as 3; 3 mm < ulcer length ≤ 4 mm was scored as 3; ulcer length > 4 mm was scored as 5; and the score for ulcer width > 2 mm was doubled (23).

Statistical analysis

All data are expressed as the means ± standard deviation (X±SD). Data before and after +Gz stress exposure were evaluated using one-way analysis of variance (ANOVA) with the least significant difference (LSD) test for post hoc analysis. Correlations between all parameters were analysed using Pearson's correlation test. All analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, Illinois, USA). *P* values ≤0.05 were considered statistically significant.

RESULTS

Biochemical data

Biodynamic stress from positive (+Gz) acceleration for 40 seconds led to a significant change in the activity of certain antioxidant enzymes in the liver in the experimental group compared to the control group. Biodynamic stress led to a highly statistically significant increase ($p<0.01$) of CAT activity compared to untreated animals (control group), in which the activity of CAT was decreased, and a statistically significant increase ($p<0.05$) in GSH-Px activity (4.79 ± 1.67) was observed compared to untreated animals (3.17 ± 1.23). The level of the LPx (3.44 ± 0.5) was significantly decreased ($p<0.05$) compared to control (4.52 ± 1:05), but the content of GSH and the activities of other enzymes were not significantly changed. The levels of all enzymes examined in the G group of animals exposed to biodynamic stress by positive (+Gz) acceleration, compared to the levels in the control group, are shown in Fig. 1. In the control group, Px activity was positively correlated with the levels of LPx ($r=0.86$; $p<0.001$). In addition, XOD activity was positively correlated with the GSH content ($r=0.90$; $p<0.001$) (Tables 1 and 2). Interestingly, in the G group, which was exposed to positive (+Gz) acceleration, the CAT activity was negatively correlated with the GSH-Px activity ($r=0.71$; $p<0.05$) (Fig. 2).

Table 1 - The correlation matrix of parameters of oxidative stress in the livers of healthy animals (control group) (*p<0.05; ***p<0.01).

Control group	GSH (nmol/mg protein)	LPx (nmol/mg protein)	Px (nmol/mg protein·min^{-1})	CAT (nmol/mg protein·min^{-1})	XOD (μmol/mg protein·min^{-1})
LPx (nmol/mg protein)	0.34				
Px (nmol/mg protein·min^{-1})	0.34	***0.86*****			
CAT (nmol/mg protein·min^{-1})	-0.35	-0.46	-0.35		
XOD (μmol/mg protein·min^{-1})	***0.9*****	0.56	0.59	-0.43	
GSH-Px (nmol/mg protein·min^{-1})	0.63	0.05	-0.03	-0.22	0.38

Table 2 - Correlation matrix of the studied parameters of oxidative stress in the livers of animals in the G group exposed to positive (+7 Gz) acceleration (*p<0.05; ***p<0.01).

+Gz group	GSH (nmol/mg protein)	LPx (nmol/mg protein)	Px (nmol/mg protein·min^{-1})	CAT (nmol/mg protein·min^{-1})	XOD (μmol/mg protein·min^{-1})
LPx (nmol/mg protein)	-0.19				
Px (nmol/mg protein·min^{-1})	-0.02	0.16			
CAT (nmol/mg protein·min^{-1})	-0.14	0.4	0.19		
XOD (μmol/mg protein·min^{-1})	-0.05	0.47	0.48	-0.11	
GSH-Px (nmol/mg protein·min^{-1})	0.19	-0.10	0.09	***-0.71****	0.18

Pathological morphological analysis

In the control and experimental groups, macroscopic and microscopic changes were observed in the heart, liver and gastric tissues.

In the livers of the animals in the experimental group, macroscopic alterations were not observed, while significant microscopic changes were observed in the liver tissue. The liver cells showed unclear boundaries, strong hyperaemia expression, vacuolysed cells and a high volume of liquids reflecting degenerative changes (Fig. 3). The blood vessels were filled with compacted platelets, reminiscent of thrombotic changes. A large, organized thrombus was observed in the lumen of an artery. Inflammatory lesions, including lymphocytic infiltration, granuloma, macrophage aggregates and variable glycogen content, were not observed in the hepatic tissues of animals in the experimental group. Generally, hepatic parenchyma showed moderate interstitial haemorrhages (Fig. 4).

In the heart tissue of the experimental group, significant macroscopic alterations were not observed, compared to healthy animals. However, in histological preparations of the heart, we observed altered and unclear cell borders, with the loss of the transverse leaf striping in the vertical direction (Fig. 5). These microscopic myocardial lesions were mild and nonspecific and did not contribute to an aetiological diagnosis of the cardiac disease.

Gastric mucosa from the control group showed that the epithelium was intact, and the cells had no hyperaemia and oedema, and the submucosa and muscularis contained no inflammatory cells. In five of the ten animals exposed to positive (+ 7Gz) stress, we observed significant macroscopic changes in the gastric mucosa, with a total ulcer index of UI=1.54 mm ± 0.02 and a score of 2 (Fig. 6). Moreover, the stress-induced gastric ulcers in the experimental group showed that the structure of the gastric mucosa was completely damaged, with gastric gland cell necrosis, cells displaying hyperaemia and oedema, and several inflamma-

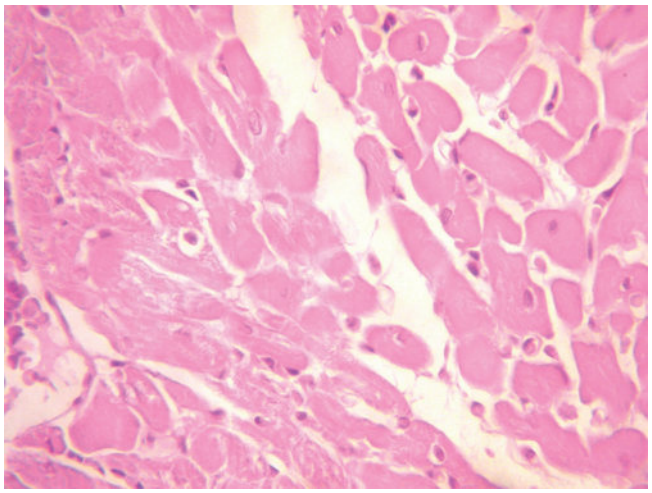

Fig. 3 - Macroscopic appearance of the livers of animals exposed to positive (+7 Gz) acceleration (magnification 10x)

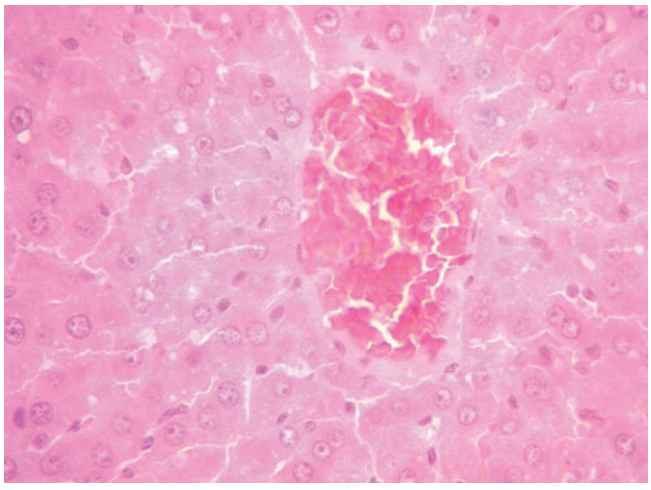

Fig. 4 - Microscopic appearance of the livers of animals exposed to positive (+7 Gz) acceleration (magnification 100x)

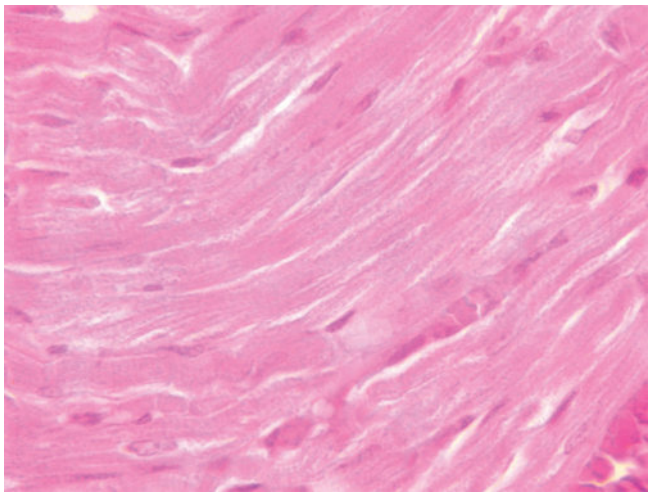

Fig. 5 - Microscopic appearance of the hearts of animals exposed to positive (+7 Gz) acceleration (magnification 100x)

tory cells infiltrating the mucosa, submucosa and muscularis (Fig. 7).

DISCUSSION

The objective of the present study was to reveal the effects, including morphological effects, and investigate the role of antioxidative enzyme system under biodynamic stress in the liver, heart and gastric mucosa as a result of high-magnitude +Gz exposure in a rat model.

In the present study, we examined the influence of acute biodynamic stress (+7 Gz) on the dynamics of the parameters of oxidative stress in the liver, such as enzyme activity of xanthine oxidase (XOD), catalase (CAT), peroxidase (Px), glutathione peroxidase (GSH-Px), total content of glutathione (GSH), lipid peroxidation (LPx) and histopathological alterations in the liver, cardiac muscle and the mucosa of the stomach.

Initially, we examined the effect of positive acceleration on CAT activity, GSH content and GSH-Px. We observed significantly increased CAT and GSH-Px activity in the experimental group, whereas there was a statistically significant negative correlation between these two parameters.

As the catalase (CAT) enzyme is involved in the degradation of H_2O_2 into water and oxygen, we assumed that in the state of the positive (+Gz) acceleration generated large amounts of hydrogen peroxide (34). This idea was confirmed by the increased activity of GSH-Px, which by other metabolic pathways, in cooperation with GSHR, reduces hydrogen peroxide into water. This reduction of hydrogen peroxide was followed by the conversion of the reduced form of glutathione (GSH) into the oxidized form (GSSG) (22, 34). In addition, the increased activity of catalase (CAT), as an enzyme for antioxidant protection, was observed in the liver of rats exposed to immobilization stress, alcoholic stress (99% ethanol) and stress induced by indomethacin, while the activity of GSH-Px in these three stress models was reduced in the liver (30-32).

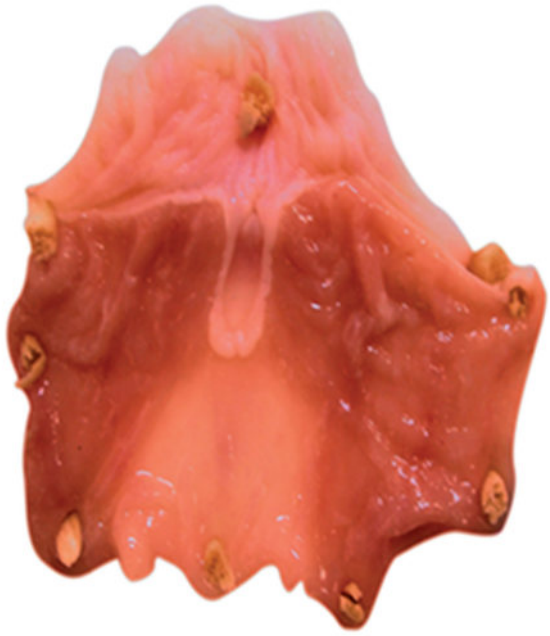

Fig. 6 - Macroscopic appearance of the gastric mucosa of animals exposed to positive (+ 7 Gz) acceleration (magnification 10x)

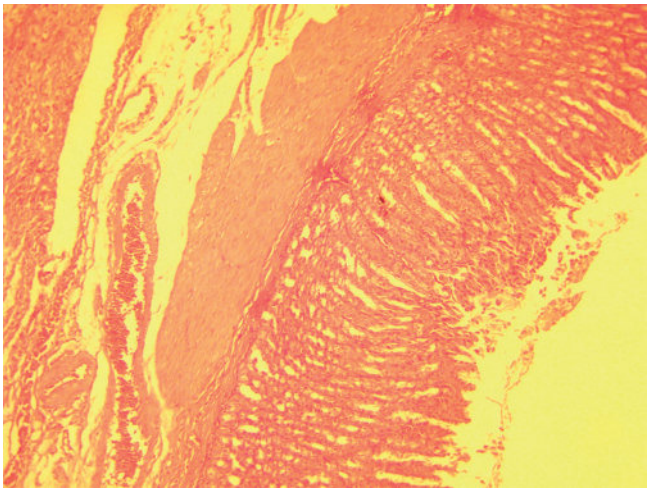

Fig. 7 - Microscopic appearance of the gastric mucosa of animals exposed to positive (+ 7 Gz) acceleration (magnification 100x)

The levels of GSH were decreased, but not significantly, likely reflecting the increased activity of GSH-Px, which was not be able to significantly "spend" reduced glutathione. Important non-enzymatic compounds of protection, such as GSH in the liver, were not changed compared to the control group, and under acute stress the protective role of these compounds was confirmed. *Chen L et al* investigated the effects of high-sustained positive acceleration (+Gz) for 15 days on rats. Interestingly, the activities of its antioxidant enzymes SOD and GSH-Px were decreased but the formation of malondialdehyde (MDA) was increased (24). These results are not consistent with the results of the present study, and the difficulty in interpreting experimental data on animals

(mice and rats) lies in the diversity of these experiments (the diversity of additional substances, which show different properties, different mice/rats by sex and age, duration of +Gz exposing, level used, type and scope). Thus, acute biodynamic stress induced by positive acceleration (+7 Gz) is likely insufficient to significantly induce decreased levels of antioxidant enzymes, such as GSH (24).

Generally, acute stress resulting from acceleration at high amplitude in exposed organism directly disturbs proper cell functioning, leading to vessel collapse and multiple organ ischaemia. Indeed, these processes are similar to those of ischaemia and reperfusion, as a major generator of reactive oxygen species (37). During the stress of positive acceleration forces, resulting in many ischaemia/reperfusion injuries, it is important to evaluate the levels of antioxidant enzymes, such as the enzymes mentioned above.

Thus, cytosolic xanthine oxidase has been considered for many years as the main source of superoxide anion during the reperfusion of ischaemic tissues. Many authors have proposed that the mechanism of post-ischaemic reperfusion damage is mediated by xanthine oxidase (36). In the present study, the levels of Px and XOD were not significantly changed compared to control conditions, and this finding confirms that the acute stress induced by acceleration for 40 seconds did unlikely generate significant ROS production, and under these conditions, the liver likely does not suffer oxidative stress.

Furthermore, levels of lipid peroxidation (LPx) were significantly decreased compared to the control, which was positively correlated with Px activity in the control group. Little is known about the acute effects of +Gz stress on lipid peroxidation; thus, it is difficult to compare these results with the results of other authors. *Zhang et al* described the importance of changes in the dynamic of LPx in various organs of rats exposed to chronic high +Gz stress (+10 Gz) for three weeks. This study showed significantly increased concentration of MDA in mitochondria infarction after exposure to +10 Gz-in, while the activity of SOD in liver homogenates and mitochondria in the kidney was significantly reduced, but repeated +10 Gz acceleration increased mitochondria LPx in hearts and affected the metabolism of free radicals in the liver and kidneys of rats (25). Moreover, *Zhan et al* investigated the effects of repeated +10 Gz stress on cerebral lipid peroxidation, liver and renal function in rats. These authors showed that the lipid peroxidation in rat cerebral homogenate, mitochondria and cytoplasm was significantly increased (29). These results indicated that repeated high +Gz stress could induce peroxidative injury in the brain and generate harmful effects on renal function. Thus, chronic biodynamic stress can induce more deleterious effects on the lipid components of the membrane compared to acute +Gz stress (25).

To completely evaluate the effects of acute +Gz acceleration, we observed macroscopic and microscopic alterations in the liver, heart and gastric tissue of rats. There is limited data concerning the morphological changes in different organs and tissues (liver and heart) under high positive acceleration, and it is difficult to compare the results of the present study with those of other studies. We detected significant microscopic changes in all tissue samples mentioned above, but macroscopic changes were verified only in the gastric mucosa. *Li J* and co-workers studied the impacts of positive acceleration (+Gz) on the gastric mucosal tissues in cases of acute gastric mucosal injury and explored the role of oxygen free radicals (27). The results suggested that +Gz exposure might aggravate the acute gastric mucosal injury, and changes in MDA and SOD contents in the gastric tissues indicated that the oxygen free radicals play an important role in this regard. The authors also concluded that the damage increased with increasing positive acceleration (27). Some authors investigated the role of the exogenous administration of antioxidant enzymes under positive acceleration (+5 and +10 Gz) on gastric mucosal tissues and concluded that this pretreatment reduces gastric mucosal injury (28).

Thus, the basic question is whether these antioxidant enzymes are indicators of oxidative stress and damage in the liver, heart or gastric mucosa?

In healthy animals with no macroscopic and microscopic alterations, the levels of Px activity were positively correlated with the levels of LPx. In the experimental group, the activity of these parameters was changed, and the activity of LPx was decreased while the activity of Px was insignificantly increased, with significant micro and macroscopic alterations in the mentioned tissues. To our knowledge, peroxidases (Px) are enzymes localized in cellular organelles, such as peroxisomes, which is also the location of many reactive oxygen species (ROS) (34, 35). Under acute stress, we expected increased ROS and Px production, consistent with the results of the present study. However, the LPx level was paradoxical decreased, likely reflecting short-term biodynamic stress, while repeated stress induced increased levels of LPx (29).

Under oxidative stress, we expected the increased activity of all antioxidant enzymes, such as GSH, CAT, XOD and GSH-Px, but the duration of the stress period was definitely a limiting factor in the present study. GSH activity was unexpectedly decreased, and the levels of other enzymes were insignificantly increased. Interestingly, these primarily enzymatic changes are excellent indicators of tissue damage but are not specific, as the values of parameters confirm the existence of damage but do not explain the extent and type of damage.

However, it is important to know the mechanisms by which positive acceleration induces harmful effects on different cells in organisms. Exposure to high-sustained positive acceleration (+Gz) has a pathophysiological effect on the heart of the rat. As critical regulators of cardiac myocyte survival and death, mitochondria may be crucially involved in +Gz-induced pathogenesis (33-37). *Chen* and co-workers investigated myocardial mitochondrial ultrastructure, respiratory function, and antioxidant capacity in rats after exposure to +10 Gz for 5 min (33). The results showed that high +Gz stress could damage the mitochondrial ultrastruc-

ture, evidenced as swollen, degenerated, and reduced mitochondria, and broken or disappeared mitochondrial cristae. These effects resulted in significant changes in quantitative indicators of mitochondria morphometry, for example increased surface density, volume density, average volume, and average surface area, and reduced numerical density. Other studies have also revealed that exposure to +Gz stress induced the dysfunction of the mitochondrial respiratory chain, reduced the activity of antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase), and increased the malondialdehyde content (30-37).

CONCLUSION

The results of the present clearly indicate that +Gz acceleration alters biochemical systems. However, these results tend to suggest that these alterations in cellular processes may be mediated by influences other than hypoxia or ischaemia via changes in the antioxidant capacity.

However, the molecular mechanisms by which oxidative stress is produced and the time course of the phenomenon remain unclear, and additional studies with repeated or chronic biodynamic stress after acute biodynamic stress are necessary to clearly explain and provide more precise answers concerning the mechanisms and dynamics of antioxidant enzymes in oxidative stress induced by positive acceleration.

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BACTERIAL FLORA PLAY IMPORTANT ROLES IN ACUTE DEXTRAN SULPHATE SODIUM-INDUCED COLITIS BUT ARE NOT INVOLVED IN GAL-3 DEPENDENT MODULATION OF COLON INFLAMMATION

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BAKTERIJSKA FLORA IGRA VAŽNU ULOGU U AKUTNOM KOLITISU IZAZVANOM DEKSTRAN NATRIJUM SULFATOM, ALI NIJE POVEZANA SA GAL-3 ZAVISNOM MODULACIJOM INFLAMACIJE U KOLONU

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ABSTRACT

An altered immune response to normal gut microflora is important for the pathogenesis of ulcerative colitis (UC). Galectin-3 (Gal-3) is an endogenous lectin that plays an important pro-inflammatory role in the induction phase of acute colitis by promoting activation of the NLRP3 inflammasome and production of IL-1β in macrophages. By using dextran sulphate sodium (DSS) induced colitis, a well-established animal model of UC, we determined whether Gal-3 affects the function of colon infiltrating macrophages by interfering with intestinal microflora.

Our results showed that genetic deletion of Gal-3 significantly attenuates DSS-induced colitis by down-regulating infiltration of phagocytic cells (neutrophils, macrophages and dendritic cells) in colon tissue of DSS-treated mice, and this correlated with differences in bacterial flora of the gut. Antibiotic treatment attenuates DSS-induced colitis in WT and Gal-3$^{-/-}$ mice without affecting differences between the groups.

In conclusion, Gram negative bacterial flora play an important role in DSS-induced acute colitis of mice but are not involved in Gal-3 dependent modulation of colon inflammation.

Keywords: *acute DSS-induced colitis, microflora, Gal-3*

SAŽETAK

Promenjen imunski odgovor na komensalne bakterije u gastrointestinalnom traktu je važan za patogenezu ulceroznog kolitisa. Galektin-3 (engl. Galectin-3, Gal-3) je endogeni lektin, koji igra važnu proinflamacijsku ulogu u inicijalnoj fazi akutnog kolitisa, tako što promoviše aktivaciju NLRP3 inflamazoma i produkciju IL-1β u makrofagima.

U ovom istraživanju ispitivan je uticaj Gal-3 na mikrofloru u patogenezi akutnog kolitisa izazvanog dekstran natrijum sulfatom (engl. Dextran sulphate sodium, DSS).

Delecija gena za Gal-3 je znacajno smanjila oštećenje tkiva kolona životinja tretiranih DSS-om. U poređenju sa WT miševima, u tkivu kolona DSS-tretiranih Gal-3$^{-/-}$ životinja je bila značajno manja zastupljenost fagocita (neutrofila, makrofaga i dendritskih ćelija) sto je koreliralo sa promenama u sastavu bakterijske crevne flore. Primenom antibiotika ublazio se razvoj akutnog kolitisa, ali bez uticaja na već postojeću razliku u bolesti između WT i Gal-3$^{-/-}$ miševa.

Gram negativna bakterijska flora igra važnu ulogu u akutnom kolitisu izazvanom DSS-ом, ali ne učestvuje u Gal-3 zavisnoj modulaciji inflamacije kolona.

Ključne reči: *akutni kolitis, mikroflora, Gal-3, DSS*

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disorder of the gastrointestinal tract (1). The etiology of UC is still elusive, and many factors have been reported to be involved in the development of this disease, including epithelial cell destruction, genetic susceptibility and modulation of bacterial flora in the intestinal environment. Therefore, murine models have become essential tools to investigate the pathophysiological mechanisms and immunological processes underlying chronic mucosal inflammation in the colon (2). Dextran sulphate sodium (DSS)-induced colitis is a frequently used animal model of UC (3). Intestinal microflora play an important role in the susceptibility and responsiveness to DSS-induced colitis (4). DSS induces mucosal injury and inflammation, initially through a direct toxic effect on epithelial cells, followed by invasion of intestinal bacteria into subepithelial tissue and subsequent recruitment and activation of inflammatory cells: neutrophils, macrophages, eosinophils, mast cells, dendritic cells (DCs), and NKT cells, accompanied by production of inflammatory mediators, and leading to the development of severe colitis (4).

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Galectin-3 (Gal-3) is an endogenous lectin that exerts both pro- and anti-inflammatory effects, depending on the disease condition. It plays an important disease-exacerbating role in autoimmune/inflammatory and malignant diseases (5-9) but has a protective role in obesity-induced inflammation and type 2 diabetes, as well as in primary biliary cirrhosis (10, 11). Recently, several clinical studies showed a correlation between the serum level of Gal-3 and exacerbation of acute colitis, indicating the importance of Gal-3 as a potential marker for this disease (12-14). Serum concentrations of Gal-3 were significantly increased in specimens from patients with the active form of UC (12). Recently, we showed that Gal-3 plays an important pro-inflammatory role in the induction phase of acute DSS-induced colitis by promoting activation of the NLRP3 inflammasome and production of IL-1β in macrophages (7).

In this study, we investigated whether Gal-3 affects the function of colon infiltrating macrophages by interfering with intestinal microflora and whether these interactions are important in the development and progression of DSS-induced colitis.

MATERIALS AND METHODS

Animals

Male, 6-8-week-old wild type (WT) and Gal-3$^{-/-}$ C57BL/6 mice (provided by Dr Daniel Hsu, University of California, Sacramento, CA) were used for the induction of DSS-induced colitis. Targeted disruption of the mouse Gal-3 gene was performed in C57BL/6 embryonic stem cells, and mice homozygous for the disrupted gene were obtained (15). Breeding pairs of Gal-3$^{-/-}$ and WT Gal-3$^{+/+}$ C57BL/6 mice of the same substrain were maintained in the animal facilities of the Faculty of Medical Sciences, University of Kragujevac, Serbia. All animals received humane care, and all experiments were approved by and conducted in accordance with the Guidelines of the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia. Mice were housed in a temperature-controlled environment with a 12-h light-dark cycle and were administered standard laboratory chow and water *ad libitum*.

Induction of acute colitis

Colitis was induced with 3% w/v DSS (molecular weight 40 kDa; TdB Consultancy, Uppsala, Sweden) dissolved in drinking water given *ad libitum* for up to 7 days, as previously described (3). Control mice were given DSS-free water.

Assessment of the severity of colitis

The Disease Activity Index (DAI) was used to assess clinical signs of colitis (16). Body weight measurements, analysis of stool consistency, and faecal occult blood tests were performed daily. Body weight was measured daily and compared with the body weight measured on day 0 (the 1st day of DSS administration). The results are presented as ±% body weight loss.

Histology

For histological analysis, colons were removed from euthanised mice, rinsed with phosphate buffer solution (PBS), and cut longitudinally before being rolled into a 'Swiss roll', as previously described (17). Swiss rolled colons were fixed in formalin and embedded in paraffin, and 5-µm sections were stained with haematoxylin-eosin (H&E) and examined in a blinded manner by a pathologist. Sections were analysed for damage to the epithelium including damage to crypts, submucosal oedema, haemorrhage, and infiltration by immune cells. The histology score for each mouse was calculated as the sum of 'Infiltration' and 'Damage of Epithelium' sub-scores, as previously described (18).

Flow cytometry analysis of colon infiltrating cells

Isolation of immune cells from the lamina propria and flow cytometry analysis were conducted as previously described (7). Briefly, each colon was dissected away from the caecum. The colon was cut into pieces 3 cm long and then cut longitudinally, so that 3 x 3 cm flaps of colonic tissue were made. The flaps were placed in a 50 ml conical tube and washed three to five times with 30 ml cold HBSS, calcium and magnesium free. After decanting the supernatant, the pieces were incubated in 20 ml HBSS/EDTA for 30 min in a 37°C water bath. Each tube was shaken regularly during the incubation to ensure that epithelial cells were disrupted from the mucosa. The pieces were sedimented, and the supernatant was decanted. The remaining EDTA was washed out with 40 ml HBSS, calcium and magnesium free. The fragments of colonic tissue were placed in a 5 cm Petri dish and cut into smaller pieces with a scalpel. The pieces were aspirated with a pipette, transferred to a fresh 50 ml conical tube and filled to 20 ml with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS). Then, 1 ml of 4000 Mandl units (3 x 10^6 Wünsch units)/ml collagenase D and 200 µl of 1 mg/ml DNase were added to the tube and incubated for 1 h in a 37°C water bath. The supernatant was filtered through a 100 µm nylon cell strainer into a clean 50 ml conical tube. Cold HBSS, calcium and magnesium free, was added to a volume of 50 ml. Cells were pelleted by centrifuging 10 min at 450 g, at 4°C. The pellet was disrupted, and cells were re-suspended in 50 ml HBSS, calcium and magnesium free, and filtered through a 40-µm nylon cell strainer into a clean 50 ml conical tube. Cells were again pelleted by centrifuging 10 min at 450 g, at 4°C. The pellet was disrupted, and cells were re-suspended in 20 ml of 30% Percoll. Then, the cell suspension was carefully layered over 25 ml of 70% Percoll in a 50-ml conical tube and centrifuged for 20 min at 1100 x g, room temperature, with as low an acceleration rate as possible and with the brake off. Clumping of cells was prevented by the addition of 1 mM EDTA to the solution. Epithelial cells floated on the 30% Percoll layer, and immune cells were found between the 30% and 70% layers.

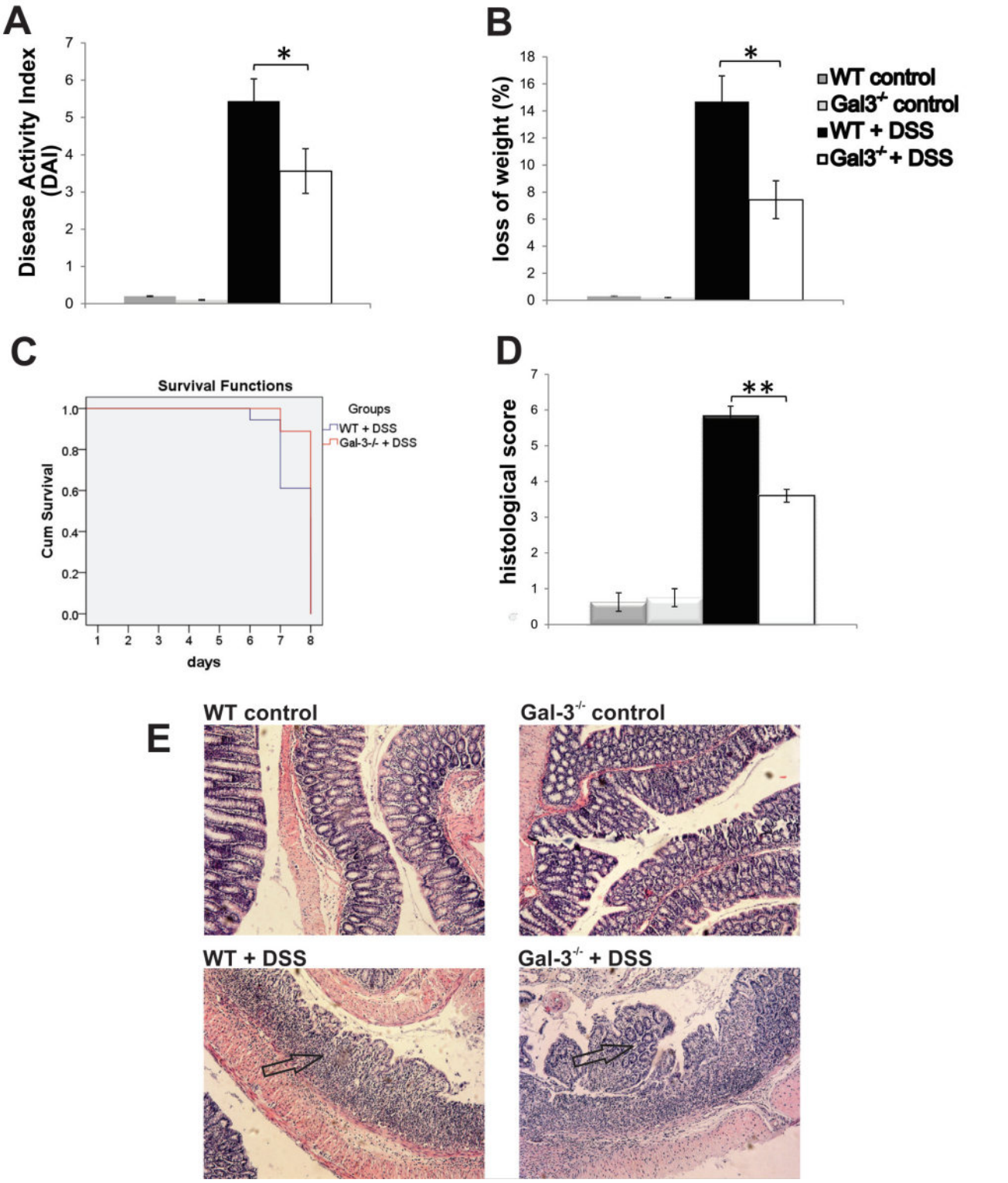

Figure 1. Galectin-3 deficiency attenuates DSS-induced colitis. Water with 3% DSS was given to mice for 7 days; regular drinking water was fed to control mice. Disease Activity Index (DAI) was scored at day 7 using the following parameters: weight loss, stool consistency, and rectal bleeding (**A, B**). Survival rate of mice with colitis (**C**). Histological examination was performed with haematoxylin and eosin (H&E) staining. H&E stained images of representative colon tissues are shown at the same magnifications (100x) (**D, E**). Data presented as the mean ± standard error of the mean (SEM); n = 10 mice per experimental group. *p<0.05, **p<0.001.

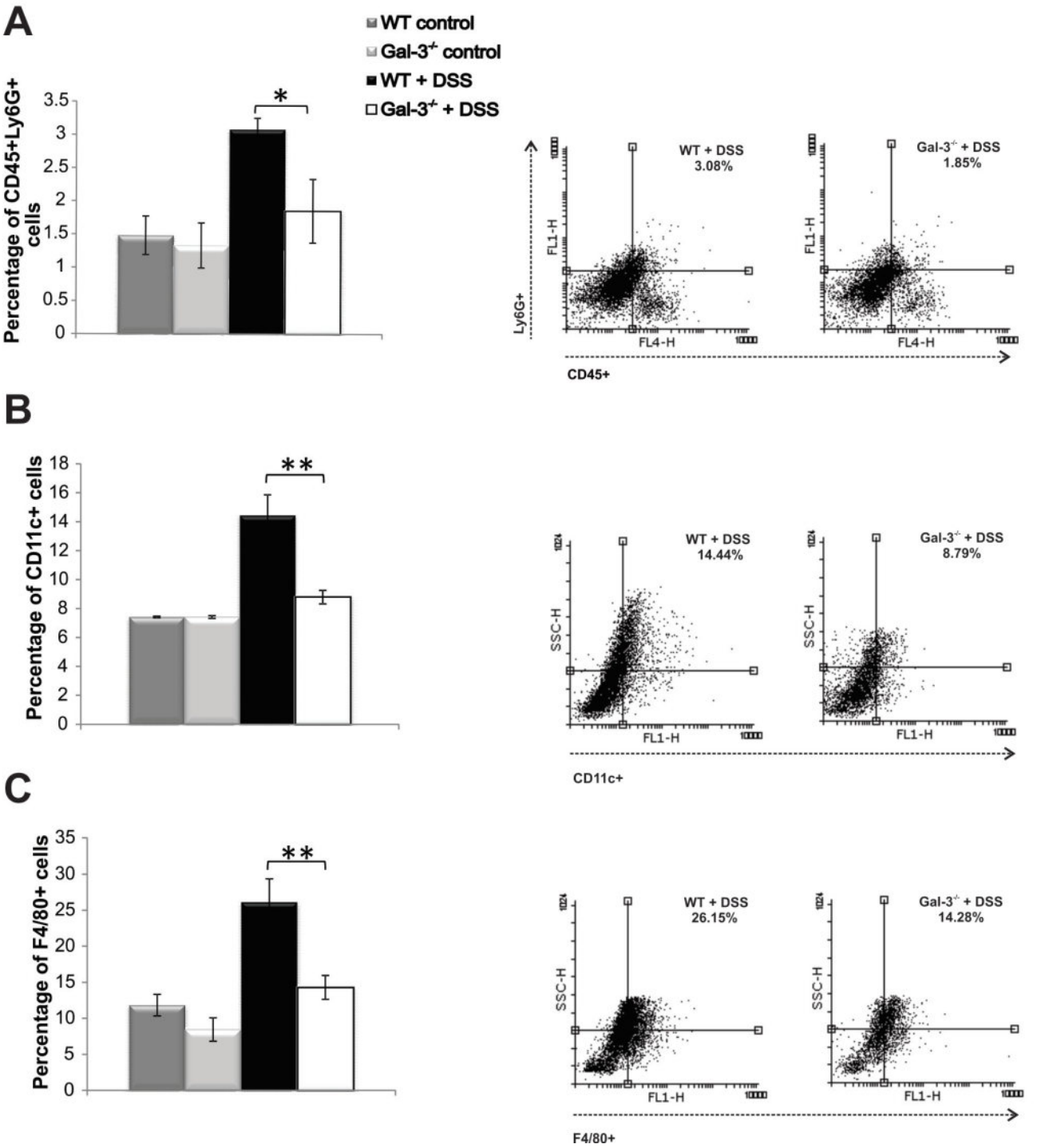

Figure 2. Genetic deletion of Gal-3 decreases the percentage of neutrophils, dendritic cells and macrophages in the colons of DSS-treated mice. The percentage of neutrophils (CD45+Ly6G+), CD11c+ dendritic cells, and F4/80+ macrophages was significantly lower in colons of DSS-treated Gal-3$^{-/-}$ mice (**A-C**). Representative flow cytometry dot plots are shown. Values are the mean ± standard error of the mean (SEM) (n = 10 per group). *p<0.05, **p<0.001.

Debris and dead cells were pelleted at the bottom of the conical tube.

Flow cytometry followed routine procedures by using 1x10^6 cells per sampwhichle, which were incubated with anti-mouse F4/80, CD11c, CD45, and Ly6G conjugated to fluorescein isothiocyanate (FITC; BD Biosciences, Franklin Lakes, NJ), or allophycocyanin (APC; BD Biosciences). Flow cytometric analysis was conducted on a BD Biosciences FACSCalibur and analysed using the Flowing software analysis program.

Antibiotic treatment

To investigate the potential role of the microbiota and their relationship with Gal-3 in acute DSS-induced colitis,

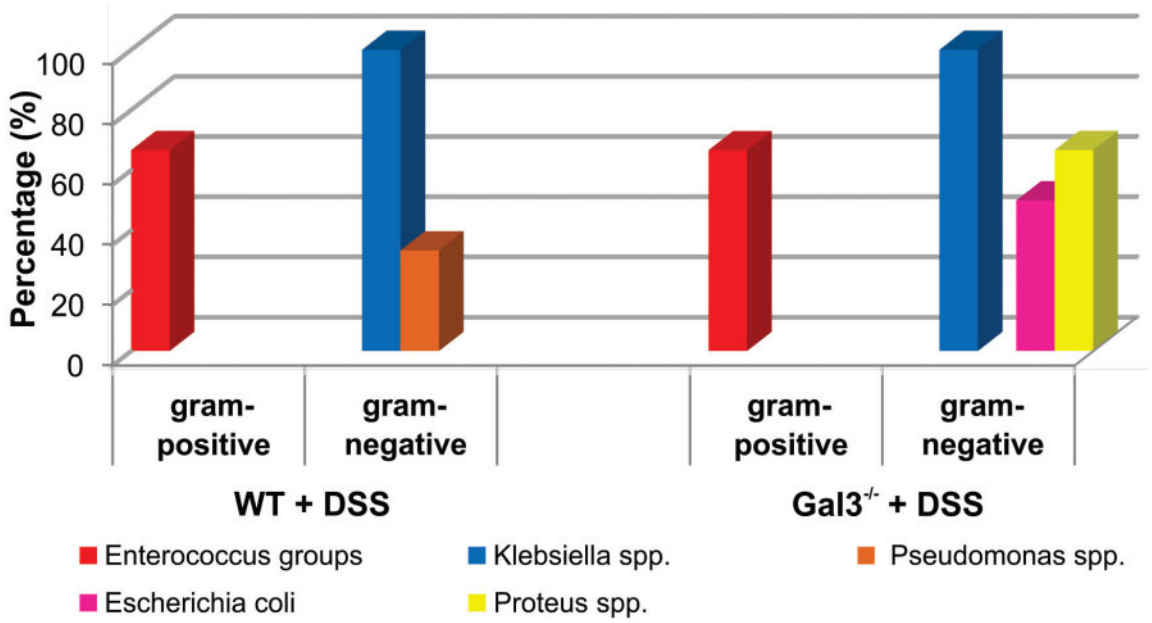

Figure 3. Bacterial strains isolated from the stools of WT and Gal-3$^{-/-}$ DSS-treated mice. The graphs show the percentage of mice in whose stools different bacterial strains were isolated. Red bars represent Enterococcus groups, blue bars represent Klebsiella spp., orange bars represent Pseudomonas spp., pink bars represent Escherichia coli, and yellow bars represent Proteus spp.

experimental animals received water supplemented with antibiotics. Mice were treated daily with antibiotics (metronidazole (1 mg/g) and ciprofloxacin (0.5 mg/g)), intraperitoneally injected from day 0 to the last day of the experiment. (19). A fresh stool sample was collected in a clean tube and processed for bacteria determination. Samples were then placed in a special dish, which was filled with a gel that boosts the growth of bacteria, as previously described (20).

Statistics

Data are expressed as the mean ± SEM for each group. We tested for normality using the Shapiro-Wilk's test and for homogeneity of variances using Levine's test. A paired samples *t*-test was used to compare the two matched groups. The independent samples Student's *t*-test was otherwise used to compare two groups with a Gaussian distribution. Fisher's exact test was used to assess survival differences between groups. Statistical analyses were performed using SPSS 21.0 for Windows (SPSS, Inc., Chicago, IL). All reported p values were 2-sided, $p<0.05$ was considered statistically significant, and $p<0.001$ was considered highly significant.

RESULTS

Genetic deletion of Gal-3 significantly attenuated DSS- induced colitis

Genetic deletion of Gal-3 attenuated DSS-induced colitis, according to the clinical parameters assessed (Figure 1A), loss of weight (Figure 1B), and the survival rate (Figure 1C). All DSS treated WT mice developed severe colitis with similar clinical symptoms: diarrhoea, rectal bleeding, and weight loss. The presence of blood in the faeces was detected one to two days after the start of DSS treatment, whereas gross bleeding and diarrhoea were observed from day 4. Significant body weight loss (>5%) became prominent after five days of DSS treatment. These observations were confirmed by histological analysis (Figure 1D). The DSS-treated group clearly exhibited a severe mucosal inflammatory cell infiltration and a disruption of crypt architecture (epithelial ulcerations and loss of goblet cells), whereas lesions were prevented in Gal-3$^{-/-}$ animals treated with DSS (Figure 1E, low panel).

Gal-3 deletion significantly reduced the presence of phagocytic cells in DSS-injured colons

Genetic deletion of Gal-3 markedly decreased the numbers of neutrophils, dendritic cells and macrophages in colon tissue. Flow cytometry analysis showed a significantly lower percentage of CD45+Ly6G+ neutrophils, CD11c+ DCs and F4/80+ macrophages in colon tissue of DSS-treated Gal-3$^{-/-}$ mice (Figure 2), indicating the important role that Gal-3 plays in the infiltration of phagocytic cells in DSS-injured colons.

To assess the differences in bacterial microflora between DSS-treated WT and Gal-3$^{-/-}$ mice, microflora of the gut were analysed. There were no differences in Gram positive bacteria, while significant differences in Gram negative bacterial flora of the gut were observed. *Enterococcus* and *Klebsiella* species were found in both DSS-treated WT and Gal-3$^{-/-}$ mice. Pseudomonas species were found only in the DSS-treated WT mice, while *Escherichia coli* and Proteus species were found only in the DSS-treated Gal-3$^{-/-}$ mice (Figure 3).

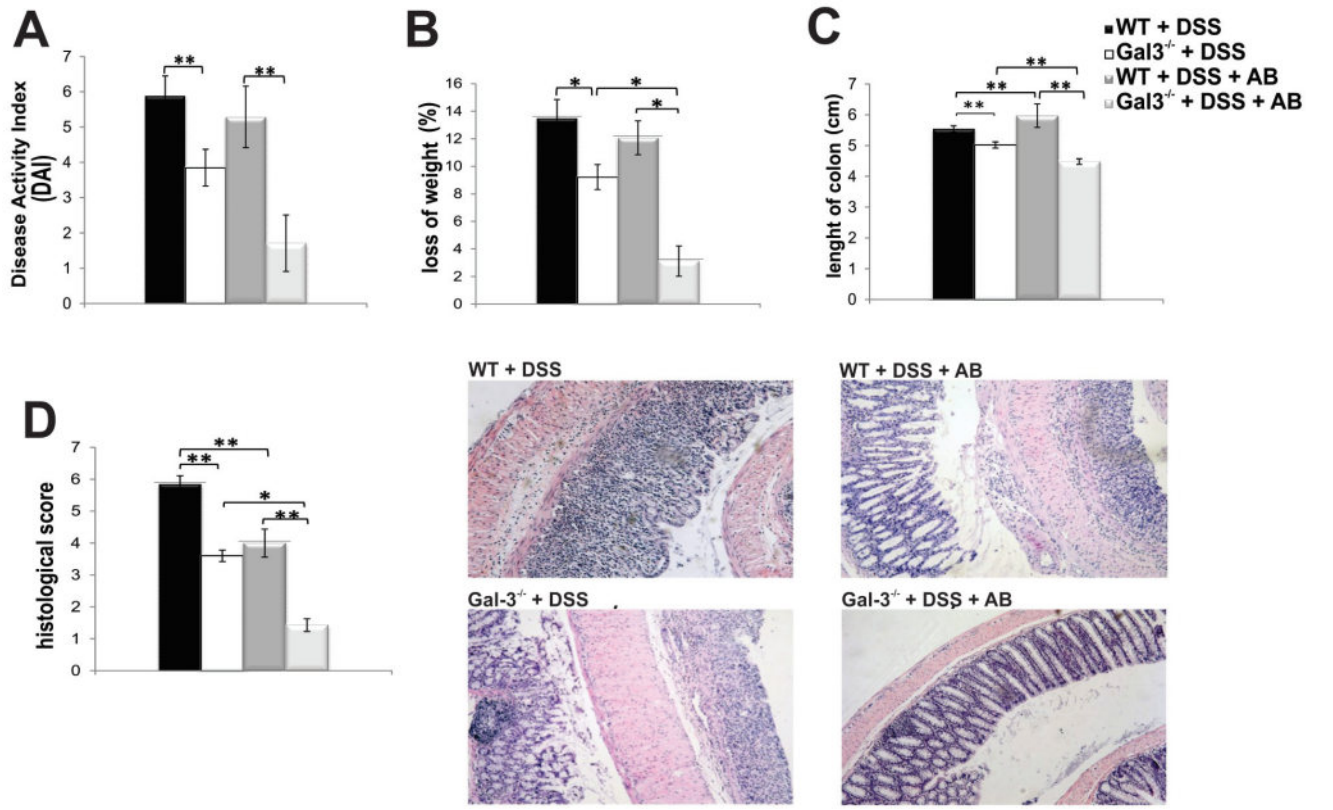

Figure 4. Antibiotic treatment ameliorated DSS-induced colitis. Antibiotics were intraperitoneally administered to DSS-treated mice. After 7 days, the DAI was evaluated, and colon length was measured (**A-C**). Histological score as well as representative photomicrographs (×100 magnification) of colon sections are shown (**D**). Values are the mean ± standard error of the mean (SEM) (n = 10 per group). *p<0.05, **p<0.001.

Antibiotic treatment significantly attenuated DSS-induced colitis without affecting differences between WT and Gal-3$^{-/-}$ mice

The use of antibiotics, which are effective against Gram negative bacteria (19), attenuated DSS-induced colitis, ameliorated diarrhoea and rectal bleeding, and reduced the loss of body weight (Figure 4A, B). Colon shortening was significantly lower in the DSS-treated Gal-3$^{-/-}$ mice compared to the WT DSS-treated animals (Figure 4C). These findings were confirmed by histological analysis. In DSS-treated mice that received antibiotics, the colonic mucosa showed only slight pathological changes, including a reduced extent of colon damage and reduced infiltration of inflammatory cells (Figure 4D). The structure of the crypt epithelial cells remained intact, and the histopathological injury scores were significantly decreased when compared with DSS-only treated animals (Figure 4D). Most importantly, it appears that antibiotic treatment has a protective effect in DSS-induced colitis but does not affect the differences between the DSS-treated WT and Gal-3$^{-/-}$ mice. The difference in clinical and histological parameters of DSS-induced colitis remained statistically significant between the antibiotic treated WT and Gal-3$^{-/-}$ animals (Figure 4).

DISCUSSION

There is increasing evidence that intestinal microflora play an important role in the pathogenesis of UC. Several studies in animal models of intestinal inflammation suggest that the inflammatory responses are triggered by normally nonpathogenic microbial flora. Bacteria are of prime importance for the onset of UC in IL-2-deficient mice, which are free of symptoms when they are kept in a germ-free environment (21). Additionally, IL-10-deficient mice develop an attenuated disease when they are kept in a facility with a defined microbial environment (22, 23). Indirect evidence for the involvement of microorganisms in these findings includes the increased infiltration of phagocytic cells into the inflamed colons, which is followed by an uptake of luminal antigens (24) and the onset of inflammation in injured colons (24, 25).

It is well known that Gal-3 is involved in migration of phagocytic cells (26, 27). Accordingly, we found a significantly lower percentage of neutrophils, DCs and macrophages in the DSS-injured colons of Gal-3$^{-/-}$ mice that was accompanied by a significant decrease in epithelial cell damage, oedema, ulceration and destruction of crypts (Figure 1D, E). These findings are in line with our recently

published data in which we showed that Gal-3 promotes activation of the NLRP3 inflammasome and production of IL-1β in colon-infiltrating macrophages (7) and that pharmacological inhibition of Gal-3 enhances the capacity of mesenchymal stromal cells to promote alternative activation of macrophages in DSS-induced colitis (28).

Several bacterial species were isolated from colons of UC patents, and some of them may modulate the activity of DSS, playing important roles in the induction of colitis (29, 30, 31). An increased number of *Escherichia coli* species were isolated from colons of UC patients (29, 30). DSS is depolymerized in mouse faeces under aerobic conditions, and among all intestinal bacteria, *Proteus mirabilis* has the best ability to desulphonate and depolymerize DSS (32). In line with these findings, we noticed a significantly higher presence of *Escherichia Coli* and *Proteus* species in DSS-treated Gal-3$^{-/-}$ mice (Figure 3) that correlated with attenuated disease in these animals.

Since metronidazole alone or in combination with ciprofloxacin may attenuate chronic TNBS-induced colitis in rats (33) and carrageenan-induced colitis in guinea pigs (34), and since a combination of these antibiotics is effective against Gram negative bacteria such as *Escherichia coli* and *Proteus*, we used these antibiotics to determine the possible role of Gal-3 in the e interactions between intestinal microflora and the activity of phagocytic cells in acute colitis. We observed that the combination of metronidazole and ciprofloxacin significantly attenuated acute DSS-induced colitis in WT and Gal-3$^{-/-}$ mice, as determined by clinical and histological scores (Figure 4). Nevertheless, the differences in clinical and histological scores between DSS-injured WT and Gal-3$^{-/-}$ mice were still evident in the antibiotic treated animals, indicating that the Gal-3 dependent decrease in the presence of phagocytic cells in the inflamed colons was not directly related to the content of intestinal microflora.

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ANATOMICAL PARAMETERS OF THE ACETABULUM IN HEAVY VEHICLE OPERATORS

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ANATOMSKI PARAMETRI ACETABULUMA KOD VOZAČA TEŠKIH VOZILA

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ABSTRACT

It has been suggested that long-term exposure by heavy vehicle operators to whole-body vibration (WBV) may be related to an increased risk of pathological changes in the anatomical parameters of the hip. The aim of this study was to explore the difference in anatomical parameters of acetabulum in drivers of heavy vehicles (experimental group; n=60) and subjects who have not been exposed to WBV (control group; n=60). The anteroposterior radiographic view of the hips was used to measure the following parameters: the vertical centre edge (VCE), the 'horizontal toit externe' angle (HTE), the neck shaft angle (NSA) and the acetabular depth (AD). Compared with the control group, the mean VCE angle values and AD were significantly lower, while the average HTE and NSA values were significantly higher in the experimental group. This study supports the hypothesis that exposure to whole-body vibration during operation of a vehicle causes an increased risk of acetabular dysplasia.

Keywords: *Whole-body vibration, Drivers, Hip joint, Dysplasia*

SAŽETAK

S obzirom da izloženost vibracijama celog tela, usled upravljanja teškim vozilima, može biti u vezi sa povećanim rizikom od nastanka patoloških promena zgloba kuka, cilj studije je bio da ispita razlike u anatomskim parametrima acetabuluma kod vozača teških vozila (eksperimentalna grupa; n=60) i ispitanika koji nisu izloženi vibracijama (kontrolna grupa; n=60). Anteriorno-posteriorni radiografski snimak kuka korišćen je za merenje sledećih parametara: ugao lateralizacije femura (Vibergov ugao), nagib krova acetabuluma, kolodijafizalni ugao femura i dubina acetabuluma. Srednje vrednosti Vibergovog ugla i dubine acetabuluma bile su značajno niže, a nagib acetabuluma i kolodijafizalni ugao značajno viši, u eksperimentalnoj nego u kontrolnoj grupi. Studija potvrđuje hipotezu da izloženost vibracijama celog tela predstavlja rizik za nastanak acetabularne displazije.

Ključne reči: *Vibracije celog tela, Vozači, Zglob kuka, Displazija*

ABBREVIATIONS

AD - acetabular depth,
AP- anteroposterior,
HTE - horizontal toit externe angle,
NSA - neck shaft angle,
OA – osteoarthritis,
VCE - vertical centre edge angle,
WBV- whole-body vibration

INTRODUCTION

Osteoarthritis (OA) is one of the most common joint disorders worldwide (1). In addition to damage and loss of articular cartilage, patients present with remodelling of subarticular bone, osteophyte formation, ligamentous laxity, weakening of periarticular muscles, and, in some cases, synovial inflammation (2). Despite the prevalence of OA, the extremely long and indolent course that characterizes the disease has resulted in great difficulty in evaluating its natural history (3). The pathological changes of acetabular anatomical parameters are considered as prevalent predisposing factors for the development of hip OA. Risk factors for acetabular dysplasia can be divided into person-level fac-

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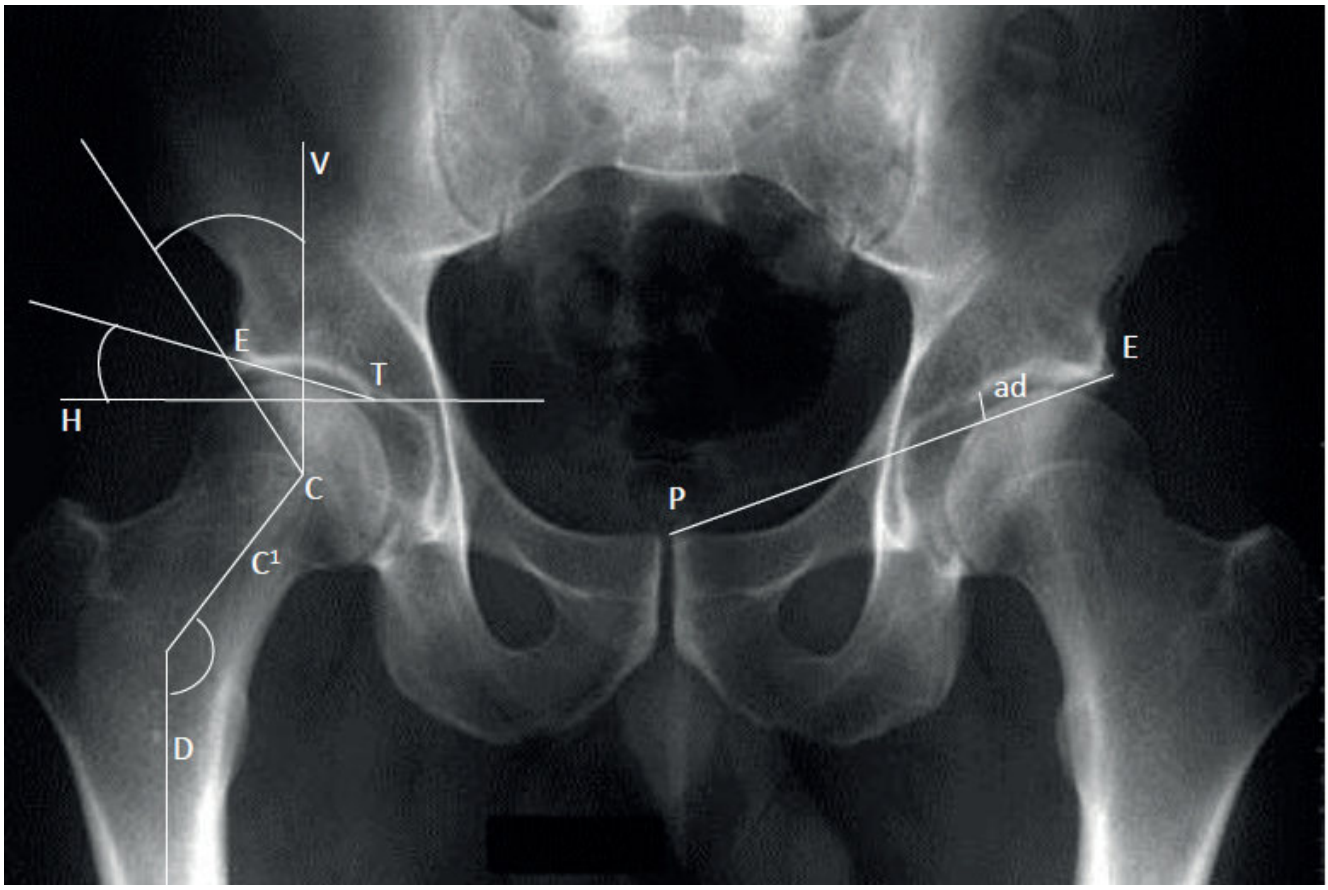

Figure 1. The VCE, HTE and neck shaft angles and acetabular depth.

tors (age, gender, obesity, genetics and diet) and joint-level factors (injury, malalignment and abnormal loading of the joints), which interact in a complex manner (1).

OA of the hip, the large weight-bearing joint, is a major source of pain and disability and represents the most frequent indication for total hip arthroplasty (4,5). Recent reviews have concluded that there is evidence of a causal relationship between occupational mechanical exposures and primary hip OA (6-8). Workers with whole-body vibration exposure report a variety of physical disorders, both musculoskeletal and neurological (9). For example, some studies have found an association between working as a farmer and an increased risk of OA of the hip (10, 11). It has been suggested that exposure to the whole-body vibration caused by driving tractors and jumping up and down from the tractor cabin are of importance. On the other hand, a study by Järvholm and colleagues (12) showed no increased risk of OA in the hip for drivers of heavy vehicles such as tractor drivers.

The aim of this study was to investigate the relationship between the exposure of drivers of heavy vehicles to whole-body vibration and pathological changes in the anatomical parameters of acetabulum. We hypothesized that exposure to the whole-body vibration in drivers of heavy vehicles will be related with significant changes in the anatomical parameters of the hip components.

SUBJECTS AND METHODS

The study was performed among 120 subjects who were divided into two groups: the experimental group and the control group. The experimental group consisted of 60 male operators of heavy vehicles (workers employed in the car industry: three paving machine operators, 12 earth movers, 24 tractor and loading machine operators, 17 dumper drivers, two roller operators, and two roller graders) who were diagnosed with lumbar syndrome and were and were 52.2±2.5 years old. The control group consisted of 60 male subjects of similar age, randomly selected from the general population, who were also diagnosed with lumbar syndrome.

Patients with a history of hip fracture and with hip deformities were excluded from the study.

The study was approved by the ethical committee of Clinical Center Kragujevac. After an explanation of the study's purposes, risks and benefits, all patients gave a written informed consent for participation in the study.

Anteroposterior (AP) radiographic views of the hips, standardized for position of the beam and radiographic penetration, were taken in the supine position with legs extended and internally rotated by 15°, with a distance of 101.6 cm between the x-ray source and the radiographic film. The central radiographic ray was aligned to be perpendicular to the cassette, entering 5.08 cm superior to the pubic symphysis. Films with incorrect patient positioning were excluded from the analysis.

Using a Plexiglas instrument with a ruler for joint space width measurement and protractors appropriate for measuring hip architectural angles (13,14), the following parameters were measured (Fig 1):

1) The vertical centre edge angle (VCE), i.e., Wiberg's angle, is the angle formed by the vertical line drawn through the centre of the femoral head (point C) and the line CE (point E being the acetabular roof lateral rim). It measures the lateral covering of the femoral head by the acetabular roof and is considered insufficient (congenital dysplasia) when it is ≤20° and excessive (coxa profunda) when it is ≥40°.
2) The 'horizontal toit externe' angle (HTE), i.e., the Tönnis angle, is the angle formed by the horizontal line drawn through point T (medial extremity of the acetabular roof) and the line TE. It measures acetabular roof acclivity and is considered too oblique when it is ≥12°.
3) The neck shaft angle (NSA) is formed by the neck axis and axis of the femoral diaphysis. It is indicative of coxa valga when it is ≥140°.
4) The acetabular depth (AD) is defined as the segment 'ad' that stretches from the deepest point of the acetabulum and the line drawn from the lateral extremity of the acetabular roof to the superior pubic angle. When the AD is ≤9 mm, this is a criterion of dysplasia (acetabular insufficiency).

Statistical analyses were performed with statistical package SPSS 19.0 for Windows. After checking the normality of the data, the data were analysed using the t-test. The results are expressed as the mean ± standard deviation. The alpha level for significance was set at $P < 0.05$.

RESULTS

According to the results presented in Table 1, the incidence rate of the pathological changes of acetabular parameters in the experimental group was higher than the incidence rate in the control group.

The mean values of VCE angles and the average values of AD in the experimental group were significantly lower than in the control group; however, in comparison with the control group, the average values of HTE angles and NSA were significantly higher in the experimental group.

Table 1. Values of the VCE angle, HTE angle, neck shaft angle, and of acetabular depth.

Parameter	Experimental group	Control group	Significant difference
VCE angle (degrees)	19.74 ± 3.69	25.8 ± 4.82	P=0.000
HTE angle (degrees)	17.43 ± 3.69	12.17 ± 4.19	P=0.000
Neck – shaft angle (degrees)	141.16 ± 6.90	134.93 ± 6.52	P=0.000
Acetabular depth (mm)	11.02 ± 3.38	13.26 ± 1.86	P=0.000

Acetabular dysplasia, considered as the condition when the VCE angle is <20°, was found in 14 subjects in the experimental group and five subjects of the control group and was unilateral in all cases. Acetabular dysplasia, defined as a VCE angle <20° with an AD <9 mm, was found in eight subjects from the experimental group.

DISCUSSION

OA is a multifactorial disease involving multiple systemic factors, such as metabolism, hormones, genetics, age, and sex, as well as local biomechanical factors, such as mechanical workload, body mass index (BMI), and acetabular dysplasia (15). Acetabular dysplasia is a condition wherein the acetabular roof is underdeveloped and remains vertically oriented and shallow, which results in a smaller surface area available for weight-bearing (16). The weight-bearing surface therefore receives a much larger force per unit area during walking and may experience early degeneration (16).

Numerous studies have shown that patients with marked hip dysplasia have an increased risk of hip OA since acetabulum abnormality leads to increased cumulative joint contact stress (17). Recent studies suggest that the mild acetabular dysplasia that persists into adult life may also be a significant factor in OA aetiology (18, 19). This aspect is especially important if a subject performs heavy physically demanding work. The presence of a subtle biomechanical abnormality, secondary to either joint incongruity (smaller acetabular depth) or decreased joint surface area (smaller CE angle), may increase joint stresses and consequently lead to OA (15). Studies on athletes from sports that subject joints to repeated high loading studies and individuals with physically demanding occupations (farmers, construction workers, metal workers, miners, pneumatic drill operators, etc.) support this assumption (20-24). Thus, the aim of our study was to explore the anatomical parameters of acetabulum in heavy vehicle operators in the car industry and note the risks of OA in this population.

To explore hip dysplasia, we used four parameters related to the shape of acetabulum and femur (VCE, HTE and NSA angle, AD) measured on AP radiographs of hips. The AP view of the pelvis is the single most important view for defining acetabular dysplasia and is the first radiographic step in the exploration of adult hip pain (14).

The VCE angle was first described by Wiberg in 1939 (25). This angle measures femoral head lateralization on the AP view of the pelvis and reports normal values if above 25°. Values between 20° and 25° are considered borderline, while a VCE angle of less than 20° is considered diagnostic of acetabular dysplasia (14). Fourteen subjects from our experimental group had hip dysplasia according to the VCE angle value, resulting in an average VCE value of 19.74 ± 3.69 in this group. The incidence of a VCE angle <20° was signifi-

cantly lower in the control group (five out of 60 subjects), resulting in a significant difference in the average value of this parameter between the two groups. In a Rotterdam study, the VCE angle was more strongly correlated with acetabular dysplasia in subjects performing high versus low physically demanding work (15), suggesting that this parameter is valid for hip dysplasia diagnosis in our experimental subjects.

In addition to a low Wiberg's angle, the radiographic diagnosis of hip dysplasia is usually made when the AD is lower than 9 mm (26). In our study, compared to controls, the experimental group had significantly lower AD values and eight out of 60 subjects fulfilled both requirements for dysplasia diagnosis (low VCE angle and AD). Previous studies have shown that there is a significant correlation between VCE angle and AD but also that both of these parameters are independent risk factors for hip dysplasia (15, 19). McWilliams and colleagues showed that as the CE angle and AD decreased, the risk of hip OA increased (19).

The Tönnis angle is used to evaluate the orientation of the acetabular roof in a coronal plane and the superior lateral coverage of the femoral head (14). It measures the angle of the weight-bearing surface and is considered normal when its value is approximately 10º, while values above 12º point to a too oblique roof acclivity (27, 28). Values of the HTE angle above 10° are frequently found in acetabular dysplasia (14). The results of our study show that the HTE angle was significantly increased in the experimental group and significantly different from the angle measured in controls. This puts much more stress on the affected hip, which is especially important in subjects performing hard work or subjects subjected to long-term WBV.

The mechanics of the hip joint are dependent on the relationship between the femoral head and the acetabulum (29). The NSA, also known as the caput-collum-diaphyseal angle or inclination angle, is an important anatomical measure for the evaluation and description of the geometry of the proximal femur and hip joint (30). Previous studies have proven its biomechanical and clinical significance in a number of orthopaedic conditions, such as hip dysplasia and OA, among other conditions. (31, 32). Femora are usually categorized as coxa vara when the NSA is <120°, physiologicall when the NSA is ≥120° to <135°, and coxa valga when the NSA is ≥135° (33, 34). A recently published systematic review (35) revealed that the mean NSA in healthy adults (5,089 hips) is 128.8° (98–180°), while in patients with OA (1,230 hips) it is 131.5° (115–155°). Such a huge variance, the authors explained, is due to the central issue of inconsistency in the published methods of measurement. However, the average NSA angles in our study were pretty high in both groups and significantly higher in the experimental group, contributing to the overall pathological status of subjects exposed to WBV.

All the presented results suggest that pathological values of the measured anatomical parameters, i.e., hip dysplasia, are very common in populations working on heavy machines that produce WBV. Since cross-sectional studies in European populations have supported an association between hip dysplasia and hip OA (36-39), we thus may conclude that subjects from the experimental group are at a higher risk of developing OA than those from the control group.

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HIGHER SERUM URIC ACID LEVELS IN MULTIPLE SCLEROSIS PATIENTS AFTER LONG-TERM INTERFERON BETA TREATMENT

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POVIŠEN NIVO MOKRAĆNE KISELINE KOD OBOLELIH OD MULTIPLE SKLEROZE NAKON DUGOTRAJOG LEČENJA INTERFERONOM BETA

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ABSTRACT

Interferon beta is a safe and efficacious treatment for relapsing multiple sclerosis (MS). However, there is some evidence that uric acid, a scavenger of peroxynitrite, is involved in MS pathology and that increasing serum uric acid levels might have beneficial therapeutic effects. The aim of this study is to investigate serum uric acid levels in MS patients before and after long-term interferon beta treatment.

Blood samples from 101 MS patients (53 receiving interferon beta 1a treatment and 48 receiving interferon beta 1b treatment; 28 male and 73 female; mean age at treatment onset 32,4±7,3 years; mean duration of disease at treatment onset 5,1±3,2 years; mean EDSS 2±1,3) before and after interferon beta treatment (mean treatment duration 3±2 years) were analysed. Serum uric acid levels were measured using a quantitative enzymatic assay (Elitech Diagnostic, Sees, France). MS patients had significantly increased serum uric acid levels after treatment compared with those at the beginning of treatment (272,31±78,21 μmol/l vs. 210,17±53,65 μmol/l; p=0,019, Wilcoxon Mann-Whitney U-test). We did not find significant differences in serum uric acid levels between the interferon beta 1a and interferon beta 1b groups (p=0.98).

These results indicate that one of the beneficial effects of interferon beta in MS might be based on the elevation of serum uric acid levels as a natural scavenger of peroxynitrite.

Keywords: *multiple sclerosis, interferon beta, uric acid*

SAŽETAK

Interferon beta je bezbedan i efikasan lek kod relapsno-remitentnog tipa multiple skleroze (MS). Međutim, postoje dokazi da je mokraćna kiselina, koja uklanja peroksinitrit, uključena u patologiju MS i da povišen nivo mokraćne kiseline može da ima korisne terapijske efekte. Cilj ovog istraživanja je da ispita nivo mokraćne kiseline kod obolelih od MS pre i posle dugotrajne terapije interferonom beta. Analizirani su krvni rezultati uzeti od 101 pacijenta (53 je primalo interferon beta-1a, a 48 je primalo interferon beta-1b; 28 muškaraca i 73 žene; prosečna starost na početku lečenja je 32,4 ± 7,3 godine, srednje trajanje bolesti na početku lečenja 5,1 ± 3,2 godine, srednji EDSS 2 ± 1,3) pre i posle lečenja interferonom beta (srednje trajanje lečenja 3 ± 2 godine). Nivo mokraćne kiseline u serumu je meren pomoću kvantitativnog enzimskog testa (Elitech Diagnostic, Sees, France). Oboleli od MS imaju značajno povišen nivo mokraćne kiseline nakon primene terapije u poređenju sa nivoima na početku bolesti (272,31 ± 78,21 μmol/l vs. 210,17 ± 53,65 μmol/l; p=0,019, Wilcoxon Mann-Whitney U-test). Nismo utvrdili značajnu razliku u nivoima mokraćne kiseline između grupa pacijenata sa terapijom interferon beta-1a pacijenata sa terapijom interferon beta-1b (p=0.98). Naši rezultati pokazuju da se jedan od korisnih efekata terapije interferonom beta kod MS može bazirati na povišenom nivou mokraćne kiseline koji prirodno uklanja peroksinitrit.

Ključne reči: *multipla skleroza, interferon beta, mokraćna kiselina*

ABBREVIATIONS

MS – multiple sclerosis,
EDSS– Expanded Disability Status Scale score,
CNS - central nervous system

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INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) with unknown aetiology. In recent years, there have been advances in understanding the pathogenesis of MS. There is significant evidence that oxidative stress is involved in MS pathology (1, 2). The proximal agents of neuronal cell damage might be nitric oxide and peroxynitrate (forming in a reaction between nitric oxide and superoxide anion radicals), which can compromise antioxidant defences and cause oxidative damage to tissues (3, 4). Uric acid is an important physiological antioxidant in the total antioxidant capacity of blood plasma (5, 6, 7). There is some evidence that MS patients have lower serum uric acid levels (8, 9, 10, 11, 12, 13, 14) and that MS and gout (chronic hyperuricaemia) are mutually exclusive diagnoses (15, 16).

Interferon beta is an efficacious and safe first line treatment for relapsing MS. It is the first therapeutic intervention demonstrated to modify the natural history of MS (reduces the relapse rate, decreases radiological disease activity, and slows progression of MS). Interferon beta has a range of effects on the immune system, which could explain its positive effects on MS (17, 18, 19, 20).

The aim of this study is to estimate changes in serum uric acid levels before and after long-term interferon beta treatment.

MATERIALS AND METHODS

Serum samples of 101 patients with definite relapsing remitting MS, according to the criteria set forth by McDonald, admitted to the Clinic of Neurology, Clinical Center Kragujevac, were analysed. Forty-eight patients with MS were treated with interferon beta-1b (Betaferon^{R}) in doses of 8 MIU s.c. every second day, while 55 MS patients were treated with interferon beta-1a (Rebif^{R}) in doses of 44 mg s.c. three times weekly, from January 2004 to January 2011.

The inclusion criteria for treatment included being over 18 years of age, having had at least two relapses in last two years, and having an EDSS score (Expanded Disability Status Scale score) (22) of less than or equal to 3.5 (criteria for including MS patients receiving interferon beta treatment in Serbia). During this seven-year period, 26 patients stopped the treatment (16 interferon beta 1a and 10 interferon beta 1b patients). We investigated the serum uric acid levels of the MS patients before initiating the treatment and at the end of the treatment (in cases of treatment discontinuation) or in January 2011 for the patients who continued the treatment.

All blood samples were taken as part of a routine laboratory screening for interferon beta treatment (looking for possible adverse events every six months or at the end of the treatment). Serum uric acid levels were measured using a quantitative enzymatic assay (Elitech Diagnostic, Sees, France) according to the manufacturer's protocol, and the results were standardized using a commercial uric acid standard solution. Blood samples were obtained from all subjects after overnight fasting. Because of the circadian fluctuation of uric acid, all blood samples were taken at the same time (at 8 am) (23). There were no patients with chronic renal disease or diabetes mellitus, nor were there any receiving acetylsalicylic acid and thiazide diuretics. In Clinical Center Kragujevac, the normal range of serum uric acid levels was 150 to 350 μmol/l for females and 210 to 420 μmol/l for males. All MS patients consented to be involved in the study. The local Ethical Committee approved this investigation.

Table 1. Demographic and clinical characteristics of MS patients

	Total	Interferon beta 1a	Interferon beta 1b	p
Number of patients	101	53	48	ns
Male/Female	73/28	41/12	32/16	ns
Age at treatment onset: years (Mean±SD) Range	32.4±7.3 19-56	32.5±8.1 19-56	32.5±6.3 23-47	ns
Disease duration at onset of treatment (Mean±SD) Range	5.1±3.2 1-15	4.6±2.5 1-11	5.6±3.7 1-15	ns
EDSS score at treatment onset (Mean±SD) Range	2.1±1.3 0-3.5	2.3±1.2 0-3.5	1.9±1.5 0-3.5	ns
Mean follow up period	3.9±2.0	3.73±2.14	3.9±1.8	ns

EDSS- Expanded Disability Status Score; ns- not significant

The clinical and demographic characteristics of the MS patients are presented in Table 1.

The SYSTAT program was used for statistical analysis. Significant differences between groups was calculated by the Wilcoxon Mann-Whitney U test. Differences in mean values were calculated using t-test. Correlations were analysed using Spearman's rank correlation. The results are given as mean ± SD.

RESULTS

Serum uric acid levels before and after treatments with interferon beta (i.e., the follow-up period) are pre-

Table 2. Uric acid levels in serum of MS patients before and after interferon beta treatment

MS patients	Serum uric acid levels umol/l ± SD	p
Before onset of treatment	210.17±53.65	
After follow up period Total Interferon beta 1b Interferon beta 1a	 272.31±78.21* 281.33±52.28* 269.89±93.71*	 0.019 0.015 0.022

* significant in comparison with serum uric acid levels before treatment

sented in Table 2. The MS patients were found to have significantly higher mean serum uric acid levels after a mean of 3,9 years of interferon beta treatment compared with pretreatment serum uric acid levels (272,31±78,21 μmol/l *vs.* 210,17±53,65 μmol/l; p=0,019, Wilcoxon Mann-Whitney U-test). On the other hand, there were no significant differences in serum uric acid levels in MS patients after treatment with interferon beta 1b and interferon beta 1a (281,33±52,28 μmol/l vs. 269,89±93,71 μmol/l, respectively; Wilcoxon Mann-Whitney U-test, p=0,298).

Pretreatment serum uric acid levels increased in 88 patients, decreased in five patients and were unchanged in eight patients after a mean 3,9 years of treatment. We also found a significant linear correlation between the duration of interferon beta treatment and changes (increasing) in serum uric acid levels (p=0,021).

DISCUSSION

Interferon beta is a well-established and safe first line treatment for MS. To our knowledge, this is the first study to compare changes in serum uric acid levels before and after long-term interferon beta treatment. We previously reported higher uric acid levels after one year of interferon beta 1b treatment in a small preliminary sample of patients (24).

Several studies have demonstrated lower serum uric acid levels in MS patients and suggest that higher levels of uric acid may offer protection against the development of MS (5-16). There are some opinions that the lower urate levels among multiple sclerosis cases could be a consequence rather than a cause of the disease (25). Consequently, treatment attempts in human MS with a precursor of uric acid have been reported (26, 27). A few studies have demonstrated elevated serum uric acid levels after the administration of some drugs, such as glatiramer acetate (28) and high-dose methylprednisolone (29), indicating that some beneficial effects of these drugs might be due to the elevation of serum uric acid levels.

Interferon beta reduces the exacerbation rate in patients with relapsing remitting (RR) MS, decreases disease activity in the brain (measured as the identification of new or enlarging lesions in serial brain magnetic resonance imaging (MRI)), and slows the progression of MS. Interferon beta exerts a large range of effects on the immune system, which can explain its positive effects on MS, including antagonism of the proinflammatory cytokine interferon gamma (17, 18), inhibition of the production of chemokines and matrix metalloproteinases (19), and increased production of Interleukin 10 (20).

The results of this study indicate that one of the beneficial effects of interferon beta might be based on the elevation of serum levels of uric acid as a natural scavenger of peroxynitrite.

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QUALITY OF LIFE IN PRIMARY INSOMNIA: THREE-WEEK TREATMENT WITH ZOLPIDEM VS. LORAZEPAM

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KVALITET ŽIVOTA U PRIMARNOJ NESANICI: POREĐENJE TRONEDELJNOG TRETMANA ZOLPIDEMOM I LORAZEPAMOM

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ABSTRACT

Insomnia is a condition of inadequate quality or quantity of sleep that has extremely adverse effects on daytime activities. The aim of this study was to compare the quality of life in patients with primary insomnia before and after a 3-week treatment with lorazepam (n=20) and zolpidem (n=21) and to compare the potential differences in dysfunctional beliefs and attitudes regarding patients' sleep between the two groups. The diagnosis of primary insomnia was established using the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision criteria, and patients had to complete a specially designed sleep log every day; on scheduled visits, we also administered a Visual Analogue Scale for quality of life and a self-evaluation questionnaire about Dysfunctional Beliefs and Attitudes related to Sleep at the beginning and end of this study.

In summary, the examinees in our study had significantly decreased parameters of quality of life, quite lower than expected based on previous findings in this area. However, by the end of the study, quality of life significantly improved with treatment: it improved by approximately 2/3 in the Lorazepam group and more than twice in the Zolpidem group, with a significant difference in favour of Zolpidem (p=0.047). This finding is most likely a consequence of its better safety profile and in part its better efficiency in terms of influence on certain domains of sleep itself, as previously discussed. Further specialized studies in this area with larger samples and a more detailed methodology are clearly warranted.

Keywords: *primary insomnia, zolpidem, quality of life*

SAŽETAK

Nesanica je stanje neadekvatnog kvaliteta ili kvantiteta sna, koje ima izuzetno negativne efekte na dnevne aktivnosti. Cilj ovog istrazivanja je bio da se uporedi kvalitet života pacijenata sa primarnom nesanicom pre i nakon tronedeljnog tretmana lorazepamom (n=20) i zolpidemom (n=21) i da se uporede potencijalna disfunkcionalna verovanja i stavovi o spavanju između ove dve grupe. Dijagnoza primarne nesanice je postavljena korišćenjem kriterijuma Dijagnostičkog i statističkog priručnika za mentalne poremećaje, četvrto izdanje, a pacijent je svakog dana morao da kompletira specijalno dizajniran dnevnik spavanja dok je na zakazanim posetama primenjivana Vizuelna analogna skala za kvalitet života i upitnik samo-evaluacije o Disfunkcionalnim verovanjima i stavovima povezanim sa spavanjem, i to na početku i na kraju ove studije.

Utvrdili smo da su ispitanici u našem ispitivanju imali značajno snižene parametare kvaliteta života, više od očekivanog, ceneći dosadašnje nalaze iz ove oblasti. Međutim, do kraja ispitivanja, kvalitet života je značajno poboljšan uz primenjenu terapiju: kvalitet života je povećan približno za 2/3 u grupi na Lorazepamu, a skoro 2 puta u grupi na Zolpidemu, sa značajnom razlikom u korist Zolpidema (p=0.047). Ovakav nalaz je najverovatnije posledica njegove bolje sigurnosti, a delom i zbog njegove bolje efikasnosti u pogledu uticaja na pojedine oblasti sna, što je prethodno diskutovano.

Dalja istraživanja, specijalizovana u ovoj oblasti, sa većim uzorcima i detaljnijom metodologijom su apsolutno opravdane.

Ključne reči: *primarna nesanica, Zolpidem, kvalitet života*

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INTRODUCTION

Insomnia is a condition of inadequate quality or quantity of sleep (1) that most commonly occurs due to difficulties falling asleep and maintaining sleep and early awakening. Poor sleep does not enable rest, which reflects extremely adversely on daytime activities (2).

To be exact, insomnia is a consequence of numerous illnesses and conditions included under one general term called "sleep disorders". In many individuals with chronic insomnia, it is not possible to determine a clear cause for the condition. The majority of them most likely suffer from primary insomnia, a disorder with a frequency in the general population of 1.3% (3) to 5% (4). Problems with primary insomnia often last over a year (5,6). In some cases, the problems occur early in childhood, while in others, they occur later in life, especially after a stressful event. One study suggested that the average duration of primary insomnia was approximately 14 years, and the onset of complaints was reported to be approximately at the age of 40 years (4). A thorough analysis suggested that primary insomnia is basically a heterogeneous entity that consists of several specific disorders such as psychophysiological insomnia, idiopathic insomnia, and sleep state misperception (7). Chronic stress, inadequate sleep hygiene, and internal distress can also contribute to the onset of primary insomnia (8).

The social aspects of the illness suggest that insomnia can be related to significant daytime effects such as fatigue, irritability, poor concentration and mood changes, and many life activities are disturbed as well (9). For instance, there is also a belief that insomnia is related to greater work absence (10), and it might be associated with a greater risk of car accidents (11). After relieving any pain, the physician's next obligation is to enable adequate sleep for patients in primary healthcare (12). The results published to date clearly indicate that insomnia has pervasive effects on numerous aspects of health-related quality of life and that this connection, to various extents, occurs independently of the comorbidities present (13).

The combined use of a pharmacologic and non-pharmacologic approach (cognitive-behavioural interventions) to treat insomnia provides better results than each of them separately (14). Among the several medications labelled as benzodiazepine hypnotics, the most suitable candidates include temazepam, estazolam, loprazolam, lormetazepam, oxazepam and lorazepam. However, the first 4 hypnotics are not available in Serbia, and oxazepam penetrates the brain relatively slowly, causing a delayed hypnotic action (15). This pharmacological profile makes lorazepam, similar to temazepam, one of the most prescribed hypnotics (16).

The aim of this study was to compare the quality of life of patients with primary insomnia before and after a 3-week treatment with lorazepam and zolpidem and to compare the potential differences in dysfunctional beliefs and attitudes regarding patients' sleep between the two examined groups.

MATERIAL AND METHODS

Patients and Treatment

The study was conducted at the Psychiatric Clinic of the Clinical Centre Kragujevac from September 2003 until May 2010, and it included patients who met the following inclusion criteria: ambulatory patients of both genders, age 18 to 65 years, with a diagnosis of primary insomnia according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (17). Patients' somatic state and laboratory analysis had to be within physiological ranges or clinically insignificant, and patients were not previously treated with any psychotropic medications. Before joining the study, the patient or his or her legal representative had to sign, voluntarily and individually, the written informed consent approved by the independent Ethical Committee of Clinical Centre Kragujevac.

The study included 3 weeks of active treatment for each patient, and during this period, the patients received the investigated medications. The duration of active treatment and the dosage were determined according to guidelines on the rational use of hypnotics. The number of patients evaluated to join the study was 49, 41 of whom were included in active treatment and then randomly divided into the treatment groups by clinical pharmacologist. Treatment groups were prepared in accordance with a randomization code list. Qualified patients were randomly placed into group 1 or group 2: group 1 received a 10 mg oral tablet of zolpidem prior to sleep, and group 2 received a 2 mg oral tablet of lorazepam prior to sleep.

From Visit 1, during week 1 of active treatment, the investigated medications were administered continuously, i.e., every night prior to sleep. Since the continuous use of hypnotics can be related to the development of tolerance, as well as rebound effects, we used an intermittent dosage after Visit 2 (during week 2) and after Visit 3 (during week 3 of active treatment). Specifically, the dose was decreased as follows: in week 2, the patients received 5 capsules each, and in week 3, they received 3 capsules each, which they used during the week according to their own needs, until Visit 4 at the end of the study.

Psychiatric Assessment

A diagnosis of primary insomnia can be established when all obvious causes are carefully excluded (17). DSM-IVTR criteria should be used to confirm the diagnosis: complaints lasting for at least a month and causing significant problems in social, professional and all other aspects of life. It is mandatory to exclude other specific sleep disorders (narcolepsy, difficulty breathing, parasomnias, and disorders of circadian rhythm), mental and somatic illnesses, as well as substance abuse (taking drugs) (18).

The examinees completed a specially designed sleep log every day (obtained by combining the Athena Insomnia Scale, 5 items (AIS-5) (19), and the Visual Analogue

Scale (VAS) for quality of life (20)), while on scheduled visits, we also administered the VAS for quality of life and a self-evaluation questionnaire about Dysfunctional Beliefs and Attitudes related to Sleep (DBAS) at Visit 1 and Visit 4.

A Visual Analogue Scale was used to assess patient's quality of life related to his or her current health condition. This scale consists of a 100 millimetre line, with the beginning of the line (0 mm) defined as ²I am completely ill, tired and exhausted, and bad health has completely ruined my life² and the end of the line (100 mm) defined as ²I am completely healthy, rested and fresh, and my great health enables me to fully commit to life². The examinees were asked, ²How did your health condition affect your quality of life during the past week?², and they were instructed to mark the line with a vertical dash, corresponding to where they felt was the appropriate answer. The number of millimetres from the beginning of the line to the spot where the examinee had marked the line was measured with a ruler, and the length obtained corresponded to the score. Higher scores represent a better quality of life (21).

As insomnia also implies a disorder in cognitive functioning, we used a list of 30 items (statements) to obtain a self-evaluation of beliefs and attitudes regarding sleep and difficulties maintaining sleep – the Dysfunctional Beliefs and Attitudes about Sleep (DBAS). The examinee rates all items from 0 to 10. The final score is obtained by adding the points from all items and dividing the total sum by the number of items. This questionnaire is used to establish and assess the changes in cognitive functioning due to sleep and insomnia (beliefs, attitudes, expectations, evaluations, characteristics) (22).

To carefully establish the timeline of quality of life affected by the study medications, in our study, we applied the concept of days adjusted by quality of life (Quality-Adjusted Life Days- QALD). This approach was used analogously to the widely accepted and used concept referring to the period of one year (Quality Adjusted Life Years- QALY), which was primarily developed for chronic illnesses (23). Our methodology was determined by the short-term study period and was also based on similar previous studies that referred to illnesses and conditions with a limited time duration, such as antibiotic prophylaxis (24), prevention of acute respiratory infections (25) and therapy of acute pyelonephritis (26). The basis for the QALD assessment was the previously mentioned utility scores established using the VAS.

Statistical Analysis

All statistical tests were performed using appropriate software (SPSS, 22.0, Chicago, IL, USA). The following descriptive methods were used: measures of central tendency (x), measures of variability (SD) and relative numbers (indicators of structure). Regarding methods for hypothesis testing, the following tests were used: Chi-square test, t-test, Mann-Whitney test, Wilcoxon test, Analysis of variance and Friedman test. Statistical hypotheses were tested at a level of significance of $p<0.05$.

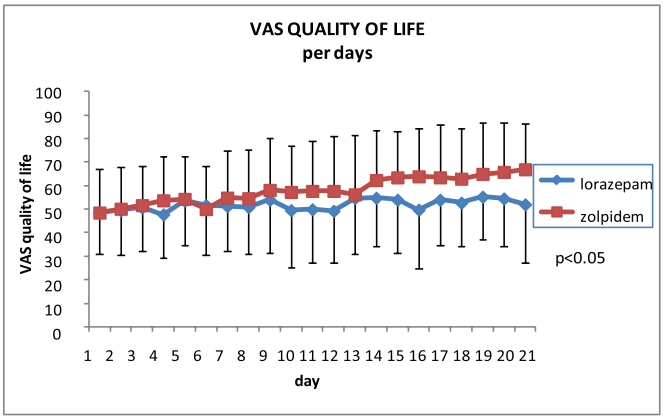

Figure 1. VAS quality of life per day

Based on the daily assessment of quality of life, using the VAS scale as part of the sleep log, we calculated the percentage differences in quality of life (utility score = VAS per day/100), as well as the number of days of quality life (QALD, Quality-adjusted life days = utility score * 21).

RESULTS

Demographic Data

The majority of patients, approximately 2/3, were middle-aged, at the beginning of the fifth decade of their life. A significantly smaller number of patients was younger than 30 years of age (approximately every fifth examinee) or older than 60 years (approximately every 10th examinee). Two-thirds of the investigated population were female. Additionally, approximately 2/3 patients were from an urban environment, whereas every 6th was from a rural setting. Regarding age, gender, place of birth and place of residence, there were no statistically significant differences between the investigated groups.

VAS - Quality of life per day

The comparison of changes in VAS quality of life scores obtained through the 21 days of the study in both groups of patients is presented in Table 1 and Figure 1. There were some significant changes in VAS score over time in both groups of examinees, i.e., the Lorazepam group ($p=0.002$) and Zolpidem group ($p<0.001$). There was a statistically significant difference in VAS quality of life scores between the investigated groups for Day 20 ($p=0.049$) and Day 21 ($p=0.043$), with examinees from the Zolpidem group having significantly better quality of life on those days than the Lorazepam group.

VAS - Quality of life per visit

Repeated-measures single factor analysis of variance was used to compare the changes in VAS scores of quality of life obtained during Visits 1, 2, 3 and 4 in both the

Table 1. VAS quality of life per day

VAS Patient self-evaluation	lorazepam (n=20)	zolpidem (n=21)	p lorazepam vs zolpidem
	$\bar{x}$±SD	$\bar{x}$±SD	
Day 1	48,6±17,61	48,52±18,34	0,896
Day 2	49,45±18,78	50,14±17,78	0,904
Day 3	50,65±18,44	51,66±16,43	0,853
Day 4	47,75±18,33	53,71±18,76	0,31
Day 5	53,55±18,87	54,33±18,13	0,927
Day 6	51,65±21,1	50±18,35	0,79
Day 7	51,25±19,1	54,86±19,8	0,556
Day 8	50,85±19,89	54,62±20,76	0,557
Day 9	53,95±22,67	58,14±22,1	0,522
Day 10	49,55±24,32	57,28±19,66	0,269
Day 11	49,9±22,7	57,71±21,27	0,239
Day 12	49,25±21,98	57,71±23,29	0,188
Day 13	54,7±23,66	56,24±25,3	0,774
Day 14	54,8±20,4	62,33±21,14	0,253
Day 15	53,95±22,65	63,47±19,37	0,155
Day 16	49,75±24,84	63,9±20,53	0,053
Day 17	53,95±19,11	63,48±22,57	0,144
Day 18	52,7±18,57	62,9±21,21	0,064
Day 19	55,25±18,3	64,95±21,58	0,144
Day 20	54,5±20,1	65,8±20,99	0,049
Day 21	52±24,9	66,9±19,22	0,043
p change in time	0,002	0,001	

VAS- Visual Analog Scale; n- number of patients; $\bar{x}$- mean value; SD – standard deviation;

Table 2. VAS - quality of life per visits

Visit	lorazepam (n=20)	zolpidem (n=21)	p lorazepam vs zolpidem
	$\bar{x}$±SD	$\bar{x}$±SD	
Visit 1	32,6±16,24	30,76±15,15	0,676
Visit 2	49,5±17	56,19±18,84	0,134
Visit 3	53,5±17,79	62,76±19,64	0,14
Visit 4	54,95±19,29	67,85±20,1	0,025
p change in time	p<0,001	p<0,001	

VAS- Visual Analog Scale, n- number of patients; $\bar{x}$- mean value; SD – standard deviation

Lorazepam and Zolpidem groups (Table 2). Significant changes over time were identified in the Lorazepam group (p<0.001), as well as in the Zolpidem group (p<0.001).

There was a significant difference in VAS quality of life scores between the investigated groups at Visit 4 (p=0.025), indicating that Zolpidem patients had a better quality of life than Lorazepam patients at that visit. The changes in scores of the VAS quality of life item between visits in both groups of examinees (lorazepam and zolpidem), as well as their statistical significance, are shown in Table 3.

There were significant changes in the Lorazepam group, p<0.05, at Visit 1 compared to Visit 2 (p=0.001), Visit 3 (p<0.001) and Visit 4 (p<0.001). There were also significant changes in the Zolpidem group during Visit 1 compared to Visit 2 (p<0.001), Visit 3 (p<0.001) and Visit 4 (p<0.001) and during Visit 2 compared to Visit 3 (p=0.001) and Visit 4 (p=0.001).

Overall assessment of quality of life

The mean values, standard deviations and significance of changes in total scores for the VAS item- quality of life obtained in the daily evaluations with sleep logs and weekly evaluations during the visits are shown in Table 4.

There were no significant differences based on the daily evaluations, but there was a significant difference in the change in VAS score per day of quality of life between groups in favour of the Zolpidem group (p=0.047) according to the evaluations at visits, which means that the Zolpidem patients had an overall better quality of life.

Based on the daily assessments with sleep logs, the percentage differences in quality of life (utility score), as well as the number of quality life days (QALD) were calculated. The mean values, deviations and significance of the scores of these variables are shown in Table 5.

There were no statistically significant differences in the change in total VAS score, utility score or QALD value between the investigated groups. However, the results showed that there was a difference in VAS quality of life score in favour of Zolpidem patients. During the 3 weeks of treatment, Zolpidem patients had 1.3 more days of quality life than the Lorazepam patients.

DBAS assessment

Changes in cognitive sleep experience at the beginning and at the end of the study were assessed by the DBAS. The results obtained are presented in Table 6.

There was a significant change in DBAS score in the Lorazepam group at p<0.05 on Day 0 compared to Day 21 (p=0.006). A significant change in DBAS score was also present in the Zolpidem group (p<0.001). There was no statistically significant difference in the change in DBAS score between the investigated groups at Visit 1 (p=0.173) and Visit 4 (p=0.392).

DISCUSSION

The concept of quality of life is becoming very important in modern medicine (27). Although there is no universal, generally accepted definition of this term, it most often comprises a subjective and objective assessment of an individual's current level of functioning compared to what that individual perceives is possible or ideal (28).

The effect of a disease on quality of life is determined using different instruments of clinical assessment (29).

Table 3. VAS quality of life between visits

VAS Quality of life	lorazepam (n=20)	zolpidem (n=21)
	p	
V1-V2	0,001	0,001
V1-V3	0,001	0,001
V1-V4	0,001	0,001
V2-V3	0,297	0,001
V2-V4	0,767	0,001
V3-V4	1	0,073

VAS- Visual Analog Scale; n- number of patients; x;¯- mean value; SD – standard deviation;

Table 4. VAS per day of quality of life

Type of assessment	lorazepam (n=20)	zolpidem (n=21)	p
	x;¯±SD	x;¯±SD	
Evaluation at visits	52,78±16,78	62,55±18,16	0,047
Everyday evaluation	51,81±17,47	58±18,79	0,278

VAS- Visual Analog Scale; n- number of patients; x;¯- mean value; SD – standard deviation;

Table 5. VAS score, utility score and QALD of investigated groups

Quality of life variable	lorazepam (n=20)	zolpidem (n=21)	p
	x;¯±SD	x;¯±SD	
VAS total score	1088±366,98	1218,71±394,48	0,197
Utility score*	0,52±0,17	0,58±0,18	0,197
QALD**	10,88±3,67	12,18±3,94	0,196

VAS- Visual Analog Scale; n- number of patients; x;¯- mean value; SD- standard deviation;
*Utility score = VAS per day/100;
**Quality adjusted life days = utility score • 21

Table 6. DBAS score at the first and at the final visit

DBAS	lorazepam (n=20)	zolpidem (n=21)	Statistical significance among groups
	x;¯±SD	x;¯±SD	
Visit 1	5,18±0,98	5,59±0,89	p=0,173
Visit 4	4,4±1,27	4,1±1,01	p=0,392
Statistical significance of changes in time	p=0,006	p<0,001	

VAS- Visual Analog Scale; DBAS- Dysfunctional Beliefs and Attitudes related to Sleep; n- number of patients; x;¯- mean value; SD- standard deviation

Considering the numerous modalities of living and the absence of a unique definition and classification in this area, the precise objectification of quality of life remains a significant investigational challenge. However, by using different methodological approaches and compiling the known findings, relatively good insight into the influence of many diseases on quality of life has been achieved.

There are few studies on insomnia that have investigated quality of life, especially considering the conditions studied in randomized clinical studies. Some of the potential reasons for this scarcity of research are as follows: the large number of different clinical manifestations of sleep disorders, the presence of comorbidity especially from the group of mental illnesses, the absence of a unique classification of treatment, the significant number of different therapeutic approaches, and the demographic, social, economic and cultural differences in different environments. However, based on previously developed instruments such as the QOLI (Quality of Life of Insomniacs) and Leeds Sleep Evaluation Questionnaire, it has been indicated that the presence of insomnia significantly disturbs many domains of quality of life (30,31). Subsequent methodological improvements, such as the SF-36 questionnaire (Medical Outcomes Study Short-Form Health Survey 36), Quality of Life Enjoyment and Satisfaction Questionnaire (Q-LES-Q), Hotel Dieu 16 (HD-16) and others, have enabled further insight into the relationship between insomnia and quality of life.

In this study, we performed a global assessment of quality of life using the Visual Analogue Scale on a daily basis (using the sleep log) as well as weekly at study visits. At the beginning of the study, it was established that insomnia has an extremely negative influence on quality of life (quality of life was rated at only 1/3 of the ideal). However, by the end of the study, quality of life had significantly improved with treatment, by approximately 2/3 in the Lorazepam group and more than twice in the Zolpidem group, with significant difference in favour of Zolpidem. Similar results were achieved in the daily assessments of quality of life.

The estimated quality of life was significantly lower than those of other similar studies. For instance, in a sample of 35,527 Ontario residents in Canada, the utility index for mental illnesses (indirect measure of quality of life) was estimated to be between 0.846 and 0.850 out of a maximum score of 1.0, while the lowest values were recorded in individuals who survived a traumatic event (0.765 - 0.790) (32). In a different study, quality of life was evaluated as relatively good in individuals with insomnia: 70% in individuals with chronic insomnia, 81% in individuals with occasional sleep disturbances, and 96% in individuals without insomnia (9). The lower quality of life in our study can be explained by the influence of at least 3 factors. First, the specific social-economic conditions in our country in previous decades had a significant influence, leading to a decrease in quality of both health and living compared to other, more developed countries, as shown by many domestic studies (33,34,35) and as recognized by the international public as well (36,37).

Second, there is a certain degree of methodological limitations to our research regarding the measurement of quality of life. Although the VAS scale, which was used in our study, is a widely used instrument, it does have some significant shortcomings (38). Accordingly, the simultaneous use of several techniques such as Time-to-Trade Off (TTO),

Standard-Gamble (SG) or generic questionnaires such as the SF-36 is recommended for a more precise assessment of quality of life. In studies of patients with insomnia treated with zolpidem, the SF-36 scale showed a significant improvement in quality of life (39,40). However, our study was not specifically directed and designed to evaluate quality of life. Instead, that part of the analysis was primarily predicted by the needs of an economic model, i.e., the identification of utility scores for a subsequent analysis of cost-utility. Indeed, there are almost no valid studies for individuals with insomnia that have established the exact values of utility scores, especially in therapeutic trials (41).

Third, it is known that quality life is negatively correlated with the severity of insomnia (42). The inclusion of the general population in the assessment of the index, that is, utility scores, and the heterogeneity in terms of various clinical forms of insomnia represent significant methodological differences between our research and other studies, and thus a more reliable comparison of results is needed. Our study sample was strictly limited to individuals with primary insomnia, and the presence of more than two-thirds of those with a severe form of insomnia might be the reason for the significantly worse quality of life scores than expected.

In our study, the group that received zolpidem had higher QALD scores; more precisely, every zolpidem examinee had 1-2 more days of quality living than individuals in the control group. Although this difference was not statistically significant and seems relatively modest, it could be of greater interest over a longer period of time. For example, a six-month duration of insomnia is the minimum period for diagnosing chronic primary insomnia beyond any doubt (43). If individuals are treated with zolpidem throughout this entire period, as in previous clinical studies (44,45), then they could have at least 8 to 16 more days of quality life than if they were not taking any hypnotic medication or were treated with lorazepam or similar benzodiazepines. As chronic insomnias usually last a year, even longer (6), this gain would become progressively larger over a longer period of time.

CONCLUSION

In summary, the examinees in our study had a significantly decreased quality of life, quite lower than expected based on previous findings in this area. Generally, individuals treated with zolpidem showed a trend of improving quality of life, which reached statistical significance in some aspects by the end of the study monitoring period. This finding is most likely a consequence of its better safety profile and partially its better efficiency in terms of influence on certain domains of sleep itself, as discussed previously. The small study sample, methodological limitations of evaluating quality of life, as well as the relatively short period of time are probable reasons the superiority of zolpidem in this domain of clinical efficiency was not definitively proven. Furthermore, specialized studies in this area with larger sample sizes and a more detailed methodology are clearly necessary. Based on our results, treating insomnia can improve quality of life domains to a significant extent, although the final effects depend on many factors such as the investigated population, the assessment method used, and the therapeutic modality.

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Conflict of Interest
None.

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PREVALENCE OF RISK FACTORS AMONG WOMEN WITH OSTEOPOROSIS

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PREVALENCA FAKTORA RIZIKA KOD ŽENA SA OSTEOPOROZOM

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ABSTRACT

Osteoporosis is a progressive bone disorder that can be influenced by many different factors. A cross-sectional study has been conducted with the aim to assess the prevalence of risk factors as well as to identify the possible causes of improvement of the disease. The study population consisted of 97 women older than 35 who had previously been diagnosed with osteoporosis. Dual-energy X-ray Absorptiometry (DXA) scan was used to determine bone mineral density (BMD) in order to assess the current state of the disease. The participants were asked to complete a standardized IOF (International Osteoporosis Foundation) questionnaire. According to BMD measurements, 24.7% of women had normal bone density while 18.6% had T-score lower than -2.5. There was a statistically significant correlation between T-score and the history of previous bone fractures. Besides, a relatively high prevalence of certain risk factors (such as underweight, early menopause, oophorectomy, thyroid and parathyroid disorders etc.) was observed in woman with osteoporosis.

Keywords: *osteoporosis; osteopenia; bone mineral density; T-score; risk factors*

SAŽETAK

Osteoporoza predstavlja progresivni poremećaj kostiju koji nastaje pod uticajem velikog broja faktora.Studija preseka je sprovedena sa ciljem da se proceni prevalenca faktora rizika, kao i da se identifikuju eventualni uzroci poboljšanja bolesti. Studija je obuhvatila 97 žena, starijih od 35 godina, sa prethodno dijagnostikovanom osteoporozom. Metod dvostruke apsorpciometrije X-zraka (DXA) je upotrebljen za određivanje mineralne gustine kostiju (BMD) i procenu trenutnog stanja bolesti. Učesnice su popunjavale standardizovani IOF upitnik. Na osnovu merenja BMD, 24.7% žena je imalo normalnu gustinu kostiju, dok je kod 18,6% određen T-skor niži od -2,5. Dobijena je statistički značajna povezanost T-skora sa istorijom preloma. Osim toga, dobijena je relativno visoka prevalenca određenih faktora rizika (kao što su pothranjenost, rana menopauza, ooforektomija, poremećaji tiroidne i paratiroidne žlezde itd) kod žena sa osteoporozom.

Ključne reči: *osteoporoza; osteopenija; mineralna gustina kostiju; T-skor; faktori rizika*

ABBREVIATIONS

BMD – bone mineral density **BMI** – body mass index

INTRODUCTION

Osteoporosis is a progressive skeletal disorder characterized by low bone mass, compromised bone structure and increased risk of fractures (1). It affects more than 75 million people in Europe, Japan and the USA (2), and therefore it represents a serious public health problem. Every year it causes about 9 million of fractures worldwide (3). According to statistics, 1 in 3 women as well as 1 in 5 men aged over 50 experience osteoporotic fractures. Based on bone mineral density (BMD), the following categories have been established (4):

- Normal bone structure – BMD is less than 1 standard deviation (SD) below the young adult mean value (T-score ≥ -1);
- Osteopenia – BMD is between 1 and 2.5 SD below the young adult mean value (-2.5 < T-score < -1);
- Osteoporosis – BMD is more than 2.5 SD below the young adult mean value (T-score ≤ -2.5);
- Severe osteoporosis – BMD is 2.5 SD or more below the young adult reference mean in the presence of fragility fractures.

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Several unchangeable predictors of osteoporosis have been identified, such as genetics, age, gender, some chronic diseases etc. On the other hand, there are some factors that can be controlled in order to prevent the disease and decrease the occurrence of fractures. These are dietary patterns, nutritional status, cigarette smoking, physical activity, alcohol use etc. The aim of this study was to investigate the current state of the disease as well as the prevalence and the role of certain risk factors among patients who had previously been diagnosed with osteoporosis.

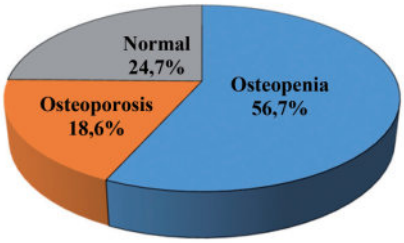

Figure 1. Classification of the participants according to the measured T-score value

MATHERIAL AND METHODS

A cross-sectional study has been carried out using a simple random sampling method. The study population consisted of 97 female patients older than 35, who had previously been diagnosed with osteoporosis. The size of the sample was determined using G-power 3.0.10 (with an assumed power of 0.80 and significance level α=0.05). The study was conducted during the year 2014-2015, in the Health Centre Kragujevac, Serbia.

All participants were subjected to Dual-energy X-ray Absorptiometry (DXA) scan in order to determine BMD, T-score (deviation of patient's BMD from the reference BMD of a healthy 30-year old adult) and Z-score (deviation of BMD from age-matched reference value). In addition, the participants were also asked to fill out a standardized IOF (International Osteoporosis Foundation) questionnaire (5). It included questions regarding family anamnesis, lifestyles, history of fractures, diseases and medications. Anthropometric measurements were performed in order to calculate body mass index (BMI) of all respondents.

Statistical analyzes were done using SPSS 20.0 computer software. Data were tested for normality using Kolmogorov-Smirnov test. Spearman bivariate correlation analyzes were applied in order to investigate the correlations between quantitative variables, while chi-square (χ^2) test was used for analyzing categorical data. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Table 1 presents descriptive statistics of quantitative variables investigated in the study. The age of the participants ranged from 38 to 83, with a mean of 62.5 ± 8.7. T-score and Z-score were determined based on measured BMD. According to current T-score, 24.7% of women had normal bone density, while 18.6% retained the diagnoses of osteoporosis (Figure 1). The rest of the participants were classified as osteopenic.

Table 1. Descriptive statistics of quantitative variables

	Minimum	Maximum	Average	Standard deviation
Age	38	83	62.5	8.7
Menopause	20	58	42.8	14.1
BMD	0.19	0.85	0.41	0.13
T-score	-3.60	0.50	-1.68	0.94
Z-score	-3.20	1.20	-0.86	0.92

Table 2. Spearman correlation analyzes

	Age	Beginning of menopause
T-score	-0,339	-0,097
Z-score	0,085	0,068

The results of analyzing correlations between continuous variables are given in Table 2. T-score was moderately negatively correlated with the age of the participants. There was a weak correlation of T- and Z-score with the age of beginning of menopause. Figure 2 presents T-score classification of the participants belonging to different age groups.

Table 3 presents the results of analyzing the data obtained using IOF questionnaire. Two categories (df=1) were considered while performing χ^2 test: women with normal BMD (T-score > -1) and women with osteopenia or osteoporosis (T-score < -1). As expected, a significantly higher percentage of women with T-score < -1 experienced bone fractures after a minor fall. No other statistically significant difference was found between these two categories.

DISCUSSION

According to BMD measurements, a significant improvement of the disease was observed in 24.7% of patients who had previously been diagnosed with osteoporosis. In addition, 56.7% of women were classified as osteopenic. This amelioration could be the result of combined effects of many different factors that can influence the development of the disease. As expected, the ratio of normal to osteoporotic cases decreased with increasing age of the participants (Figure 2).

Previous studies have indicated that bone mineral density is highly (60-80%) dependent on genetic factors (6, 7). About 24% of all respondents participating in our study have reported having one or both parents with the diag-

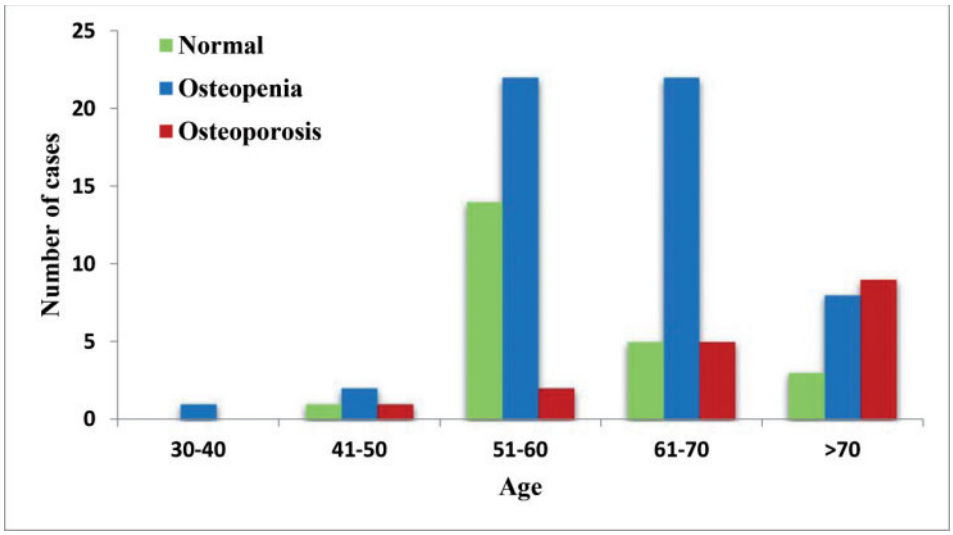

Figure 2. Number of participants with normal BMD, osteopenia and osteoporosis in different age intervals

nosis of osteoporosis. The prevalence of such cases was higher among the women with T-score < -1. Besides, more women with osteopenia or osteoporosis had bone fractures in the past (in comparison to those with normal BMD). This difference was statistically significant (p=0.028) and it was in agreement with some previous studies that also found a strong correlation of BMD with the history of falls and fractures [8].

Table 3. The analyzes of categorical data obtained from IOF One-Minute Test

IOF One-Minute Test	Normal [%]		Osteopenia [%]		Osteoporosis [%]		χ^2	p
	YES	NO	YES	NO	YES	NO		
1. Have either of your parents been diagnosed with osteoporosis or broken a bone after a minor fall?	17.4	82.6	24.5	75.5	25.0	75.0	0.112	0.737
2. Did either of your parents have a stooped back (dowager's hump)?	8.7	91.3	7.4	92.6	23.5	76.5	0.000	1.000
3. Are you 40 years old or older?	100.0	0.0	98.2	1.8	100.0	0.0	0.000	1.000
4. Have you ever broken a bone after a minor fall, as an adult?	20.8	79.2	50.0	50.0	52.9	47.1	4.807	**0.028**
5. Do you fall frequently (more than once in the last year) or do you have a fear of falling?	33.3	66.7	25.9	74.1	41.2	58.8	0.044	0.834
6. After the age of 40, have you lost more than 3 cm in height (just over 1 inch)?	39.1	60.9	51.0	49.0	76.5	23.5	1.233	0.267
7. Are you underweight (BMI < 19 kg/m^2)?	22.7	77.3	17.6	82.4	41.2	58.8	0.017	0.772
8. Have you ever taken corticosteroid tablets for more than 3 consecutive months?	8.3	91.7	17.3	82.7	18.8	81.3	0.482	0.505
9. Have you ever been diagnosed with rheumatoid arthritis?	13.6	86.4	13.2	86.8	11.8	88.2	0.000	1.000
10. Have you been diagnosed with an overactive thyroid or overactive parathyroid glands?	18.2	81.8	27.3	72.7	23.5	76.5	0.159	0.690
11. Did your menopause occur before the age of 45?	41.7	58.3	42.6	57.4	40.0	60.0	0.000	1.000
12. Have your periods ever stopped for 12 consecutive months or more?	0	100	9.1	90.9	0	100	0.546	0.335
13. Were your ovaries removed before age 50, without you taking Hormone Replacement Therapy?	8.3	91.7	18.2	81.8	12.5	87.5	0.389	0.505
14. Do you regularly drink alcohol in excess of safe drinking limits (more than 2 units a day)?	0	100	0	100	0	100	-	-
15. Do you currently, or have you ever, smoked cigarettes?	30.0	70.0	41.2	58.8	35.7	64.3	0.157	0.692
16. Is your daily level of physical activity less than 30 minutes per day?	45.0	55.0	56.9	43.1	28.6	71.4	0.163	0.686
17. Do you avoid, or are you allergic to milk or dairy products, without taking any calcium supplements?	10.0	90.0	11.5	88.5	14.3	85.7	0.000	1.000
18. Do you spend less than 10 minutes per day outdoors, without taking vitamin D supplements?	26.3	73.7	19.2	80.8	23.1	76.9	0.000	1.000

Low body mass index (BMI) has been identified as an important risk factor for development of osteoporosis due to low intake of energy and micronutrients (9, 10). Besides, estrogens have positive effects on preserving bone mass by affecting the metabolism of Ca, P and vitamin D [11]. Aromatase (an enzyme expressed in gonads and fat tissue) synthesizes estrogens from androgen precursors. Synthesis in adipose tissue is the dominant source of estrogens in postmenopausal women therefore a high body mass index was assumed to have protective effect on bone degradation [12, 13]. In this study, 41.2% of women with T-score < -2.5 had BMI lower than 19 kg/m^2. Totally 24.2% of all participants were underweight, which is rather high in comparison to the prevalence of underweight reported by Grujic et al. for healthy adult females in Serbia (14). This confirms the fact that individuals with low BMI have higher susceptibility to osteoporotic disorders. Besides, 76.5% of women with T-score < -2.5 had lost more than 3 cm in height after the age of 40, which is almost double the percentage obtained for the women with normal BMD (39.1%).

Furthermore, many studies have suggested that different lifestyles can affect bone mineral density. Smoking and alcohol consumption are usually considered to promote development of the disease (8, 15). The results of our study could not confirm this assumption since all the participants denied consumption of alcoholic drinks. Besides, the percentage of smokers was almost equal in all T-score categories and it was comparable to the data obtained in healthy population (16). Moreover, extensive studies have supported positive effects of physical activity on bone formation, reduction of bone loss and prevention of fractures (17, 18). Totally, 48.8% of women in this study reported having less than 30 minutes of physical activities per day (including housework, gardening, walking, running etc.). Unexpectedly, women with T-score < -2.5 appeared to be more physically active than others. However, a statistically significant correlation between physical activity and T-score was not found.

Hormonal changes occurring in menopause increase the rate of bone degradation. The decrease in estrogen levels leads to enhanced risk of osteoporosis (19). Reduced production of hormones is also responsible for increased susceptibility to osteoporotic disorders in women with removed ovaries. Totally 41.5% of women participating in our study had entered menopause before the age of 45. Besides, 14.6% of women reported having had oophorectomy before the age of 50 without using hormonal substitution. The prevalence of oophorectomy was slightly higher among women with T-score < -1 although this difference was not statistically significant.

Long-term corticosteroid therapy induces bone loss by causing osteoblastic suppression and increased bone resorption (20). The percentage of woman who had taken corticosteroid tablets for more than 3 consecutive months was more than two times higher in the group with T-score < -2.5 (18.8%) compared to the group with normal BMD (8.3%).

Thyroid and parathyroid hormones have direct catabolic effect on bone mineral homeostasis; they stimulate bone mineral resorption and calcium loss (21). In our study, 24.2% of all participants reported having overactive thyroid or parathyroid glands. There was no strong correlation between T-score and the incidence of these disorders. However, the prevalence of hyperthyroidism and hyperparathyroidism was rather high in comparison to the prevalence of 0.5-2% obtained for general female population (22, 23).

Calcium and vitamin D have been identified as the most important micronutrients that enable achieving peak bone mass in youth and prevention of bone loss with aging. Dairy products are the main sources of calcium due to the high content and high absorption rate (24). Besides, calcium and vitamin D supplementation are extensively used in treatment of osteoporosis. 12.6% of the respondents in this study reported avoiding milk and milk products as well as calcium supplements. In addition, 21.2% of women spent less than 10 minutes per day outdoors, without using vitamin D supplementation.

CONCLUSION

Comparing to general population data, a high prevalence of some risk factors was obtained in a group of women diagnosed with osteoporosis. There is a significant correlation between T-score and the history of bone fractures. The improvement of the disease is probably the result of many different factors, including lifestyles and habits, history of diseases and medications, as well as therapy adherence and persistence. Therefore, a special care should be devoted to informing women with osteopenia and osteoporosis about all possible factors that might affect the course of the disease and improve the quality of life.

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EVALUATION OF ANTIBIOTIC CONSUMPTION AT RAKOVICA COMMUNITY HEALTH CENTER FROM 2011 TO 2015

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EVALUACIJA POTROŠNJE ANTIBIOTIKA U DOMU ZDRAVLJA RAKOVICA U PERIODU 2011_2015. GODINA

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ABSTRACT

Antibacterial drugs are among the major discoveries of the 20th century because they significantly reduced the rate of morbidity and mortality as well as the risk of infections related to invasive medical procedures. Indiscriminate and wrongful use of these powerful life-saving drugs has led to the development of resistance of numerous microorganisms, resulting in an increase in the number of hospital-acquired infections with a fatal outcome. Thus, it is very important to establish the volume of antibiotic consumption and surveillance of antimicrobial resistance in order to rationalize the use of this important group of medications. The usage unique ATC/DDD methodology results expressed as Defined Daily Doses (DDD)/1000 inhabitants per day (DID) has enabled the comparison of antibiotic consumption in Serbia to that in other countries for a better understanding of our results. The community health center in Rakovica provides treatment for approximately 70,820 patients. The volume of overall antibiotic consumption has been calculated as well as the use of certain antibiotics in the total consumption and comparison of the guides for good clinical practice. The most prescribed antibiotics were antibiotics for diseases of the respiratory system. The most prescribed groups of antibiotics were penicillin drugs, which are an optimal choice as per the guides for good clinical practice. Amoxicillin are the most frequently prescribed individual antibiotic. A yearly increase in prescribing penicillin was observed. A rise in consumption of all generations of quinolones was observed, particularly for levofloxacine, which is not in accordance with the recommendations.

Keywords: *Rational use of antibiotics, DID, antibiotic consumption*

SAŽETAK

Antibakterijski lekovi su među najvećim otkrićima 20. veka, jer su znatno smanjili stopu obolevanja i smrtnosti od infektivnih bolesti i rizik od infekcije kod invazivnih medicinskih procedura. Neodgovorna i pogrešna upotreba ovih moćnih lekova koji spasavaju život dovela je do razvoja rezistencije mnogih mikroorganizama na njih, a rezultat toga je i porast bolničkih infekcija sa smrtnim ishodom. Veoma je važno utvrđivanje obima potrošnje antibiotika i nadzora nad antimikrobnom rezistencijom, radi racionalizacije upotrebe ove važne grupe lekova. Primenjena je jedinstvena ATC/DDD metodologija, rezultati su izraženi kao broj upotrebljenih definisanih dnevnih doza (DDD)/1000 stanovnika /dan (DID) omogućila je poređenje potrošnje antibiotika u Srbiji sa drugim zemljama i bolje razumevanje naših rezultata. U Domu zdravlja Rakovica leči se oko 70820 pacijenata. Izračunat je obim ukupne potrošnje antibiotika kao i ucešće pojedinih antibiotika u ukupnoj potrošnji i poređenje sa vodičima dobre kliničke prakse.

Najviše su propisivani antibiotici za bolesti respiratornog sistema. Najpropisivanija grupa antibiotika su penicilini, što je u skladu sa vodičima dobre kliničke prakse. Zapažen je porast propisivanja penicilina iz godine u godinu sa dominacijom amoksicilina. Zapaža se i porast propisivanja hinolona, posebno levofloksacina, što nije u skladu sa preporukama vodiča dobre kliničke prakse.

Ključne reči: *racionalna upotreba antibiotika, DID, potrošnja antibiotika*

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INTRODUCTION

In 1985, the World Health Organization (WHO) defined the rational use of drugs as a process in which the patients obtain medications that suit their needs, in doses suitable for them, within an appropriate length of time and at the lowest cost for them and the society they live in (1).

Currently, the irrational use of medications, with all of its negative implications, represents a continuing process that takes on increasing proportions and is thus considered to be one of the biggest global public health issues (2).

Of all the drugs, antibiotics have played the greatest role in indiscriminate drug prescriptions. The discovery and use of antimicrobial drugs for the treatment of infections constitutes the biggest success of modern medicine. Approximately 80 % of all antibiotics prescribed in health care institutions are being prescribed within primary health care settings and most frequently for respiratory tract infections. A non-clinical factor, such as the pressure that is exerted on doctors by the patients, also has a large impact on antibiotic prescriptions, but the doctors themselves prescribe antibiotics quite often and unjustifiably (3). A study has been performed involving patients with symptoms of cough where antibiotics are quite frequently administered, showing that there is no difference in the degree of recovery between the patients treated with antibiotics and the ones who did not receive them (4).

The introduction of a mandatory continuing medical education (CME) requirement for health workers in the Republic of Serbia regulated by the Law on Health Care and Rules will probably have an impact on solving this problem.

Apart from other public health measures that have led to the extension of life expectancy, the use of antibiotics is certainly of great importance. However, the success in treatment of infections is compromised by their irrational use, which has led to bacterial resistance of these medications. Irrational use is defined as microbiologically inefficient antimicrobial therapy that can have adverse effects on the outcome of treatment. The irrational use of antimicrobial drugs is important not only from the clinical aspect, i.e. because of the outcome of the patient treatment, but also from the public health aspect since it represents one of the main factors for the emergence of resistance of infectious agents (5).

With an aim at preventing resistance, antimicrobial drugs should be administered rationally, which according to current concepts means that their usage should not be empirical but rather targeted and based on diagnostic evidence.

Over the last 30 years, the development of new antibiotics has considerably decreased, while the options to treat infections caused by resistant agents, which are increasingly on the rise, have become increasingly limited. Tens of thousands of people die each year from infections caused by resistant bacteria. The reasons for delayed development of antibiotics are simple: drug development is risky and expensive, while medicines used to treat infections are not as profitable as the ones that treat chronic illnesses. Indiscriminate use of antibiotics has led to the emergence of multidrug resistant microorganisms–MRSA, VRSA, VRE, etc. The problem of the increasing resistance of microorganisms to antibiotics has become a global health issue (6).

The choice of therapy should rely either on the culture and identification of bacterial pathogens and the results of the sensitivity test (directed therapy) or on the familiar common pathogens in the given state and their common forms of resistance (empirical therapy).

The basic principles of the rational use of antibiotics are as follows:

- Based on the localization of the infection, the causative agent that is in question can be assumed.
- An empirical antibiotic choice during the initial patient contact should be made.
- Sampling for microbiological survey should be conducted prior to administering antibiotics.
- Within 48-72 hours, the effectiveness of antibiotics should be reconsidered, and in view of microbiological findings, the choice of whether to continue or change the application of antibiotics should be decided.
- Apply the antibiotic for a sufficient length of time to treat the infection in question.

One of the ways to achieve this goal is to evaluate and correct the antibiotic prescribing habits in all health care institutions, principally in the primary health care system such as community health centers (7). This study has examined the protocols on antibiotic prescribing at Rakovica Community Health Center, Belgrade.

GOAL

The main goal of this study is to provide insight into the volume of consumption of antibiotics and the participation of certain antibiotics in their overall consumption at Rakovica Community Health Center, as well as to compare the volume to the national guidelines effective in Serbia and the ESAC (European Surveillance of Antimicrobial Consumption) recommendations.

METHODOLOGY

The monitoring of antibiotic use refers to a five-year period (2011 -2015) involving patients over 18 years of age at the adult health care service at Rakovica Community Health Center. The community health center in Rakovica provides treatment for approximately 70,820 patients (30,016 male and 40,657 female).

In order to assess the quality of medicinal treatment (type and scope of unreasonable pharmacotherapy), multiple and varied objective methods have been established in practice, several of which have been standardized and structured by the World Health Organization and International Network for Rational Use of Drugs (INRUD) (8).

Consumption is expressed by the Anatomical Therapeutic Chemical (ATC) /Defined Daily Doses (DDD) methodology recommended by WHO and by the number of DDD/1000 inhabitants per day (DID). The internationally accepted classification system for medicines is the Anatomical Therapeutic Chemical (ATC) classification prescribed by the World Health Organization. Each non-proprietary name of the drug code corresponds to seven alphanumeric characters that are divided into five levels of classification. J01 is a subgroup of the System for Anatomical Therapeutic Chemical (ATC) classification. These are antibiotics that are intended for systemic use. Subgroup J01 is a part of anatomical group J (anti-infective drugs for systemic use). Subgroup J01 is furthermore divided into J01A - tetracyclines, JO1C - beta lactam antibiotics and penicillins, J01D - other beta lactam antibiotics and cephalosporins, J01F - macrolides, J01M - quinolone antibiotics, J01E - sulfonamides and trimethoprim, and J01G - aminoglycoside antibiotics.

ATC/DDD methodology has been proven beneficial in overcoming the differences, and the WHO proposed that this methodology should become a European criterion in 1981, whereas it became a world criterion in 1993.

Data from the community health center in Rakovica have been obtained by the Heliant programme. The Heliant programme has enabled us to gain insight into overall antibiotic consumption, which serve as an overview of indications for which antibiotics were prescribed as well as an insight into antibiotic consumption according to the age and gender of patients.

Within the framework of the ATC/DDD methodology, the existing (real) and expected consumption of medications can be compared. Moreover, the ATC/DDD methodology enables us to compare the use and consumption of drugs among various healthcare institutions, regions and states.

Additionally, the rate of adherence to national guidelines and the rate of consumption of the recommended antibiotics is calculated in compliance with the European Surveillance of Antimicrobial Consumption (ESAC) recommendations.

The ESAC has proposed a list of disease-specific quality indicators (DSQI) for outpatient antibiotic prescriptions: 1. acute bronchitis/bronchiolitis, 2. acute upper-respiratory infection, 3. cystitis, 4. acute tonsillitis, 5. sinusitis, 6. acute otitis media, and 7. pneumonia (10).

The rate of adherence to national guides and ESAC recommendations for pneumonia has been calculated in this study.

RESULTS

In the period from 2011-2015, at Rakovica Community Health Center, the most prescribed antibiotics were for diseases of the respiratory system, followed by urinary infections, and then skin and soft tissue infections (Figure 1). As for respiratory system infections, amoxicillin was the most frequently prescribed individual antibiotic, followed by azithromycin, then amoxicillin and clavulanic acid (Figure 2). These prescriptions for respiratory system infections were most frequently prescribed for diagnosis of J02 (acute pharyngitis), then J20 (acute bronchitis) and J03 (acute tonsillitis). For urinary tract infections, the most widely prescribed medications were cephalexin, ciprofloxacin and pipemidic acid (Figure 2).

When considering the use of antibiotics in relation to age, the most widely prescribed antibiotics were penicillin and macrolides for patients under 65, whereas cephalosporins and penicillin drugs were predominant for those over 65 years of age.

Overall use of antibiotics (ATC group J01) is expressed in the form of DID at Rakovica Community Health Center. An increase in total antibiotic consumption can be noticed in the period from 2011 to 2015 (from 8,7 DID in 2011 up to 12,4 DID in 2015). Consumption per year for the last five years has been calculated for each group and subgroup of antibiotics. The most prescribed groups of antibiotics

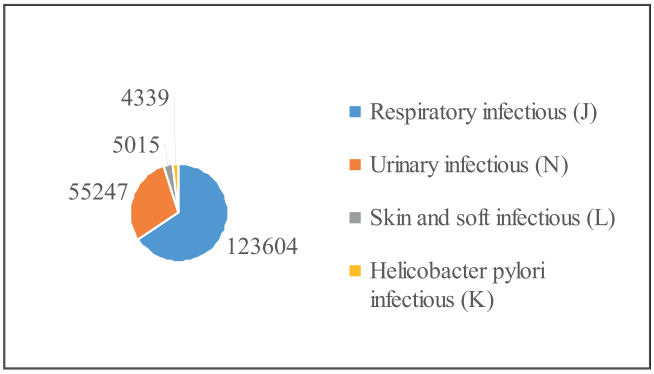

Figure 1. Number of dispensig packages according to indications over the period 2011-2015

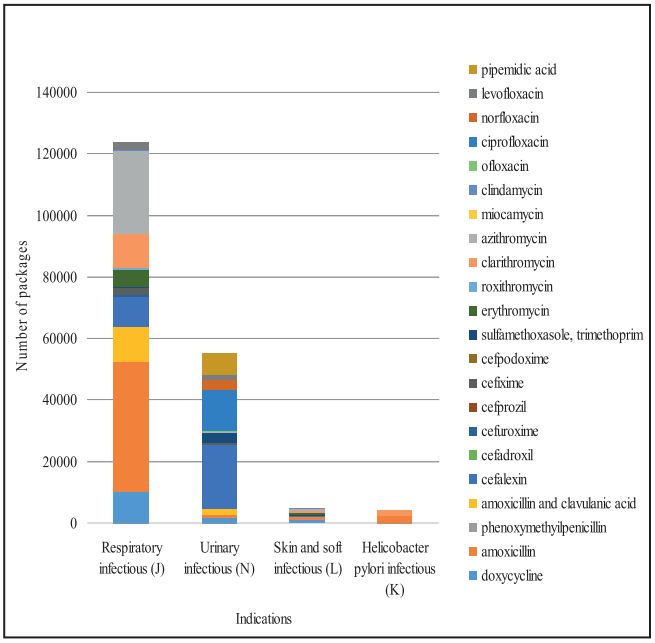

Figure 2. The most frequently prescribing antibiotics according to indications over the period 2011-2015

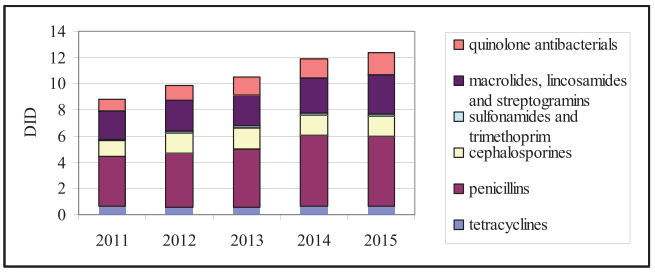

Figure 3. Consumption of antibiotics for systemic use -J01 (3rd level of ATC) defined as DID over the period 2011-2015

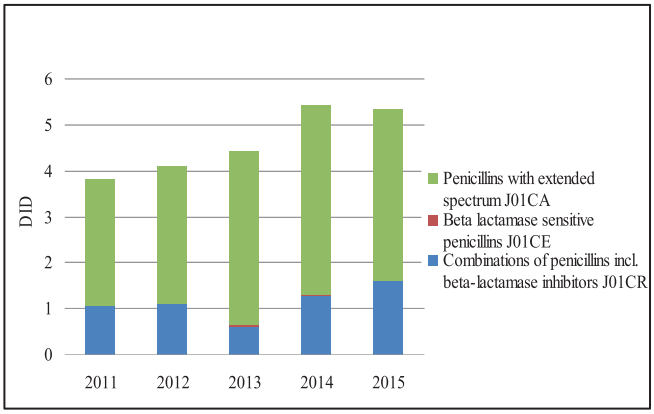

Figure 4. Consumption of penicillins -J01C (4rd level of ATC) defined as DID over the period 2011-2015

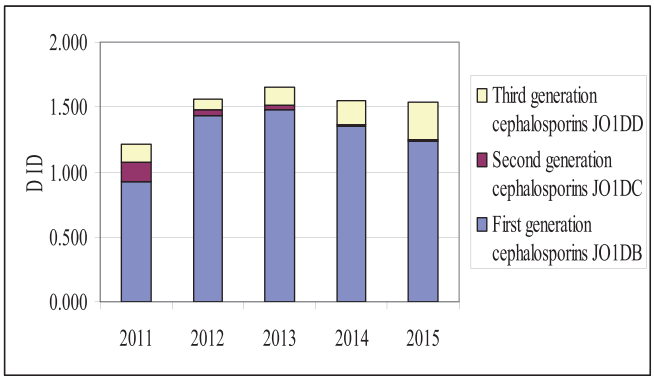

Figure 5. Consumption of cephalosporins -J01D (4rd level of ATC) defined as DID over the period 2011-2015

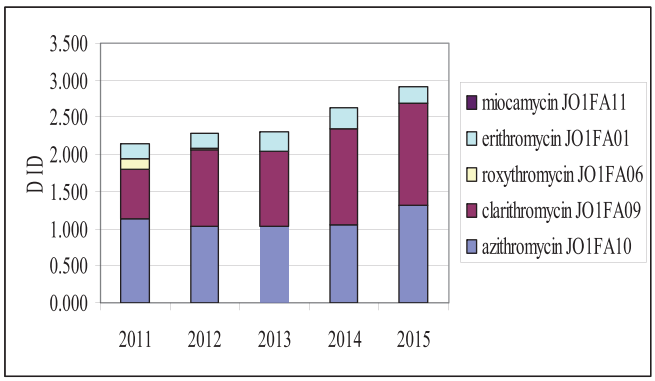

Figure 6. Consumption of macrolides -J01F (5rd level of ATC) defined as DID over the period 2011-2015

were penicillin drugs, then macrolides, followed by cephalosporins (Figure 3).

As for tetracyclines (ATC group J01A), a decrease in consumption was evident in 2013 (0,57 DID), with a new increase in 2015 (0,62 DID). The most widely used tetracycline was doxycycline.

Penicillin drugs (ATC group J01C) were the most prescribed antibiotics in all respective years, and an increase in prescribing penicillin every year was observed, with amoxicillin being the predominant drug (Figure 4).

As for cephalosporins, the most frequently prescribed medication was cephalosporins of the 1st generation (J01DE). In the previous period, a decline in the use of cephalosporins of the 2nd generation and a rise in prescribing of cephalosporins of the 3rd generation were evidenced (Figure 5).

With regard to sulfamethoxazole trimethoprim, its consumption was nearly doubled in the period from 2011 to 2015 (from 0.08 DID up to 0.18 DID).

In relation to the group of macrolides, an increase in prescribing these medications is also noticeable from 2.17 DID in 2011 up to 2.95 DID in 2015. The most frequently prescribed macrolides were azithromycin, whereas the use of clarithromycin nearly doubled (from 0.06 DID in 2011 up to 1.3 DID in 2015) (Figure 6).

The most widely prescribed quinolones were fluoroquinolones, with the leading position being that of ciprofloxacin. A rise in consumption of all generations of quinolones was observed, but the most noticeable increase was an increase in the use of levofloxacine. (from 0.01 DID in 2011 up to 0.62 DID in 2015) (Figure 7).

DISCUSSION

In comparison with other European countries, Serbia is among the countries with an above average antibiotic use, a finding that was published in *The Lancet Infectious Diseases*.[11] The use of antibiotics at Rakovica Community Health Center has been increasing over the years (from 8.7 DID in 2011 up

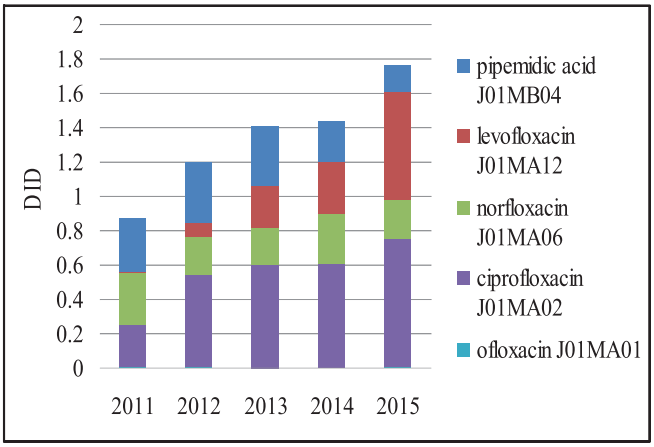

Figure 7. Consumption of quinolones -J01M (5rd level of ATC) defined as DID over the period 2011-2015

Table 1. Top INN used for pneumonia treatment in comparison with National Serbian Guideline (NSG)

ATC	INN	DID	% from total antibiotics use for pneumonia	NSG recommended as first choice	NSG recommended as second choice
J01MA12	levofloxacin	0.0617	49.04		X
J01DD08	cefixime	0.0199	15.82		
J01FA09	clarithromycin	0.0119	9.42	X	
J01CA04	amoxicillin	0.0074	5.86		
J01FA10	azithromycin	0.0072	5.69	X	
J01AA02	doxycycline	0.0055	4.33		X
J01MA02	ciprofloxacin	0.0050	4.00		X
J01CR02	amoxicillin and clavulanic acid	0.0031	2.43		
J01FA01	erythromycin	0.0010	0.79		
J01EE01	sulfamethoxasole, trimethoprim	0.0008	0.65		
J01FA06	roxithromycin	0.0006	0.46		
J01DD13	cefpodoxime	0.0005	0.40		
J01FF01	clindamycin	0.0004	0.35		
J01DC02	cefprozil	0.0003	0.28		
J01DB01	cefalexin	0.0002	0.17		
J01DC02	cefuroxime	0.0002	0.15		
J01CE02	phenoxymethyilpenicillin	0.0001	0.10		
J01MA01	ofloxacin	0.0001	0.06		
	total	0.1259	100	15.11%	57.37%

Table 2. Top INN used for pneumonia treatment in comparison with ESAC disease specific quality indicators (DSQI)

ATC	INN	DID	% from total antibiotics use for pneumonia	DSQI7b*	DSQI7c**
J01MA12	levofloxacin	0.0617	49.04		X
J01DD08	cefixime	0.0199	15.82		
J01FA09	clarithromycin	0.0119	9.42		
J01CA04	amoxicillin	0.0074	5.86	X	
J01FA10	azithromycin	0.0072	5.69		
J01AA02	doxycycline	0.0055	4.33	X	
J01MA02	ciprofloxacin	0.0050	4.00		X
J01CR02	amoxicillin and clavulanic acid	0.0031	2.43		
J01FA01	erythromycin	0.0010	0.79		
J01EE01	sulfamethoxasole, trimethoprim	0.0008	0.65		
J01FA06	roxithromycin	0.0006	0.46		
J01DD13	cefpodoxime	0.0005	0.40		
J01FF01	clindamycin	0.0004	0.35		
J01DC02	cefprozil	0.0003	0.28		
J01DB01	cefalexin	0.0002	0.17		
J01DC02	cefuroxime	0.0002	0.15		
J01CE02	phenoxymethyilpenicillin	0.0001	0.10		
J01MA01	ofloxacin	0.0001	0.06		
	total	0.1259	100	10.18%	53.04%

to 12.4 DID in 2015). Antibiotics are most frequently used for treating respiratory tract infections, followed by urinary tract infections and then skin and soft tissues infections. The consumption of penicillin drugs is prevailing, which is an optimal choice as per the guides for good clinical practice. The use of beta lactamase sensitive penicillins (cliacil) is very low, which is considered to be irrational use. It is likely that the lack of this drug's availability on the market has led to its reduced consumption. However, the use of macrolides is high both at Rakovica Community Health Center and in the whole of Serbia, which differs from other countries. Physicians decide to prescribe macrolides more frequently probably because of their good compliance (once a day regimen) and due to their good safety profile. In the previous period, an increase in prescribing cephalosporins of the 3rd generation and quinolones (of all generations) has been observed, which is not in accordance with the recommendations because these are reserve antibiotics.

In order to evaluate the approximate prescribing habits and adherence to the national and European guidelines, testing has been conducted with antibiotics that are prescribed to treat pneumonia.

Over the observed period from 2011-2015, the average use of antibiotics to treat pneumonia in a patient population over the age of 18 at Rakovica Community Health Center was 0.13 DID. The most prescribed antibiotic to treat pneumonia at Rakovica Community Health Center was levofloxacin (approximately 49%), which is not consistent with the guides for good clinical practice as a first-line therapy. Levofloxacin was followed by cefixime, clarithromycin, amoxicillin, azithromycin, doxycycline, ciprofloxacin, amoxicillin and clavulanic acid, erythromycin and sulfamethoxazole, and trimethoprim. Adherence to the principles of good clinical practice amounted to 15 % for first-line therapies that are used to treat pneumonia, whereas it was cca 57 % for alternative therapy (Table 1).

The ESAC recommended antibiotic use (doxycycline and amoxicillin) in CAP at Rakovica Community Health Center was 10 %, in contrast to the recommended range of 80-100 %. The consumption of fluoroquinolones was 53 %, which exceeded the recommended range of 0-5%, pursuant to ESAC recommendations (Table 2).

Apart from well-known factors influencing the choice and efficacy of antibiotic therapy, these drugs are not being used rationally in our country.

These results indicate the need for a more detailed analysis and monitoring of antibiotic consumption (considering not only quantity-wise but also disease-specific quality indicators), with an aim at the rationalization of prescribing practices.

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NEW THERAPEUTIC CONCEPTS IN POST-RESUSCITATION CARE

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NOVI TERAPIJSKI KONCEPTI U POSTRESUSCITACIONOM LEČENJU

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ABSTRACT

After the return of spontaneous circulation (ROSC), as a result of global ischaemia due to cardiac arrest followed by reperfusion, a condition develops called post-cardiac arrest syndrome. It manifests, alongside the pathology that caused the cardiac arrest, as a systemic inflammatory response, including severe cardio-circulatory and neurological dysfunction, leading to a fatal outcome. The aim of post-resuscitation care is to reduce the consequences of circulatory arrest, reperfusion, and the inflammatory response of the body on vital organ functions. The basis of post-resuscitation care comprises application of therapeutic hypothermia and early coronary angiography with PCI. However, after the initial enthusiasm, the validity of applying these aggressive methods in all comatose post-cardiac arrest patients was questioned. Currently, instead of therapeutic hypothermia, a strategy of maintaining a targeted body temperature, usually 36 °C, is being applied because there is no clear evidence of benefit for maintaining a lower body temperature in relation to the outcome. Additionally, patients with an obvious cardiac aetiology of cardiac arrest do not undergo early coronarography unless there is a clear indication of coronary artery occlusion. In the post-resuscitation period, the maintenance of adequate ventilation, maintaining levels of oxygen and carbon dioxide in the normal range, haemodynamic stability, control of blood glucose and electrolytes, and epileptic attack prevention are all strongly recommended measures. There is no evidence to suggest that the application of the so-called neuroprotective agents affects the outcome of cardiac arrest.

Keywords: *heart failure, outcome, post-resuscitation care, therapeutic hypothermia, percutaneous coronary intervention*

SAŽETAK

Nakon povratka spontane cirkulacije (ROSC), kao posledica globalne ishemije usled srčanog zastoja, a potom reperfuzije, dolazi do razvoja Post-cardiac arrest sindroma. On se manifestuje sistemskim inflamatornim odgovorom organizma, teškom kardiocirkulatornom i neurološkom disfunkcijom, uz prisutnu patologiju koja je dovela do srčanog zastoja, sto vodi smrtnom ishodu. Cilj postresuscitacionog lečenja je da smanji posledice prestanaka cirkulacije/reperfuzije i inflamatornog odgovora organizma na funkcionisanje vitalnih organa. Osnovu postresuscitacionog lečenja činili su primena terapijske hipotermije i rane koronarografije sa PCI. Međutim, nakon početne euforije, dovedena je u sumnju opravdanost primene ovih agresivnih metoda kod svih komatoznih pacijenata nakon srčanog zastoja. Danas se umesto terapijske hipotermije primenjuje strategija održavanja ciljane telesne temperature, uobičajeno 36°C, jer nema jasnih dokaza o benefitu održavanja nižih telesnih temperatura u odnosu na ishod. Takođe se rana koronarografija ne primenjuje više kod svih pacijenata nakon srčanog zastoja očigledne kardijalne etiologije, već samo ukoliko postoji jasna sumnja na okluziju koronarnih arterija. U post-resustitacionom periodu se takođe snažno preporučuje održavanje adekvatne ventilacije, uz održavanje kiseonika i ugljen dioksida u krvi u okvru normalnih vrednosti, hemodinamske stabilnosti, kontrola glikemije, elektrolitnog statusa i sprečavanje epi-napada. Nema dokaza da primena tkz. neuroprotektivnih lekova utiče na ishod srčanog zastoja.

Ključne reči: *srčani zastoj, ishod, post-resuscitaciono lečenje, terapijska hipotermija, perkutana koronarna intervencija*

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INTRODUCTION

The leading cause of death in Europe is out-of-hospital cardiac arrest (1). During the last decade, there has been significant progress in pre-hospital resuscitation, and a significant increase in the percentage of patients arriving to the hospital with re-established heartbeats after cardiac arrest. However, the ultimate outcome of cardiac arrest is still poor. Approximately 25-35% of patients with out-of-hospital cardiac arrest experience a return of spontaneous circulation (ROSC) after cardiopulmonary resuscitation (CPR), while the percentage of survivors discharged from the hospital is approximately 10%. However, the percentage of survivors with good neurological outcomes is even lower (2). It is clear that to improve the prognosis of cardiac arrest, in addition to measures related to the improvement of prehospital resuscitation and informing the community about the basics of CPR, emphasis should be placed on post-resuscitation care. For a good end result following cardiac arrest, it is necessary to provide adequate protocol- and team-based continuation of treatment for these patients in the hospital.

After the return of spontaneous circulation, following a period of global hypoxia and ischaemia that occur due to cardiac arrest with compensatory processes, there is further reperfusion damage. Ischaemia / reperfusion injury occurs as a consequence of complex processes happening as a result of the synthesis and release of a number of inflammatory cytokines, activation of the complement cascade, haemotaxis and activation of polymorphonuclear leukocytes. The consequences of these processes are platelet activation, intravascular coagulation, endothelial damage and increased vascular permeability. Intracellular anoxia leads to disruption of oxidative phosphorylation in the mitochondria, thereby starting the process of anaerobic glycolysis, and subsequently resulting in an increase in lactate levels and intracellular acidosis with further electrolyte disorders (3). These complex events are actually a form of systemic inflammatory response syndrome (SIRS) of the organism, which leads to progressive destruction of the cells and multiple organ dysfunction, a condition known as post-cardiac arrest syndrome. Post-resuscitation care should be aimed at minimizing post-cardiac arrest syndrome. According to the treatment recommendations of the International Liaison Committee on Resuscitation (ILCOR) from 2015 (4), besides maintaining the target temperatures (targeted temperature management), the most important goals of post-resuscitation care are early treatment of the causes of cardiac arrest, haemodynamic stabilization, adequate oxygenation and ventilation, and regulation of blood glucose and electrolyte status.

TARGET TEMPERATURE MANAGEMENT

The application of therapeutic hypothermia after cardiac arrest leads to a reduction of cerebral metabolism with the preservation of high-energy phosphate reserves and reduced release of inflammatory cytokines and excitatory amino acids. Additionally, there is a stabilization of the endothelial membrane and reduction of brain edema after the application of therapeutic hypothermia. Two published, randomized, prospective, controlled trials have shown improved survival and improved neurological outcomes following the application of therapeutic hypothermia after VF in out-of-hospital cardiac arrest (5, 6). Afterwards, in the 2010 ERC cardiopulmonary guidelines, therapeutic hypothermia was highly recommended in post-resuscitation care. The 2010 guidelines state that patients should be cooled at 32-34 °C for a duration of 12-24 hours. Cooling should start as soon as possible, even before arriving at the hospital, if possible. However, two recently published randomized, controlled studies challenge this approach.

In the first of these studies, published in JAMA (7), which included 1,359 patients, the authors showed that pre-hospital cooling with 2 litres of 4 °C saline immediately after ROSC led neither to increased survival upon discharge from the hospital nor to better neurological outcomes compared to the group of patients cooled at the moment of hospital admission. However, in this group of patients, there were significantly more repeated cardiac arrests in the field, as well as more frequent pulmonary oedemas on the first chest radiography. Diao came to the same conclusion in his meta-analysis of 5 randomized, controlled studies that included a total of 633 patients (8).

Another important multicenter, randomized study (9) included 939 unconscious patients after out-of-hospital cardiac arrest. Patients were randomized in two groups: patients whose body temperature was maintained at 33 °C and a group of patients whose body temperature was maintained at 36 °C. Among the groups there was no statistically significant difference in survival and neurological outcome. After the follow-up period of 180 days, in the group maintained at 33 °C, 54% of patients died or had poor neurological outcomes according to the CPC (Cerebral Performance Category) scale compared to 52% of patients with a fatal outcome in the group maintained at 36 °C.

This research indicates that, for a good outcome, it is sufficient to maintain the target body temperature within certain limits and to prevent fever during the first 3 days after cardiac arrest; therefore, it is not necessary to cool down patients to 32-34 °C. We should not forget that hypothermia affects all organ systems, which can lead to unintended consequences in some patients; therefore, it would be better to apply a less aggressive approach. Numerous studies show that hyperthermia is to blame for the poor outcome of cardiac arrest, whether it occurs after application of therapeutic hypothermia (rebound pyrexia) or in patients who are not cooled (10,11,12,13).

The 2015 ERC recommendations for cardiopulmonary resuscitation state that it is necessary to maintain a target body temperature between 32 °C and 36 °C.

CORONARY ANGIOGRAPHY AND PCI

Removing causes that lead to cardiac arrest is an important segment of post-resuscitation treatment because it significantly reduces the probability of cardiac arrest recurrence and worsening of the disease. In the case of cardiac arrest caused by myocardial infarction with ST-segment elevation (STEMI), the ERC 2015 guidelines strongly recommend urgent (within 90 min) coronary angiography and, if necessary, percutaneous coronary intervention (PCI), which would enable myocardial reperfusion and thus preservation of myocardial contractility and improved perfusion of the brain. There are no randomized clinical studies to confirm the benefits of this approach, but on the basis of numerous observational studies, it was concluded that the urgent PCI in STEMI doubles the chances for survival and contributes to good neurological outcomes (14).

However, there is a dilemma concerning what to do when there is no ST-segment elevation in an ECG obtained after cardiac arrest. The absence of ST-segment elevation and symptoms of acute coronary syndrome after resuscitation are not solid proof that there is no significant occlusion of the coronary arteries. In patients who had out-of-hospital cardiac arrests with cardiac aetiologies, the incidence of coronary artery occlusion is very high, even when there is no ST-segment elevation present in the post-cardiac arrest ECG. If every patient were to undergo emergency coronary angiography after cardiac arrest, regardless of whether they have obvious clinical and ECG signs of coronary artery disease, it would enable early myocardial revascularization and all the clinical benefits that it brings in cases with coronary artery occlusion. On the other hand, such a non-selective approach would expose patients who had no occlusive disease to a very invasive and potentially dangerous procedure that is unnecessary. Randomized controlled trials that assess the effects of the implementation of early coronary angiography and PCI in patients with cardiac arrest without ST-segment elevation do not exist, and the results of retrospective observational studies are contradictory. Some of these studies show that early coronary angiography and PCI increase survival (15, 16, 17), while others report no benefits from these methods in patients in whom there are not clear signs of coronary occlusion (18).

CARDIOVASCULAR STABILIZATION

Cardiovascular instability is common after cardiac arrest. It usually resolves spontaneously after 48-72 hours (19), but it is associated with poor neurological outcomes in survivors. Therefore, it is necessary to maintain an adequate mean arterial pressure (MAP) during the post-resuscitation period. Target blood pressure during the post-resuscitation period is not precisely defined, but it must be sufficient to ensure good brain perfusion, whose auto-regulatory mechanisms are disturbed during this period. In addition to not being low, blood pressure also should not be too high because it increases myocardial work and myocardial oxygen demands. Stabilization of the cardiovascular system is achieved by fluid replacement and the use of inotropic and vasoactive drugs. In experimental animal models, dobutamine showed the best effects on improving cardiac systolic and diastolic function after a cardiac arrest. Tests of other inotropic and vasoactive drugs did not demonstrate comparative advantages in relation to dobutamine (20). The combination of dobutamine and noradrenaline was shown to be especially good. When drug therapy is not effective, mechanical support, such as an intra-aortic balloon pump, can be used. In severe ventricular failure, mechanical support often is not sufficient. In these cases, a percutaneous cardiopulmonary bypass with extracorporeal oxygenation can be used. Currently, there are available portable extracorporeal blood oxygenators, whose application is possible in the field in the cases of long-lasting resistant cardiac arrest (21, 22.23).

Hemodynamic monitoring (heart rate, blood pressure, cardiac output, SvO2 or ScvO2, lactate and arterial gas analysis) is required to achieve the optimization of circulation and adequate oxygen delivery to tissues.

VENTILATION AND OXYGENATION

Experimental studies in animals have shown that hyperoxia after ROSC promotes the production of reactive oxygen species, which leads to oxygenation of lipids and proteins and disrupts the integrity of cell membranes and normal enzyme activities. Ultimately, hyperoxia leads to greater neuronal damage and poor neurological outcomes. Large multicenter, retrospective, cohort studies have shown that patients who have been hyperoxic in the post-resuscitation period had significantly higher mortality compared to normoxic and even hypoxic patients (24, 25). In the post-resuscitation period, the percentage of inspired oxygen should be adjusted to maintain oxygen saturation at 94-98%, and not above these values.

Hypocapnia and hypercapnia are common after ROSC, and both are associated with poor neurological outcomes (26). Therefore, maintenance of normocapnia is recommended during post-resuscitation care.

METABOLIC CONTROL

Because of the neurogenic and endocrine responses to stress after cardiac arrest, in the period after ROSC, hyperglycaemia is a common finding. Although numerous studies suggest a connection between hyperglycaemia and death in critically ill patients, strict glycemic control in patients after cardiac arrest cannot be recommended. In this group of critically ill patients, hypoglycaemia has a much worse effect on the neurological outcome than hyperglycaemia; therefore, it must be avoided at all costs. For

that reason, the concept of intensive insulin therapy has been abandoned (27). Variations in the levels of blood glucose have unfavourable effects on these patients and they should be avoided (28). The level of blood glucose should be measured frequently, especially when a patient is in therapeutic hypothermia. It is sufficient to maintain glycaemia below 10 mmol/l.

Electrolyte imbalance is also possible and should be avoided, especially because it might lead to a cardiac arrest. Magnesium levels in the blood should also be taken into account because magnesium affects the functioning of the central nervous and cardiovascular systems, as well as the levels of other electrolytes in the blood.

CONCLUSION

The outcome of cardiac arrest largely depends on the speed of establishing and maintaining circulation. Education of specific target groups of the population and telephone-guided CPR contributed to a higher percentage of high-quality chest compressions administered immediately after cardiac arrest. Timely CPR and the increased availability of automated external defibrillators in public places have increased the rate of initial survival after cardiac arrest. To increase long-term survival with good neurological outcomes, it is necessary to treat the complications of post-cardiac arrest syndrome and provide neuroprotection.

Implementation of measures to maintain target temperature, urgent coronary angiography and PCI, maintenance of haemodynamic and metabolic homeostasis, and adequate oxygenation and ventilation are the most important treatment modalities of post-resuscitation care.

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EXTRAMEDULLARY INVOLVEMENT OF LYMPH NODES IN MULTIPLE MYELOMA

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EKSTRAMEDULARNA ZAHVAĆENOST LIMFNIH ČVOROVA U MULTIPLOM MIJELOMU

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ABSTRACT

Myeloma multiplex is a malignant disease of bone marrow plasma cells. It is usually confined to the bone marrow, but in rare cases, patients can develop extramedullary disease. The involvement of lymph nodes is rare and can be a diagnostic challenge.

Here, we describe a 36-year-old male patient who presented with abdominal pain and discomfort initially. An abdominal ultrasound followed by computed tomography (CT) revealed retroperitoneal and mesenteric lymph node enlargement. Biopsies of the abdominal lymph node and infiltrated colon showed a plasma cell infiltrate positive for CD79α, CD38, CD138, kappa light chain and VEGF2. Multiple myeloma with extramedullary localization was diagnosed. After six cycles of chemotherapy consisting of doxorubicin, dexamethasone and thalidomide followed by autologous haematopoietic cell transplantation, the patient achieved complete remission. Specifically, a CT scan after therapy showed enlarged lymph nodes in the abdomen, but PET CT scans did not detect any metabolically active foci. Three years after the completion of therapy, the patient remains in remission.

This case illustrates a rare presentation of extramedullary myeloma involving the abdominal lymph nodes, which could have been potentially mistaken for a lymphoid malignancy.

Keywords: *multiple myeloma, extramedullary disease, lymph nodes, thalidomide*

SAŽETAK

Multipli mijelom je maligna bolest plazmocita kostne srži. Obično je ograničen na kostnu srž, ali u retkim slučajevima kod pacijenata se može javiti ektramedularna lokalizacija bolesti. Zahvaćenost limfnh čvorova je retko i može predstavljati dijagnostički izazov.

Prikazali smo pacijena starog 36 godina, muškog pola koji je imao inicijalne tegobe u vidu nelagodnosti i bola u abdomenu. Ultrazvuk i kompjuterizovana tomografija abdomena pokazali su uvećanje retroperitonealnih I mezenterijalnih limfnih čvorova. Biopsija abdominalnog limfnog čvora i zahvaćenog dela debelog creva pokazala je infiltraciju plazmocitima pozitivnim na CD79α, CD38, CD138, kappa i VEGF2 i pacijentu je postavljena dijagnoza multiplog mijeloma sa ekstramedularnom lokalizacijom. Pacijent je primio šest ciklusa hemioterapije sa doksorubicinom, deksametazonom i talidomidom nakon čega mu je urađena autologa transplantacija matičnih ćelija hematopoeze posle čega je registrovana kompletna remisija bolesti. Naime, na CT snimcima registrovani su uvećani limfni čvorovi u abdomenu, ali PET CT snimci nisu detektovali postojanje metabolički aktivnih fokusa.

Ovaj prikaz slučaja ilustruje retku prezentaciju ekstramedularnog mijeloma u abdominalnim limfnim čvorovima koji potencijalno može biti pogrešno protumačen kao limfoidna neoplazma.

Ključne reči: *multpli mijelom, ekstramedularna lokalizacija bolesti, limfni čvorovi, talidomid*

ABBREVIATIONS

MM: myeloma multiplex **EMD**: extramedullary disease

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INTRODUCTION

Myeloma multiplex (MM) is characterized by proliferation of malignant plasma cells. MM represents 13% of all haematologic malignancies (1). Although it usually is confined to the bone marrow and surrounding bones, some patients develop extramedullary disease (EMD) in the form of soft tissue plasmacytomas. Local growth of MM within the bone marrow combined with direct spreading of the disease into the soft tissue surrounding the involved bones is the main mechanism of EMD development. Haematogenous metastatic spreading is the second, not so common mechanism of EMD development (2). Haematogenous spreading usually involves skin, liver, breast, or kidney (3) and in rare cases, lymph nodes (less than 1% of all EMD) (4). Here, we present the case of a patient with EMD involving the retroperitoneal and mesenteric lymph nodes at the time of MM diagnosis.

CASE REPORT

A previously healthy 36-year-old man initially presented with abdominal pain and discomfort reported to have been occurring over the previous two months. Ultrasound revealed abdominal lymphadenopathy, and the patient was referred to the Hematology Department of Clinical Centre in Kragujevac with suspicion of a lymphoproliferative disease. Abdominal computed tomography showed mesenteric and retroperitoneal lymphadenopathy (the largest lymph node was 57 mm in diameter) without the formation of a conglomerate mass (Figure 1). Superficial lymph nodes were not palpable. The patient's haemoglobin was 8.3 g/100 ml; white blood cell and platelets counts were 5.100 and 213.000 per cubic millimetre, respectively; and the erythrocyte sedimentation rate was 104 mm/h. His serum lactate-dehydrogenase level was elevated at 331 U/L (normal range: 94–250 U/L) as was his serum globulin at 42 g/l (normal range: 20-35 g/l); other values for biochemical analyses and parameters of haemostasis were within the normal range. Tests for HIV (human immunodeficiency virus) and HCV (hepatitis C virus) antibodies and HBV (hepatitis B virus) antigen were negative. Explorative laparotomy was performed, and biopsies of the abdominal lymph node and infiltrated colon were taken. Histological examination of the specimens revealed diffuse infiltration of the lymph node and colon with regular plasma cells, which were positive for CD79α, CD38, CD138, kappa light chain and VEGF2 (Figure 2). A bone marrow biopsy showed a slightly hypercellular marrow with 20-30% CD38+, CD138+, MUM-1+ plasma-cells. Serum protein electrophoresis with immunofixation identified an immunoglobulin G kappa monoclonal gammopathy (monoclonal spike [M-spike]: 2.5 g/dL) and a free kappa protein band. The serum kappa free light chain was elevated at 9361 mg/L (normal range: 5.71-26.3 mg/L), with the serum lambda free light chain within normal range. The beta-2 microglobulin level was 3.72 mg/L (normal range: 0.7-1.8 mg/L). Any bone destruction could not be detected by X-ray examination. Therefore, the patient was diagnosed with multiple myeloma with extramedullary localization in the colon and abdominal and retroperitoneal lymph nodes, corresponding to clinical stage IIA in the Durie & Salmon staging system and clinical stage II in the International Staging System of myeloma. The patient was treated with six cycles of chemotherapy consisting of doxorubicin (17 mg days 1-4), dexamethasone (40 mg days 1-4), and thalidomide (100 mg daily), followed by high doses of melphalan with autologous haematopoietic cell transplantation. Bone marrow biopsy after treatment did not show any signs of disease, and paraproteins were not detected by serum protein electrophoresis. A CT scan showed enlarged lymph nodes in the abdomen, but PET CT scans did not detect any metabolically active foci. As such, complete remission was achieved. Three years after the completion of therapy, the patient remained in complete remission.

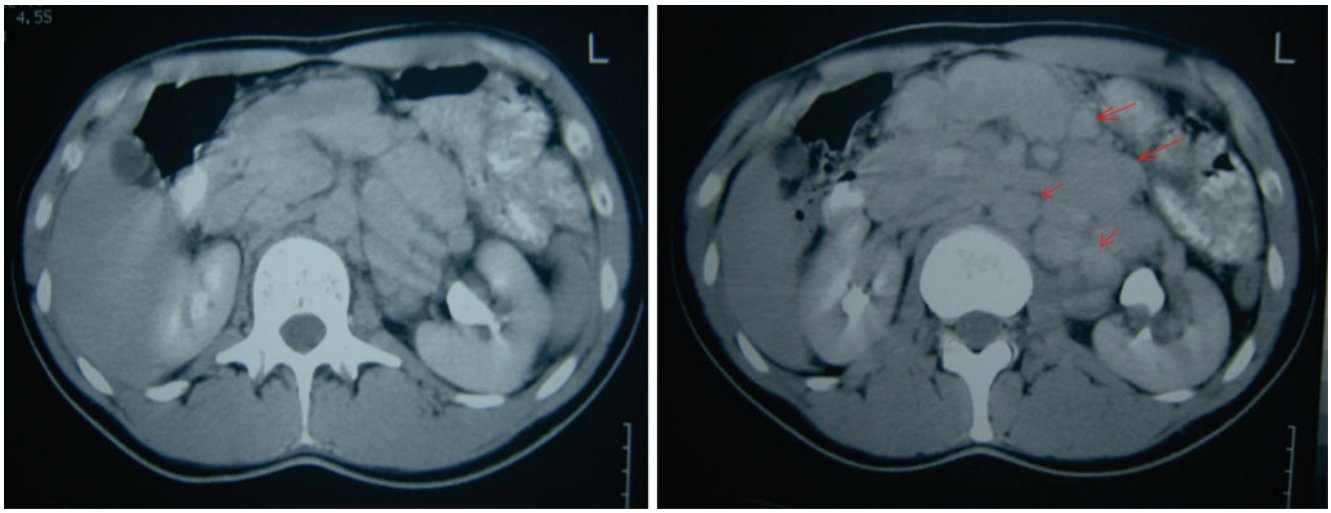

Figure 1. Abdominal computed tomography (CT). CT scans showing massive abdominal and retroperitoneal lymphadenopathy.

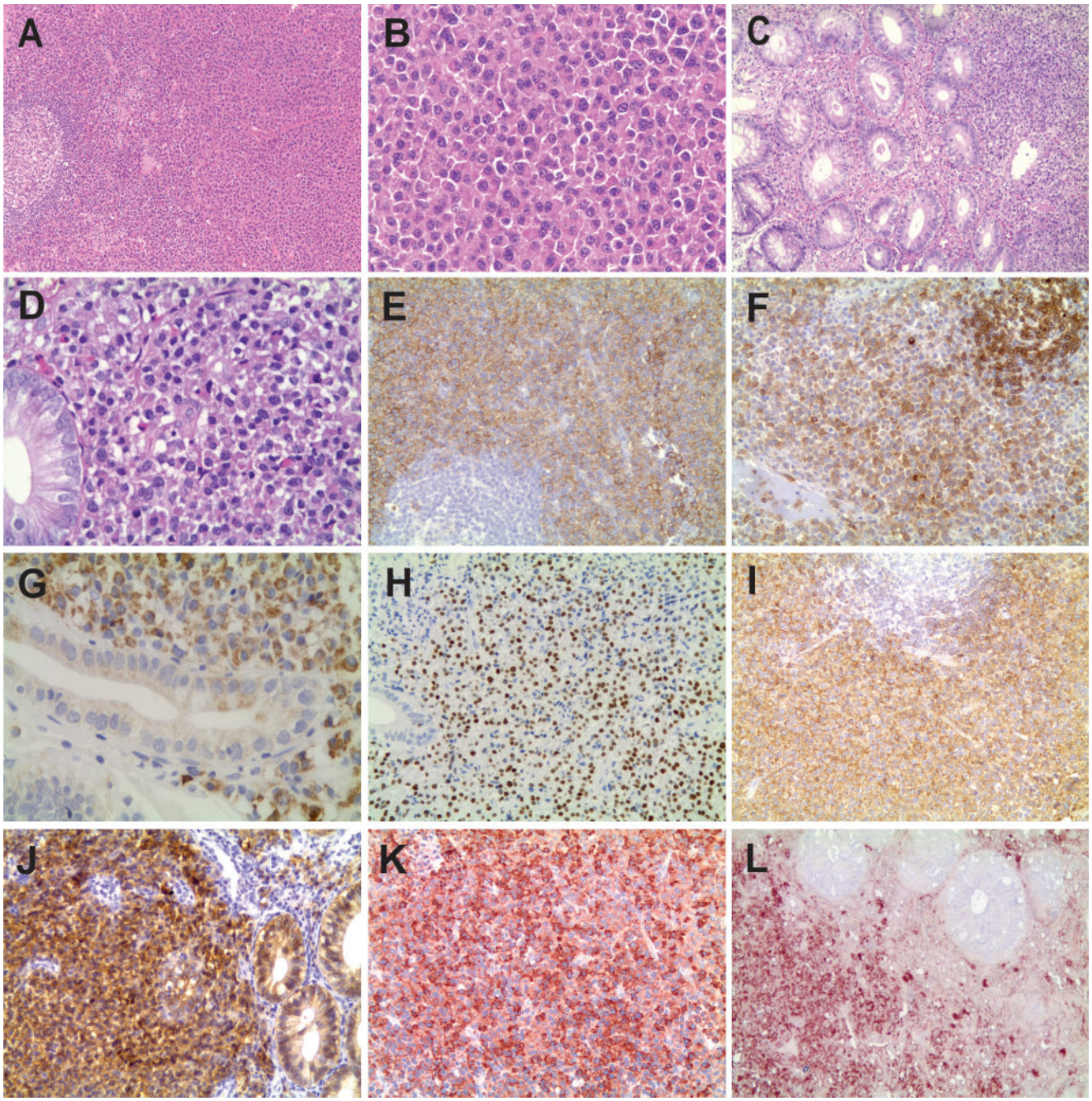

Figure 2. Histological and immunohistochemical examination of the abdominal lymph node and infiltrated colon. Haematoxylin and Eosin staining showing diffuse infiltration of the lymph node (A, B) and colon (C, D) with regular plasma cells. By immunohistochemistry, plasma cells were positive for CD38 (E), CD79α (F, G), MUM-1 (H), CD138 (I, J), and kappa light chain (K, L).

DISCUSSION

The incidence of EMD seems to have increased over recent years. At the time of the diagnosis, 7-18% of patients with MM had EMD according to different studies (5-8). An additional 6% to 20% of patients develop plasmacytomas later in the course of disease (7, 8). One explanation for this could be the more frequent use and availability of sensitive imaging techniques, such as magnetic resonance imaging, computed tomography, and occasionally positron emission tomography. Additionally, the prolonged survival of myeloma patients due to the introduction of novel agents, such as proteasome inhibitors and immune modulators, in treatment has increased the incidence of EMD (5).

The infiltration of lymph nodes by malignant plasma cells could occur as a result of primary lymph node plasmacytomas. These can be diagnosed only after the exclusion of primary bone marrow involvement (9), and would represent a rare localization of primary plasmacytomas. On the other hand, lymph nodes could be involved in the

extramedullary spreading of myeloma (10). The extramedullary involvement of lymph nodes is not frequently detected in living patients (3, 11, 12) but is discovered more often in autopsies (13). An explanation for this could be the absence of symptoms in most cases. The patient that we presented here had dyspepsia and occasionally gastric pain that together with a high value of C-reactive protein and a high erythrocyte sedimentation rate led to further investigation, which revealed mesenteric and retroperitoneal lymphadenopathy, first by ultrasound and then with computed tomography, after which a diagnosis was established based on the results of the biopsies.

Our patient was treated with a thalidomide-based regimen in six cycles, followed by autologous haematopoietic cell transplantation, and complete remission was achieved. Studies have shown that extramedullary progression of MM is associated with a worse prognosis (5, 6). In fact, patients with EMD at the time of the diagnosis, despite a lower International Scoring System (ISS) score, have poorer outcome regardless of first-line treatment (14). For de novo EMD patients who are eligible for stem cell transplantation, a triplet induction therapy approach (bortezomib-lenalidomide-dexamethasone) is suggested, followed by high-dose melphalan with autologous haematopoietic cell transplantation, triplet consolidation therapy (bortezomib-lenalidomide-dexamethasone), and maintenance treatment consisting of at least lenalidomide (15). Using thalidomide in the treatment of patients with EMD is controversial. Rosinol et al showed that patients with EMD did not respond to therapy with single agent thalidomide (16), which correlates with other studies that showed EMD progression under thalidomide treatment, despite a good bone marrow response (17, 18). In contrast, some cases of EMD responded well to thalidomide and dexamethasone therapy (19, 20). Based on the patient's age, good cardiac ejection fraction, large tumour mass, and contraindications for using bortezomib and lenalidomide as first-line therapy, we started to treat the patient with a combination of thalidomide, dexamethasone and doxorubicin, followed by high doses of melphalan and autologous haematopoietic cell transplantation. Using this regimen, the patient achieved complete remission.

To conclude, extramedullary myeloma is a heterogeneous entity that affects almost 15% of MM patients during their overall disease course. The clinical presentation can be very unusual and efforts should be made to optimally detect extramedullary disease. PET-CT is an important tool at diagnosis and during follow-up. After the treatment of EMD, patients are still exceedingly poorly managed, which is particularly challenging. It is necessary to define innovative treatment strategies to improve the outcomes of this subgroup of myeloma patients.

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PRIMARY SMALL CELL CARCINOMA OF LUNG WITH METACHRONOUS BREAST METASTASIS

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PRIMARNI MIKROCELULARNI KARCINOM PLUĆA SA METAHRONOM METASTAZOM U DOJCI

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ABSTRACT

Breast metastases from an extra-mammary malignancy are rare. Among the lung malignancies that metastasise in the breasts, previous literature has described approximately 30 cases of NSCLC and only a few cases of SCLC. Here, we present a 54-year-old woman with metachronous breast metastasis from pulmonary small cell carcinoma. She presented with a soft tissue mass in the right lung hilum. After bronchoscopy with biopsy, SCLC was verified. The patient was given 4 cycles of etoposide and cisplatin followed by radiation therapy. Seven months after the diagnosis of primary lung cancer, the patient palpated a mass in her right breast. Clinical examination and further diagnostics revealed the suspected malignancy, and a radical mastectomy was performed. Immunohistochemical findings suggested metastatic SCLC in the breast. Differentiation between primary and metastatic cancer in the breast is very important for therapeutic planning.

Keywords: *small cell lung carcinoma, metachronous metastasis, breast carcinoma*

SAŽETAK

Dojka predstavlja retko mesto ekstramamarnih metastaza. Među karcinomima pluća koji su prezentovani sa metastazom u dojci, u literaturi je opisano oko 30 slučajeva NSCLC i samo nekoliko slučajeva SCLC. Mi smo prikazali pacijentkinju starosti 54 godine sa metahronom metastazom sitnoćelijskog carcinoma pluća u dojku. Pacijentkinji je inicijalno dijagnostikovana mekotkivna masa u desnom hilusu pluća. Nakon bronhoskopije i biopsije tumourskog tkiva, postavljena je dijagnoza sitnoćelijskog karcinoma pluća. Pacijentkinja je lečena sa 4 ciklusa hemioterapije po protokolu etopozid i cisplatina, nakon čega je nastavljena radioterapija. Sedam meseci nakon postavljanja dijagnoze primarnog carcinoma pluća, pacijentkinja je napipala čvor u desnoj dojci. Klinički pregled i dodatna dijagnostika su pokazali da se najverovatnije radi o malignom tumouru. Urađena je radikalna mestektomija. Imunohistohemijska analiza je ukazala na metastazu sitnoćelijskog karcinoma u dojku. Diferencijalna dijagnoza između primarnog i metastatskog karcionoma dojke je veoma bitna radi daljeg planiranja terapije.

Ključne reči: *sitnoćelijski karcinom pluća, metahrone metastaze, karcinom dojke*

ABBREVIATIONS

SCLC: small cell lung carcinoma

NSCLC: non-small cell lung carcinoma

LD: limited disease

INTRODUCTION

Small cell lung cancer (SCLC) is an aggressive malignancy that comprises 13-15% of all lung cancers (1). At the time of diagnosis, SCLC presents itself as a metastatic disease in 60% of cases. The most common places for metastasis are the liver (35%), brain (47%), bones (25%), adrenal gland (8%) and lungs (12%) (2). Metastasis in the breasts represents an extremely rare phenomenon, and it occurs in 0,4-1,3% of cases (3). Primary malignancies that usually metastasise in the breasts are melanoma (29,8%), lung carcinoma (16,4%), ovarian and endometrial cancer (12,7%), intestinal carcinoma (9,9%), leukaemia and lymphoma (8,4%), rhabdomyosarcoma (7,3%) and renal cell carci-

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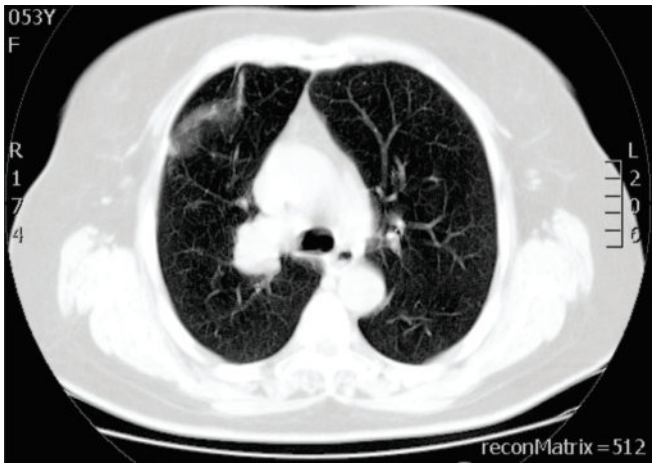

Figure 1. CT scan of the thorax shows a soft tissue mass in the right lung hilum with dimensions of 24×30 mm.

noma (1,5%) (4). Among lung cancers that metastasise in the breasts, the literature has described approximately 30 cases of NSCLC and only a few cases of SCLC (5-9). Here, we report the case of a 54-year-old woman with breast metastasis from SCLC.

CASE REPORT

A 54-year-old woman who smoked 20 cigarettes a day for 40 years presented with dyspnoea, orthopnoea, exhaustion, coughing and subfebrile temperature (up to 37,5°C). She had been experiencing these ailments for two months. A computed tomography (CT) scan of the thorax showed a soft tissue mass in the right lung hilum with dimensions of 24×30 mm, without mediastinal lymphadenopathy and without contents in the pleural spaces (Figure 1). The liver, spleen, adrenal glands and other abdominal organs did not exhibit morphological changes. After bronchoscopy with biopsy, SCLC was verified (Figure 2). As the tumour mass was only on one side of the chest and can be treated with a single radiation field, it was staged as limited disease (LD). The patient was given 4 cycles of etoposide and cisplatin followed by radiation therapy.

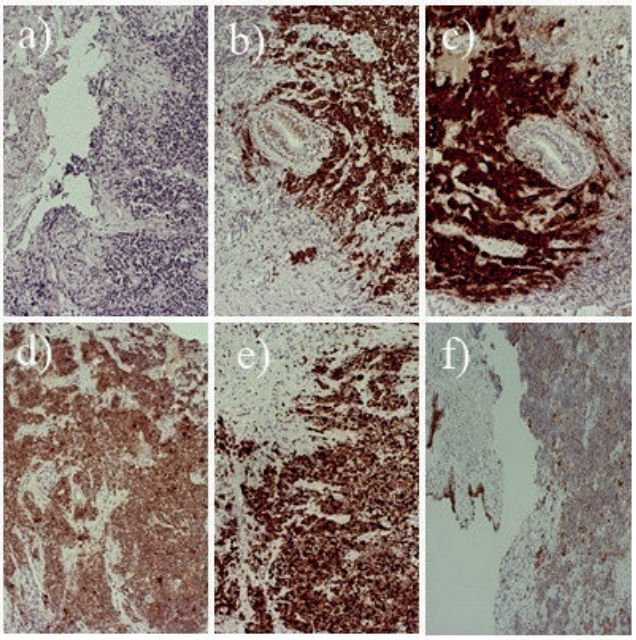

Figure 2. Primary small cell carcinoma of the lung: a) haematoxylin and eosin staining shows fragments of bronchial mucosa imbued with irregular tumour sheets and ribbon tumour arrangements consisting of easy polymorphic cells, about twice the size of lymphocytes with scant cytoplasm and large hyperchromatic nuclei. Immunohistochemical tumour cells were diffusely positive for b) TTF-1, c) CD56, and d) chromogranin. e) The proliferation index was high; around 90% of the tumour cell nuclei are expressing Ki67. f) CK7 was partially positive.

Seven months after the diagnosis of the primary lung cancer, the patient palpated a mass in her right breast. Clinical examination revealed a change in the lower lateral quadrant of the right breast, approximately 40 mm in size, that was hard, painless and had a fixed base. Breast ultrasound revealed a heterogeneous, lobulated mass that was consistent with malignancy. Considering that the ex tempore biopsy showed a malignancy with the impression of primary breast carcinoma, a radical mastectomy was performed. The tumour cells were positive for CD56, chromogranin, synaptophysin, and neuron-specific enolase (NSE) and did not express receptors for oestrogen (ER), progesterone (PR) or *human epidermal growth factor receptor 2* (HER-2). The cells were diffusely positive for thyroid transcription factor-1 (TTF-1) and p53 and focally positive for EMA, cytokeratin 7 (CK7) and CK8, while they were negative for mammaglobin, gross cystic disease fluid protein 15 (GCDFP-15), CK5/6, CK14, CK20, BerEP4, vimentin and p63; the cells also revealed a high proliferation index (Ki67 90%). No evidence of in situ carcinoma was observed. The histomorphological image together with the obtained immunophenotype of the cancer cells corresponded to small cell cancer metastasis, which was primarily localised in the lungs (Figure 3).

Due to postural instability and headaches, another cranial CT examination was performed one month after the operation. The results of CT demonstrated metastatic changes. Palliative radiotherapy was administered at a dosage 25 Gy in 5 fractions. Because of the poor general health of the patient, further treatment was continued with symptomatic and supportive therapy. The patient died 15 months after the diagnosis of primary lung cancer and three months after the diagnosis of breast metastasis.

DISCUSSION

In rare cases, the breasts could be a location for metastasis from the contralateral side, while extra-mammary primary neoplasm metastases are exceptionally rare. The reason for this metastasis can be a large surface of fibrous tissue in the breast and a relatively poor blood supply (10). Mirrielees et al. published a systematic review of the literature on breast metastasis from primary lung carci-

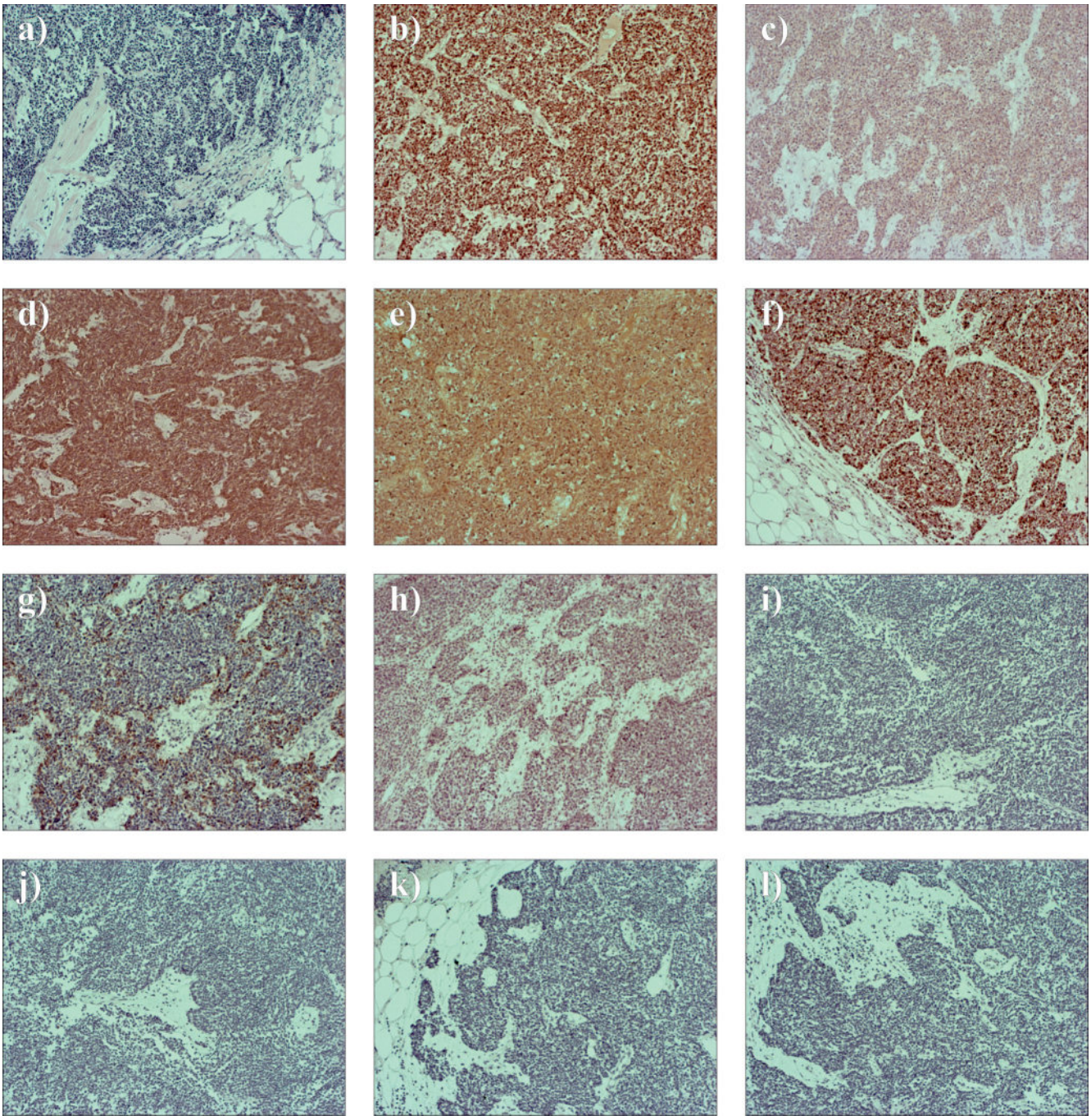

Figure 3. Metastases of small cell lung cancer in the breast: a) haematoxylin and eosin staining of the metastatic breast tumour showed irregular tumour sheets consisting of easy polymorphic cells, about twice the size of lymphocytes with scant cytoplasm and large hyperchromatic nuclei. Immunohistochemical tumour cells were positive for b) TTF-1, c) synaptophysin, d) CK8, and e) NSE. f) The proliferation index was high; around 90% of the tumour cell nuclei are expressing Ki67. g) CK7 was partially positive, while other markers were negative, including h) CK5/6, i) mammaglobin, j) ER, k) PR, and l) HER2.

noma. Thirty-one cases of non-small cell lung carcinoma (NSCLC) were identified with metastases in the breasts, and eight cases of SCLC were identified with metastases in the breasts. Sixty-seven percent of the presented breast metastases of NSCLC patients were metachronous, while in 80% of the primary SCLC cases, breast metastases were synchronous (11).

The influence of oestrogen was indicated as a risk factor for the appearance of breast metastasis, especially in younger women, which increases vascularisation and loosens the breast stoma. In the literature, breast metastases were described in men who were treated with oestrogen hormonal therapy due to primary prostate carcinoma (12).

Metastasis to the breast occurs either by direct invasion, pleural seeding, haematogenous dissemination or lymphatic spreading. With lymphatic spreading, there is an assumption that the lung cancer cells first metastasise to the ipsilateral axillar lymph nodules, then retrogradely

spread to the intra-mammary lymphatic system, and finally establish breast metastasis (13). Clinically, a breast metastasis is presented as a palpable, quickly growing, well-limited, painless tumour mass whose preferred location is the upper outer quadrant of the breast. Retraction of the skin above the affected part of the breast or the nipple is not a characteristic of the metastasis. A mammogram shows the breast metastasis as a limited mass, while irregular edges, spiculations and micro-classifications are rarely observed. Breast metastases are localised in subcutaneous tissue unlike primary carcinoma, which develops in the breast glandular parenchyma.

Differential diagnosis between primary and secondary breast carcinoma often presents a challenge, especially in the case of poorly differentiated tumours. Histological detection of an in situ intra-ductal component is of great importance because supports primary breast carcinoma. Metastatic deposits are sharply limited compared to surrounding breast tissue. Elastosis and calcification are characteristics of primary breast carcinoma but rarely of metastases. SCLC cells express neuroendocrine differentiation markers, usually CD56, synaptophysin, NSE or chromogranin. TTF-1 has a role in the regulation of protein expression in the thyroid gland, lungs and diencephalon, and as such represents a specific and sensitive marker for diagnosis of lung adenocarcinoma (14).

TTF-1 was positive in 93% of SCLCs and negative in breast adenocarcinomas (15,16). According to the literature, 2,8% of primary breast carcinomas can show weak or focal expression of TTF-1, and those cases are usually associated with CK5/6 or P-cadherin positivity, grade 3 tumours and the 'triple negative' (ER-, PR- and HER2-negative) phenotype. Napsin A is a sensitive marker for lung adenocarcinoma, which is why the combination of Napsin A and TTF-1 is better for establishing or excluding metastasis in the primary lung carcinoma of the breast compared to using only TTF-1 (17,18).

The 8G7G3/1 clone of TTF-1 is less sensitive but more specific with regard to SPT24, which is why it is recommended for routine use in order to avoid doubts when determining an adequate diagnosis.

Markers that are in favour of primary breast cancer are receptors for ER, PR, GCDFP-15 and mammoglobin. Receptors for ER are expressed in 80% and GCDFP-15 in 45-53% of primary breast carcinomas. Expression of ER in lung carcinoma is low (7,6-14,1%) as well as expression of GCDFP-15 (5,2-15%). Mammoglobin is expressed in 48-72,1% of breast carcinomas and is negative in lung carcinomas (19-22).

The combination of CK7 and CK20 is irreplaceable in a crude differentiation with regard to the origin of cancer. Breast carcinomas are mostly CK7+ and CK20-, while it is less probable that the malignancy is a breast carcinoma if it is CK20+ and CK7- (23).

Breast metastases from non-breast solid malignancies are associated with a poor outcome; the median survival from the time of breast metastasis diagnosis is 10 months. Significantly better survival was observed in patients who had no evidence of another disease at the time of diagnosis, in patients with neuroendocrine tumours, and in patients who underwent surgical resection for breast metastases (24).

Surgical treatment of the metastatic change in a breast represents an option for the treatment of metachronous metastases, although it is not completely clear if surgical treatment may influence the prognosis of the disease. For patients presenting synchronous lung cancer with breast metastasis, removal of the breast lesion offers no benefit to the patient (11).

In our case, we presented a patient with breast cancer in the T2-N0 clinical stage. Cancer metastases in the breast are extremely rare, and taking into account the high prevalence of primary breast cancer, a palpable tumour mass was considered a new primary breast tumour. Fine-needle aspiration (FNA) cytology and needle core biopsies with immunohistochemical analyses should be the diagnostic procedures of choice in patients with a palpable tumour mass in the breast, especially in those with a previously diagnosed malignant disease, in order to avoid unnecessary radical surgery. If the axillary lymph nodes are not palpable, axillary imaging with ultrasound should be considered, and suspicious nodes should be sampled by FNA or core biopsy. Neoadjuvant chemotherapy with possible surgical resection is a therapeutic option in those patients without distant metastases.

CONCLUSION

Breast metastases from SCLC represent an extremely rare phenomenon. These metastases mainly occur in the contralateral breast, while in only 0,5% of cases, the metastases result from extra-mammary primary tumours. Since the treatment and prognosis of the diseases are different, it is important that the differentiation be made between primary and metastatic breast carcinoma. Information about previously diagnosed malignancies is of great importance, especially regarding metachronous metastases, in order to carry out the necessary diagnostic procedures so that the most adequate therapeutic modality can be provided to the patient.

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A complete reference should give the reader enough information to find the relevant article. And most importantly, complete and correct references may allow automatic creation of active links by the MetaPress technology that we use for making the electronic version of our journal. Active reference linking is regarded as the greatest benefit of electronic publishing and it adds a lot of value to your publication.

2.Book

a. Book (one author)

Format:

Author. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Baxter, R. (1982). *Exactly Solvable Models in Statistical Mechanics.* New York: Academic Press.

b. Book (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Kleiner, F.S., Mamiya C.J. & Tansey R.G. (2001). *Gardner's art through the ages* (11th ed.). Fort Worth, USA: Harcourt College Publishers.

c. Book chapter or article in an edited book

Format:

Author(s) of chapter. (Year of publication). Chapter title. In Editors of the book (Eds.), *Book title* (Chapter page range). Place of publication: Publisher.

Example:

Roll, W.P. (1976). ESP and memory. In J.M.O. Wheatley & H.L. Edge (Eds.), *Philosophical dimensions of parapsychology* (pp. 154-184). Springfield, IL: American Psychiatric Press.

d. Proceedings from a conference

Format:

Author(s). (Year of publication). Title. In Conference name, Date (Page range). Place of publication: Publisher.

Example:

Field, G. (2001). Rethinking reference rethought. In Revelling in Reference: Reference and Information Services Section Symposium, 12-14 October 2001 (pp. 59-64). Melbourne, Victoria, Australia: Australian Library and Information Association.

e. ebook

Format:

Author(s). (Year of publication). *Title*. Publisher. Retrieving date, http address. DOI.

Example:

Johnson, A. (2000). *Abstract Computing Machines*. Springer Berlin Heidelberg. Retrieved March 30, 2006, from SpringerLink http://springerlink.com/content/w25154. DOI: 10.1007/b138965.

f. Thesis

Format:

Author(s). (Year of publication). *Title*. Information, Place of publication.

Example:

Begg, M. M. (2001). *Dairy farm women in the Waikato 1946-1996: Fifty years of social and structural change*. Unpublished doctoral dissertation, University of Waikato, Hamilton, New Zealand.

g. Report

Format:

Author(s). (Year of publication). *Title*. Place of publication: Publisher. (Report number)

Example:

Osgood, D. W., & Wilson, J. K. (1990). *Covariation of adolescent health problems*. Lincoln: University of Nebraska. (NTIS No. PB 91-154 377/AS)

h. Government publication

Format:

Institution name. (Year of publication). *Title.* Place of publication: Publisher.

Example:

Ministerial Council on Drug Strategy. (1997). *The national drug strategy: Mapping the future*. Canberra: Australian Government Publishing Service.

TABLES

Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

FIGURES AND FIGURE LEGENDS

All illustrations (photographs, graphs, diagrams) will be considered as figures, and numbered consecutively in Arabic numerals. The number of figures included should be the least required to convey the message of the paper, and no figure should duplicate the data presented in the tables or text. Figures should not have titles. Letters, numerals and symbols must be clear, in proportion to each other, and large enough to be readable when reduced for publication. Figures should be submitted as near to their printed size as possible. Figures are reproduced in one of the following width sizes: 8 cm, 12 cm or 17 cm, and with a maximal length of 20 cm. Legends for figures should be given on separate pages.

If magnification is significant (photomicrographs) it should be indicated by a calibration bar on the print, not by a magnification factor in the figure legend. The length of the bar should be indicated on the figure or in the figure legend.

Two complete sets of high quality unmounted glossy prints should be submitted in two separate envelopes, and shielded by an appropriate cardboard. The backs of single or grouped illustrations (plates) should bear the first authors last name, figure number, and an arrow indicating the top. This information should be penciled in lightly or placed on a typed self-adhesive label in order to prevent marking the front surface of the illustration.

Photographs of identifiable patients must be accompanied by written permission from the patient.

For figures published previously the original source should be acknowledged, and written permission from the copyright holder to reproduce it submitted.

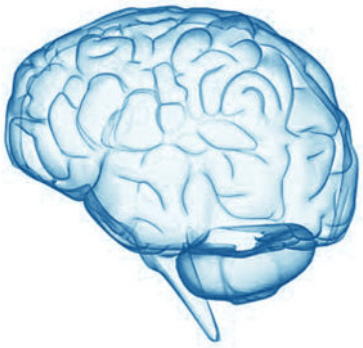
Color prints are available by request at the authors expense.

LETTERS TO THE EDITOR

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.

PROOFS

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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