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KOMPLEKSI PLATINE SA EDDA ETILENDIAMIN *N, N'* DIACETAT LIGANDIMA KAO POTENCIJALNI ANTITUMORSKI AGENSI

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ABSTRACT

e design of platinum based drugs is not a new field of interest. Platinum complexes are widely used as anticancer agents and currently, approximately 30 platinum(II) and platinum(IV) entered into some of the phases of clinical trials. A special place in today's research belongs to platinum complexes with diammine ligands. A large number of edda (ethylenediamine-N, N'-diacetate)-type ligands and their corresponding metal complexes has been successfully synthesized. is article summarizes recent progress in research on edda-type-platinum complexes. Some of these agents achieves better e ect compared to the gold standard (cisplatin). It has been shown that there is a possible relationship between the length of the ligand ester group carbon chain and its cytotoxic e ect. In most cases the longer the ester chain is the greater is the antitumor activity. Of particular interest are the noticeable e ects of some new platinum compound with edda-type ligand on cell lines that are known to have a high level of cisplatin-resistance. Exanimate complexes appear to have a di erent mode of mechanism of action compared with cisplatin which includes apoptotic and necrotic cell death. ere are indications that further investigations of these compounds may be very useful in overcoming the problems associated global cancer statistic.

Key words: *platinum complexes, edda ligand, cytotoxicity*

SAŽETAK

Kompleski platine koriste se kao osnova za dizajn novih lekova. Oni su u širokoj upotrebi kao antitumorski agensi i do danas je oko 30 komplesa platine(II) i platine(IV) u nekoj od faza kliničkog ispitivanja. Posebno mesto u današnjim istaživanjima zauzimaju kompleksi metala sa edda ligandima. Uspešno je sintetisan veliki broj novih edda liganda i odgovarajućih kompleksa. Neki od ovih agensa pokazuju bolju aktivnost od zlatnog standarda, cisplatine. Pokazano je da postoji moguća veza između dužine ugljovodiničnog lanca estraske grupe liganda i citotoksičnog efekta. U većini slučajeva dužina lanca direktno korelira sa antitumorskom aktivnošću. Zabeležena je efikasnija citotoksična aktivnost određenih kompleska platine sa edda ligandima na ćelijskim linijama tumora koji pokazju odgovarajući stepen rezistencije na cisplatinu. Ispitivani komleksi imaju različit mehanizam dejstva od cisplatine, koji uključuje elemente nekrotične i programirane ćelijske smrti. Postoje nagoveštaji da dalja istraživanja ovih agensa mogu biti značajna za prevazilaženje globalnog problema sa kojim se svet danas suočava, a koji se odnosi na stalni porast osoba obolelih od karcinoma.

Ključne reči: *kompleksi platine, edda ligandi, citotoksičnost*

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INTRODUCTION

The era of modern medical chemistry, which includes drugs based on metals, began with discovery of cisplatin (*cis*-diaminedichloroplatinum(II)) (1). It appears that metal complexes are a solid basis for the design of new drugs. A vast number of geometric isomers and different coordination numbers of metallic ions enable fine-tuning of both kinetic (ligands substitution rate) and thermodynamic (strength of metal-ligand bonds, electrode potential) parameters during synthesis of metal complexes – a potential drug (2-5). Ligands play a significant role in design and synthesis of novel complexes, both due to their ability to recognize sites where a complex should bind in a target cell, and the redox processes involved when a ligand that may be released in the cell (6-10).

Platinum complexes are widely used as anticancer agents and currently, approximately 30 platinum(II) and platinum(IV) complexes have entered into some phase of clinical trial (11). A special place in current research belongs to platinum complexes with diamine ligands. A large number of edda (ethylenediamine-*N, N'*-diacetate)-type ligands and their corresponding metal complexes (platinum, ruthenium, cobalt and palladium) have been successfully synthesized (12). Among them, platinum and ruthenium complexes stand out due to their anticancer effects, which have been confirmed on a large panel of different tumour cell lines.

DESIGN AND BIOLOGICAL EVOLUTION OF PLATINUM BASED DRUG

Cisplatin is the first platinum-based drug with anticancer effect approved by the FDA (Food and Drug Administration) and it is most efficient in treatment of many tumors including testicular, ovarian, kidney and neck cancer (2,13). After intravenous administration, cisplatin remains structurally unaltereddue to the high concentration of hloride in blood plasma (100 mM). It reaches tumor cells by either simple diffusion through cell membrane or active transport by copper transporter CTR1 (14-16). Due to much lower concentration of chloride ions in cytosol (3-20 mM) compared to extracellular fluid, there is rapid hydrolysis and substitution of chloride ligands by modified water molecules. After hydrolysis, the platinum cationic complex ($[Pt(NH_3)_2(H_2O)_2]^{2+}$) enters nucleus where it forms a coordinative bond with nitrogen atoms of nucleic bases of DNA, usually guanine (17,18). A bifunctional GG macrochelate is formed through coordination with guanine nitrogen atoms from adjacent DNA chains. $[Pt(NH_3)_2(H_2O)_2]^{2+}$ represents a chain between DNA strands. The modified DNA is permanently damaged and impossible to be used for transcription and replication, resulting cell cycle arrest and consequently apoptosis (18-21).

Despite extraordinary success of cisplatin, this drug has a number of drawbacks (22). For example cisplatin does not show sufficient selectivity towards tumor cells and cause nephrotoxicity, ototoxicity, or anemia (23,24). However, from the chemical standpoint, the platinum(II) complex is highly reactive. It may react with sulphur-containing amino acids (Cys and Met), such as metallothionein and albumin. In the cell, in the $[Pt(NH_3)_2(H_2O)_2]^{2+}$ form, it can react with different chemical classes, carbonate ions, phosphates, methionine, glutathione or metallothioneins. All this greatly reduces efficiency and utilization of this drug (3,19,24-27). It has therefore been necessary to synthesize more selective and less reactive molecule. This led to platinum complexes of the second and third generation (28-30). Complexes of the second generation are structural analogs of cisplatin, designed to overcome the toxicity of cisplatin, while the third generation complexes were created as even more advanced analogues with the main task to act on tumor cells resistant to cisplatin. Since the FDA approved cisplatin as drug, seven more platinum(II) complexes have been introduced to clinical use: 2 of them worldwide (carboplatin and oxaliplatin) and 5 of them in certain countries (nedaplatin, loboplatin, heptaplatin, miriplatin and cycloplatin) (31-33).

It has been found that each ligand has a role in the structure-activity relationship of synthesized complex compound. L-ligands, permanent ligands, form the strongest bond with platinum and remain intact in the final compound of the complex and DNA (34,35). The resistance of tumor cells to the drug mainly depends on these ligands. Oxaliplatin is an analogue of cisplatin, which has a more voluminous and hydrophobic diaminocyclohexyl ligand that "fits" in a major DNA groove thus preventing access to enzymes which "fix" DNA. The main advantage of oxaliplatin compared to cisplatin is that it acts on tumor cells resistant to cisplatin. Currently, it is the drug of choice for colorectal cancer (21,36). Pt-X bond (X is an outgoing ligand) is the weakest, and this is the place of possible hydrolysis in the cell. Therefore, this ligand directly affects the kinetics of reaction between the drug and DNA (34). Modification of these X-ligands can be achieved by reducing the number of side reactions in the cell. Both L and X ligand groups affect lipophilicity and solubility of the complex. Carboplatin (*cis*-diammine-1,1-cyclobutanedicarboxylateplatinum(II)) has in its structure bidentate cyclobutane dicarboxylate ligand which has impact on reduction of number of side reactions of this drug in the cell. These changes eliminated nephrotoxicity of carboplatin (23).

Due to the many side reactions of cisplatin and its analogues in cells, QSAR (quantitative structure activity-relationship) assessments of platinum(IV) complex are beginning. These complexes, $5d^6$ low spin electron configurations of Pt(IV) ion have octahedral geometry, which compared to platinum(II) complexes provides two new axial ligands, thereby increasing the kinetic stability and reducing the reactivity of these complexes compared to platinum(II) complexes. These ligands should be lipophilic to facilitate easier complex passage through the membrane

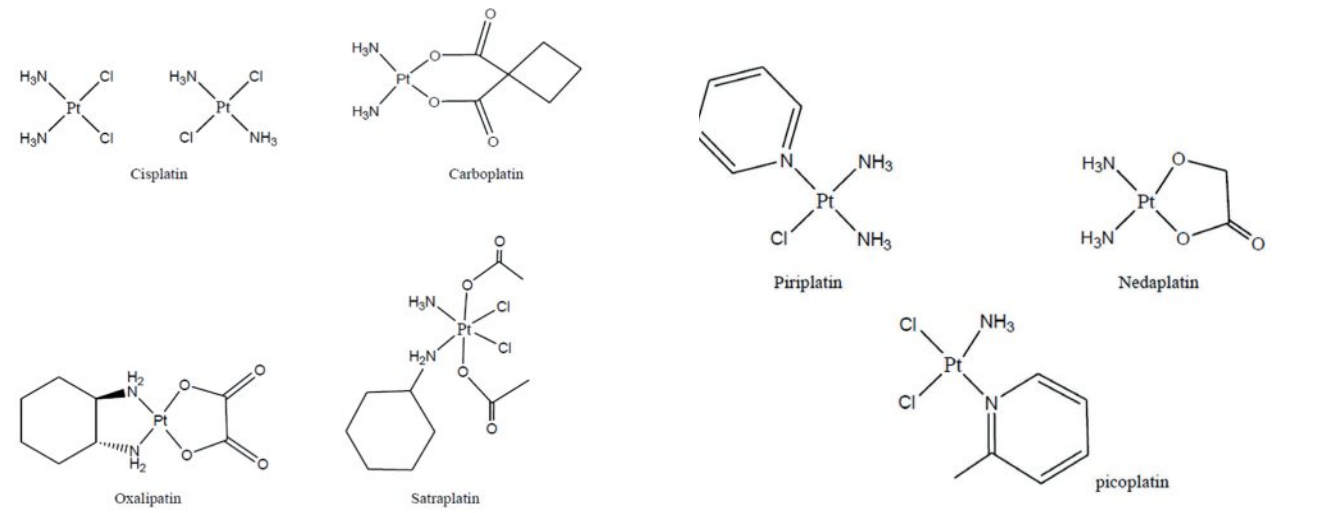

Figure 1. Structural formulae of platinum drug

and to make Pt-ligand bond stronger, so there would not be any hydrolysis and side reactions. Furthermore, these ligands are potential binding sites for so-called carriers in cells, nanoparticles that allow smooth passage of the drug to target site in a cell. It is assumed that this structure of platinum(IV) complex affects stability of the complex, which is the basis for their potential oral use (34, 37).

It was believed that octahedral platinum(IV) complexes are more inert in blood circulation and that they will be activated when they enter the cell. By cell entering Pt(IV) complexes will lose Pt(II) species which are responsible for cytotoxicity (38). It was believed that this fact will allow platinum(IV) complexes to be superior over the platinum(II) complexes regarding the degree of resistance, side effects and possible oral administration. The first attempt to synthesize a whole new drug platinum(IV) based line, in context of prodrugs, has been made by Rosenberg (6). Platinum(IV) complexes *cis*-[Pt(NH_3)$_2$$Cl_4$], *trans*-[Pt($NH_3$)$_2$$Cl_4$] and [Pt(en)$Cl_4$] were soon abandoned because they showed less anticancer activity then cisplatin. By today, two most prosperous agents are satraplatin (JM216) and LA-12 (Figure 1) (39, 40). The greatest success was with complex of JM216 - satraplatin. Satraplatin is a lipophilic molecule, easily enters the cell, it is inert and stable, and because of all this has potential for oral administration. It is reduced within the cell by the cytochrome C, and then by hemoglobin as well in the presence of NADH. Satraplatin is characterized by a comfort drug use (can be used orally) for patient unlike other Pt(II) drugs that can only be used intravenously. The presence of intracellular agents as glutathione, ascorbic acid and others is required for reduction of satraplatin and therefore for its activation (41). Also satraplatin can be used for treatment of prostate, lung and ovarian tumors with little signs of nephro-, neuro- and ototoxicity. LA12 is a satraplatin analogue and future investigation will probably demonstrate that it may be used for ovarian carcinoma resistant on cisplatin, or even colorectal tumors (42,43).

A series of platinum(IV) complexes similar to JM216 has also been synthesized, with aliphatic, aromatic and alicyclic amines, with straight and branched chain, which showed higher activity compared to cisplatin. Despite major efforts and detailed studies and predictions, none of platinum(IV) complexes, including JM216, has not been approved for clinical use. Very good results of biological tests of oxaliplatin and satraplatin encouraged the idea of synthesis of platinum(II) and platinum(IV) complex with edda type ligands as their analogues, in order to obtain better anti-cancer agents (42,43).

R_2EDDA TYPE LIGAND

Since the beginning of platinum derivates exploration, less attention has been given to aminocarboxylate ligand complexes. Liu (44) was the first who showed the coordination of ethylenediamine-*N,N'*-diacetate with platinum(II). Unfortunately, he obtained [Pt(H_2edda)Cl_2] complex in which both carboxylate groups were protonate, mainly due to synthesis conditions, which left platinum(II) coordination sphere the same as in [Pt(en)Cl_2]. Over the coming years, investigations of complexes with edda and related ligands have attracted the attention, chiefly because the good chelating ability of the ligands which may indicate a variety of complexes' stereochemical and physical properties. Ethylenediamine-*N*-*N*'-diacetic acid (edda) contains two nitrogens and also two oxygens as donor atoms. It acts as a tetradentate ligand in the case of complete coordination. R_2 edda ligand type belongs to the dialkil esters group of ethylenediamine-*N*-*N*'-diacetic acid (H_2edda), di(izo)propionic acid (H_2eddp, H_2eddip), di-2-(3-cyclohexyl)-propanoic acid (H_2eddch), di-2-(3methyl)-butanoic adic (H_2eddv), di-2-(4-methyl)-pentanoic acid (H_2eddl), as well as propilendiamine-*N*-*N*'-diacetic acid (H_2pdda) (Figure 2). Edda ligands can very easily be esterified, and during the complete coordination edda- ligands type esters mainly behave as

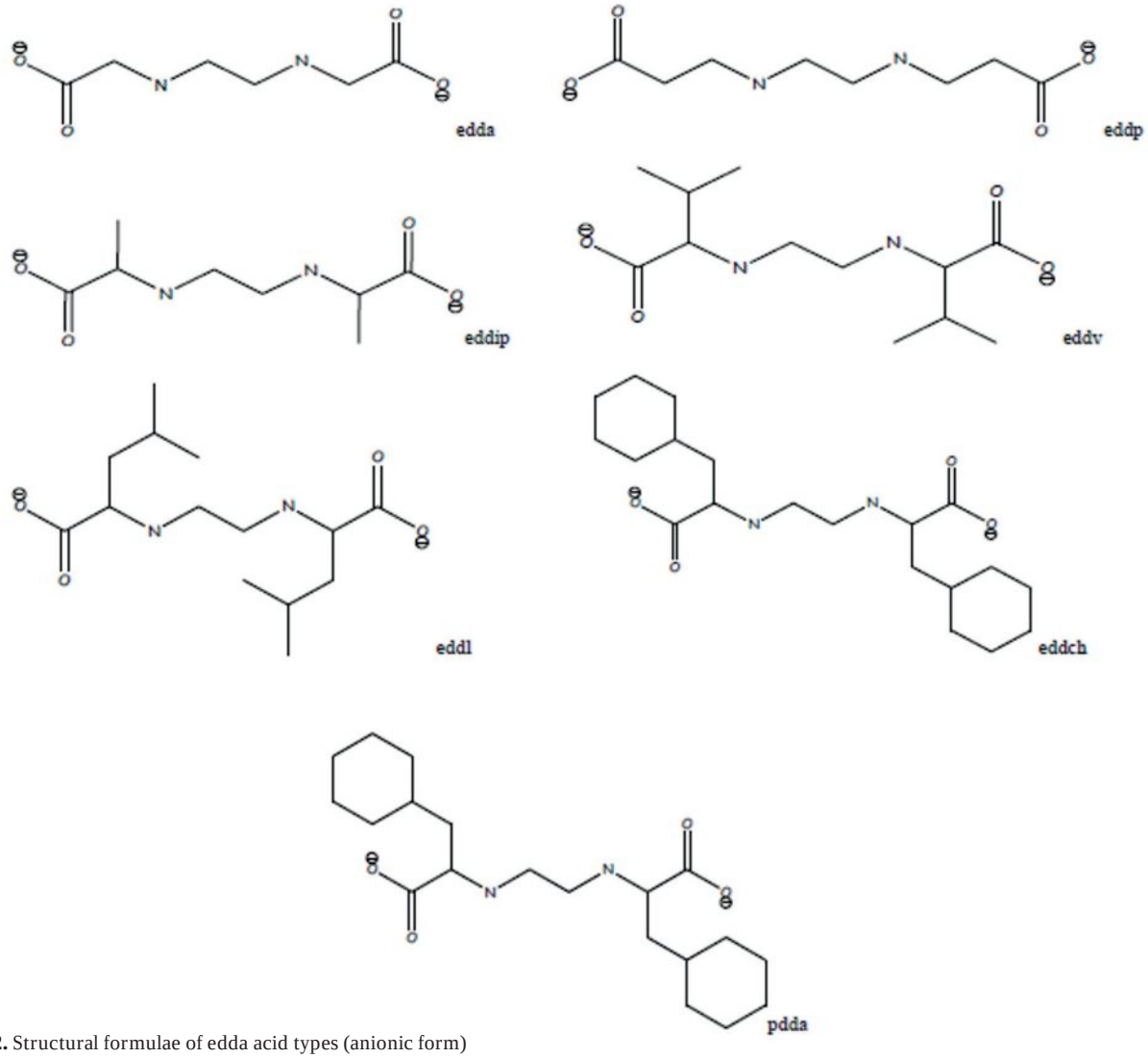

Figure 2. Structural formulae of edda acid types (anionic form)

bidentate ligands. In some cases, hydrolysis of one or both of ester groups occurs, thus the ligands may behave as bidentate or tridentate, respectively (45).

CYTOTOXICITY OF PLATINUM COMPLEXES WITH EDDA TYPE LIGANDS

Platinum complexes with edda-type ligands

Platinum(II) and platinum(IV) complexes with ehtylenediamine ligands, *N*-(2-hydroxyethyl)ethylenediamine (heen), *N-N'-bis*(2-hydroxyethyl)ehtylenediamine (he2n), ehtylenediamine-*N, N'*-diacetic acid (H_2edda) and ehtylenediamine-*N*-monoacetic acid (hedma) were examined in order to reveal their cytotoxicity on different cell lines of human ovarian carcinoma (Table 1) (45). These complexes have temperate cytotoxic effects, through they were significantly lower than those of cisplatin and JM-216. It has been proven that platinum(II)/(IV) complexes with multidentate ligands *N*-(2-hydroxyethyl)ethane-1,2-diamine (NNOH) and ethylenediamine-*N,N'*-diacetic acid (H_2edda) have a different influence on CH1, 41M and Skov-3 cell line than on cisplatin resistant cell lines (Table 1) (46). While platinum(IV) complex with *NN'* donor set is 2-5 five times more potent against cisplatin sensitive/resistant cell lines, in comparison with platinum(II) complex, but with complexes with edda ligand situation is entirely different - platinum(II) complexes are far more active. Platinum(IV) complexes with ligands dialkyl esters of ethylenediamine-*N, N'*-diacetic acid (R_2edda) [$PtCl_4$(R_2edda)] x H_2O (R = Me, Et, *n*-Pr) were also investigated primarily to clarify influence at length of carbon chain in ester group on antiproliferative effect *in vitro* (Figure 3) (47). Examinations were performed on severe human tumor cell lines (Table 1). It was shown that by replacing methyl group in ester chain by ethyl or propyl group cytotoxic effect will be increased - the longer the ester chain is, the greater is the antitumor activity. The absence of this trend is observed on DLD-1 cell line. Complexes [$PtCl_4$(Et_2edda)] and [$PtCl_4$(Pr_2edda)] achieved highest cytotoxic activity on cisplatin-resistant

Table 1. Cytotoxic e ect of Platinum complexes with edda ligand type

Ligand type	Pt complex	Carbon chain in ester group (R)	Cell line	Cytotoxic e ect comparing to cisplatin		Study
edda	IV	Me, Et, *n*-Pr	testicular germ cell tumors (1411HP, H12.1), colon carcinoma (DLD-1), melanoma (518A2), liposarcoma and lung carcinoma (A549)	lower	stronger cytotoxic e cacy in cisplatin-resistant 1411HP cells (compared to other cell lines) [ca. 35–40 μM] (vs. cisplatin[IC_{50} 2.7 μM])	Kaludjerovic et al (2008) [47]
	II	Me, Et, *n*-Pr	melanoma (5182A), human thyroid carcinoma (8505C), head and neck tumor (A253), cervix (A431), lung (A549), ovarian (A2780), breast (MCF-7) and all colon (HT-29, HCT-8, DLD-1, SW1736)	lower	*n*-Pr complex showed the highest action against ovarian (A2780) cells [IC_{50} value of 51 μM] (vs. cisplatin [IC_{50} 0.55 μM])	Kaludjerovic et al (2014) [48]
eddp	IV	Br,J	human ovarian cancer (A2780/A2780cisR)	lower	A2780 cell line: Pt complexes[IC_{50} value ca. 30-90 μM] (vs. cisplatin [IC_{50} 0.2 μM]) A2780cisR cell line: Pt complexes[IC_{50} value ca. 90-270 μM] (vs. cisplatin [IC_{50} 3.5 μM])	Sabo et al (2004) [49]
		Et and *n*-Pr	ovarian (A2780), cervix (A431), melanoma (518A2), lung (A549), head and neck (FaDu), colon (HT-29, HCT-8, DLD-1, 8505C, SW480)	lower	$PtCl_4$(*n*-Pr_2eddp)] has highest e ect on A2780, 518A2 and A549 cell lines[IC_{50} value 8.6 μM / 17.99 μM / 20.81 μM] (vs. cisplatin [IC_{50} 0.5 μM / 1.5 μM / 1.5 μM])	Kaludjerovic et al (2009) [52]
		n-Bu	melanoma (B16)	more potently	N/A	Maksimovic-Ivanic et al (2012) [53]
	II and IV	*n*-Bu, *n*-Pe/ Br,J	human cervix adenocarcinoma (HeLa), human myelogenous leukemia (K562)	lower	Pt(dveddp)Cl_4 e ect on K562 cell line is closest to cisplatin [IC_{50} value 5.87 μM] (vs. cisplatin [IC_{50} 5.0 μM])	Kaludjerovic et al (2005) [50]
eddp and pdda	IV	*n*-Bu, *n*-Pe/ Br	mouse fibrosarcoma (L929), human astrocytoma (U251)	comparable	best results were gained with platinum(IV) complexes [$PtCl_4$(R_2eddp) (K562 cell line [IC_{50} value 5.87 μM] vs. cisplatin [IC_{50} 5.0 μM])	Kaludjerovic et al (2005) [51]
(S,S) eddp	II and IV	*i*-Pr, *i*-Bu;	human cervix adenocarcinoma(HeLa), human myelogenous leukemia (K562), malignant melanoma (Fem-x cell)	lower	Pt(IV) isopropyl (*S,S*)eddp complex is most active [IC_{50} value 30.48μM / 12.26 μM / 13.68 μM] (vs. cisplatin [IC_{50} 4.47 μM / 5.77 μM / 4.7 μM])	Krajcinocic et al (2008) [54]
	IV	Br,J	rat glioma cell line(C6), human glioma cell line(U251), mouse fibrosarcoma cell line (L929)	lower	IC_{50} value of cisplatin 9.8 μM / 23.6 μM / 19.3 μM vs. IC_{50} values of Pt(IV) complexes were over then 100 μM	Djinovic et al (2010) [55]
		n-Pr, *n*-Bu, *n*-Pe	the colon cancer adnocarcinoma cell line (HTC-116), breast cancer cell line (MDA-MB-231)	more potently (*n*-Pr and *n*-Pe)	*n*-Pr e ect on HCT-116 cells - [IC_{50} value of 77.68 μM] (vs. cisplatin [IC_{50} 263.66 μM]) and on MDA-MB-231 cells (72h) [IC_{50} value of 64.21 μM] (vs. cisplatin [IC_{50} 114.11 μM]) *n*-Pe e ect on HCT-116 cells - [IC_{50} value of 96.08 μM] (vs. cisplatin [IC_{50} 263.66 μM]) and on MDA-MB-231 cells (24h) [IC_{50} value of 238.60 μM] (vs. cisplatin [IC_{50} 425.32 μM])	Stojkovic et al (2014) [56]
eddp and *(S,S)* eddp	II and IV	*i*-Pr, *i*-Bu; cyclopentyl	mouse colon cancer (CT26CL25), colon cancer (HTC116 and SW620), prostate cancer (PC3 and LNCaP), glioblastoma (U251), human melanoma (A375), and murine melanoma (B16))	more potently (Platinum(IV) complexes)	IC_{50} value of platinum(IV) complexes is up to 3 times lower than that of the corresponding platinum(II) complexes Platinum(IV) complexes on CT26CL25, HCT116, SW620, and B16 cell lines) [IC_{50} ca. 35–100 μM] (vs. cisplatin[IC_{50} ca. 45-120 μM])	Kaludjerovic et al (2012) [57]
eddip	II	Et, *n*-Pr, *n*-Bu, *n*-Pe	human colon cancer cell lines (HCT116, SW480 and CaCo-2)	more potently	*n*-Bu Pt(II) complex has highest e ect [IC_{50} value 11.23 μM / 5.09 μM / 4.02 μM] (vs. cisplatin [IC_{50} 161.25 μM / 51.64 μM / 64.74 μM])	Volarevic et al (2013) [58]
meddch	IV	H, Me, Et, *n*-Pr and *n*-Bu	Glioma (C6 and U251), fibrosarcoma (L929) and melanoma (B16)	more potently	IC_{50} value 1.9–8.7 μM compare to cisplatin (IC_{50} 10.9–67.0 μM)	Lazić et al (2010) [59]
		Me, Et, *n*-Pr and *n*-Bu	human melanoma (A375), human glioblastoma (U251), human prostate cancer (PC3), human colon cancer (HCT116), mouse melanoma (B16) and mouse colon cancer (CT26CL25) cells	more potently	IC_{50} value of Pt complexes ca. 2.9-21.3 μM (vs. cisplatin [IC_{50} ca. 12.5-120 μM])	Mihajlovic et al (2012) [63]
eddl	II	Et, *n*-Pr, *n*-Bu, *n*-Pe	chronic lymphocytic leukemia (CLL)	more potently	IC_{50} value (form Et to *n*-Pe) 22.35 μM / 9.85 μM / 5.39 μM / 10.37 μM (vs. cisplatin [IC_{50} 263.75 μM])	Vujic et al (2011) [60]
	IV	Et, *n*-Pr, *n*-Bu, *n*-Pe	human breast cancer (MDA-MB-361 and MDA-MB-453), T-leukemia (Jurkat), chronic myelogenous leukemia (K562), colorectal cancer (SW480) and CLL lymphocytes	more potently only for SW480 cells, for other cell types lower or comparable	SW480 cells: IC_{50} value of Pt complexes (form Et to *n*-Pe) 5.09 μM / 2.32 μM / 3.95 μM / 0.74 μM (vs. cisplatin [IC_{50} 31.92 μM])	Vujic et al (2012) [61]
eddch	IV	Me, Et, *n*-Pr, *n*-Bu	human glioblastoma (U251), mouse melanoma (B16)	more potently	U251 cells: IC_{50} value of Pt complexes ca. 1.9-17.5 μM (vs. cisplatin [IC_{50} ca. 20.0 μM]) B16 cells: IC_{50} value of Pt complexes ca. 3.1-21.3 μM (vs. cisplatin [IC_{50} ca. 94.3 μM])	Mihajlovic et al (2013) [64]
(S,S)-1,3-propanediamine-*N,N'*-di-2-(3-cyclohexyl) propanoic acid	II	*i*-Bu; *n*-Pe and *i*-Pe/J	human neoplastic cell lines (HeLa, A549, MDA-MB-231, LS-174, EA.hy 926), and human fetal lung fibroblast cell line (MRC-5)	more potently (*n*-Pe Pt(II) complex)	*n*-Pe Pt(II) complex IC_{50} value 5.4 μM / 11.1 μM / 11.9 μM / 12.2 μM/ 5.9 μM (vs. cisplatin [IC_{50} 6.9 μM/ 17.2 μM / 15.4 μM/ 21.9 μM/ 22.4 μM])	Savic et al (2014) [65]
(*S,S*)-eddba	IV	Et, *n* - Pr, *n*-Bu	chronic lymphocytic leukemia (CLL)	more potently	IC_{50} value of Pt complexes (form Et to *n*-Bu) 5.04 μM / 6.08 μM / 25.28 μM (vs. cisplatin [IC_{50} 331.61 μM])	Dimitrijevci et al (2013) [66]

N/A- not applicable

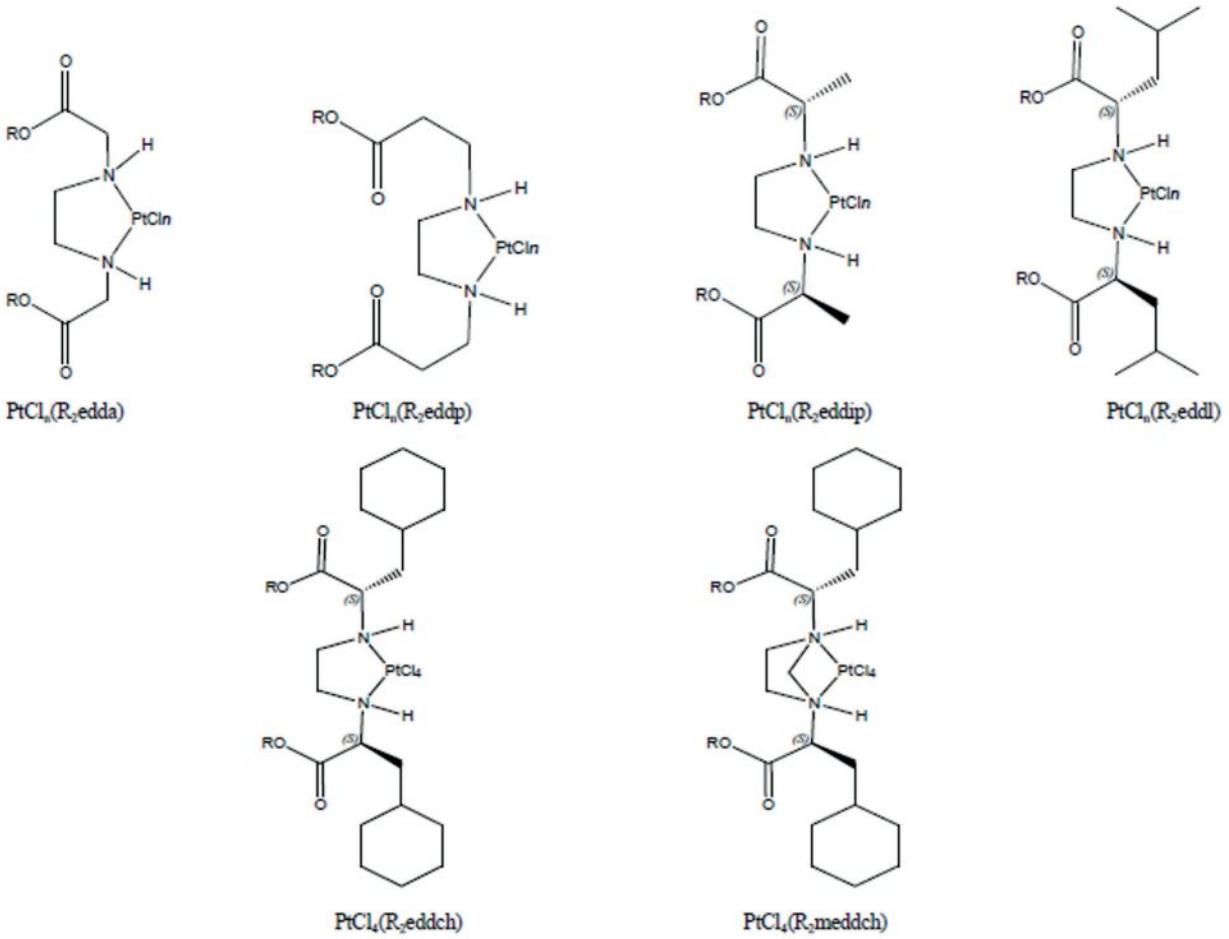

Figure 3. Structural formulae of platinum compounds with R2edda ligand type

1411HP cell line. All of them induce apoptosis and their effect is dose-dependent. Platinum(II) complexes with bidentate edda ligand type [$PtCl_2(R_2edda)$] (R = Me, Et, *n*-Pr; edda = ethylenediamine-*N*, *N*'-diacetate) were also questioned (48). *In vitro* cytotoxic effect was studied on various cell lines to generate new evidence regarding the cytotoxic effect of these complexes, (Table 1). The aforementioned trend of the impact of alkyl chain (in ester group) length on antitumor activity can also be applied in this case, except of HTC-8 and HT-29 cells. Thus, the highest cytotoxic effect has been achieved by [$PtCl_2$(*n*-Pr_2edda)] against A2780 cells. However, the activities of all these new platinum (II) compounds is lower compared to the appropriate platinum(IV) complexes, as well as cisplatin.

Platinum complexes with eddp and eddip-type ligands

Grow inhibition which had been provided by influence of two new platinum(IV) complexes ([PtX_2(eddp)] x *n* H_20; X= Cl/Br; n = 1 or 1,24; eddp=ethylenediamine-*N*, *N*'-di-3-propionate) on A2780/A2780cisR pair of human ovarian cancer cell lines, showed that trans-[PtX_2(eddp)] (x= Cl^-, Br^-) complexes have far less cytotoxic affinity compared to cisplatin and complexes with edda ligands (*cis*-[$PtCl_2$(edda)]) (49). There is a high probability that difference in effects stems from otherwise complex geometry. These two complexes will obtain dissimilar adducts with cell molecules (DNA nucleic bases) by direct interaction. However, if reduction process (Pt(IV) to Pt(II)) occurs before interaction with nucleic bases, *trans*-[PtX_2(eddp)] complexes will form tetracoordinated platinum(II) complexes where eddp ligand occupies all four coordination positions preventing in that way reaction with DNA nucleic bases (49). In the contrary, reduction of *cis*-[$PtCl_2$(edda)] complex, by formatting tetracoordinated platinum(II) complexes which contain biscoordinated edda ligand and leaving two chloro ligands which could easily be replaced and make a link with DNA (49). Also, some studies of platinum(II)/(IV) complexes ([$PtCl_4(Bu_2eddp)$], [$PtBr_3Cl(Bu_2eddp)$], [$PtCl_2I_2(Bu_2eddp)$], [$PtCl_4(Pe_2eddp)$], [$PtCl_2(Bu_2eddp)$]) have demonstrated that these complexes had five times weaker cytotoxic effect on HeLa (human cervix adenocarcinoma) cell line compared to cisplatin, but the effect on K562 (human myelogenous leukemia) cell line was almost equal to the effect of cisplatin (Table 1) (50). It was concluded that the exchange of two chloro ions for two iodo ions in pres-

ent complexes will only decrease the antitumor activity of the complexes. It has been shown that these complexes induce apoptosis, but in some cells secondary necrosis was detected (50). Several complexes of platinum(II)/(IV) were investigated in the light of *in vitro* antitumor activity against some mouse and human cell lines (Table 1) (51). Of all of the complexes whose efficiency was studied (*trans*-[$PtCl_2$(pdda)], *trans*- [$PtBr_2$(pdda)], *trans*- [$PtCl_2$(eddp)], *trans*- [$PtBr_2$(eddp)], [$PtCl_2$(H_2eddp)], [$PtCl_4$(Pe_2eddp)], [$PtCl_2$(Bu_2eddp)], [$PtCl_4$(Bu_2eddp)]), best results were gained with platinum(IV) complexes [$PtCl_4$(R_2eddp)] (R= Bu or Pe). These two complexes showed the cytotoxic activity was dose-depended and comparable with cisplatin, but also that they archive cytotoxic effect more rapidly than cisplatin. Further examination of toxicity of [$PtCl_4$(Pe_2eddp)] and [$PtCl_4$(Bu_2eddp)] pointed out that these complexes cause ROI (reactive oxygen intermediates)-dependent, ERK (extracellular signal-regulated kinase)-independent induction of tumor cell necrosis as opposed to cisplatin - it induced ROI-independent apoptotic death of tumor cells (48). Cytotoxic effect of two more platinum(IV) complexes, [$PtCl_4$(Et_2eddp)] and [$PtCl_4$(*n*-Pr_2eddp)], has been investigated on severe cell lines but each one of them showed less activity *in vitro* in regard to cisplatin (Table 1) (52). Kaludjerovic et al. (52) also established that there is an interaction between plasmid pBR322 DNA and platinum(II)/(IV) complexes, in the presence or absence of ascorbic acid. From all of the platinum (eddp) complexes, [$PtCl_4$(*n*-Bu_2eddp)] is the only one which has *in vivo* anti-tumor activity demonstrated (53). Cytotoxic effect of [$PtCl_4$(*n*-Bu_2eddp)] is dose and time dependent, and this complex shows its effect faster then cisplatin against B16 melanoma cells. Investigation on mice examined platinum(IV) complex demonstrated greater efficiency then cisplatin in terms of reducing volume of a tumor in its appropriate doses. The greater advantage over the cisplatin is reflected in the absence of kidney damage; [$PtCl_4$(*n*-Bu_2eddp)] did not show any sign of nephrotoxicity (53). A new modification of platinum(II/IV) complexes with R_2(*S*,*S*)eddp ligand type (*O*,*O*'-di-isopropyl or *O*,*O*'-di-isobutyl- (*S*,*S*)-ethylenediamide-*N*, *N*'-di-2-propionate) were synthetized (Table 1) (54). The results showed that the Pt(IV)complexes were followed with better cytotoxic activity. Also, it has been noted that if in platinum(II) complexes with this ligand type occur the interchange isopropyl group for isobutyl group, cytotoxic activity will be increased. On the other hand, if this exchange occurs in platinum(IV) complexes, the cytotoxic activity will be decreased, regardless of the type of cell line. However, the best activity has been demonstrated by platinum(IV) complex with *O*,*O*'-di-isopropyl-(*S*,*S*)-ethylenediamide-*N*, *N*'-di-2-propionate ligand against K526 and Fem-x cell lines, unfortunately each one of these complexes had lower cytotoxic activity (2-5 times) in comparison to the corresponding cisplatin. Complexes [$PtCl_2${(*S*,*S*)-*i*Bu_2eddip}], [$PtCl_4${(*S*,*S*)-*i*Pr_2eddip}] and [$PtCl_4${(*S*,*S*)-*i*Bu_2eddip}] induce apoptosis. Complex [$PtCl_2${(*S*,*S*)-*i*Pr_2eddip}] led to chromatin condensation in HeLa cells, but contrary to previous mentioned complexes which cause rounding of cells, this complex caused more irregular cell shapes which may indicate that disruption of cytoskeleton and/or plasma membrane may be occurred. Two new platinum(IV) complexes, and there *in vitro* activities, were demonstrated - [PtX_2(*S*,*S*-eddp)] x nH_2O (*S*,*S*-eddp = ethylenediamine-*N*, *N*'-di-*S*,*S*-2-propanoate ion, X = chlorido or bromido, n = 4, 0) (55). The complexes displayed significantly lower cytotoxicity on severe cell lines and have a quite different mechanism of action compared to cisplatin (Table 1). Also exchange of tetradentate eddp ligand with bidentate eddp ester ligand will lead to enhancement in cytotoxicity of platinum(IV) complexes. Stojkovic et al. (56) synthesized three new platinum(IV) complexes with bidentate *N*, *N*'-ligands, [$PtCl_4$(R_2-*S*,*S*-eddp)] (R=*n*-Pr, *n*-Bu, *n*-Pe). It has been shown that all three new complexes have a dose and time-dependent grow-inhibition effect. [$PtCl_4$(*n*-Pr_2-*S*,*S*-eddp)] and [$PtCl_4$(*n*-Pe_2-*S*,*S*-eddp)] had much higher antiproliferative activity in comparison with cisplatin. Also, MDA-MD-231 cell line showed to be less sensitive to the treatment with all these complexes, including cisplatin. The greatest cytotoxic effect on HTC-116 cell line was demonstrated by [$PtCl_4$(*n*-Pe_2-*S*,*S*-eddp)] and on the other side greatest effect on a MDA-MB-231 was made by [$PtCl_4$(*n*-Pr_2-*S*,*S*-eddp)]. All this compounds induced a type of programmed cell death, but the third complex ([$PtCl_4$(*n*-Pe_2-*S*,*S*-eddp)]) had highest proapoptotic effect. Cytotoxic effect of two platinum(IV) complexes ([$PtCl_4$(R_2eddp)]) (R= *i*Pr (isopropyl) or *i*Bu (isobutyl)) and three platinum(II)/(IV) complexes ([$PtCl_{2/4}$(R_2eddip)]) (R= *i*Pr (isopropyl), *i*Bu (isobutyl) or *c*-Pe (cyclopentyl)) was examined on various cancer cell lines (Table 1) (57). Increasing the number of hydrophobic alkyl side chains appears to result in enhancement of cytotoxicity, in fact complexes with isopropyl group had less activity then those with isobutyl or cyclopentyl group. Platinum(II) (eddip) complexes with isobutyl group have proven to be more effective on HCT116 and SW620 cells then cisplatin, as was the effect of these types of complexes with cyclopentyl group on CT-26CL25, HCT116, SW6220 and B16 cells (Table 1). No signs of toxicity on normal primary cells (fibroblasts and keratinocytes) of complexes was found. All of these new platinum compounds induce caspase-dependent apoptosis. Moreover, ROS (reactive oxygen species) and RNS (reactive nitrogen species) are not being singled out as the main mediators of toxicity. On the same human colon cancer cell lines Volarevic et al. (58) examined the antiproliferative effect of four new platinum(II) complexes with *O*,*O*'-dialkyl esters of (*S*,*S*)-ethylenediamine- *N*, *N*'-di-2-(4-methyl) pentanoic acid (alkyl, ethyl, propyl, *n*-butyl, *n*-pentyl). In comparison to cisplatin all these new complexes have shown a higher cytotoxic activity. Conclusion of this study indicated that the shorter the ester chain in complex is, the complex will show less cytotoxic activity. Thus, the greatest impact was expected from platinum(II) complex with *O*,*O*'-dipentyl esters. Still, highest impact on human

colon cancer cells (especially on HTC116) has been made by platinum(II) complex with *O,O'*-dibutyl esters. It is thought that the reason for this is the superior intercellular accumulation (59).

Platinum complexes with meddch-, eddl-, eddch and eddba- type ligands

Recently, platinum(II) and platinum(IV) complexes with (*S,S*)-R_2eddl ligand type have been synthetized (60,61). Highest activity had come from complexes with n- butyl group in ester chain [$PtCl_n$((*S,S*)-R_2eddl)] (R= Et, Pr, *n*-Bu or *n*-Pe; n=2 or 4; *O,O'*-diethyl-(*S,S*)-ethylenediamine-*N, N'*-di-2-(4-methyl)-pentanoate)platinum), although cytotoxic effect increases with the increase of ester chain length, as previously mentioned. This type of platinum(II) complexes were found to display much higher antitumor activities on CLL cells in comparison to cisplatin (60), especially [$PtCl_2$((*S,S*)-*n*-Bu_2eddl)], which is the bearer of the highest antitumor activity of them all. Platinum(IV) compounds with (*S,S*)-R_2eddl ligand type were appraised for their cytotoxic effect (Table 1) (61). Very potent complex was also the one with *n*-butyl group in ester chain [$PtCl_4$((*S,S*)-*n*-Bu_2eddl)]. It is interesting that CLL cells is the only cell line more sensitive on platinum(II) complex. Lazic et al. (59), synthesized a new platinum(IV) compound with tetradentate coordinated (*S,S*)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl) propanoate (cyclohexyl edda/eddch). The cytotoxic effect of these complexes [$PtCl_4$((*S,S*)-R_2eddch)] (R= Me, Et, *n*-Pr, *n*-Bu) were tested against various cell lines (Table 1). All compounds were clearly more cytotoxic than cisplatin, especially against cisplatin-resistant B16 cells. They also suggested that the length of alkyl chain has different effect than is the case with other platinum complexes. The longer the alkyl chain is, the poorer is the antitumor activity. They also cleared the difference which existed between mechanisms of action of these complexes and golden standard. Cisplatin brings about caspase-dependent apoptosis realized by an autophagy response. On the other hand, new octahedral platinum(IV) complexes induce necrosis like cell death. Soon after, there was a report of octahedral Pt(IV) complex with di-*n*-propyl-(*S,S*)-ethylenediamine-*N, N'*-di-2-(3-cyclohexyl)propanoato ligand and its effect on immune cells (SPC and LNC) (62). It has been shown that this platinum(IV) complex, in concentrations which have been proven effective on tumor cells, does not notably affect viability of immune cells. Also, this complex disenables synthesis of IFN-γ, IL-17 and NO in immune cells. The new prospective platinum(IV) drugs were synthesized with the novel *N,N'*-methylene modified cyclohexyl ethylenediamine-*N,N'*-diacetate (edda)-type ligands, [$PtCl_4$((*S,S*)-R_2meddch)] (R= Me, Et, *n*-Pr, *n*-Bu) (63). All of these compounds, with the exemption of [$PtCl_4$((*S,S*)-Me-2meddch)], demonstrated higher citotoxic activity then cisplatin on every cell line, especially on HCT116 and CT26CL25 cell lines resistant or poorly responsive to treatment with cisplatin (Table 1). These platinum(IV) complexes with eddch ligand type induce apoptosis, but in lower dose range, and it has been shown that they affect primary keratinocytes and fibroblasts less then cisplatin, which may be indicative of their selectivity. Furthermore, series of complex electrochemical tests were performed by cyclic voltammetry and differential pulse voltammetry (64). This study indicated that the reduction of these complexes is performed as two-electron process followed by the loss of axial chloride ligand and the length of the C atom chain in esters part affects the reduction potential. Correlation between redox potentials and IC_{50} (half maximal inhibitory concentration) values was not established. Pt(II)-iododo complexes with derivatives of ethylenediamine-*N, N'*-diacetate (edda)-type of ligands, (esters of (*S,S*)-1,3-propanediamine-*N, N'*-di-2-(3-cyclohexyl)propanoic acid) are also a new potential anticancer substance (65). Cytotoxic effect of isobutyl, *n*-pentyl and isopentyl esters of these compounds were examined against various human cell lines (Table 1). Although summary effect of all these compounds was better in regard to cisplatin, in LS-174 cells effect was 3 to 4 times higher than golden standard. However, exanimate complexes seem to have a different mode of mechanism which includes apoptotic and necrotic elements of cell death. These complexes also evince better affinity for DNA binding then cisplatin and enter cells efficiently, which may be an important advantage in respect of avoiding cell resistance. Better intracellular accumulation and DNA binding are probably the result of substitution kinetics of iodide ligands and proper lipophilicity of an edda ligand type. The cytotoxic effect against freshly isolated CLL cells is achieved by [$PtCl_4$(R_2-*S,S*-eddba)] (R= Et, Pr or Bu) (eddba-ethylenediamine-*N, N'*-di-*S,S*-(2,2'-dibenzyl)acetic acid) complex, as reported by Dimitrijevic et al. (66). The cytotoxic influence of [$PtCl_4$(Et_2-*S,S*-eddba)] and [$PtCl_4$(Pr_2-*S,S*-eddba)] was better than complex with *n*-butyl group in ester chain, but still, all of them have considerably higher antiproliferative ability against CLL cells then cisplatin (Table 1).

CONCLUSION

The design of platinum based drugs is not a new field of interest. This article summarizes recent research progress in research on edda-type-platinum complexes - new type of platinum based drugs. Some of these compounds achieves better effect compared with the gold standard (cisplatin). Of particular interest are the noticeable effect of some new platinum compound with edda ligand type on cell lines which are known to have a high level of cisplatin-resistance. There are indications that further investigations of these compounds may be very useful in overcoming the problems with global cancer statistic. Further preclinical and clinical researches might give some useful information which can help in overcoming the main problems related to platinum based drugs (including tumor resistance and less serious side effect).

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Conflicts of interest

The authors declare no financial or commercial conflicts of interest.

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MORPHOLOGY OF HUMAN NUCLEUS ACCUMBENS NEURONS BASED ON THE IMMUNOHISTOCHEMICAL EXPRESSION OF GAD67

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MORFOLOGIJA NEURONA HUMANOG NUKLEUSA AKUMBENSA ZASNOVANA NA IMUNOHISTOHEMIJSKOJ EKSPRESIJI GAD67

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ABSTRACT

e nucleus accumbens is a part of the ventral striatum along with the caudate nucleus and putamen. e role of the human nucleus accumbens in drug addiction and other psychiatric disorders is of great importance. e aim of this study was to characterize medium spiny neurons in the nucleus accumbens according to the immunohistochemical expression of GAD67.

is study was conducted on twenty human brains of both sexes between the ages of 20 and 75. e expression of GAD67 was assessed immunohistochemically, and the characterization of the neurons was based on the shape and size of the soma and the number of impregnated primary dendrites.

We showed that neurons of the human nucleus accumbens expressed GAD67 in the neuron soma and in the primary dendrites. An analysis of the cell body morphology revealed the following four di erent types of neurons: fusiform neurons, fusiform neurons with lateral dendrites, pyramidal neurons and multipolar neurons.

An immunohistochemical analysis showed a strong GAD67 expression in GABAergic medium spiny neurons, which could be classified into four di erent types, and these neurons morphologically correlated with those described by the Golgi study.

Keywords: *nucleus accumbens; GAD67; immunohistochemistry*

SAŽETAK

Nukleus akumbens je jedro koje predstavlja deo ventralnog strijatuma, zajedno sa nukleusom kaudatusom i putamenom. Ima značajnu ulogu u nastanku bolesti zavisnosti i drugih psihijatrijskih poremećaja. Cilj ove studije je tipizacija GABAergičkih neurona srednje veličine sa spinama na osnovu imunohistohemijske ekspresije GAD67.

Istraživanje smo izveli na 20 humanih mozgova, oba pola, starosti od 20 do 75 godina. Ekspresija GAD67 jeurađena imunohistohemijskom metodom, a tipizacija neurona srednje veličine sa spinama je izvršena na osnovu oblika i veličine some i broja impregnisanih primarnih dendrita.

Ovim istraživanjem smo pokazali da neuroni srednje veličine sa spinama u humanom nukleusu akumbensu eksprimiraju GAD67 u citoplazmi neurona, kao i u primarnim dendritima. Analizom morfologije perikariona utvdili smo prisustvo četiri tipa neurona: fuziformni neuroni, fuziformni sa bočnim dendritom, piramidalni neuroni i multipolarni neuroni.

Ovom metodologijom smo potvrdili jaku ekspresiju GAD67 u GABAergičkim neuronima srednje veličine sa spinama, koji se mogu klasifikovati u četiri morfološka tipa, što korelira sa rezultatima prethodno sprovedene Goldži studije.

Ključne reči: *nukleus akumbens; GAD67; imunohistohemija*

ABBREVIATIONS

GABA – gamma-Aminobutyric acid **GAD67** – glutamate decarboxylase

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INTRODUCTION

The nucleus accumbens is an important region of the brain that is involved in many reward-conditioned behaviours. As a part of the limbic system, this nucleus is positioned between the rostral part of the striatum and the lateral borders of the septal nuclei in the human telencephalon. The nucleus accumbens, in mammals, is sparsely integrated with the lateral striatum (1). The striatum is the main location of the basal ganglia and the subcortical brain region that plays a key role in active control, learning and memory (2). In mammals, based on morphological, histological and immunohistochemical criteria, the nucleus accumbens comprises several subterritories, of which two of the main parts are defined as the core and the shell (3-5). The nucleus accumbens plays a very important role in motivation and planned motor activities related to food and drug reward, sexual arousal and stress response (6-7). Dysfunction of this structure causes various mental disorders, such as obsessive-compulsive disorder, depression and drug addiction (8-10).

GABA is one of the most common neurotransmitters in the CNS, and it plays a key role in motion processes, neurogenesis and the development of tissue. Almost 95% of neurons in the nucleus accumbens are GABAergic neurons, and the remainder are cholinergic interneurons. The GABAergic neurons of the nucleus accumbens are identified as medium spiny neurons (11). GABA is the major inhibitory neurotransmitter in invertebrates and vertebrates. GABAergic neurons form the efferent output projections from the nucleus accumbens core and shell. Defects in the regulation of GABA homeostasis cause neurological disorders, such as epilepsy, Parkinson's disease, schizophrenia, anxiety disorders, autism, bipolar disorder and post-traumatic syndrome (12-18). Glutamate decarboxylase (GAD) catalyses the production of GABA molecules, which, in comparison with any other inhibitory neurotransmitters, are widespread in the vertebrate brain. There are two forms of GAD, GAD65 and GAD67, with enzymatic characteristics and a subcellular distribution stimulated by the two genes (19).

Using antiserum specific to GAD67 and monoclonal antibodies specific to GAD65, it was shown that the two forms of GAD differ in distribution. GAD67 is widely distributed in the soma and primary dendrites of neurons, while GAD65 is primarily distributed in the axon endings of neurons in the animal brain (20-22).

Generally, in the striatum, there are several specific morphological types of neurons, which are classified by the size of the cell body (large vs. medium) and the presence or absence of spines. The most common and well-studied type of neurons in mice and rats are GABAergic medium spiny neurons (23-24). Due to the small number of studies on the human nucleus accumbens and the enormous importance of GABAergic medium spiny neurons, this study aimed to investigate this cell population and classify it based on the shape and size of the cell bodies by using the immunohistochemical expression of GAD67.

MATERIAL AND METHODS

The study included 20 adult human brains of both genders (11 males and 9 females) between the ages of 20 and 75 years (average 36.7 ± 2.4). All of the brains were obtained within 12–18 hours after death. Only normal brains with no visible malformations and without any neuropathological changes or neuropsychiatric history were used. The brains were fixed in 10% neutral phosphate buffered formalin (3.7% formaldehyde) over a period of at least 3 months.

We investigated all parts of the nucleus accumbens and sliced the brain tissue in coronal sections rostrocaudally, where this nucleus merges without a clear border with the medial septal nucleus dorsomedially and ventrolaterally with the basal nucleus and substantia innominata.

The standard streptavidin-biotin-peroxidase complex method was used for the immunohistochemical analysis, according to the manufacturer's instructions (Dako, LSAB+® system). Serial sections 3-5 μm thick, obtained from paraffin tissue blocks of 40 hemispheres, were deparaffinized in xylene and rehydrated in graded alcohol, and the endogenous peroxidase activity was blocked. Antigen retrieval was achieved using a microwave oven at 750 W, 3 times for 6 minutes in a high pH citrate buffer.

Immunostaining of GAD67 was performed with a monoclonal anti-glutamic acid decarboxylase 67 antibody (clone K-87, purified mouse immunoglobulin, product number G5419, at a dilution of 1:500, SIGMA, USA). The slides were incubated in the primary antibody for 60 min, in a secondary antibody for 30 min and finally in streptavidin-HRP for 20 minutes. The visualization was performed with 3,3'-diaminobenzidine as the chromogen, and the slides were counterstained with Mayer's haematoxylin. The addition of all of the reagents, except the primary antibody, was used as the negative control.

A positive reaction for GAD67 was defined as a discrete or strong localization of the brown chromogen in the cytoplasm. The classification of the immunohistochemically impregnated neurons was performed according to the shape and size of the cell bodies and the number of impregnated primary dendrites. The morphometrical analysis involved the following two parameters: the maximal (Dmax) and minimal (Dmin) diameter of the pericarion. The criteria for the selection of the neurons for the analysis were a full appearance of the cell body and a clearly visible nucleus. The measurements of the neurons were performed using a Zeiss AxioVision 3.0.6. Three researchers assessed all of the slides independently. The results are presented as the mean ± standard deviation.

RESULTS

GAD67 expression in the nucleus accumbens was diffusely spread in a large number of neurons, their soma and their primary dendrites, with a granular appearance and a high intensity (Fig. 1). We were able to distinguish the fol-

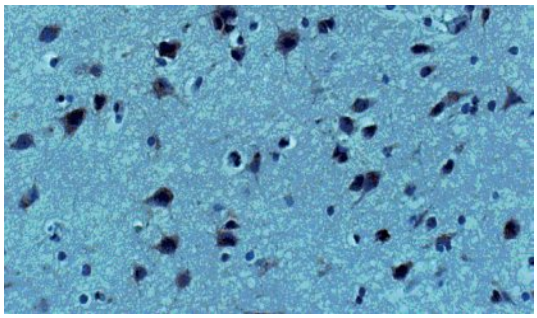

Fig. 1. e expression of GAD67 in medium spiny neurons of the human nucleus accumbens, x 200 magnification

lowing four types of human nucleus accumbens neurons according to the shape of the soma and the number of primary dendrites: fusiform neurons, fusiform neurons with lateral dendrites, pyramidal neurons and multipolar neurons.

Type I - Fusiform neurons

In the fusiform type of neurons, there was a strong immunoreactive expression of GAD67 in the elongated (spindle) soma, which had thick primary dendrites at both ends. The neuronal cytoplasm was filled with brown granules that partially covered the nucleus and showed a high intensity of GAD67 immunohistochemical expression. This type of neuron was observed in the shell of the nuclei. The dimensions of this type of soma are as follows: Dmax 23.02 ± 3.12 μm and Dmin 10.8 ± 0.94 μm (Fig. 2).

Type II - Fusiform neurons with lateral dendrites

This type of neuron was also detected in the shell. A thick lateral dendrite leaves the elongated (spindle) soma, usually in the middle part of the soma. The fusiform neuron with lateral dendrites showed a high intensity and expression of the GAD67 immunoreactive granules, which were extended to the beginning of the primary dendrite. The dimensions of the soma of this type of neuron are as follows: Dmax 22.51 ± 3.12 μm and Dmin 12.27 ± 0.8 μm (Fig. 3).

Type III – Pyramidal neurons

This type of neuron has a triangular soma, which varies from clearly pyramidal to an elongated pyramid. The pyramidal type of neuron is dominantly observed in the core of the nuclei. One of the dominant characteristics of this type of neuron is the presence of a strong apical dendrite, while the two basal dendrites are of a smaller diameter. The pyramidal type of neuron showed a high intensity and expression of the GAD67 immunoreactivity granules. The dimensions of this type of soma are as follows: Dmax 21.92 ± 3.2 μm and Dmin 14.28 ± 1.1 μm (Fig. 4).

Type IV – Multipolar neurons

The multipolar neuron was one of the most dominant medium spiny neurons of the nucleus accumbens. Approximately six (from 3-8) primary dendrites rose from the different soma forms. The multipolar type of neurons showed a high intensity expression of the GAD67 immu-

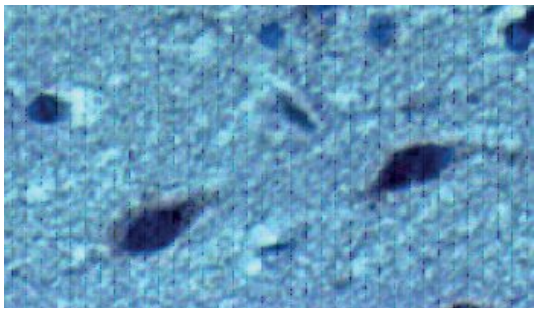

Fig. 2. e expression of GAD67 in fusiform medium spiny neurons of the human nucleus accumbens, x 400 magnification

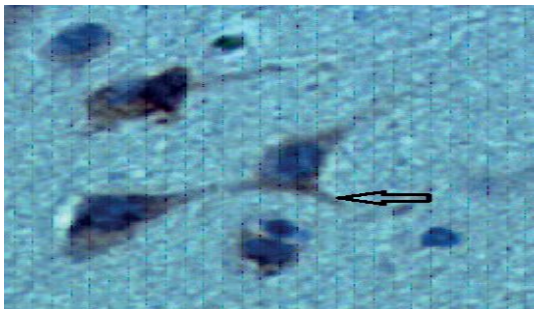

Fig. 3. e expression of GAD67 in fusiform medium spiny neurons with lateral dendrites of the human nucleus accumbens; the arrow indicates the lateral dendrite, x 400 magnification

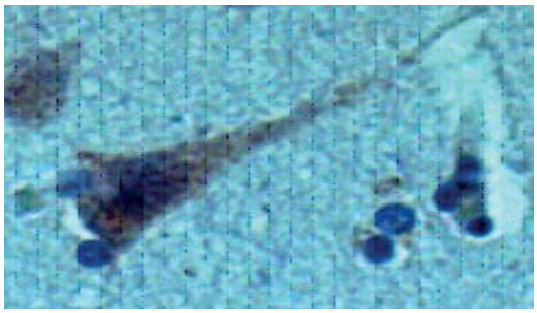

Fig. 4. e expression of GAD67 in pyramidal medium spiny neurons of the human nucleus accumbens, x 400 magnification

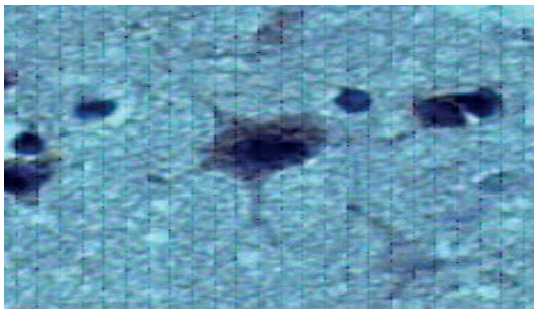

Fig. 5. e expression of GAD67 in multipolar medium spiny neurons of the human nucleus accumbens, x 400 magnification

noreactive granules. This type of neuron was predominantly registered in the core. The dimensions of this type of soma are as follows: Dmax 22.73 ± 3. 1 μm and Dmin 15.23 ± 1.3 μm (Fig. 5).

DISCUSSION

The nucleus accumbens is a critical brain region involved in many reward-related behaviours. This nucleus comprises major compartments, including the core and the shell, which encompass several subterritories. GABAergic medium spiny neurons constitute the output neurons of the nucleus accumbens core and shell (24).

Drugs of abuse exert potent molecular and cellular alterations in the nucleus accumbens, and many of these changes occur in the medium spiny neurons, the principal projection neurons of the nucleus accumbens, which account for 90–95% of all of the neurons in these regions (25).

Bolam et al. (26) used two methods, Golgi-Cox and immunohistochemistry, to identify two types of rat striatal medium spiny neurons, small and large. They found a strong GABAergic expression in the soma and primary dendrites in the major group of the medium spiny neurons.

In our study, we found 4 types of neurons that expressed GAD67 in the soma and, mainly, in the primary dendrites. In our previous morphological investigations (using the Golgi-Cox method), we found 4 types of medium spiny neurons in the human nucleus accumbens (3), which correlate to the immunohistochemical staining method used here.

The findings of animal studies of the GAD-immunoreactive neurons in the rat septum show that the soma of the medium spiny neurons had an oval, fusiform, pyramidal or multipolar shape. These types of neurons of the rat striatum (mostly medium spiny) show an intense expression of GAD67 that is localized in the soma and primary dendrites (27-30).

Trifonov et al (31) also found expression of GAD67 predominantly in the medium spiny neurons of the striatal region in rodents that were mainly of an oval soma shape.

Investigating the GABAergic expression of the rat striatal medium spiny neurons, Cuzon Carlson at al (32) found two morphological types. The first type had an oval soma shape, and the second type had a fusiform soma shape. The oval soma shape could be related to our type III and type IV neurons, while the fusiform neurons from their study could correlate with type I and type II neurons described in our study.

CONCLUSION

In our previous studies, we investigated the morphology of the medium spiny neurons of the human nucleus accumbens (soma shape, dendritic arborization, spines, axonal orientation, and diameter) using the Golgi-Cox method (3). Furthermore, we were interested in the morphology of the GABAergic medium spiny neurons based on the immunohistochemical expression of GAD67, an enzyme important in the synthesis of this major inhibitory neurotransmitter. Four types of medium spiny neurons of the human nucleus accumbens (fusiform, fusiform with lateral dendrites, pyramidal and multipolar), localized in both compartments of the nucleus core and shell, could be distinguished, and they morphologically correlate with those described by the Golgi study.

The significance of this study is to gain better insight into the basic morphology of the human nucleus accumbens, which completes the morphological and immunohistochemical profile of the limbic system.

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THE EFFECTS OF HIGH DOSES OF NANDROLONE DECANOATE ON CARDIAC MUSCLE TISSUE

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EFEKTI VISOKIH DOZA NANDROLON DEKANOATA NA SRČANO MIŠIĆNO TKIVO

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ABSTRACT

In recent decades, steroid abuse has become very popular and widespread among professional and recreational athletes. e aim of this study was to examine the chronic e ects of training combined with high doses of nandrolone decanoate on cardiac muscle tissue. e study included 32 Wistar albino rats divided into 4 groups: control (T-N-), steroid (T-N+), exercise-training (T+N-) and exercise plus steroid (T+N+) groups. e T+N- and T+N+ group swam for 4 weeks, 1 hour per day, 5 days per week. e N+ (nandrolone positive groups) received nandrolone decanoate (20 mg/kg) once per week, subcutaneously. After 4 weeks of training, the rats were sacrificed. Heart biopsy specimens were routinely fixed and embedded in para n. Five-micrometre thick sections were stained with haematoxylin and eosin (H/E) and Masson-Trichrome dyes. Captured microscopic images were processed by special software for image analysis to quantify results. Our results showed that the combination of nandrolone and training causes left ventricular wall thickening of 30%. Average cardiac muscle cell longitudinal diameter was increased by 6% in the T-N+ group, by 16% in the T+N- group and by 25% in the T+N+ group. e cross sectional muscle cell area was increased in the T+N+ group by 33%. Heart collagen content was increased in the nandrolone group compared to the control group by 261%. Collagen content was decreased in the T+N+ group by 34%. High doses of AAS induced left ventricle hypertrophy and excessive heart collagen deposition.

Keywords: *nandrolone decanoate, high doses, exercise, heart muscle*

SAŽETAK

Poslednjih nekoliko decenija, zloupotreba AAS je postala veoma popularna i široko rasprostranjena među rekreativnim i profesionalnim sportistima. Cilj istrazivanja je da pokaže hronične efekte treninga u kombinaciji sa visokim dozama nandrolon dekanoata na srčano mišićno tkivo. U studiju je ukljceno 32 mužjaka pacova, Wistar albino soja, podeljenih u 4 grupe: kontrola (T-N-), grupa na steroidima (T-N+), grupa koja trenira (T+N-) i grupa koja trenira i prima steroide (T+N+). Grupe T+N- i T+N+, plivale su 4 nedelje, 1h dnevno, 5 dana u nedelji. Grupe koje primaju steroide (N+), dobijale su jednom nedeljno nandolon u dozi od 20 mg/kg, subkutano. Nakon četvoronedeljnog treninga životinje su žrtvovane. Kompletna srca su rutinski fiksirana u ukalupljena u parafinske blokove. Preseci debljine 5 µm bojeni su H/E i Mason Trihrom tehnikom. Mikrofotografije dobijenih preparata su obrađivanje specijalizovanim softverom za analizu slike u cilju kvantifikacije rezultata. Naši rezultati su pokazali da kombinacija treninga i nandrolona izaziva zadebljanje zida leve komore za 30%. Srednji dijametar duznog preseka srčanog mišića se povećao za 6% u T-N+ grupi, za 16% u T+N- i za 25% u T+N+ grupi. Poprečni presek je povećani za 33% u T+N+ grupi. Depoziti kolagena u srcu bili su povecani u grupi T-N+ za 261% u poređenju sa kontrolnom grupom. Depoziti kolagena bili su sniženi u T+N+ grupi za 34%. Visoke doze AAS izazivaju hipertrofiju zida leve komore i prekomerno deponovanje kolagena.

Ključne reči: *Nandrolon, visoke doze, trening, srčani mišić.*

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INTRODUCTION

Anabolic androgenic steroids (AAS), synthetic derivatives of testosterone, were created as an attempt to synthesize a testosterone-like steroid that would have strong anabolic effects (1). Because of these anabolic effects, AAS became very popular among professional and recreational athletes, even though they were banned in many organized sports (2, 3). The hormone is most frequently used among weightlifters, bodybuilders, swimmers and cyclists to improve their physical performance (4), strength and muscle mass (5). However, these individuals may take doses of up to 100 time higher doses than the therapeutic range, which leads to severe adverse effects on many organ systems (6).

Nandrolone is a derivate of 19-nortestosterone and is most often administered in the form of depot preparations. Today, it is the one of the most popular AAS among athletes. It has a stronger anabolic effect than testosterone, but with long-term usage (7), severe adverse effects such as structural and functional alterations of the liver and even hepatocellular adenoma can be detected (8). Furthermore, chronic abuse of AAS can lead to pathological left ventricular hypertrophy via androgenic receptors (9) located in the cytoplasmic compartment of the cardiac muscle cells (10, 11), as well as abnormalities of impulse conduction and contractility (12, 13). Studies have shown that abuse of AAS can induce hypertrophy of the left ventricle with disproportionate accumulation of extracellular collagen and interstitial fibrosis (14-17). Previous reports have further shown that chronic administration of supraphysiological doses of AAS can lead to increased interventricular septum thickness, dilated cardiomyopathy, arrhythmia, heart failure and sudden cardiac death (9, 14, 18). On the other hand, exercise and endurance training are known to induce significant changes in the morphology of the heart, including increased left ventricular chamber size, wall thickness and increased mass of the heart, known as an "athlete's heart" (19). This is a balanced and reversible physiological modification that is followed by necessary neovascularization because muscle hypertrophy occurs as a result of the adaptation of the cardiac muscle to increased physical activity (20).

A review of available literature showed that majority of the studies aimed at revealing the effects of AAS on cardiac muscle tissue (with or without training) were designed to investigate the influence of supraphysiological doses (up to 10 mg/kg) of steroids on the heart (5, 14, 17, 21). However, considering that some individuals, in attempt to rapidly increase their muscle mass and performance, are taking up to 20 mg/kg of AAS, we wanted to gain a better understanding of the effects on cardiac muscle tissue morphology of high-dose nandrolone decanoate administered either alone or in combination with training over a short-term interval.

MATERIAL AND METHODS

Experimental animals

This study included 32 male Wistar albino rats (10 weeks old, weighing 220-280 g). Rats were housed in collective cages (eight rats per cage). Food and water were provided *ad libitum*. The room temperature was kept at 25±1°C with 12-h alternating light and dark cycles. The rats were randomly divided into four groups: T-N- (sedentary rats with no administration of nandrolone decanoate or exercise training; control group), T-N+ (sedentary rats with weekly administration of nandrolone decanoate (depot preparation, 20 mg/kg s.c. during a period of 4 weeks), T+N- (exercising rats (swimming 1 h/day) with no administration of nandrolone decanoate) and T+N+ (exercising rats (swimming training 1 h/day) with weekly administration of nandrolone decanoate depot preparation 20 mg/kg s.c. during a period of 4 weeks). Over the course of 4 weeks, the steroid and exercise-training plus steroid group received nandrolone decanoate (DECA DURABOLIN ®, Organon, Holland) administered by subcutaneous injection once per week in doses of 20 mg/kg. The initial and final body weights (BW) and heart weight (HW) were measured. During the 4 weeks, rats were swimming in a pool of 120 x 80 x 50-cm length/depth/width) for 1 hour per day, 5 days per week. The swimming was performed every exercise day starting at 9 a.m. The water temperature was 37 °C. After the experimental period, the rats were sacrificed. After short-term anaesthesia (ketamine 100 mg/kg and xilazid 10 mg/kg), the animals were premedicated with heparin as an anticoagulant and were sacrificed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986 UK), and their hearts were surgically removed for histological examination. This protocol was approved by the Ethics committee, Faculty of Medical Science, University of Kragujevac.

Histological and image analysis

The isolated rat hearts were halved so that the left and right ventricular walls were fully exposed. The organs were fixed in 4% formalin, routinely processed and embedded in paraffin. Sections, 5 µm thin, were stained with H/E, for the visualization of tissue structures, and with Masson-Trichrome dye for collagen detection and quantification. Images of tissue sections were captured with a digital camera attached to the Olympus BX51 microscope. Morphometric analysis was performed by calibrated Axiovision software (Zeiss, USA), as well as with Image Pro-Plus (Media Cybernetics, USA). All measurements were made in triplicate and average values were considered exact. From each half of the heart, 100 serial tissue sections were made. Odd sections were stained with H/E, and even ones were stained with Masson-Trichrome. Cross-sectional area, longitudinal-section diameter measurement of the cardiac muscle cells and collagen segmentation were performed

Table 1. Body and heart weight measurements presented as the mean ±SD

	Initial body weight (g)	Final body weight (g)	Heart weight (g)	BW to HW ratio
T-N-	252±22.57	438±35.04	1.170±0.05	374:1
T-N+	248±37.04	401±23.12	1.132±0.03	354:1
T+N-	258±29.54	406±30.55	1.203±0.04	337:1
T+N+	270±46.77	416±49.44	1.334±0.09	312:1

on 10 tissue sections for each specimen. Collagen quantification and image segmentation was done by two independent researchers, and mean values were considered exact.

Statistical Analysis

The ANOVA test was used for statistical comparison of data. P values $p<0.05$ were considered statistically significant. All statistical calculations were made with the SPSS computer program, version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as the means ± standard deviations (SD).

RESULTS

Body weight (BW) and heart weight (HW) measurement

After the four-week experimental period, we found an increase in body weight compared to initial weight in all groups. The largest increase in body weight in the control group was 73%, versus 55% in the T-N+ group, 57% in the T+N- group and 54% in T+N+ group (Table 1). When BW to HW ratio was calculated at the end of the experiment, results showed that the lowest BW/HW values occurred in groups where animals were exposed to training (with or without nandrolone administration), suggesting that training rather than nandrolone was the most influential factor (Table 1).

Heart morphometry

During the experiment, morphometry analysis was performed to estimate the effects of training and/or nandrolone administration on left ventricular wall thickness, heart muscle cell size and collagen content in the connective tissue of the heart.

Our findings showed that both nandrolone and exercise affected the left ventricular wall. Increased thickness was observed in all experimental groups, and the percentage of thickening ranged from 6% (only nandrolone administration) to almost 30% (nandrolone and training together) compared to the control group (Table 2). Exercise only also led to a greater degree of wall thickening (16%) than that induced by nandrolone administration alone (Fig 1A). Low-magnification overview of the left ventricular wall revealed that the thickening almost exclusively involved myocardium and did not significantly affect the other layers of the heart. Considering that the myocardium is mostly composed of contractile heart muscle cells, our results, unsurprisingly, showed a marked change in their diameter. Both longitudinal section diameter (LD) and cross-section area (CSA) were increased in all experimental groups compared to the control group. The group receiving nandrolone administration only revealed increases of 6% in LD and 13% in CSA, whereas the group receiving nandrolone and training together revealed increases of 25% and 33% (LD and CSA, respectively) compared to the control group (Table 1). Training alone induced increases in the size of heart muscle cells of 21% (LD) and 23% (CSA) (Fig 1b and 1c). There was a similar tendency in the results of left ventricular thickening, with average values positioned just between those of the T-N+ group and T+N+ group.

Collagen content

Results related to the collagen content of heart connective tissue showed that the strongest deposition was observed in the hearts of the animals that were exposed to nandrolone administration without training (261% increase related to the control group). On the contrary, training alone and training combined with nandrolone administration caused a decrease in heart collagen content compared to the control group (45% for the T+N- group and 34 % for the T+N+ group) (Table 2) (Fig. 1d). It was therefore clear that training alone induced the most significant decrease in collagen content of the heart.

DISCUSSION

Anabolic androgenic steroids (AAS), although banned in sports, are still widely used by professional and recreational athletes who intend to quickly gain muscle mass and improve cardiovascular system performance. In the available

Table 2. Measurement of morphometric parameters and heart collagen content, mean ±SD

	Left ventricle tickness (μM^2)	Longitudinal section diametar (μM)	Cross-over area (μM^2)	Collagen content (%)
T-N-	1704.306±180.309	15±2.02	152±17.074	0.8±0.201
T-N+	1807.5±200.904	16±1.87	175±19.802	2.02±0.159
T+N-	2011.25±250.633	19±3.27	197±20.861	0.39±0.203
T+N+	2385±300.985	20±2.66	225±22.268	0.53±0.194

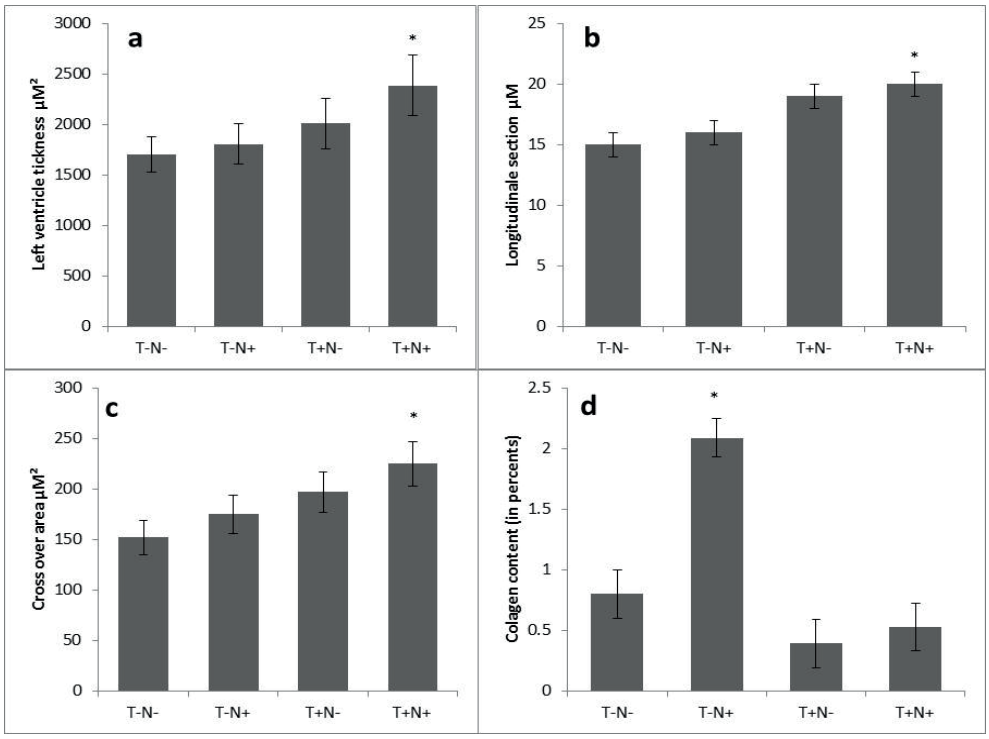

Figure 1. Values of left ventricle morphometric parameters after four weeks of study: a) Left ventricle thickness, b) Longitudinal section, c) Cross-sectional area, d) Collagen content. Each value is represented as the mean ±SD. (*, p<0,05)

literature, there is a plethora of information concerning the effects of chronic AAS abuse on the function and structure of numerous organs (8, 10, 11, 16). Similarly, researchers have shown that steroid abuse also induces changes in heart structure, ventricular thickness and size (4, 11, 15, 22, 23), as well as heart connective tissue content (5, 15, 17). Our results are in accordance with previous findings, as nandrolone, and especially nandrolone with training, induced significant increases of left ventricular thickness. We demonstrated that the effect of left ventricular hypertrophy occurring in animals exposed to nandrolone and training was 32% stronger than that in animals treated with nandrolone alone, and 40% stronger than in the control group. Furthermore researchers agree that steroids with or without training affect ventricular thickness in a dose-dependent manner, but in their studies, the most frequently investigated effects were the effects of supraphysiological doses of nandrolone (from 1-10 mg/kg per week) on the heart (24). However, due to dissimilarity of the administered doses of AAS, training processes and the durations of experimental treatments in various studies, it is difficult to draw a solid conclusion about the true effect of specific doses of steroids on heart tissue (5, 11, 14, 15, 17). We demonstrated that high doses of steroids (up to 20 mg/kg per week) as used in our study (many times higher than in some studies) caused thickening of the heart wall, but the degree of this change was not significantly higher than in earlier studies (5, 11, 14, 15, 17). This means simply that the administration of steroids during training affects the heart in dose-dependent manner, but limits exists when doses of 20 mg/kg or similar are reached. This is significant in our opinion because athletes who use steroids must be aware that higher doses of steroids or their derivatives will not improve the performance of cardiovascular system. Considering that the majority of the heart wall is composed of cardiac muscle cells, our findings related to the measurement of cell diameter confirm these findings. Unfortunately, we were unable to find sufficient data to compare the direct effects of various doses of steroids on cardiac myocyte size. A study published by Ren et al (2012) showed that steroids alone can induce cardiomyocyte hypertrophy (up to 35% at 100nM of dexamethasone) a dose much higher than that used in our study (15% increase with nandrolone alone). The divergence may be the result of different study design (e.g., their study was performed in cell culture conditions); therefore, the doses of the hormones may not be comparable. Nevertheless, the increases in cardiomyocyte size observed in our experiment were consistent with the ventricular wall thickening also observed. This was particularly obvious when the cross-area of the heart muscle cells was measured.

Another adverse effect of nandrolone abuse is increased heart collagen content. Our results showed that the most significant collagen deposition between cardiac myocytes was observed in the animals that were treated with nandrolone alone. Furthermore, the lowest collagen content was observed in the hearts of the animals that were exposed to training alone or in animals exposed to both nandrolone and training. This proves that training is the most significant way to reduce the collagen-related adverse effects of steroids. These results coincide with the findings of previous studies (5) but are in conflict with the findings of Tano et al (2011), who stated that the collagen content is highest in hearts of animals that were exposed to combined nandrolone and training. On the other hand, our results are in accordance with those of Franquni et al (2013). They found that nandrolone alone, administered in the same dose as in our study but twice a week, caused a

10-fold increase of heart collagen. The increase in heart collagen content observed in our study among animals exposed only to nandrolone was 2.5-fold compared to controls (261% increase). These significant differences in results only show that further investigation is needed on the effects of AAS on heart collagen deposition. Nevertheless, our findings and those of Franquni et al lead us to the conclusion that dose-dependent heart collagen deposition is not limited, even at the highest doses. In contrast to the nandrolone effect, our study showed that training induces a decrease in collagen content of the heart. Similar findings were reported by other researchers (5), which demonstrates that exercise can minimize the adverse collagen deposition effect of steroids.

In general, our study demonstrates that nandrolone abuse has serious adverse effects on heart morphology. High doses of steroids, used by some misinformed athletes during the training process, induces significant collagen deposition and heart fibrosis, but does not significantly increase left ventricular thickness compared to lower supraphysiological doses.

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EFFECTS OF DIVALENT CATIONS ON OUTWARD POTASSIUM CURRENTS IN LEECH RETZIUS NERVE CELLS

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EFEKTI DVOVALENTNIH KATJONA NA IZLAZNE KALIJUMSKE STRUJE RETZIUSOVIH NERVNIH ĆELIJA PIJAVICE

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ABSTRACT

e present study examines the e ects of divalent metals, cadmium (Cd^{2+}) and manganese (Mn^{2+}), on the outward potassium currents of Retzius cells in the hirudinid leeches Haemopis sanguisuga using conventional two-microelectrode voltage-clamp techniques. e outward potassium current is activated by depolarization and plays an important role in determining both the neuronal excitability and action potential duration. A strong inhibition of the fast current and a clear reduction in the late currents of the outward current with 1 mM Cd^{2+} were obtained, which indicated that both components are sensitive to this metal. Complete blockage of the fast and partial reduction of the slow outward currents was observed after adding 1 mM Mn^{2+} to the extracellular fluid. ese data show that the outward K^+ current in leech Retzius nerve cells comprises at least two components: a voltage-dependent K^+ current and a Ca^{2+}-activated K^+ current. ese observations also indicate that Cd^{2+} is more e ective than Mn^{2+} in blocking ion flow through these channels and that suppressing Ca^{2+}-activated K^+ outward currents can prolong the action potential in nerve cells.

Key word: *Retzius neuron, cadmium, manganese, outward potassium currents*

SAŽETAK

Ispitivana su dejstva dvovalentnih katjona, kadmijuma (Cd^{2+}) i mangana (Mn^{2+}) na izlazne ispravljačke kalijumske struje Retziusovih nervnih ćelija pijavice, Haemopis sanguisuga, tehnikom nametnutog napona sa dve mikroelektrode. Depolarizacijom izazvane izlazne kalijumske struje imaju značajnu ulogu u determinaciji nadražljivosti nervnih ćelija kao i trajanja akcionih potencijala. 1 mM Cd^{2+} je izazvao blokadu brze i redukciju kasne komponente izlazne kalijumske struje, što ukazuje da su obe komponente senzitivne na Cd^{2+}. Drugi dvovalentni katjon, 1 mM Mn^{2+} je doveo do kompletne blokade brze i delimične inhibicije spore komponente izlazne kalijumske struje. Rezultati ovog istraživanja ukazuju da su izlazne kalijumske struje u Retziusovim nervnim ćelijama pijavice sastavljene od najmanje dve komponente: naponsko-zavisne i kalcijumsko-aktivisane kalijumske struje. Rezultati studije potvrđuju da je Cd^{2+} efikasniji blokator izlaznih kalijumskih kanala od Mn^{2+}, kao i da inhibicija kalcijumsko-aktivisanih kalijumskih struja može biti odgovorna za prolongiranje trajanja akcionih potencijala.

Ključne reči: *Retziusov neuron, kadmijum, mangan, izlazne kalijumske struje.*

INTRODUCTION

Divalent cations (such as Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+}) are known as nonspecific calcium (Ca^{2+}) channel antagonists that block Ca^{2+} channels to varying degrees. Additionally, divalent metals are among the classical tools that have been used to investigate the activation and deactivation kinetics and the permeation of the potassium (K^+) current (1). Cadmium (Cd^{2+}) is a nonessential divalent metal ion that can cause cytotoxicity in multiple organs, including the brain. Recently, a great amount of scientific data has been used in attempts to define the mechanism of action of Cd^{2+} in brain. However, the mechanisms underlying Cd^{2+} neurotoxicity remain not entirely understood. Multiple scientific studies have shown that the toxic effects of Cd^{2+} on the nervous system are manifold. For example, Lopez et al (2) demonstrated that in cultured cortical neurons, a low concentration of Cd^{2+} (100 nM) induced apop-

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tosis, whereas higher concentrations (100 mM) produced necrotic cell death. More recent studies (3) have reported that Cd^{2+} toxicity in cerebral cortical neurons is mediated by intracellular Ca^{2+} elevation, which triggers the activation of the apoptotic signalling pathway in mitochondria. Additionally, the cellular toxicity generated by Cd^{2+} is due, in part, to the generation of reactive oxygen species (ROS) that depolarize the mitochondrial membrane potential and decrease the ATP levels (4).

Some novel studies have revealed the endoplasmic reticulum to be the cellular target of Cd^{2+} toxicity. The Cd^{2+}-induced release of Ca^{2+} from endoplasmic reticulum occurs via the inositol trisphosphate (IP_3) pathway (5). In contrast, Cd^{2+} inhibition of the electron transport chain in mitochondria generates reactive oxygen species (ROS) and activates caspase-9. These pathways appear to be simultaneously activated, and their synergistic activation can promote apoptosis through the production of ROS and Ca^{2+}-mitochondria signalling (6). According to Smith et al. (7), Cd^{2+} rapidly increases IP_3, which is known to mobilize stored Ca^{2+}. Furthermore, they showed that Cd^{2+} and other divalent metals increased IP_3 and mobilized intracellular Ca^{2+}. Yang et al (8) demonstrated that Cd^{2+} induces necrotic cell death by increasing both the intracellular Ca^{2+} concentration and the ROS level. Experimental studies have shown that Cd^{2+}-induced apoptosis is mediated by the Ca^{2+} signalling pathway and that Ca^{2+}-mediated apoptosis occurs through the mitochondria-caspase signalling pathway (3). Recent studies have reported that, similar to other toxic metals, Cd^{2+} impairs neurogenesis and physiological signal transduction (9). Additionally, evidence is growing that Cd^{2+} exposure can alter gene expression and cause an epigenetic effect. One possible mechanistic pathway for Cd^{2+}-induced toxicity is through the modification of hormone levels by affecting the hypothalamic-pituitary-gonadal axis (10, 11).

In contrast to Cd^{2+}, which is a nonessential metal, manganese (Mn^{2+}) is an essential trace element that is ubiquitous and pivotal for normal cell function and metabolism. Nevertheless, excessive accumulation of Mn^{2+} in the brain may lead to a condition known as manganism, a neurodegenerative disorder associated with dysfunctions in the basal ganglia that causes parkinsonian-like symptoms (12-14). Recent studies have reported that Mn^{2+}-induced neurotoxicity is mediated, at least in part, by the generation of ROS, depletion of antioxidant defence mechanisms and mitochondrial dysfunction (15).

The purpose of this study was to investigate the components of the somatic outward current and determine the relationship between the Ca^{2+} and voltage-dependent K^+ currents on the basis of ion-substitution experiments. According to the activation and deactivation kinetics, the Ca^{2+}-activated K^+ current could be separated into two distinct components: a fast transient and a slow current. The leech served as an appropriate model for studying ion channels because of the extreme structural simplicity of its nervous system.

MATERIALS AND METHODS

Experimental animals

The experiments were performed utilizing *Retzius* nerve cells from the first ten abdominal ganglia of the leech, *Haemopis sanguisuga* (commonly known as the horse-leech). The dissection method was similar to that described previously (17, 18). The 21 segmental ganglia contain ~400 neurons arranged in six packets. Retzius neurons are the largest cells (~60 μm diameter of the soma) located on the ventral side of the ganglia. They were identified by their large cell bodies, position and firing properties (17, 19). Isolated ganglia of the Retzius neurons have resting potentials that range from - 40 to -60 mV and amplitude action potentials that range from 30 to 50 mV, and they fire spontaneously at a slow rate (0.2-3 APs/sec).

Electrophysiological technique

All electrophysiological recordings were made at room temperature (20-24°C) under two-electrode voltage-clamp conditions. The electrical arrangement for voltage clamping utilized was based on the theoretical principles developed for different cellular membranes (20). Retzius neurons were impaled with two electrolyte-filled microelectrodes to record membrane potential and to perform the current injection. For the intracellular recordings, we used glass micropipettes pulled from borosilicate glass (1.5 mm outside diameter, 0.6 mm inside diameter, Clark Electromedical Instruments, Edenbridge, UK) filled with 3 M KC1 (resistance ~ 20 MΩ). Electrodes were connected to a voltage-clamp amplifier (Bioelectric Instrument model DS2C). The bath was grounded *via* an agar bridge. Command pulses were derived from a Tektronix 161 pulse generator. Voltage and current recordings were displaced on a Tektronix 564 oscilloscope. Output signals were digitized by an A/D converter (Axon Instruments, Jakarta, Indonesia) and were saved before being analysed on a computer running in-house acquisition software.

Briefly, the difference between the membrane potential and the command pulse was amplified, and the resulting voltage was used to drive the current across the membrane via the microelectrode. The membrane potential was measured between the second electrode and the ground. In the voltage-clamp recording mode, depolarization from a holding potential of -70 mV elicited outward currents that increased to steady-state values.

Solutions

During the experiment, isolated ganglia were superfused, initially with normal leech saline of the following composition (in mM): 115 NaCl, 4 KCI, 2 $CaCl_2$, 1.2 Na_2HPO_4, 0.3 NaH_2PO_4 (pH 7.2). The sodium-free Ringer contained 115 mM TRIS-Cl (Tris Ringer) instead of NaCl and phosphate buffer. $CdCl_2$ (1 mM) and $MnCl_2$ (1 mM) were

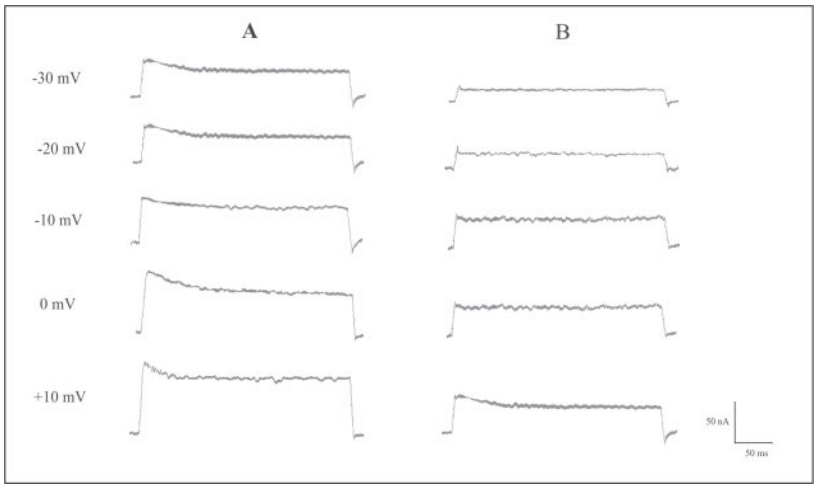

Figure 1. Representative current recordings obtained under long-lasting stimulation (300 ms) in Tris Ringer (A) and at 10 min after adding 1 mM $CdCl_2$ to the Tris Ringer (B) during the displacement of the holding potential from -70 mV to the potential given at each trace.

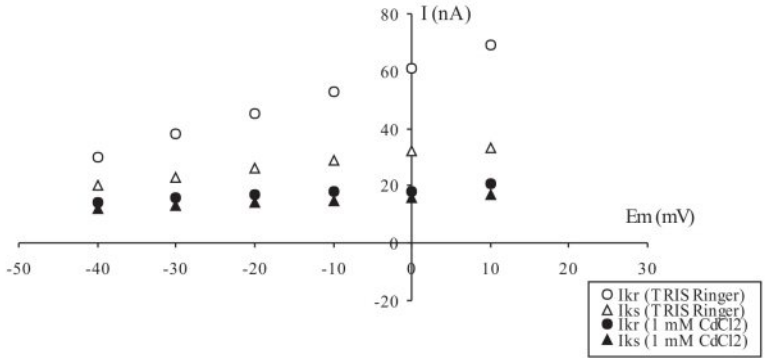

Figure 2. e current-voltage relationship at the peak of the outward K^+ current in the absence (open symbols) and presence (solid symbols) of 1 mM $CdCl_2$.
Ikr- rapid outward K^+ current; Iks-slow outward K^+ current.

used as blockers of outward potassium channels and added to the normal or Tris Ringer solution. $CdCl_2$ and $MnCl_2$ were purchased from Sigma-Aldrich (St. Louis, MO, USA). The bath volume was 2 ml, and the solution changes were completed within 30 sec.

Statistical analysis

Statistical analysis was performed using Student's t-test for paired correlated samples. Currents (in nA) in the presence and absence of $CdCl_2$ and $MnCl_2$ were compared for each Retzius nerve cell.

RESULTS

The effect of Cd^{2+} on the outward K^+ current of leech *Retzius* nerve cells

In the first series of experiments, we examined the effect of the Ca^{2+} channel blocker, Cd^{2+} (1 mM), on the membrane K^+ current in Retzius neurons. The outward K^+ currents, which contribute to the resting membrane potential and repolarization of the action potential, were studied in voltage-clamped leech Retzius neurons. The K^+ current was activated by depolarization with the sodium-free Ringer (TRIS Ringer) with long-lasting stimulation (300 ms) by an activated outward current. From -70 mV, depolarizing steps to a potential of approximately -40 mV elicited a fast outward current. This transient part of the total outward current had rapid activation kinetics and inactivated within 50 ms. Higher depolarization produced a larger current and a slow outward current that showed little inactivation. The measurements of the outward K^+ currents (in nA) in the control condition (TRIS Ringer) were compared with those in the Cd^{2+} condition for each Retzius cell.

The voltage-clamp experiments demonstrated that Cd^{2+} (1 mM) reduced both types of outward currents, with different efficacies. The fast outward current, activated by small depolarizations (i.e., at -35 mV), was more sensitive to Cd^{2+} than was the slow part of the outward current activated by larger depolarizations. Typical current-voltage records were obtained upon replacing sodium with Tris (A) and at 10 min after adding 1 mM Cd^{2+} (B) to Tris Ringer after displacing the membrane potential from a resting level of -40 mV, as shown in Fig. 1. A strong inhibition of the fast current and an evident reduction in the late currents of the outward current with 1 mM Cd^{2+} were observed, which indicated that both components are sensitive to this metal.

To investigate the relationship between the change in the outward potassium current with the change in voltage (I–V), the membrane potential was increased from a holding potential of -70 mV to five potentials, ranging from -40 to +10 mV in 10 mV increments (Fig. 2). In the presence

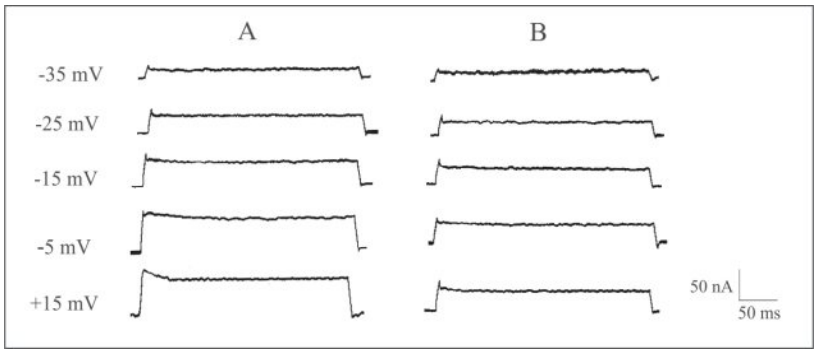

Figure 3. Patterns of voltage clamp current recordings in a leech Retzius nerve cell in Tris Ringer (A) and at 10 min after adding 1 mM $MnCl_2$ (B). Potentials were increased from the holding potential (-70 mV) to the potential given on each trace.

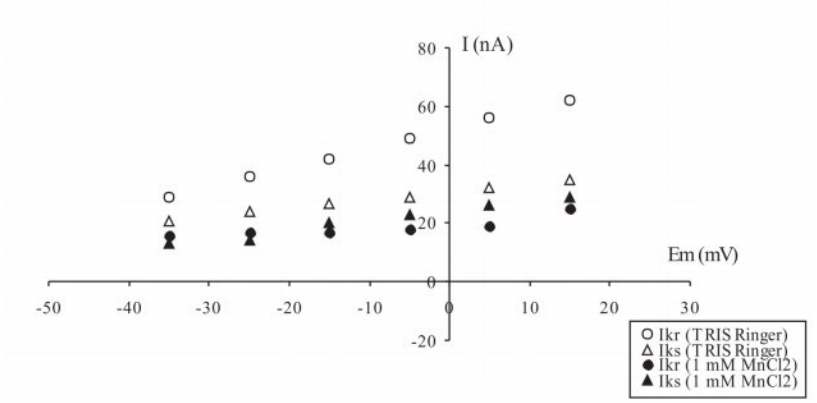

Figure 4. e current-voltage relationship at the peak of the outward K^+ current in the absence (open symbols) and presence (solid symbols) of 1 mM $MnCl_2$.
Ikr- rapid outward K^+ current; Iks-slow outward K^+ current.

of 1 mM Cd^{2+}, at the test potential of +10 mV, the fast and slow parts of the K^+ outward current were reduced from 69 to 21 nA (69.57%) and from 33 to 17 nA (48.49%), respectively.

The effect of Mn^{2+} on the outward K^+ current of leech Retzius nerve cells

To determine the total outward current in terms of their voltage and calcium-dependent components, experiments using another Ca^{2+} channel blocker, Mn^{2+}, were performed.

In contrast to the previous experiments with Cd^{2+}, Mn^{2+} was effective only in blocking the fast but not the late outward current. Voltage-clamp experiments were performed on freshly dissociated Retzius neurons in the TRIS Ringer (A), and at 10 min after adding Mn^{2+} (1 mM) to the Tris Ringer solution (B). Typical membrane current pattern curves that were produced by increasing the depolarization from a steady holding level of -70 mV are shown in Fig 3. Clearly, both components were affected, and the fast transient part was completely reduced.

Figure 4 shows the corresponding current-voltage (I-V) relationship that was obtained with 1 mM Mn^{2+}. In the presence of Mn^{2+}, at the test potential of +15 mV, the fast and slow parts of the K^+ outward current were reduced from 62 to 25 nA (59.68%) and from 35 to 29 nA (17.15%), respectively. The data obtained with Mn^{2+} support the view that both the fast and partially slowed outward current are Ca^{2+} activated.

DISCUSSION

The results reported in this paper show that the outward K^+ current in leech Retzius nerve cells is composed of two distinct components: a voltage-dependent current and a Ca^{2+}-activated K^+ current. The Ca^{2+}-activated K^+ current plays a key role in regulating neuronal excitability. In this study, the addition of inorganic Ca^{2+} channel blockers (Cd^{2+} or Mn^{2+}) was used to demonstrate the dependence of Ca^{2+} on outward K^+ currents.

Recent, much of our knowledge of membrane transporters has been acquired by studying the interaction of blocking agents and ionic channels. The voltage dependent conductance of electrically excitable membranes has been extensively studied by several different approaches. The classic study by Hodgkin and Huxley (16) led to a conclusion on the existence of two independent membrane conductance: early Na^+ and late K^+ conductance. However, the conductances of other tissues appeared to be more numerous than those found in the squid giant axon. In spite of the difficulties in separation, several outward currents have been identified, such as the slow and fast K^+ currents and Ca^{2+}-activated K^+ currents.

Leech ganglion cells have been recognized as a promising model system for electrophysiological studies because the size and accessibility of these neurons allow them to be readily implanted with several microelectrodes. In leech Retzius nerve cells, Ca^{2+} activated K^+ currents are large outward K^+ currents (17, 18). Electro-

physiological studies have shown that membrane transport proteins are susceptible to divalent cations (such as Cd^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+}). To date, most research has focused on the toxic effects of Cd^{2+} on the brain, with only a few experimental studies on the effects of Mn^{2+} on outward K^+ currents. Although the molecular mechanisms of action of Cd^{2+} on ion transporters are not fully understood, several hypotheses have been proposed. One possibility is that Cd^{2+} may mimic other metals (and elements) at the site of the membrane transporters or channels in the nerve cell membrane (21). Several lines of evidence suggest that Cd^{2+} is transported into neurons and other cells via transporters for naturally occurring cations, such as Ca^{2+} ("ionic mimicry") (21, 22). Zalups and Ahmad (22) proposed the mechanism of Cd^{2+} transport into a cell. This uptake has been recently proposed to occur through a mechanism of ionic mimicry, whereby Cd^{2+} mimics the divalent cation species of one or more of these nutritive metals at the binding site of one or more carrier proteins and/or channels that transport these metals. One possible pathway for Cd^{2+} entry into a cell is via Ca^{2+} channels in the plasma membrane (including both voltage-gated and receptor-dependent Ca^{2+} channels), thus leading to the accumulation of Cd^{2+} in the cell and, finally, the induction of caspase-12-mediated apoptosis (5).

In recent years, new electrophysiological studies have indicated that Cd^{2+} may interact with ion channels and transporters. Considering nerve cell function, Cd^{2+} can attack ion channels either directly or indirectly by disrupting the physiological signal cascades (5, 23, 24). Cd^{2+} is a well-known specific blocker of Ca^{2+} channels and inhibits Ca^{2+} cellular uptake. Electrophysiological investigations in various experimental models have shown that Cd^{2+} is commonly used to block voltage-dependent Ca^{2+} currents (25) and Ca^{2+}-activated K^+ currents (26-29). In rat pyramidal neurons (26), 200 µM cadmium was found to markedly reduce the early and late components of Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). Cd^{2+} is classically considered a Ca^{2+} channel blocker, but previous studies have demonstrated that Cd^{2+} (200 µM) does not affect the depolarization-activated outward current (27). However, Sah et al. (30) demonstrated that Cd^{2+} at concentrations of 0.1-0.5 mM had no effect on the K^+ current of guinea pig neurons, whereas at higher concentrations (2 mM), it induced a reduction of the outward current. In the present study, we found that Cd^{2+} significantly affected the fast and late membrane currents. In contrast, Mn^{2+} blocked the fast transient outward current in leech Retzius nerve cells. The results obtained with Mn^{2+} support the theory that the fast and partially slow outward currents are Ca^{2+} activated. Our voltage-clamp data support the early proposal of Beleslin (17) and Stewart (18) on the existence of Ca^{2+}-activated K^+ channels in leech neurons. Interestingly, similar results were found by Mitra and Morad (28), who reported that nifedipine and cadmium reduced the Ca^{2+}-activated K^+ current.

Jow and Numann (31) show that 5 mM Mn^{2+} reduced large inward rectifier current [$I_{K(IR)}$] by 33% in human capillary endothelial cells, and similar results in rat pyramidal neurons were obtained by Castelli et al (32) using the patch-clamp technique. Castelli et al. reported that Mn^{2+}, in addition to performing a blocking action on high-voltage-activated Ca^{2+} channels, modified the Ca^{2+} current activation and deactivation kinetics.

Taken together, we can conclude that the total outward K^+ current is composed of two distinct components in leech Retzius nerve cells, i.e., voltage dependent and Ca^{2+}-activated K^+ currents, and that the Ca^{2+} activated K^+ current is a large outward K^+ current**.**

Conflict of Interest

All of the authors declare no conflict of interest.

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PREVALENCE OF ASYMPTOMATIC ABDOMINAL AORTIC ANEURYSM IN PATIENTS WITH CAROTID STENOSIS

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PREVALENCA ASIMPTOMATSKE ANEURIZME ABDOMINALNE AORTE KOD PACIJENATA SA KAROTIDNOM STENOZOM

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ABSTRACT

The aim of this study was to demonstrate the prevalence of abdominal aortic aneurysm in patients with carotid disease and to analyse the influence of cardiovascular risk factors for abdominal aortic aneurysm. Methods: Ultrasound for abdominal aortic aneurysm was performed in 200 patients (112 men and 88 women, mean age 65.72±7.71 years) with known carotid disease. The primary cardiovascular risk factors (age, sex, hypertension, diabetes, dyslipidaemia and smoking) were analysed. Results: We found that 15.5% of patients with carotid stenosis also had abdominal aortic aneurysm. The prevalence of abdominal aortic aneurysm was higher in men (22.23%) than in women (6.81%). There was no correlation between the severity of carotid disease and the diameter of the abdominal aortic aneurysm (p>0.05). Advanced age and smoking were independent risk factors for abdominal aortic aneurysm. Conclusion: These results demonstrate that the prevalence of abdominal aortic aneurysm is higher in patients with carotid disease than in the general population. Patients with known carotid disease may be candidates for selective screening for abdominal aortic aneurysm detection.

Keywords: *abdominal aortic aneurysm, carotid stenosis, risk factors*

SAŽETAK

Cilj ovog istraživanja jeste utvrđivanje prevalence aneurizme abdominalne aorte kod pacijenata sa karotidnom stenozom i analiza uticaja glavnih kardiovaskularnih faktora rizika na razvoj aneurizme abdominalne aorte.

Ultrazvuk stomaka za otkrivanje aneurizme abdominalne aorte je primenjen na 200 pacijenata (112 muškaraca i 88 žena), prosečne starosti od 65.72±7.71 godine sa već potvrđenom bolešću karotidnih arterija. Analizirani su glavni kardiovaskularni faktori rizika (godine, pol, hipertenzija, dijabetes, dislipidemija i pušenje).

Pronašli smo da 15.5% pacijenata sa karotidnim stenozom ima aneurizmu abdominalne aorte. Prevalenca aneurizme abdominalne aorte bila je veća kod muškaraca (22.23%) nego kod žena (6.81%). Nije bilo korelacije između težine karotidne stenoze i dimenzija aneurizme abdominalne aorte (p>0.005). Starija životna dob i pušenje su nezavisni faktori rizika za nastanak aneurizme abdominalne aorte.

Rezultati ukazuju da je prevalenca aneurizme abdominalne aorte veća kod pacijenata sa karotidnom stenozom nego u opštoj populaciji. Pacijenti sa potvrđenom karotidnom stenozom mogu biti ciljna grupa za skrining pregled za postojanje aneurizme abdominalne aorte.

Ključne reči: *aneurizma abdominalne aorte, karotidna stenoza, faktori rizika*

INTRODUCTION

Carotid stenosis and abdominal aortic aneurysm (AAA) are frequent ailments in the elderly. It was previously thought that these illnesses were caused by atherosclerosis. However, despite that these two illnesses share the majority of their risk factors, research has shown that aneurysmal disease pathogenetically differs from occlusive artery disease (1-3). Occlusive artery disease affects the intima and partially media and is strongly associated with atherosclerosis. On the other hand, aortic aneurysmal disease affects the media and adventitia and leads to degeneration of the elastin and collagen; however, compared to occlusive disease, it has a weaker association with atherosclerosis (4). The disease is usually asymptomatic until the aneurysm ruptures, in which case the mortality rate is high, even with surgical intervention. Early diagnosis of asymptomatic AAA significantly reduces mortality in these patients.

Ultrasonography of the abdomen is an accurate, realistic and widely accepted method for detecting AAA

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that has a sensitivity and specificity of almost 100% (5). Screening of the general population with elective surgical intervention reduces the mortality caused by AAA, but the screening process is expensive. Furthermore, there is some disagreement as to which subpopulations should be screened for AAA (6).

The aim of this study is to explore the prevalence of AAA in patients who were previously diagnosed with carotid disease and to analyse the influence of major cardiovascular risk factors for the development of AAA.

MATERIALS AND METHODS

Between January 2006 and October 2008, ultrasound of the abdominal aorta and carotid arteries was performed in 200 patients (112 males and 88 females) who were previously diagnosed carotid disease. The mean age for all patients was 65.72±7.71 (range from 45 to 81; 65.55±7.91 for men and 65.94±7.48 for women).

The registered risk factors were common for atherosclerosis and included the following: hypertension, diabetes, dyslipidaemia and smoking. Patients were considered hypertensive if they were taking anti-hypertensive therapy (drugs or diet) or had a blood pressure over 140/90 mm Hg on at least two different occasions. A diabetes diagnosis was made for patients who were undergoing endocrinology therapy or were on a modified diet; with an overall cholesterol value over 5.7 mmol/L, LDL cholesterol over 3.5 mmol/L and HDL cholesterol below 1.81 mmol/L. Patients were classified as current smokers, former smokers (smoked in the past, but not smoking for at least 5 years) or non-smokers.

Carotid disease was diagnosed using a 7.5 MHz probe with a Shimatzu SDU 2200 ultrasound device according to the criteria for determining the level of carotid artery stenosis based on the peak systolic velocity and end-diastolic velocity. Patients with carotid stenosis were assigned to one of two groups, the symptomatic or asymptomatic carotid stenosis group, according to initial reports on the carotid arteries, anamnesis and neurological status.

Symptomatic patients had a history of stroke or a transitory ischaemic attack, or they had a focal neurological disorder.

Asymptomatic patients were recruited from a group of patients who had visited the vascular surgeon because of peripheral vascular disease, on which occasion their carotid arteries were examined and asymptomatic carotid disease was diagnosed. Symptomatic carotid stenosis was diagnosed in 86 patients (47 men and 39 women; mean age of 66.98±6.72; range from 55 to 79; men 66.61±6.83; women 67.41±6.64), whereas asymptomatic carotid stenosis was diagnosed in 114 patients (62 men and 49 women; mean age of 64.78±8.28; range from 45 to 81; men 64.78±8.34; women 64.77±8.22). The patients with carotid stenosis were further divided into 7 subgroups according to the following criteria provided by Filis et al. 2002 (7): a reduced lumen in at least one carotid artery resulting from occlusion within the following ranges: up to 50%, 50-59%, 60-69%, 70-79%, 80-89%, 90-99% or complete occlusion (100%). If a patient had bilateral carotid disease, only the carotid artery with more pronounced stenosis was included in the statistics. The patient distribution by sex, age and type of carotid stenosis is presented in Table 1.

Table 1. Distribution of patients with carotid stenosis by sex and age

	<55	55-64	65-74	>74	Total
Asymptomatic carotid stenosis Men/Women	9 6/3	22 13/9	47 25/22	8 3/5	86 47/39
Symptomatic carotid stenosis Men/Women	21 14/7	34 17/17	50 29/21	9 5/4	114 65/49
Total	30	56	97	17	200

A 3.5 MHz probe with a Shimatzu SDU 2200 ultrasound device measured the largest transverse and anteroposterior diameters of the infrarenal abdominal aorta. The patients were classified according to the findings in the following 3 categories (7): normal aorta (diameter less than 27 mm), dilated aorta (diameter of 27-29 mm) or aneurysm (diameter over 29 mm). The patients with aneurysm were classified according to the aneurysm diameter into the following 3 categories: diameter of 30-39 mm, diameter of 40-49 mm, and diameter equal to or exceeding 50 mm. Patients with an AAA over 40 mm underwent a subsequent CT of the abdomen, and patients with an aneurysm exceeding 50 mm were considered for surgical intervention. The finding of an abdominal aorta was correlated with the most significant risk factors for atherosclerosis.

Statistical analysis

SPSS v7.5 was used for the statistical analysis. As for the descriptive statistics, the mean ± standard deviation were used for continuous variables, whereas the number and percentage were used for nominal variables. The X^2 test was used for comparisons between nominal variables, whereas the t-test was used for comparisons between continuous variables. Correlations were calculated using a univariate correlation analysis. A multivariate correlation model was used to examine the variables that independently affected the onset of AAA.

RESULTS

The average diameter of the abdominal aorta in the 200 examined patients was 28.14±10.34 mm (range from 18 to 70 mm). On average, men had a significantly larger abdominal aorta diameter compared with women (30.19±11.24 vs. 25.52±8.45; t-test, p=0.001). The majority of the patients (135 patients, 67.5%; 62 men and 73 women) had a normal aorta diameter, with an average of 22.35±2.95 mm. An ectatic aorta was found in 34 patients (17%; 15 men

Table 2. Aortic diameters in patients with carotid stenosis

	Definition (mm)	Total	%
Normal aorta	< 27	135	67.5
Ectatic aorta	27-29	34	17
AAA	>29	31	15.5

Table 3. Prevalence of abdominal aortic aneurysm related to sex and age

Age	Total	AAA	Prevalence %
<55 Men Women	30 20 10	1 1 -	3.33 5 -
55-64 Men Women	56 30 26	3 2 1	5.36 6.67 3.85
65-74 Men Women	97 54 43	22 19 3	22.67 35.18 6.98
>75 Men Women	17 8 9	5 3 2	29.41 37.5 22.22

Table 4. Risk factors and development of abdominal aortic aneurysm

Variables	r	p
Sex	0.199	0.04*
Hypertension	0.292.	0.007**
Diabetes	-0.265	0.06
Dyslipidemia	0.175	0.360
Asymptomatic carotid stenosis	0.285	0.009**
Smoking	0.317	0.002**
Age	0.648	0.000**

* statistical significance at 0.05 level;
** statistical significance at 0.01 level

Table 5. Variables significantly correlate to development of abdominal aortic aneurysm

Analyzed variables	t	Beta Coe cient	SE	p
Sex	0.819	0.071	0.148	0.415
Hypertension	0.947	0.124	0.196	0.234
Smoking	6.528	0.592	0.012	0.002**
Age	6.958	0.619	0.007	0.000**
Asymptomatic carotid stenosis	0.477	0.045	0.151	0.639

** statistical significance at 0.01 level

and 19 women), with an average diameter of 27.12±1.62 mm (Table 2).

Aneurysm of the abdominal aorta was found in 31 patients (25 men and 6 women), or 15.5% of all patients with average diameter of 43.73±10.39 mm, i.e., 22.32% men with average diameter of 44.1±9.59 mm and 6.81% women with average diameter of 42.73±12.77 mm. The mean age of patients with AAA was 76.34±7.49 mm. On average, men with AAA were significantly younger than women with AAA (73.97±7.49 mm vs. 77.36±6.36 mm; t-test, p=0.018). Small aneurysm, i.e., aneurysm with a 30 to 49 mm diameter, was found in 19 patients (14 men and 5 women), or 9.5% of the total number of patients, whereas AAA above 49 mm was found in 12 patients (11 men and 1 woman), or 6% of the total number of patients. Ten patients (9 men and 1 woman), or 5% of the total number of patients, underwent a successful surgery.

The prevalence of AAA increased significantly with age ($\chi 2$ test, p=0.008). The increase was most pronounced above 65 years in men and 75 years in women (Table 3).

The patients with asymptomatic carotid stenosis had a significantly larger average diameter of the abdominal aorta compared to patients who had symptomatic carotid stenosis (29.03±11.62 mm vs. 26.95±8.29 mm; t-test; p=0.005). AAA had a significantly higher prevalence in patients with asymptomatic carotid stenosis (20 vs. 11, $\chi 2$ test, p=0,023). This difference was significant with men (17 vs. 8; $\chi 2$ test, p=0,039) but not women.

The correlation analysis showed no correlation between the extent of carotid stenosis and AAA (correlation test, p<0.05). That is, there was no correlation between patients with a severe type of carotid stenosis and an increased prevalence of AAA or vice versa.

Patients with both carotid stenosis and AAA had a significantly higher prevalence of hypertension (24 or 77.42%, $\chi 2$ test, p=0.005) and smoking (28 or 90.32%, $\chi 2$ test, p=0.000). The average diameter of the aortic aneurysm did not differ between current smokers (46.79±11.4 mm) and former smokers (43.89±8.75 mm) (t-test, p=0.628); however, both of these groups had aortic aneurysm diameters that were significantly larger than those of non-smokers (36.33±3.21 mm; t-test, p=0.021 for current smokers and p=0.035 for former smokers). Patients with diabetes (40.82±9.15 mm) had a significantly smaller average diameter of AAA than patients who did not have diabetes (47.20±10.62 mm; t-test, p=0.047).

The univariate correlation analysis showed that asymptomatic carotid stenosis (r=0.285, p=0.009), smoking (r=0.317, p=0.002), hypertension (r=0.292, p=0.007), a male sex (r=0.199, p=0.04) and an increasing age (r=0.648, p=0.000) had significant positive correlations with the onset of AAA in patients with carotid stenosis. Dyslipidaemia (r=0.175, p=0.360) was not presented as a risk factor that had a significant correlation with the onset of AAA. Diabetes presented a negative correlation with the onset of AAA (r=-0.265, p=0.06), but the difference did not reach statistical significance (Table 4). The multivariate regression analysis indicated that age (p=0.000) and smoking (p=0.002) were risk factors that independently affected the onset of AAA (Table 5).

DISCUSSION

To date, abdominal ultrasound is the most practical method for identifying AAA (8). The time needed to perform abdominal ultrasound and measure the aortic di-

ameter is less than 10 minutes (9). The best therapy for AAA is presymptomatic elective surgery of carefully chosen patients. Despite having different methodologies, a recent meta-analysis and a randomised general population screening study aimed at detecting asymptomatic AAA and timely intervention both showed a significant reduction in AAA-related mortality (8, 10). However, a recent analysis of 16 studies that investigated the cost efficiency of AAA screening (11) failed to provide definitive recommendations for the population groups in which abdominal aorta screening should be performed.

The prevalence of AAA in the general population is approximately 4-10% for men over 60 years of age and 0.5-3% for women over 80 years of age (8, 12-15). However, it is very difficult to compare the results of these studies because of the differences in the study samples, methodology, and definitions of AAA.

The comorbidity of carotid stenosis and AAA and the prevalence of AAA in patients with carotid disease have been rarely studied. It was shown that carotid intima media thickening in patients with AAA was higher than that in the healthy population but lower than that in patients with peripheral artery disease (16, 17). The 15.5% prevalence of AAA in patients with carotid stenosis that we found in this study is higher than the prevalence found by Carty et al. (11%) (18) and Kurvers et al. (6.5%) (19), lower than the prevalence found by Karanjia et al. (20.22%) (20) and Barba et al. (21.2%) (21) and similar to the prevalence found by Kang et al. (22) (patients with carotid stenosis over 50%, 18.2%; patients with carotid stenosis below 50%, 12.2%; only patients without diabetes were included in the study). However, if one takes into consideration that our study included a similar number of men and women, whereas the number of men was much higher in the previous studies, then one can consider that the present results are in agreement with the findings of Karanjia et al. and Barba et al. (20, 21). In our study, the prevalence of AAA was significantly higher in men (22.32%) than in women (6.81%). However, this can be explained by the fact that AAA occurs much later in women than in men (23, 24), and the mean age was similar between men and women in this study. Indeed, men with aneurysm were significantly younger than women with AAA in this study. The prevalence of AAA significantly increases with age in both sexes, but the largest number of aneurysms was found in men over 65 years of age and in women over 75 years of age.

The average AAA diameter was significantly larger in patients with asymptomatic carotid stenosis than in patients with symptomatic carotid stenosis. The patients with asymptomatic carotid stenosis had been diagnosed with peripheral arterial disease, whereas the patients with symptomatic carotid stenosis had been diagnosed with cerebrovascular disease. Previous studies have shown that patients with peripheral arterial disease have a high prevalence of AAA (21), but the same was not shown with cerebrovascular disease.

Most of our patients (9.5% of total number of examined patients, and 61.29% of patients with AAA) who had AAA also had a small AAA, with a typical diameter of 30 to 49 mm. Aneurysms of this size do not require surgical intervention, but only periodic monitoring of the aneurysm growth, which had been performed. A small number of patients (12,6% of all examined patients, and 38.71% of patients with AAA) had an aneurysm with a diameter of 49 mm. Of these 12 patients, 10 (9 men and one woman, 5% of all patients) underwent successful surgery. One patient did not undergo surgery for his AAA, which did not exceed 5.5 mm, whereas another patient did not undergo surgery due to a recent myocardial infarction and a poor cardiovascular status.

No correlation was found between the size of an AAA and the severity of carotid stenosis, i.e., it was not shown that patients with a more severe form of carotid stenosis also had a higher prevalence of AAA and vice versa. This finding is consistent with the data published by Kang et al. (22).

Carotid disease and AAA share many risk factors. Our results show a significant positive correlation between hypertension, smoking, age, male sex and asymptomatic carotid stenosis and the development of AAA. Furthermore, the male sex, age and smoking are factors that independently influence the development of AAA. The presence of diabetes showed a negative correlation with the development of AAA, but the difference was not statistically significant.

All studies so far have shown that AAA has a much higher prevalence above the age of 65. It is interesting that there are many studies dealing with development of AAA only in men, which likely stems from the previous opinions that AAA is an extremely rare event in women and that aneurysmal arterial disease in women differs from that in men. The fact is that AAA develops in women after menopause, myocardial infarction, or stroke. However, the latest research (23) confirmed AAA is usually not diagnosed or is misdiagnosed in women and that operated women had a far worse postoperative outcome with procedures for a ruptured AAA. The risk factors in women do not differ significantly than those in men.

We have indicated that smoking is an independent risk factor for the development of AAA, and this finding is in compliance with other studies. An analysis of ten studies with over one million patients who smoked reported a prevalence of AAA that was 2.5 and 3.5 times higher than those for coronary disease and cerebrovascular disease, respectively (25). It appears that smoking is a greater risk factor for the development of aneurysms than for obstructive arterial disease. The smoking-mediated dysfunction of endothelium could be the underlying mechanism through which smoking triggers the development and growth of an AAA, resulting in a reduction in tissue-type plasminogen activator, which in turn leads to hypercoagulability (26). There are indicators that smoking cigarettes is a cause of inflammation, which is expressed through increased lev-

els of circulating leucocytes, C-reactive protein, interleukin 6 and fibrinogen (27). Signs of inflammation were also present in passive smokers (28). Smoking cigarettes deteriorates the elasticity of the ascending aorta (29), whereas long-term smokers have reduced distensibility of the ascending aorta compared to non-smokers (29).

Diabetes is the most interesting rick factor for the development of AAA. Among the patients who had an abdominal aorta examined for the presence of aneurysm, 54.5% also had diabetes. However, if only patients with AAA are considered, then the diabetes prevalence drops to a mere 16.13%. On the other hand, in the patients with AAA, those with diabetes had a significantly smaller AAA diameter than the AAA patients without diabetes. The univariate correlation analysis revealed a negative correlation between diabetes and the development of AAA, but that difference was not significant. In other words, the results of this study indicated the presence of diabetes has a protective effect on the development of AAA. The connection between AAA and diabetes is not clear, but it is thought that reduced monocyte activity and reduced production of matrix metalloproteinases, as observed in diabetes, may play a protective role in AAA (30). A negative association between diabetes and AAA, meaning that diabetes plays a protective role in the development and expansion of AAA, was noted early as 1995 (31), and this was confirmed in a number of patients (32). It was recently shown that the diameter of the aorta has an inverse relationship with the serum glucose concentration (33) and that diabetes reduces the progression of AAA (30).

Aneurysm of abdominal aorta is a disease that differs by its aetiology, pathogenesis, clinical picture, risk factors and therapy from arterial occlusive disease (such as carotid disease), although the both diseases include degenerative atherosclerotic changes within the blood vessel wall. However, numerous factors affect the course of events in developing arterial occlusive disease (disease of endothelium) or aneurysmal arterial disease (collagen and elastin disease).

The results of this study and a small number of other studies that focused on the prevalence of AAA in patients with carotid stenosis indicate different prevalence rates for AAA in these patients, even for patients with stenosis of less than 50%, which does not have to be considered as clinically significant. Therefore, screening these patients for the existence of AAA could be useful in reducing the mortality resulting from the rupture of AAA.

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COMPARISON OF BIOMETRIC VALUES AND INTRAOCULAR LENS POWER CALCULATIONS OBTAINED BY ULTRASOUND AND OPTICAL BIOMETRY

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POREĐENJE BIOMETRIJSKIH VREDNOSTI I KALKULACIJE JAČINE INTRAOKULARNOG SOČIVA DOBIJENIH ULTRAZVUČNOM I OPTIČKOM BIOMETRIJOM

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ABSTRACT

is study sought to compare the biometric values and intraocular lens (IOL) power obtained by standard ultrasound and optical biometry.

We examined 29 eyes in preparation for cataract surgery. None of the patients had refractive surgery or corneal anomaly. In all patients, the horizontal and vertical refractive power of the cornea was determined using a keratometer (Bausch&Lomb). e axial length of the eye was determined via A-scan ultrasound (BVI-compact-V-plus) using Hollady's formula. e IOL power and complete biometric measurements were obtained via an IOL Master-500-Zeiss using the Hollady-2 formula. All obtained values were compared and analysed using the statistical program SPSS 20.

e average age of treated patients was 71.21±1.68 years. In 16 patients with dense cataracts (55.17%), it was not possible to determine the IOL power by optical biometry. Optical biometry obtained significantly increased axial length values of 24.04±0.29 mm compared with those obtained with ultrasound biometry (23.89±0.28 mm, p=0.003). e mean refractive cornea power values of the horizontal meridian measured using a keratometer (42.50±0.47 D) and an IOL Master (42.69±0.49 D) were not statistically different (p=0.187). e mean values of the refractive cornea power of the vertical meridian obtained using a keratometer (42.62±0.48D) and an IOL Master (43.36±0.51 D) exhibited a statistically significant di erence (p=0.000). e keratometer obtained statistically significant lower mean values of corneal refractive power (42.73±0.32 D) compared with those obtained with optical biometry (43.22±0.35 D, p=0.000). Ultrasound biometry obtained significantly increased the mean values of IOL power (20.19±0.48D) compared with those obtained with optical biometry (19.71±0.48 D, p=0.018).

e large number of patients who receive an operation for dense cataracts indicate the need for representation of both biometric methods in our clinical practice.

Key words: *axial length, intraocular lens power, ultrasound biometry, optical biometry*

SAŽETAK

Cilj je poređenje vrednosti biometrijskih podataka i jačine intraokularnog sočiva (IOL) dobijenih standardnom ultrazvučnom i optičkom biometrijom.

Pregledano je 29 očiju u okviru pripreme za operaciju katarakte. Niko od pacijenata nije imao refraktivnu operaciju, ni anomaliju rožnjače. Svim pacijentima je pomoću keratometra (Baush&Lomb) određena horizontalna i vertikalna prelomna moć rožnjače, A-scan ultrazvukom (BVI-compact-V-plus) aksijalna dužina oka, pomoću Holladyeve formule jačina intraokularnog sočiva kao i kompletno biometrijsko merenje IOL Master 500- Zeiss uz upotrebu Hollady-2 formule. Sve dobijene vrednosti su poređene i obrađene statističkim programom SPSS 20.

Prosečna starost pacijenata je bila 71.21±1.68 godina. Kod 16 pacijenata sa gustom kataraktom (55.17%) nije bilo moguće odrediti jačinu intraokularnog sočiva optičkom biometrijom. Optičkom biometrijom se dobijaju statistički značajno više vrednosti aksijalne dužine 24.04±0.29 mm, nego ultrazvučnom biometrijom 23.89±0.28 mm, p=0.003. Između srednje vrednosti jačine prelamanja rožnjače po horizontalnom meridijanu izmerene keratometrom, 42.50±0.47 D i IOL Master-om, 42.69±0.49 D nije uočena statistički značajna razlika, p=0.187. Dobijene srednje vrednosti jačine prelamanja rožnjače po vertikalnom meridijanu određene keratometrom 42.62±0.48 D i IOL Master-om 43.36±0.51 D, pokazuju statistički značajnu razliku, p=0,000. Keratometrom se dobijaju statistički značajno niže srednje vrednosti prelamanja rožnjače, 42.73±0.32 D nego optičkom biometrijom 43.22±0.35 (38.34-46.62 D), p=0.000. Ultrazvučnom biometrijom dobija se statistički značajno viša srednja vrednost jačine IOL-a 20.19±0.48 D (17.0-23.0 D), nego optičkom biometrijom 19.71± 0.48 D, (16.0-22.5 D), p=0.018.

Veliki broj pacijenata koji se operišu u stadijumu guste katarkte ukazuju na potrebu zastupljenosti obe metode biometrije u našoj kliničkoj praksi.

Ključne reči: *aksijalna dužina, jačina intraokularnog sočiva, ultrazvučna biometrija, optička biometrija*

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ABBREVIATIONS

IOL - intraocular lens
AL - axial length of the eye
ACD - anterior chamber depth
K - refractive power of the cornea, keratometric value
K1 - refractive power of the cornea by the horizontal meridian
K2 - refractive power of the cornea by the vertical meridian
US - ultrasound

INTRODUCTION

Modern cataract surgery requires the achievement of ideal postoperative refractive results.

In addition to good operational techniques, intraocular lenses (IOL) quality and retina identification, a precise calculation of the intraocular lens power is of crucial importance to achieve good results after refractive cataract surgery (1, 2). Accurate calculations primarily depend on the accuracy of preoperative biometric data, including the axial length of the eye (AL), the anterior chamber depth (ACD), and the keratometry values (K), and the precision of the formula applied to calculate IOL power (3-6). Incorrect calculation of the lens power is the main reason for patient dissatisfaction and lens replacement in modern cataract surgery (7).

Intraocular lens power calculations are possible with standard ultrasound biometry and modern contactless optical biometry (IOL Master, Zeiss).

Optical biometry provides more comfort for the physician and the patient because it is a fast, non-invasive, non-contact approach that does not require local anaesthesia. In addition, there is no risk of trauma and infection of the cornea (3, 8, 9).

The purpose of this study is to compare the biometric values and IOL power obtained by standard ultrasound and optical biometry and to consider the advantages and disadvantages of optical and ultrasound biometry.

MATERIALS AND METHODS

A comparative study was conducted. We examined 29 eyes of 29 patients in preparation for cataract surgery. Biometric measurement and IOL power calculation were performed for all patients by ultrasound (standard) and optical biometry. None of the examined patients underwent refractive surgery or exhibited corneal anomalies.

The horizontal and vertical refractive power values of the cornea were determined using a keratometer (Bausch and Lomb). The axial length of the eye was determined using A-scan ultrasound (BVI compact V plus), and the intraocular lens power was determined using Hollady's formula.

We did complet biometric measurements by optical biometry, IOL Master 500, Zeiss camera using coherent light interference: the axial length, keratometry, anterior chamber depth and IOL power. We used Hollady-2 formula for calculating intraocular lens power.

Our study included a comparison of the axial length of the eye (Student's t-test), the refractive power of the cornea by the horizontal meridian K1 (Wilcoxon test), the refractive power of the cornea by the vertical meridian on K2 (Student's t-test), the refractive power by the cornea K (Student's t-test) and the intraocular lens power (Wilcoxon test) as obtained by a standard ultrasound and modern optical biometry.

For statistical data analysis, the statistical program SPSS 20 was used, and p-values <0.05 were considered statistically significant.

RESULTS

The measurement was performed on one eye of 29 patients who were preparing for cataract surgery. Examined patients were 62 to 83 years of age, with a mean age of 71.21 ± 1.68 years. The frequency of patients of a certain age was determined by the Xi^2 test. Two patients (6.90%) in the age range of 50 to 59 years were examined, 5 (17:24%) patients in the age range of 60 to 69 years were examined, 15 (51.72%) patients in the age range 70 to 79 years were examined, and 7 patients (24.14%) in the age range 80 to 89 years were examined. Most patients were 70 to 79 years of age. No statistically significant differences were noted in the frequency of patients of a certain age (Xi^2=7.621, p=0.974).

In 16 patients (55.17%), it was not possible to determine the intraocular lens power using optical biometry. Of these patients, 2 were 50 to 59 years of age (100% of the patients examined at that age), 7 patients were 70 to 79 years of age (46.67% of the patients examined at that age) and 6 patients were 80 to 89 years of age (85.71% of the patients examined at that age). For all patients 60 to 69 years of age, the IOL power was determined using both apparatuses (Table 1).

The mean value of the axial length of the eye measured by ultrasound was 23.89 ± 0.28 mm in the range 21.62-26.35 mm, and by optical biometry 24.04 ± 0.29 mm in the range 22.52-26.67 mm, as measured by optical biometry. A statistically significant difference between the axial length

Table 1. Age of patients undergoing preoperative preparations for cataract surgery

Age range	Number of examined patients	% of examined patients	I0L Master calculation could not be performed	% of patients in the age range in whom I0L Master measurements could not be obtained
50 – 59	2	6.9	2	100%
60 – 69	5	17.24	0	0%
70 – 79	15	51.72	7	46.67%
80 – 89	7	24.14	6	85.71%
90 – 100	0			
Total	29	100	15 (51.72%)	

Table 2. e axial length of the eye measured by ultrasound and optical biometry

	Ultrasound biometry	Optical biometry	Statistical significance
Axial length of the eye ± SD (min-max)	23.89 mm±0.28 mm 22.43 mm-26.3 mm	24.04 mm±0.29 mm 22.52 mm-26.67 mm	p = 0.003 Student's T-test

of the eye measured by the standard ultrasound method compared to modern optical biometry via the IOL Master (t-test, p=0.003) was observed. Optical biometry provided greater axial eye length values (Table 2).

The mean value of the refractive cornea power of the horizontal meridian (K1) as measured by the Bausch & Lomb keratometer was 42.50 ± 0.47 D. The minimum measured value was 38.00 D, and the maximum value was 45.00 D. The mean value of the refractive cornea power of the horizontal meridian K1 as measured by the IOL Master was 42.69 ± 0.49 D. The minimum value was 38.1 D, and the maximum value was 44.64 D. The obtained keratometric values of the horizontal meridian K1 as calculated by the Bausch & Lomb keratometer and IOL Master did not exhibit a statistically significant difference (the Wilcoxon test, p=0.187) (Table 3).

The mean value of the refractive cornea power of the vertical meridian as assessed by the Bausch & Lomb keratometer was 42.62 ± 0.48 D (minimum 38.01 D, maximum 44.75 D). The mean value determined by IOL Master was 43.36 ± 0.51 D (minimum 38.66 D, maximum 45.67 D). Vertical refractive cornea power (K2) values as determined by these two apparatuses exhibited a statistically significant difference (Student's t-test, p <0.01) (Table 3).

The mean value of corneal refractive power (K) as determined by the Bausch & Lomb keratometer was 42.73 ± 0:32 D (minimum 38.25 D, maximum 45.88 D). The mean value determined by the IOL Master mean value was 43.22 ± 0.35 D (minimum 38.34 D, maximum 46.62 D). A statistically significant difference was noted between the obtained measurements. Measurements obtained with the

Table 3. Keratometric values

	Bausch&Lomb	IOL Master	Statistical significance
K1 Middle value ± SD (min-max)	42.56 ± 0.47 D (38.0 D - 44.64 D)	42.69 ± 0.49 D (38.1 D - 44.64 D)	p = 0.187 Wilcoxon test
K2 Middle value ± SD (min-max)	42.62 ± 0.48 D (38.1 D - 44.75 D)	43.36 ± 0.51 D (38.66 D - 45.67 D)	p < 0.01 Student's T-test
K Middle value ± SD (min-max)	42.73 ± 0.32 D (38.25 D - 45.88 D)	43.22 ± 0.35 D (38.34 D - 46.62 D)	p = 0.000 Student's T-test

Table 4. Intraocular lens power as determined by ultrasound and optical biometry

	Ultrasound biometry	Optical biometry	Statistical significance
Mean value of IOL power ± SD	20.19 ± 0.48 D	19.71 ± 0.48 D	p =0.018
(min-max)	17.00 D-23.00 D	16.00 D-22.5 D	p < 0.05
			Wilcoxon test

Bausch & Lomb keratometer produce lower values (Student's t-test, p=0.000) (Table 3).

The mean value of the intraocular lens power obtained by standard ultrasound biometrics was 20.19 ± 0.48 D (minimum 17.0 D, maximum 23.0 D). The mean value of the intraocular lens power measured by optical biometry was 19.71 ± 0.48 D (the minimum IOL power was 16.0 D, the maximum was 22.50 D). A statistically significant difference was noted between the intraocular lens power values observed by standard ultrasound compared to optical biometry (Wilcoxon test; p=0.018, p < 0.05) (Table 4).

DISCUSSION

The axial length of the eye can be measured by ultrasound (contact and immersion techniques) and optical biometry (IOL Master or Lenstar). The study of preoperative and postoperative ultrasound biometry revealed that 54% of errors in predicting the refractive power after IOL implantation can be attributed to errors in measuring the axial length of the eye (10-12). Therefore, it is very important to carefully and accurately obtain measurements at this stage (13). An error of 100 micrometres in axial length can lead to postoperative refractive errors from 0.28 D (14).

Noncontact optical biometry has become the gold standard given its ease of performance, accuracy and reproducibility (14). In addition to high-precision, noncontact and non-invasive measurements, the advantages of optical biometry include speed and patient comfort. Given that IOL power calculations by optic biometry do not require anaesthesia, there is no risk of corneal trauma and infections (3, 8, 9). In addition, mydriasis is not required to perform this technique. The main drawback of this technique is the inability to measure the axial length in 10% of patients with dense posterior subcapsular cataract (15). In these patients, the contact ultrasonic method is the method of choice, and the immersion method is rarely used. It is also not possible to perform measurements in patients with severe corneal pathology, eyelid abnormalities, macular degeneration and eccentric fixation. In these patients, it is possible to obtain eye biometrics using ultrasound (16). Two main causes of errors when using applanation ultrasound biometry include mistakes in measuring axial length that arise from the indentation of the eyeball and axial measurements of axial length (13). The immersion ultrasonic technique avoids these drawbacks. Applanation ultrasound techniques achieve better refractive results (17). Compared with ultrasound biometry, where the IOL power calculation depends on the experience of the performer, optical biometry measures the axial length of the eye along the visual axis no identacije eyeball. The measurement is less dependent on the person who is performing the measurement (13, 18). Optical biometry also has an advantage in patients with silicone oil and rear staphyloma (16).

A large number of authors suggest that measurements of AL and the IOL power using the IOL Master are comparable or more precise with respect to the use of the applanation ultrasound method in the normal population (3, 13, 19-21). In addition, numerous studies indicate that both methods exhibit high accuracy and reproducibility (22, 23). Modern optical biometry achieves optimal visual acuity after cataract surgery in 90% of patients ± 1 D and in greater than 60% ± 0.5 D of best corrected visual acuity (14).

Our results confirm previous research results and indicate the inability to use optical biometry on patients with dense cataracts. The percentage of patients in whom it was not possible to measure the axial length of the eye and intraocular lens optical biometry in our study was 51.72%. In all the patients, clinical examination revealed a dense cataract or dense posterior subcapsular cataract. Such a high inability to perform optical biometry is not consistent with data in the literature that range from 8% to 10% (20, 24), but it is possible to explain the results of studies by showing that the failure to execute biometric measurements correlates with the existence of last subcapsular cataracts (14, 16, 19). The results of our research are understandable if we consider the peculiarities of our environment, including a large number of patients who are waiting for cataract surgery. According to data for the month of July of the current year, the average wait for cataract surgery is 537.3 days. Given the long wait, a large number of patients have dense cataracts for whom surgery is not possible due to technical characteristics of the apparatus used to perform optical biometry measurements.

Our study confirmed previous findings that compared with optical biometry, contact ultrasound biometry provides a reduced axial length (14, 24-26). Previous studies demonstrate that contact ultrasound biometry provides reduced AL values compared with immersion ultrasound biometry (27). IOL power calculations depend on the precision of the applied formula. The most commonly used formulas for calculating IOL power lens (Hoffer Q (28), Holladay 1 (29), and SRK/T (30)) use two biometric measurements (axial length and keratometry) and an IOL constant (31). The conclusion of previous studies suggests that no single formula is suitable for all eyes (14). According to Aristodemou and associates who tested Hoffer Q, Holladay 1, and the SRK/T formulas in 8108 eyes, Hoffer Q is best for axial lengths below 21.5 mm, whereas SRK/T is ideal for those with axial lengths greater than 26.0 mm. For axial length values that fall between these values, no statistically significant differences were noted among the formulas. However, Holladay 1 has certain advantages (32). In our study, the axial length of the eye measured by ultrasound biometry was in the range of 21.62 to 26.35 mm; therefore, the Holladay formula was used. Based on optical biometry, the axial length was in the range of 22.52 to 26.67 mm, and the Holladay-2 formula was used for calculating the IOL power.

Although optical biometry exhibits high precision and reproducible measurements (22-23), unfortunately, the high equipment cost and the limited equipment availability explain the current preference for ultrasonic methods of biometrics.

CONCLUSION

High patient expectations in terms of achieving good refractive results after cataract surgery indicate the need for continuous improvement of surgeons' operating techniques, the design of intraocular lenses and accurate biometric measurements.

In comparison with the standard biometry (Bausch & Lomb and ultrasound), optical biometry in our study showed significantly higher axial length of the eye, significantly higher refractive power of the cornea K and statisticly significantly higher refractive cornea power by vertical meridian K2, and significantly lower power lenses that need to be implanted in the posterior chamber.

The peculiarities of our environment, including a long period of waiting for cataract surgery and a large number of patients with dense cataracts who receive an operation, indicate the need for the use of both methods in clinical practice.

Declaration of interests

The authors declare no conflicts of interests.

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HOSPITAL ACQUIRED PNEUMONIA IN NEWBORNS WITH BIRTH WEIGHT LESS THAN 1500 GRAMS: RISK FACTORS AND CAUSES

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BOLNIČKE PNEUMONIJE KOD NOVOROĐENČADI ROĐENIH SA TELE SNOM MASOM MANJOM OD 1500 GRAMA: FAKTORI RIZIKA I UZROČNICI

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ABSTRACT

Low birth weight newborns (≤ 1500 grams) are at a high risk of acquiring hospital infections due to the immaturity of the immune system, lack of e cient structural barriers, and an incomplete development of endogenous microbial flora.

e aim of this study was to reveal the potential risk factors for hospital-acquired pneumonia in low birth weight newborns.

is study was a prospective cohort design with a nested case-control study and was conducted between January 1st, 2012 and June 30th, 2015 at the Neonatology Department, Clinical Centre Kragujevac, Serbia. ere were 1140 newborns hospitalized at the Neonatology Department for longer than 48 hours during the study period, and 169 of them (14.82%) weighed less than 1500 grams at birth. In total, 73 (43.19%) newborns with low birth weights developed HIs. e most prevalent HI was hospital pneumonia (n=64, 87.67%).

Although univariate analyses identified many risk factors with a significant influence on the occurrence of hospital pneumonia, multivariate analysis identified only the following two independent risk factors for hospital pneumonia in newborns with birth weights below 1500 grams: mechanical ventilation (p=0.003, OR=68.893, 95% CI=4.285-1107.699) and longer hospitalization (p=0.003, OR=1.052, 95% CI=1.017-1.088). Almost all of the pathogens isolated from the patients with pneumonia were gram-negative bacteria (98.50%). More than half of all of the isolates were Acinetobacter spp (37.50%) and Enterobacter spp (18.75%).

Our study showed that mechanical ventilation and prolonged hospitalization were significant risk factors for the development of hospital pneumonia in newborns with birth weights below 1500 grams.

Keywords: *Newborns; low birth weight; nosocomial infections; pneumonia*

SAŽETAK

Novorođenčad sa malom telesnom masom na rođenju (≤ 1500 grama) odlikuju se prisustvom visokog rizika za razvoj bolničkih infekcija usled nezrelosti imunog sistema, nedostatka funkcionalno razvijenih strukturalnih barijera kao i nepotpuno razvijene endogene mikrobne flore.

Utvrditi potencijalne faktore rizika za razvoj bolničkih pneumonija kod novorođenčadi rođenih sa malom telesnom masom.

Sprovedena je prospektivna kohortna studija sa ugnježdenom studijom tipa slučaj-kontrola u periodu od 1. januara 2012. godine do 30. juna 2015. godine u Centru za neonatologiju Kliničkog centra Kragujevac, Republika Srbija. Ukupno je u pomenutom periodu praćenja bilo 1140 novorođenčadi hospitalizovanih u Centru za neonatologiju duže od 48 časova, od kojih je njih 169 (14,82%) imalo telesnu masu na rođenju manju od 1500 grama. Intrahospitalnu infekciju iz pomenute grupe od interesa razvilo je 73 (43,19%) novorođenčadi sa malom telesnom masom na rođenju, i to najčešće pneumoniju (n=64; 87,67%).

Mada je univarijantna analiza ukazala na značajan uticaj brojnih faktora rizika na razvoj intrahospitalnih upala pluća, multivarijantnom analizom su, pak, identifikovana samo dva nezavisna faktora rizika za razvoj bolničkih pneumonija kod novorođenčadi sa telesnom masom ispod 1500 grama na rođenju: mehanička ventilacija (p=0,003, OR=68,893, 95% CI= 4,285-1107,699) i produžena hospitalizacija (p=0,003, OR=1,052, 95% CI=1,017-1,088). Većina patogenih uzročnika infekcija izolovanih kod pacijenata sa upalom pluća bili su iz grupe Gram negativnih bakterija (98,5%). Više od polovine svih izolata pripadali su vrstama Acinetobacter (37,50%) odnosno Enterobacter (18,75%) bakterija.

Naše istraživanje je pokazalo da mehanička ventilacija i produžena hospitalizacija predstavljaju značajne faktore rizika za razvoj bolničke pneumonije kod novorođenčadi sa telesnom masom na rođenju manjom od 1500 grama.

Ključne reči. *Neonatus; mala telesna masa na rođenju; bolničke infekcije, pneumonija*

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INTRODUCTION

Hospital infections (HIs) are the main cause of morbidity and mortality in neonatal intensive care units (NICUs) and are accompanied by major utilization of resources, prolonged hospitalization and increased costs (1,2). More than 30% of hospitalized newborns develop HIs, sometimes followed by a lasting impairment of health, which places HIs on the short list of the most serious public health problems (3-5).

Premature newborns and low birth weight newborns (≤ 1500 grams) have the highest risk of acquiring HIs due to the immaturity of their immune systems, lack of efficient structural barriers, and an incomplete development of endogenous microbial flora (6-8). They are also exposed to numerous invasive diagnostic and therapeutic procedures, which often open the doors for the entrance of pathogens (placement of venous and urinary catheters, endotracheal intubation, mechanical ventilation, and total parenteral nutrition). Although the most frequent type of HI is intrahospital pneumonia, there are only a few published studies regarding the epidemiology of HIs.

The aim of this study was to reveal the potential risk factors for hospital-acquired pneumonia in low birth weight newborns (b.w. ≤ 1500 g) and establish a basis for planning preventive measures.

MATERIALS AND METHODS

Our study was a prospective cohort design with a nested case-control study and was conducted between January 1st, 2012 and June 30th, 2015 at the Neonatology Department, Clinical Centre Kragujevac, Serbia. This department has 30 beds (15 devoted to intensive care and 15 to special care), occupying 6 rooms in total. The personnel wash their hands with a 0.75% solution of povidone iodine, and rapid disinfection is performed with various alcohols. In total, eight physicians take care of the patients, and there is one nurse per three intensive care beds, and one nurse per four special care beds. The following measures for prevention of HIs are routinely conducted in this department: hand hygiene, installation of alcohol-based hydrogels for hand disinfection in the ward, performing environmental cultures, assessing staffing, and grouping infected or colonized patients.

The study was approved by Ethics Committee of the Clinical Centre Kragujevac.

The inclusion criteria were a birth weight below 1500 grams and hospitalization longer than 48 hours. Newborns with hospital-acquired pneumonia were classified as cases, and newborns without pneumonia were classified as control. Other types of hospital infections were among the exclusion criteria for this study.

The diagnosis of HI and the determination of the exact anatomical location were established according to the standard diagnostic criteria of the Center for Prevention and Control of Diseases (CDC) in Atlanta (9), which considers all neonatal infections, whether acquired during delivery or hospitalization, as hospital-acquired unless evidence indicated transplacental acquisition.

All newborns admitted to this department were submitted to the following diagnostic tests: peripheral blood leukocyte count, platelet count, C-reactive protein, and swabs and blood cultures if required. On the third day of hospitalization, the newborns were routinely examined for signs of pneumonia with laboratory tests; clinical signs, such as fever; respiratory problems (apnea, tachypnea, bradycardia, wheezing, rhonchi, cough, increased production of respiratory secretions, and new onset of purulent sputum or change in character of sputum); chest X-ray when requested by paediatricians; and microbial cultures.

Relevant study data were taken from the patients' files (patient's history, patient's charts, reports from laboratories, etc.), by examination of the patients and from the paediatricians in charge of the patients. Each case was analysed separately by a representative of the Department of Prevention and Hospital Infections Control of the Clinical Centre at Kragujevac, and complex cases were elaborated by the group of investigators. The patients were followed until they were cured, discharged from the hospital or died.

The data on the potential risk factors were collected by means of special epidemiological questionnaires, which included the following:

1. Details about the mother and the pregnancy: maternal age at the moment of delivery, maternal diseases, and whether it was singleton or twin pregnancy;
2. Details about the delivery: date, type (vaginal or by Caesarean section), whether there was rupture of the membranes or placental detachment, macroscopic inspection of the amniotic fluid (milky, meconium stained or green) and term of delivery; and
3. Details about the newborn: sex, gestational age (determined by the ultrasound examination), birth weight (grams), Apgar score values in the 1st and 5th minute after birth, reasons for admission to the hospital, diagnostic and therapeutic procedures that were undertaken (venous catheters, mechanical ventilation) and the results of the laboratory tests upon admission.

Isolation and identification of bacterial pathogens were performed in the Department for Microbiology at the Clinical Centre Kragujevac by means of conventional biochemical methods (10).

Collected data were analysed using the Statistical Package for Social Science for Windows (SPSS), version 18. At first, descriptive statistics were calculated, including measures of central tendency (mean and median), measures of variability (standard deviation), and relative numbers. After checking for normality of the data distribution with the Kolmogorov-Smirnov test, the differences between the groups in continuous variables were tested by Student's t-test, and the differences in frequencies were tested by Chi-square test. The differences were considered significant if the probability of the null hypothesis was less than 0.05. A multivariate logistic regression model was constructed to assess the simultaneous

influence of the independent variables in the development of hospital pneumonia and their mutual interaction.

RESULTS

There were 1140 newborns hospitalized at the Neonatology Department for longer than 48 hours during the study period, and 169 of them (14.82%) weighed less than 1500 grams at birth. In total, 73 (43.19%) newborns developed HIs. The most prevalent HI was hospital pneumonia (n=64, 87.67%), and only 9 newborns had some other type of HI (6 cases of urinary tract infection, 2 cases of sepsis and 1 case of omphalitis), and they were excluded from further study.

The median gestational age of newborns weighing less than 1500 grams was 29.58 weeks (range, 23-38 weeks), and the median birth weight was 1195 grams (range, 500-1500 grams). The reasons for admission to the Neonatology Department were premature birth (96.25%), respiratory distress syndrome (70.0%), asphyxiation (66.25%), infection at birth (8.0%), necrotizing enterocolitis (7.5%) and congenital anomalies (3.1%).

The results of the univariate analysis of risk factors for hospital pneumonia in newborns with birth weights below

Table 1. Risk factors for hospital pneumonia in newborns with birth weights below 1500 grams (univariate logistic regression analysis)

Variable	Neonates		χ^2 / t	*p* value
	Cases (n=64)	Controls (n=96)		
Maternal age (years)	29.66±6.53	30.29±6.24	t=0.619	0.537
Rupture of membranes	15 (23.4)	17 (17.7)	χ^2=0.788	0.375
≤ 24 hours	12 (18.8)	6 (6.3)	χ^2=6.009	0.014*
Caesarean section	30 (46.9)	47 (48.9)	χ^2=0.067	0.796
Placental detachment	8 (12.5)	3 (3.1)	χ^2=5.272	0.022*
Changed appearance of amniotic fluid	16 (25.0)	15 (15.6)	χ^2=2.161	0.142
Maternal disease in pregnancy**	17 (26.6)	17 (17.7)	χ^2=1.799	0.180
Vaginitis and urine tract infection in mother	9 (14.1)	4 (4.2)	χ^2=5.038	0.025*
Male gender	31 (48.4)	43 (44.8)	χ^2=0.205	0.650
Gestational age (weeks)	28.77±2.42	29.92±3.43	t=2.325	0.021*
≤27	20 (31.3)	22 (22.9)	χ^2=1.377	0.241
28-31	39 (60.9)	41(42.7)	χ^2=5.107	0.024*
32-36	5 (7.8)	30 (31.3)	χ^2=12.343	<0.001*
37-41	1 (1.6)	2 (2.1)	χ^2=0.037	0.812
Birth weight (grams)	1175.78±230.05	1196.30±284.43	t=0.482	0.637
≤1000 grams	17 (26.6)	24 (25.0)	χ^2=0.049	0.824
Apgar score in the first minute	4.44±2.25	5.53±2.33	t=2.137	0.034*
≤3	26 (40.6)	24 (25.0)	χ^2=4.364	0.037*
4-6	24 (37.5)	37 (38.5)	χ^2=0.018	0.894
7-10	14 (21.9)	35 (36.5)	χ^2=3.844	0.050
Apgar score in the fifth minute	4.95±1.91	5.92±2.06	t=2.982	0.003*
≤3	18 (28.1)	13 (13.5)	χ^2=5.228	0.022*
4-6	30 (46.9)	35 (36.5)	χ^2=1.727	0.189
7-10	17 (26.6)	46 (47.9)	χ^2=7.335	0.007*
Twin pregnancy	13 (20.3)	26 (27.1)	χ^2=0.955	0.328
Delivery before term	64 (100.0)	90 (93.8)	χ^2=4.156	0.041*
Respiratory distress syndrome	52 (81.3)	55 (57.3)	χ^2=9.950	0.002*
Asphyxia	44 (68.8)	59 (61.5)	χ^2=0.890	0.345
Stay of peripheral venous catheter (days)	34.72±16.35	12.07±7.80	t =-11.731	<0.001*
Mechanical ventilation	63 (98.4)	44 (45.8)	χ^2=47.968	<0.001*
Duration of mechanical ventilation (days)	20.86±14.17	7.16±5.76	t=-6.069	<0.001*
White cells count (x10^9/L)	18.71±12.55	18.26±14.00	t=-0.211	0.833
C-reactive protein (mg/L)	5.23±9.97	5.89±11.36	t=0.397	0.705
Durationof hospitalization (days)	73.22±27.74	27.48±21.62	t=-1.690	<0.001*

NOTE: Results are presented as $\bar{x}$ ±SD, if not otherwise indicated;
* significant di erence
** Diseases include: anemia, gestational diabetes mellitus, hypertension, vaginitis and urine tract infection

Table 2. Multivariate analysis (logistic regression) of risk factors for hospital pneumonia in newborns with birth weight below 1500 grams

Risk factors	B	OR	95% CI	p
Mechanical ventilation	4.233	68.893	4.285-1107.699	0.003
Duration of hospitalization (days)	0.051	1.052	1.017-1.088	0.003

NOTE: Only significant factors are presented
B – coe cient of logistic regression analysis; OR – Odds Ratio; CI – confidence interval

1500 grams are shown in Table 1. The following risk factors for hospital pneumonia reached the level of statistical significance: rupture of the membranes ≤ 24 hours before delivery (p=0.014), placental detachment (p=0.022), maternal urinary tract infection or vaginitis (p=0.025), lower gestational age (p=0.021), lower Apgar score in the first (p=0.034) and the fifth minute (p=0.003) after birth, premature birth (p=0.041), respiratory distress syndrome (p=0.002), longer stay with peripheral venous catheter (p<0.001), mechanical ventilation (p<0.001), longer duration of mechanical ventilation (p<0.001) and longer hospitalization (p<0.001).

Multivariate analysis identified only the following two independent risk factors for hospital pneumonia in newborns with birth weights below 1500 grams: mechanical ventilation (p=0.003, OR=68.893, 95% CI=4.285-1107.699) and longer hospitalization (p=0.003, OR=1.052, 95% CI=1.017-1.088) (Table 2).

Almost all of the pathogens isolated from the patients with pneumonia belonged to gram-negative bacteria (98.44%). More than half of all of the isolates were *Acinetobacter spp* (37.50%) and *Enterobacter spp* (18.75%). A detailed distribution of isolated pathogens is shown in Table 3.

DISCUSSION

Our study included a large number of potential risk factors for hospital pneumonia in newborns with birth weights below 1500 grams. However, although the univariate analysis identified many significant risk factors, the multivariate analysis showed that there were only two independent risk factors for hospital pneumonia as follows: mechanical ventilation and longer hospitalization.

Table 3. Distribution of isolates from respiratory tract in newborns with birth weight less than 1500 grams, who developed hospital pneumonia

Pathogens	n (%)
Acinetobacter spp	23 (37.50)
Enterobacter spp	12 (18.75)
Klebsiella spp	10 (15.63)
Escherichia coli	8 (12.50)
Stenotrophomonas maltophilia	6 (9.38)
Pseudomonas aeruginosa	4 (6.25)
Coagulase negative staphylococcus	1 (1.56)
Total	64 (100.0)

It was not surprising that mechanical ventilation was a risk factor for hospital pneumonia in our patients because the same was shown in other studies (11-13). Yalaz and associates (14) had found that the highest incidence of hospital pneumonia was observed in infants with birth weights ≤1000 grams who were on mechanical ventilation. This intervention is often necessary for infants with respiratory failure, poor gas exchange, increased effort for breathing, apnea of prematurity, and/or the need for surfactant-replacement therapy. However, invasive respiratory support is associated with lung injury and adverse neurologic outcomes, and it sometimes allows entry of infectious agents that cause pneumonia. It was recommended that exposure to mechanical ventilation should be limited (15, 16). Some authors suggest that significant reduction in the frequency of pneumonia could be achieved by non-invasive ventilation instead, such as nasal continuous positive pressure ventilation or nasal synchronized intermittent mandatory ventilation (14).

Patients on mechanical ventilation need frequent and intensive contact with medical staff, which may disrupt protective barriers. Additionally, these patient are frequently submitted to more invasive diagnostic and therapeutic procedures (venous catheterization, urinary bladder catheterization, etc.), which create additional opportunities for the entrance of bacterial pathogens (17). Patients in countries with limited health resources are especially vulnerable because understaffing and work overload lead to errors and omissions in aseptic techniques and introduction of bacteria into blood or the respiratory or urinary tract.

Our study also linked the occurrence of hospital pneumonia with prolonged hospitalization (OR=1.052, 95% CI = 1.017-1.088), which was expected because previous studies had demonstrated associations of this factor with other types of HIs (18-20). The patients in the hospital frequently become colonized with multiresistant strains of bacteria, and when the delicate balance of the normal bodily microbial flora is disrupted by indiscriminate use of antimicrobial agents and normal body defences are impaired by the underlying disease, they become easy prey for such strains. The hospital environment, especially that of intensive care units, is home of many multiresistant bacterial strains. It was noted that in newborns who were hospitalized for prolonged periods, the normal microbial flora was replaced by multiresistant bacterial strains from the hospital environment (21). Additionally, prolonged hospitalization increases chances for transfer of multiresistant bacteria from newborns with infection to those without.

It is interesting that none of the factors that were related to the mother, the pregnancy or the newborns themselves had a significant influence on the emergence of hospital pneumonia in this group of patients. Such results support the theory that hospital pneumonia in newborns with birth weights below 1500 grams is primarily a "device-associated, health care-associated infection". To decrease the frequency of hospital pneumonia in this group of newborns, utilization of mechanical ventilation should be limited to when absolutely necessary.

The distribution of bacterial pathogens in our study was similar to the distributions described in other studies from developing countries, and gram-negative bacteria were the most frequent causative agents of hospital pneumonia (11, 22, 23). It is important to emphasize that infections with these pathogens are associated with high mortality rates. In our study, more than half of the isolated strains were *Acinetobacter spp* (37.50%) and *Enterobacter spp* (18.75%). The geographical variation in the prevalence of certain gram-negative microorganisms may be caused by the frequency of mechanical ventilation, inappropriate sampling of respiratory tract secretions, and biofilm formation. However, in developed countries in Europe and North America, the predominant cause of pneumonia in the NICUs are gram-positive cocci, which are now more often resistant to many antibiotics than previously described (e.g., methicillin-resistant *S. aureus* (MRSA)) (24-26). Because every unit has its own unique endemic flora, active surveillance for HIs is critical to guiding empiric antibiotic therapy and implementing effective preventive strategies.

It was proven that simple and low-cost measures can reduce the incidence of HIs (22, 27). The lessons learned from Western European countries (28) support our efforts in organization and regular training of the infection control team at our hospital. Although infection control teams at hospitals have only recently become obligatory in Serbia, we had established an infection control team at our hospital (with qualified epidemiologist and infection control nurses) five years ago. This team is responsible for the daily surveillance of all newborns, early detection of symptoms and signs of an infection in close collaboration with responsible clinicians and organization of infection control and prevention measures. Additional study is needed to estimate the effectiveness of these measures in our hospital.

Our study had certain limitations. First, the study was conducted in a single hospital; therefore, the results could be influenced by the choice of patients and the peculiarities of local medical practices. Second, we could not reliably estimate the occurrence of the transfer of pathogens between the patients.

In conclusion, our study showed that mechanical ventilation and prolonged hospitalization were significant risk factors for the development of hospital pneumonia in newborns with birth weights below 1500 grams. Low birth weight newborns with these risk factors should receive more stringent care with administration of special preventive measures to avoid development of hospital pneumonia.

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CORRELATIONS BETWEEN CLINICAL PARAMETERS AND HEALTH RELATED QUALITY OF LIFE IN POSTMENOPAUSAL OSTEOPOROTIC WOMEN

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KORELACIJA IZMEĐU KLINIČKIH PARAMETARA I KVALITETA ŽIVOTA KOD PACIJENTKINJA SA OSTEOPOROZOM U POSTMENOPAUZI

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ABSTRACT

e purpose of this study was to assess the correlation between health-related quality of life (HRQoL) and clinically relevant osteodensitometric and biochemical parameters in postmenopausal osteoporotic women. Bone mineral density (BMD) and T scores of the lumbar vertebrae and femoral neck were assessed in 100 osteoporotic women (56 without previous fractures and 44 with previous fractures) using dual x-ray absorptiometry. e Fracture Risk Assessment Tool (FRAX) index for major osteoporotic and hip fractures was calculated based on demographic data and hip BMD. Venous blood samples were taken from each subject for biochemical analysis (serum calcium, phosphorus, alkaline phosphatase and vitamin D levels). HRQoL was assessed using the QUALEFFO-41 questionnaire (domains: Health perception, Pain, and Physical, Social and Mental function). Basic participant characteristics (age, menopause length, body mass index, smoking habits, hereditary tendency towards fracture, fracture history) correlated with some of the QUALEFFO-41 domains, but the correlation coe cients were low (r<0.3), except in the case of the correlation between Pain and fracture history (r=0.638). Of the six variables included in the multiple regression model, fracture history was shown to be the most significant predictor with respect to the following three QUALEFFO-41 domains: Pain (b=20.511), Social function (b=2.548) and Health perception (b=3.185). Correlation analysis showed that after adjustment for basic characteristics, BMD and T score of the femoral neck and Pain (r=0.331 and r=0.449, respectively), Social function (r=0.422 and r=0.419) and Health perception (r=0.434 for T score of the femoral neck) exhibited the strongest correlations. Vitamin D was negatively correlated with Mental function, while the other biochemical parameters exhibited variable correlations with the QUALEFFO-41 domains (r≈0.2-0.5). Our study confirmed the previously established relationship between BMD of the femoral neck and HRQoL in patients with osteoporosis and demonstrated correlations between various blood bone metabolism parameters and HRQoL that have not been previously investigated.

Keywords: osteoporosis; quality of life; bone mineral density; FRAX; Vitamin D

SAŽETAK

Cilj studije bio je pronalaženje korelacija između kvaliteta života postmenopauzičnih pacijentkinja sa osteoporozom i klinički relevantnih osteodenzitometrijskih i biohemijskih parametara. Mineralna gustina kostiju i T skor u nivou lumbalne kičme i vrata butne kosti mereni su, putem dvoenergetske apsorpciometrije X zracima, na uzorku od 100 osteoporotičnih žena. Procena rizika od nastanka glavnih osteoporotičnih fraktura i frakture kuka je vršena na osnovu demografskih podataka i mineralne gustine kosti u nivou vrata butne kosti (FRAX indeks). Svakoj pacijentkinji je uzet uzorak venske krvi radi biohemijskih analiza (serumski nivoi kalcijuma, fosfora, alkalne fosfataze i vitamina D). Kvalitet života procenjen je na osnovu QUALEFFO-41 upitnika (domeni: Percepcija zdravlja, Bol, Fizičko, Socijalno i Mentalno funkcionisanje). Bazične karakteristike pacijentkinja (starost, trajanje menopauze, indeks telesne mase, pušenje, pozitivan hereditet za frakturu butne kosti, prethodna fraktura) korelirale su sa nekim domenima QUALEFFO-41 upitnika, ali su koeficijenti korelacije bili niski (r<0.3), osim u slučaju veze između domena Bol i prethodne frakture (r=0.638). Od ovih šest odabranih prediktivnih varijabli u modelu multiple regresije, prethodna fraktura se pokazala kao najznačajnija prediktivna varijabla za tri QUALEFFO-41 domena: Bol (b=20.511), Socijalna funkcija (b=2.548) i Percepcija zdravlja (b=3.185). Korelaciona analiza je pokazala da su, nakon prilagođavanja bazičnim karakteristikama ispitanica, najjače korelacije pronađene između mineralne gustine kosti i T skora u nivou vrata butne kosti i domena Bol (r=0.331 and r=0.449, respektivno), Socijalna funkcija (r=0.422 i r=0.419) i Percepcija zdravlja (r=0.434 za T skor u nivou vrata butne kosti). Vitamin D je negativno korelirao sa domenom Mentalna funkcija, dok su drugi biohemijski parametri sporadično korelirali sa domenima QUALEFFO-41 (r≈0.2-0.5). Rezultati naše studije potvrđuju vezu između mineralne gustine kosti u nivou vrata butne kosti i kvaliteta života pacijentkinja sa osteoporozom, i ukazuju na korelacije između biohemijskih parametara koštanog metabolizma u krvi i kvaliteta života, što u ranijim studijama nije ispitivano.

Ključne reči: *osteoporoza; kvalitet života; mineralna gustina kosti; FRAX indeks; vitamin D*

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ABBREVIATIONS

BMD - Bone Mineral Density
DXA - Dual X-ray Absorptiometry
FRAX - Fracture Risk Assessment Tool
FRAX FNF - FRAX index for Femoral Neck Fracture
FRAX MOF - FRAX index for Major Osteoporotic Fracture
HRQoL – Health-Related Quality of Life
QoL - Quality of Life
QUALEFFO - Quality of Life Questionnaire of the European Foundation for Osteoporosis

INTRODUCTION

Osteoporosis is a condition characterized by systemic microarchitectural deterioration of bone tissue, impairments of bone mass and strength, and increases in bone fragility and fracture susceptibility (1). Osteoporosis and its associated fractures have been documented for many years and have commonly been viewed as inevitable consequences of the ageing process (2). Given the current prevalence of the condition (3) the ageing of the population, and the clinical, economic, and social aspects of the disease, osteoporosis may be considered an emerging major public health burden (2, 4).

Low bone mineral density (BMD) is one of the most significant risk factors for fracture (3). BMD can be assessed with dual x-ray absorptiometry (DXA), and osteoporosis is defined as a BMD of 2.5 standard deviations or more below peak bone mass, which is represented by the T score (5). Some new fracture risk assessment tools have been developed (6), including the World Health Organization fracture risk assessment tool (FRAX index), an algorithm that integrates clinical risk factors with BMD to predict an individual's 10-year risk of sustaining a hip fracture or another major osteoporotic fracture (7, 8). Although osteoporosis may exist without fractures in many individuals, in postmenopausal women, osteoporotic fractures typically occur in the distal forearm, the spine and the upper femur (9). From a patient perspective, fractures and the subsequent loss of mobility and autonomy caused by fractures often result in a major decrease in quality of life (QoL). Thus, QoL measures have gained increasing attention as clinically relevant patient-centred endpoints in clinical trials (10).

The effects of osteoporosis and osteoporosis-related fractures on QoL are commonly assessed using health-related QoL (HRQoL) tools, such as the Quality of Life Questionnaire of the European Foundation for Osteoporosis (QUALEFFO) (11). The relationship between HRQoL and osteoporotic fracture has been extensively researched and reviewed (12), while the relationship between HRQoL and osteoporosis without fractures has been investigated in only a few studies, the results of which are ambiguous (9, 13). It has been suggested that patients often experience pain with osteoporosis even before fractures occur (13). This pain may be related to fatigue or bone microarchitectural failure, which may be associated with low BMD (14). The available literature shows that BMD of the upper femur is correlated with some HRQoL domains (10, 15), while the relationship between HRQoL and BMD of the lumbar spine is not as clear (15, 16).

Vitamin D plays a critical role in bone metabolism (17); thus, its deficiency represents a major determinant of osteoporosis fracture risk (18-21). It has been reported that inadequate serum vitamin D levels are associated with low calcium absorption, secondary hyperparathyroidism, increased bone turnover, bone loss and low BMD (17, 21-23). The increase in fracture risk caused by inadequate vitamin D levels is not merely the result of decreases in BMD. In recent years, vitamin D deficiency has been associated with loss of muscle mass, muscle weakness, and musculoskeletal pain (24-27), all of which significantly contribute to falls. Generally, little is known regarding the relationship between serum vitamin D levels and HRQoL in osteoporosis, but a very recent study suggested that low serum vitamin D levels were a significant determinant of HRQoL in osteoporotic women independent of the conventional factors that reduce HRQoL (28).

The aim of our research was to assess the correlations between HRQoL and clinically relevant osteodensitometric (BMD and T scores of the lumbar spine and femoral neck and FRAX indices of the hip and other major osteoporotic fractures) and biochemical parameters (serum calcium, phosphorus, alkaline phosphatase and vitamin D levels) in postmenopausal osteoporotic women. We also aimed to explore the influences of basic subject characteristics, especially fracture history, on osteoporotic patient quality of life.

PATIENTS AND METHODS

This cross-sectional study included a group of 100 osteoporotic patients (56 without a history of fracture and 44 with a history of fracture) from the Clinical Centre Kragujevac, Serbia. Data collection was performed from January to December 2012.

The study was approved by the Ethics Committee of the Clinical Centre Kragujevac. After being informed of the study's purpose, risks and benefits, all patients provided written informed consent to participate in the study.

Diagnoses of osteoporosis (T score ≤-2.5) were based on the results of DXA scans performed by an experienced rheumatologist using a Discovery TM osteodensitometer

(Hologic, Bedford, MA). Patients with secondary osteoporosis or some other chronic disease affecting quality of life (tumour, chronic renal failure, chronic pulmonary insufficiency, cardiovascular disease, uncontrolled hypertension, diabetes, rheumatoid arthritis, and severe hearing, vision, and cognitive function disturbances), as well as patients receiving glucocorticoids and patients with a history of fracture within the last 6 months, were excluded from the study.

The study comprised the following steps: 1) anamnesis (age, menopause occurrence and length, fracture occurrence, hereditary tendency towards fracture, smoking habits, and alcohol consumption), 2) patients completed the QUALEFFO-41 questionnaire (the questionnaire was described and validated for use in Serbian populations by Tadic and colleagues (29)), 3) patients underwent osteodensitometric measurements (BMD, T score) of the femoral neck (BMD FN, T score FN) and lumbar vertebrae (BMD LV, T score LV), 4) patients underwent anthropometric measurements (body mass index (BMI)), 5) patient 10-year fracture risks (FRAX index) for major osteoporotic fracture (spine, forearm, hip or shoulder fracture; FRAX MOF) and femoral neck fracture (FRAX FNF) were calculated based on the data obtained using the abovementioned measurements and a computer program (30), and 6) patients provided blood samples for biochemical analysis (serum vitamin D (Vit D), calcium (Ca), phosphorus (P), alkaline phosphatase (ALP)), which was performed using routine methods in the Central Laboratory of the Clinical Centre Kragujevac.

The QUALEFFO-41 questionnaire comprises the following five domains (41 items in total): Pain (5 items), Physical function (17 items), Social function (7 items), Health perception (3 items) and Mental function (9 items). Most of the items have 5 answer options, although some have 3 or 4 answer options. Most items are scored in reverse order, i.e., the lowest number on the scale corresponds to the best answer, and the highest number corresponds to the worst answer (with the exception of 6 items). The score for each domain is calculated as the average value of all the answered items, which are linearly transformed on a scale of 0-100. Higher scores are indicative of worse HRQoL.

Statistical analysis was performed using SPSS 19.0 for Windows. Data were analysed using two independent samples tests, bivariate correlation, partial correlation and multiple linear regression. The results are expressed as means ± standard deviations and as numbers and percentages. The alpha level for significance was set to $p<0.05$.

RESULTS

Correlation and multiple regression analyses were conducted to examine the relationships between QUELEFFO-41 domains and basic patient characteristics. Tables 1 summarize the descriptive statistics and analysis results.

As seen in Table 1, all parameters except hereditary tendency towards fracture were significantly correlated with the Pain domain, but the coefficients of those correlations were poor, except in the case of fracture history, whose relationship with the Pain domain was moderately strong. The multiple regression model significantly predicted Pain scores based on fracture history after adjustment for the other variables included in the model, indicating that patients with fracture histories were likely to have higher Pain scores. Two independent samples tests showed that patients without and with fracture histories differed significantly with respect to their scores in this domain (45.53±8.97 vs 65.45±15.98, P=0.000). No other osteodensitometric variables contributed to the multiple regression model.

Physical function domain scores were not significantly correlated with any basic patient characteristics, and the multiple regression model did not significantly predict the score of this domain.

Regarding the Social function domain, body mass index was significantly negatively associated with social function, and fracture history was positively correlated with social function; however, the strength of this correlation was poor. The multiple regression model significantly predicted the score of this domain. Age exhibited significant negative regression weight, and fracture history exhibited positive regression weight; thus, after adjustment for the other variables in the model, older patients were expected to have lower Social function scores, while patients with fracture histories were expected to have higher Social function scores. Two independent samples tests showed that patients without and with fracture histories significantly differed with respect to their scores in this domain (31.07±4.97 *vs.* 33.45±3.60, p=0.012).

Hereditary tendency towards fracture was negatively correlated with the Health perception domain, and fracture history was positively correlated with the Health perception domain (poor correlation strength). The multiple regression model significantly predicted the score in this domain. Patients with hereditary tendency towards fracture were expected to have lower Health perception scores, and patients with history of fracture were expected to have higher Health perception scores. Two independent samples test showed that patients without and with fracture histories significantly differed with respect to their scores in this domain (31.21±5.21 *vs.* 34.63±7.01, p=0.006).

The Mental function domain was significantly correlated only with smoking (very low negative correlation). The multiple regression model did not significantly predict scores in this domain.

The mean scores that patients achieved in each QUELEFFO-41 domain are shown in Table 1, while the mean values of patient osteodensitometric parameters and biochemical parameters are shown in Table 2.

Correlation analysis (Table 3) showed that among the 100 osteoporotic women enrolled in this study, the Pain domain was significantly negatively correlated with T

Table 1. Summary statistics, correlations and regression analysis results: a prediction model based on basic patient characteristics (*p<0.05).

Prediction model: Basic participant characteristics					
Age (X±SD)	Menopause duration (X±SD)	BMI (X±SD)	Smoking (no/yes)	Hereditary tendency towards fracture (no/yes)	Fracture history (no/yes)
65.39±7.69	18.25±7.81	24.59±2.85	72/28	94/6	56/44

Pain score (X±SD): 54.166±16.256 R^2 = 0.428, F(6, 89) = 11.095, p = 0.000			
Variable	Correlation coe cient	Multiple regression weights	
		b	β
Age	0.187*	-0.308	-0.146
Menopause duration	0.174*	0.184	0.089
BMI	-0.330*	-0.410	-0.072
Smoking	0.192*	0.091	0.003
Hereditary tendency towards fracture	-0.168	-5.482	-0.082
Fracture history	0.638*	20.511*	0.629

Physical function score (X±SD): 7.416±0.634 R^2 = 0.064, F(6, 89) = 1.010, p = 0.424			
Variable	Correlation coe cient	Multiple regression weights	
		b	β
Age	-0.091	0.001	0.018
Menopause duration	-0.140	-0.016	-0.202
BMI	-0.068	-0.023	-0.103
Smoking	0.155	0.235	0.170
Hereditary tendency towards fracture	0.020	0.004	0.002
Fracture history	0.090	0.053	0.042

Social function score (X±SD): 31.979±4.572 R^2 = 0.171, F(6, 89) = 3.061, p = 0.009			
Variable	Correlation coe cient	Multiple regression weights	
		b	β
Age	-0.074	-0.290*	-0.479
Menopause duration	0.019	0.188	0.316
BMI	-0.264*	-0.292	-0.180
Smoking	0.087	0.720	0.071
Hereditary tendency towards fracture	0.007	0.937	0.049
Fracture history	0.276*	2.548*	0.273

Health perception score (X±SD): 32.677±6.356 R^2 = 0.143, F(6, 89) = 2.474, p = 0.029			
Variable	Correlation coe cient	Multiple regression weights	
		b	β
Age	0.116	-0.133	-0.161
Menopause duration	0.137	0.144	0.178
BMI	-0.162	0.016	0.007
Smoking	0.042	0.230	0.017
Hereditary tendency towards fracture	-0.273*	-6.495*	-0.250
Fracture history	0.277*	3.185	0.251

Mental function score (X±SD): 30.604±2.568 R^2 = 0.102, F(6, 89) = 1.685, p = 0.134			
Variable	Correlation coe cient	Multiple regression weights	
		b	β
Age	-0.143	-0.083	-0.240
Menopause duration	-0.048	0.064	0.187
BMI	-0.164	-0.220*	-0.235
Smoking	-0.170*	-0.360	-0.062
Hereditary tendency towards fracture	0.042	0.968	0.088
Fracture history	-0.145	-0.887	-0.165

Table 2. Participants' osteodensitometric and biochemical parameters (X±SD).

BMD LV (gr/cm²)	T score LV (SD)	BMD FN (gr/cm²)	T score FN (SD)	FRAX MOF (%)	FRAX FNF (%)	Vit D (ng/ml)	Ca (mmol/l)	P (mmol/l)	ALP (U/l)
0.74±0.09	-2.65±0.94	0.69±0.10	-2.14±1.30	12.29±10.94	3.24±7.46	21.31±6.05	2.31±0.21	1.18±0.25	70.09±14.92

Table 3. Correlations between clinical parameters and QUELEFFO-41 domains in 100 osteoporotic patients (†Adjusted for age, menopause duration, BMI, smoking habits, hereditary tendency towards fracture and fracture history; *p<0.05).

QUALEFFO-41 domain	Correlation coe cient	BMD LV	T score LV	BMD FN	T score FN	FRAX MOF	FRAX FNF	Vit D	Ca	P	ALP
Pain	Crude	-0.224*	-0.283*	0.144	0.004	0.270*	0.130	-0.106	0.101	-0.243	-0.015
	Adjusted†	-0.090	-0.042	0.331*	0.449*	-0.155	-0.094	-0.090	-0.042	0.331*	0.449*
Physical function	Crude	-0.074	-0.147	-0.004	-0.039	0.087	0.087	0.222	-0.233	-0.414*	-0.062
	Adjusted†	-0.141	-0.096	-0.045	-00.050	0.143	0.101	-0.141	-0.096	-0.045	-0.050
Social function	Crude	-0.113	-0.195	0.345*	0.317*	-0.066	-0.095	0.002	0.304*	-0.263	-0.135
	Adjusted†	-0.069	0.025	0.422*	0.419*	-0.205	-0.144	-0.069	0.025	-0.422*	0.419*
Health perception	Crude	-0.097	-0.092	0.189	0.009	0.069	-0.061	-0.137	-0.026	0.163	0.065
	Adjusted†	-0.027	-0.043	0.176	0.434*	-0.363*	-0.277*	-0.027	-0.043	0.176	0.434*
Mental function	Crude	-0.221*	-0.187	0.198	0.162	-0.226*	-0.154	-0.418*	0.263*	-0.380*	-0.302*
	Adjusted†	-0.245*	-0.253*	0.166	0.252*	-0.259*	-0.165	-0.245*	0.253*	0.166	0.252*

score LV and BMD LV and was positively correlated with FRAX MOF; however, these correlation coefficients were very low and were not statistically significant following adjustment for possible confounding variables (basic patient characteristics). However, after adjustment for basic patient characteristics, BMD FN, T score FN and P and ALP levels exhibited significant positive correlations with Pain scores, and the strength of the linear relationship was fair (correlation coefficient value 0.3-0.5).

Physical function domain scores were significantly negatively correlated with P levels, and the strength of the correlation coefficient was fair; however, after adjustment for confounding variables, this correlation lost its significance.

Social function domain scores were significantly positively correlated with BMD FN, T score FN and Ca levels. After adjustment, the correlation coefficients pertaining to the relationships between the Social function domain and BMD FN and T score FN increased, while Ca levels were found to have a non-significant correlation with this QUELEFFO-41 domain. Furthermore, after adjustment for confounding variables, P and ALP exhibited significant positive correlations with the Social function domain, and the strengths of these correlation coefficients were fair.

Before adjustment for confounding variables, Health perception scores were not significantly correlated with any osteodensitometric or biochemical variables; however, after adjustment for confounding variables, T score FN and ALP were positively correlated with the Social function domain (fair correlation strength), and FRAX MOF and FRAX FNF were negatively correlated with the Social function domain (low to fair correlation strength).

Finally, Mental function domain scores were significantly negatively correlated with BMD LV, FRAX MOF and Vit D, P, and ALP levels and positively correlated with Ca (poor to fair correlation strength). After adjustment for confounding factors, the correlation coefficients pertaining to the relationships between Mental function and the indicated osteodensitometric and biochemical parameters (BMD LV, T score LV, T score FN, FRAX MOF, and Vit D, Ca, ALP levels) were poor.

DISCUSSION

HRQoL represents an aspect of QoL that is related to patient physical, emotional, and social well-being. Previous studies have shown that HRQoL is generally impaired in women who are being treated for postmenopausal osteoporosis, but the extent to which HRQoL correlates with different clinical parameters has not been determined. Thus, the aim of our research was to evaluate the relationships between clinical (osteodensitometric and biochemical) osteoporosis markers and various HRQoL domains and to evaluate the influences of basic patient characteristics (age, BMI, and fracture history) on HRQoL.

The results of our study showed that some of the six basic participant characteristics (age, menopause length, body mass index, smoking habits, hereditary tendency toward fracture, fracture history) were significantly correlated with some QUELEFFO-41 domains; however, with the exception of the correlation between fracture history and pain score, those correlations were very weak (correlation coefficients lower than 0.3). The correlation coefficient pertaining to the relationship between fracture histories and Pain scores was 0.638, indicating that this relationship was moderately strong. This was confirmed by multiple regression analysis, which showed that fracture history had a significantly positive regression weight; thus, if the

other five variables remained constant, having a fracture would increase the pain score by approximately twenty points (i.e., patients with fractures would tend to exhibit worse pain scores). This finding is consistent with those of a study by Bianchi et al. (13), who reported that 66 % of osteoporotic women with history of fracture reported pain, while 40 % of women who have never experienced a fracture reported pain. This is understandable, as pain caused by a vertebral fracture may last for three years or more (31, 32) because vertebral fractures cannot be repaired and can cause permanent spinal deformities. Hip fracture, the most serious osteoporotic fracture (2), is even more painful. Furthermore, hip fracture is associated with a slow and often incomplete recovery (33).

Fracture history was also significantly correlated with the Social function and Health perception domains in our study, although the strengths of these correlations were weaker than that of the correlation between fracture history and the Pain domain. These results are consistent with those of numerous other studies showing that among patients with postmenopausal osteoporosis, women who have previously experienced fractures have lower HRQoL than women who have not experienced fractures (34-38). For example, Guillemin and colleagues (39) explored how a range of clinical characteristics may contribute to reductions in HRQoL and showed that previous fractures were associated with lower health utility scores by 10.3 % and that fear of falling, which may induce fractures, was also significantly related to worse HRQoL. Osteoporotic fractures affect the musculoskeletal system and cause chronic pain, functional disability, mood changes and HRQoL impairment, but knowledge of an increased fracture risk can also affect the daily activities and HRQoL of subjects with low BMD (9). Pain, which may exist even before fractures occur, as well as numerous other comorbidities, may be responsible for this. These patients are deeply worried and anxious and consequently experience psychosocial behaviour changes resulting in depression and low self-esteem, which have negative effects on health perception (40).

The possibility that HRQoL may be affected by low BMD has not been extensively investigated, and many studies do not differentiate between osteoporosis (low BMD) and fracture. Many cross-sectional prospective population studies indicate that the risk of fracture increases by a factor of 1.5 to 3.0 for each standard deviation decrease in BMD (41). However, although bone mass is an important component of fracture risk, several risk factors, such as age, BMI, previous fragility fractures, parental hip fracture history, rheumatoid arthritis, glucocorticoid use, and active cigarette smoking, must also be taken into account (5). Thus, we attempted to determine the correlations between BMD, T score and FRAX index and patient HRQoL. Previously, Bianchi et al. showed that the correlations between BMD T scores and QUALEFFO-41 scores were significant after adjustment for age, social status (e.g., education, marriage, and living alone or not) and lifestyle habits (e.g., smoking and drinking) in osteoporotic patients and that the correlations between BMD T scores and the Physical function, Social function and Health perception domains were independent of the presence of fractures (13). Correlation analysis in our study showed that correlation coefficients increased in most cases when the correlations between osteodensitometric parameters and HRQoL were adjusted for possible confounding variables (basic participant characteristics). However, those coefficients were still poor (<0.3) or fair (0.3 - 0.5). The highest correlation coefficients pertained to the relationships between BMD FN and T score FN and Pain, Social function and Health perception scores. Wilson's analysis reported that four out of six data sets pertaining to the relationship between HRQoL and BMD of the upper femur demonstrated significant associations between these parameters (9). Additionally, multivariate analysis performed in a study by Romagnoli et al. showed that BMD of the upper femur was associated with Physical function and Health perception scores, as well as total QUALEFFO-41 scores (10). The association between BMD of the lumbar spine and HRQoL was previously reported in only one study and only in the control group (15). The absence of a correlation between HRQoL and BMD of the lumbar spine in previous studies may be explained by the incidence of degenerative change in the lumbar spine region, which can cause artificial increases in BMD, as measured by DXA (42).

Vitamin D insufficiency and deficiency are directly associated with various morbidities in elderly people (24); however, little is known regarding the relationship between serum vitamin D levels and HRQoL in osteoporosis. Although there is no consensus regarding optimal vitamin D levels, it is recommended that patients maintain serum vitamin D levels of more than 30 ng/ml to ensure maximum calcium absorption and optimal health (23). Levels below 20 ng/ml are considered vitamin D insufficiency, while levels below 12 ng/ml are considered vitamin D deficiency (5). Several studies have found that more than 50 % of patients with osteoporosis have inadequate vitamin D levels (19). A systematic review regarding vitamin D inadequacy among postmenopausal women reported ranges of 12.5-76 % for vitamin D deficiency and 1.6-86 % for vitamin D insufficiency among osteoporotic women (43). Given the role of vitamin D in bone metabolism (17), as well as the effects of vitamin D on the neuromuscular system (44, 45), the absence of strong literature data regarding the relationship between its levels and osteoporotic patient HRQoL is surprising. Few subjects in recent years have been the source of as much discussion in the medical community as vitamin D; thus, we included its measurement in our study. Furthermore, we included the measurements of other important bone health-related biochemical parameters, such as minerals (calcium and phosphorus) and the enzyme alkaline phosphatase, a marker of bone formation.

A recently published study by Ohta and coworkers (28) reported that serum vitamin D levels were a significant determinant of QoL in osteoporotic women independent of the conventional factors that reduce QoL. The results of

our study showed that vitamin D levels were significantly correlated with only Mental function, both before and after adjusting for potential confounding variables. This finding is consistent with those of some previous studies, as vitamin D deficiency has been shown to be related to depression, which may contribute to decreases in QoL (19). Basaran and coworkers also reported a significant relationship between vitamin D and Physical function (19), which was not noted in our study. Generally, many studies have shown associations between vitamin D and physical functions (46, 47), but few have directly investigated the relationship between vitamin D status and HRQoL in osteoporosis patients.

More than 99 % of Ca and 80-85 % of P in the human body are located in bones; however, these elements may also be found in blood and cells and play multiple important roles elsewhere in the body (48). For instance, Ca plays an important role in neuromuscular activity, while P plays an important role in energy metabolism. Thus, we hypothesized that the levels of these elements may be correlated with Physical function scores. In our study, Physical function scores correlated significantly (negatively) with only P levels; however, after adjustment for basic characteristics, this relationship lost its significance. Although Ca plays a role in both pain and antinociception (49), it was not correlated with Pain scores, while P and ALP, after adjustment for potential confounding variables, were positively correlated with Pain, and the strength of the correlation coefficient was fair. Correlation analysis suggested that patients with higher P levels exhibited better mental and psychosocial health, while patients with higher serum Ca levels exhibited worse mental and psychosocial health. In the general population, high serum Ca levels are associated with faster declines in cognitive function in individuals over the age of 75 (50), which may explain the positive correlations between Ca and Mental and Social function. The correlation coefficients for the abovementioned relationships ranged from poor to fair ($r \approx 0.2$-0.4), which may be explained by the ages of our subjects and the fact that all of the biochemical parameters of our subjects were within their reference ranges. The strongest correlation coefficients ($r \approx 0.4$-0.5) between biochemical parameters and quality of life that were noted in our study were those pertaining to the relationship between ALP, an important marker of bone formation (51), and Pain, Health perception, Social function and Mental function. Our results indicate that after adjustment for basic subject characteristics, higher ALP levels were correlated with worse Pain, Health perception, Social function and Mental function scores. Ross et al. (52) showed that mean baseline serum bone ALP levels were significantly higher among women with a history of osteoporotic fracture than among women without a history of fracture and that in separate age-adjusted logistic regression models, serum bone ALP and calcaneus BMD were each significantly associated with new fractures. The results of that investigation indicated that increased bone turnover is significantly associated with an increased risk of osteoporotic fracture in postmenopausal women and that this association is similar in magnitude to and independent of the association between BMD and QUALEFO-41 domains (52). Thus, the positive correlations between ALP and QUALEFFO-41 domains that were observed in this study are similar to those observed between BMD FN and T score FN and QUALEFFO-41 domains and thus seem logical.

Our study included a limited number of patients compared to larger multinational investigations, but it is among first to explore this public burden in our country. This is important, as nutrition varies differently among races and geographical areas, and dietary intake of minerals and vitamins, especially Ca, P and vitamin D, is important for bone metabolism.

Our study confirmed the existence of a previously established relationship between BMD of the femoral neck and HRQoL in patients with osteoporosis and demonstrated correlations between blood biochemical bone metabolism parameters and HRQoL that have not been previously investigated.

The findings of our study highlight the need to include HRQoL measures in evaluations of the risk-benefit profiles of osteoporosis medications, as well as in fracture prevention programs. HRQoL should be included in comprehensive assessments of the costs of osteoporosis, including the costs associated with fracture-related morbidity. Differences in HRQoL should be taken into account when setting the priorities of health care programs. HRQoL assessments are useful for performing clinical trials and for assessing disease burdens.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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OXIDATIVE STRESS IN TRAINING, OVERTRAINING AND DETRAINING: FROM EXPERIMENTAL TO APPLIED RESEARCH

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OKSIDATIVNI STRES U TRENIRANOSTI, PRETRENIRANOSTI I DETRENIRANOSTI: OD EKSPERIMENTALNIIH DO PRIMENJENIH ISTRAŽIVANJA

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ABSTRACT

According to the hormesis theory, the responses of biological systems to stressors in exercise training may be explained by a U-shaped curve with inactivity and overtraining as the two endpoints. Both of these endpoints decrease physiological functions. Markers of oxidative stress may be important parameters for biological monitoring of athletes. Numerous studies have shown that acute exercise has the potential to induce oxidative stress, but regular exposure to an increased level of prooxidants leads to upregulation of the endogenous antioxidative defence system (ADS) of an athlete. Studies that explored the redox state in athletes during the competitive season showed that the antioxidative status changes depending on the training load and training phase. During the training season, a state of fatigue known as overtraining may occur, which results from an excessive training load. Oxidative stress has been suggested as one of the causes of overtraining syndrome. Based on the existing studies, it can be said that a connection exists, but whether oxidative stress is a cause or a consequence of overtraining is yet to be clarified. Furthermore, detraining (training reduction or cessation) leads to a partial or complete loss of training-induced anatomical, physiological and performance adaptations; therefore, it seems reasonable to assume that changes in ADS are also reversible.

Keywords: *redox state, athletes, overtraining syndrome, detraining, adaptation*

SAŽETAK

Prema teoriji hormezije, odgovori bioloških sistema na stresore koji deluju tokom treninga mogu da se objasne "U" krivom, čija su dva kraja neaktivnost i pretreniranost. Obe navedene krajnosti imaju za posledicu pogoršanje fizioloških funkcija. Markeri oksidativnog stresa mogu biti važni parametri biološkog praćenja sportista. Veliki broj studija je pokazao da akutno vežbanje ima potencijal da izazove oksidativni stres, ali da redovno izlaganje tela povišenom nivou prooksidanata vodi ushodnoj regulaciji endogenog antioksidativnog sistema sportiste. Studije koje su ispitivale redoks status sportista tokom takmičarske sezone pokazale su da se antioksidativni status menja u zavisnosti od trenažnog opterećenja i trenažne faze. Tokom trenažne sezone, kao posledica preteranog opterećenja, može nastati stanje umora poznato kao pretreniranost. Oksidativni stres je predložen kao jedan od uzroka sindroma pretreniranosti. Na osnovu postojećih studija, može se reći da veza postoji, ali da li je oksidativni stres uzrok ili posledica pretreniranosti tek treba da bude razjašnjeno. S druge strane, detreniranost (smanjenje ili redukcija treninga) izaziva parcijalni ili totalni gubitak anatomskih, fizioloških i takmičarskih adaptacija izazvanih treningom, pa je razumno očekivati da su promene u antioksidativnom sistemu sportiste takođe reverzibilne..

Ključne reči: *redoks status, sportisti, sindrom pretreniranosti, detreniranost, adaptacija*

INTRODUCTION

Oxidative stress is a condition in which the delicate balance that exists between the production of prooxidants and their subsequent amelioration via the antioxidant defence system (ADS) becomes skewed in favour of prooxidants (1). Exercise provides an excellent model to study the dynamic balance between oxidative challenges and antioxidant defence in biological systems. From the work published during the previous 3 decades, it is now known that acute exercise of sufficient volume, intensity and duration can increase reactive oxygen and nitrogen species (RONS) production, which can lead to the oxidation of several biological molecules (1-4). However, repeated exposure of the system to increased RONS production from chronic exercise training leads to an upregulation in the endogenous

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ADS (1-4). This provides adaptive protection from RONS during subsequent training sessions, as well as during non-exercise related conditions.

According to the hormesis theory, the response of a biological system to stressors during exercise training can be explained by a U-shaped curve for which the two endpoints are inactivity and overtraining (5). Both of these endpoints result in decreased physiological functions (3). Markers of oxidative stress may be important parameters of biological monitoring of athletes (6). Studies that have explored the redox state in athletes during the competitive season have shown that the antioxidative status changes depending on the training load and training phase (7-13). Periods of intensive training may lead to a decrease in antioxidative capacity and a consequent increase in oxidative stress. Although there is no clear evidence yet, oxidative stress has been suggested as one of the underlying mechanisms of overtraining syndrome (14). However, detraining (training reduction or cessation) leads to a partial or complete loss of training-induced anatomical, physiological and performance adaptations (15,16). Thus, it seems reasonable to assume that changes in the ADS are also reversible.

OXIDATIVE STRESS IN TRAINING

The relationship between physical activity and oxidative stress has gained the attention of scientists, and as a result, hundreds of papers have been published on this topic. In those studies, oxidative stress was estimated using various methods and parameters (direct detection of free radicals; measuring radical-mediated damage of lipids, proteins and DNA; and measuring the activity of antioxidative enzymes or the concentrations of nonenzymatic antioxidants). Some studies have been conducted under laboratory conditions, e.g., athletes subjected to (sub)maximal exercise protocols on a treadmill, cycling ergometer or some other ergometer, whereas others followed changes in the redox state of athletes following sport-specific physical activity (training or competition). The majority of investigators have explored oxidative stress induced by aerobic exercise (running, swimming, cycling, triathlon, etc.), but there is a somewhat smaller number of studies on the relationship between anaerobic exercise and oxidative stress (sprinting, jumping, training with resistance, etc.). A few of these papers examined oxidative stress in team sports characterised by a combination of aerobic and anaerobic capabilities (basketball, handball, football, etc.).

When exploring the relationship between oxidative stress and exercise, one must distinguish between the effect of an acute exercise session and a regular training process. A detailed review of the existing literature on the relationship between acute and/or chronic exercise and oxidative stress is beyond the scope of this paper, and readers are referred to some excellent review papers, such as those by Ji (17), Bloomer and Goldfarb (18), Finaud and colleagues (2), Fisher-Wellman and Bloomer (1), and Lewis et al. (6). Generally, the data on the acute effects of exercise on redox homeostasis in humans are equivocal because many types of exercise and experimental conditions were used in previous studies. Some studies showed an increase in the levels of lipid peroxidation after (sub)maximal exercise, whereas some did not report this. As a consequence of increased free radical production, the activity of antioxidative enzymes also changed in the majority of studies. However, the results of some studies are conflicting in regard to the direction and extent of change in the activity of different enzymes. Some studies reported increased activity of superoxide dismutase, catalase and glutathione peroxidase, whereas others reported their activity decreased or showed no significant change after acute exercise. The levels of nonenzymatic antioxidants in plasma, urine or tissues also changed with exercise. Many studies measured the levels of reduced glutathione and found that they were decreased after aerobic exercise, whereas the levels of the oxidised form of glutathione were increased. Because of these inconsistencies in the literature, we can only conclude that both acute aerobic and anaerobic exercises have the potential to increase free radical production, which may or may not result in acute oxidative stress (1). The extent of oxidative stress induced by an acute bout of exercise depends on many factors, such as the exercise mode, intensity and duration; the participant's state of training; gender; age; nutrition habits, etc. For example, our research team showed that sport-specific and sport-nonspecific bouts of exercise induce different redox responses in athletes (19). It seems that unaccustomed, short, and intensive physical activity may induce oxidative stress in trained athletes, whereas sport-specific activity of longer durations with a proper warm-up period may not (19). Interestingly, basal levels of antioxidants may also affect an individual's response to exercise—we have shown that, in a group of young handball players, the levels of hydrogen peroxide, nitric oxide, superoxide dismutase and catalase were changed after exercise only in athletes with the lowest basal superoxide dismutase activity (20). Different basal (rest) levels of antioxidants are certainly a consequence of the training process, and consequently, the fitness level of an individual. Different types of sports affect the basal and acute redox states differently (21). Although exercise-induced increase in RONS production has the potential to result in significant cell disturbances, in accordance with the theory of hormesis, a low grade oxidative stress appears necessary for various physiological adaptations to take place (3). There is increasing evidence that low-to-moderate levels of cellular prooxidants play an important role in the modulation of muscle force, control of cell signalling pathways and regulation of gene expression (22-25). Regular exposure to an increased level of prooxidants leads to upregulation of the endogenous antioxidative system, which shifts the redox state of an athlete towards a more reducing environment that is suitable for cells (1, 3). However, to induce adaptive changes, i.e., improve the efficacy of the antioxidative defence, a training programme must be long enough and have adequate intensity. A well-planned and controlled aerobic exercise training

programme was shown to induce an increase in antioxidative enzyme activity in plasma and other tissues (especially in initially unfit subjects), although this adaptation was not always correlated with an increase in the maximal oxygen consumption. However, this does not mean there is no relationship between cardiorespiratory fitness and the redox state of an individual (26). Many studies have compared the antioxidant status of trained and untrained subjects at rest and revealed a higher antioxidant capacity in the blood and muscles of athletes. This basal difference certainly affects the response of the redox system to acute exercise. Well-trained subjects experience less oxidative stress than untrained subjects when exposed to exercise (27).

OXIDATIVE STRESS IN OVERTRAINING

During the training season, a state of fatigue resulting from an excessive training load known as overtraining can occur. To induce structural and functional adaptations that enable improvements in sport performance, athletes and coaches manipulate the training load through adjustments in intensity, duration and frequency, or through a reduction of the regenerative period (28). Every athletic training program includes a component of repetitive overloading, but with an inadequate recovery time or an abrupt increase in training load, overloading may produce undesired effects, such as chronic fatigue or a lack of performance improvements, i.e., overtraining (29).

Although there is no direct evidence yet, oxidative stress has been suggested as one of the causes of overtraining syndrome (30-33). Cellular damage, especially on the muscular level, is associated with the inflammatory processes that occur during the repair of damaged tissue, and this inflammation can lead to further production of prooxidants by neutrophils and macrophages (34). On the other hand, nitric oxide and reactive oxygen species play a key role in the regulation of immune functions, affecting virtually every step in the development of inflammation (35). For example, low concentrations of nitric oxide inhibit cytokine expression, whereas high levels of nitric oxide can be toxic and proinflammatory (35). Thus, monitoring the markers of oxidative stress during the season can be useful for timely detection of athletes who are at increased risk for overtraining, and steps can be taken to avoid adverse consequences, especially in regard to the health and sport performance of athletes (12).

The number of studies that have examined the relationship between oxidative stress and overtraining is very small. Human studies have shown that overtrained athletes have higher levels of oxidative stress markers at rest compared to controls, and these levels increase with exercise (30, 33). Generally, investigation of the mechanisms behind overtraining syndrome in humans is difficult because performing longitudinal studies in which athletes are trained in such a manner that they develop overtraining syndrome is unethical. Thus, animal overtraining models have been developed (36, 37). Animal (rat) models often allow for more invasive, extensive, and homogenous experimental designs than those used with human subjects. Using rat experimental models enables the manipulation of individual and external variables that are commonly investigated in human studies and allows invasive analyses; therefore, they can precisely characterise the adaptations that occur.

Ferraresso and colleagues (38) recently published the results of a study conducted on rats that were subjected to an 11-week training protocol designed to cause overtraining. Compared to rats in the control group and rats that did not develop overtraining, overtrained rats had significantly increased values of lipid peroxidation, as well as increased activity of superoxide dismutase, catalase and glutathione reductase in both muscle and blood (38). The authors concluded that the increased level of antioxidant protection represents an adaptive mechanism that protected the overtrained rats from increased ROS production. Using the same protocol for inducing overtraining, Dong and colleagues (39) investigated oxidative damage in the neutrophils of rats. They concluded that overtraining can activate NADPH oxidase-mediated overproduction of RONS (which they quantified through levels of malondialdehyde in the blood) and that NADPH oxidase was responsible for apoptosis of neutrophils and lymphocyte DNA damage in overtrained rats. Ogonovszky and colleagues investigated the effects of moderate, high and excessive training loads on the markers of oxidative stress and DNA damage in the brain (40) and liver (41) of rats. They found that overtraining did not induce oxidative stress in the brain (40), but it led to oxidative damage to nuclear DNA in the liver (41). Zoppi and Macedo (42) found that oxidative stress was dependent on fibre type in different muscles of overtrained rats. Pereira and colleagues (43) showed that overtraining induced in Swiss mice through training sessions of downhill running was associated with oxidative stress in skeletal muscle cells and total blood. Our research team recently conducted a study that aimed to induce overtraining in rats through an experimental swimming protocol and explored the redox state of rats at both systemic (in blood) and local levels (in the coronary effluent of an isolated rat heart, unpublished data). Although the differences in the levels of pro/antioxidants in the blood of moderately trained rats and rats trained according to an experimental overtraining protocol were not statistically significant. The overtrained rats had the highest levels of the three measured endogenous antioxidants, which was probably an adaptation that occurred to cope with increased oxidative demands of multiple daily training sessions (44). Furthermore, compared to controls, the hearts of those rats produced lower levels of superoxide anion radicals and hydrogen peroxide.

OXIDATIVE STRESS IN DETRAINING

According to the principle of reversibility, training-induced physiological adaptations are transitory and may disappear when the training load is not sufficient. This can

result from training reductions (a progressive or nonprogressive reduction of the training load during a variable period of time to reduce the physiological and psychological stress of daily training), training cessation (a temporary discontinuation or complete abandonment of a systematic programme of physical conditioning), or confinement to bed rest due to illness or injury (15). The characteristics of detraining may differ depending on the duration of training cessation or insufficient training (15, 16).

The number of studies on the reversibility of adaptations in the antioxidative system in athletes is even smaller than the number of studies on the effects of overtraining. Fatouros and coauthors (45) explored oxidative stress in older men during training (16 weeks, walking/running 3 times a week) and detraining (after 4 months) and showed that endurance training lowered lipid peroxidation and increased glutathione peroxidase activity and total antioxidant capacity, but detraining abolished these adaptations. The reversibility of positive training-induced changes in the redox state of exercisers was also confirmed in a study by Radak and colleagues (46) who explored oxidative stress in the brains of rats subjected to training and detraining. Agarwal and coauthors (47) showed that regular exercise improves superoxide dismutase levels in the paraventricular nucleus of rats, but two weeks of detraining caused a reversal of exercise-induced improvement in antioxidant status within the paraventricular nucleus of hypertensive rats. In contrast, exercise or detraining did not affect superoxide dismutase levels in normotensive rats (47). Finally, Rodrigues and coauthors (48) evaluated the effects of exercise training and detraining on inflammatory and metabolic profiles after myocardial infarction in rats. They observed no expressive changes in oxidative stress in adipose tissue in the experimental groups (control, sedentary infarcted, trained infarcted, detrained infarcted) (48). Our research team recently designed a project aiming to elucidate sex differences in cardiac adaptations to training and detraining in rats, as well as the extent, mechanisms and speed of changes of selected cardiodynamic, morphological parameters and oxidative stress parameters in the heart and blood of rats. However, those experiments are still in progress.

CONCLUSION

Although the results of hundreds of studies on the relationship between oxidative stress and exercise training are equivocal because of the many types of exercises and experimental conditions used in those studies, it is generally accepted that regular exercise improves the redox state of exercisers. Because of a small number of studies, the importance of oxidative stress in the development of overtraining is still unclear. Based on the existing studies, it can be said that a connection exists, but whether oxidative stress is a cause or consequence of overtraining is yet to be clarified. Detraining abolishes adaptations in the ADS of exercisers; however, the precise characteristics of such changes in the ADS due to detraining also cannot be clearly defined by exploring the existing literature.

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Conflict of interests:
None declared.

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SECONDARY HYPERTENSION: DIFFERENTIAL DIAGNOSIS AND BASIC PRINCIPLES OF TREATMENT

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SEKUNDARNA HIPERTENZIJA: DIFERENCIJALNA DIJAGNOZA I OSNOVNI PRINCIPI LEČENJA

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ABSTRACT

Secondary hypertension occurs in 5-10% of cases in the patient population with primary hypertension. e most common forms of secondary hypertension are as follows: parenchymal renal disease (renoparenchymal hypertension), renal artery stenosis (renovascular hypertension), adrenal gland adenoma (primary hyperaldosteronism), a tumour of the adrenal gland marrow (pheochromocytoma) and adenoma of adrenal and pituitary glands (Cushing's syndrome). In patients with a typical clinical picture of secondary hypertension, the appropriate diagnostic tests should be conducted based on the suspected form of secondary hypertension. Determining a diagnosis of secondary hypertension is gradual. First, the appropriate screening tests are performed. If the screening test is positive, then additional tests to confirm the forms of secondary hypertension are conducted. Once a diagnosis of the appropriate form of secondary hypertension is confirmed, tests to distinguish causes and laterality tests to determine the precise localisation of the pathological process are applied to evaluate the response to therapy. Analysing the results of endocrine diagnostic tests provides an accurate diagnosis and selection of optimal therapeutic procedures.

Keywords: *secondary hypertension, renoparenchymal hypertension, renovascular hypertension, primary aldosteronism, pheochromocytoma, Cushing's syndrome*

SAŽETAK

Sekundarna hipertenzija se javlja kod 5-10% slučajeva u populaciji bolesnika sa hipertenzijom. Najčešći uzroci sekundarne hipertenzije su: parenhimska bolest bubrega (renoparenhimska hipertenzija), stenoza renalne arterije (renovaskularna hipertenzija), adenom nadbubrežne žlezde (primarni hiperaldosteronizam), tumour srži nadbubrežne žlezde (feohromocitom) i adenom nadbubrežne žlezde i hipofize (Kušingov sindrom). Kod bolesnika sa tipičnom kliničkom slikom pojedinih oblika sekundarne hipertenzije, primenjuju se odgovarajući dijagnostički testovi. Dijagnostikovanje sekundarne hipertenzije je stepenasto. Prvo se primenjuju odgovarajući skrining testovi. Ukoliko je skrining test pozitivan, primenjuju se testovi za potvrđivanje pojedinih oblika sekundarne hipertenzije. Kada se potvrdi dijagnoza odgovarajućeg oblika sekundarne hipertenzije, primenjuju se testovi za razlikovanje uzroka, kao i testovi lateralizacije, za preciznu lokalizaciju patološkog procesa i procenu odgovora na terapiju. Analiziranje rezultata više endokrinoloških dijagnostičkih testova obezbeđuje ispravnu dijagnozu i izbor optimalnog terapijskog postupka.

Ključne reči: *sekundarna hipertenzija, renoparenhimska hipertenzija, renovaskularna hipertenzija, primarni aldosteronizam, feohromocitom, Cushing-ov sindrom*

INTRODUCTION

Secondary hypertension is defined as hypertension that occurs due to detectable factors. In the population of patients who are suffering from hypertension (blood pressure >140/90 mmHg), the prevalence of secondary hypertension is 5-10%. However, in the population of patients with resistant hypertension (the impossibility of realizing a target arterial blood pressure plus the optimum dose of three antihypertensive medicines, including a diuretic), the prevalence of secondary hypertension is up to 85% (1, 2). The most common forms of secondary hypertension are renoparenchymal hypertension, renovascular hypertension, hypertension due to primary aldosteronism, pheochromocytoma and Cushing's syndrome (1, 2).

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Differential Diagnosis and Treatment of Secondary Hypertension

Most common causes of secondary hypertension are renal parenchymal disease, renal artery stenosis, adrenal gland adenoma (primary aldosteronism), tumor of the adrenal gland marrow (pheochromocytoma) and adenoma of the adrenal and pituitary glands (Cushing's syndrome) (1, 2).

Renoparenchymal Hypertension

Renoparenchymal hypertension is defined as hypertension occurring in chronic kidney disease. The prevalence of renoparenchymal hypertension in patients with hypertension is 1.6 to 8%, and in the population of patients with resistant hypertension, it is 2-10% (3, 4). The pathogenesis of renoparenchymal hypertension is complex and multifactorial (3, 4). The two most important pathogenetic mechanisms are an increased volume of extracellular fluid (the dependence of volume) and/or an increased activation of the renin-angiotensin system (dependence of angiotensin 2) (3, 4). Other factors that influence the development of renoparenchymal hypertension include the following: increased activity of the sympathetic nervous system, endothelial dysfunction, increased production of endothelin-1, increased oxidative stress, reduced concentration of endothelium-relaxing substances (nitric oxide - NO), changes in the arterial wall structure and sleep apnea (3, 4). Clinical and experimental research in recent years indicates the growing importance of increased activity of the sympathetic nervous system and obstructive sleep apnea in the development of renoparenchymal hypertension (3, 4). Increased activity of the sympathetic nervous system in chronic kidney disease is due to the following: activation of chemoreceptors in the kidney (renal ischaemia), decreased baroreceptor sensitivity in the kidney, decreased central dopaminergic tone, and decreased renalase activity in the kidney; additionally, renalase is flavin adenine dinucleotide-dependent amine oxidase, which degrades the catecholamines. In chronic renal disease, its activity has been reduced, and this results in the enhanced activity of the sympathetic nervous system (3, 4).

The measurement of serum creatinine and urine analysis (erythrocytes, cylinders and proteins) is used as the screening test for parenchymal kidney disease. If the result is positive, the endogenous creatinine clearance, 24-hour proteinuria, microalbuminuria and renal ultrasound (kidney size, the volume of renal parenchymal echogenicity and thickness, the ratio of parenchyma and the pelvis, and the atrophy index) should be determined. The atrophy index (AI) represents the ratio of the longitudinal sinus diameter and the longitudinal kidney diameter. A normal value of the atrophy index is AI≤0.7 (5). An AI>0.70 and a resistance index (RI)>0.70, as measured from the blood flow curve through intrarenal segmental arteries or interlobar arteries, point to tubulointerstitial chronic kidney disease (tubulointerstitial injury) (5).

The treatment consists of the application of the renin-angiotensin system blockers, beta-blockers and diuretics. Thiazide diuretics are used, and loop diuretics will be applied in patients with creatinine clearance less than 30 ml/min (a contraindication to the use of potassium-sparing diuretics) (3, 4).

Obstructive sleep apnea (OSA) is defined as a breathing disorder, which is characterized by recurrent obstructive apneas and hypopnea, caused by the collapse of the upper airway during sleep (sleep apnea). It often occurs in patients with chronic kidney disease (in 21-47% of patients with end stage renal disease), and the main pathogenetic mechanism for the development of hypertension is the increased activity of the sympathetic nervous system (3, 4). Obstructive sleep apnea causes a disorder of the circadian rhythm of blood pressure. Blood pressure is not reduced during the night in > 10% ("non-dippers"), and in patients suffering from chronic kidney disease, the prevalence of the "non-dippers" status is 74-82% (3, 4). Typical clinical characteristics include obesity, large neck (neck circumference in men greater than 42 cm and in women greater than 39 cm), macroglossia and daytime sleepiness. A history of daytime sleepiness should always arouse suspicion of obstructive sleep apnea. In these patients, an interruption of breathing during sleep is determined by using the Epworth Sleepiness Scale (ESS). Patients with an ESS score of ≥ 10 are at high risk of obstructive sleep apnea. In these cases, polysomnography should be conducted, and based on the apnea-hypopnea index (AHI; number of apneas and hypopnea in one hour of sleep), OSA severity can be assessed. Obstructive sleep apnea (OSA) is mild if the AHI index is from 5-15/h, moderate if AHI = 16-30/h and severe if AHI > 30/h (1, 3, 4). Patients who are diagnosed with OSA require echocardiography to assess the morphology and function of the left and right chambers and an estimation of the pulmonary artery pressure (pulmonary hypertension) (1, 3, 4).

Renovascular Hypertension

Renovascular hypertension is defined as hypertension that is caused by renal artery stenosis. In the patient population with hypertension, the prevalence of renovascular hypertension amounts to 1-8%, and in the patient population with resistant hypertension, its prevalence is 2.5 to 20% (6-8). The two main causes of renal artery stenosis (RAS) are atherosclerosis (90%) and fibromuscular dysplasia 10% (9, 10). Atherosclerosis affects the starting point and proximal part of the main trunk of the renal artery, which results in the development of renovascular hypertension, ischaemic nephropathy and repetitive "*flash*" pulmonary edema (bilateral atherosclerotic renal artery disease) (9, 10). Fibromuscular dysplasia (FMD) is a non-aterosklerotic, noninflammatory disease of the arteries, most commonly affecting the renal (distal part of the main trunk) and internal carotid artery (11, 12). Fibromuscular dysplasia of the media (70-95% of cases) is distinguished

by its characteristic constrictions and extensions ("*string of beads*") in the angiography findings (11, 12). In most patients, the disease is asymptomatic in the course of several years, which makes its early detection hard, and it may clinically present itself with renovascular hypertension and ischaemic nephropathy (severity of the clinical picture depends on the degree of stenosis and type of fibromuscular dysplasia) (11, 12).

Renovascular hypertension should be suspected in patients with the following: severe hypertension (diastolic blood pressure ≥120 mmHg), resistant hypertension (optimal control inability, using three antihypertensive medicines, including a diuretic), finding a hum in the auscultation of the abdomen (renal artery), moderate (diastolic blood pressure ≥ 105 mmHg) or severe hypertension (diastolic blood pressure ≥120 mmHg) and a difference in the size of the kidneys (the difference between the longitudinal diameter of the right and left kidney > 1.5-2.0 cm), hypertension and an increase in serum creatinine concentration after the administration of angiotensin-converting-enzyme blockers 1 (ACE) and or angiotensin 2 receptor blockers (ARB) (increase of creatinine serum concentrations of 0.5-1.0 mg/dl (44.2-88.4 μmol/l) after administration of ACE/ARB) (13, 14).

The following screening tests are performed in these patients for the diagnosis of renovascular hypertension: plasma-renin activity (PRA), Captopril test and Color Doppler ultrasonography of the renal artery (CDU) (13, 14). Low levels of plasma renin activity (PRA ≤ 0.65 ng/ml/h) with hypokalemia (K^+< 3.5 mmol/l) indicate the primary aldosteronism. Moderate levels of plasma renin (PRA = 0.65 to 3.2 ng/ml/h) require a conduction of tests for the confirmation of renovascular hypertension (CT angiography, MR angiography). Plasma-renin activity (PRA) ≥ 3.2 ng/ml/h indicates a high probability of the existence of renovascular hypertension and requires direct renovasography (13, 14).

The most sensitive test for the detection of renovascular hypertension is the Captopril test. Two to three weeks prior to testing, medicines affecting the PRA should be discontinued (angiotensin-converting-enzyme blockers 1, angiotensin 2 receptor blockers, beta blockers, direct renin blocker). Alpha 1-blockers (*doxazosin*) have no influence on the rennin concentration in plasma and plasma-renin activity. The patient needs to rest at least 30 minutes before sampling. A blood sample for the determination of the plasma-renin activity is taken 30 minutes before and 60 minutes after the administration of Captopril tablets at a dose of 25-50 mg. The test is positive if the stimulated plasma-renin activity is ≥ 12 ng/ml/h or if the absolute increase of plasma-renin activity is ≥ 10 ng/ml/h (13, 14). A Color Doppler ultrasonography of the renal arteries is performed in all patients with moderate or high probability for renovascular hypertension, in which the plasma-renin activity is ≥ 1.6 ng/ml/h (15-17). The Direct Doppler criteria for renovascular hypertension includes the following: peak systolic blood flow velocity (VmaxS) > 180 cm/s, end-diastolic flow velocity (VendD) > 50 cm/s, reno-aortic index (RAR) > 3.5 and reno-renal index (RRR) > 4.0 (14-16). The indirect Doppler criteria for renovascular hypertension include: loss of early systolic peak (ESP), resistance index (RI) < 0.45, acceleration time (AT) > 70 ms, acceleration (Acc) < 300 cm/s^2, the difference in resistance index (ΔRI) > 0.05 (> 5%), the peak systolic velocity measured from the blood flow curve through interlobar arteries < 15 cm/s and the ratio of peak systolic velocity measured from the blood flow through the interlobar arteries of both kidneys (RIR) > 5 (renal interlobar ratio - RIR) (15-18).

If one of the screening tests for renovascular hypertension is positive, tests to confirm renovascular hypertension (computed tomographic angiography (CTA) or magnetic resonance angiography (MRA) or conventional digital subtraction angiography (DSA)) will be performed (14). These diagnostic methods require the use of a contrast agent (CTA, DSA) and gadolinium (MRA), and they are not indicated in patients with a glomerular filtration rate of less than 30 ml/min/1.73 m^2 due to the risk of contrast nephropathy (CN) and nephrogenic systemic fibrosis (NSF) (19, 20). Digital subtraction angiography is the gold standard for the diagnosis of renovascular hypertension. It offers the possibility of the direct assessment of haemodynamic significance of renal artery stenosis [peak systolic translesion pressure gradient (ΔP) ≥ 20 mmHg, the ratio of pressure in the distal renal artery (Pd) and the aorta (Pa) is less than 0.9 (Pd/Pa < 0.9)] (21).

When patients are diagnosed with renovascular hypertension, lateralization tests are applied for assessing the response after renal artery revascularization. The higher the degree of lateralization, the greater the likelihood of optimal blood pressure control and prevention of the progression of ischaemic nephropathy after renal artery revascularization (13, 14). The lateralization tests include a ratio of plasma-renin activity in both renal veins, Captopril scintigraphy and resistance index measured from the flow curve through intrarenal arterial segments (CDU) (13, 14). A ratio of plasma-renin activity from the renal vein stenosis of the renal artery and renal vein without renal artery stenosis greater than 1.5 indicates a good response after revascularization (lateralization of plasma-renin activity indicates haemodynamically significant stenosis) (13, 14). Captopril scintigraphy is used for the diagnosis of renovascular hypertension and for assessing the functionality of stenosis (positive lateralization). Firstly, dynamic renal scintigraphy without Captoprilis is performed in patients (^{99m}Tc-DTPA - diethylene triamine pentaacetic acid), and the next day, renal scintigraphy is performed after taking Captopril, *per os*, at a dose of 25-50 mg. The finding is positive if the decline in glomerular filtration rate (GFR) of the affected kidney (^{99m}Tc-DTPA) is > 10% (13, 14). Positive Captopril scintigraphy (positive lateralization test) indicates a positive response after revascularization (optimal control of blood pressure, prevention of loss of kidney function) (13, 14).

In patients with stenosis of one renal artery, angiotensin-converting-enzyme blockers 1 and or angiotensin 2 receptor blockers are applied, with appropriate monitoring (serum creatinine concentration) (22). The application of these medicines is contraindicated if there is a stenosis of both renal arteries, due to the high risk of developing acute kidney damage (23-26). The indications for renal artery revascularization (angioplasty with or without stenting, surgical by-pass) include: renal artery stenosis ≥ 50% (systolic pressure gradient ΔP ≥ 20 mmHg and the ratio of Pd/Pa < 0.9 indicate haemodynamically significant stenosis > 60%), resistant hypertension, loss of renal function after administration of angiotensin-converting-enzyme blockers 1 (ACEI) and or angiotensin 2 receptor blockers (ARB) (reduction of GFR ≥ 30% compared with the value before applying ACEI/ARB, an increase in serum creatinine concentration of 0.5 - 1.0 mg/dl (44.2 to 88.4 μmol/l) after administration of ACEI/ARB), and recurrent "flash pulmonary oedema" associated with bilateral renal artery stenosis (23-26). The contraindications for renal artery revascularization include the following: longitudinal diameter of the affected kidney less than 8.0 cm, resistance index measured from the blood flow curve through the segmental arteries (RI) > 0.8, chronic kidney disease (GFR < 30 ml/min/1.73 m^2) and negative Captopril scintigraphy (absence of lateralization) (23-26).

Primary Hyperaldosteronism

Primary aldosteronism is the most common form of secondary hypertension. The main causes of the development of primary aldosteronism are adrenal gland adenoma, which secretes aldosterone, and idiopathic bilateral adrenal gland hyperplasia (27-29). The prevalence of primary aldosteronism in the patient population with hypertension is 3.5% (27-29). Screening for primary aldosteronism should be performed in patients with: resistant hypertension, moderate or severe hypertension, hypertension and hypokalemia, hypertension that is complicated by cerebrovascular events in patients younger than 40 years and patients with hypertension and swellings (27-29).

The determining of the plasma aldosterone concentration (PAC: ng/dl) and plasma renin activity (PRA: ng/ml/h) and the measurement of the plasma aldosterone concentration (PAC) will be conducted as the screening test for the diagnosis of primary aldosteronism (27-29). The conduction of the test requires the adequate preparation of patients: two weeks before the test patients must discontinue antihypertensive medicines that affect the renin and aldosterone concentrations in plasma (angiotensin-converting-enzyme blockers 1, angiotensin 2 receptor blockers, beta blockers, direct renin blockers), and spironolactone should be discontinued for at least two months before blood sampling for screening tests (27-29). Alpha-1-blockers (doxazosin), hydralazine, and calcium channel blockers (verapamil) have the least impact on the concentration of renin and aldosterone in the plasma (27-29). If the ratio of PAC/PRA is > 20 and plasma aldosterone concentration is > 15 ng/dL (> 416 pmol/l), the test is positive (27-29).

Tests to confirm (confirmatory testing) primary aldosteronism should be performed in patients with positive screening tests, such as the *per os* sodium suppression test, IV infusion sodium suppression test and Captopril suppression test (27-29). The first test involves the *per os* use of NaCl (in addition to the normal intake of sodium of 9.0 g NaCl/24-hours, an additional 3 x 2.0 g of NaCl/24-hours are applied, over the course of three days) and measurement of the aldosterone and sodium concentration in the urine on the fourth day. The test is positive if the aldosterone concentration (aldosterone-18-glucoronide) in the urine is > 12μg/24 h (> 33.3 nmol/24 h) with the sodium concentration in urine being > 200 mmol/24 h. If tetrahydroaldosterone is determined, the test is positive if the concentration in urine is > 70 μg/24 h (27-29). In the IV infusion sodium suppression test, the plasma aldosterone concentration is determined before and after the IV infusion of 2,000 ml of 0.9% NaCl solution. An IV infusion is administered in the period from 8-12 am (contraindications for IV infusion: heart weakness, history of myocardial infarction, severe and poorly controlled hypertension), the blood sample is taken at 8 am and 12 am, and the blood sample testing will determine the concentration of aldosterone, renin and cortisol in the plasma. In patients with no autonomous secretion of aldosterone, the infusion of 0.9% NaCl solution blocks the secretion of aldosterone. Normal suppression is defined as the plasma concentration of aldosterone < 10 ng/dl (< 277 pmol/l). If the plasma aldosterone concentration, after IV infusion of 0.9% NaCl solution over the course of 4 h, is greater than 10 ng/dL (> 277 pmol/l), the test is positive (confirms the diagnosis of primary aldosteronism) (27-29). In the Captopril suppression test (CST), the aldosterone concentration in serum is measured prior to and two hours after the administration of Captopril in a dose of 25 mg. The test is positive if the aldosterone concentration in the serum is (PAC) > 15 ng/dl (> 416 pmol/l) (27-29).

If the screening test and confirmatory testing for primary aldosteronism are positive, tests for differential diagnosis of primary aldosteronism are applied: a display of adrenal glands (adrenal computed tomography (CT) or nuclear magnetic resonance (NMR)), as well as tests for the assessment of lateralization (concentration of aldosterone in the blood sample from the adrenal vein). Adrenal vein catheterization is conducted in centres that perform at least 10 catheterizations within one year. A sample of blood from the right and left adrenal vein and vena cava inferior is taken. In all the blood samples, the concentration of aldosterone and cortisol is determined. The selectivity index (SI) is the ratio of the concentration of cortisol in the blood sample from the adrenal vein (Ca) and a blood sample from the vena cava inferior (Cvci) (Ca/Cvci). A selectivity index ≥ 2 represents a good position of the catheter (catheterization of adrenal veins) (27-29). The lateralization index represents the ratio of aldosterone and

cortisol in both adrenal veins ((A/Cd)/A/Cl)). A lateralization index ≥ 4.0 indicates the existence of a unilateral adrenal gland adenoma, which increased the secretion of aldosterone. In idiopathic hyperaldosteronism (bilateral hyperplasia), the aldosterone/cortisol ratio from the adrenal veins is not higher than the ratio of aldosterone/cortisol from the vena cava inferior sample (27-29).

In patients with adrenal gland adenoma (positive lateralization test), the unilateral laparoscopic adrenalectomy is performed, and in patients with idiopathic bilateral hyperplasia, medicine therapy is administered (mineral corticoid receptor antagonists: spironolactone and eplerenone) (27-29). Before operative treatment, spironolactone is administered in a dose of 50-100 mg/day for four weeks, and postoperatively, fludrocortisone is often applied in a dose of 50-100 μg/day (28). Spironolactone is administered for the treatment of bilateral adrenal hyperplasia, at a dose of 12.5-25 mg/day, and the dose is gradually increased up to a dose of 100 mg/day. A dose of spironolactone greater than 100 mg/day is associated with side effects, such as: gynaecomastia, loss of libido, erectile dysfunction in men and disorder of cyclic menses in a woman. Regular monitoring of patients includes an assessment of renal function and potassium in serum (28). The alternative is the spironolactone is eplerenone, which is administered at a dose of 50-100 mg/day. In case of failure to achieve optimal control of blood pressure (targeted blood pressure of less than 140/90 mmHg) and hygienic-dietary regime (restriction of salt intake: < 2.0 g/day NaCl), potassium-sparing diuretics (amiloride or triamterene) or calcium channel blockers are administered (29).

Pheochromocytoma

Pheochromocytoma is a tumour made up of chromaffin cells of the adrenal marrow, which have the ability to produce and secrete catecholamines (29, 30). It is also referred to as the intra-adrenal paraganglioma (intraglandulary paraganglioma), unlike extra-adrenal sympathetic and parasympathetic paraganglioma (extra-glandular paraganglioma) (29, 30). The prevalence of pheochromocytoma in hypertensive patients is 0.1-0.5%. Due to the paroxysmal release and increased catecholamine concentration in the serum, the clinical picture is characterized by the "5P"s: paroxysmal hypertension, palpitation, increased perspiration, paleness and pulsating headache (29, 30). Paroxysmal hypertension may occur spontaneously or after physical exertion, after administration of certain medicines (β-blockers, metoclopramide, glucagon, ACTH) or as a result of an increase of the intra-abdominal pressure (29, 30).

Screening for pheochromocytoma should be performed in patients with resistant hypertension, a characteristic clinical picture ("5P"), hypertension after administration of β-blockers (paradoxical increase in arterial blood pressure after administration of β-blockers), hypertension while using antidepressants, as well as in patients with a jump in blood pressure during anaesthesia, surgery or angiographic procedures, patients with a family history of pheochromocytoma, and in patients with a history of genetic syndromes that are known to be associated with pheochromocytoma (multiple endocrine neoplasia type 2 (MEN 2), von Hippel Lindau syndrome (VHL syndrome) and neurofibromatosis type 1) (29, 30).

There are two screening tests available: the determination of the concentration of metanephrines and normetanephrines in the plasma and the 24-hour urine sample. Before performing the screening test, it is necessary to discontinue treatment with certain medicines (antidepressants, beta blockers, amphetamines, levodopa, phenoxybenzamine) and stimulants (alcohol, nicotine, caffeine). A concentration of metanephrines >0.31 nmol/l and normetanephrines >0.61 nmol/l in the plasma suggests an increased catecholamine secretion. A concentration of metanephrines >0.7μmol/24 h and normetanephrines >1.7μmol/24 h in the urine confirms the existence of pheochromocytoma (29, 30).

If one of the screening tests is positive, the following confirmatory testing is performed to verify the pheochromocytoma: clonidine suppression test and chromogranin concentration in plasma. The clonidine suppression test is performed by measuring the concentrations of catecholamines and metanephrines in the plasma before and 180 minutes after the administration of clonidine (clonidine *per os*, at a dose of 300 μg). The test is positive if the concentration of noradrenaline in the plasma is reduced by less than 50% or the normetanephrine concentration in the plasma by less than 40% (maintaining increased concentrations of noradrenaline and normetanephrine in the plasma after the administration of clonidine) (29, 30). A chromogranin plasma concentration > 270 ng/ml confirms the diagnosis of pheochromocytoma (29). Once the diagnosis of pheochromocytoma is confirmed, tests for the differential diagnosis of pheochromocytoma are conducted. Distinguishing unilateral from bilateral disease is achieved by a combination of techniques of display/visualization (computerized tomography (CT) or nuclear magnetic resonance (NMR) of adrenal glands, MIBG scintigraphy of adrenal glands (^{123}I or ^{131}I-*metaiodbenzylguanidine*) and PET scans of adrenal glands (*fluorodopamine* PET)). Pheochromocytoma is 3-6 cm in size and usually localized in the marrow of adrenal glands (80%). If the pheochromocytoma is greater than 5.0 cm, it is the malignant form of pheochromocytoma. MIBG scintigraphy is used to distinguish intra-adrenal (core of adrenal gland) from extra-glandular pheochromocytoma (paraganglioma) (29, 30). The radiological diagnosis of the abdomen, chest and neck should be performed to detect extra-adrenal pheochromocytoma. If MIBG scintigraphy is negative, and the suspected pheochromocytoma presence is big, a fluorodopamine PET scan should be conducted (29, 30).

Treatment is surgical and requires appropriate preoperative preparation. α-Adrenoceptor antagonists (doxazosin, α_1 blocker) are applied 7-14 days prior to surgery to block effects of suddenly liberated catecholamines (pre-

venting the development of hypertensive crisis and cardiac rhythm disorders) (29-31). Treatment begins with the administration of phenoxybenzamine at a dose of 10 mg twice a day, with a gradual increase in the dose of 10-20 mg/day, every two to three days (total dose is approximately 1.0 mg/kg/day) (30, 31). In patients with tachycardia, beta blockers can be administered only after the alpha blockers. Atenolol is administered in a dose of 12.5-25 mg, twice a day, or metoprolol is administered in a dose of 25-50 mg, two to three times a day (30, 31).

Cushing's Syndrome

Cushing's syndrome is characterized by many symptoms and signs, which have been caused by increased concentrations of cortisol in the body tissue (purple stretch marks, plethoric appearance of the face, weakness of the proximal muscles, spontaneous bruising, unexplained osteoporosis, hypertension) (32-35). Depending on the aetiology, Cushing's syndrome can be distinguished into several types: one dependent of the adrenocorticotropic hormone (ACTH) (pituitary adenoma that secretes ACTH (Cushing's disease)) and the syndrome of ectopic secretion of ACTH (neuroendocrine tumours (bronchial carcinoids)), and Cushing's syndrome independent of ACTH (adenoma or cancer of the adrenal gland, adrenal hyperplasia) (32-35). The prevalence of Cushing's syndrome in patients with hypertension is 0.5-1.0%. The mechanisms for hypertension in patients with Cushing's syndrome are multiple and include the following: mineralocorticosteroid activity of cortisol, activation of the renin-angiotensin-aldosterone system, increased reactivity to the vasoconstrictor effect (angiotensin 2, catecholamines, vasopressin), and reduced activity of the vasodilatory system (NO synthase, prostacyclin, kallikrein-kinin system) (32-35).

Testing for Cushing's syndrome is indicated in patients with the following factors: a typical clinical picture (hypertension and osteoporosis that are not in accordance with the age of patients) and resistant hypertension. Treatment for the iatrogenic Cushing's syndrome and medicines that affect the metabolism of dexamethasone and cortisol (phenytoin, phenobarbital, carbamazepine, rifampin, pioglitazone, diltiazem and cimetidine, fenofibrate, synthetic glucocorticoids) should be discontinued before the screening test (33-35).

For initial testing of patients with Cushing's syndrome, one of the following tests is recommended: the concentration of free cortisol in a 24-hour urine sample (UFC) (at least two measurements), the concentration of cortisol in saliva samples at night (11-12 pm) (two measurements), a night dexamethasone suppression test with 1.0 mg of dexamethasone and a prolonged low-dose dexamethasone suppression test (dexamethasone suppression test (LDDST): 2.0 mg/day, for 48 h) (32-35). A concentration of free cortisol levels in the 24-hour urine sample > 250 µg/24 h points to Cushing's syndrome. The night dexamethasone test is performed by having the patient take 1.0 mg of dexamethasone *per os*, at night between 11 pm and midnight, and then a blood sample is taken in the morning between 8 am and 9 am. A serum cortisol concentration greater than 1.8 µg/dl (> 50 nmol/l) indicates a positive test (Cushing's syndrome). Cushing's syndrome is indicated by a concentration of cortisol in a saliva specimen (11-12 pm) greater than 145 ng/dl (> 4 nmol/l) (32-34). Most endocrinologists recommended the low-dose dexamethasone suppression test (LDDST test: 0.5 mg/6 h/day, 48 h) for the screening test. The test is performed in such a way that the patient is taking dexamethasone in a dose of 0.5 mg every 6 hours (9 am, 3 pm, 9 pm, 3 am). Serum cortisol concentration is determined at 9 am (6 h after the last dose of dexamethasone). The test is positive if the concentration of serum cortisol levels is greater than 1.8 µg/dl (> 50 nmol/l) (33-35).

In patients with one positive screening test, testing to confirm Cushing's syndrome is performed. These tests include the low-dose suppression test (LDDST: 2.0 mg/day, 48 h) in combination with a corticotrophin releasing factor (corticotrophin releasing hormone-CRH) (LDDST-CRH test). The test is performed in such a way that 2 hours after the last dose of dexamethasone, CRH is administered in a dose of 1.0 µg/kg IV, and cortisol levels are measured after 15 minutes. The test is positive if the concentration of serum cortisol levels, 15 minutes after CRH administration, is greater than 1.4 µg/dl (>38 nmol/l) (33-35).

In patients who are diagnosed with Cushing's syndrome, tests are performed to diagnose subtypes of Cushing's syndrome (ACTH dependant Cushing's syndrome, ACTH independent Cushing's syndrome). Determining the ACTH concentration in the plasma is used for differential diagnosis of Cushing's syndrome. ACTH concentration in plasma of less than 5.0 pg/ml (< 1.1 pmol/l) points to the Cushing's syndrome that is ACTH independent (Cushing's syndrome dependent on the adrenal glands; non-pituitary form of Cushing's syndrome). Increased production and release of cortisol from the adenoma/carcinoma of the adrenal gland (increased concentration of serum cortisol) and a negative feedback mechanism reduce the formation and secretion of CRH from the hypothalamus and pituitary ACTH (reduced release of ACTH resulting in atrophy of the adrenal gland). If the ACTH concentration in the serum is greater than 20 pg/ml (> 4.4 pmol/l), it implies a diagnosis of ACTH dependent Cushing's syndrome (pituitary tumour, ectopic tumour). A CRH stimulation test should be performed for the patients in whom the ACTH concentration in serum is between 5-20 pg/ml (1.1 to 4.4 pmol/l). The test is positive if the ACTH concentration in serum, after a CRH stimulation, is less than 30 pg/ml (< 6.6 pmol/l) (34, 35).

After the confirmation of ACTH independent Cushing's syndrome (ACTH decreased concentration in serum), it is necessary to perform a CT or NMR of the adrenal glands (to determine the location of adrenal hyper secretion of cortisol). A CT or NMR of the endocranium (pituitary) and blood sampling from the pituitary gland vein (pituitary vein) are used for differential diagnosis of

the pituitary from non-pituitary (ectopic) ACTH secretion. In clinical practice, venous blood to determine the ACTH concentration is taken from the petrosal sinus (IPS - inferior petrosal sinus) simultaneously from both sides. Blood from the anterior pituitary gland is directly flowing in these sinuses. For simultaneous evaluation of ACTH in systemic circulation, the venous blood sample is taken from the inferior vena cava (IVC). A significant ACTH gradient (IPS/IVC ratio ≥ 2.0) indicates the secretion of ACTH from the pituitary gland adenoma (Cushing's disease). If the ACTH gradient is not important, ACTH is ectopically secreted (not from the pituitary gland) (34, 35). Analysing the results of endocrine diagnostic tests will provide accurate diagnosis of Cushing's syndrome.

Treatment is surgical and with medicines. Transsphenoidal pituitary surgery (removal of pituitary corticotropic adenoma) is indicated in patients who are diagnosed with Cushing's disease. If an adrenal gland tumour (adenoma/carcinoma) is diagnosed, the unilateral laparoscopic adrenalectomy will be performed. Before surgical intervention, the administration of glucocorticoids is indicated for the prevention of sudden death due to secondary adrenal insufficiency (cortisol concentration increased, through a negative feedback mechanism, leads to atrophy of the contralateral adrenal gland) (34-36). In addition to surgical treatment, it is important to administer medicines that block the production of ACTH in the pituitary gland (pasireotide is a somatostatin analogue, to block the release of ACTH from the cells of pituitary adenomas), the creation of cortisol in the adrenal gland, and the effects of cortisol in the peripheral tissues (mifepristone is a receptor antagonist for glucocorticosteroids, and it has a higher affinity for the glucocorticosteroid receptor in relation to cortisol) (34-36).

CONCLUSION

Early diagnosis of secondary hypertension and a timely implementation of appropriate therapeutic procedures will ensure the optimum control of arterial blood pressure and prevent the development of cardiovascular morbidity and mortality in the patient population with hypertension.

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UNUSUAL RESPIRATORY MANIFESTATIONS OF ANKYLOSING SPONDYLITIS A CASE REPORT

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NEUOBIČAJENE RESPIRATORNE MANIFESTACIJE ANKILOZIRAJUĆEG SPONDILITISA PRIKAZ SLUČAJA

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ABSTRACT

A male patient, 54 years old, was initially admitted to the hospital because of fatigue he felt during the last month and swelling of the lower legs. Upon hospital admittance, gas exchange analysis showed global respiratory failure: pO_2=6.1 kPa, pCO_2=10.9 kPa, pH=7.35, A-a gradient = 1.0. Due to the existence of hypercapnia and decompensated respiratory acidosis, the patient was connected to a device for non-invasive mechanical ventilation. Reduced chest mobility was noticed, and the respiratory index value was decreased. Radiographs of the chest and thoracic and lumbo-sacral spine showed marked changes on the spine attributable to ankylosing spondylitis (AS). Radiographs of the sacroiliac joints showed reduced sacroiliac joint intraarticular space with signs of subchondral sclerosis. e diagnosis of AS was set on the basis of New York Criteria (bilateral sacroiliitis, grade 3) and clinical criteria (back pain, lumbar spine limitation and chest expansion limitation). HLA typing (HLA B27 +) confirmed the diagnosis of AS. Pulmonary function test proved severe restrictive syndrome. Polysomnography verified the existence of severe obstructive sleep apnoea (AHI =73). is was a patient with newly diagnosed AS, with consequent severe restrictive syndrome and global respiratory failure with severe obstructive sleep apnoea. e patient was discharged from the hospital with a NIV (global respiratory failure) device for home use during the night.

Keywords: *respiratory acidosis, ankylosing spondylitis, subchondral sclerosis.*

SAŽETAK

Muškarac, star 54 godine, hospitalizovan zbog umora koji je osećao u poslednjih mesec dana i otoka nogu. Gasne analize na prijemu ukazuju na postojanje globalne respiratorne insuficijencije pO_2=6.1Kpa, pCO_2=10.9KPa. Zbog postojanja hiperkapnije i dekompenzovane respiratorne acidoze, pacijent je priključen na aparat za neinvazivnu mehaničku ventilaciju. Zapažena je smanjena pokretljivost grudnog koša i smanjena vrednost respiratornog indeksa. Na rendgengrafiji grudnog koša, torakalne i lumbo-sakralne kičme registrovane su promene koje bi mogle biti u sklopu ankilozirajućeg spondilitisa. Rendgenografijom sakroilijačnih zglobova registrovan je smanjen intraartikularni prostor sa znacima subhondralne skleroze. Dijagnoza ankilozirajućeg spondilitisa postavljena je na osnovu njujorških kriterijuma (bilateralni sakroileitis, treći stadijum) i kliničkih kriterijuma (bol u leđima, ograničena pokretljivost lumbalnog dela kičme i ograničena ekspanzija grudnog koša). HLA tipizacija (HLA B27 +) je potvrdila dijagnozu. Testovi plućne funkcije pokazali su težak restriktivni poremećaj. Polisomnografija je verifikovala postojanje teške opstruktivne sleep apnee (AHI=73) . Ovo je bio pacijent sa novodijagnostikovanim AS što je za posledicu imalo težak restriktivni poremećaj sa značajno smanjenim respiratornim indeksom i razvoj hronične globalne respiratorne insuficijencije. Pacijent je otpušten iz bolnice i preveden na lečenje sa aparatom za neinvazivnu ventilaciju u kućnim uslovima.

Ključne reči: *respiratorna acidoza, ankilozirajući spondilitis, subhondralna skleroza.*

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INTRODUCTION

Ankylosing spondylitis (AS), or spondyloarthropathy, is a chronic, multisystem inflammatory disease that primarily affects the sacroiliac joints and the axial skeleton. AS can affect the tracheobronchial tree and pulmonary parenchyma, cause spontaneous pneumothorax and is associated with several unique pulmonary manifestations, such as upper lobe fibrocystic disease.

CASE REPORT

A male patient, 54 years old, was initially admitted to the hospital section of the Center for Emergency Medicine because of fatigue he felt during the last month and swelling of the lower legs. He denies any existence of chronic illness and does not take any medication. The result of gas exchange analysis upon admittance to the hospital showed global respiratory failure: pO_2 = 6.1 kPa, pCO_2 = 10.9 kPa, pH = 7.35, Aa gradient = 1.0.

Chest radiography showed reduced transparency of lung parenchyma in the left basal area, as well as minor bilateral pleural effusion. ECG on admission registered sinus rhythm and incomplete right bundle branch block. Echocardiography examination was performed and showed excessive load and enlargement of the right atrium and ventricle of the heart (right ventricle = 40 mm, average pressure in the right ventricle = 55 mmHg, tricuspidal regurgitation 4+). For further treatment and diagnosis, the patient was moved to the Clinic for Pulmonology. Subsequently, during detailed discussion with the patient, he reported in medical history that he had pain and limited mobility of the lumbar spine, significant daytime sleepiness, and loss of body weight (> 10 kg during the last few months). General overview of the patient showed that he was conscious, with signs of central cyanosis and dyspnoea at rest, using auxiliary respiratory musculature and underweight (BMI = 18.4 kg / m^2), with signs of systemic venous stasis. All haematology and biochemistry analysis, as well as thyroid hormones, showed results that were within normal ranges. Due to the existence of hypercapnia and decompensated respiratory acidosis, the patient was connected to a device for non-invasive mechanical ventilation (NIV). On the fifth day of hospitalization, despite the use of NIV (PSV = 24 cmH_2O) and good patient compliance, respiratory acidosis was registered again, with worsening hypercapnia (pH = – 7.29, $PaCO_2$ = 14.9 kPa). During further inspection, we noticed reduced chest mobility. The value of the respiratory index was decreased – 1.5 cm. Due to the reduced respiratory index and worsening state of the patient's health, radiographic examinations of the chest and thoracic and lumbo-sacral spine were done, which showed antero and laterocorporal spondylophyte and ossification of the longitudinal anterior, posterior and lateral ligament (Figure 1).

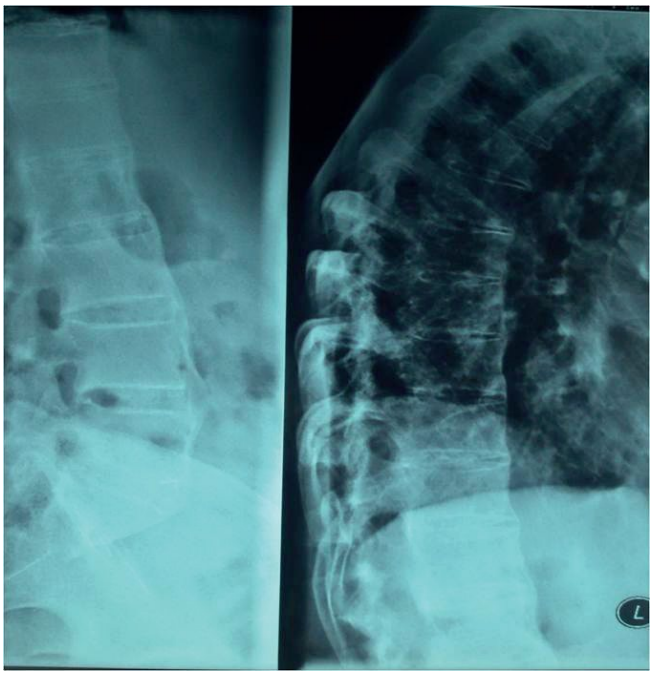

Figure 1. Radiograph of lumbosacral and thoracolumbal spine

A radiograph of the cervical spine was also taken and showed anterocorporal osteophytes and signs of anterior ligament calcification. Radiographs of the sacroiliac joints showed reduced bilateral sacroiliac joint intraarticular space with signs of subchondral sclerosis (Figure 2).

Chest HRCT was performed, which excluded changes in lung parenchyma and confirmed involvement of thoracic vertebrae. The diagnosis of AS was set on the basis of New York Criteria (bilateral sacroiliitis, grade 3) and clinical criteria (back pain, lumbal spine limitation and chest expansion limitation). HLA typing (HLA B27+) was subsequently performed and confirmed the diagnosis of ankylosing spondylitis.

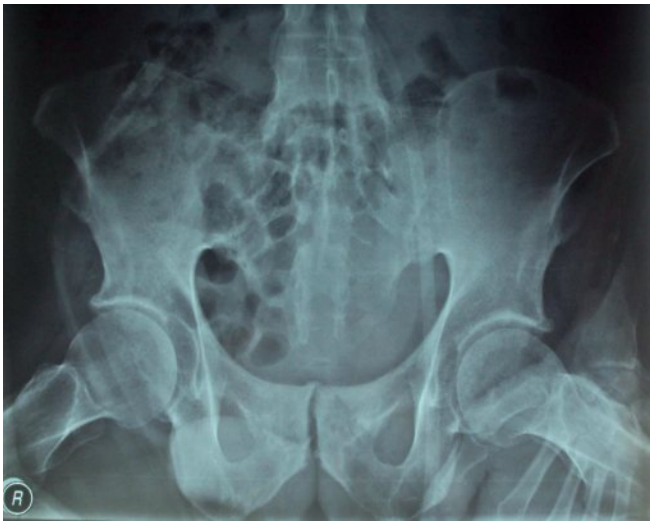

Figure 2. Radiograph of sacroiliac joints

Figure 3 shows the consequences of AS on the costovertebral, costotransversal, sternoclavicular and sternomanubrial joints.

Pulmonary function test results indicated a severe restrictive disorder of the lungs (FVC = 40%, FEV1 = 48%, FEV1/FVC = 96.4; body pletysmography: TLC = 65%). The testing of respiratory muscle force showed significant reduction in force, primarily of the inspirium muscles (Pimax = 20.2%, Pemax = 45%). Due to excessive daytime sleepiness, a sleep study (polysomnography) was done that verified the existence of severe obstructive sleep apnoea (AHI = 73, mean $SatO_2$ = 81%, minimal $SatO_2$ = 51%, while $SatO_2$ < 90% was registered over 76% of the recorded time).

After cardiorespiratory stabilisation, the patient was discharged from the hospital with a NIV (bilevel) device for home use during the night. Respiratory rehabilitation was recommended.

DISCUSSION

In the present case, AS was diagnosed in the late stage of the disease with the development of chronic respiratory failure based on observed changes on radiography of the vertebral column and positive HLA (human leukocyte antigen) typing. (1). This patient was diagnosed with AS at the age of 54, which places him in a group of 5% of the patients who became symptomatic after the age of 45. The involvement of thoracic vertebrae, costovertebral, costotransverse, sternoclavicular and sternomanubrial joints results in an increase in dorsal kyphosis and rigidity of the thorax in patients with AS (2). Cerrahoglu et al. reported that sacroiliac joint and costovertebral joint involvement showed a statistically significant correlation with decreased chest expansion, longer disease duration and increased morning stiffness in AS (3).

The simplest way to estimate reduced respiratory movement of the thorax is by measuring respiratory index (RI) on the level of the 4th intercostal space (in our patient it was 1.5 cm). A review of the literature shows that the mean value of RI in patients with AS is 3.9 + - 2.2 cm, which is significantly lower than in the control group (5.6 + -0.6 cm) without AS (4). The same study found restrictive disorder in 33.3% of patients with AS. Additionally, there was a significant positive correlation between VC and FVC with the expansion of the thorax (RI) values (4). Results of the study from Vanderschueren (5) also showed that a restrictive lung function disorder is commonly associated with reduced expansion of the thorax. On the contrary, the study from Cerrahoglu (3) showed that the parameters of pulmonary function did not correlate significantly with the involvement of costotransversal and costovertebral joints and decreased expansion of the thorax.

Reduced respiratory muscle strength (especially inspirium muscles) was found in our patient, which further contributes to reduced mobility of the chest and restrictive syndrome. In clinical practice, respiratory muscle function is evaluated with maximum inspiratory pressure and maximum expiratory pressure. Chest wall mobility is often measured in clinical practice, but the correlations between chest wall mobility and respiratory muscle strength are unknown in patients with AS. In a study from de Cordoba et al., significant positive correlation was found between the strength of respiratory muscles (IPmax) and the mobility of the chest (thoracic cirtometry at the top of the processus xyphoideus) (6).

Our patient was also diagnosed with severe obstructive sleep apnoea (OSA). Literature review shows that the prevalence of OSA in patients with AS is from 12 to 22.6%, which is significantly higher than the prevalence in the general population (7, 8). Significantly higher prevalence of OSA in AS was found in patients older than 35 years and with disease duration longer than 5 years (8). The assumed mechanisms that contribute to the occurrence of OSA in AS are reduced airway diameter at the level of the oropharynx due to the affection of the temporomandibular joint and compression of the trachea and pharynx due to the affection of the cervical spine.

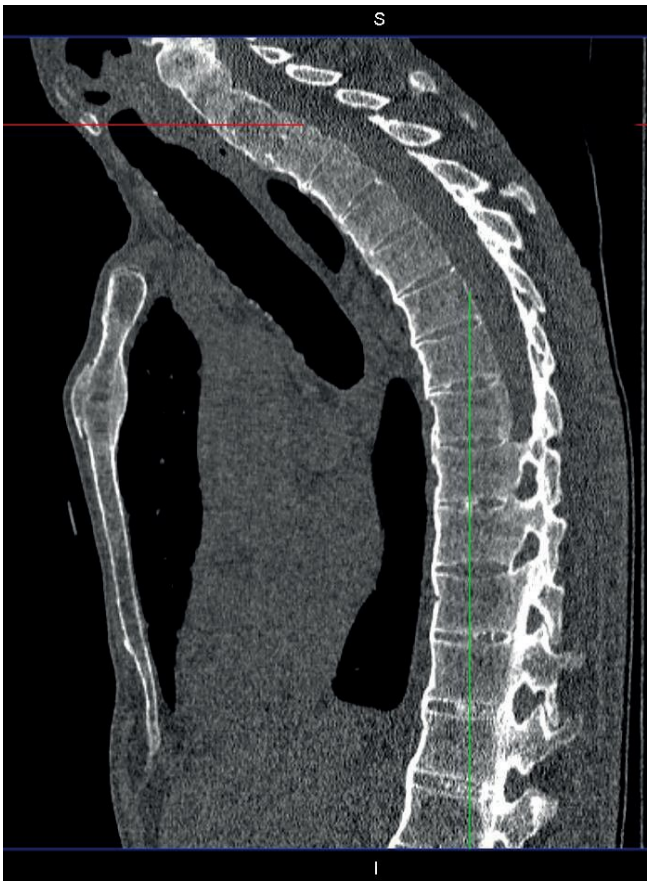

Figure 3. Spine and sternum CT scan

CONCLUSION

It could be concluded that this is a patient with newly diagnosed ankylosing spondylitis at the age of 54, with consequent severe restrictive syndrome, secondary atrophy of respiratory muscles and global respiratory failure global respiratory failure with severe obstructive sleep apnoea.

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APPLICATION OF THE VIRTUAL BRONCHOSCOPY IN CHILDREN WITH SUSPECTED ASPIRATION OF THE FOREIGN BODY CASE REPORT

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PRIMENA VIRTUALNE BRONHOSKOPIJE KOD DECE POD SUMNJOM NA ASPIRACIJU STRANOG TELA PRIKAZ SLUČAJA

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ABSTRACT

In diagnosing the aspiration of the foreign body (AFB) in children most important are: medical history, clinical signs and positive radiography of the lungs. Common dilemmas in the di erential diagnosis are life–threatening asthma attacks or di cult pneumonia. Conventional rigid bronchoscopy (RB) is not recommended as a routine method. Virtual bronchoscopy (VB) can be a diagnostic tool for solving dilemmas. Fiber–optic bronchoscopy (FOB) has a therapeutic stake in severe cases. Herein, we describe a girl, at the age of 6, who was hospitalized due to rapid bronchoconstriction and based on the anamnesis, clinical symptoms and physical findings the suspicion was that she aspirated the foreign body. Due to the poor general condition and possible sequel, the idea of RB was dropped out. Multidetector computed tomography of the chest and VB was performed and AFB was not found. Due to positive epidemiological situation, virus H1N1 was excluded. FOB established that the foreign body does not exist in the airways. During bronchoscopy numerous castings are aspirated from the peripheral airways which lead to faster final recovery. With additional procedures, the diagnosis of asthma was confirmed and for girl that was the first attack. Along with inhaled corticosteroids as prevention she feels well.

Virtual bronchoscopy can be successfully used as a valid diagnostic procedure in suspected foreign body in the children's lungs, but fiber–optic bronchoscopy remains most important diagnostic and therapeutic method.

Keywords: *asthma, children, foreign body, virtual bronchoscopy.*

SAŽETAK

U dijagnostikovanju aspiracije stranog tela (AFB) kod dece najvažniji su: anamneza, klinički znaci i pozitivna radiografija pluća. Česte dileme u diferencijalnoj dijagnozi su po život opasni napadi astme ili teška upala pluća. Konvencionalna rigidna bronhoskopija (RB) se ne preporučuje kao rutinska metoda dok virtuelna bronhoskopija (VB) može biti dijagnostička metoda za rešavanje dilema. Fiberopticka bronhoskopija (FOB) ima terapijskog udela u težim slučajevima. Ovde, opisujemo devojčicu, uzrasta 6 godina, koja je hospitalizovna zbog naglo nastale bronhokonstrikcije i na osnovu anamneze, kliničkih simptoma i fizikalnog nalaza posumnjano je da se radi o aspiraciji stranog tela. Zbog lošeg opšteg stanja i eventualnih komplikacija, odustalo se od RB. Urađena je multidetektor kompjuterizovana tomografija (MDCT) grudnog koša i virtulena bronhoskopija (VB) i AFB nije pronađena. Zbog pozitivne epidemiološke situacije, virus H1N1 je isključen. FOB je utvrdila da strano telo ne postoji u disajnim putevima. U toku bronhoskopije aspirirani su brojni odlivci u perifernim disajnim putevima što je i dovelo do konačnog bržeg oporavka. Dodatnim procedurama devojčici je dijagnostikovana astma i to je bio prvi po život opasan napad. Uz prevenciju sa inhaliranim kortikosteroidima ona je nakon tog napada bila bez tegoba.

Virtuelna bronhoskopija može se uspešno koristiti kao validna dijagnostička procedura kod sumnje na strano telo u plućima kod dece ali fiberoptička bronhoskopija ostaje suverena metoda kako u dijagonstici tako i u njenom terapijskom učinku .

Ključne reči: *astma, deca, strano telo, virtuelna bronhoskopija.*

ABBREVIATIONS

AFB – aspiration of the foreign body
BDT – bronchodilator test
ECP – eosinophilic cationic protein
FEV1 – forced expiratory volume in first second
FOB – fiber–optic bronchoscopy
IMC – Institute for Health Protection of Mother and Child of Serbia "Dr Vukan Cupic"
MDCT– multidetector computed tomography
RDG– radiography of chest
RB– rigid bronchoscopy
VB – virtual bronchoscopy

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INTRODUCTION

The aspiration of the foreign body (AFB) into the tracheobronchial treeis unfortunatelly still very common especially in children that are less then tree years old (1). In diagnosing AFB of very great importance are: information on suspicion of aspiration, clinical signs (choking, wheezing, stridor, paroxysmal cough), followed by data on recurrent infections of the lower respiratory tract and positive lungs radiography (RDG) (2, 3).The symptoms and signs are often not so obvious, and in over 30% of the patients the RDG is normal and often children are then treated under a diagnosis of asthma or difficultpneumonia (3).

Although the rigid bronchoscopy (RB) is the conventional method for the diagnosis and removal of foreign bodies in the airways, it is invasive, can cause many complications and it can happen that the suspected foreign body is not confirmed after procedure, therefore RB is not recommended as a routine method (4, 5). On the other hand, asthma is the most frequent chronic disease in children with increasing tendency and hastily formed bronchoconstriction is often caused by a severe asthma attack which raises doubts about the justification of bronchoscopy (6). Fiber–optic bronchoscopy (FOB) is considered safe and it has a therapeutic stake in severe cases life–threatening asthma attacks and in freeing the airways of mucous plugs. Therefore, in children age, there is a common dilemma in the differential diagnosis of asthma and aspiration of foreign body and vice versa, so it is necessary to react quickly (7).

Because of all this, today the more attention ispaid to non–invasive methods such as multidetector computed tomography (MDCT) and virtual bronchoscopy (VB). They are much more applicable for pediatric patients and represent the pinnacle of the new techniques in the diagnosis of foreign body aspiration in children (8, 9).

CASE REPORT

Six years old female child was hospitalized in the Pediatric Clinic, Clinical Centre “Kragujevac” as an emergency due to coughing, cyanosis, dyspnea, extreme fatigue and collapse. In the evening before the admission, the girl had dry cough, initially occasionally, and then during the night intensively and in paroxysms. The next day, in the morning she visited the competent dispensary where she collapsed and after inhalation with beta 2 agonists immediately referred to Pediatric Clinic. Before the cough started she was healthy, but just before that she ate chocolate with hazelnuts and since she does not like the hazelnuts she said that she removed them from the chocolate. She had not sniffle or fever.

From personal history was learned that she is the first child of neat pregnancy and premature birth in the 32nd week of gestation, weighing 1,580 grams at birth, Apgar score 7. She spent one month in the Department of Neonatology, but she was not on the mechanical ventilation. Since the birth she was on the infant milk formula. Early psychomotor development was uneventful. She does not have food and medicine allergy, episodes of eczema and wheezing. Neatly vaccinated according to the calendar of vaccination and so far she was healthy. She is attending collective preschool. No family diseases of interest. Father is smoker.

Physical examination on the admission proved that the girl had normal height and nutritional status for her age (BH=118cm; BW=20kg; BMI=14.39), she was somnolent, collapsed, dispnoic, pale with signs of peripheral cyanosis. Pharynx hyperemic, tongue white coated. During the inspection, the chest was evidently cylindrical, with visible respiratory asymmetric movements and pronounced retraction of intercostal space above the left hemithorax. Auscultatory over the lungs, the asymmetry finding above the left hemithorax weakened respiratory sound with extended titration (1:3) inspiratory–expiratory wheezing and inspiratory crackles from that side. Respiratory rate increased (40/min), and oxygen saturation on room air was reduced (SaO2=87%). Cardiac action was rhythmic, tachycardic (CF=127/min), tones covered with the findings on the lungs. During the course of reviewing the girl lost her conscious which was re–establishes with the use ofoxygen.

Immediately on the admission the completed laboratory analyzes showed respiratory acidosis, PH=7.31, pO2=6,9kPa, pCO2=6.4kPa, leukocytosis with neutrophilia (Le=23.9x10^{9}/l – Neu 82%), with normal values of glycaemia, electrolytes count, erythrocytes sedimentation, C–reactive protein, erythrocyte count, hemoglobin, platelet count, urea, creatinine, alkaline phosphatase, transaminases and serum amylase.

Under the suspicion on the foreign body the RDG was immediately made. The RDG shows that the trachea has normal flow and caliber, present bilateral hyperinflation of the lung parenchyma with accented interstitial shadows on pericardial ribbon, without visible radio transparent foreign body (Figure 1a, 1b and 1c). During the X–ray diagnosis, the general health condition deteriorated, oxygen saturation falls on 75% and hypercapnia with hypoxemia was deeper. The emergency resuscitation measures are immediately applied (oxygen, bronchodilators and corticosteroid therapy), after which the situation was somewhat better. The specialist radiologist and specialist for ear, nose and throat was consulted due to the continuing lack of recovery of the child and asymmetry on the physical findings on the lungs and all under suspicion of non–transparent foreign body.

Because of the difficult health situation of the girl the RB is redrawn and after consultative review the additional diagnostic MDCT of the chest and VB were performed. For the first time in our Clinical Center the virtual bronchoscopy was performed on the younger ages child. Findings of the chest MSCT showed in the sections on both sides at the level of the lower lobe, more left, accented interlobular septum with some consolidation spotting areas, on the

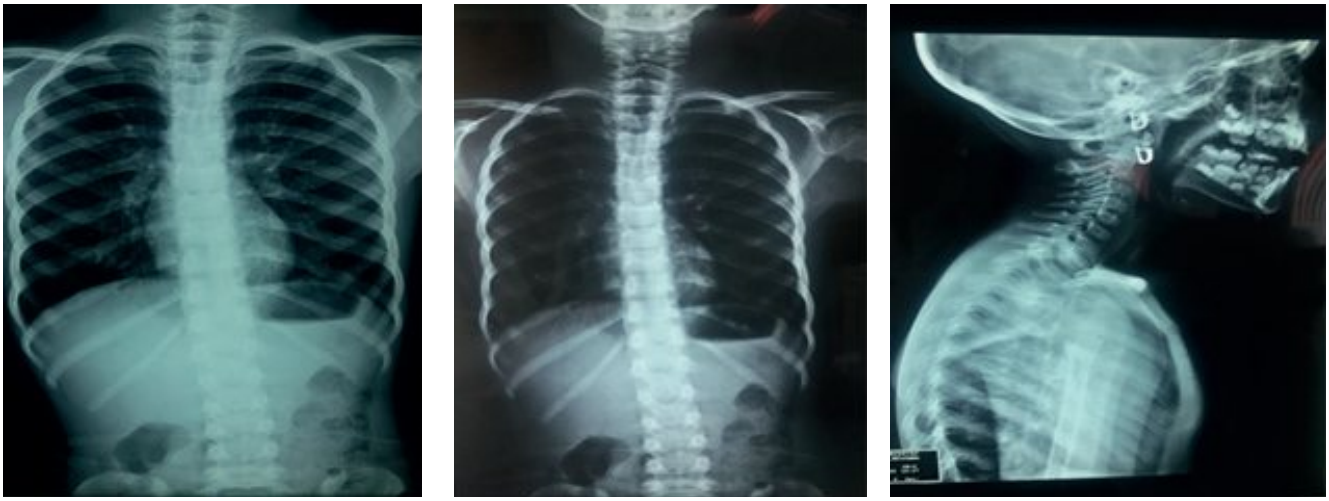

Figure 1. a) and b):RDG pulmo on admission (PA): the signs of hyperinflation and increased bronchovascular tracery on both sides; c) Profile RDG of lung – the trachea is adequate transparency and caliber

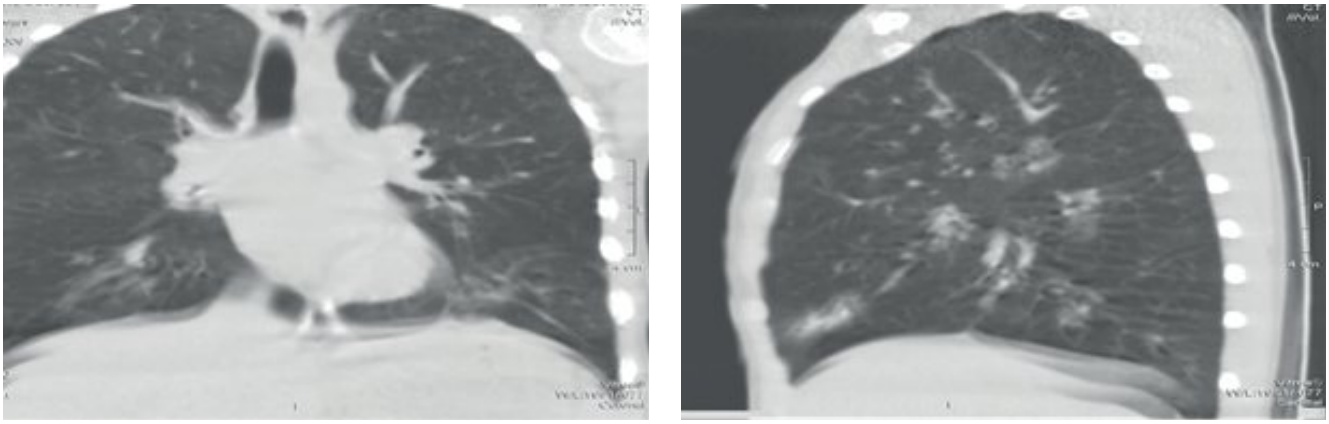

Figure 2. and 3. Multidetector computed tomography of the chest

right covers anterior basal segment and on the left lateral–posterior basal segment (Figure 2 and 3). Some right subsegment bronchi on the anterobasal level segment were filled with content (findings are going in favor of interstitial pneumonia). Both pleural space were free. By using the virtual bronchoscopy of the larynx, trachea and bronchi up to lobar the segmental fields were clear (Figure 4).

Completed VB excludes the foreign body as a cause of rapid onset of respiratory failure and after consulting the specialist for the infectious diseases and epidemiologist, because of the existence of the H1N1 virus between the population, the antiviral therapy was introduced. Since the unchanged poor condition of the patient remained the following days, she was sent to the Institute for Health Protection of Mother and Child of Serbia "Dr Vukan Cupic" (IMC), Belgrade, for further treatment and diagnostics.

During the hospitalization in IMC and after obtained analysis, the virus H1N1 was excluded. By underwent FOB the observed mucosa of the tracheobronchial trees erythematous, edematous and covered with a glassy secretion which cast in the form of numerous peripheral bronchial and aspirates on the both sides. There was no sign of a foreign body, compression and malacia of the central airways. That confirmed the previous VB finding and confirmed the suspected serious bronchial obstruction.

In IMC the patient after aspiration of the secretions showed the significant clinical and auscultative improvement, so she continued the therapy with bronchodilators

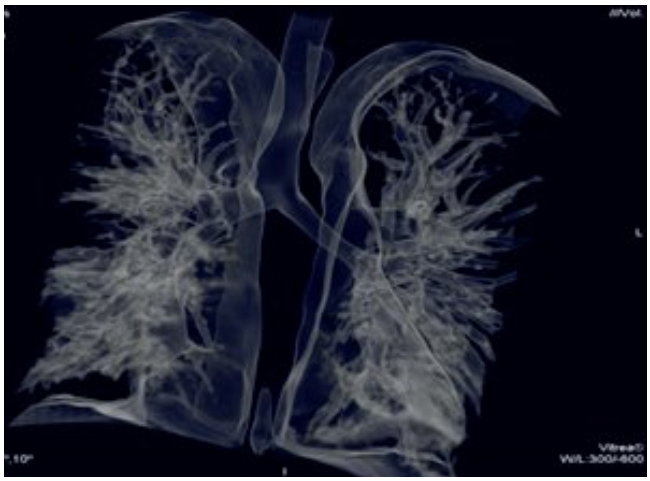

Figure 4. Virtual bronchoscopy of the larynx, trachea, bronchi and both principal lobar and segmental branches

and corticosteroids. The performed spirometry showed forced expiratory volume in first second (FEV1) 88%, with a positive bronchodilator test (BDT +25%) and skin–prick test which showed sensitization to mites and dust (+6mm and +3mm). After 6 days of hospitalization the girl was released home with a diagnosis of acute bronchoobstruction and a suspicion that this is the first really severe asthmatic attack.

In the prevention they introduced inhaled glucocorticosteroids. After a month and a half on the follow–up examination in our pulmonary ambulance, the girl was in a great general condition, pulmonary function neat, FEV1=120%, BDT negative, auscultator findings tidy. Then the food allergy test was performed and the same show sensitizations to spinach and milk (+6mm and +6mm) and was negative for other nutritive allergens, among others the peanuts and cocoa. The total immunoglobulin E (IgE) was elevated – 250 IU/mL (normal for age is <63IU/mL) and a specific IgE to peanut and hazelnut was negative (<0.35), with normal values of eosinophilic cationic protein (ECP=7.31µg/L). Since the established diagnosis was asthma, the therapy with inhaled glucocorticoids continued. After a year the girl is well with normal lung function.

DISCUSSION

Despite the precautions, AFB is one of the major causes of mortality in childhood. Approximately 500–3000 children annually die due to aspiration of a foreign body (2). The most common foreign bodies are of organic nature and the most common localization because of its anatomical features is the main right bronchus (1). In rapid onset of wheezing, particularly where the inspiratory wheezing dominates, cyanosis, cough, and where there is asymmetry of physical findings in the lungs, the possibility of aspiration of a foreign body should always be ruled out and particularly with preschool children (1, 2). A large number of studies indicated that the suppression after eating, coughing, cyanosis, dyspnea, tachypnea, inspiratory stridor, impaired breathing and asymmetry of auscultation findings are important parameters in suspected aspiration of the foreign body (10, 11). Since we have almost all that symptoms and signs with our patient we set up the suspicion of aspiration of hazelnut that was in the chocolate, that girl consumed the previous evening.

The longer retention of foreign bodies in the airways may cause the lung infection, bronchiectasis, atelectasis and pulmonary abscess and therefore early diagnosis is of great importance (12). After clinical findings, the next step in the diagnosis was RDG that could indicate the occurrence of infiltration or trapped air as indirect sign of the presence of non–transparent foreign body. Many studies agree that with the aspiration of a foreign body the chest X–ray is normal in a high percentage (30–48%) and that relying only on the X–ray of the lungs can lead to the delayed diagnosis of AFB (3, 7, 10, 11, 13).

Hillard et al. finds that the diagnosis only 45% are found during the first day and in the high percentage of 17% that the diagnosis are found after a month of aspiration (13). Taking into the consideration the fact that before the brochoscopy the clinical diagnosis of foreign body aspiration is set in about 2/3 of patients and that in 30% the same is treated as pneumonia, 2% as asthma and 2% as laryngitis. Samkani and his associates suggests that bronchoscopy must be performed in all suspicious cases (10).

Knowing all this we were thinking about further diagnostic procedures. Because of the complications that entails and based on the previous analysis, we gave up from the rigid bronchoscopy, which could lead to unnecessary potential sequels. We take into the consideration the fact that in approximately ¼ of patients with suspected foreign body aspiration RB was negative (4, 5). We decided to do MDCT of the girl's chest and virtual bronchoscopy, which for the first time was done in our Clinical Centre on children. Kosucu et al. found a high correlation of 100% between MDCT and VB with RB in the diagnosing the AFB which in our case was confirmed as correct (14).

After the findings of the VB that there is no foreign body in the airways and due to the continued poor health state of the child, we decided to send the patient to an institution of higher instance that can perform the additional diagnostic in sense of fiber–optic bronchoscopy, that is not possible in our institution and which other authors recommend as the next step in further diagnostics (15, 16). In the IMD, where the child was sent, after excluding H1N1 viral bronchopneumonia, the FOB was performed and it was confirmed that there is no foreign body in the airways.

Bronchus obstruction in very small children with asthma can occur suddenly, as is seen in the so–called fatal asthma attack or "brittle" asthma. In these cases, viscous mucus hyper secretion is often dominate and leads to the formation of mucus plugs that seals the lumen of the airways. In asthmatic attack, auscultation and inspection of lung can be registered different degrees of obstruction between the right and left side, or at different levels of lung due to smooth muscle spasm, mucosal edema and mucus hypersecretion in particular. It often can lead to atelectasis or pneumothorax. This often results in suspecting the bronchopneumonia, foreign body or atelectasis on one side (7).

Findings of the mucus plugs with the extensive destruction and desquamation of the bronchial epithelium of the almost entire length of the bronchial tree of the patient that dies from severe asthma says how much this phenomenon is serious. Sometimes it is necessary to perform the bronchoscopy to free up the airways from the mucous plugs (6, 7). In our little patient, during the bronchoscopy the numerous castings in peripheral airways were aspirated which lead to a faster final recovery.

Veras and associates also stated that theVB is fast, efficient and that the classic bronchoscopyis not superior, although endobronchial secretions can sometimes create difficulties in diagnosis (17). Numerous studies agree that it is greater benefit of non–invasive diagnostic proce-

dure which allows the MDCT virtual bronchoscopy than the potential harmful effects of radiation. This method three-dimensionally shows the tracheobronchial tree in children with unexplained respiratory symptoms or who suspected of presence of a foreign body. This avoids complications that carry general anesthesia and invasive rigid bronchoscopy. It can also be very useful for correct localization of pathological changes or foreign bodies preoperatively(18–22).On the other side, even though this is an excellent method for determining intra and extra luminary changes with the possible visualization of the distal bronchi certainly is not recommended for routine execution because of the dangerous radiation (8, 9).

Although MDCT–VB is well correlated to the flexible bronchoscopy, it has its limitations. Until today the only direct way to observe the respiratory tract is still FB. FB is also an advantage in removing foreign bodies, bronchoalveolar lavage and taking a biopsy, especially in infants and young children. Flexible bronchoscopy directly evaluates and dynamics of the airway (10, 16, 22).

Additional methods, such as spirometry, skin prick test, total and specific IgE as well as on the specific food additionally helped in diagnostic and in the treatment of the underlying disease.

CONCLUSION

Virtual bronchoscopy of the larynx, trachea, bronchi and both principal lobar and segmental branches can be successfully used as a valid diagnostic procedure in suspected foreign body in the lungs in children, but fiber–optic bronchoscopy remains sovereign methods in diagnosing the respiratory insufficiency and in its therapeutic effect.

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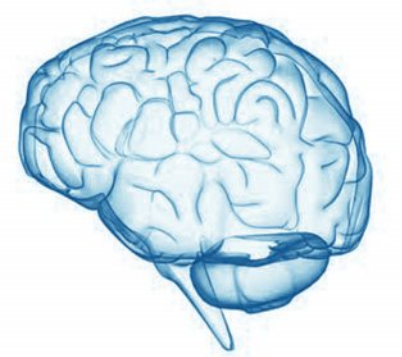

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