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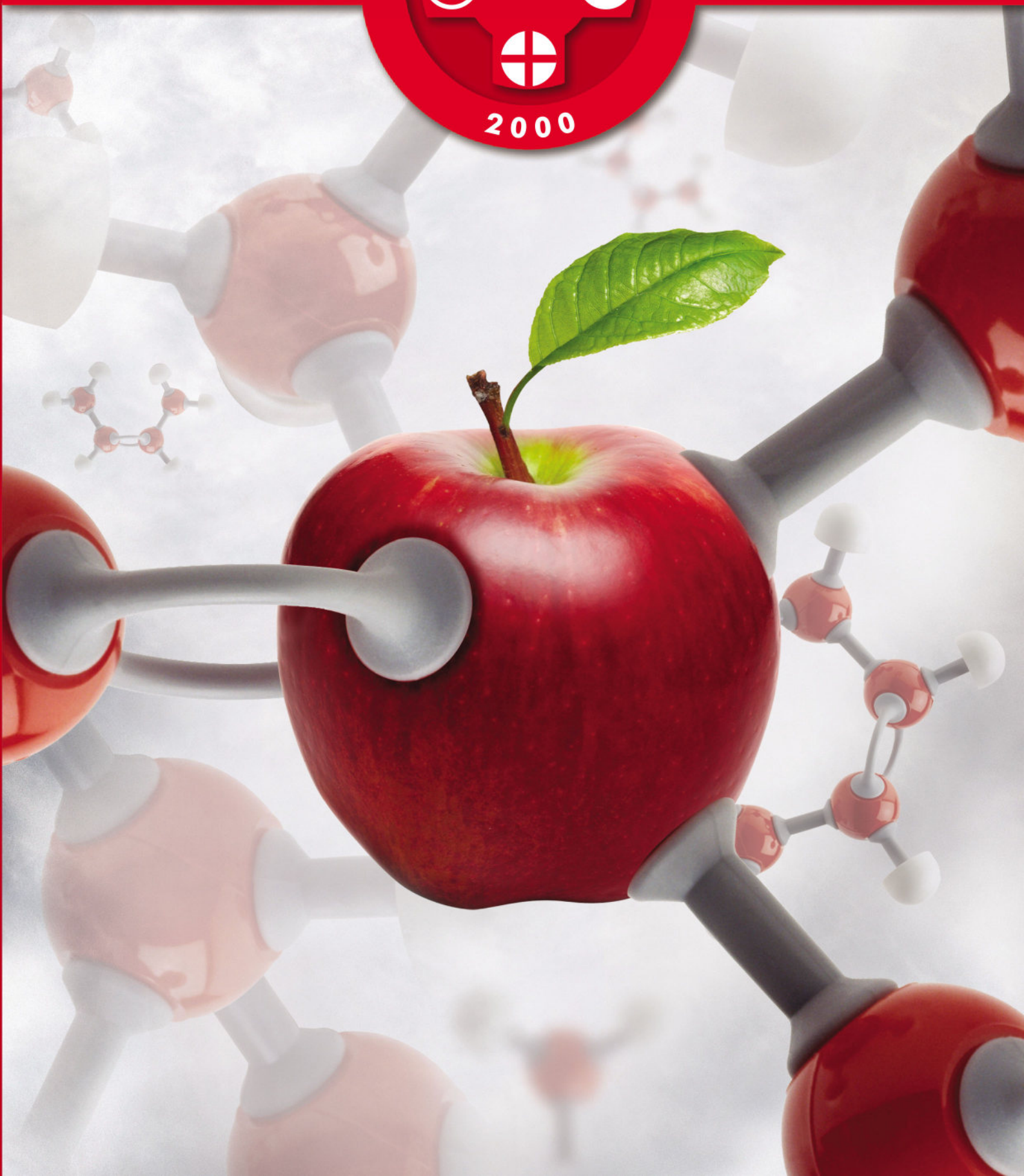

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ROLE OF CALCIUM CHANNEL BLOCKERS IN MYOCARDIAL PRECONDITIONING

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ULOGA BLOKATORA KALCIJUMSKIH KANALA U PREKONDICIONIRANJU SRCA

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ABSTRACT

Coronary heart disease is the leading cause of mortality and morbidity worldwide. The effects of coronary heart disease are usually attributable to the detrimental effects of acute myocardial ischaemia-reperfusion injury. Newer strategies such as ischaemic or pharmacological preconditioning have been shown to condition the myocardium to ischaemia-reperfusion injury and thus reduce the final infarct size. This review investigates the role of calcium channel blockers in myocardial preconditioning. Additionally, special attention is given to nicorandil whose mechanism of action may be associated with the cardioprotective effects of preconditioning. There are still many uncertainties in understanding the role of these agents in preconditioning, but future research in this direction will certainly help reduce coronary heart disease.

Keywords: *Preconditioning, Calcium channel blockers, Nicorandil*

SAŽETAK

Koronarna bolest srca je vodeći uzrok mortaliteta i morbiditeta širom sveta. Efekti koronarne bolesti srca se najčešće pripisuju štetnim efektima akutnog infarkta, odnosno ishemijsko-reperfuzionoj povredi. Novije strategije, kao što su ishemijsko i farmakološko prekondicioniranje, su pokazale da uticajem na ishemijsko-reperfuzione povrede srca konačna veličina infarkta može biti smanjena. Ovaj pregledni članak ispituje ulogu blokatora kalcijumskih kanala u prekondicioniranju srca. Takodje, posebna pažnja je posvećena nikorandilu, čiji bi mehanizam dejstva mogao biti povezan sa kardioprotektivnim efektima prekondicioniranja. Još uvek postoji mnogo nejasnoća u razumevanju uloge ovih lekova u prekondicioniranju ali buduća istraživanja u ovom pravcu bi svakako doprinela smanjenju koronarne bolesti srca.

Ključne reči: *Prekondicioniranje, Blokatori kalcijumskih kanala, Nikorandil*

ABBREVIATIONS

ATP - Adenosine triphosphate
CCBs - Calcium channel blockers
CHD - Coronary heart disease
I/R - Ischaemia-reperfusion
IPC - Ischaemic preconditioning
K(ATP) - ATP-sensitive K+
PKC - protein kinase C
ROS - reactive oxygen species

INTRODUCTION

Coronary heart disease (CHD) is the leading cause of mortality and morbidity worldwide. According to the World Health Organization, 12.8% of all deaths result from CHD. The effects of CHD are usually attributable to the detrimental effects of acute myocardial ischaemia-reperfusion (I/R) injury (1, 2). This injury includes different clinical manifestations such as myocardial necrosis, arrhythmia, myocardial stunning and endothelial and microvascular dysfunction. Depending on the severity of the condition, the patient may be treated with medications, surgery or both. The treatment includes the use of thrombolytic agents, beta-antagonists, angiotensin-converting enzyme inhibitors, calcium channel blockers (CCBs), coronary artery bypass surgery, angioplasty and stenting (3-5). Despite

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several therapeutic advances, both medical and surgical, there is still no effective therapy for preventing myocardial I/R injury.

In the past few decades, it has become clear that the myocardial response to I/R injury can be reduced. Based on recent studies, a universally accepted potential endogenous strategy for protecting the heart against acute ischaemia-reperfusion injury is preconditioning. Research into the mechanisms of preconditioning has revealed multiple receptors, pathways, and end effectors. Recent studies suggest that certain pharmacological agents may stimulate these mechanisms. Our understanding of the complex mechanism of preconditioning is still incomplete, and its disclosure could contribute to the treatment of acute myocardial infarction.

PHENOMENON OF MYOCARDIAL PRECONDITIONING

Myocardial preconditioning is a process where myocardium cells or tissues are exposed to a sublethal stimulus to protect them from a subsequent normally lethal stress. Preconditioning can attenuate the subsequent prolonged or lethal tissue injury by increasing the cell tolerance to the stress (6). The term "preconditioning" was used for the first time in 1964 by Janoff to refer to the phenomenon of stress-induced endogenous tolerance against traumatic or endotoxic insults (7). The protective effect of preconditioning in the heart can be demonstrated by the reduction in infarct size and myocardial stunning, prevention of arrhythmias, or the acceleration of the recovery of myocardial function after ischaemia (8-10). Myocardium can be preconditioned by two basic techniques including ischaemic and pharmacological preconditioning.

Ischaemic preconditioning (IPC)

Ischaemic preconditioning was described for the first time in 1986 by Murry and colleagues when they found that a "preconditioned" heart in a canine model became resistant to ischaemia-induced infarction. In fact, infarct size and the degree of reperfusion injury were reduced by single or multiple cycles of ischaemia and reperfusion (11). The same beneficial effect has since been confirmed in every species studied, independently of both the presence of collaterals in the coronary circulation and the size of the animal model (12-14), and has more recently been confirmed in the human (15).

The underlying mechanisms of IPC are still a matter of debate. Protective effects of IPC on the heart can be a consequence of the reduction in reactive oxygen species (ROS) generation, delay in adenosine triphosphate (ATP) depletion, and the reduction of the infarct size, apoptosis and neutrophil accumulation. It is possible that adenosine, noradrenaline and bradykinin play a role in this mechanism (16-18). In addition,, activation of protein kinase C (PKC), which is known to be a key player in numerous intracellular signal transduction pathways, is believed to be one of the main causative mechanisms in the protection of IPC across various species (19). Nevertheless, for many of the proposed mechanisms, a modulation of intracellular calcium ion ($[Ca^{2+}]i$) homeostasis may be the final common pathway in the protection against ischaemic injury. A rise in intracellular free $[Ca^{2+}]i$ has been postulated to be an important factor in ischaemic myocardial injury (20, 21).

Notably, there are many candidates for the mechanism of IPC. Recently it has been reported that the activation of PKC opens the ATP-sensitive K^+ (K(ATP)) channels which is currently thought to be the end-effector of many signal transduction systems related to the IPC mechanism. Therefore, it is thought that K(ATP) channel openers are also effective in protecting against human ischaemic injury (22).

Myocardial ischaemia occurs when blood flow to the heart muscle (myocardium) is obstructed by a partial or complete blockage of a coronary artery. Coronary arteries can be occluded by thrombi, atherosclerotic plaques, vasoconstriction or inflammation (23). During myocardial ischaemia, the absence of oxygen and metabolic substrates in cardiomyocytes can cause functional, structural and metabolic diseases (24). As a result, the cells switch to anaerobic metabolism, which leads to the accumulation of lactate and a drop in intracellular pH. The main result is intracellular Na^+ overloading. Therefore, diminished intracellular concentrations of ATP and creatine phosphate cause decreased activity of adenosine triphosphate-reliant ion pumps, including the Na^+/K^+ ATP-ase pump, and the exacerbation of contractile function. This induces the Na^+-H^+ exchanger to extrude H^+ and results in intracellular Na^+ overload, which activates the $2Na^+$-Ca^{2+} exchanger to function in reverse to extrude Na^+ and leads to intracellular $[Ca^{2+}]i$ overload. These processes and the generation of ROS can lead to cell death induced by ischaemic episodes (20, 25-27).

Furthermore, with reperfusion, the restoration of blood flow after an ischaemic episode may result in paradoxical cardiomyocyte dysfunction caused by ROS, intracellular and mitochondrial Ca^{2+} overload and accumulation of inflammatory cells. The phenomenon called "reperfusion injury" occurs when prompt changes in intracellular ions and normalization of pH cause cell death and greater damage than what is induced by pre-reperfusion ischaemia (26, 28).

Pharmacological preconditioning

As we mentioned above, in addition to IPC, preconditioning can be induced in the heart with pharmacological agents. However, it is still unclear whether the various forms of pharmacological preconditioning have the same molecular mechanisms as ischaemic preconditioning. In recent years, many investigators have studied myocardial

pharmacological preconditioning with various agents (29-32), but particular attention has been directed towards pharmacological agents that modulate Ca^{2+} (33, 34).

Ca^{2+} channel blockers are well known to be cardioprotective when taken after I/R injury (35, 36), but little is known about a possible preconditioning effect before ischaemia. Controversy remains about their ability to reduce infarct size or at least delay the necrotic process. With this in mind, the aim of this review was to examine the possible role of calcium channel blockers as a mediator of ischaemic preconditioning.

CALCIUM CHANNEL BLOCKING AGENTS

Calcium antagonists or calcium channel blockers (CCBs) were introduced into clinical medicine in the 1960s and were approved for the treatment of hypertension in the 1980s (37, 38). CCBs have been one of the mainstays in therapy for cardiovascular diseases, such as angina pectoris, paroxysmal supraventricular tachyarrhythmia, hypertrophic cardiomyopathy, Raynaud phenomenon, pulmonary hypertension, diffuse oesophageal spasm, and cerebral vasospasm, for many years (39). CCBs as a group are heterogeneous and, based on the chemical structure and functional distinctions, include 3 main classes: dihydropyridine, phenylalkylamine and benzothiazepine derivate (40, 41). The differences in chemical structures provide heterogeneity in the action of these agents. However, all CCBs inhibit calcium influx by binding to the α1 subunit of calcium channels and inhibit cell excitability (42). Inhibition leads to the relaxation of vascular smooth muscle cells, vasodilation and a lowering of blood pressure. In cardiac muscle, contractility is reduced, and the sinus pacemaker and atrioventricular conduction velocities are slowed (43, 44). CCBs also reduce angiotensin II–mediated vasoconstriction and decrease the angiotensin II-stimulatory effect on adrenal biosynthesis and secretion of aldosterone (45). Unlike other vasodilators, calcium antagonists induce mild natriuresis and do not cause volume retention. Thus, calcium antagonists lower blood pressure mainly by reducing peripheral vascular resistance (46).

Furthermore, in many clinical trials, there is controversial data on taking CCBs and an increase in cardiovascular mortality (47, 48). Although several theories have been offered, the mechanism by which CCBs increase cardiovascular mortality is still unknown.

However, the hypothesis that cellular calcium overload may contribute to the onset of irreversible ischaemic cell injury suggested a possible role for CCBs in the protection of ischaemic myocardium (49).

Dihydropyridine CCBs in myocardial preconditioning

Dihydropyridine CCDs include amlodipine, felodipine, isradipine, lacidipine, lercanidipine, nicardipine, nifedipine, nisoldipine, and others (40). Experimental data suggest that this group of CCBs binds to both dihydropyridine and nondihydropyridine binding sites. Dihydropyridines selectively inhibit calcium ion influx across cell membranes, with a greater effect on vascular smooth muscle cells than on cardiac muscle cells (50). Dihydropyridine CCBs have variable effects on heart rate. Acutely, these drugs tend to induce a reflex tachycardia, but long-term studies have shown similar heart rates before and during therapy (51).

Higher doses of these drugs are generally associated with an increase in heart rate. A group of authors even suggested that the use of short-acting nifedipine in moderate to high doses causes an increase in total mortality. In patients with poor or no collateral flow, nifedipine reduced ischaemic episodes, while in patients with good collateral flow nifedipine significantly increased them (52). Nicardipine and isradipine show the same effects (53).

In vitro experiments on human atrial trabeculae indicate that patients using CCBs (amlodipine n=7, diltiazem n=4, verapamil n=1) were not functionally protected by transient ischaemia. However, a difference in functional performance after I/R between atrial trabeculae with and without CCB exposure was not detected (54).

Likewise, not all studies have demonstrated increased cardiovascular mortality with CCBs. Wallbridge and colleagues studied whether pretreatment with nisoldipine could modify cardioprotective effects of IPC in pigs. Their results indicate that continuous infusion of nisoldipine throughout the entire protocol until onset of reperfusion does not attenuate the potential protective mechanism of IPC; in fact, it may have even exerted a small additional cardioprotective effect (55). These findings are in accordance with an experimental study in guinea pig isolated hearts by Camara and colleagues. They suggest that nifedipine given before ischaemia induces a preconditioning effect as shown by improved left ventricular pressure and lower $[Ca^{2+}]$ on reperfusion after ischaemia (56).

Phenylalkylamine CCBs in myocardial preconditioning

Phenylalkylamine CCBs are relatively selective for myocardium and are often used to treat angina; they also reduce myocardial oxygen demand, reverse coronary vasospasm. Within this group are verapamil, gallopamil, and others (41), which inhibit the alpha and beta subunits of voltage-dependent calcium channels. Specifically, their effect on L-type calcium channels in the heart causes a reduction in inotropy and chronotropy, reducing heart rate and blood pressure. The most commonly used drug from this group is verapamil (57).

Miyawaki and colleagues have indicated that pretreatment with verapamil alone (0,63 μmol/L; 15 minutes before I/R) did not exert a significant effect on ischaemic injury in the Langendorff-perfused rat model compared with no pretreatment in ischaemic control hearts. On the other hand, verapamil administered during IPC, at the same dose, significantly attenuated the salutary effects of IPC

(33). In addition, another study by the same authors demonstrated that ATP contents were significantly higher and cell structure was better preserved in Ca^{2+} preconditioned hearts than in ischaemic control hearts. In other groups, Ca^{2+} influx during Ca^{2+} preconditioning was inhibited with low doses of verapamil (0.2 and 0.5 μmol/L), but verapamil did not influence the cardioprotection of Ca^{2+} preconditioning. On the contrary, in hearts treated with 2 μmol/L verapamil, lactate dehydrogenase release was significantly increased, ATP content was reduced, mitochondria were swollen and partly disrupted, glycogen was depleted, and myofibrils were partly transformed into contraction bands (58).

The effects of verapamil preconditioning are controversial. Yu and colleagues found that verapamil preconditioning (20 μmol/L; 10 min) significantly improved diastolic and systolic functions and reduced the incidence of arrhythmias. One of the possible mechanisms for this effect is a reduction in the influx of [Ca^{2+}], thereby stabilizing cardiomyocytes in myocardial stunning and avoiding the occurrence of Ca^{2+}-induced [Ca^{2+}] release during I/R injury (59).

Benzothiazepine CCBs in myocardial preconditioning

This class of drugs is an intermediate class between phenylalkylamine and dihydropyridines in their selectivity for vascular calcium channels. By having both cardiac depressant and vasodilator actions, benzothiazepines are able to reduce arterial pressure without producing the same degree of reflex cardiac stimulation caused by dihydropyridines (41). The main representative of this group is diltiazem. Diltiazem is effective in the treatment of angina, and the longer-acting formulation is effective in the treatment of hypertension. It is less negatively inotropic than verapamil but should still be used cautiously with beta-blockers (60).

Okuda and colleagues have suggested that diltiazem (10 mg/kg) preconditioning leads to a reduction in the infarct area in the coronary artery of an adult mongrel dog (61). De Jong and colleagues studied the effects of diltiazem administered before or during myocardial ischaemia in the Langendorff-perfused rat heart. They observed that diltiazem decreases adenine nucleotide catabolism and presumably does not protect by negative inotropy during myocardial ischaemia. Myocardial function measured by the capacity to develop tension was decreased by diltiazem, and pretreated hearts did not show arrhythmias. Diltiazem also reduced myocardial oxygen demand, thereby diminishing the effect of flow impairment (62).

Nicorandil in myocardial preconditioning

As already mentioned, besides CCBs, K(ATP) channel openers are also effective in protecting against human ischaemic injury; therefore, we pay special attention to nicorandil.

Nicorandil is an antianginal drug whose properties lie between those of nitrates and K^+ channel openers. Activation of K(ATP) channels causes K^+ efflux, hyperpolarization of the smooth muscle cell membrane, and closure of voltage-gated Ca^{2+} channels. Closure of Ca^{2+} channels reduces intracellular levels of Ca^{2+}, resulting in relaxation of vascular smooth muscle and dilation of systemic and coronary arterioles. The nitrate moiety produces relaxation of vascular smooth muscle with dilation of systemic venous circulation and epicardial coronary arteries (63). This drug has been shown to be effective after oral administration in patients with stable angina and acute myocardial infarction (64, 65). Nicorandil allows for simultaneous dilation and relaxation of arterial and venous vasculature via its effect on smooth muscles (64).

Ohno and colleagues have shown that nicorandil preconditioning reduced the size of myocardial infarcts by opening the K(ATP) channels, and this effect was dependent on the plasma nicorandil concentrations immediately before the ischaemia induced in rabbits (66). This study corroborates the findings of Matsubara and co-authors where the preconditioning mechanism of nicorandil is explained by opening K(ATP) channels (67). Nicorandil reduces myocardial infarct size in various animal models. A chronic experiment on rabbits has shown that nicorandil (100 μg/kg bolus + 30 μg/kg–1·min–1 iv for 60 min) induces delayed cardioprotection against myocardial infarction (68). Similar findings were obtained in the study where rats were administered nicorandil (in oral dose; 3 or 6 mg/kg; 5 consecutive days). Rats were then subjected to myocardial I/R (40 min/10 min). Nicorandil was effective in attenuating the ischaemia/reperfusion-induced ventricular arrhythmias, creatine kinase-MB release, lactate accumulation and oxidative stress (69). Another possible mechanism of preconditioning is the upregulation in the expression of COX-2 and Bcl-2 as it occurs after IPC and nicorandil preconditioning (68, 70).

Several clinical studies have shown that nicorandil improves functional and clinical outcomes in patients with acute myocardial infarction (71, 72). Intravenous preadministration of nicorandil attenuates ST-segment elevation and improves lactate metabolism during coronary angioplasty, suggesting that pharmacological preconditioning is induced by nicorandil. Also a level of troponin T, one of the reliable metabolic markers of myocardial injury, is suppressed after coronary angioplasty as well as ST-segment elevation during coronary angioplasty (72). In the I-WIND trial, Kitakaze and colleagues randomized patients with an anterior ST-segment elevation myocardial infarction to receive intravenous nicorandil as a bolus or placebo after primary percutaneous coronary intervention. The overall morbidity and mortality were the same in both groups after 3 years. However, 61 patients continued on oral nicorandil after discharge, and in this group, the LV ejection fraction was better at the 6-month follow-up (73). Larger trials are needed, however, to examine the cardioprotective action of nicorandil.

Although many investigators have studied the role of calcium channel blockers in myocardial preconditioning, there are many open questions that future research should seek to answer. Nevertheless, the use of calcium channel blockers to mimic preconditioning in selected clinical settings may be a desirable future therapeutic goal. Based on current knowledge, we can say that nicorandil preconditioning is certainly worth investigation. More importantly, future studies should reveal simpler and even more effective therapeutic interventions for protecting the heart from ischaemia/reperfusion.

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ANTIPROLIFERATIVE ACTIVITY OF GOLD(III) COMPLEXES WITH ESTERS OF CYCLOHEXYL-FUNCTIONALIZED ETHYLENEDIAMINE-N,N'-DIACETATE

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ANTIPROLIFERATIVNA AKTIVNOST ZLATO(III) KOMPLEKSA SA CIKLOHEKSIL-FUNKCIONALIZOVANIM ESTRIMA ETILENDIAMIN-N,N'-DIACETATA

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ABSTRACT

Six gold(III) complexes with esters of cyclohexyl-functionalized ethylenediamine-N,N'-diacetate, general formula $[AuCl_2\{(S,S)\text{-}R_2eddch\}]PF_6$, [(S,S)-eddch = (S,S)-ethylenediamine-N,N'-di-2-(3-cyclohexyl)propanoate, R = Me, Et, n-Pr, n-Bu, i-Bu, i-Am, **1–6**, *respectively], were tested against cancer cell lines such as human melanoma Fem-x, human colon carcinoma LS174T and non-small cell lung carcinoma A549 as well as a non-cancerous human embryonic lung fibroblasts MRC-5 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with the aim of assessing in vitro antitumoral activity and selectivity. All investigated complexes showed lower cytotoxicity and better or similar selectivity in comparison to cisplatin, used as reference compound. Complex $[AuCl_2\{(S,S)\text{-}(i\text{-}Am)_2eddch\}]PF_6$ (**6**) demonstrated the highest activity against Fem-x (IC_{50} = 14.98 ± 0.34 μM). Additionally, the same complex expressed 4.5 times higher selectivity than cisplatin.*

Keywords: *cytotoxicity, gold(III) complexes, R_2edda type-ligands, selectivity*

SAŽETAK

Šest kompleksa zlata(III) sa cikloheksil-funkcionalizovanim estrima etilendiamin-N,N'-diacetata, opšte formule $[AuCl_2\{(S,S)\text{-}R_2eddch\}]PF_6$, ((S,S)-eddch = (S,S)-etilendiamin-N,N'-di-2-(3-cicloheksil)propanoat, R = Me, Et, n-Pr, n-Bu, i-Bu, i-Am, 1–6), ispitivano je na humanim ćelijskim linijama malignog melanoma Fem-x, karcinoma debelog creva LS174T, karcinoma pluća A549 kao i normalnim ćelijama MRC-5 (fetalni plućni fibroblast) korišćenjem 3-(4,5-dimetiltiazol-2-yl)-2,5-difeniltetrazolium bromid (MTT) testa u cilju procene in vitro antitumorske aktivnosti i selektivnosti. Svi ispitivani kompleksi pokazali su manju citotoksičnost i bolju ili sličnu selektivnost u odnosu na cisplatinu koja je korišćena kao referentna supstanca. Kompleks 6 je pokazao najveću aktivnost sa IC_{50} (Fem-x) vrednošću od 14,98 ± 0,34 μM. Takođe, isti kompleks pokazuje 4,5 puta veću selektivnost od cisplatine.

Ključne reči: *citotoksičnost, zlato(III) kompleksi, R_2edda tip-liganada, selektivnost*

ABBREVIATIONS

A549 - Non-small cell lung carcinoma cell line
Fem-x - Human melanoma cell line
HeLa - Human cervix adenocarcinoma cell line
K562 - Human myelogenous leukemia cell line
LS174T - Human colon carcinoma cell line
MRC-5 - Non-cancerous cell line human embryonic lung fibroblast
PBMC - Human peripheral blood mononuclear cells
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
R2edda-type ligand - *O,O'*-Dialkyl-ethylenediamine-*N,N'*-diacetate ester type ligands
R2eddch - *O,O'*-Dialkyl-(S,S)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate ester

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INTRODUCTION

Cancer is a disease of deregulated cellular behavior (1). The main processes of cancer treatment in humans are surgery, radiation, and chemotherapy. Cancer chemotherapeutic agents can often provide temporary relief of symptoms, give the patient more time and rarely cure. In recent years, a lot of research has been conducted to the synthesis of potential anticancer drugs (2–4). A successful anticancer drug should kill or inactivate cancer cells without causing excessive damage to normal cells (5, 6). Discovering of cisplatin by Rosenberg in the 1970s, platinum complexes became one of most commonly explored class of cytostatics in anticancer chemotherapy, but only a few are in worldwide clinical practice use or in clinical trials (7–10). An overview or some currently employed anticancer drugs (I–III), in clinical trials (IV, V) platinum-based compounds are illustrated in Fig.1.

The accumulation of platinum compounds in the body has deleterious effects. The two major problems associated with the use of cisplatin derivatives are the severe toxic side effects (11–15) and the intrinsic or acquired resistance manifested in various types of cancers (16). Therefore, in recent decades, a large number of new metal-based complexes have been developed and tested (17–19).

Gold complexes have recently gained a considerable attention as a class of antitumor compounds with different pharmacodynamics and kinetic properties than cisplatin with strong cell growth inhibiting effects (20, 21). Since gold(III) is isoelectronic to platinum(II) and both metals preferentially generate square planar complexes (d^8 system) a number of gold(III) complexes were synthesized and tested as an alternative to the anticancer drug cisplatin (22–27). Due to the fact that the gold(III) has high reduction potential, a range of strategies has been applied in order to obtain gold(III) complexes with sufficient stability under physiologically relevant conditions. These strategies are mostly related to appropriate ligand selection, which has been shown to be crucial in decreasing the pronounced tendency of the gold(III) metal center to be reduced to gold(I) or/and metallic gold (28). The chelation of the metallic center with multidentate ligands has shown to enhance the stability of the complex (29, 30).

The mechanisms of action of cytotoxic gold(III) complexes seem to be innovative and substantially different from that of cisplatin (31). Indeed, it is well known that DNA is a primary target for platinum(II) complexes while the gold(III) complexes act by targeting mitochondria of cancer cells (32) or by inhibiting the activities of different proteins (33, 34). Recently, it was found that thiol-containing enzymes, such as thioredoxin reductase (TrxR), can play important roles in the mechanisms of action of anticancer gold complexes (33).

Lately, we reported synthesis and characterization of gold(III) complexes with esters of cyclohexyl-functionalized ethylenediamine-*N,N'*-diacetate, general formulae $[AuCl_2\{(S,S)\text{-}R_2eddch\}]PF_6$, ((*S,S*)-eddch = (*S,S*)-ethylenediamine-*N,N'*-di-2-(3-cyclohexyl)propanoate) (Fig. 2) (22).

The *in vitro* cytotoxic evaluation of the investigated complexes against tumor cell lines: human adenocarcinoma HeLa, human myelogenous leukemia K562 and against normal stimulated and nonstimulated peripheral blood mononuclear cells PBMC, showed that the cytotoxic action of gold(III) complexes with cyclohexyl-functionalized ethylenediamine-*N,N'*-diacetate esters, (R= *i*-Bu, *i*-Am), is fairly comparable to that of cisplatin (22). Additionally, it was found that these complexes reduce to gold(I) species in two steps through short-living intermediate gold(II) (35). This is very important because it is believed that the cytotoxic activity of gold(III) complexes comes from appropriate gold(I) species produced by gold(III) reduction *in vivo* (24, 35).

Inspired by these promising results we are deeply interested in further investigations related to the *in vitro* antiproliferative activity of $[AuCl_2\{(S,S)\text{-}R_2eddch\}]PF_6$ complexes against other cancer cell lines such as human melanoma Fem-x, human colon carcinoma LS174, and non-small cell lung carcinoma A549 as well as a non-cancerous cell line human embryonic lung fibroblast MRC-5.

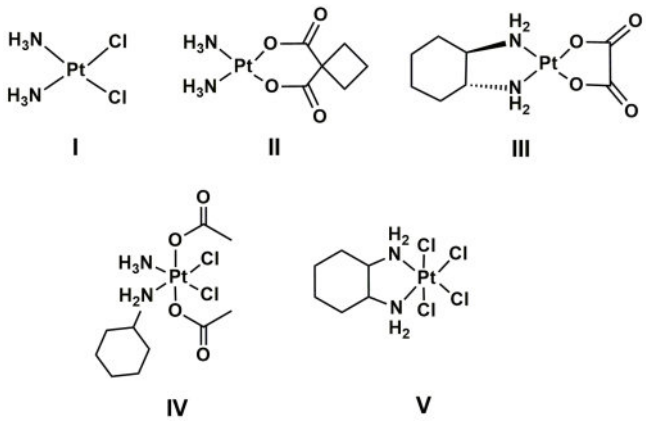
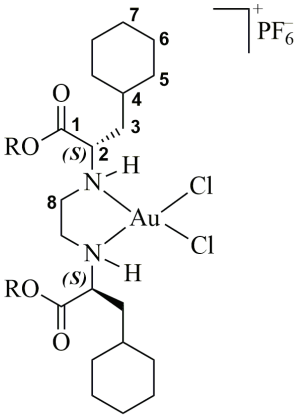


Figure 1. The structure of platinum complexes: Cisplatin (**I**); Carboplatin (**II**); Oxaliplatin (III); Satraplatin (**IV**); Tetraplatin (**V**)



R = Me, Et, *n*-Pr, *n*-Bu, *i*-Bu, *i*-Am
1-6

Figure 2. $[AuCl_2\{(S,S)\text{-}R_2eddch\}]PF_6$ complexes

MATERIALS AND METHODS

Complexes

Gold(III) complexes were synthesized according to literature procedure. Shortly, $Na[AuCl_4]$ was reacted with with an equimolar amount of corresponding ligands, methyl, ethyl, *n*-propyl, *n*-butyl, isobutyl and isoamyl esters of (*S*,*S*)-ethylenediamine-*N*,*N*'-di-2-(3-cyclohexyl)propanoic acid respectively (22). Each ligand was suspended in methanol, deprotonated with $LiOH{\cdot}H_2O$ and after stirring of 1h, a solution of $Na[AuCl_4]{\cdot}2H_2O$ in methanol was added. The desired complexes were obtained after addition of ammonium hexafluorophosphate. Purity and constitution of the obtained products were confirmed with elemental analysis, ^1H and ^{13}C NMR as well as UV/Vis spectroscopies and mass spectrometry. As examples for analytical and spectroscopic data for complex $[AuCl_2\{(S,S)\text{-}i\text{Am}_2\text{eddch}\}]PF_6$ is provided (22). Numeration of carbon atoms is shown in Fig. 2.

$[AuCl_2\{(S,S)\text{-}i\text{Am}_2\text{eddch}\}]PF_6$ (22): Yield: 66 mg, 57%. Anal. Calcd for $C_{30}H_{56}N_2O_4AuCl_2PF_6$: C, 39.09; H, 6.12; N, 3.04%. Found: C, 38.98; H, 6.13; N, 3.11%. ^1H NMR (200 MHz, $CDCl_3$): δ 0.95 (d, $(CH_3)_2CHCH_2CH_2$–OOC–, 12H; m, C^7H_2, 4H), 1.24 (m, $C^{5,6}H_2$, 8H), 1.50-1.90 (m, C^3H_2, C^4H, $C^{5,6}H_2$, $(CH_3)_2CHCH_2CH_2$–OOC– and $(CH_3)_2CHCH_2CH_2$–OOC–, 20H), 3.43 (m, C^8H_2, 4H), 3.92 (m, C^2H, 2H), 4.30 (m, $(CH_3)_2CHCH_2CH_2$–OOC–, 4H), 4.71 (s, N*H*, 2H). δ ^{13}C NMR (50 MHz, $CDCl_3$): 11.1 ($(CH_3)_2CHCH_2CH_2$–OOC–), 16.3 ($(CH_3)_2CHCH_2CH_2$–OOC–), 22.3 ($(CH_3)_2CHCH_2CH_2$–OOC–), 25.9 (C^6), 32.4 (C^4), 33.1 (C^7), 33.8 (C^5), 36.9 (C^3), 44.5 (C^8), 59.2 (C^2), 65.8 ($(CH_3)_2CHCH_2$–OOC–), 171.0 (C^1). IR (ATR): ν_{max} = 2929, 2854, 1731, 1453, 1260, 1212, 851 cm^{-1}; UV/Vis ($CHCl_3$): λ_{max} (ε, 6630 M^{-1} cm^{-1}) 320.95 nm; ESI–MS (CH_3CN), positive: m/z: 775.33 $[M]^+$, 776.33 $[M + H]^+$.

Preparation of drug solutions

The stock solutions of the investigated gold(III) complexes were prepared freshly in DMSO (Sigma-Aldrich, St. Louis, MO, USA) at the concentrations of 1 mM, and immediately diluted by nutrient medium to various working concentrations. Nutrient medium was RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (Sigma-Aldrich St. Louis, MO, USA).

Cell lines

Human melanoma Fem-x, human colon carcinoma LS174T, non-small cell lung carcinoma A549 cell lines, and non-cancerous human embryonic lung fibroblasts MRC-5 were grown in nutrient medium.

Determination of cell survival

Target cells Fem-x (5000 cells/well), LS174T (7000 cells/well), A549 (5000 cells/well), and non-cancerous MRC-5 (5000 cells/well) were seeded into the wells of a 96-well flat-bottomed microtitre plate. After 24 h, different working concentrations of investigated compounds were added to the wells, except for the controls, where only the complete medium was added. The final concentration range used in the experiments was 1–200 μM (gold(III) complexes: 12.50, 25, 50, 100, and 200). Cisplatin was used as the positive control, and the final concentrations were 2.08, 4.17, 8.33, 16.67, and 33.3 μM. The final concentration of DMSO never exceeded 0.5%, which is a non-toxic concentration for the cells. Culture medium with corresponding concentrations of investigated compounds, but without cells, was used as blank. The cultures were incubated for 72 h, and the effects of the investigated compounds on cancer cell survival were determined using the microculture tetrazolium test (MTT), according to Mosmann (36) with modification by Ohno and Abe (37). Briefly, 20 mL of MTT solution (5 mg/mL of phosphate-buffered saline, PBS) was added to each well. Samples were incubated for additional 4 h at 37 °C in a humidified atmosphere of 5% CO_2 (*v*/*v*). Afterward, 100 mL of 100 g/L sodium dodecyl sulfate (SDS) was added in order to extract the insoluble formazan, which represents the product of the conversion of the MTT dye by viable cells. The number of viable cells in each well is proportional to the intensity of the absorbance (A) of light, which was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader at 570 nm, 24 h later. IC_{50} values (±SD) were calculated using four-parameter logistic function and presented as mean from three independent experiments. IC_{50} is defined as the concentration of an agent inhibiting cell survival by 50% compared to the vehicle-treated control. As positive control cisplatin was used. All experiments were performed in triplicate.

RESULTS

The cytotoxic activity of the six investigated gold(III) complexes was evaluated in comparison with cisplatin against three different cell lines: human melanoma Fem-x, human colon cancer LS174T, and non-small cell lung carcinoma A549, as well as normal non-cancerous cell line MRC-5. The results of *in vitro* cytotoxic activity are expressed as IC_{50} and presented in Table 1. The representative graphs showing the action of various concentrations of investigated complexes on Fem-x, LS174T, A549, and non-cancerous MRC-5 cell survival, determined by MTT test, upon 72 h of continuous agent action are shown in Fig. 3. Additionally, selectivity indices are presented in Table 2.

DISCUSSION

The cytotoxic action of all investigated gold(III) complexes (Table 1), was the most pronounced against human melanoma Fem-x cells and showed the significant cytotoxicity. In comparison, tested complexes show the moderate cytotoxicity on the A549 and LS174T cells that does not differ significantly. The order of the sensitivity of the examined malignant cell lines toward gold(III) complexes was: Fem-x > LS174T > A549.

The replacement of methyl by ethyl group in ester moiety (**1** → **2**) led to increased cytotoxic activity of investigated complexes against LS174T and A549 cell lines, while the cytotoxic activity against Fem-x and non-malignant cells slightly decreased. The substitution of ethyl with *n*-propyl group (**2** → **3**) in gold(III) complexes decreased antiproliferative action against all examined cell lines except MRC-5 cells. Further substitution of *n*-propyl with *n*-butyl (**3** → **4**) led to enhancement of cytotoxic action against Fem-x cells, whereas in all other cases complexes showed lower activity. Complex with isobutyl ester moiety, **5**, have shown similar cytotoxicity in comparison to **4** against Fem-x, LS174T, and MRC-5 cell lines. On the other hand, the same complex exhibited a stronger antiproliferative effect against A549 cells. Interestingly, complex **6** expressed the highest activity against Fem-x, but it was found to be the least effective against LS174T and A549 cell lines. Generally, all examined complexes exhibited several times weaker activity than the reference compound cisplatin. Herein examined gold(III) complexes have shown lower activity against Fem-x, LS174T and A549 than on human cervix adenocarcinoma HeLa and human myelogenous leukemia K562 tumor cell lines, reported previously (22). Furthermore, it is important to note that the investigated complexes showed less toxic action against non-cancerous MRC-5 cells than on rested and stimulated normal immu-

Table 1. Concentrations of compounds that induced a 50% decrease in Fem-x, LS174T, A549, and MRC-5 cell survival rate [expressed as IC_{50} (μM)]. The cells were incubated with the compounds for 72 h

compounds	IC_{50} [μM]			
	Fem-x	LS174T	A549	MRC-5
1	23.67 ± 2.44	42.38 ± 2.41	64.91 ± 0.58	194.25 ± 3.73
2	25.16 ± 2.75	37.69 ± 0.38	45.71 ± 1.22	>200
3	28.28 ± 1.24	48.71 ± 0.96	57.86 ± 1.74	182.51 ± 2.66
4	19.77 ± 1.95	55.39 ± 1.28	68.32 ± 0.39	>200
5	21.36 ± 1.62	48.56 ± 0.86	52.18 ±1.54	188.49 ± 3.28
6	14.98 ± 0.34	72.54 ± 2.41	75.22 ±0.63	>200
cisplatin	4.82 ± 0.35	4.27 ± 0.57	10.92 ± 1.38	14.11 ± 0.72

Table 2. Selectivity indices

compounds	IC_{50} (MRC-5)/IC_{50} (tumor cell line)		
	Fem-x	LS174T	A549
1	8.21 ± 0.86	4.58 ± 0.28	2.99 ± 0.06
2	> 7.95	> 5.31	> 4.38
3	6.45 ± 0.38	3.75 ± 0.09	3.15 ± 0.11
4	> 10.12	> 3.61	> 2.93
5	8.82 ± 0.69	3.88 ± 0.10	3.61 ±0.12
6	> 13.35	> 2.76	> 2.66
cisplatin	2.93 ± 0.26	3.30 ± 0.47	1.29 ± 0.18

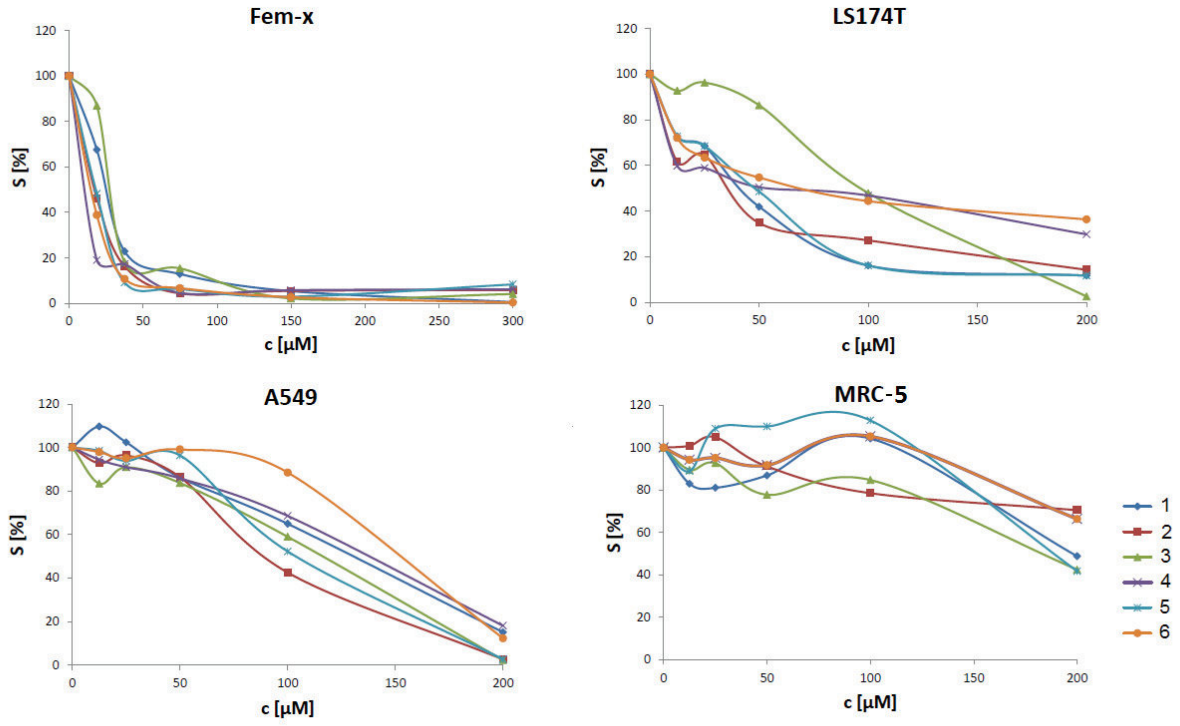


Figure 3. The survival of Fem-x, LS174T, A549 and MRC-5 cells incubated for 72 h with different concentrations of investigated gold(III) complexes (MTT assay)

nocompetent human peripheral blood mononuclear cells (PBMC) (22). This data point out that investigations concerning anticancer agents development require studies in diverse types of normal cells.

Additionally, on adherent cell lines complexes **1–6** exhibit significantly lower antitumor activity than on the nonadherent leukemic K562 cells (22). On the other hand, according to the rapidly dividing HeLa and Fem-x cells all complexes show a very similar, somewhat greater cytotoxicity (22).

Against the non-cancerous lung fibroblasts (MRC-5) all tested complexes were significantly less toxic than cisplatin. As can be seen from selectivity indices (Table 2) the selectivity of these gold(III) complexes is greater or similar than cisplatin as a reference drug. All complexes demonstrated the lowest selectivity indices on A549 cell lines, however 2–3 times higher than that of cisplatin. Complexes **1** and **2** showed the highest selectivity toward LS174T cells, even more than cisplatin, while for the other complexes it was similar to cisplatin. Considering selectivity of all complexes it was greatest toward Fem-x tumor cells. It should be noted that complex **6** shows more than 4.5 times greater selectivity on Fem-x than cisplatin and therefore it is a very promising candidate for further investigation.

CONCLUSION

The presented results showed that complex **6**, from all investigated gold(III) complexes, with isoamyl moiety in the κ^2-*N,N'* coordinated bidenatate ester ligand, exhibited the highest antitumor activity against Fem-x cell line (IC_{50} = 14.98±0.34 μM), from three cell lines tested. *In vitro* results indicate that these agents are cell type specific, for instance from the all tested tumor cell lines they exhibit the highest antitumor activity against leukemic K562 cells. Moreover, their toxicity is also cell type specific, they are less toxic against non-cancerous MRC-5 cells than on rested or stimulated PBMC. Additionally, the selectivity of these gold(III) complexes is similar or several times greater (up to 4.5 times) than cisplatin as a reference drug.

Acknowledgments

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Conflicts Of Interest

The authors declare no conflict of interest.

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MODELLING CREEP (RELAXATION) OF THE URINARY BLADDER

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MODELIRANJE PUZANJA (RELAKSACIJE) MOKRAĆNE BEŠIKE

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ABSTRACT

We first present the results of an experiment in which the passive properties of the urinary bladder were investigated using strips of rabbit bladder. Under the assumption that the urinary bladder had orthopaedic characteristics, the strips were taken in the longitudinal and in the circumferential directions. The material was subjected to uniaxial tension, and stress-stretch curves were generated for various rates of deformation. We found that the rates did not have a significantly effect on the passive response of the material. Additionally, the stress-stretch dependence during relaxation of the material when exposed to isometric conditions was determined experimentally.

Next, we measured nonlinear stress-stretch dependence to determine the coefficients for this dependence in analytical form using a standard fitting procedure. The same approach was used to obtain the coefficients for the relaxation curves from the experimental data. Two constitutive laws, the nonlinear model for passive response and the creep model, were introduced within the shell finite element for geometrically and materially nonlinear analysis. We provide descriptions of the numerical procedures that were performed by considering the urinary bladder as a thin-walled shell structure subjected to pressure loading.

The developed numerical algorithm for the incremental-iterative solution was implemented into the finite element program, PAK. The response of the urinary bladder was calculated for continuous filling, and the numerical and experimental results were compared through cystometrograms (pressure-volume relationships). We also present comparisons of the shapes and volumes of the urinary bladder obtained numerically and experimentally. Finally, the numerical results of the creep response, when placed under constant internal pressure, are provided for various stages of deformation.

Keywords: *Urinary bladder, Passive properties, Creep of material, Finite element modelling*

SAŽETAK

Prvo predstavljamo eksperimentalno određivanje osobina mokraćne bešike u pasivnom stanju korišćenjem segmenata bešike. Uzeti su segmenti u uzdužnom i poprečnom pravcu pretpostavljajući da bešika ima ortotropne karakteristike. Izvršeno je jednoosno izduživanje materijalnih segmenata i dobijene su napon-streč krive za različite brzine deformacije. Pronađeno je da brzine nemaju značajnog efekta na odgovor materijala u pasivnom stanju. Takođe je eksperimentalno određena napon-streč zavisnost tokom relaksacije materijala izloženog izometrijskim uslovima.

Zatim smo primenili izmerene nelinearne relacije napon-streč da odredimo koeficijente ove zavisnosti u analitičkoj formi korišćenjem standardne procedure fitovanja. Isti pristup je korišćen da bi odredili koeficijente za eksperimentalno dobijene krive relaksacije. Uvedena su dva konstitutivna zakona: nelinearni model za pasivni odgovor i model puzanja, korišćenjem konačnog elementa ljuske za geometrijsku i materijalno nelinearnu analizu. Dajemo opis numeričke procedure, smatrajući mokraćnu bešiku strukturom tankozidne ljuske opterećene pritiskom.

Razvijeni algoritam za inkrementalno-iterativno rešavanje je implementiran u program za konačne elemente PAK. Proračunat je odgovor mokraćne bešike za kontinualno punjenje, i numerički i eksperimentalni rezultati su upoređeni preko cistometrograma (krivih pritisak-zapremina). Takođe predstavljamo poređenje oblika i zapremina mokraćne bešike dobijenih numerički i eksperimentalno. Konačno, prikazani su numerički rezultati puzanja bešike u slučaju konstantnog unutrašnjeg pritiska, za različite faze deformacije.

Ključne reči: *Mokraćna bešika, Pasivne osobine, Puzanje materijala, Modeliranje konačnim elementima.*

ABBREVIATIONS

FEM - Finite element modelling

PAK - Finite element program for linear and nonlinear structural analysis, mass and heat transfer and biomechanics

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INTRODUCTION

The passive and active mechanical properties of the urinary bladder wall have been the subject of investigation in many studies (1, 3, 5, 7, 11, 12). Based on the experimental findings of these studies, various forms of analytical expressions have been proposed to describe the material characteristics of the urinary bladder wall and the response of the urinary bladder as a whole.

Because of the combination of modern numerical methods, such as the finite element method (FEM), and contemporary computer development, simulation of the complex three-dimensional (3D) behaviour of human organs is possible. Along this line, we analysed the dynamic responses of muscle and cartilage to the mechanical loads and physiological stimuli that are extremely important for understanding the mechano-biochemical processes that occur in biological structures (8, 9, 10). Here, we focus on the passive mechanical responses of the urinary bladder.

Our numerical analysis of the urinary bladder (under passive conditions) is based on the constitutive laws of the bladder wall that have been established based on data collected in specifically designed experiments. We defined the following two FEM material models: 1) a model for nonlinear material response derived based on the measured stress-stretch relationship; and 2) a creep material model derived based on experimental relaxation data. We modelled the urinary bladder as a shell structure undergoing large displacements and calculated the passive response of the urinary bladder. Specifically, we focused on the pressure-volume relationship (cystometrogram) and the rate of the volume change due to material creep. The predictions generated for whole organ response were compared with experimentally obtained cystometrogram data.

2. EXPERIMENTAL PROTOCOL

A total number of 20 rabbits of both sexes that weighed approximately 2.5-3 kg was used. All animals were killed by cervical dislocation according to Schedule 1 of the Animal (Scientific Procedures) Act, 1986, UK. The urinary bladders of the rabbits were emptied and dissected out, and strips of bladder were placed in an organ chamber perfused with Krebs-Ringed solution (in mM, NaCl 117, KCl 4.7, $NaHCO_3$ 24.8, $MgSO_4$ x $7H_2O$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.2 and D-glucose 11.1). The solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 and maintained at 31±1 °C to prevent spontaneous contractions. Different initial strip lengths were taken in the longitudinal and circumferential directions and strained continuously using various strain rates. Developed force was measured using an isotonic transducer (Elunit, Yugoslavia) and recorded using a PIC digital recording system (ECM, Yugoslavia). We allowed the strips to relax after various initial stretches were performed to measure relaxation under isometric conditions. We defined the strip length measured just before visible changes in tension were recorded on the PIC digital recording system as the initial length.

The filling pressure-volume curve of the urinary bladder, known as a cystometrogram (CMG), was determined by increasing the contained volume and measuring the pressure response. We used two experimental protocols to generate the cystometrograms for isolated bladders. During the first experiment, the bladders were emptied, dissected out, transferred to oxygenated Krebs-Ringer solution, and filled continuously at a velocity of 4.8 ml/min using a slow drive syringe pump (syringe pump mod 355/ Sage instr.). During the second experiment, the bladders were filled with Krebs-Ringer solution in a stepwise manner, with increases of 10 ml occurring at 20-second increments. Pressure responses were measured using a pressure transducer (Ugo Basile, USA) and recorded using a PIC digital recording system (ECM, Serbia).

3. FINITE ELEMENT MODELLING

The geometry of the urinary bladder can be closely approximated using a thin-walled shell type structure. Naturally, shell finite elements are most appropriate when generating finite element models, as shown in Fig. 1. In this article, we also describe the numerical procedures used for calculating urinary bladder responses under passive conditions using the results of the experimental investigation described in Section 2.

We assessed the large strain and large displacement formulation of the shell finite elements (2). Since our material model was based on stress-stretch curves, we first provide some necessary equations that describe the kinematics of deformation and then describe the implementation of the material models.

In nonlinear finite element analysis, the equilibrium equation is formulated as follows:

$$ {}^{t+\Delta t}\mathbf{K}^{(i-1)}\Delta\mathbf{U}^{(i)} = {}^{t+\Delta t}\mathbf{F}^{ext} - {}^{t+\Delta t}\mathbf{F}_{\text{int}}^{(i-1)} \qquad (1) $$

where is the incremental nodal displacement vector ${}^{t+\Delta t}\mathbf{K}^{(i-1)}$ is the element stiffness matrix ${}^{t+\Delta t}\mathbf{F}^{\mathbf{ext}}$ and ${}^{t+\Delta t}\mathbf{F}_{\text{int}}^{(i-1)}$ are the external and internal nodal forces, respectively; the t+Dt index denotes the end of time (load) step; and "i" represents the equilibrium iteration number for the current time step. The stiffness matrix and nodal forces were expressed as follows:

$$ {}^{t+\Delta t}\mathbf{K}^{(i-1)} = \int_V {}^{t+\Delta t}\mathbf{B}_{\mathbf{L}}^{(i-1)T}\ {}^{t+\Delta t}\mathbf{C}^{(i-1)}\ {}^{t+\Delta t}\mathbf{B}_{\mathbf{L}}^{(i-1)}\mathbf{dV} + \int_V {}^{t+\Delta t}\mathbf{B}_{\mathbf{NL}}^{(i-1)T}\ {}^{t+\Delta t}\hat{\sigma}^{(i-1)}\ {}^{t+\Delta t}\mathbf{B}_{\mathbf{NL}}^{(i-1)}\mathbf{dV} \qquad (2) $$

$$ {}^{t+\Delta t}\mathbf{F}_{\text{int}}^{(i-1)} = \int_V {}^{t+\Delta t}\mathbf{B}_{\mathbf{L}}^{(i-1)T}\ {}^{t+\Delta t}\sigma^{(i-1)}\mathbf{dV} \qquad (3) $$

where $\mathbf{B}_L$ and $\mathbf{B}_{NL}$ are the linear and nonlinear strain-displacement transformation matrices, $\mathbf{C}$ is the constitutive

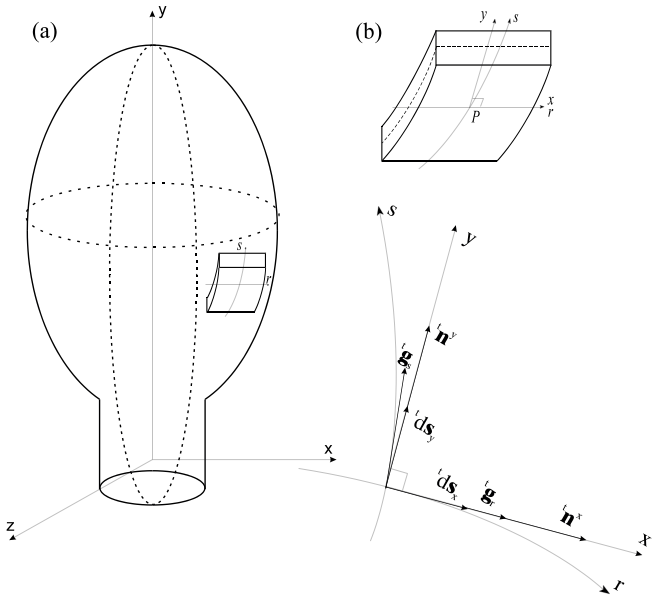


Fig. 1 Urinary bladder model
(a) Urinary bladder as a thin-walled structure
(b) Shell finite element with the principal material axes

matrix (stress-strain matrix); and $\hat{\sigma}$ and σ are the matrix and vector of the stress components for the indicated time step and iteration, respectively. $\mathbf{B}_L$ and $\mathbf{B}_{NL}$ were calculated using a standard procedure (2) and the formulation of the shell finite elements.

For the shell finite element analysis, the local coordinate system (x-y) was defined in the shell tangential plane, as shown in Fig. 1. The x-axis was taken as the direction of the (first) r-axis along the natural coordinate line, while "y" was orthogonal to the x-axis. For the current configuration (we omitted the index for iteration number to simplify the notation), we determined the rate of stretch (${}^{t+\Delta t}_{0}\lambda_x$) in the x direction using the following equations. The lengths of a material element on the r-line at time t+Dt and t=0 (Fig. 1(b)) are as follows:

$$ {}^{t+\Delta t}ds_x = \left\| {}^{t+\Delta t}d\mathbf{s}_x \right\| = \left\| \frac{\partial \mathbf{x}}{\partial \mathbf{r}} dr \right\| = \left\| {}^{t+\Delta t}\mathbf{g}_r \right\| dr \tag{4} $$

$$ {}^{0}ds_x = \left\| {}^{0}d\mathbf{s}_x \right\| = \left\| {}^{0}\mathbf{g}_r \right\| dr = \left\| {}^{0}\mathbf{J}_1 \right\| dr \tag{5} $$

where ${}^{t+\Delta t}\mathbf{g}_r$ and ${}^{0}\mathbf{g}_r$ were the base vectors of the r-curve at the current and initial configurations, respectively; and ${}^{t+\Delta t}\mathbf{J}_1$ and ${}^{0}\mathbf{J}_1$ were the first rows in the Jacobian matrix of the following coordinate transformation: $\mathbf{J} = \partial \mathbf{x} / \partial \mathbf{r}$. The rate of stretch ${}^{t+\Delta t}_{o}\lambda_x$ was determined using the following equation:

$$ {}^{t+\Delta t}_{o}\lambda_x = \frac{\left\| {}^{t+\Delta t}\mathbf{g}_r \right\|}{\left\| {}^{0}\mathbf{g}_r \right\|} = \frac{\left\| {}^{t+\Delta t}\mathbf{J}_1 \right\|}{\left\| {}^{0}\mathbf{J}_1 \right\|} \tag{6} $$

The rate of stretch (${}^{t+\Delta t}_{o}\lambda_x$) can also be calculated as follows:

$$ {}^{t+\Delta t}_{0}\lambda_x = \sqrt{ {}^{t+\Delta t}_{0}C_{ij} \; {}^{0}n_i^x \; {}^{0}n_j^x } \tag{7} $$

where ${}^{t+\Delta t}_{0}C_j$ is the right Cauchy-Green deformation tensor

$$ ({}^{t+\Delta t}_{0}\mathbf{C} = {}^{t+\Delta t}_{0}\mathbf{F}^{T} \; {}^{t+\Delta t}_{0}\mathbf{F}) \tag{8} $$

and ${}^{0}\mathbf{n}^{x}$ is the normal vector in the x direction within the reference configuration. Additionally, ${}^{t+\Delta t}_{0}\mathbf{F} \hat{=} {}^{t+\Delta t}\mathbf{x}/\partial {}^{0}\mathbf{x}$ is the deformation gradient. The stretch in the orthogonal "y"-direction is determined by using definition vectors ${}^{t+\Delta t}d\mathbf{s}_y$ and ${}^{0}d\mathbf{s}_y$ in the y direction as follows:

$$ {}^{t+\Delta t}d\mathbf{s}_y = {}^{t+\Delta t}\mathbf{g}_y \, dr = \left\{ ({}^{t+\Delta t}\mathbf{g}_y)_X \, \mathbf{i} + ({}^{t+\Delta t}\mathbf{g}_y)_Y \, \mathbf{j} + ({}^{t+\Delta t}\mathbf{g}_y)_Z \, \mathbf{k} \right\} dr \tag{9} $$

$$ {}^{0}d\mathbf{s}_y = {}^{0}_{t+\Delta t}\mathbf{F} \; {}^{t+\Delta t}d\mathbf{s}_y = {}^{0}_{t+\Delta t}\mathbf{F} \; {}^{t+\Delta t}\mathbf{g}_y \, dr \tag{10} $$

$$ \text{as } {}^{t+\Delta t}_{0}\lambda_y = \frac{ {}^{t+\Delta t}ds_y }{ {}^{0}ds_y } = \frac{ \left\| {}^{t+\Delta t}\mathbf{g}_y \right\| }{ \left\| {}^{0}_{t+\Delta t}\mathbf{F} \; {}^{t}\mathbf{g}_y \right\| } \tag{11} $$

where ${}^{0}_{t+\Delta t}\mathbf{F} = {}^{t+\Delta t}_{0}\mathbf{F}^{-1}$ is the inverse deformation gradient and

$$ {}^{t+\Delta t}\mathbf{g}_y = {}^{t+\Delta t}\mathbf{n} \times {}^{t+\Delta t}\mathbf{g}_r \tag{12} $$

is the base vector in the "y" direction; ${}^{t+\Delta t}\mathbf{n}$ is the shell normal. Additionally, the following equation may be derived based on equation (7):

$$ {}^{t+\Delta t}_{0}\lambda_y = \sqrt{ {}^{t+\Delta t}_{0}C_{ij} \; {}^{0}n_i^y \; {}^{0}n_j^y } \tag{13} $$

The numerical results for the stretches calculated using the base vectors and the Cauchy-Green deformation tensor were the same, but the first approach was more computationally efficient. The stretch (λ_z) occurring in the shell normal direction was determined based on the incompressibility condition of the material as follows:

$$ \lambda_z = \lambda_x^{-1} \, \lambda_y^{-1} \tag{14} $$

Using the λ_z calculated using the previous equation, we calculated the current shell thickness (${}^{t+\Delta t}h$) using the following equation:

$$ {}^{t+\Delta t}h = {}^{0}h \; {}^{t+\Delta t}_{o}\lambda_z \tag{15} $$

where ${}^{0}h$ is the initial thickness.

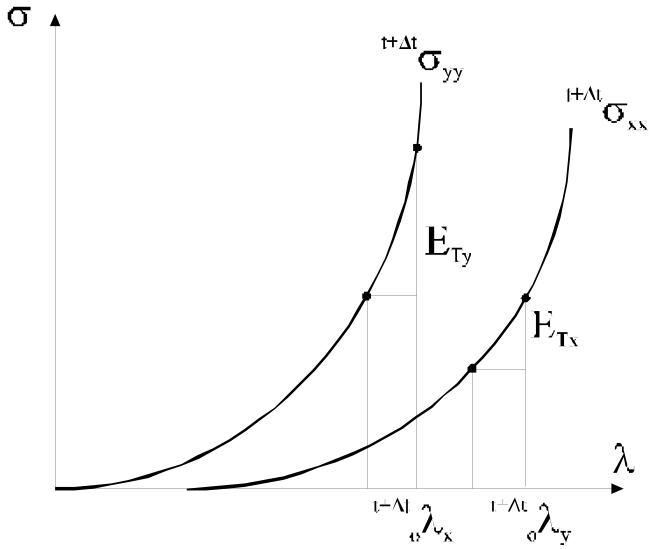


Fig. 2 Schematic representation of the material characteristics curves

3.1 THE STRESS-STRETCH MATERIAL MODEL

The stress calculated herein represents the Cauchy stress (force per unit current area). In accordance with the literature data and results of our experimental investigation, we introduced several basic assumptions to be used when formulating the material model. The assumptions were as follows:

a) The material was orthotropic;
b) The material was incompressible, as indicated by the identification of a Poisson's ratio of $\nu \approx 0.5$ in all directions; and
c) The normal stresses were dominant in the material.

The axially symmetric structure under axially symmetric loading conditions was evaluated by establishing the x-axis in the circumferential direction and "y" in the axial direction. Additionally, in accordance with assumption a), we used the experimental curves (Fig. 2) generated in these two orthogonal directions. The observations we made during the experiments suggested that the lateral strain placed on the strips during uniaxial straining was practically the same, so the model met assumption b).

The normal stresses ${}^{t+\Delta t}\sigma_x$ and ${}^{t+\Delta t}\sigma_y$ were determined according to the following procedure. First, we write notated the strains corresponding to the stress increments as follows:

$$de_{xx} = \frac{d\sigma_{xx}}{E_x} - \nu\frac{d\sigma_{yy}}{E_y} \tag{16}$$

$$de_{yy} = -\nu\frac{d\sigma_{xx}}{E_x} + \frac{d\sigma_{yy}}{E_y} \tag{17}$$

where Ex and Ey are the tangent moduli that can be expressed as

$$E_x = \frac{d\sigma_{xx}}{de_{xx}} = \frac{d\sigma_{xx}}{d\lambda_x}\frac{d\lambda_x}{de_{xx}} = E_{Tx}\ {}^t_o\lambda_x \tag{18}$$

$$E_y = \frac{d\sigma_{yy}}{de_{yy}} = \frac{d\sigma_{yy}}{d\lambda_y}\frac{d\lambda_y}{de_{yy}} = E_{Ty}\ {}^t_o\lambda_y \tag{19}$$

Here, ETx and ETy were the tangent moduli to the stress-stretch curves shown in Fig. 2. We employed the following equation to derive the aforementioned relationships:

$$d\lambda = \lambda\, de \tag{20}$$

based on the definitions for stretch (l) and small strain €. The stress-stretch curves were approximated using the constitutive laws as follows:

$$\sigma_{xx} = a_x\left(\frac{1}{b_x}(({}^t_o\lambda_x)^{b_x} - 1)\right) = a_x\, g_x({}^t_o\lambda_x) \tag{21}$$

$$\sigma_{yy} = a_y\left(\frac{1}{b_y}(({}^t_o\lambda_y)^{b_y} - 1)\right) = a_y\, g_y({}^t_o\lambda_y) \tag{22}$$

where a_x, b_x, a_y, b_y are the coefficients identified by fitting the analytical expressions to the experimental curves. We noted that the functions for $g(\lambda)$ satisfied the defined conditions necessary for reducing the stretch applied to small strain tensors in cases of small stretch as follows:

$$g(1) = 0 \tag{23}$$

$$\left(\frac{\partial g}{\partial \lambda}\right)_{\lambda_i=1} = g'(1) = 1 \tag{24}$$

Based on equations (16) and (17), we solved for the stress increments as follows:

$$\begin{Bmatrix} d\sigma_x \\ d\sigma_y \end{Bmatrix} = \frac{1}{1-\nu^2}\begin{bmatrix} E_{\bar{x}} & \nu\frac{{}^t_o\lambda_x}{{}^t_o\lambda_y}E_{\bar{x}} \\ \nu\frac{{}^t_o\lambda_y}{{}^t_o\lambda_x}E_{\bar{y}} & E_{\bar{y}} \end{bmatrix}\begin{Bmatrix} d\lambda_x \\ d\lambda_y \end{Bmatrix} \tag{25}$$

The stress increments in time step were defined as follows:

$$\begin{Bmatrix} d\sigma_x \\ d\sigma_y \end{Bmatrix} = \frac{1}{1-\nu^2}\begin{bmatrix} E_{\bar{x}} & \nu\frac{{}^t_o\lambda_x}{{}^t_o\lambda_y}E_{\bar{x}} \\ \nu\frac{{}^t_o\lambda_y}{{}^t_o\lambda_x}E_{\bar{y}} & E_{\bar{y}} \end{bmatrix}\begin{Bmatrix} d\lambda_x \\ d\lambda_y \end{Bmatrix} \tag{26}$$

$$\Delta\sigma_y = \frac{1}{1-\nu^2}\left(\int_t^{t+\Delta t}\nu\frac{{}^\tau_o\lambda_y}{{}^\tau_o\lambda_x}E_{\bar{y}}\ d\lambda_x + \int_t^{t+\Delta t}E_{\bar{y}}\ d\lambda_y\right) \tag{27}$$

Using the expressions for stress-stretch curves (equations (21) and (22)), we obtained the following equations:

$$\Delta\sigma_{xx} = \frac{1}{1-\nu^2}\left(a_x \frac{1}{b_x}\left(\left({}^{\tau}_{o}\lambda_x\right)^{b_x} - 1\right) + \nu\left(\lambda_x E_{Tx}\right)_{mean} \ln\left({}^{\tau}_{o}\lambda_y\right)\right) \quad (28)$$

$$\Delta\sigma_{xx} = \frac{1}{1-\nu^2}\left(a_x \frac{1}{b_x}\left(\left({}^{\tau}_{o}\lambda_x\right)^{b_x} - 1\right) + \nu\left(\lambda_x E_{Tx}\right)_{mean} \ln\left({}^{\tau}_{o}\lambda_y\right)\right)_{t}^{t+\Delta t} \quad (29)$$

in which we have employed the following approximations:

$$\lambda_{mean} = \frac{1}{2}\left({}^{t}_{o}\lambda + {}^{t+\Delta t}_{o}\lambda\right) \quad (30)$$

$$E_{Tmean} = \frac{1}{2}\left({}^{t}E_T + {}^{t+\Delta t}E_T\right) \quad (31)$$

Therefore, the following stresses were identified at the end of time (load) step:

$${}^{t+\Delta t}\sigma_{xx} = {}^{t}\sigma_{xx} + \Delta\sigma_{xx} \quad (32)$$

$${}^{t+\Delta t}\sigma_{y} = {}^{t}\sigma_{y} + \Delta\sigma_{y} \quad (33)$$

When $\lambda < 1$, we used the constitutive relations defined for $\lambda > 1$.

In accordance with assumption c), we calculate the shear modulus (G) as follows:

$$G = \frac{E_x + E_y}{4(1+\nu)} \quad (34)$$

which reduced the isotropic value when $E_x = E_y$. The shear stresses were determined using the Green-Lagrange shear strains, which were calculated in the standard manner (2).

3.2 CREEP MODEL

As previously described in Section 2, the creep behaviour of the material was determined via relaxation tests. We obtained a family of relaxation curves via the relaxation tests, which are shown schematically in Fig. 3. These curves can be approximated by the following expression:

$$\sigma = c_0 + c_1 e^{c_2 t} \quad (35)$$

where $c_0 = c_0(\lambda)$, $c_1 = c_1(\lambda)$, and $c_2 = c_2(\lambda)$ were the material parameters that were obtained by fitting of this equation to the experimental results.

After taking into account the creep effect at time step Dt, we proceeded as follows. The total normal stress ${}^{t+\Delta t}\sigma$ (in direction x or y) was expressed as follows:

$$\sigma = c_0 + c_1 e^{c_2 t} \quad (36)$$

where ${}^{t+\Delta t}\sigma^{t}$ is the level of stress corresponding to the transient response, and $\Delta\sigma^{c}$ represents the contribution

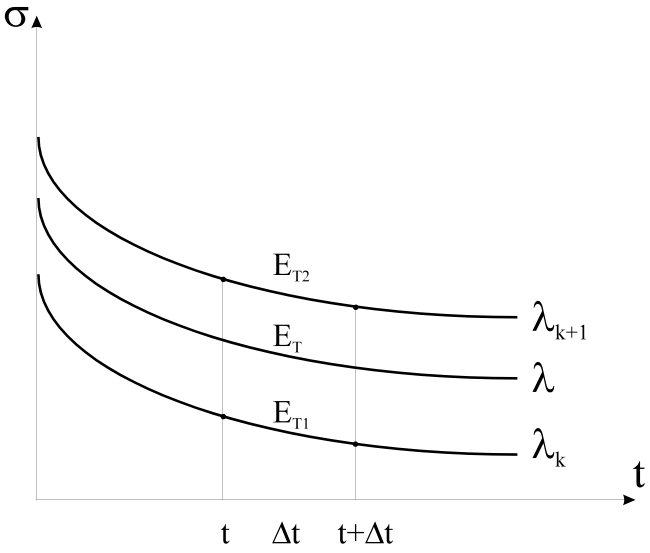


Fig. 3 Relaxation curves for various stretches λ

of the creep effect. The creep stress $\Delta\sigma^{c}$ was determined using the following equation:

$$\sigma = c_0 + c_1 e^{c_2 t} \quad (37)$$

where E_T is the slope on the relaxation curve given stretch l. The modulus E_T was interpolated based on the two measured curves (with $\lambda_k \le \lambda$ and $\lambda_{k+1} \ge \lambda$) as follows:

$$E_T = E_{T1} + \frac{E_{T2} - E_{T1}}{\lambda_{k+1} - \lambda_k}\left(\lambda - \lambda_k\right) \quad (38)$$

where E_{T1} and E_{T2} were the moduli on the curves with λ_k and λ_{k+1},

$$E_{T1} = \left(\frac{d\sigma}{dt}\right)_{\lambda=\lambda_k} = c_{11}\, c_{21}\, e^{c_{21} t} \quad (39)$$

$$E_{T2} = \left(\frac{d\sigma}{dt}\right)_{\lambda=\lambda_{k+1}} = c_{12}\, c_{22}\, e^{c_{22} t} \quad (40)$$

in which coefficients c_1, c_2 and c_2, c_2 corresponded to the curves with λ_k and λ_{k+1}, respectively.

We used the current stretch l at a given material point as the mean value identified in the current time step, as follows:

$$\lambda = \frac{1}{2}\left({}^{t}\lambda + {}^{t+\Delta t}\lambda\right) \quad (41)$$

In the proposed creep model, we did not take into consideration the creep effects on the shear modulus since normal stress/strain components are the main contributors to urinary bladder response.

4. EXPERIMENTAL RESULTS

In this section, we present the results of the experiments described in Section 2 that used the rabbit urinary bladder

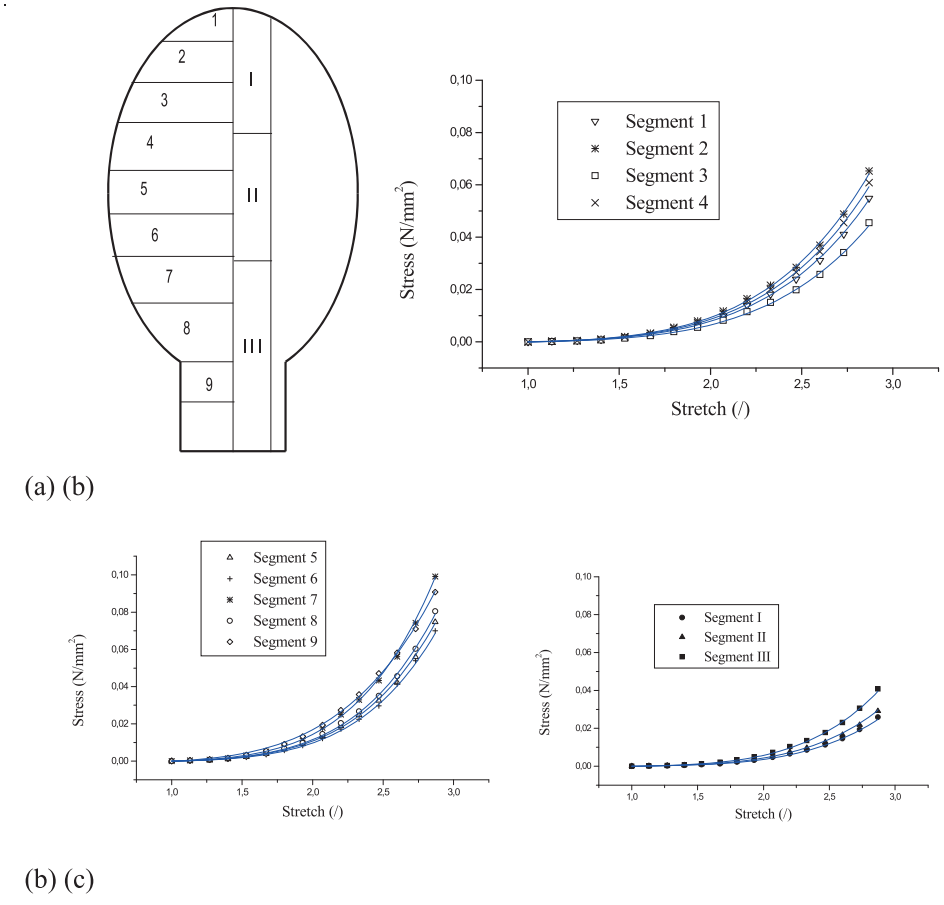


Fig. 4 Stress-stretch curves for various segments
(a) Schematic view of the urinary bladder with segments
(b) Stress-stretch dependence in the longitudinal direction
(c) Stress-stretch dependence in the circumferential direction

strips. First, we provide four typical stress-stretch curves from which the important conclusions regarding the mechanical characteristics of the material were drawn and then we present two relaxation curves. Additionally, we illustrate the mechanical behaviour of the urinary bladder using cystometrograms for continuous and stepwise loading. Based on the range of the experimentally derived strain rates $(0.5\,s^{-1} - 8\,s^{-1})$, we found that the strain rate effects could be neglected.

Longitudinal			Circumferential		
Segment	ay	by	Segment	ax	bx
I	0.0008	5.2818	1	0.0011	5.2818
II	0.0006	5.2818	2	0.0013	5.2818
III	0.0005	5.2818	3	0.0009	5.2818
			4	0.0012	5.2818
			5	0.0015	5.2818
			6	0.0013	5.3759
			7	0.0020	5.2818
			8	0.0016	5.2818
			9	0.0034	4.5690

Table 1 Constitutive coefficients for the longitudinal and circumferential directions

The nonhomogeneous and orthotropous character of the urinary bladder wall material is shown in Fig. 4. Material segments in the longitudinal and circumferential directions were taken from various positions in the urinary bladder, as shown in Fig. 4a. Based on Figs. 4b and c, one can observe that the segments in the lower part of the bladder demonstrated stiffness in both directions. Additionally, the stiffness identified in the circumferential direction was higher than the stiffness identified in the longitudinal direction. The continuous curves in these figures correspond to the equations (21) and (22), while the discrete points represent the experimental values. Table 1 provides the values of material constants "a" and "b", which were obtained using a standard curve fitting procedure.

As described in Sections 2 and 3, the creep behaviour of the material was represented via the relaxation curves. These curves were obtained by stretching the specimens to stretch l and then holding the strain constant and measuring the forces during relaxation time. Figs. 5a and 5b illustrate the relaxation curves for the longitudinal and circumferential directions, wherein the solid lines correspond to the analytical expression (36), and the experimental results are represented by the discrete points. Based on these figures, we observed that the amount of the stress relaxation

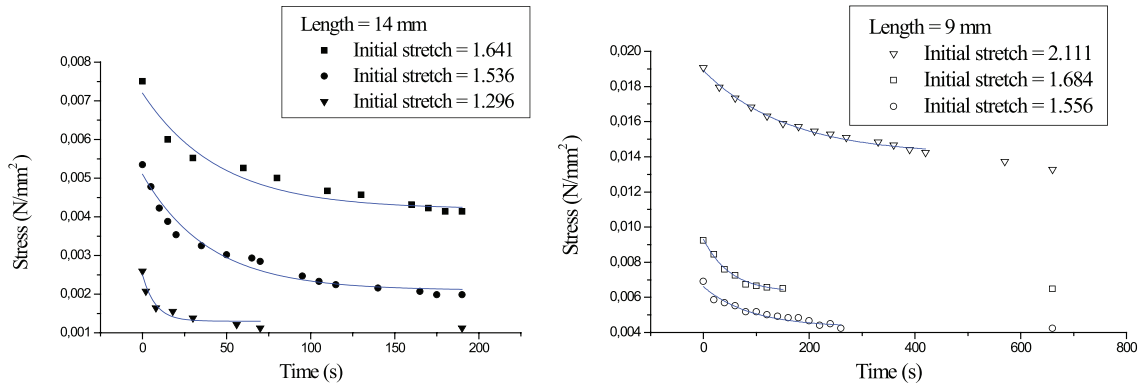


Fig. 5 Stress relaxation
(a) Longitudinal segments (solid symbols)
(b) Circumferential segments (open symbols)

Longitudinal				Circumferential			
Stretch	c0	c1	c2	Stretch	c0	c1	c2
1.641	0.0042	0.0030	-0.0220	2.111	0.0141	0.0048	-0.0063
1.536	0.0021	0.0030	-0.0253	1.684	0.0063	0.0030	-0.0198
1.296	0.0013	0.0012	-0.1297	1.556	0.0043	0.0023	-0.0111

Table 2 Coefficients derived for the longitudinal and circumferential directions using the creep model

was within approximately 40% of the initial stress levels. The material coefficients for the relaxation curves are provided in Table 2.

Finally, Fig. 6 shows the pressure-volume curves (cystometrograms) that were derived for the continuous and stepwise fillings using the procedures described in Section 2. The recorded pressure-volume curves had the same general nonlinear character as the uniaxial stress-stretch relationships observed for the bladder strips. Additionally, no significant difference was observed between the responses observed during the continuous and stepwise fillings, indicating that the creep contribution to the intervals of constant pressure was not high.

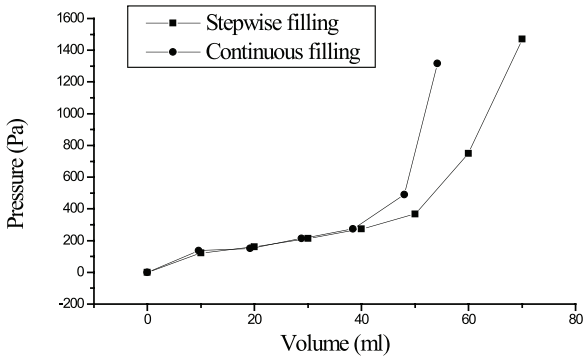


Fig. 6 Pressure-volume dependence (cystometrogram) for the continuous and stepwise fillings

5. NUMERICAL SIMULATIONS

During the numerical simulations, we demonstrated the accuracy of the numerical models when compared with experimental results by considering the whole urinary bladder as a structure and via a simple creep problem.

Uniaxial Stretch.

This example serves to demonstrate the accuracy of the numerical procedure and the calculated material response when compared with the experimental observations. The following two extreme cases are considered:

a) the material was free to contract laterally; and
b) the material was restrained in the lateral direction.

Fig. 7a shows the one four-node shell finite element with dimensions, boundary conditions and loading included in the analysis. The element was loaded with stress ${}^{t+\Delta t}\sigma_x$ that increased over time, as shown in Fig. 7b. The constitutive curves generated under the assumption that the material was orthopaedic are shown in Fig. 7c. The solutions were obtained using (1) one-step solution (Dt=1s, Dt=2s,..., Dt=6s) from the zero-stress to the current stress, and (2) multistep solution, with Dt=1 ranging from time t=0 to the current time (t=,1 s, t=2s, ..., t=6s). We observed that the one-step and multistep solutions displayed good accuracy. Additionally, the numerical solutions ${}^{t+\Delta t}\sigma_x(\lambda_x)$ laid approximately on the analytical curves for both isotropic and orthotropic material, while

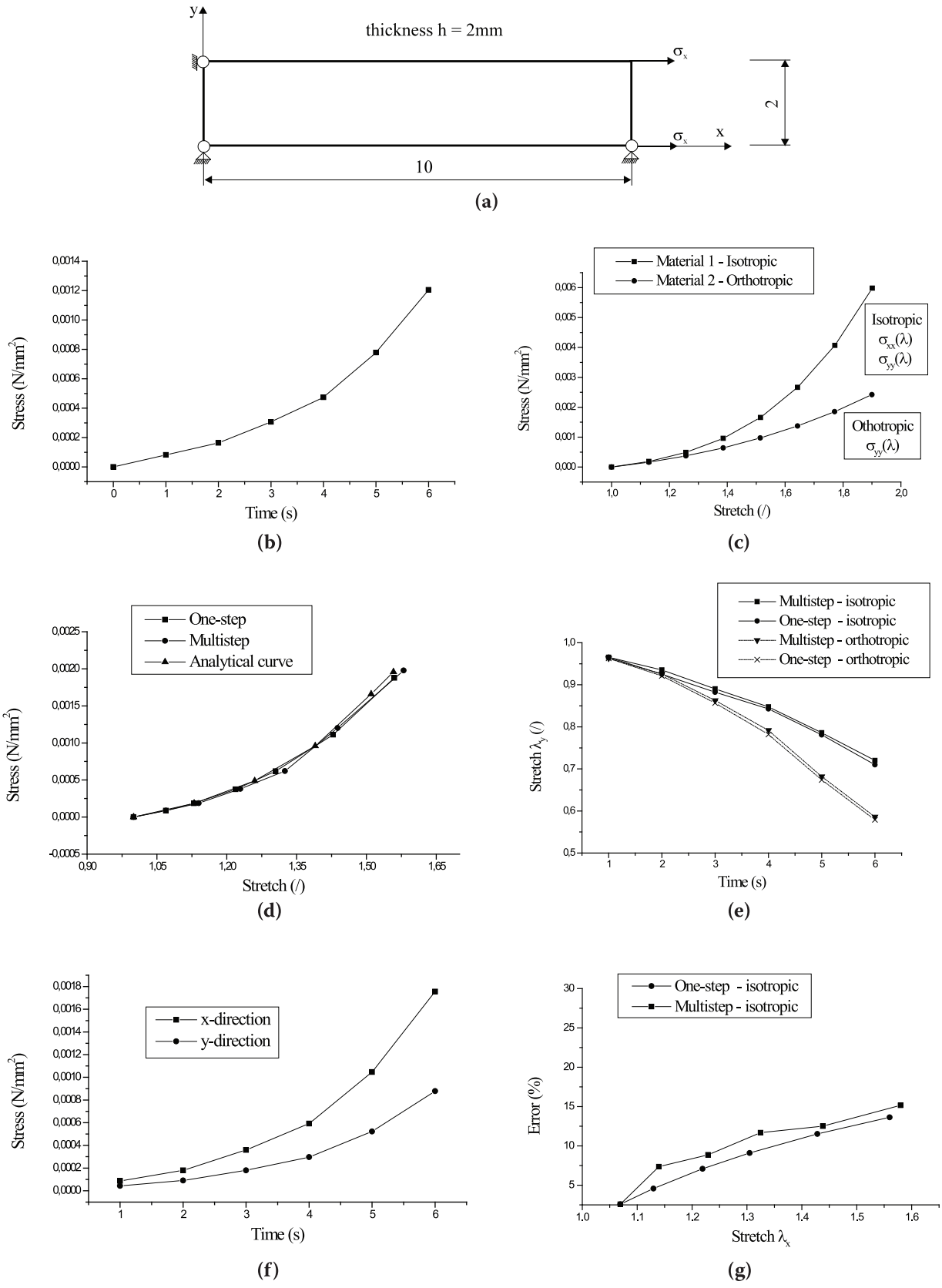
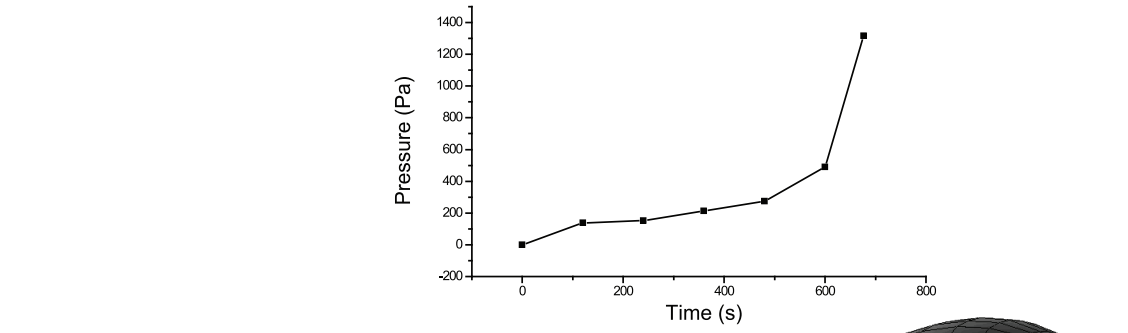
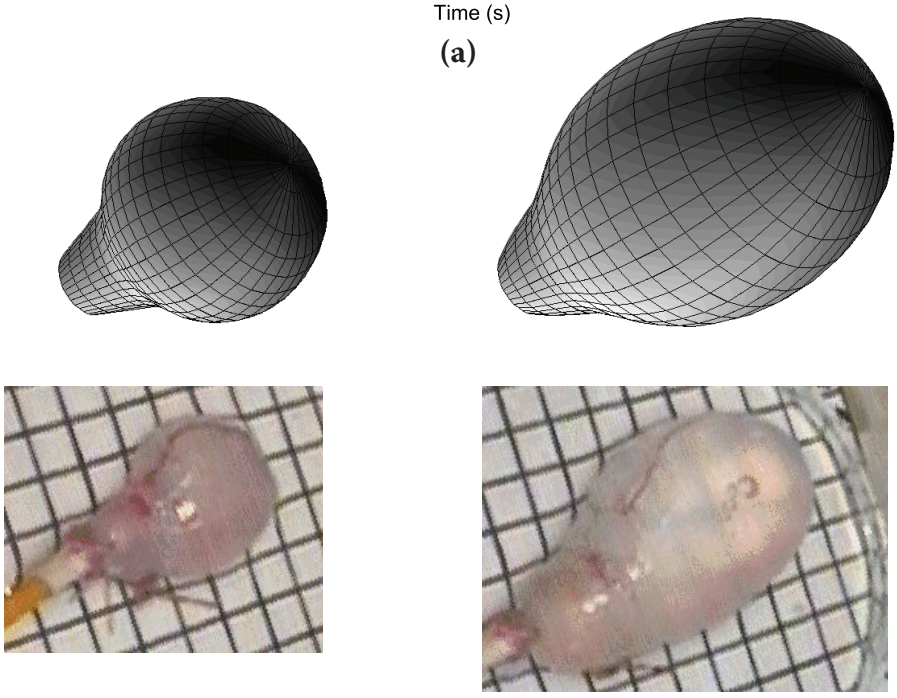


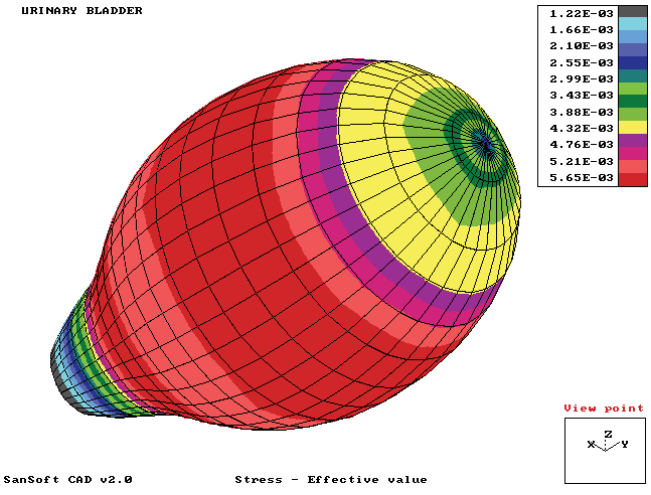
Fig. 7 Uniaxial stretch
(a) Shell finite element
(b) Stress-time relationship (prescribed)
(c) Stress-stretch relationships (prescribed)
(d) Stress-stretch numerical solution
(e) Stretch-time dependence in the y direction (computed)
(f) Stress-time dependence in the x and y directions (computed)
(g) Error- stretch dependence in the x direction (computed)



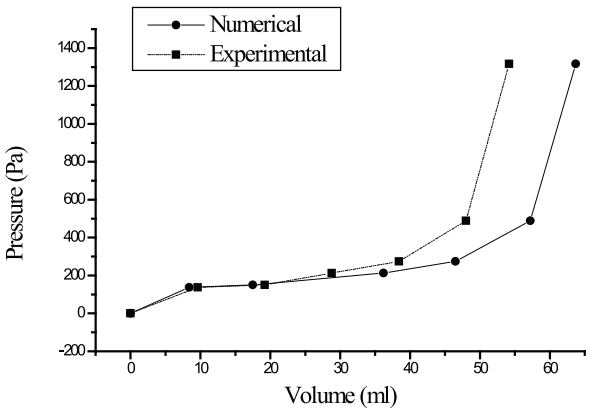
(a)



(b)



(c)



(d)

Fig. 8 Filling of the urinary bladder
(a) Pressure-time relationship
(b) Original and final configurations (computed and experimentally observed)
(c) Stress field in the urinary bladder (final configuration)
(d) Experimental and numerical cystometrograms

the lateral stretches were different for these two cases (Fig. 7d). The lateral (λ_y) and axial (λ_x) stretch values were higher under the same level of stress ($^{t+\Delta t}\sigma_{x}$) when orthotropic material was used because the $^{t+\Delta t}\sigma_{y}\ (\lambda_y)$ curve was considered to lie below the $^{t+\Delta t}\sigma_{x}\ (\lambda_x)$ curve, as seen in Fig. 7e.

In case b), stretching in the x direction produced tensional stress $^{t+\Delta t}\sigma_{y}$ in the y direction. Fig. 7f shows the multistep solutions for both the isotropic and the orthotropic material. In the both cases, we assumed that the following condition was satisfied according to the incompressibility assumption b):

$$\sigma_y = 0.5\sigma_x \tag{42}$$

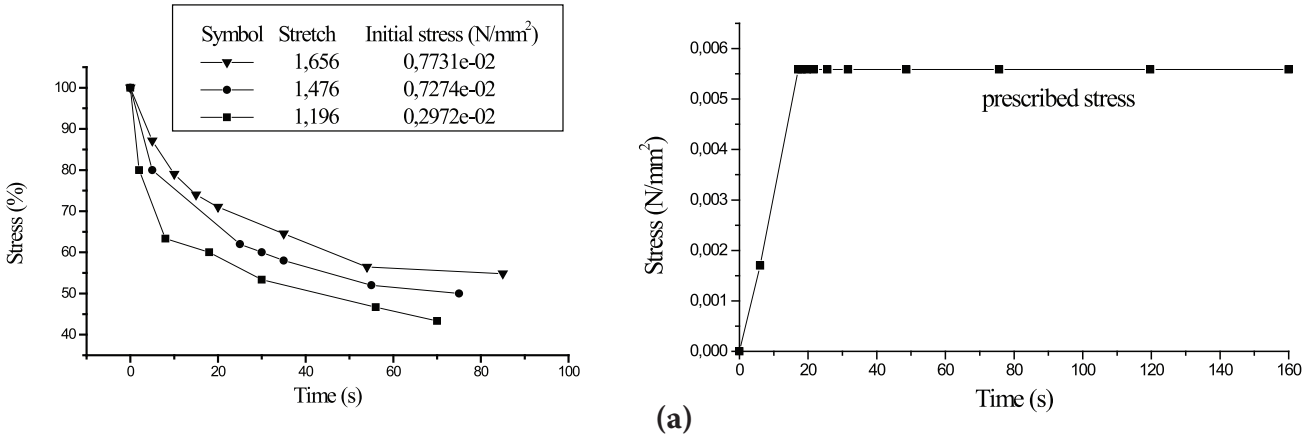
Fig. 7 g shows the departure (error) of the calculated stress from analytical value for the isotropic material, which was calculated using the following equation

$$\text{Error}\,(\%) = \frac{(\sigma_{xx})_{\text{computed}} - (\sigma_{xx})_{\text{analytical}}}{(\sigma_{xx})_{\text{analytical}}} 100$$

Filling of Urinary Bladder

We analysed urinary bladder response to the filling process. The cystometrogram shown in Fig. 6 was used to calculate the dependence of fluid pressure on time necessary for determination of structural load. In the numerical analysis, we considered continuous filling in addition to pressure loading, as shown in Fig. 8a. A schematic representation of the segment positions used in the experimental investigations is shown in Fig. 4a. The stress-stretch relationships that were employed in the material models are shown in Figs. 4b and 4c, and the coefficients of the models are provided in Table 1. Due to the observed axial symmetry, only one quarter of the structure is modelled, and 162 shell finite elements were included.

Urinary bladder response was calculated using 30 steps, with time step Dt=30s lying in the domain corresponding to the flat parts of the stress-stretch curves and Dt=5s lying in the following domain. The initial configurationwhich used the initial experimentally determined volume value (V0=9.6 ml (numerical V0=5.4 ml)), and the final structure used the final experimentally determined volume value (V0=54.16 ml (numerical V0=43.7 ml)), as shown in Fig. 8b. We noted that the problem was highly nonlinear, both in the geometrical and in the material sense, with stretches of approximately 2.4 λ identified in some regions. The real shapes of the urinary bladder at the initial stage and end of loading that were registered on camera are shown in the same figure. We observed that similar results were identified in the shape and magnitude of the urinary bladder



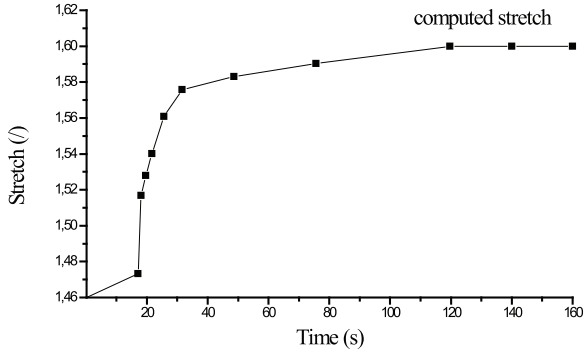


Fig. 9 Uniaxial creep
(a) Experimental relaxation curves
(b) Computed stretch-time relationship

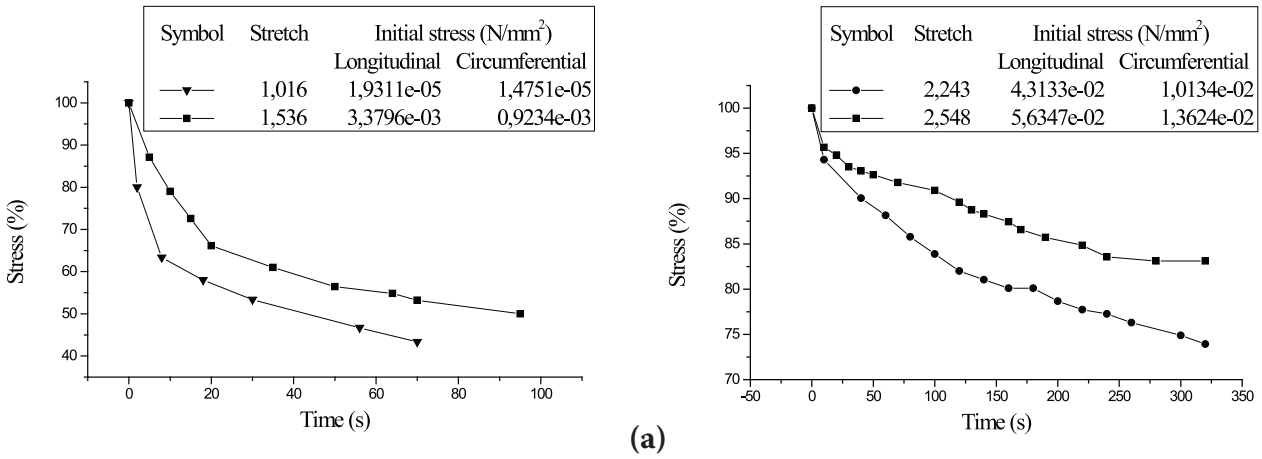


Fig. 10 Creep of urinary bladder
(a) Relaxation curves
(b) Change in volume due to creep at various initial volumes

in the numerical model and the experimental results. The von Mises stress distribution in the wall at the final configuration is presented in Fig. 8c. We noted that the zone of maximum stress was the middle section of the urinary bladder.

Finally, the cystometrograms obtained numerically and experimentally are provided in Fig. 8d. Agreement between numerical and experimental results can be observed based on this figure, verifying the numerical simulation.

Uniaxial Creep.

In this example, we investigated the accuracy of material response prediction using our creep model. In the numerical analysis, we employed the plane stress finite element under constant uniaxial loading conditions. The relaxation curves shown in Fig. 9a were obtained experimentally.

The calculated creep curve, represented as the stretch-time relationship, is shown in Fig. 9b. The figure demonstrates that creep of the material diminished with time, which was also observed experimentally. We observed that the stretch increase that occurred due to creep was relatively small.

Creep of Urinary Bladder.

We calculate the creep of the urinary bladder as a structure, considering, for simplicity, that the material was orthotropic and homogenous with respect to transient loading and creep. In the longitudinal direction, we employed the nonlinear stress-stretch relationship provided by the curve corresponding to segment 9 in Fig. 4b, while for the circumferential direction we used the curve for segment II in Fig. 4c.

The relaxation curves used in the analysis are shown in Fig. 10a, where stress (presented as a percentage of the initial stress observed) is provided as a function of time. The curves for the longitudinal and circumferential directions are the same shape but correspond to different initial stresses, as indicated in the figure.

The internal pressure changed when stepwise filling was employed, as can be seen in the diagram in Fig. 10b. The creep deformation was dominant during the constant pressure-time intervals. The increase in volume that was observed in association with time during creep at various initial bladder volumes corresponded to constant pressures 1,2,...5, as shown in Fig. 10b. We observed that the volume increase was more pronounced under higher stretch conditions (higher initial volumes).

CONCLUSIONS

We have presented the procedures used for experimental determination of the passive mechanical properties of the urinary bladder wall and the results of the numerical analysis performed using state-of-the art modern approaches and ain nonlinear finite element methodology. The stress-stretch curves and the relaxation curves obtained during the experiments were used to formulate the two material models using the analytical form: a) nonlinear model for passive response, and b) creep model. The models were incorporated into the shell finite elements to numerically simulate the transient responses of the urinary bladder under passive and creep conditions.

The results of the numerical simulations demonstrate the possibilities of modelling the complex mechanical behaviour of urinary bladder, which was treated as a thin-walled structure. The proposed methodology provides a solid basis for generating a deeper understanding of the urinary bladder response. This approach may be further generalized to assess the active phase as well, which is the subject of our current research.

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THE EFFECTS OF METHIONINE-ENRICHED AND VITAMINS (FOLATE, PYRIDOXINE AND COBALAMINE)-DEFICIENT DIET ON EXPLORATORY ACTIVITY IN RATS - A BRIEF REPORT

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EFEKTI METIONINOM-OBOGAĆENE I VITAMINIMA (FOLATI, PIRIDOKSIN I KOBALAMIN) DEFICIJENTNE DIJETE NA EKSPLORATIVNU AKTIVNOST PACOVA-KRATAK IZVEŠTAJ

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ABSTRACT

The aim of this study was to evaluate the impact of increased homocysteine levels induced by methionine nutritional overload (twice as standard) and deficiency of the vitamins folate, pyridoxine and cobalamine, which plays an important role in homocysteine metabolism in anxiety-related behaviour, expressed by means of exploratory activity in rats. Twenty-three male Wistar albino rats (4 weeks old, 100±15 g body weight) were divided into three groups: control (n=8), methionine-enriched (Meth+, 7.7 g of methionine/kg chow, n=7) and methionine-enriched vitamin-deficient (Meth+Vit-, 7.7 g of methionine/kg chow, deficient in folate, pyridoxine and cobalamine - 0.08, 0.01 and 0.01 mg/kg, n=8). All animals had free access to food and water for 30 days. Behavioural testing was performed using the elevated plus maze (EPM) test. Standard parameters for vertical exploratory activity, the number of rearings and the number of head-dippings, as well as the total exploratory activity (summarizing overall exploratory activity in the EPM) were significantly reduced following 30 days of methionine nutritional overload ($p<0.05$, $p<0.05$ and $p<0.01$, respectively). A methionine-enriched diet coupled with a reduction in some B vitamins resulted in a more pronounced decline in exploratory drive observed in the EPM test compared to the control ($p<0.01$). The decline in total exploratory activity associated with vitamin deficiency was significant compared to the Meth+ group ($p<0.05$). The results of this study highlight the important role of homocysteine in the modulation of exploratory activity in rats. Decreased exploratory drive induced by both a methionine-enriched and vitamin-deficient diet could be attributed to an anxiogenic effect of hyperhomocysteinemia.

Keywords: *methionine, homocysteine, folate, pyridoxine, cobalamine, exploratory activity, anxiety, rats*

SAŽETAK

Cilj ovog istraživanja je bio ispitivanje uticaja povišenih nivoa homocisteina, uzrokovanih povećanim sadržajem metionina u hrani (dvostruko u odnosu na standard), kao i deficijencijom vitamina (folati, piridoksin, kobalamin) koji imaju značajnu ulogu u metabolizmu homocisteina, na nivo anksioznosti koji se iskazuje u ponašanju. Dvadeset tri Wistar albino pacova muškog pola (4 nedelje starosti, 100±15g telesne mase) je podeljeno u 3 grupe: kontrola (n=8), metioninom obogaćena (Meth+, 7,7 g metionina/kg hrane, n=7) i metioninom obogaćena i deficijentna u sadržaju vitamina (Meth+Vit-, 7,7 g metionina/kg hrane, deficijentna u sadržaju folata, piridoksina i kobalamina - 0,08, 0,01 i 0,01 mg/kg, n=8). Sve životinje su imale slobodan pristup hrani i vodi tokom 30 dana. Bihejvioralno testiranje je sprovedeno u testu uzdignutog krstastog lavirinta (UKL). Standardni parametri vertikalne eksplorativne aktivnosti, broj uspravljanja i broj naginjanja, kao i ukupna eksplorativna aktivnost (sumacija sveukupne eksplorativne aktivnosti u UKL), su bili značajno smanjeni nakon 30 dana povećanog unosa metionina ($p<0,05$, $p<0,05$ i $p<0,01$). Metioninom obogaćena ishrana, udružena sa smanjenjem pojedinih B vitamina, je uzrokovala dodatno smanjenje eksplorativne aktivnosti u UKL testu u odnosu na kontrolu ($p<0,01$). Smanjenje ukupne eksplorativne aktivnosti uzrokovano dodatnom deficijencijom vitamina je bilo značajno u odnosu na Meth+ grupu ($p<0,05$). Rezultati ove studije naglašavaju značajnu ulogu homocisteina u modulaciji eksplorativne aktivnosti kod pacova. Smanjena eksplorativna aktivnost uzrokovana metioninom obogaćenom ishranom i deficijencijom vitamina u hrani se može smatrati anksiogenim efektom hiperhomocisteinemije.

Ključne reči: *metionin, homocistein, folati, piridoksin, kobalamin, eksplorativna aktivnost, anksioznost, pacovi*

ABBREVIATIONS

BHMT - betaine-homocysteine methyl-transferase
CBS - cystathionine-beta-synthase
CNS - central nervous system
CSE - cystathionine-gamma-lyase
EPM - elevated plus maze
Hcy - homocysteine
SAM - S-adenosylmethionine
TEA - total exploratory activity

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INTRODUCTION

Homocysteine (Hcy) is a non-protein-forming, sulfur-containing, non-essential amino acid. Increased Hcy levels have been associated with a diversity of diseases, including cardiovascular diseases (1, 2), malignancies (3), birth defects and pregnancy complications (4, 5), as well as with central nervous system (CNS) dysfunction (6, 7).

Hcy is synthesized by transmethylation of the (essential) diet-derived amino acid methionine. Its metabolism may be represented as the intersection of two metabolic pathways: remethylation and transsulfuration (8). In the remethylation pathway, Hcy is recycled to methionine under the catalysis of methionine syntase or betaine-homocysteine methyltransferase (BHMT). In the transsulfuration pathway, Hcy is condensed with serine, forming cystathionine (by enzyme cystathionine-beta-synthase, CBS), which is subsequently hydrolized to cysteine (by enzyme cystathionine-gamma-lyase, CSE), essential for the formation of glutathione, or finally decomposed to taurine (9-11).

Folic acid (B_9), pyridoxine (B_6) and cobalamine (B_{12}) are crucially involved in processes important for homocysteine metabolism. B_6-dependent enzymes CBS and CSE are basically involved in the transsulfuration pathway, as previously described (12). Folate and vitamin B_{12} are involved in the remethylation reactions of Hcy back to methionine to complete the methyl cycle (folate/B_{12}-dependent remethylation). Folate, in the form of coenzyme N-5-methyl tetrahydrofolate, can donate a methyl group to Hcy in a reaction catalyzed by the vitamin B_{12}-dependent enzyme methionine synthase. The remethylation reactions of Hcy to methionine may also include folate/B_{12}-independent remethylation, which involves betaine-homocysteine methyltransferase (BHMT). Therefore, vitamins B_9 and B_{12} play an important role in Hcy content within the cell and, subsequently, in plasma level circulation (12).

Hyperhomocysteinemia represents a risk factor for numerous disorders affecting the CNS, such as neurological cognitive deficit, mental retardation, demyelination (13, 14), Alzheimer's disease (15), Parkinson's disease (16), stroke (17) and schizophrenia (18). The strong causal connectivity between elevated Hcy levels and this diversity of CNS disorders may be found in some differences in Hcy metabolism that are specific for brain tissue. It has been reported that transmethylation of Hcy in the brain is limited exclusively to the folate/B_{12}-dependent pathway due to lack of BHMT in brain tissue. The other metabolic pathway of Hcy, transsulfuration, in brain tissue is also limited due to lack of CSE (also observed in adipose tissue). Therefore, the metabolic processes that can limit Hcy accumulation in brain tissue are limited only to folate/B_{12}-dependent remethylation, which may explain the higher vulnerability of the CNS to increased Hcy levels (19). In addition, the methylation reactions that are critical for the proper synthesis of serotonin, other monoamine neurotransmitters and catecholamines (20), which play an important role in mood regulation, largely rely on B vitamin cofactors (B_6, B_9 and B_{12}).

Numerous clinical trials have provided additional information on the importance of the factors that may affect Hcy metabolism in the genesis of various mood disorders. Therefore, it has been reported that patients with depression had lower serum levels of folate and vitamin B_{12} (21, 22). Additionally, numerous anxiety disorders are related to impaired one-carbon metabolism (23-26).

The aim of this study was to evaluate the impact of increased Hcy levels induced by methionine nutritional overload, as well as by deficiency in vitamins (folate, pyridoxine and cobalamine) that play an important role in homocysteine metabolism in anxiety-related behaviour, expressed by means of exploratory activity in rats.

MATERIALS AND METHODS

Animals and treatment

The dietary protocols were performed on 23 male Wistar albino rats (4 weeks old, 100±15 g body weight). Rats were housed in standard environmental conditions (12/12 h light/dark cycle, 23±1 °C) in polycarbonate cages (2 animals per cage). The rats were randomly divided into three groups: control (n=8), methionine-enriched (Meth+, n=7) and methionine-enriched vitamin-deficient (Meth+Vit-, n=8). The animals in the control group were fed with standard chow, whereas the animals in the two other groups were fed with commercial methionine-enriched chow (7.7 g of methionine/kg) combined with variable vitamin B content (Mucedola SRL., Milan, Italy). Methionine content in enriched diets was twice as high compared to that in standard chow (3.85 g of methionine/kg, (27). The Meth+ group received methionine-enriched chow with regular vitamin B content (folate, pyridoxine and cobalamine: 2.0, 70.0 and 0.03 mg/kg, respectively). The Meth+Vit- group was fed with the methionine-enriched chow that was deficient in folate, pyridoxine and cobalamine (0.08, 0.01 and 0.01 mg/kg, respectively). All animals had free access to food and water. Applied dietary protocols lasted for 30 days.

Behavioural testing

Behavioural testing was performed 24 h following the completion of dietary pretreatment. Rats were transported in their home cages to the testing room (approx. at 9 a.m.) and allowed to acclimate 1 hour prior to testing. Testing was performed in the elevated plus maze (EPM), as the EPM test is commonly considered a standard for measuring anxiety levels in rodents (28). EPM consisted of two opposite open arms (50x20 cm) and two opposite enclosed arms (50x20x30 cm), elevated 100 cm from the floor. Each rat was placed in the centre of the elevated plus maze facing the open arm and was allowed 5 minutes of free exploration. The following parameters of exploratory activity in the EPM test were estimated: the number of rearings, the number of head-dippings and the total exploratory activity (TEA). The number of rearings and the number of

head-dippings are considered indicators of anxiety level (28, 29) because exploratory drive is in conflict with fear of the unknown, i.e., anxiety (30). To estimate overall exploratory activity in the EPM test, we used a recently proposed parameter—total exploratory activity (31)—which includes patterns of exploratory activity observed in different zones of the EPM (closed and open arms). TEA is calculated as the sum of the numbers of rearings and head-dippings during 5 minutes of testing in the EPM. EPM tests were recorded by digital camera, mounted above mazes at the appropriate height, under proper conditions of silence and illumination. Video files were analysed using Ethovision software XT 12, Noldus Information Technology, the Netherlands.

All research procedures were carried out in accordance with the European Directive for the welfare of laboratory animals No: 2010/63/EU and Principles of Good Laboratory Practice (GLP). The protocol for the current study was approved by the Ethics Committee for experimental animal wellbeing of the Faculty of Medical Sciences of the University of Kragujevac, Serbia (No: 01-11794).

RESULTS

Both the methionine-enriched diet and the methionine-enriched, folate-, vitamin B_6- and B_{12}-deficient diet resulted in significant decline in exploratory activity in the EPM test by means of the number of rearings ($F=9.011$, $df=2$), the number of head-dippings ($F=10.851$, $df=2$), and total exploratory activity ($F=19.779$, $df=2$).

As shown in Fig. 1, increased methionine intake for one month induced significant decreases in vertical exploratory activity in the EPM test, expressed as the number of rearings, compared to the control ($p<0.05$). The methionine-enriched diet coupled with the total restriction of folate, vitamin B_6 and vitamin B_{12} intake also reduced the number of rearings in the EPM test compared to the control ($p<0.01$), but this additional decline in exploratory activity was not significant when compared to the group on the methionine-enriched diet.

The prolonged intake of methionine-enriched chow also reduced the other pattern of vertical exploratory activity in the EPM test, the number of head-dippings (Fig. 2), compared to the control ($p<0.05$). Folate, vitamin B_6 and B_{12} deficiency, simultaneously applied with the methionine-enriched diet, resulted in an even greater reduction in the number of head-dippings when compared to the control group ($p<0.01$). However, such a decrease in this behavioural pattern was not significant compared to the effects of the methionine-enriched diet with sufficient folate, vitamin B_6 and B_{12} intake.

The total exploratory activity in the EPM, expressed by means of the sum of the number of rearings and the number of head-dippings, was confirmed to be a more sensitive marker of exploratory activity in the EPM test, as it covers both patterns of vertical exploratory activity (Fig. 3). Therefore, both applied dietary protocols resulted in significant reductions in the total exploratory activity compared to the control ($p<0.01$). Moreover, unlike the indi-

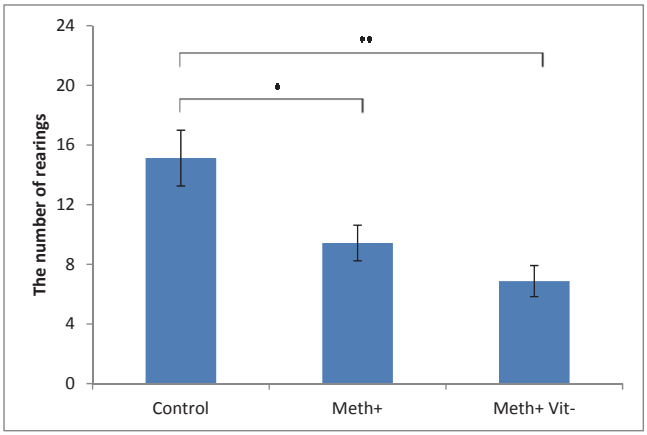

Figure 1 The number of rearings (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

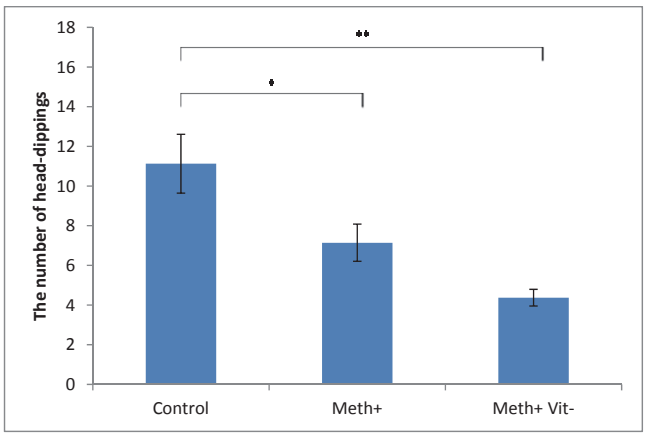

Figure 2 The number of head-dippings (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

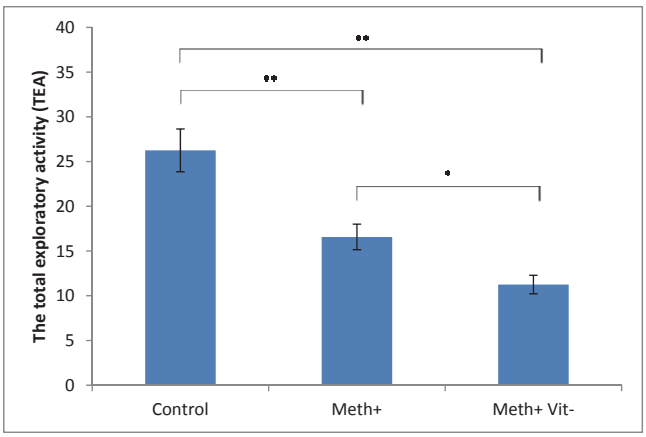

Figure 3 The total exploratory activity (Mean ± SEM, *denotes a significant difference $p<0.05$, **denotes a significant difference $p<0.01$). Control: control group, Meth+: methionine-enriched diet group, Meth+Vit-: methionine-enriched vitamin-deficient diet group.

vidual markers of exploratory activity in the EPM test, the total exploratory activity revealed that total restriction in folate, vitamin B_6 and B_{12} accompanied with methionine-enriched diet significantly diminished total exploratory activity compared to the group with adequate folate, vitamin B_6 and B_{12} intake ($p<0.05$).

DISCUSSION

Results of this study showed that dietary protocols designed to provide significant imbalance in Hcy metabolism can alter some specific behavioural patterns, such as exploratory activity in rats. On the other hand, exploratory activity obtained in the EPM test has been widely used as an indicator of anxiety (32-34). Because the reduction in exploratory activity has been interpreted as an augmented anxiety response in rodents (35, 36), our results confirmed the role of Hcy in maintaining anxiety state level.

Both standard parameters for vertical exploratory activity, the number of rearings (which mostly occur in the closed arms of the EPM) and the number of head-dippings (which occur exclusively in the open arms of the EPM), as well as the total exploratory activity (which summarizes overall exploratory activity in the EPM), were significantly reduced following 30 days of methionine nutritional overload. Although there are very few reports on the effects of high methionine intake on exploratory activity in rodents, our results correspond to previous research on the decreased number of rearings in the open field test following 30 days of nutritional overload with methionine (27). However, it seems that an acute increase in methionine-enriched food intake had no effect on exploratory drive, whereas a prolonged dietary protocol (5 and 10 mg/kg of L-methionine daily) resulted in significant decline in the number of rearings after 21 days (37). The proposed explanation for observed behavioural alterations was found in the fact that increased methionine intake induced lowering of S-adenosylmethionine (SAM, the compound that is involved in methylation cycle, affecting the formation of Hcy) in some specific brain regions that are responsible for the control of various brain functions (38). Methionine content in the applied diet was sufficient to produce an increase (4-5 folds) in serum Hcy levels (27). It is well known that the neurotoxic effects of hyperhomocysteinemia may be provoked directly by inducing DNA damage, resulting in increased apoptosis (39), as well as via N-methyl-D-aspartate and group I metabotropic glutamate receptors (40). Additionally, a methionine-enriched diet has been reported to significantly enhance the oxidative damage in different brain regions, such as the hippocampus, that have a crucial role in maintaining anxiety state levels (27). The reported alterations in brain oxidative status following methionine nutritional overload are mainly based on the reduction of antioxidant enzyme activity and depletion of glutathione levels (27). It has also been reported that increased reactive oxygen species production in specific brain regions is accompanied by increased anxiety levels in rodents (41) and in humans (42).

A methionine-enriched diet (for one month) accompanied with a reduction in some B vitamins (folate, pyridoxine and cobalamine) that are crucial for Hcy metabolism resulted in even more pronounced decline in exploratory drive observed in the EPM test compared to the control. Although the additional reduction in the number of rearings and the number of head-dippings was not significant comparing to methionine-enriched diet alone, diminishment of total exploratory activity (the most comprehensive indicator of exploratory drive in the EPM) revealed that deficiency in certain B vitamins can seriously inhibit exploratory activity in rats. There is no existing data on the impact of deficiency in the named vitamins (along with methionine overload) on the genesis of anxiety disorders by means of parameters of exploratory activity. However, our results are in line with previous reports of decreased exploratory drive in B vitamin (B_2, B_9, B_{12}, choline) deficiency in mice (43). The observed anxiogenic effect following B vitamin deficiency was achieved after 8 weeks of treatment and was accompanied by increased homocysteine plasma levels. Hyperhomocysteinemia, which may underlie the anxiogenic effect (such as that observed in this study), was also reported following dietary protocols with B_9 (44). Additionally, vitamin B_6 deficiency in rat chow resulted in hyperhomocysteinemia and enhanced lipid peroxidation accompanied by decreased SAM and glutathione levels in plasma and liver (45). Furthermore, the beneficial effects of B_{12} supplementation on hyperhomocysteinemia in aged rats have confirmed the importance of cobalamine in methionine metabolism in the brain (46). Although, numerous studies have pointed to the key role of hyperhomocysteinemia-induced neurotoxicity in various brain regions, recent research has presented the crucial possibility that hyperhomocysteinemia may be just a result of, rather than a cause of, at least some mood disorders, such as depression under chronic stress (47).

Based on reports that different patterns of exploratory activity can be considered reliable indicators of anxiety (48-50), we can also compare our results with clinical trials that analyse the connection between impaired one-carbon metabolism and anxiety disorders, such as obsessive-compulsive disorder (23, 24, 26).

In summary, the results of this study suggest the important role of Hcy in the modulation of exploratory activity in rats. Even more, because exploratory drive represents a reliable indicator of anxiety state level, it seems that future investigations considering protocols that can influence homocysteine metabolism (especially in the brain) may be a useful tool in the prevention and cure of some anxiety-related disorders.

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REGULARITIES OF OXIDATIVE STRESS COURSE IN CEREBRAL STROKE

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SMER I TOK PROCESA OKSIDATIVNOG STRESA PRI CEREBROVASKULARNOM UDARU

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ABSTRACT

Objective of the article: to improve diagnosis and treatment results of patients with ischaemic and haemorrhagic strokes by means of a comprehensive in-depth review of free radical processes and the defining of patterns of their course under the conditions of stroke. During the study, the authors established the regularities for the course of free radical processes in stroke with the development of oxidative stress and the severity of peroxide-lipid component, which increases in proportion to the severity of ischaemic or haemorrhagic stroke with maximum intensity in cases of adverse outcomes. Multi-stage mathematical modelling allowed for the determination of a highly effective formula for early stroke prognosis, which includes only 5 indicators used for estimation at hospitalization: consciousness level, blood glucose level, number of leukocytes in venous blood, antiperoxide activity of plasma and malondialdehyde. It was found that each of these parameters is an independent marker of hospital mortality. The consideration of all these indicators makes it possible to carry out early prognostic diagnostics with 90% probability and to timely correct treatment. We have also established digital boundaries, which are indications for the administration of energy correct therapy, the proper implementation of which has significantly improved the results of hospital treatment.

Keywords: *stroke, oxidative stress, apoplectic attack, free radical processes, early prediction, predicative model.*

SAŽETAK

Cilj ovog rada je poboljšanje dijagnoze i ishoda tretmana pacijenata sa ishemijskim i hemoragijskim moždanim udarom sa osvrtom na patofiziološke mehanizme nastanka posredstvom slobodnoradikalskih procesa kao smera tih procesa u pomenutim stanjima. U ovoj studiji, autori su ustanovili važnost slobodnih radikala u generisanju oksidativnog stresa i proceni stepena lipidne peroksidacije, cija je produkcija proporcionalna težini ishemijskog ili hemoragijskog udara kao i prisustvu negativnih komplikacija. Multidimenzionalna matematička jednačina nam je omogućila procenu prognozu i praćenje ovih pacijenata, koja se zasniva na 5 indikatora procene pri hospitalizaciji: stanje svesti, nivo glukoze u krvi, broj leukocita u krvi, antiperoksidna aktivnost markera plazme i nivo malonildiladehida. Utvrđeno je da je svaki od ovih pet indikatora nezavistan marker hospitalnog mortaliteta. Uzimanje u obzir svih ovih indikatora omogućava rano dijagnostikovanje u 90% slučajeva i blagovremeno lečenje. Pored toga, utvrdili smo digitalne granice, koje su indikaciono područje za primenu energetske terapije, kao značajne smernice koja može poboljšati rezultate bolničkog lečenja.

Kljucne reci: *moždani udar, oksidativni stres, apopleksija, slobodnoradikalski proces, rano prepoznavanje, model predviđanja*

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INTRODUCTION

Oxidative stress is an important component of many serious diseases [Liu Z, Zhou T, Ziegler AC, 2017; Bjørklund, G., Chirumbolo, S., 2016], and it also plays a role in the process of ageing [Bonomini, F., Rodella, L.F., Rezzani, R, 2015]. It has been proven that the imbalance of free radical processes (FRP) with the predominance of one of its components (oxidative stress) leads to accumulation of pathological genetic aberrations [Wang D, Feng JF, Yuan GY et al. 2017; Vijayalakshmi, P., Geetha, C.S., Mohanan, P.V., 2013] and provokes inflammation [8-9] and disorders of different organs and body system functioning [Azizova, O.A., Gao, L.N., Dumikyan, A.Sh. et al. 2011; Silina, E.V., Rumyantseva, S.A., Bolevich, S.B. et al. 2011; Asmat, U., Abad, K., Ismail, K., 2016]. It is known that the development of acute conditions, including socially significant diseases, is often preceded by a long asymptomatic stage, during which the parameters of FRP change [Aliev, N.A., Bobiev, A.B., Khamidov, D.B. et al. 2015; Giam, B., Kaye, D.M., & Rajapakse, N.W., 2016]. At the same time, many molecular mechanisms of cell pathology foundations during the development and treatment of major socially significant diseases, (the main of which is cerebral stroke), still remain unstudied [Chatzopoulos A, Tzani AI, Doulamis IP et al. 2017].

For a long time during the study of free radical cells and tissue damage, oxidative stress was considered only in terms of ascertaining facts about changes in levels of certain parameters, without revealing the patterns of their dynamics [Chehaibi K, Trabelsi I, Mahdouani K, Slimane MN, 2016, Žitňanová I, Šiarnik P, Kollár B et al. 2016]. There is no unified approach to the use of FRP parameters, as prognosis markers, and assessing the feasibility and effectiveness of conducting various types of corrective therapy [Chamorro Á, Dirnagl U, Urra X, 2016]. Stroke is the leading cause of disability in the population and the second cause of mortality in the world [Strong, K., Mathers, C., Bonita, R., 2007]. This prevalence makes stroke the most important problem not only for clinical angioneurology but also as a key social problem, which requires the development of maximally effective treatment methods, based on a comprehensive study of pathogenesis aspects.

The aim of this scientific work is to develop a pathophysiological-ly based strategy for treating patients with stroke, based on studying the regularities of FRP course.

MATERIALS AND METHODS

During the study, we conducted a prospective clinical-instrumental study, which included 383 patients with acute stroke verified by tomography (CT/MRI). In 302 (78.9%) cases, patients had ischaemic stroke (IS), and in 81 (21.1%) cases patients had haemorrhagic stroke (HS). In addition to stroke, 96% of patients had other cardiovascular disease diagnoses, which indicate a high level of vascular comorbidity. The distribution of patients by sex, age, nature and severity of disease is presented in Table 1. From the table below, it can be seen that HS is a significantly more serious disease than IS.

All patients were hospitalized in the intensive care unit and received complex therapy. When included in the study, patients were divided into two groups: 106 patients received standard therapy, and 277 patients additionally received EC therapy; Ascorbic Acid (AA) – 97 (32.1%) patients with IS and 23 (28.4%) patients with HS; Cytoflavin – 67 (22.2%) patients with IS and 32 (39.5%) patients with HS; Reamberin – 9 (3.0%) patients with IS; Ethylmethyl hydroxypiperidine succinate – 29 (9.6%) patients with IS and 11 (13.6%) patients with HS. A combination of two or more energy-correctors was provided to 52 (17.2%) patients with IS and 14 (17.3%) with HS. Patient groups were comparable at the time of hospitalization (Table 2).

All patients underwent clinical and instrumental monitoring in dynamics (until day 21 of hospitalization), which included the study of anamnesis and complaints, clinical somatic monitoring with daily monitoring of blood pressure, heart rate, respiratory rate and body temperature. Neurological status, which includes the consciousness disorder level and motor deficiency, was estimated in detail according to Glasgow Coma Scale (GCS), NIH, the Bartel social adaptation index and the Rankin modified scale. MRT/CT scan of the brain was conducted on all patients during the first hours after hospitalization; thereafter, 34 patients with IS additionally underwent CT/MRT in dynamics; on the 1st, 5th and 20th day in T1, T2 and Flair regimens, respectively. The data on blood and urine analysis, biochemical analysis, coagulograms and the acid-base state of arterial and venous blood were studied in dynamics. Additionally, we also examined the FRP in blood plasma in dynamics. FRP were studied in terms of generation of active oxygen forms (GAOF) by: leukocytes, the indicators of basal chemiluminescence intensity (CLIb) and zymosan-stimulated chemiluminescence intensity (CLIs), the activity coefficient (AC), spontaneous chemiluminescence (SpCL) and hydrogen peroxide-induced chemiluminescence (IndCL) of secondary plasma, antiperoxide plasma activity (APA), and by-products of lipid peroxidation (LPO), the main component of which, is malondialdehyde (MDA). The values studied in 33 cases of healthy people and donors were taken as the normal index for FRP.

The **statistical processing** of data was carried out using the SPSS 17.0 and Statistica 6.0 programmes with implementation of standard parametric and nonparametric criteria for assessing significant differences. The differences were considered to be significant at $p < 0.05$. The descriptive statistics of qualitative parameters are presented in the form of frequencies (abs, %), while the quantitative parameters are presented, in the form of the median (Me) and average ± standard deviation; these parameters include the lower and upper quartile, in case a parameter had a far non-normal distribution function. To compare two independent nonparametric samples, we used the

Table 1. Characteristic of patients with apoplectic attack.

Characteristic	Ischaemic stroke (n=302)	Haemorrhagic stroke (n=81)	**Overall (n=383)**
Average age*, years (M±m) Min-max	65.06±10.32 36-87	61.01±13.77 28-94	63.20±12.54 28-94
Sex/age*: - male - female	159 (52.7%), 63 y.o. 143 (47.3%), 69 y.o.	45 (55.6%), 52 y.o. 36 (44.4%), 67 y.o.	204 (53.3%), 59 y.o. 179 (46.7%), 69 y.o.
Duration of hospitalization*: < 6 hours 6-24 hours 24-48 hours >48 hours	 57 (18.87%) 70 (23.18%) 91 (30.13%) 84 (27.82%)	 24 (29.63%) 26 (32.10%) 21 (25.92%) 10 (12.35%)	 81 (21.15%) 96 (25.07%) 112 (29.24%) 94 (24.54%)
Consciousness level*: - intact - sleepiness - somnolentia - semi-coma - coma	 221 (73.18%) 24 (7.95%) 25 (8.28%) 21 (6.95%) 11 (3.64%)	 34 (41.98%) 12 (14.81%) 9 (11.11%) 19 (23.46%) 7 (8.64%)	 255 (66.58%) 36 (9.40%) 34 (8.88%) 40 (10.44%) 18 (4.70%)
Scope of damage (according to CT/MRT data)	<10 cm^3 – 107(35.4%) 10-50 cm^3 – 106(35.1%) >50 cm^3 – 89 (29.5%)	<10 cm^3 – 22 (27.2%) 10-30 cm^3 – 31 (38.3%) >30 cm^3 – 28 (34.5%)	129 (33.7%) 137 (35.8%) 117 (30.5%)
Arterial hypertension	291 (96.69%)	75 (92.59%)	366 (95.56%)
CHD, Cardiosclerosis	212 (70.20%)	52 (64.20%)	264 (69.92%)
Pneumofibrosis, emphysema	106 (35.10%)	27 (33.33%)	133 (34.73%)
Ciliary arrhythmia *	93 (30.79%)	7 (8.64%)	100 (26.11%)
Diabetes mellitus *	78 (25.83%)	9 (11.11%)	87 (22.72%)
Obesity *	63 (20.86%)	8 (9.88%)	71 (18.54%)
Postinfarction cardiosclerosis *	57 (18.87%)	5 (6.17%)	62 (16.19%)
Stenocardia	45 (14.90%)	10 (12.35%)	55 (14.36%)
GIT diseases	41 (13.57%)	13 (16.05%)	54 (14.10%)
Repeated Acute Cerebrovascular Event *	55 (18.21%)	4 (4.94%)	59 (15.40%)

Note: * – the difference between groups is significant, $p < 0.05$

Table 2. Groups of patients with apoplectic attack.

	Ischaemic stroke (n=302)		p	Haemorrhagic stroke (n=81)		p	Overall (n=383)		p
	Group I (n=81)	Group II (n=221)		Group I (n=25)	Group II (n=56)		Group I (n=106)	Group II (n=277)	
Average age	66.8±1.1	64.1±0.7	0.120	59.8±1.5	62.2±1.5	0.265	63.4±1.0	63.1±0.8	0.576
Sex: -male -female	44(53.3%) 37(45.7%)	115(52.0%) 106(48.0%)	0.624	13 (52.0%) 12 (48.0%)	32(57.1%) 24(42.9%)	0.851	57(53.8%) 49(46.2%)	147(53.1%) 130(46.9%)	0.993
Admission: <24 h 24-48 h >48 h	 28(34.6%) 27(33.3%) 26(32.1%)	 99(44.8%) 64(29.0%) 58(26.2%)	 0.275	 15(60.0%) 7(28.0%) 3(12.0%)	 35(62.5%) 14(25.0%) 7(12.5%)	 0.960	 43(40.6%) 34(32.1%) 29(27.3%)	 134(48.4%) 78(28.2%) 65(23.4%)	 0.389
Conscious alteration of consciousness	57(70.3%) 24(29.6%)	164(74.2%) 57(25.8%)	0.603	11(44.0%) 14(56.0%)	23(41.1%) 33(58.9%)	0.978	68(64.2%) 38(35.8%)	187(67.5%) 90(32.5%)	0.616
Volume-(cm^3): Me 25%/75% Q N(%): <10 10-30/50 >30/50	38.46±7.51 11.70 2.40/39.56 26(32.1%) 30(37.0%) 25(30.9%)	34.83±3.8 17.26 1.58/50.78 81(36.7%) 76(34.4%) 64(28.9%)	0.731 0.764	37.96±5.26 27.58 11.64/51.18 7(28.0%) 9(36.0%) 9(36.0%)	28.23±2.57 24.80 13.76/38.90 15(26.8%) 22(39.3%) 19(33.9%)	0.314 0.961	38.29±5.1 20/51 7.52/51.00 33(31.1%) 39(36.8%) 34(32.1%)	31.9±3.1 20.87 4.59/47.18 96(34.6%) 98(35.4%) 83(30.0%)	0.434 0.804

Mann-Whitney test, while for multiple comparisons we used the Kruskal-Wallis test. To compare two dependent nonparametric samples, we used the Wilcoxon signed-rank test, and for the multiple comparisons we used the Friedman test. The qualitative variables were compared using the χ^2 test (Pearson's chi-squared test, for the analysis of contingency tables). The stratification of obtained results was carried out by multifactor analysis, the basis of which was the correlation matrix (Pearson and Spearman methods). To build this matrix, we determined the characteristic values and corresponding vectors with correlation coefficients $r > 0.2$; $p < 0.05$. To determine important factors, we used the principal component method. The number of counted complexes was determined by means

of a point chart of normalized stress, which estimates the total weight of variables included in the complex. To select indicators with a high factor load, we used the Varimax orthogonal rotation method. The prognostic modelling was carried out using discriminant analysis and binary logistic regression. Differences were considered to be significant, when $p < 0.05$.

RESULTS

Positive significant imbalance of FRP was detected on the 1st day of hospitalization of patients with stroke; however, the degree of severity and the direction of this imbalance varied (Table 3). It was established that among the patients with acute IS, the imbalance of FRP mainly affects the peroxide-lipid component markers in the form of a reliable increase in the MDA indicators average of 1.27 times ($p < 0.01$), SpCL – 1.05 times ($p < 0.05$) and IndCL – 1.23 times ($p < 0.001$). The level of APA among patients with IS was reliably ($p < 0.05$) reduced on average 1.07 times. In contradistinction from patients with IS, when hospitalized, patients with HS were diagnosed with an increase in oxygen markers: CLIb – 1.27 times ($p < 0.01$) and CLIs – 1.42 times ($p < 0.01$). At the same time, patients with HS had a marked imbalance and lipid components of FRP in the form of a significant increase in the level of MDA by 1.36 times ($p < 0.01$), SpCL – 1.11 times ($p < 0.001$) and IndCL – 1.32 times ($p < 0.001$); in the background, a decrease in APA of 1.15 times ($p < 0.01$) occurred. The comparative analysis of FRP indexes among patients with strokes of a different nature revealed significant differences, mainly in markers of oxygen stages; according to CLIb ($p < 0.001$), which on average was 45% higher among patients with HS, AC ($p < 0.01$) was 55% higher among patients with IS, and SpCL index ($p < 0.05$) was 26% more under HS. As to the other markers, we have noted the tendencies for more pronounced imbalances of all FRP and HS stages, the pathogenesis of which is more multifaceted.

The consolidated mechanism was the aggravation of free radical imbalance, as the condition of the patient became more severe, with the displacement of the disregulation vector into the peroxide-lipid side. Thus, the level of consciousness disorders, as the main clinical criterion of severity, were chosen for the patients with stroke. In the patients with moderate IS, who were admitted into the hospital without signs of altered consciousness, the parameters of the oxygen stages of FRP significantly exceeded the norm: CLIs on average by 1.18 times ($p < 0.05$) and IndCL – by 1.25 times ($p < 0.001$). The patients with severe IS, who had a consciousness disorder when hospitalized, were diagnosed with a significant increase in the parameters of

Table 3. Imbalance of free radical processes in critical states of different genesis.

FRP OXYGEN markers	**CLIb (mV/sec×10⁶ L)**	**CLIs (mV/sec×10⁶ L)**	**Activity ratio (CLIs/CLIb)**
Health (n=33)	*63.37±5.04* *62.50* *41.61/80.30*	*435.83±32.49* *469.85* *307.55/564.43*	*8.28±1.19* *6.89* *3.99/11.01*
Stroke (n=383)	91.14±7.39 64.23 37.10/136.18	774.89±54.96 **571.30** * 322.20/1186.00	10.41±2.05 6.70 3.11/12.02
Ischaemic stroke (n=302)	87.89±7.65 54.90 23.24/125.70	721.81±61.17 544.50 264.40/1245.00	14.67±3.19 7.65 4.08/22.02
Haemorrhagic stroke (n=81)	119.05±12.04 **79.45** * 49.99/160.58	781.66±70.39 **667.20** * 404.35/1028.50	7.32±1.20 4.94 3.02/9.19

ALKALINE-LIPIDIC	**SpCL of the secondary plasma**	**H_2O_2 IndCL of the secondary plasma**	**IndCL/SpCL (k/APA)**	**MDA (μM)**
Health (n=33)	*0.820±0.013* *Me=0.802* *0.788/0.857*	*2.13±0.10* *Me=2.09* *1.62/2.48*	*2.73±0.14* *Me=2.78* *2.06/3.19*	*2.92±0.17* *Me=2.75* *2.52/3.70*
Stroke (n=383)	0.854±0.007 **0.850** * 0.803/0.929	2.77±0.05 **2.62** * 2.11/3.30	3.25±0.08 **3.02** * 2.55/3.98	3.75±0.11 **3.60** * 2.89/4.47
Ischaemic stroke (n=302)	0.857±0.008 **0.840** * 0.793/0.910	2.73±0.07 **2.58*** 2.24/3.29	3.24±0.09 **2.98** * 2.57/4.10	3.68±0.12 **3.49*** 2.91/4.48
Haemorrhagic stroke (n=81)	0.889±0.012 **0.889*** 0.812/0.952	2.93±1.13 **2.75*** 2.09/3.32	3.41±0.16 **3.19*** 2.51/3.84	4.03±0.24 **3.74** * 2.86/4.54

Statistical results: M±m; Median; Quartiles 25%/75%.
** – significant for p <0.05 difference of indicator from the norm.*

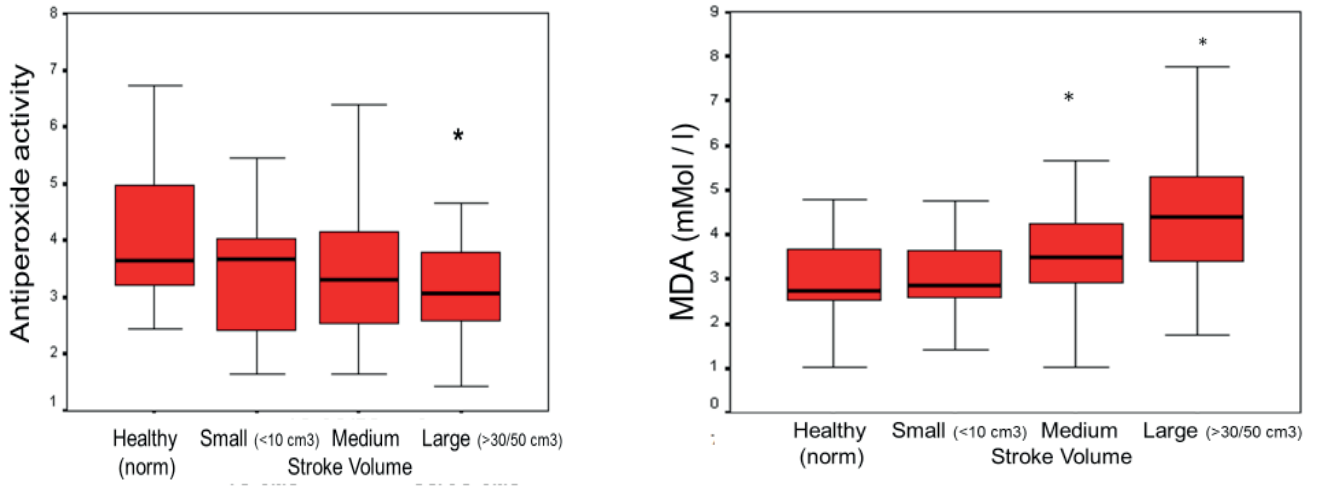

Figure 1. Comparative analysis of TBA-RP (MDA) and k/[IndCL/SpCL] (APA) indicators among patients with stroke of different scopes during hospitalization.
** – p <0.05 – difference from the norm*

FRP peroxide-lipid component: SpCL – by 1.11 times ($p < 0.01$), IndCL – by 1.23 times ($p < 0.001$), IndCL/SpCL – by 1.06 times ($p < 0.05$) and MDA – by 1.41 times ($p < 0.01$). On the 1st day of hospitalization, the inhibition activity in oxygen markers among patients with altered consciousness states were registered. The obtained data prove that the defence-adaptive role of active oxygen forms release, as a stimulant of activity of its own antiradical systems, under conditions of ischaemia, as well as the pathological role of uncontrolled hyperactivation of peroxide-lipid component of FRP (increasing MDA with decreasing APA) in cell destruction, death by apoptosis and necrosis. Similar changes were also typical for patients with HS. The comparative analysis of FRP indexes determined the significance of the MDA-titer increasing with the increase in severity of health conditions among patients with HS.

In the case of various types of stroke, the TBA-RP (MDA) and APA levels were taken as an early marker of scope and severity of damage. The results of FRP index analysis for different scopes of brain damage show a gradual aggravation of FRP imbalance severity with a shift towards peroxide processes alongside a decrease in antiradical systems activity under the increased scope of stroke. Thus, with a small IS scope (less than 10 cm^3), significant changes in the free radical status affected the oxygen part of the oxidative stress spectrum (the increase in CLIs and AC), and thereafter a tendency towards an increase in the defensive APA was noted. With an average scope of IS (10-50 cm^3), a significant disruption of peroxide lipid markers was observed against the background of APA decrease ($p < 0.05$), which sharply aggravated with an extensive IS (more than 50 cm^3), illustrating the MDA-titer increase on average by 60% and APA depression by 40% ($p < 0.05$). In the case of an HS of more than 30 cm^3, the highest imbalance in both the oxygen and peroxide stages of FRP was noted, which led to the progression of secondary ischaemia.

The analysis of FRP characteristics performed among the patients who were admitted to the hospital at different periods from the moment of first appearance of clinical symptoms made it possible to reveal the patterns of free radical imbalance development in the absence of drug correction, as staying at home, the patients did not receive adequate therapy. Mostly, the FRP imbalance was expressed among the patients hospitalized on the 2nd-3rd day of the disease. At the same time, a gradual change in free radical reactions from oxygen to peroxide lipid in the form of MDA growth was noted against the background of APA decrease with formation of a vicious circle initiated by active oxygen forms and enhanced LPO, i.e., tissue destruction.

The next stage of our study was the differentiation of patients into subgroups with benign (discharged from inpatient department, n = 305, 79.6%) and adverse outcomes (hospital mortality, n = 78, 20.4%, including 48 (15.9%) with IS and 30 (37.0%) with HS). The correlation analysis helped us to determine that the adverse stroke outcome was associated with a high level of neurological insufficiency (according to the NIH scale in dynamics), functional insufficiency (according to Rankin and Bartel scales), depressed level of consciousness, large disease focus (more than 37 cm^3), coexisting somatic pathology (CHD, cardiosclerosis, body-weight index increase > 30.5 kg/m^2), high diastolic blood pressure figures (> 95 mm Hg), heart rate (> 85/min), and high respiratory rate (> 20/min) at the time of hospitalization and with the prevalence of complications (pneumonia, stress-protective ulcers of digestive haemorrhage (DH), and venous thromboembolism). Standard laboratory markers examined at the time of hospitalization of patients with stroke were significantly ($p < 0.05$) correlated with death and are given below in Table 4. The obtained data allowed conducting an interval analysis of these indicators with the following definition of threshold values and the boundaries of low and high risks of death.

Table 4. Prognosis of the risk of adverse outcome by laboratory parameters during the first day of hospitalization.

Index	Low risk	Average risk	High risk
Thrombocytes at hospitalization (ths.)*	<162	162-355.4	>355.4
Leukocytes at hospitalization*	<4.9	4.9-16.8	>16.8
Glucose at hospitalization*	<4.2	4.2-11.1	>11.1
Leukocytes at the 1st day*	<5.3	5.3-14.6	>14.6
Banded neutrophils at the 1st day (%)*	<2	2-11	>11
Stab neutrophils at the 1st day (%)*	<54	54-82	>82
Lymphocytes (%)*	>29	8-29	<8
ESR at the 1st day (mm/h)*	<4	4.0-41.3	>41.3
Glucose at the 1st day (mmol/L)*	<5.1	5.1-15.6	>15.6
Urea at the 1st day*	<2.9	2.9-12.3	>12.3
Direct bilirubin at the 1st day*	<4.9	4.9-19.5	>19.5
Lactate dehydrogenase at the 1st day*		235.7-833.5	>833.5
Sodium at the 1st day*	<129.8	129.8-148.0	>148
Thrombin time at the 1st day*	<20.50	20.50-31.38	>31.38
Prothrombin ratio at the 1st day*	>103.5	72.2-103.5	<72.2

*Note: * – significant difference under $p < 0.05$ after the outcome of a stroke.*

When conducting a comparative background analysis of FRP markers and stroke outcome, it was found that the most pronounced imbalance of free radical status at the 1st day of hospitalization was registered under an adverse outcome. Thus, CLIb was sharply reduced in the case of adverse IS outcome ($p < 0.05$). The adverse HS outcome was characterized by the intensification of CLIb (critical values – 488.24 mV/s × 10^6 leukocytes, above which all cases of HS ended fatally). With CLIs, a tendency towards greater activation was observed in the case of adverse outcomes ($p > 0.05$). The outcome model was unambiguously reflected by the indicators of MDA and APA (Table 5). Thus, IndCL/SpCL index at the time of hospitalization was already strongly reduced among deceased patients with IS ($p < 0.05$). The critical values of IndCL/SpCL index were 5.87 among patients with IS and 6.09 among patients with HS. The plasma level of MDA was drastically increased among deceased patients ($p > 0.05$) with critical values of MDA: 6.39 μmol/l among patients with IS and 5.41 μmol/l among patients with HS. Exceeding these values reflected the absolute risk of hospital mortality. The performed analysis allows the recommendation of the use of these indicators as the early prognostic markers of stroke course and outcomes.

A comparative analysis of FRP dynamics among patients with stroke of different genesis who received no EC/AO revealed a marked imbalance of both oxygen and lipid FRP markers throughout the whole period of in-patient follow-up. In the 2nd-3rd week, when diurnal infusions were replaced by the use of tablets, the tendency towards activation of FRP had been noted.

The analysis of FRP dynamics revealed the following characteristics under IS: rapid activation in the period from the 1st to the 5th day, according to CLIb – by 1.61 times, CLIs – by 2.32 times, and AC – by 2.99 times. Henceforth, there was a regression of indicators with CLIb normalizing, but CLIs and AC did not normalize even by the 20th day. APA significantly increased by the 5th day – by 1.15 times, having a tendency to normalize by the 10th day, while during the period from the 10th to the 20th day, we noted a 1.36-time decrease in APA. MDA throughout the whole follow-up period gradually increased (by 1.11 times) during the period from the 1st to the 20th day.

The dynamics of FRP among patients with HS had the following characteristics: according to CLIb and CLIs, we observed the regression during the period from the 1st to the 10th day by 1.88 and 1.29 times, respectively; AC increased during the period from the 1st to the 5th day by 1.74

Table 5. Prognosis of mortality risk in terms of FRP among patients with stroke of different natures examined on the 1st day of hospitalization.

	Low risk	Average risk	High risk	Absolute risk
IndCL	1.28-1.84	1.85-4.24	>4.24	>5.18
IndCL/SpCL (k/APA)	1.33-1.92	1.93-5.38	>5.38	>6.09
MDA	1.01-1.51	1.52-5.30	>5.30	>6.39

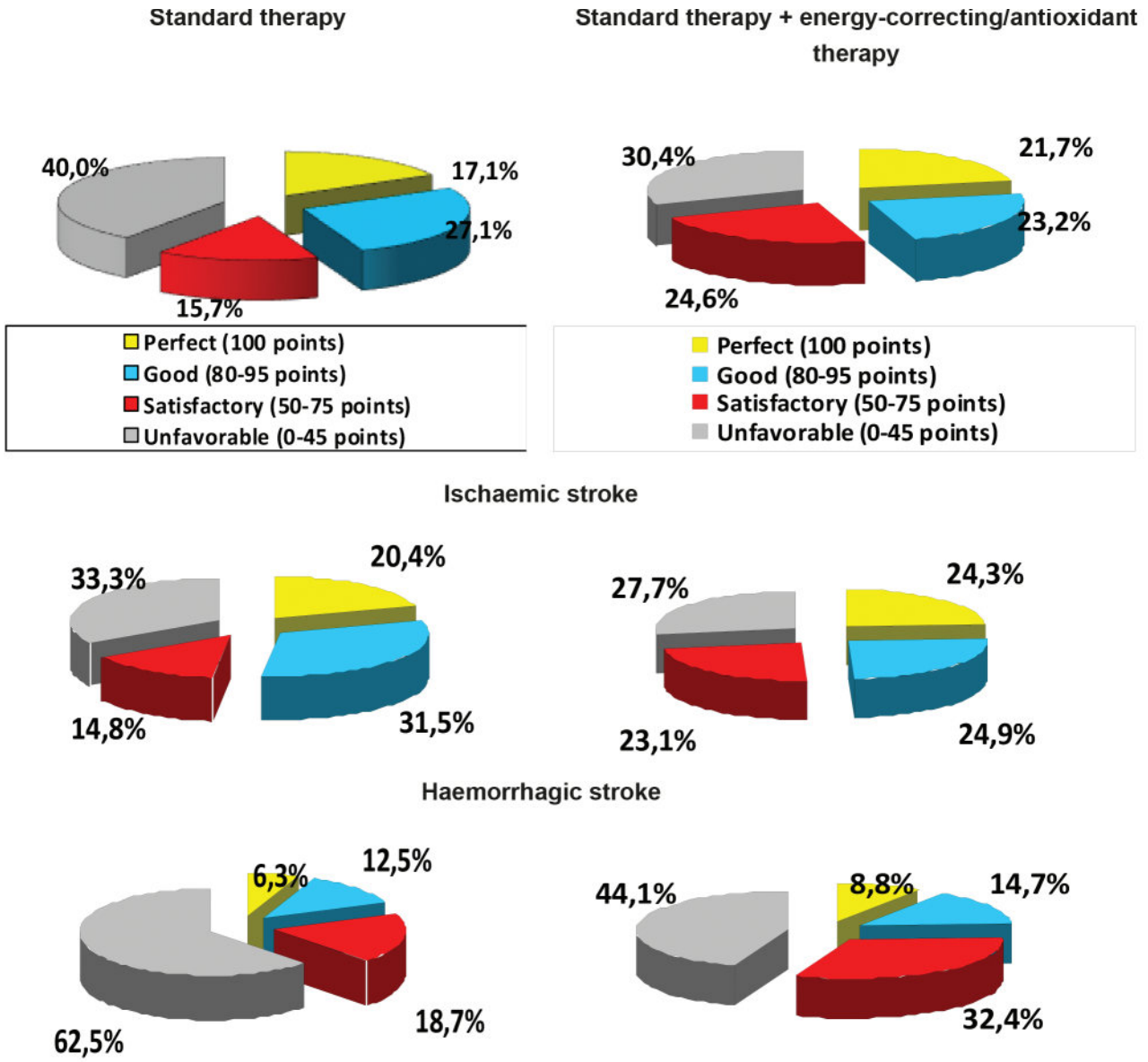

Figure 2. The structure of functional outcomes among patients with strokes of different characteristics according to the Bartel index for the 20th day of hospitalization in groups of patients with and without energy-correcting/antioxidant therapy

times; APA increased in the period from the 1st to the 10th day on average by 1.39 times, normalizing by the 5th day, but during the 10th-20th day, we noted the APA tended to decrease by 1.15 times; plasma MDA had increased in the period from the 1st to the 5th day by 1.20 times, from the 5th to the 10th day – by 1.13 times, and during the 10th-20th days – by 1.09 times.

The administration of EC/AO therapy from the very 1st day to patients with stroke of a different genesis led to positive dynamics in FRP. Thus, the pronounced regression of CLIb and CLIs, noticeable already by the 3rd-5th day, was maintained up to the 7th-10th day, i.e., up to the moment of the last infusion of energy correctors; this result meant that the production of active forms of oxygen subsided, and their concentration decreased, resulting from the interaction with an antioxidant. The second stage in the positive effect of EC therapy was a decrease in the severity of LPO reactions, which was registered by the dynamics of MDA regression and significant growth of APA by the 3rd-5th-7th-10th day. It should be noted that there was a tendency of FRP activation after the end of EC therapy (by the time of discharge), which indicated the need for longer (more than 10 days) EC/AO therapy.

The use of EC therapy not only contributed to the normalization of FRP parameters but also allowed the improvement of the treatment results. The inclusion of EC/AO therapy into the complex therapy of patients with stroke resulted in a more rapid regression (than in the comparative group) of consciousness disturbances ($p < 0.05$) as well as focal neurologic symptoms with authentically more significant regression by the time of discharge ($p < 0.05$).

The final stage of the study was the development of a mathematical model for stroke prognosis. To this end, we used discriminant analysis (DA), and with the help of ,several characteristics, an individual could be assigned to one of the given groups. The DA core was the construction of the discriminant function: $D = b_1x_1+b_2x_2+... + b_nx_n+a$, where x_1 and x_n were the values of variables that corresponded to the examined cases; *a* was constant; $\mathbf{b_1}$-b_n were coefficients, which were estimated using DA. During the study, we determined the values of D; it was then possible to carry out the division into groups for stroke prognosis with a maximum accuracy.

To form the prognosis model of FRP parameters, we initially used all studied variables: CLIb, CLIs, AC, SpCL, IndCL, APA and MDA. Later, their number was reduced to two (APA, MDA) without critical loss of prognosis significance. Thus, according to the DA results, when D ≥ 0.55, the patient fell into the 'adverse outcome' group, and when D ≤ -0.3, the patient fell into the 'benign outcome' group. The prognosis accuracy was 68.6% ($p < 0.05$).

$D = -2.665 + 0.021X_1 + 0.672X_2$	
where the constant = -2.665; X_1 – APA; X_2 – MDA.	
Adverse outcome: D > 0.54 [-2; 3.5]	Benign outcome: D < -0.29 [-2; 1.5]

However, the obtained accuracy did not satisfy us. Therefore, we later conducted a multifactor analysis of all clinical laboratory parameters assessed in dynamics among patients with stroke. The purpose of this analysis, first, was to detect the persistent correlations between variables, mainly for the further construction of an accurate prognostic model of disease outcome. The initial array of observations was formed on the basis of data from 383 patients with cerebral stroke. At the first step of the factor analysis procedure, we selected 119 indicators (including dynamics up to the 20th day of observation) that had a proven or presumed influence on the course and outcome of stroke, and then we standardized the set values of variables (z-transformation). Based on the analysis of the total variance, we received 191 factors, including 79 factors excelling in strength;, the indexes were grouped by their strengths of influence on the general picture of indicator variability and explained 95.18% of the total variance. For further exclusion criteria, we used the Cattell scree test, and as a result, we determined 4 HS. After performing 8 rotations by the Varamax method, the significant factors were united into the main components in descending order, which allowed for choosing and reducing them in the future.

Significant multiple correlation coefficients confirmed the descriptiveness and prognosis value of selected clinical and biochemical parameters complemented by the parameters of instrumental methods. Based on the multivariate analysis results, for our further DA and for the future development of an IS prognosis model, we selected the 22 most significant factors studied during the hospitalization of patients and reflected them in the main components, which characterized the outcome (death or discharge): CLIb, CLIs, AC, APA and MDA; consciousness level by Glasgow Coma Scale (GCS); Bartel index; modified Rankin scale score; NIH; blood pressure; heart rate; white blood cell count; lymphocyte, glucose, thrombocytes, creatinine, urea, potassium, sodium and fibrinogen levels; and ALT, AST, LDH, PTI, INR and prothrombin time. The further step-to-step minimization of their number (up to 5 major factors) allowed for performing DA with reliable accuracy and formulating a mathematical model of ischaemic stroke outcome.

$D= -1.463 + 1.235X_1 – 0.055X_2 – 0.099X_3 + 0.038X_4 + 0.555X_5$		
where the constant = -1.463; X_1 – level of consciousness (0 – intact; 1 – sleepiness; 2 – somnolencia; 3 – semi-coma; 4 – coma); X_2 – blood glucose level; X_3 – number of leukocytes in blood (thous.); X_4 – IndCL/SpCL; X_5 –MDA (μmol/l);		
Adverse outcome:	**Benign outcome:**	**Prognostic significance:**
D > 1.210	D < -0.355	**85.1%** ($p < 0.001$)
D > 3	D < -1	**99.9%**

The research performed made it possible to develop an algorithm for pathogenetically grounded therapy of FRP course disorders under critical states of various geneses. We have established that the indications for EC-therapy are as follows: an increase in CLIb > 130 mV/s × 10^6 leukocytes, CLIs with zymosan > 750 mV/s × 10^6 leukocytes, AC > 26, IndCL/SpCL (k/APA) > 3, and TBC-RP (MDA) > 4 μmol/l.

CONCLUSION

The performed study has shown that in the case of stroke of different natures, accompanied by syndromes of tissue ischaemia, hypoxia, and local and systemic inflammatory reactions, we can observe the same sequence in the development of oxygen imbalance and lipid stages of FRP; we can further observe the rate of unfolding of these syndromes, and which convey the dynamics of clinical and standard laboratory indicators. The earliest markers of FRP imbalance severity are the CLIs and CLIb, which characterize the imbalance of the FRP oxygen component. In cases of increasing ischaemia, the greatest imbalance is revealed by the levels of parameters reflecting the stage of lipid peroxidation (decrease of APA and growth of MDA).

Among the patients with ischaemic stroke, the imbalance markers of FRP lipid phase were as follows: MDA increased by 1.27 times, and APA decreased by 1.07 times. In cases of haemorrhagic stroke, CLIb increased by 1.27 times, CLIs – by 1.42 times, and MDA – by 1.36 times; however, APA decreased by 1.15 times. The levels of critical MDA values were as follows: 6.39 μmol/l – in IS; 5.41 μmol/l - in HS.

The study of FRP index dynamics, among patients with stroke of different genesis who received no EC therapy, has shown a marked imbalance in both the oxygen and lipid stages throughout the whole period of in-patient

follow-up, with a tendency to activate FRP by the time of discharge. The early inclusion of AC/AO therapy into the complex treatment of patients with critical states of various natures and severity promotes the activation of consciousness, which advances the comparison group, the regression of neurological insufficiency by means of reducing the cerebral ischaemia zone, the reduction of disability with a change in its structure by means of decrease of severe, and the increase of good functional outcome.

The established digital boundaries of risk of adverse outcomes for various laboratory indicators became a key result of the study, making it possible to recommend the use of these indicators, as early prognostic markers of stroke course and outcomesto and to carry out the early diagnosis and prognosis of stroke. The multifactor analysis of more than 700 criteria (indicators evaluated in dynamics among patients with stroke), discriminant analysis, logistic regression and mathematical modelling allowed the identification of the formula that includes only 5 factors, the assessment of which, on the 1st day of hospitalization, makes it possible to predict with a high degree of accuracy (more than 88%) the outcome of a stroke. These results gives us an opportunity to timely optimize stroke therapy and to improve treatment results.

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AQUEOUS EXTRACT OF CARICA PAPAYA LINN ROOTS HALTS SODIUM ARSENITE-INDUCED RENAL INFLAMMATION THROUGH INHIBITING ADENOSINE DEAMINASE, 8-HYDROXY-2′-DEOXYGUANOSINE, C-REACTIVE PROTEIN AND INDUCIBLE NITRIC OXIDE SYNTHASE ACTIVITY

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VODENI EKSTRAKT KORENA CARICA PAPAYA L. ZAUSTAVLJA INFLAMACIJU BUBREGA IZAZVANU NATRIJUM-ARSENITOM, INHIBIRAJUĆI ADENOZIN DEAMINAZU, 8-HIDROKSI-2'-DEOKSI-GUANOZIN, C-REAKTIVNI PROTEIN, INOS AKTIVNOST

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Running title: Anti-inflammatory activity of *Carica papaya root*
Kraći naslov: Antiinflamatorna aktivnost korena *Carica papaya*

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ABSTRACT

Objectives: *Inflammation plays a crucial role in many of the metabolic abnormalities. The prototypic marker of inflammation is C-reactive protein (CRP), Nitric Oxide (NO), inducible nitric oxide synthase (iNOS) and their inhibition is considered a promising strategy to combat inflammation. Here, we report the anti-inflammatory mechanism of Carica papaya root aqueous extract in sodium arsenic-induced renal dysfunction.*

Methodology: *Thirty-five rats were used for the experiments. Griess assay was used to evaluate the inhibitory effect of Carica papaya roots aqueous extract on the overproduction of nitric oxide (NO). ELISA was used to determine the level of pro-inflammatory markers including c-reactive protein (CRP). ELISA was used to analyze 8-OHdG. The inhibitory effect on the enzymatic activity of inducible nitric oxide synthase (iNOS), adenosine deaminase (ADA), malondialdehyde (MDA) was tested by enzyme activity assay kits.*

Results: *Carica papaya roots aqueous extract suppressed sodium arsenite-stimulated NO production and pro-inflammatory secretion, such as CRP. Carica papaya roots aqueous extract significantly ($p < 0.05$) decrease the activities of iNOS, 8-OHdG, ADA and MDA.*

Conclusion: *These results indicated that potent inhibition on CRP, NO, iNOS, ADA, 8-OHdG might constitute the anti-inflammatory mechanism of Carica papaya roots aqueous extract.*

Keywords: *Carica papaya Linn. roots, anti-inflammatory biomarkers, renal inflammation*

SAŽETAK

Ciljevi: *Inflamacija ima ključnu ulogu u brojnim metaboličkim poremećajima. Smatra se da inhibicija faktora inflamacije kao što su C-reaktivni protein (CRP), azot monoksid (NO), inducibilna azot monoksid sintetaza (iNOS) predstavlja obećavajuću strategiju u borbi sa inflamacijom. U ovom radu smo izvestili o antiinflamatornom mehanizmu vodenog ekstrakta korena Carica papaya kod natrijum-arsenitom izazvane bubrežne disfunkcije.*

Metodologija: *U eksperimentima je korišćeno 35 pacova. Griess-ov rastvor je korišćen da ispita inhibitorni efekat vodenog ekstrakta korena Carica papaya na povećanu produkciju azot monoksida. Za određivanje nivoa proinflamatornog markera CRP-a je korišćena elisa. Elisa je korišćena za analizu 8-hidroksi-2'-deoksiguanozina (8-OHdG). Esej za analizu enzimske aktivnosti je koričen za određivanje aktivnosti iNOS, adenozin deaminaze (ADA) i malonildialdehida (MDA).*

Rezultati: *Vodeni ekstrakt korena Carica papaya suprimira natrijum-arsenitom stimulisanu produkciju NO i sekreciju proinflamatornih molekula kao što je CRP. Vodeni ekstrakt korena Carica papaya je statistički značajno ($p<0,05$) smanjio akivnost iNOS, 8-OHdG, ADA i MDA.*

Zaključak: *Ovi rezultati pokazuju da jaka inhibicija CRP, NO, iNOS, ADA, 8-OhdG može predstavljati potencijalan mehanizam antiinflamatornog delovanja vodenog ekstrakta korena Carica papaya.*

Ključne reči: *koren Carica papaya L., antiinflamatorni biomarkeri, bubrežna inflamacija*

ABBREVIATIONS

CRP – C reactive protein; **ADA** – Adenosine deaminase;
NO – Nitric oxide; **8-OHdG** - 8-hydroxy-2′-deoxyguanosine;
iNOS – inducible nitric oxide synthase; **MDA** - malonaldehyde

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INTRODUCTION

Arsenic is a ubiquitous element present in food, soil, water and air, and it is released into the environment from both anthropogenic and artificial sources (1, 2). The foremost inorganic forms of arsenic comprise of the trivalent meta arsenite As^{3+} and the pentavalent arsenate As^{5+}. Trivalent arsenic form has a higher affinity for thiol groups and is more cytotoxic and genotoxic than As^{5+} (3, 4). Organisms that store the trivalent intermediates are thought to be at greater risk of arsenic- induced ailments (4). Some of the organic forms contain the methylated metabolites monomethylarsonic acid (MMA), dimethylarsenic acid (DMA) and trimethylarsine oxide (TMAO) as well as arsenocholine, arsenobetaine (AsB) and arsenosugars. Over 80% of commercially employed arsenic compounds are used to manufacture products with agricultural applications for examples herbicides, fungicides, insecticides, algaecides, wood preservatives, dyestuffs, sheep dips and medicines for the eradication of tapeworms in sheep and cattle. Arsenic compounds have been used for at least a century in the treatment of yaws, amoebic dysentery, syphilis and trypanosomiasis (5).

Toxic effects of arsenic accredited to production of reactive oxygen species (ROS) and oxidative stress results in the alteration of the antioxidant defense system, increased oxidative stress and cell death (6, 7). Liver and kidney are target organs for arsenic toxicity in rats, though, concentration of arsenic was found to vary in these organs (8). Arsenic induced nephrotoxicity results in disturbing the antioxidant defense system, protein oxidation and lipid peroxidation products. It also leads to renal functional deterioration (9). Though, its high affinity for sulphydryl groups of protein has a tendency to readily react with the sulfhydryl groups of proteins and this turn inhibit biochemical pathways and considered to be the biologically active form and the major source to arsenic toxicity (10). Lipid peroxidation leading to decrease glomerular filtration rate and to elevate nitrogenous waste such as urea, uric acid and creatinine (11, 12). 8-OHdG is formed from deoxyguanosine in DNA by hydroxyl free radicals. Because of its stability, 8-OHdG is known as one of the most reliable markers of oxidative DNA damage (13).

Recent studies have implicated the endogenous signaling molecule called adenosine in renal function. Adenosine is produced by enzymatic phosphohydrolysis of its precursor molecules, particularly adenosine triphosphate (ATP) and Adenosine monophosphate (AMP) (14, 15, 16). Hence, inhibition of ADA activity has been proposed to be a good therapeutic approach for the management or prevention of kidney dysfunction.

Carica papaya belongs to the family of *Caricaceae*. Papaya is an herbaceous succulent plant that possess self-supporting stems (17). It is a large perennial herb with a swift growth rate. The plants are usually short-lived, but can produce fruit for more than 20 years (18). The papaya has a rather complex means of reproduction. The plant was originally derived from the southern part of Mexico. *C. papaya* is a perennial plant, and it is presently distributed over the whole tropical area. In particular, *C. papaya* fruit circulates widely, and it is accepted as food. In Nigeria, pawpaw is one of the most popular, cheapest, economically important fruit tree grown and consumed for its nutritional content (19). *Papaya* is not a tree but an herbaceous succulent plant that possesses self-supporting stems (17). The top ten countries that produces *Carica papaya* plants worldwide are; Brazil (25%), Nigeria (15%), India (12%), Mexico (11%), Indonesia (10%), Ethiopia (4%), Congo (4%), Peru (3%), Venezuela (3%), and lastly China (2%) (FAO, 2004). Hence, this study investigated the effect of *Carica papaya* root extract on the sodium arsenite-renal inflammation through inhibition of anti-inflammatory biomarkers.

MATERIALS AND METHODS

Plant material

Roots of *C. papaya* Linn. was freshly collected in May, 2016 from Ijadu farm, Ado-Ekiti, Ekiti State, Nigeria. The plant was identified and authenticated by a senior taxonomist at the herbarium unit of the Department of Biological Science, Afe Babalola University, Ado-Ekiti, Nigeria and a voucher specimen number ABUAD/040 was deposited accordingly. All plant names in this manuscript are formatted according to the latest revision in "The Plant List", and correspond to the good practices in publishing studies on herbal materials, as described by (20).

Preparation of aqueous extract of C. papaya root

The aqueous extract of the powered *C. papaya* root was prepared using method described previously (12). The roots were air-dried in the laboratory at ambient temperature (30 ± 2 °C) for 28 days, minced using a laboratory mechanical grinder and the powders obtained stored until further use. 50 g of the powdered sample were extracted with distilled water (via maceration) for 48 h. The percentage yield extraction calculated as follows:

$$\% \text{ yield} = \frac{\text{Weight of the dry extract}}{\text{Weight of powdered leaves}} \times 100\%$$

Experimental Animals

Eight weeks old male albino rats of Wistar stain with initial mean body weight (bw) (155.25 ± 11.52 g) were used in this study. The animals were obtained from Animal house, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria. The animals were housed in large clean spacious cages and were given food and water *ad libitum*. The animal room was well ventilated with a 12 h light/dark cycle, throughout the period of the experiment. They were fed *ad libitum* on rat pellets (Top Feeds, Nigeria) and water. The handling of animals was conformed to the standards

of National Institute of Health (NIH publication 85-23, 1985) for experimental animal maintenance. Animals were maintained according to the rules and regulations of the Experimental Animal Research Ethics Committee of Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria (Ethical approval number: ABUAD/ACA/121).

Animal distribution

Animals were randomly divided into five groups as follows: Group I Rats that received vehicles alone (served as control), Group II Rats that received arsenic as sodium arsenite in drinking water at a concentration of 100 ppm. Group III Rats that were treated with arsenic along with ascorbic acid (200 mg/kg body wt. dissolved in water) given by oral gavage once a day. Group IV Rats that were given arsenic along with aqueous extract of *C. papaya* roots (100 mg/kg bw) by oral gavage once a day. Group V Rats that were administered arsenic along with aqueous extract of *C. papaya* roots (150 mg/kg bw) by oral gavage once a day for 21 days.

Isolation of blood and organs

At the end of the experimental period, animals were euthanized by diethyl ether and blood and organ samples were collected. The whole blood of each animal was collected via cardiac puncture and immediately preserved in a refrigerator until further processing. The blood samples were centrifuged at 3000 rpm for 15 min and serum from each blood sample was separated and preserved at -30 °C for further analysis. The kidney was collected from each animal, washed with normal saline, wiped with filter paper, weighed and preserved at -30 °C until subsequent analysis.

Estimation of Plasma 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Blood Plasma samples from each group were diluted twice and filtered through 20 µm filters and subsequently filtered sample was used for 8-OHdG analysis. The 8-OHdG analysis was performed using the NWLSTM 8-OHdG ELISA kit (Northwest Life Science Specialties, LLC, Vancouver, WA). The protein content in each sample was estimated using DC protein Assay kit. The result was expressed as 8-OHdG (ng/mg of protein).

Estimation of Adenosine deaminase

ADA activity determination was performed as method described previously (21) which is based on the direct measurement of the formation of ammonia, produced when adenosine deaminase acts in excess of adenosine. 50 µL of kidney homogenates reacted with 21 mmol/L of adenosine, pH 6.5, and was incubated at 37 °C for 60 min. The protein content used for the platelet experiment was adjusted to between 0.7 and 0.9 mg/mL. Results were expressed in units per liter (U/L). One unit (1 U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

Assay for Serum C-reactive Protein

Serum C-reactive protein (CRP) was measured according to the method described previously (22) by ELISA technique using kits purchased from DIA MED (Belgium).

Quantification of Nitric Oxide (NO)

Nitric Oxide content in kidney homogenates was estimated in a medium containing 400 mL of 2% vanadium chloride (VCl_3) in 5% HCl, 200 mL of 0.1% N-(l-naphthyl) ethylenediaminedihydrochloride, and 200 mL of 2% sulfanilamide (in 5% HCl). After incubating at 37 °C for 60 minutes, nitrite levels, which correspond to an estimative of levels of NO, was determined spectrophotometrically at 540 nm, based on the reduction of nitrate to nitrite by VCl_3 using method described previously (23). Kidney nitrite and nitrate levels were expressed as nanomoles of NO per milligram of protein.

Assay of Serum Inducible Nitric Oxide Synthase (iNOS) enzymatic activity

The activity of iNOS in the serum was measured by an iNOS activity assay kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology) using a fluorescence microplate reader with excitation/emission wavelength of 495/515 nm.

Determination of Pro-Antioxidant Marker

The non-antioxidant markers such as malonaldehyde (MDA) was measured via adopting the method described previously (24).

Estimation of arsenic

Tissue/blood samples were digested according to the method described previously (25). To 100 mg of tissues/1 ml of blood, 1 ml of concentrated nitric acid was added, followed by 1ml of perchloric acid. The sample was then digested over a sand bath until the solution turned yellow in color. If the color of the digest was brown, more nitric acid and perchloric acid were added and the oxidation was repeated. The digest was made up to known volume with deionized water. Aliquots of this were used to estimate arsenic by using the atomic absorption spectrophotometer. The concentration of arsenic was expressed as µg/ dl blood or µg/ g tissue.

Data analysis

Data are presented as the mean ± SEM (n= 7). Data were analyzed by using a statistical software package (SPSS for Windows, version 20.0, IBM Corporation, NY, USA) using One-Way ANOVA, Duncan multiple range *post-hoc* test (DMRT). Values were considered significantly different at $p < 0.05$.

RESULTS

The result for the marker of DNA in serum are presented in Fig 1. The 8-OHdG level was significantly ($p <$

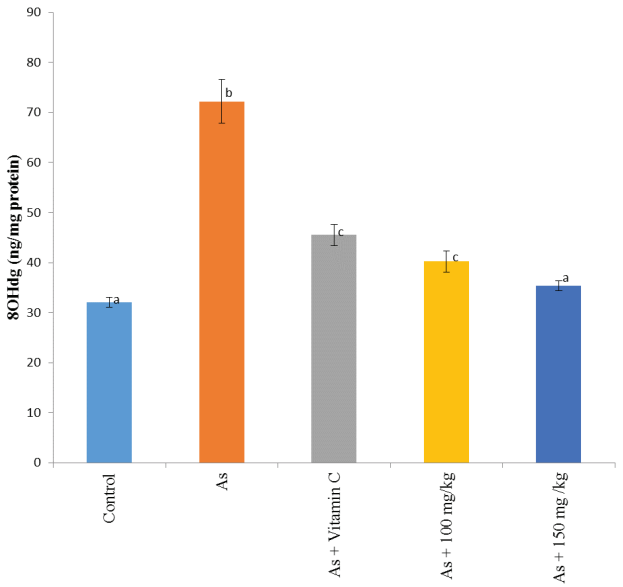

Figure 1: Effect of aqueous extract of *C. papaya* roots and arsenic in the levels of serum 8-OHdG level (ng/mg protein) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p < 0.05 (DMRT), As; Arsenic.

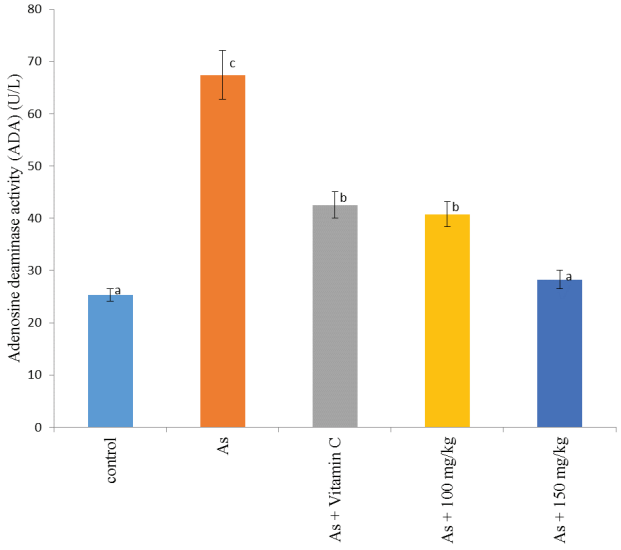

Figure 2: *C. papaya* root inhibited Adenosine deaminase (ADA) enzymatic activity (U/L) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p < 0.05 (DMRT), As; Arsenic.

0.05) increased in arsenic treated rats compared to normal control rats. However, treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight significantly decreased the level of 8-OHdG in serum compared with arsenic untreated rats. Vitamin C administration significantly decreased the levels of 8-OHdG ($p < 0.05$) (Fig.1). These effects were found to be pronounced in the arsenic + 150 mg/kg body weight *C. papaya* root aqueous extract.

The data for adenosine deaminase (ADA) in serum are presented in Fig 2. As seen from the (Figure 2), ADA activity was significantly higher in arsenic only treated rats ($p < 0.05$) compared to normal control rats. Treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight all exerted significant decrease inhibitions ($p < 0.05$) on the ADA activity compared with arsenic untreated rats. Vitamin C administration significantly decrease ADA activity ($p < 0.05$).

The data for serum C-reactive protein (CRP) are presented in Fig 3. The serum C-reactive protein concentration was significantly higher in arsenic untreated rats compared to normal control rats ($p < 0.05$). Treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight significantly decreased ($p < 0.05$) the concentration of serum C-reactive protein compared with arsenic untreated rats. Vitamin C administration significantly decreased concentration of serum C-reactive protein ($p < 0.05$) (Fig.3).

The levels of nitric oxide (NO) as a measure of enhanced oxidative stress has been measured and presented in Fig 4. Rat exposed to arsenic exhibited significant decrease in nitric oxide levels compared to normal control ($p < 0.05$). Simultaneous treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C significantly increases the nitric oxide levels ($p < 0.05$) in serum compared to those treated with arsenic alone (Fig. 4).

Furthermore, the inhibitory effect of aqueous extract of *C. papaya* root on the activity of iNOS enzyme was examined. As shown in Fig. 5, arsenic untreated rats caused a significant ($p < 0.05$) increment of iNOS enzymatic activity compared to normal control. Administration of aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C strongly inhibited ($p < 0.05$) the iNOS enzymatic activation in arsenic treated rats compared to arsenic alone.

As shown in Figure 6, compared with the control group, arsenic significantly increased ($p < 0.05$) the levels of MDA in arsenic untreated rats compared to normal control. When aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C was administered to arsenic, the levels of MDA were significantly reduced ($p < 0.05$) compared to arsenic untreated rats.

The arsenic concentrations in blood and kidney tissue are present in Figure 7. Results showed that arsenic accumulated preferentially in the kidney and serum. Arsenic concentrations in the blood and kidney tissues were significantly higher ($p < 0.05$) in arsenic untreated rats compared to normal control rats. Administration with 100 mg/kg body weight of *C. papaya* root aqueous extract and 150 mg/kg body weight of *C. papaya* root aqueous extract groups and vitamin C significantly ($p < 0.05$) decreased the concentrations.

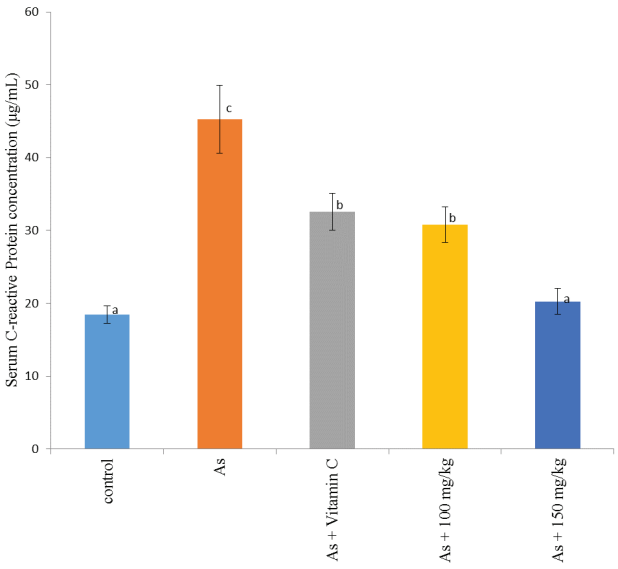

Figure 3: *C. papaya* root effect on Serum C-reactive protein concentration (µg/mL) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (ace) differ significantly at p < 0.05 (DMRT), As; Arsenic.

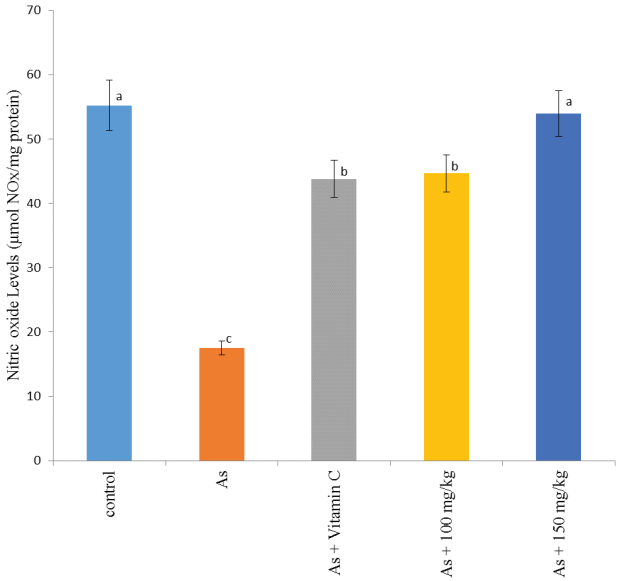

Figure 4: *C. papaya* root effect on the levels of nitric oxide (NO) (µmol NOx/mg protein) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p < 0.05 (DMRT), As; Arsenic.

DISCUSSION:

At the moment, therapeutic options to prevent or manage acute renal injury associated with heavy metal poisoning are tremendously restricted, hence, the look for novel therapeutic interventions is an area of intense investigation. 8-OHdG, may be a novel biomarker for arsenic-induced renal inflammation and oxidative stress. It was reported that elevated levels of 8-OHdG in plasma will cause DNA damage in arsenic exposed rats (26). In this study, levels of 8-OHdG in plasma from arsenic only treated rats were significantly increased compared to control rats, which is similar to the reports of (26). Increased levels of 8-OHdG could also be as a result of oxidative stress induced by the arsenic (27). Treatment with *C. papaya* root aqueous extract at 150 and 100 mg/kg body weight respectively, or vitamin C, to arsenic-induced rats considerably reduced the 8-OHdG levels in rat plasma compared to arsenic only rats which might be due to the antioxidant activities of *C. papaya* root and its ability to ameliorate oxidative stress induced by arsenic as well as present phytochemical components such as phenolic, flavonoids and saponins (28).

Of recent, studies involve the endogenous signal molecule adenosine in renal function or protection. Thus, enzymatic production of nucleoside adenosine from its precursor molecules adenosine triphosphate and adenosine monophosphate, and regulation of its level by ADA play a vital role in decreasing renal damage and conserving the functionality of the kidney during incidents of renal heavy metal poisoning (15, 16). The results of this study revealed that ADA activity was considerably increased in the kidney of arsenic only treated rats compared with the control ($p < 0.05$). Earlier researches have confirmed upregulation in the ADA activity in renal injury (29, 30, 31). Hence, increase ADA activity found in this study might lead to a reduction in the level of adenosine, a renoprotector molecule (15,16). Numerous studies have linked the levels of adenosine in hypoxia, inflammation, or acute renal injury (15, 32). Treatment with aqueous *C. papaya* roots was able to prevent an increase in ADA activity in arsenic-treated rats (Figure 2). This implies that aqueous *C. papaya* roots

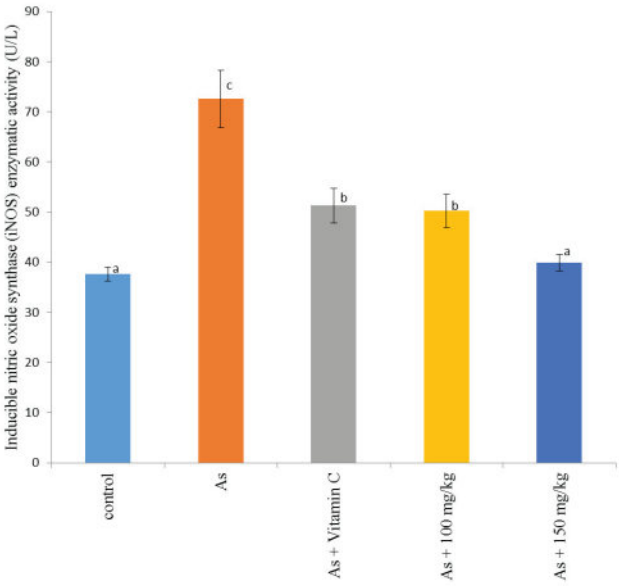

Figure 5: *C. papaya* root inhibited inducible nitric oxide synthase (iNOS) enzymatic activity (U/L) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p < 0.05 (DMRT), As; Arsenic.

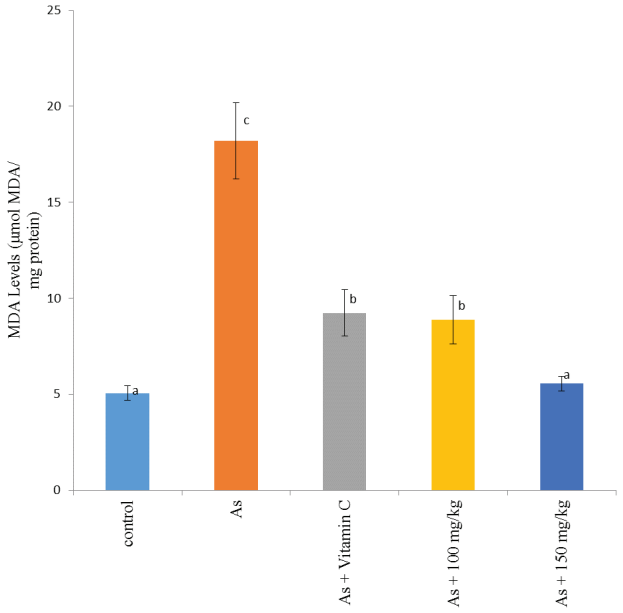

Figure 6: *C. papaya* root effect MDA levels (µmol MDA/protein) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at p < 0.05 (DMRT), As; Arsenic.

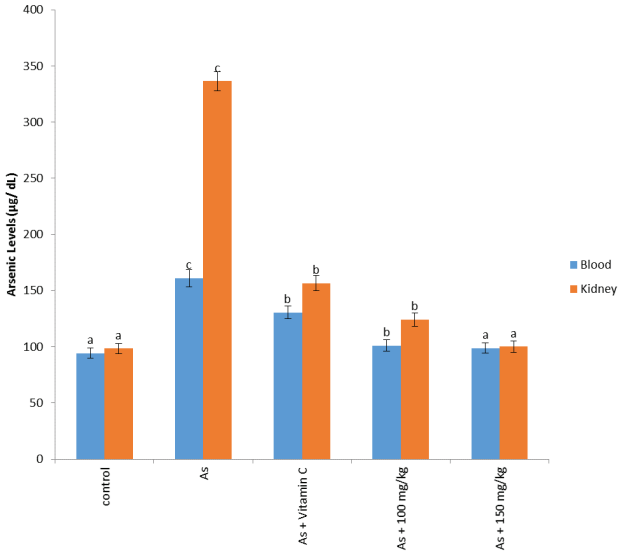

Figure 7: *C. papaya* root effect Arsenic levels (µg/ dL) of control and experimental rats. Values are mean ± SEM for 7 rats in each group. Values not sharing a common superscript letter (a–e) differ significantly at p < 0.05 (DMRT), As; Arsenic.

has a protective role against arsenic poisoning, and also the probable mechanism may well be because of their inhibitory effect on renal ADA activity, thereby leading to an increase in the level of adenosine.

Cytokines synthetized by neutrophils and macrophages stimulate the production of acute-phase proteins, like C-reactive protein (CRP). CRP is synthesized by the hepatocytes. CRP levels in serum increase during infection and inflammation (33). C-reactive protein is a marker, which appears during the late phase of infection (34, 35). In the present study rats chronically exposed to a wide range of arsenic concentrations through drinking water, arsenic exposure was associated with increased serum CRP levels in arsenic-treated rats compared with the control ($p < 0.05$) (Figure 3). Arsenic exposure has been proven to cause inflammation; this may be a mechanism for arsenic-induced diseases, as inflammation is involved in the pathogenesis of many chronic diseases, including cardiovascular disease, metabolic syndrome (36), chronic kidney disease (37), and cancer (38). Chronic exposure of rats to 100 ppm sodium arsenite in drinking water resulted in an increase in the acute-phase protein C-reactive protein (CRP) in the kidney (39).

In this present study, it revealed a significant decrease in the levels of nitric oxide (NO) in arsenic-induced renal injury (Figure 4). Though, treatment with aqueous *C. papaya* roots, restores the level of NO in arsenic treated rats. This increase in nitric oxide could be a result of the fact that *C. papaya* roots exhibited an inhibitory effect on the activity (40). However, an increasing evidence that nitric oxide, a potent vasodilator, is one amongst the foremost crucial paracrine modulators and mediators in the regulation of renal functions, for examples total and regional renal blood flow, renal autoregulation, glomerular filtration rate, renin secretion, and salt excretion (41). Nitric oxide also plays a vital role in the pathogenesis of several renal syndromes, for instance diabetic nephropathy, inflammatory glomerular disorders, acute renal failure, and nephrotoxicity of drugs or heavy metals, assigning both beneficial effects through its hemodynamic functions (41).

Aqueous C. papaya roots inhibits arsenic induced expression of inflammation markers inducible nitric oxide synthase (iNOS) activity. In this study, arsenic untreated rats caused a significant ($p < 0.05$) increment of iNOS enzymatic activity compared to normal control (Figure 5). Though, treatment with aqueous extract of *C. papaya* root at 100 mg/kg body weight and 150 mg/kg body weight and vitamin C strongly inhibited ($p < 0.05$) the iNOS enzymatic activation in arsenic treated rats compared to arsenic alone. This result is in agreement with Figure 4, where we observed a decrease in the levels of nitric oxide in arsenic treated rats (42).

The mechanism of arsenic-induced oxidative stress involves an imbalance between generation and removal of reactive oxygen species in tissues and cellular elements, inflicting harm to membranes, DNA, and proteins (43). Reactive oxygen species generated by arsenic initiate lipid peroxidation of the membrane-bound polyunsaturated fatty acids, resulting in impairment of the membrane structural and functional integrity, an interaction between free radicals of numerous pedigrees and unsaturated fatty acids that are typical in membrane lipids. Degradation of polyunsaturated fatty acids in cell bio-membranes by reactive oxygen species, induced by arsenic, ends up in the destruction of bio-membranes and therefore the formation of thiobarbituric acid reactive species, MDA, or conjugated dienes as indicators of lipid peroxidation (44, 45). In this study, the level of MDA was measured as an indicator of lipid peroxidation (Figure 6). The level of MDA was considerably increased in the kid-

ney tissue of arsenic treated rats. The experimental findings suggest that oxidative stress plays a very crucial role in arsenic-induced renal injuries. Our result is in accordance with other findings (44, 45, 46,47). The increased MDA levels in renal tissue is a sign of over accumulation of lipid peroxides in tissue, causing overconsumption.

Statistics generated revealed that arsenic accumulated preferentially in the kidney tissues. Arsenic concentrations in the kidney tissues (Figure 7) were considerably higher ($p < 0.05$) in arsenic treated rats alone. However, treatment with *C. papaya* root aqueous extract at 150 and 100 mg/kg body weight considerably decrease the levels of arsenic in the tissues. Vitamin C administration also reduced the levels of arsenic in the tissues compared to the arsenic-only exposed rats. These results are in accordance with results reported by (48) which showed that arsenic dispersal in tissues depends on the route of administration and its form.

CONCLUSION

In conclusion, *C. papaya* roots halts arsenic-induced renal inflammation by inhibiting anti-inflammatory biomarkers in the kidney. Hence, these activities could suggest some probable mechanisms of action for its renoprotective activity.

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Declaration of Conflict of Interest

We declare that we have no conflict of interest.

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ANALYSIS OF RISK FACTORS FOR DEVELOPMENT OF COGNITIVE DISORDERS IN MAINTENANCE HEMODIALYSIS PATIENTS – PILOT STUDY

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ANALIZA FAKTORA RIZIKA ZA RAZVOJ KOGNITIVNIH POREMEĆAJA KOD BOLESNIKA NA HEMODIJALIZI - PILOT STUDIJA

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ABSTRACT

Prevalence of cognitive disorders is high in maintenance hemodialysis patients. Montreal cognitive assessment (MoCA) is used for detecting and evaluation of cognitive disorder degree in this patient population. In examined patient population, only 5 (12.5%) of them had normal cognitive function (MoCA ≥26). Mild cognitive impairment (MoCA 18-26) was found in 65.9% (29) patients, while moderate cognitive disorder (MoCA 10-17) was detected in 6 (21.6%) patients. Major cognitive disorder wasn't detected in examined population. Statistically significant correlation was not established between laboratory parameters and overall MoCA score. Statistically significant correlation, however, was established between MoCA item that evaluates space and time orientation and intermediate secondary hyperparathyroidism and space and time orientation and severe secondary hyperparathyroidism. Hemodynamic instability during hemodialysis and silent ischemia of the brain are increasing risk of appearance of cognitive disorders in maintenance hemodialysis patients.

Keywords: *hemodialysis, cognition, MoCA, secondary hyperparathyroidism*

SAŽETAK

Prevalenca kognitivnih premećaja je visoka kod pacijenata koji su na redovnoj hemodijalizi. Od ukupnog broja ispitivanih bolesnika, normalnu funkciju kognicije (MoSA ≥ 26) je imalo svega 5 bolesnika (12,5%). Blagi poremećaj kognicije (MoSA18-26) ima 29 bolesnika (65,9%), dok je 6 bolesnika (21,6%) imalo umereno težak poremećaj kognitivne funkcije (MoSa skor 10-17). Težak kognitivni poremećaj nije otkriven ni kod jednog ispitivanog bolesnika. Ne postoji statistički značajna korelacija između ukupnog MoSA skora i ispitivanih laboratorijskih parametara. Statistički značajna korelacija postoji između dela MoSA skora koji ispituje funkciju orijentacije u prostoru i vremenu i umerenog i teškog sekundarnog hiperparatireoidizma. Hemodinamska nestabilnost u toku hemodijalize i nemi infarkti mozga povećavaju rizik od nastanka poremećaja funkcije kognicije u populaciji bolesnika koji se leče redovnom hemodijalizom.

Ključne reči: *hemodijaliza, kognicija, MoSA skor, sekundarni hiperparatireoidizam*

INTRODUCTION

Cognitive disorders represent a wide variety of disorders; not solely memory disorders. Those are acquired disorders, going from mild cognitive impairment that is barely noticeable and doesn't impact every day activities; to dementia that is not compatible with independent life. Common characteristic for these disorders is clinically significant memory loss. Prevalence of dementia grows with years, from 5-7% until 65 years of life, to over 50% in older than 85 years (1). In general population, older patients have more distinct correlation of cognitive impairment with decreased GFR (*glomerular filtration rate*) (2, 3). Cognitive disturbances are present in all stages of CKD (chronic kidney disease) and are very common in maintenance hemodialysis patients: up to 60% of this population can meet the criteria for some of cognitive disorders (4, 5). MCI prevalence in maintenance hemodialysis patients reach up

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to 20%, and major cognitive disorders prevalence is about 10% (6, 7). Presence of mild cognitive disorder is relative contraindication for initiation of hemodialysis treatment. This comes from the fact that patient has to understand the hemodialysis process itself; and he or she must be able to cooperate with the staff (8). Cognitive disorders diminish quality of life and life expectancy of maintenance hemodialysis patients. In general population, in elder than 75 and with diagnosed Alzheimer disease, life expectancy is about 7.5 years; while life expectancy in maintenance hemodialysis with Alzheimer's is significantly smaller – only 3.3 years (9, 10, 11). This study had multiple goals: aside of determining of prevalence and severity of established cognitive disorder, we wanted to examine whether gender, maintenance hemodialysis duration, anemia, number of intradialytic hypotension and severity of secondary hyperparathyroidism have impact and in what level on cognitive function.

METODOLOGY

The research was conducted in Clinic for Urology, Nephrology and Dialysis; in Center for Nephrology and Dialysis of Clinical center "Kragujevac", with design of cross-sectional, analytic study. Before onset of the research, all necessary permissions were acquired from local Ethic Comity. Total of 40 maintenance hemodialysis patients that are treated in the Center participated voluntary. Inclusion criteria were: age of 18 years and older, maintenance hemodialysis treatment longer than 3 months, and patients without previous diagnosis of MCI or dementia. Exclusion criterion was any diagnosed cognitive disorder.

Montreal cognitive assessment (MoCA)

Montreal cognitive assessment (MoCA) is one of the cognition assessment tools, and its revised version has been recently validated for maintenance hemodialysis patients (12). In this population, most common cognitive disorders are in executive functions, lower speed of information processing, lower speech fluency, as well as short - term memory of verbal and non-verbal data (13, 14). Total score counts 30 points, and it can be obtained by simple addition of max value which can be found next to every item. Lower limit for normal cognition is 26, and every value lower than this one expresses severity of cognitive disorder. Patients with MCI get between 18-26 points; patients with 10-17 point are the ones with medium severity cognitive disorder; and those with less than 10 points have major cognitive disorder. In patients with less than 12 years of education, we add 1 point on the observed score (15).

Diagnostic criteria

Main criterion for diagnosis of dementia was memory disorder (criterion A1) and any of these: aphasia (A2a), apraxia (A2b), agnosia (A2c), executive function disorders (criterion A2d). All represented disorders must be more severe than previously registered cognitive state and cannot be explained by any other psychiatric disease. Along with these symptoms, space and time disorientation, alopersonal and autopersonal disorientation, hallucinations, delusions, disinhibited behavior, cursing, mood and sleep disorders could be presented. Cognitive disorder cannot be diagnosed if listed symptoms represent exclusively during delirium.

Variables

The primary variable was value of total score of Montreal cognitive assessment (MoCA). Independent variable were: gender, maintenance hemodialysis duration of treatment (from 3 months to 5 years, 5-10 years, and over 10 years) and laboratory parameters: anemia (hemoglobin (Hb) level <110 g/l), nutritive status (total serum protein levels and albumins), inflammation markers – CRP (C-reactive protein), quality of dialysis index (Kt/V), impact of secondary hyperparathyroidism (mild – PTH up to 500 ng/l; intermediate – PTH 500-800 ng/l and severe – PTH over 800 ng/l), intradialytic hypotension (every decrease of systolic blood pressure for 20 mmHg and more regarded to systolic pressure at the beginning of hemodialysis) had significant impact on cognition.

Statistics

Data was analyzed in SPSS version 21.0. Laboratory parameters were calculated as median of three measurements for last three months before onset of data gathering. Data were analyzed with descriptive statistics, normality tests; and according to data distribution pattern, we conducted parametric or non-parametric tests. For correlation determination we used Pearson's correlation coefficient, which significance was determined as significant if p value was less than 0.05 ($p<0.05$). Multivariable analysis was preformed to determine which of the risk factors can be used as predictor of cognitive decline.

RESULTS

Our study included 40 maintenance hemodialysis patients, 67.5% were men and 32.5% women, with the average age 59.6±9.2 years. Demographic characteristics are presented in Table 1. Besides of listed main reasons of CKD; there is one patient with endemic nephropathy, one with chronic pyelonephritis, one with rectal carcinoma, and two patients with obstructive nephropathy. Average total MoCA score was 21.75 ±3.5 points. There were no statistically significant differences between genders. MCI (MoCA 18-26) had 29 patients (65.9%); while intermediate severity cogni-

Table 1. Basic demographic data on the participants

Variables		Values
		%, Xsr ± SD
Gender (m, f)		67.5:32.5%
Age (yrs.)		59.65±9.15
Inhabitance	Urban	65.0%
	Rural	35.0%
Years of education	Male	12.63±3.42
	Female	10.85±1.91
	Urban	12.27±3.36
	Rural	11.33±2.50
Duration of HD treatment (months)		74.00±77.05
Primary kidney disease		
Renal polycystic disease		27.5%
Chronic glomerulonephritis		17.5%
Nephroangiosclerosis		37.5%
Diabetic nephropathy		15.0%
Underlying disease		
Diabetes type 2		15.0%
Myocardial infarction		7.5%
COPD*		10.0%
Hepatitis B		7.5%
Hepatitis C		17.5%.
Stroke		2.5%

* Chronic obstructive pulmonary disease

tive disorder (ICI) (MoCA 10-17) had 6 patients (21.6%). We didn't register patients with major cognitive disorder (MoCA ≤10). Patients that had lowest MoCA score in our study had least time spent in education and longest time spent on hemodialysis treatment. Patients didn't connect numbers with letters (57.5%); 37.5% had drawn clock correctly, 27.5% made more than 2 mistakes during deduction of numbers, and only 30% heard letter A in line. Average count of letter S was 6-8. Only 35% of participant finished whole task. 35% of participants made mistake when asked about similarities between two objects. There were no statistically significant differences in laboratory parameters between participants with MCI and ICI (Table 2). There were no statistically significant correlation between laboratory parameters and total MoCA score. Difference in total MoCA score in patients with normal and elevated CRP levels is 1.6 points (22.70:21.04). Total MoCA score doesn't correlate with total PTH levels, while significant correlation is found in those with intermediate SHPTH (Pearson's correlation -0.993, p <0.001) and severe SHPTH (Pearson's correlation -0.887, p <0.005) and part of MoCA that assesses space and time orientation. Multivariable analysis showed that only level of ionized iron in blood can be used as predictive factor for cognitive decline (0.707; p=0.016) with high sensitivity (0.97) and medium specificity (0.60).

DISCUSSION

Participants in this study were predominantly male, of average age of 60 years. Their address was mainly located in town, and they spent around 12 years in education. They have spent 74.0±77.0 months on maintenance hemodialysis treatment. Normal cognitive function (MoCA≥26), according to MoCA, had only 12.5% (5) patients. It is well known fact that maintenance hemodialysis patients have cognitive disorders, and it is commonly under recognized. In different studies, percentage of these disorders goes from 30-87%; and only 3% is recognized (16). This pathology is common in CKD in all stages (17). Around 65% of MCI patients later get diagnosed with dementia, and in about 24% of them dies 11 years after getting diagnosed with dementia (18). Clock drawing test can be very informative when talked about differentiation of dementia (19). Some groups of questions can clearly distinct healthy persons from ones with MCI (20). Our findings correlate with other similar studies (21). Level of CRP in our study did not correlate significantly with global cognitive function unlike previous findings (22-25). Our study showed that elevated PTH levels and severity of secondary hyperparathyroidism statistically significantly correlates with space and time orientation disorders. Influence of elevated PTH levels in primary

Table 2. Influence of risk factors on degree of cognitive disorder

Variable	MCI Xsr+SD	ICI Xsr+SD	P value
Erythrocyte count	3.37±0.50	3.26±0.36	>0.05
Hemglobin (g/l)	102.40±10.25	94.70±8.21	>0.05
Hematocrit	0.319±0.33	0.299±0.24	>0.05
Iron	10.83±3.26	9.07±2.82	>0.05
Ferritine	781.14±317.05	788.06±316.77	>0.05
TSAT	23.34±8.76	29.25±10.80	>0.05
CRP	11.8±15.86	11.06±8.77	>0.05
Blood glucose	6.09±4.45	5.81±1.81	>0.05
Creatinine	850.08±191.05	850.84±181.97	>0.05
Uric acid	366.74±70.3	354.25±98.73	>0.05
Kt/V	0.99±0.22	1.21±0.42	>0.05
Total proteins	62.33±5.40	57.92±11.80	>0.05
Albumin	35.84±4.21	37.63±3.03	>0.05
Ca	2.16±0.20	2.0±0.24	>0.05
PO4	1.66±0.46	1.91±0.40	>0.05
CaxP	3.45±1.19	3.16±1.48	>0.05
iPTH	312.54±353.19	687.30±685.72	<0.01
Hypotension number(3 months)	17.42±7.94	14.0±11.20	>0.05
Average systolic pressure	131.41±15.58	121.70±9.6	>0.05

hyperparathyroidism is documented, when total cognitive function elevation was detected after parathyroidectomy (26, 27). There was one case of dementia as presentation of primary hyperparathyroidism (28). In SHPTH, in maintenance hemodialysis population, growth of global cognitive function was noted after parathyroidectomy (29). In general population, elevated PTH levels can predict decrease of global cognitive function during 5 years, independent from renal function (30).

Cause of these disorders is still unrecognized. Anatomically, in hemodialysis patients' population more frequent is cerebral atrophy, hippocampal atrophy and silent ischemia (31). Taken that predominantly executive and orientation function are affected; it is indicated that risk factors are the same as in vascular diseases (4). Hemodynamic instability during hemodialysis, frequent asymptomatic intradialytic hypotension, especially in elder patients and those with longer treatment period have important role in functionality of sensitive anatomic structures (32-34).

Strengths and limitations

The results of our study should be considered into its limitations such as not enough examined risk factors, and the design of study that doesn't allow patient follow-up. On the other side, the study strengths were use of MoCA score, which is clinically significant, as well as it is highly sensitive for MCI detection. Further study should be longitudinal, have more details in anamnesis, and have more detailed cognition testing.

CONCLUSION

Cognitive disorders are very common in maintenance hemodialysis patient. In spite of that; they are highly under recognized. Being as they are, they represent occult risk factor; they make doctor – patient cooperation difficult, they aggravate education process and adaptation of patient to treatment itself, so it makes them increased mortality factor in this patient population.

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THE PREVALENCE OF PHYSICAL AND PSYCHOLOGICAL ABUSE AND ITS CORRELATION WITH DEPRESSIVE AND ANXIETY SYMPTOMS AMONG STUDENTS

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PREVALENCIJA FIZIČKOG I PSIHIČKOG ZLOSTAVLJANJA I NJENA POVEZANOST SA NASTANKOM DEPRESIVNOSTI I ANKSIOZNOSTI KOD STUDENTSKE POPULACIJE

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ABSTRACT

Abuse in younger populations has been an issue of growing concern globally since youth already face various life situations that can heighten the occurrence of depression and anxiety disorders. The aim of this study was to determine the prevalence of physical and psychological abuse and its correlation with depressive and anxiety symptoms among students.

This research was conducted as an epidemiological study of a sample of 1,940 university students using a standardized questionnaire by the World Health Organization. The survey, in addition to questions related to the abuse of youth, also included the Beck Depression Inventory and Beck Anxiety Inventory.

Based on the results of this study, psychological abuse had a prevalence of 17.1%, while the prevalence of physical abuse was approximately 1.8%. Depressive symptoms were significantly related to physical ($p<0.001$) and psychological abuse ($p<0.005$), and anxiety symptoms were also significantly related to both physical ($p=0.003$) and psychological abuse ($p<0.005$).

The results of this study indicated the importance of the early detection of abuse and depressive and anxiety symptoms among university students, which is essential for mental health promotion and the prevention of mental disorders.

Keywords: *University students, depressive symptoms, anxiety symptoms, physical abuse, psychological abuse*

SAŽETAK

Zlostavljanje kod populacije mladih, postaje sve više sfera interesovanja na globalnom nivou, jer sučeljavanje sa brojnim životnim situacijama koje nosi ovaj period života ubrzava nastanak depresivnih i anksioznih poremećaja. Cilj našeg istraživanja je da se utvrdi prevalencija fizičkog i psihičkog zlostavljanja, i njena povezanost sa nastankom depresivnosti i anksioznosti kod ispitivane studentske populacije.

Istraživanje je sprovedeno kao epidemiološka studija preseka na uzorku od 1940 studenata, korišćenjem standardizovanog upitnika Svetske zdravstvene organizacije koji je pored aspekta vezanog za zlostavljanjene kod mladih, obuhvatao Bekovu skalu za procenu depresivnosti i Bekovu skalu za procenu anksioznosti.

Na ispitivanom uzorku studenata, prevalencija psihičkog zlostavljanja iznosi 17,1% a prevalencija fizičkog zlostavljanja 1,8%. Utvrđeno je da postoji povezanost između nastanka depresivnih simptoma sa fizičkim zlostavljanjem ($p<0,001$) kao i psihičkim zlostavljanjem ($p<0,005$). U pogledu anksioznih simptoma, takodje je nadjena povezanost kako sa fizičkim zlostavljanjem ($p<0,003$) tako i sa psihičkim zlostavljanjem ($p<0,005$).

Dobijeni rezultati ukazuju na značaj ranog prepoznavanja zlostavljanja kao i depresivne odnosno anksiozne simptomatologije kod mladih, u cilju kreiranja programa promocije mentalnog zdravlja i prevencije mentalnih poremećaja.

Ključne reči: *studenti, depresivni simptomi, anksiozni simptomi, fizičko zlostavljanje, psihičko zlostavljanje.*

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INTRODUCTION

From a socio-cultural point of view, an individual is a product of his/her social relationships and social life. An individual is also a member of certain social communities, whose principles and values he/she acquires while also gaining skills to participate in collective interactions. To accept the cultural characteristics of his/her community, an individual moves through a physical and psychological developmental process. The outcomes of all these integrated processes, which are under the influence of one's biological and psychological development, help an individual build his/her own identity and become an autonomous person (1). Mental disorders are one of the most important predictors of the development of disorders related to social health and the exclusion of individuals as functional members of society (2).

Both general and expert public discussion surround the concept of zero tolerance for violence, and special attention is given to the abuse of children, women and adolescents.

The abuse of young people includes the following: all forms of physical and/or emotional abuse, sexual abuse, neglectful or negligent treatment, and commercial or other forms of exploitation. All of these abuses lead to actual or potential harm to one's health. In Serbia, the data on the extent of harassment and abuse are still insufficient. Due to the lack of an integrated database and the difficulty of comparing results from different sectors, precise and complete data are still non-existent (3).

In approximately 80% of cases, the abusers are parents or guardians. The current literature on the topic states that potential risk factors are as follows: poverty, low levels of education and addiction (4). Predictors of psychological and physical abuse and harassment, which increase the risks for mental disorders, cannot be generalized to all cultural environments. In developed countries, the primary risk factors are divorce between parents, relationship with a stepfather or stepmother and parents' addictions. Studies conducted in multicultural environments, especially in North America, often include cultural and ethnic differences as risk factors, but the same cannot apply to predominantly ethnically homogenous countries such as Japan or China (5). The results of abuse and negligence can be divided into early (at an early age) or late (at an adult age) stages.

Mental health problems, which may occur as a consequence of abuse, can differ; they may start as changes in cognitive functioning (retardation, developmental disharmony, intellectual inhibition, difficulty with concentration) and psychological functioning (fear, anxiety, aggressiveness, anger, hostility, guilt, shame, changes in self-perception and self-destructive behaviour) and end up as mental disorders in adulthood such as depression and anxiety disorders, personality disorders, addictions and suicidal behaviour (2, 3, 6).

Depression and anxiety typically have their first onset while an individual is of university age, making mental disorders a salient problem area for university students. From a public health perspective, the early detection of depression and anxiety is essential, especially in young adults, to implement appropriate screening and prevention programmes (6).

The aim of this study was to determine the prevalence of physical and psychological abuse and its correlation with depressive and anxiety symptoms among students.

MATERIALS AND METHODS

Study design and participants

The research we conducted used a cross-sectional survey of students attending the University of Kragujevac, Serbia, during a two-year period (2013-2015). The University of Kragujevac, with its twelve departmental faculties, is a state-owned university in Central Serbia. Six of its faculties are located in Kragujevac, while the other six faculties are located in five towns within Central Serbia, thus covering an area with more than 2,500,000 inhabitants. All twelve departmental faculties were selected for the survey: Faculty of Agronomy, Faculty of Economics, Faculty of Engineering, Faculty of Mechanical and Civil Engineering, Faculty of Medical Sciences, Faculty of Education, Faculty of Law, Faculty of Natural Sciences and Mathematics, Faculty of Technical Sciences, Teachers Training Faculty, Faculty of Philology and Arts, Faculty of Hotel Management and Tourism.

The sample for this cross-sectional survey comprised 1,940 randomly selected students from the total of 18,123 students at the University of Kragujevac. The students were randomly selected from every study year of each departmental faculty in proportion to the faculty's size and in relation to the total number of students at the university. The students were sorted using the university's student database according to a previously generated random order (random computer function).

Procedure: The Beck Depression Inventory (BDI-IA), the Beck Anxiety Inventory (BAI) and a self-administered anonymous questionnaire assessing social life characteristics and demographic and socioeconomic variables were used. Approval for the study was obtained from the Faculty of Medical Sciences Ethics Committee. Participation in the study was completely voluntary with no economic or other motivation. Informed consent was obtained, and confidentiality of the responses was assured. The study was conducted in the participants' own classrooms by the lead researcher. Those who were absent during the distribution of questionnaires were excluded.

Instruments: To determine the variables, a self-assessment questionnaire with detailed subdomain questions was used. The symptoms of depression were evaluated using the BDI-IA scale. This scale was developed in the 1960s and is one of the most widely used instruments for measuring the severity of depression, with a

Table 1. Distribution of physical and psychological abuse in student population

Variables	Total number	(%)
Have you been physically harassed during the last six months?	(N = 1857)	
Yes	34	1.8
No	1792	96.5
I don't remember	31	1.7
Have you been psychologically harassed during the last six months?	(N = 1904)	
Yes	332	17.4
No	1513	79.4
I don't remember	59	3.2

focus on the behavioural and cognitive aspects of depressive disorders (7).

The questionnaire was designed to assess a variety of depressive symptoms that the individual may have experienced over the preceding week. It consisted of 21 items, with each response receiving a score on a scale ranging from 0 to 3. The total score had a minimum of 0 and a maximum of 63. The internal consistency for the BDI-IA was good, with an average alpha coefficient of 0.81 for non-psychiatric samples and with highly inter-correlated items (8). The symptoms of anxiety were evaluated using the BAI scale, a short list that described 21 anxiety symptoms that were experienced over the preceding week. The scale consisted of 21 items with each response receiving a score on a scale ranging from 0 to 3 (9).

Statistical analysis and assessment

The data analysis was carried out using the IBM Statistical Package for the Social Sciences (SPSS) software version 19.0. Data cleaning was performed to detect any missing values, coding error or any illogical data values. The qualitative variables were presented as numbers and percentages. The continuous variables (scores on depression, anxiety and symptoms), were presented as the means and standard deviations (SD). The descriptive statistics for all the variables and the participants' depressive and anxiety symptoms were calculated and appropriately expressed as frequencies, mean values and standard deviations. A chi-squared test was used to find any existing associations between abuse characteristics and anxiety and depressive symptoms. All tests were 2-tailed, and the level of significance was set at $p<0.05$.

RESULTS

Of the 1,968 distributed questionnaires, a total of 1,940 students completed the questionnaires, leading to a response rate of 98.6%. The mean age of the participating students was 21.04 (SD = ± 2.23) years with a range of 18–57 years. The distribution of physical and psychological abuses of the sample is summarized in Table 1.

This study revealed that the prevalence of depressive symptoms among physically abused students was 53.1%, while the prevalence of anxiety symptoms was 47.6%.

The prevalences of depressive and anxiety symptoms in psychologically abused students were 54.8% and 61.2%, respectively.

Depressive symptoms were significantly related to physical ($p<0.001$) and psychological abuse ($p<0.005$). Anxiety symptoms were also significantly related to both physical ($p=0.003$) and psychological abuse ($p<0.005$).

The association between depressive and anxiety symptoms with potential risk factors are summarized in Table 2.

DISCUSSION

The results of this study indicated that the prevalence of psychological abuse among the student population was 17.1%, while the prevalence of physical abuse was 1.8%.

Based on data from the Institute of Public Health of Serbia, approximately 6% of young people aged 15–29 were exposed to physical abuse at some point in their lives. Within this age group, males (8.7%) were more exposed to physical violence compared to females (3.4%). Exposure to physical violence decreased with age and was the highest in the 15-19 age group (8.0%), while it was the lowest in the 20-29 age group (2.9%). At the same time, 11.6% of young people aged 15-29 were exposed to mental abuse. The highest percentage of people who were subjected to psychological violence was found in the 15-19 age group (12.9%) and 20-24 age group (12.5%), while it was significantly lower in the 25-29 age group (8.5%) (2).

The prevalence of physical abuse in developed countries ranges from 4% to 16%, while the prevalence of psychological abuse is approximately 10% (6).

The results of a national epidemiological survey conducted in the United States on a sample of 1,058 young people aged between 14 and 21 show that approximately 51% of females and 43% of males have survived at least one of the three types of abuse (physical, psychological or sexual) (10).

Table 2. Association of depressive and anxiety symptoms with physically and psychologically abuse

Variable	Non	Mild	Moderate & severe	x^2	Df	P
Depressive symptoms						
Have you been physically harassed during the last six months?						
Yes	46.9%	28.1%	25.0%	19.943	4	**<0.001**
No	73.4%	17.8%	8.7%			
Have you been psychologically harassed during last six months?						
Yes	52.5%	26.6%	21.0%	139.130	6	**<0.005**
No	81.8%	12.8%	5.4%			
Anxiety symptoms						
Have you been physically harassed during the last six months?						
Yes	45.2%	25.8%	29.0%	16.323	4	**<0.003**
No	67.6%	22.4%	9.9%			
Have you been psychologically harassed during last six months?						
Yes	38.9%	29.1%	32.1%	198.524	6	**<0.005**
No	32.1%	6.5%	8.3%			

*Bold values show and emphasis the significance of the factors

Al–Fayez and colleagues conducted research on a sample of 4,467 high school students and determined that 18% of young people were abused by a mother, 15% by a father and 18% by a third-party person during the previous six months. For the time period of the past 12 months, the values were 4.3%, 5.8%, and 6.4%, respectively. The values obtained for lifetime experiences of these abuses were 3.4%, 5.3% and 5.8%, respectively. The authors found that child abuse was closely associated with parental divorce, high scores for anxiety/depression, difficulty studying and difficulty establishing social relationships. It was also found that psychological abuse by a mother was the most important predictor of depression, anxiety and low self-esteem (11.5–19.7% of variance) (11).

The present study showed that physical and psychological abuse were significantly associated with depressive and anxiety symptoms. More than one-quarter of the respondents were physically abused (28.1%), and more than one-third of those who were psychologically abused had mild depressive symptoms (26.6%).

This study assessed the association between physical and psychological abuse and anxiety symptoms. A little less than one-third of students who were psychologically abused (25.8%) and about one-third of students (29.1%) who were physically abused manifest mild anxiety symptoms.

Similar to our results, the results of other studies have also shown a correlation between various types of violence and psychological distress, depression (12-15), anxiety (16-20), and depression and anxiety (5, 21-23).

Norman and colleagues indicated that physically abused individuals are at higher risk of depressive disorders (54%) and anxiety disorders (especially panic and post- traumatic stress disorder) (51%) (4).

Some studies have reported that individuals who were physically abused during childhood have a 1.5 higher likelihood of suffering from mental disorders later on in life (24). More precisely, Gal and colleagues indicated that physical abuse in early life stages increases the risk of anxiety disorders in adolescence. This claim was confirmed by a study conducted in Israel with a sample of 4,589 adolescents (19). It is widely understood that physical abuse is almost always associated with other types of violence. Young people are often exposed to different types of violence. Adolescents who have experienced more than one type of harassment (physical, psychological, sexual) more commonly have mental health problems (i.e., depression, anxiety and behaviour disorders) (5). This finding is consistent with the results shown in this study.

The culmination of enduring violence, in addition to consequent mental disorders, often trigger suicide attempts. Skapinakis et al. state that victims of harassment have suicidal thoughts more often, especially if violence happens once a week or more (25). The most essential step in violence prevention is directed toward the general population. Achieving zero tolerance of violence requires breaking taboos about family violence, destigmatizing abused individuals and employing active multidisciplinary processes that coordinate legal and executive activities with the maximum involvement of institutions and individuals.

Healthcare systems have a unique role in these activities and require training on the specific procedures for identifying abused and harassed individuals and helping them access adequate treatment. Depression and anxiety in younger populations can be integral to the development of multiple symptoms and can arise as a consequence of reactivating early childhood traumas, such as forms of violence (6).

The primary limitation of the study was its cross-sectional design, which did not permit inferences about possible causal relations between the explanatory variables and the disorders of interest. Moreover, as the survey was completed anonymously, it was not possible to assess the test–retest reliability of the BDI/BAI in this sample. Another limitation was the self-reported nature of this study. Finally, our sample comprised a group of students from one university in Serbia, which limited the ability to generalize the results to other universities. According to Strategy 2020 by the World Health Organization strengthening mental health promotion programmes is highly relevant (26, 27).

CONCLUSION

These results demonstrate that the high rates of depressive and anxiety symptoms among university students are related to physical and psychological abuse. The past several years have provided data that highlight this neglected public health problem in institutes of higher education.

The importance of early identification, especially of the minor signs of depression, could prevent or reduce its severity and chronicity. From a public health perspective, the onset and development of mental illness in students is a potentially critical area for intervention programmes. A particular challenge is to promote the early diagnosis of depression by initiating community-based intervention programmes and to reduce the stigma of mood disorders. Such efforts hold substantial promise for the development of interventions that may have a positive impact on the health and well-being of university students.

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CONFLICT OF INTEREST

The authors fully declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research described.

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COST-EFFECTIVENESS ANALYSIS OF ETANERCEPT IN COMBINATION WITH METHOTREXATE FOR RHEUMATOID ARTHRITIS - MARKOV MODEL BASED ON DATA FROM SERBIA

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ANALIZA ODNOSA TROŠKOVA I EFEKATA ETARNECEPTA U KOMBINACIJI SA METOTREKSATOM U LEČENJU REUMATOIDNOG ARTRITISA - MARKOVLJEV MODEL BAZIRAN NA PODACIMA IZ SRBIJE

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ABSTRACT

Biological therapeutic strategies have shown positive benefits for chronic and progressive rheumatoid arthritis (RA) in clinical and radiological outcomes. Despite these results, the use of biological drugs in the treatment of RA is limited by high costs. The aim of this study was to compare the cost effectiveness of etanercept in combination with methotrexate and methotrexate alone in patients with RA in the socioeconomic environment of a Balkan country.

We conducted a cost-effectiveness study using a Markov model from a societal perspective with a time horizon of 480 months. The cycle duration was set to one month. The basic transition probabilities and data on therapeutic efficacy were estimated from the available literature, while costs were calculated using the medical documents of patients with RA treated at the Clinical Center Kragujevac.

Our results indicated that treatment of patients with RA using methotrexate alone is more cost effective, with a cost-effectiveness ratio of 1.446.640,78 RSD/QALY, than treatment with a combination of methotrexate and etanercept, with a cost-effectiveness ratio of 5.882.714,57 RSD/QALY.

The use of etanercept to treat RA is not cost effective in the socioeconomic environment of Serbia. The cost-effectiveness ratio of biological drugs would be more favourable if special strategies for the pricing policy of biological drugs were established on the basis of local pharmacoeconomic studies.

Keywords: *rheumatoid arthritis; biological therapy; etanercept, cost-effectiveness analysis*

SAŽETAK

Primena bioloških lekova u lečenju reumatoidnog artritisa doprinosi promeni progresivnog toka ove bolesti kako u kliničkom tako i u radiološkom smislu a uz to popravlja i funkcionalnu sposobnost obolelih. Pa ipak, primena biološke terapije u lečenju reumatoidnog artritisa je ograničena visokim troškovima koji prate upotrebu ovakvih lekova. Cilj ovog istraživanja je bio da se uporede troškovi i efekti kombinacije etanercepta i metotreksata sa primenom metotreksata u lečenju reumatoidnog artritisa u farmakoekonomskim uslovima u Srbiji.

Za potrebe ovog istraživanja konstruisan je Markovljev model u kojem je predstavljena hronična priroda i progresivan tok reumatoidnog artritisa, a bazične, tranzicione verovatnoće i efikasnost etanercepta i metotreksata su procenjene na osnovu podataka iz dostupne literature. Za potrebe ovog istraživanja procenjeni su troškovi lečenja pacijenata obolelih od reumatoidnog artritisa, a na osnovu dostupne dokumentacije iz Kliničkog centra Kragujevac. Istraživanje je sprovedeno sa aspekta društva u celini, a u troškove i ishode je uključena diskontna stopa od 3%. Vremenski horizont je iznosio 40 godina, a jedan ciklus u modelu je trajao 1 mesec.

Ukoliko se sagleda odnos troškova i efekata, lečenje reumatoidnog artritisa metotreksatom je povoljnije nego lečenje iste bolesti kombinacijom metotreksata i etanercepta s' obzirom da vrednost odnosa troškova i efekata za etanercept u kombinaciji sa metotreksatom iznosi 5.882.714,57 RSD/QALY dok vrednost odnosa troškova i efekata za monoterapiju metotreksatom iznosi 1.446.640,78 RSD/QALY.

Odnos troškova i efekata etanercepta u kombinaciji sa metotreksatom ukazuje da je primena biološke terapije u lečenju reumatoidnog artritisa neisplativa u socio-ekonomskim uslovima u Srbiji.

Ključne reči: *reumatoidni artritis; biološka terapija; etarnecept; analiza odnosa troškova i efekata*

ABBREVIATIONS

ACR - American College of Rheumatology
bDMARDs - Biological Disease-modifying antirheumatic drugs
cDMARDS-conventional DMARD-s
DAS28 - Disease Activity Score
DMARDs - Disease-modifying antirheumatic drugs
IL-1 - Interleukin-1
IL-6 - Interleukin-6
ICER - Incremental cost-effectiveness ratio
HAQ - Health Assessment Questionnaire
NICE - National Institute for Clinical Excellence
QALY - Quality-adjusted life year
RA - Rheumatoid arthritis

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INTRODUCTION

Rheumatoid arthritis (RA) is a systematic, autoimmune disease with chronic course that mainly affect joints but also leads to deterioration of multiple organs, decreasing the quality of life of patients causing increased mortality (1). RA affects 0.5-1% of the general population. As is the case with many autoimmune diseases, the aetiology of RA is partly known. The main aetiological factors are heredity, gender, environmental factors and infectious agents (2). RA affects women 3 to 4 times more often than men, with tendency to increase with age (3). Since the onset of RA occurs during the economically productive phase of life, the socioeconomic burden in terms of costs (direct and indirect), reduced work ability, long-term disability and morbidity, is substantial (4, 5). RA has a significant impact on the quality of life of patients and results in a considerable burden for patients. Early diagnosis of RA is crucial for timely introduction of drugs to achieve and maintain remission in patients (2).

In 1978, the American College of Rheumatology (ACR) established criteria to diagnose RA, where 4 of the 7 following criteria must be present: morning stiffness, arthritis in 3 or more joint areas, arthritis of the hand joints (more than 1 joint), symmetrical arthritis, rheumatoid nodules, elevated serum rheumatoid factor and typical radiographic changes (with the exception for the two last criteria, the changes must persist for at least 6 weeks) (6). In 2010, the European League for Rheumatoid Arthritis recommended amendments to the ACR criteria since they lack criteria for early arthritis and for newly diagnosed patients with clinical presentation with synovitis and edema within one joint and patients with synovitis where aetiology is not determinate (7). RA is a clinical entity whose chronic nature and progressive course leads to structural and functional damage of both the affected joints and the surrounding tissue and bones. Due to the variable and progressive nature of RA, different measurement instruments have been developed to estimate disease activity and the disability of patients and to monitor treatment outcomes. The Disease Activity Score (DAS28) is the "gold standard" for estimating disease activity, with values greater than 5.1 for very active disease, from 3.6 to 5.1 for moderate disease activity and less than 3.6 for inactive disease. DAS28 can also been used to assess a patient's response to therapy and achieving a state of remission. The Health Assesment Questionaire (HAQ score) is the dominant technique used to assess the functional inability of patients with RA in terms of inability, pain and discomfort, adverse reactions to drugs and economic sphere of treating RA, with values ranging from zero to three, where zero represents a state without disability and three represents a state of full disability (8, 9).

The new therapeutic concept of RA ("treat to target") is directed to better control disease activity by modifying the dose and course of therapy according to the values of disease activity. Therapeutic strategies used in RA include a wide palette of drugs known as disease-modifying antirheumatic drugs (DMARDs), which can be divided into synthetic DMARDs and biological DMARDs. Synthetic DMARDs include two large groups: conventional DMARDs (cDMARDs) and targeted DMARDs (2). The core of therapy for RA is the cDMARDs i, methotrexate, but the final response of patients can be inadequate due to inefficacy or toxicity (10). Biological DMARDs target parts of the immunopathogenic pathway in the pathogenesis of RA, such as tumour necrosis factor α (TNF-α) (infliximab, etanercept, adalimumab), interleukin-1 (IL-1) (anankira), interleukin-6 (IL-6) (tocilizumab), and costimulatory factors CD 20 (rituximab) (10, 11). Despite proven efficacy, the introduction of biological therapy is limited by its high costs in countries with recent socioeconomic transition and in other countries, with recommendations similar to those given by the National Institute for Clinical Excellence (NICE) from the U.K.: treatment with biologic medicine (mostly with a TNF blocker) is given to a patient whose response to methotrexate is poor and incomplete; if there is no response to the first biologic medicine after 3 to 6 months of treatment, the patient should be switched to another biologic medicine (2).

Cost-effectiveness analyses are crucial to estimate the costs and efficacy of biological drugs, such as etanercept.

The aim of this study was to compare the cost-effectiveness of two therapeutic strategies in patients with RA: treatment with cDMARDs alone or in combination with etanercept, using a Markov model based on efficacy data from published clinical trials and costs sampled from the economic environment in Serbia

MATERIAL AND METHODS

We constructed a Markov model to compare the cost effectiveness of etanercept in combination with MTX and MTX alone in patients with RA. The model was constructed using TreeAge Pro instead® software, version 2006 (12). Therapeutic strategies were compared using a set of scenarios representing a chronic and progressive course of RA. In our model, five health states were presented using HAQ, similar to the study of Kobelt et al. (15). These states represent the chronic nature and variable course of RA: HAQ score less than 0.6, HAQ score from 0.6 to 1.1, HAQ score from 1.1 to 1.6, HAQ score from 1.6 to 2.1 and HAQ scores higher than 2.1. For every state, we included death as a potential state. Disease activity was incorporated into the model by dividing each health state into two sub-states: one with high and another with low activity. In our model, death was the only definitive state, so a virtual cohort of 1000 patient could move from one state to another depending on the natural course of the disease and experiences from clinical trials. For every therapeutic strategy compared in the model, we assigned the initial patient distributions, transitional probabilities, utilities, and effectiveness, which were obtained from the available literature (13, 14). The economic aspects of each therapeutic

strategy were represented by the costs of the health states and were calculated using the records of actual patients treated at the Clinical Center Kragujevac, Serbia. Pharmacoecomic modelling research requires definition of the time aspects in which the compared therapeutic strategies are analysed; in our model, the time horizon was set to 40 years (480 months), with a cycle duration of one month because of the chronic nature of RA. The perspective in our model was societal since direct and indirect costs were included due to their importance in the socioeconomic burden of RA. All costs and outcomes were discounted by 3% annually. For the purposes of modelling, we conducted a pilot study to estimate the costs of RA. Patients were randomly selected from the population of patients with RA treated at the Clinical Center Kragujevac, Serbia, during one year (from June 2009 to June 2010). Patients were offered the opportunity to participate in an interview regarding potential resource items. The patients entered their data anonymously to protect their identities. No patient received financial reward for participating. Using interview techniques, we estimated data about direct costs (costs of medical exams, diagnostic procedures, medicines, and hospitalizations) and indirect cost (e.g., costs of transportation and lost wages). Data on health service utilizations were estimated separately from the medical files of patients for every HAQ state in our model and every disease activity sub-state. The sources for the prices of health services were the databases from the Republic Institute for Health Insurance (RIHI) Tariff Book, and the prices of medicines were obtained from the list of medicines financed by the RIHI issued in 2010 (15). All costs were expressed in Serbian dinars (RSD). We followed the following outcomes: gains in utility for each therapy option expressed as QALYs gained and total and mean costs for every therapeutic option in our model. The modelling process requires a definition of willingness to pay, i.e., how much a society is willing to pay for one quality-adjusted life year (QALY) gained from a specific disease treatment. In our model, we used the recommendation that the value of willingness to pay should be equal to two to three times the gross national income *per* capita. In the case of Serbia, the gross national income *per* capita (GDP/capita) was 563,400 dinars (RSD) (16). We also used the average monthly net income in Serbia to calculate the costs of lost wages.

We performed Monte Carlo simulations using a microsimulation trial, where cohorts of 1,000 virtual patients passed through all hypothetical scenarios. The results of the Monte Carlo simulation were summarized using the incremental cost-effectiveness ratio (ICER) (17, 18). For each therapeutic option, we calculated the mean costs and the mean effects and expressed them as the cost-effectiveness ratio. To determine the robustness of the results of our model, we performed two-way sensitivity analysis.

RESULTS:

The results of the pilot study indicate that the cost of treating patients with cDMARDs (MTX) for one year was on average 261,945.42 RSD or 2,113.26 Euro, and the total cost for treating patients with etanercept in combination with MTX was on average 1 509 533,002 RSD (12,178.26 Euro) (Figure 1).

We used the cost-effectiveness calculation method to compare both RA therapeutic strategies in terms of the total costs and QALY. The results of this analysis show that cDMARDs (MTX) require much less investment than the biological drug etanercept in combination with MTX. The total gain expressed in QALY was higher in the group with etanercept in combination with MTX.

The total cost for one statistical patient treated with MTX for the total time horizon of 40 years was 7,788,768.97 RSD, and the total cost for one statistical patient treated with etanercept in combination with MTX for the total time horizon of 40 years was 34,870,470.29 RSD. Total gain expressed in QALY in the same setting was 5.93

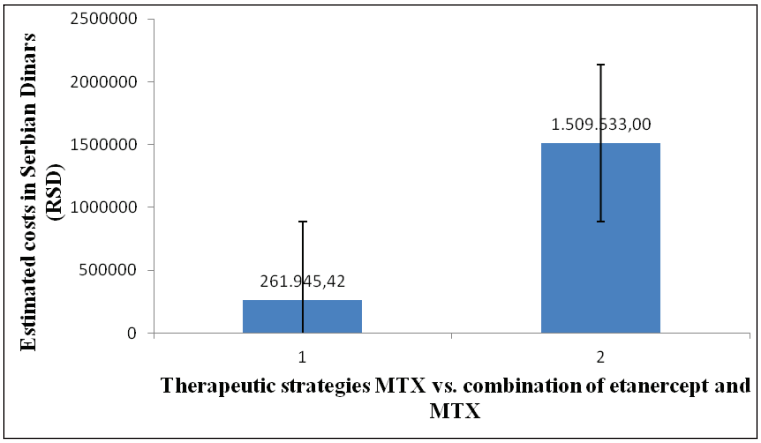

Figure 1. Total costs for one year of treatment (2009–2010) *per* patient for MTX monotherapy and a combination of etanercept and methotrexate

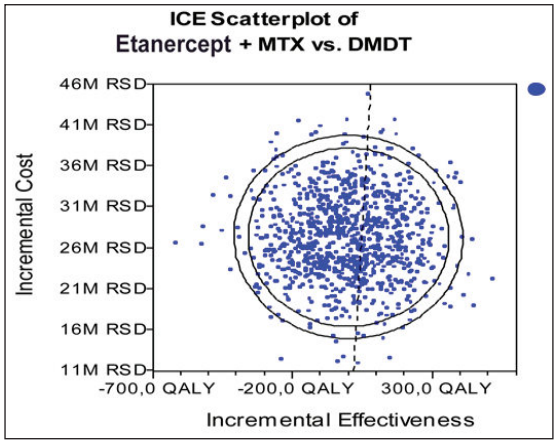

Figure 2. Distributions of the incremental cost-effectiveness ratio (ICER) calculated using Monte Carlo simulations for the total costs *per* quality-adjusted life years (QALY) for etanercept in combination with methotrexate and MTX monotherapy

Table 1. Distribution of the incremental cost-effectiveness ratios (ICERs) calculated using Monte Carlo simulations (using a cohort of 1,000 virtual patients) for the total costs per QALY shown in Figure 2.

Therapeutic strategy	Costs (RSD)	Difference in costs	Effectiveness	Difference in effectiveness	Cost/effectiveness ratio C/E	Incremental cost-effectiveness ratio Incr C/E (ICER)
cDMARD-d (MTX)	7.788.768,97 RSD		5.38 QALY		1.446.640, 78 RSD/ QALY	
Etanercept + MTX	3.487.470, 29 RSD	27.081.701, 32 RSD	5.93 QALY	0.54 QALY	5.882.714, 57 RSD/ QALY	49.821.232, 70 RSD/ QALY

QALY when the patient was treated with etanercept and MTX, while for monotherapy with MTX, the total effectiveness was 5.38 QALY.

The results of the cost-effectiveness analysis are shown in Table 1.

For etanercept in combination with MTX, the calculated ICER (with only methotrexate as the baseline comparator) for the majority of virtual patients was on the left side of the willingness-to-pay line, which indicates that this type of biological therapy for RA in the socioeconomic environment of Serbia is not cost effective.

The results of the sensitivity analysis indicate that variables from the model related to the severe form of RA with HAQ state greater than 2.1 are present more than other variables. As they change, the value of the net monetary benefit becomes negative, within the range of -7.3 to -2.8 million Serbian dinars, which makes our conclusion susceptible to changes in costs and treatment effects of etanercept in patients with more severe forms of R.

DISCUSSION:

The benefits of biological DMARDs in patients with RA have been proven in numerous clinical studies (19-22). Due to the chronic and progressive course of RA, lifelong treatment with biological drugs is associated with increased burden of RA, not only in countries in the Balkan region but also in countries with stable economies. NICE recommends prescribing these drugs only in cases where therapy with cDMARDs has failed, with continuous monitoring due to the potential side effects of these drugs. The EULAR recommendations are based on the new concept of treating RA, where achieving remission should be followed by a reduction in dose of bDMARDs (2, 23). The new goal in treating RA is discontinuation of bDMARDs in remission, which should result in decreased side effects and costs of treating RA (1).

The efficacy of etanercept in combination with MTX was proven in recent literature, where etanercept demonstrated beneficial effects, such as radiological and clinical responses (20, 21, 24).Our results indicate that the total cost of treating RA with etanercept in combination with MTX is on average 1.509.533, 002 RSD (12.178, 26 Euro), which is lower than the results from the USA (25-27). Due to the high costs of bDMARDs, the use of etanercept or similar drugs within the Serbian health system is not part of regular clinical practice, and it is limited by restrictive guidelines, which are common in the health systems of European countries (28).

The results of our model indicate that etanercept in combination with MTX compared to monotherapy with MTX is not a cost-effective therapeutic strategy, despite the higher gain since the costs of etanercept plus MTX are higher. In similar studies in countries with higher thresholds, etanercept was also not a cost-effective therapeutic strategy for most scenarios in patients with RA. TNF inhibitors have favourable cost-effectiveness ratios when the threshold is from 50 000 to 100 000 €/QALY, and if the threshold is 35000 €/QALY, rituximab is the most cost-effective alternative among biologics in the patients with an insufficient response to TNF inhibitors (13, 28)

In the socioeconomic sphere of Serbia, the gain of etanercept in patients with RA is not related to cost savings. The main reason for this outcome is that the prices of medicine are regulated by pharmaceutical companies and are similar in Serbia and in developed European countries, but the prices of health care services are 10-100 times lower in Serbia country than in developed European countries. This duality in the process of price allocation makes the cost-effectiveness of biological drugs unfavourable for the health systems in the Balkan countries, which are undergoing socioeconomic transition.

In the circumstances of the decreasing the price of etanercept, we would expect that etanercept would reach the point of an advantageous cost-effectiveness ratio.

CONCLUSIONS

To make the cost-effectiveness ratio of biological drugs such as etanercept more favourable international pharmaceutical companies need to create special pricing strategies for these drugs based on local pharmacoeconomic studies. Further surveys are needed to identify the portion of the population of patients with RA where etanercept could be an effective therapeutic strategy with cost savings.

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MECHANISM AND CLINICAL IMPORTANCE OF RESPIRATORY FAILURE INDUCED BY ANTICHOLINESTERASES

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MEHANIZAM I KLINIČKA VAŽNOST RESPIRATORNE INSUFICIJENCIJE IZAZVANE ANTIHOLINESTERAZAMA

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ABSTRACT

Respiratory failure is the predominant cause of death in humans and animals poisoned with anticholinesterases. Organophosphorus and carbamate anticholinesterases inhibit acetylcholinesterase irreversibly and reversibly, respectively. Some of them contain a quaternary atom that makes them lipophobic, limiting their action at the periphery, i.e. outside the central nervous system. They impair respiratory function primarily by inducing a desensitization block of nicotinic receptors in the neuromuscular synapse. Lipophilic anticholinesterases inhibit the acetylcholinesterase both in the brain and in other tissues, including respiratory muscles. Their doses needed for cessation of central respiratory drive are significantly less than doses needed for paralysis of the neuromuscular transmission. Antagonist of muscarinic receptors atropine blocks both the central and peripheral muscarinic receptors and effectively antagonizes the central respiratory depression produced by anticholinesterases. To manage the peripheral nicotinic receptor hyperstimulation phenomena, oximes as acetylcholinesterase reactivators are used. Addition of diazepam is useful for treatment of seizures, since they are cholinergic only in their initial phase and can contribute to the occurrence of central respiratory depression. Possible involvement of central nicotinic receptors as well as the other neurotransmitter systems – glutamatergic, opioidergic – necessitates further research of additional antidotes.

Keywords: *Anticholinesterase, Acetylcholinesterase, Acetylcholine, Atropine, Oxime, Diazepam, Respiratory depression, Muscarinic receptors, Nicotinic receptors*

SAŽETAK

Respiratorna insuficijencija je dominantan uzrok smrti kod ljudi i životinja trovanih antiholinesterazama. Organofosphorne i karbamatske antiholinesteraze inhibišu acetilholinesterazu ireverzibilno, odnosno reverzibilno. Neke od njih sadrže kvaternerni atom koji ih čini lipofobnim, čime im ograničava delovanje na periferiju, tj. van centralnog nervnog sistema. One oštećuju respiratornu funkciju primarno izazivajući desenzitizujući blok nikotinskih receptora u neuromuskularnoj sinapsi. Lipofilne antiholinesteraze inhibišu acetilholinesterazu i u mozgu i u drugim tkivima, uključujući i respiratorne mišiće. Njihove doze neophodne za prekidanje centralnog respiratornog generatora impulsa su značajno niže od doza potrebnih za paralizu neuromuskularne transmisije. Antagonist muskarinskih receptora atropin blokira i centralne i periferne muskarinske receptore i efektiveno antagonizuje centralnu respiratornu depresiju izazvanu antiholinesterazama. U cilju kupiranjahiperstimulacije perifernih nikotinskih receptora koriste se oksimi, kao reaktivatori acetilholinesteraze. Dodavanje diazepama je korisno u tretmanu konvulzija, pošto su one holinergičke samo u svojoj početnoj fazi i mogu da doprinesu pojavi respiratorne depresije. Moguća umešanost centralnih nikotinskih receptora, kao i drugih neurotransmiterskih sistema – glutamatergičkog i opioidergičkog – zahteva dalje istraživanje dodatnih antidota.

Ključne reči: *Antiholinesteraze, Acetilholinesteraza, Acetilholin, Atropin, Oksim, Diazepam, Respiratorna depresija, Muskarinski receptori, Nikotinski receptori*

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ACETYLCHOLINE, ACETYLCHOLINESTERASE AND THEIR PHYSIOLOGICAL FUNCTIONS

Acetylcholine is a neurotransmitter of vital importance in the central nervous system (CNS), but also peripherally, i.e. in the vegetative nervous system (VNS) ganglia and at the endings of the postganglionic parasympathetic fibers, such as heart, smooth muscle cells and exocrine glands (1). A specifically important role of acetylcholine is to mediate transmission at the neuromuscular junction of skeletal muscles (2).

When the action potential reaches the motoneuron ending, it opens the voltage-dependent calcium channels and causes the influx of calcium ions into the nerve ending. As a result, acetylcholine vesicles fuse with the presynaptic membrane and the neurotransmitter is released into the synaptic cleft by exocytosis. After reaching the postsynaptic membrane, acetylcholine binds to nicotinic receptors and opens the sodium channels. The ensuing influx of sodium cations into the skeletal muscle cell triggers the action potential that reaches the myofibrils and causes a muscle contraction (3). All these details are shown in Figure 1.

Acetylcholinesterase (AChE) is an enzyme located in cholinergic synapses within the synaptic cleft. It is very active, which means that it breaks down the molecules of acetylcholine into choline and acetate in split-second assuring thus that there is no surplus of acetylcholine to induce the overstimulation of the cholinergic receptors located at the postsynaptic membrane (2).

While in the CNS the types of cholinergic receptors or cholinoceptors through which acetylcholine exerts its action are believed to be both muscarinic and nicotinic, this division is much simpler at the periphery – muscarinic receptors are located at the endings of postganglionic parasympathetic fibers, while nicotinic ones are located in the both sympathetic and parasympathetic ganglia and at the neuromuscular junction (1, 2).

ANTICHOLINESTERASES AND THEIR MODE OF ACTION

Acetylcholinesterase (AChE) inhibitors or anticholinesterases comprise various chemical entities whose com-

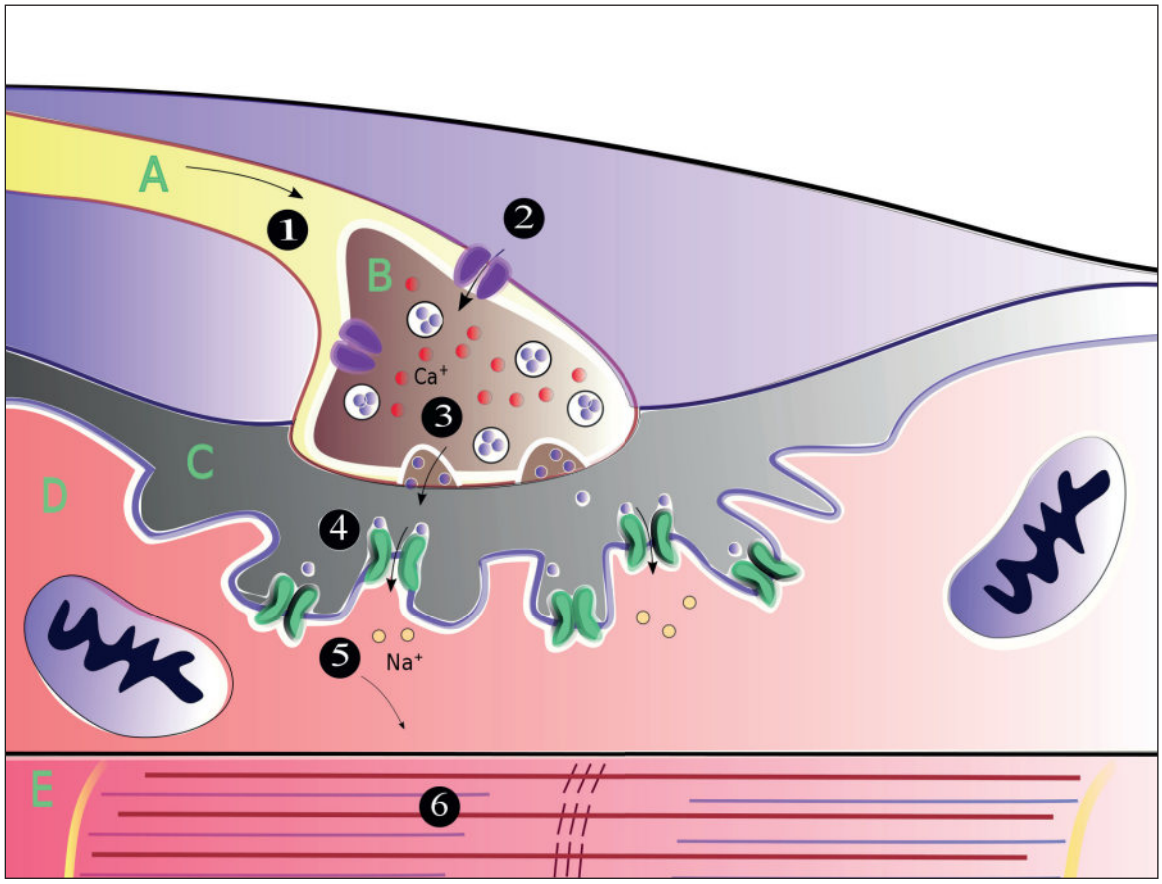

Figure 1. Schematic presentation of neuromuscular junction. (1) Nerve impulse reaches the nerve ending. (2) It opens the voltage calcium channels triggering a calcium ion influx. (3) Vesicles with acetylcholine fuse with the presynaptic membrane and the transmitter is excreted into the synaptic cleft. (4) Acetylcholine binds to the postsynaptic nicotinic receptors and opens the sodium ion channels. (5) Sodium ions enter the muscle fiber. (6) Sodium ion influx triggers the action potential in the myofibrils and causes muscle contraction. A = motor neuron axon, B = axon terminal, C = synaptic cleft, D = muscle cell, E = myofibril.

mon characteristics is ability to inhibit AChE, an enzyme crucial for the breakdown of acetylcholine. They can be divided into irreversible and reversible inhibitors, with organophosphates belonging to the former group and carbamates belonging to the latter one. Some of them have a role in medicine as therapeutic agents and are used (i) in treatment of poisonings with anticholinergic drugs - physostigmine, (ii) Alzheimer's disease - rivastigmine, and donepezil, (iii) glaucoma - phospholine (echothiophate), (iv) for antagonizing competitive neuromuscular blockade after the end of the operations - neostigmine and (v) for treatment of myasthenia gravis and (vi) in prophylaxis of the intoxications with nerve agents – pyridostigmine (4-6), while the others are being used as insecticides – parathion, paraoxon, malathion, dichlorvos, carbaryl, carbofuran (7, 8). A special group of organophosphorus anticholinesterases are nerve agents – tabun, sarin, soman and O-ethyl S-[2-(diisopropylamino)ethyl] methylphosphonothioate (VX) – that have a potential military use as chemical weapons of mass destruction (2, 9).

The physiological role of AChE is to terminate action of acetylcholine, in order to prevent the overstimulation of the cholinoceptors. As a consequence, the inhibition of AChE leads to this overstimulation and various phenomena, signs and symptoms occur as a result of this action (10, 11). Clinical picture of anticholinesterase poisoning depends on the route of exposure (12) and on the quantity of the anticholinesterase in the organism, but includes miosis, bronchoconstriction, hypersalivation, bronchorrhoea, skeletal muscle fasciculations, bradycardia, hypotension, seizures and respiratory failure, the latter being the main cause of death (9, 13).

The topic of this mini-review is to elaborate on the mechanism of the anticholinesterase-induced respiratory failure.

PERIPHERAL VERSUS CENTRAL ANTICHOLINESTERASES

Ever since the organophosphorus nerve agents became known to mankind in the 1940s, a considerable body of literature was published on the clinical picture of intoxications induced by organophosphorus compounds (OPCs) and specifically nerve agents. Cause of death in most of the animal species investigated was respiratory failure (14), although is many publications a considerable attention was drawn to the cardiovascular collapse as an independent factor contributing to a lethal outcome (15).

Anticholinesterases impair the respiratory function in mammals depending on their ability to pass the haematoencephalic or blood-brain barrier (BBB). The ones that contain a quaternary N-atom in their molecules are strongly ionized and hence hydrated and as such cannot pass the BBB, exerting their AChE-inhibiting effect only in the periphery, i.e. outside the CNS. Among the OPCs such examples are phospholine (echothiophate) iodide (16), while the most famous peripherally-acting carbamates are neostigmine and pyridostigmine, the latter being also used in prophylaxis against nerve agents (17).

The remaining anticholinesterases are, more or less, lipophilic and readily pass the BBB inhibiting thus the brain AChE, as the most important target. Best examples of such molecules are nerve agents tabun, sarin, soman and VX, insecticides dichlorvos (DDVP) and paraoxon (metabolite of parathion) and the oldest known anticholinesterase carbamate, physostigmine, which had become a model-substance in pharmacological research (15, 18). Soman is so highly lipophilic, that it reaches the brain circulation just 1 min after IV injection and completely distributes throughout the brain tissue in only 3 min (19).

PERIPHERAL COMPONENT OF ANTICHOLINESTERASE-INDUCED RESPIRATORY DEPRESSION

Exclusively peripherally acting anticholinesterases inhibit AChE in peripheral tissues, including bronchi and neuromuscular synapse. The pulmonary consequences include failure of AChE to destruct acetylcholine, leading to overstimulation of muscarinic receptors in smooth muscles of bronchi and in bronchial exocrine glands, leading to bronchoconstriction and bronchorrhoea (20).

Although this pulmonary muscarinic syndrome compromises the alveolar gas exchange and lead to hypoxaemia, the results of significant inhibition of AChE in diaphragm and intercostal muscles is considered more serious and more important for survival, although it depends on the animal species studied (14). Anticholinesterases usually in the beginning induce a slight increase in contractions of diaphragm, but longer-lasting surplus of acetylcholine in the vicinity of nicotinic receptors eventually lead to a Wedensky-type of depolarization block (21). The final outcome is a flaccid paralysis of respiratory muscles and death due to an asphyxia (22). In such cases, peripheral respiratory paralysis usually occurs after the animal was pretreated with atropine, while normal phrenic nerve discharges still can be recorded (21, 23).

NAUROANATOMY OF RESPIRATORY NEURONS

Respiration is a complex function driven by the groups of cholinergic neurons in the CNS. They can be divided into three groups: dorsal, ventral and pontine (24). Dorsal neurons are located in the medulla, close to Nucleus tractus solitarii and receive sensory signals from the vagal nerve (25). Ventral neurons are located in ventrolateral medulla and are divided into rostral, intermediate and caudal ones. Rostral neurons contain the pre-Bötzinger complex and are very important generator of central respiratory drive. As a matter of fact, pre-Bötzinger complex is a respiratory oscillator. Bilateral injections of OPCs into this group of neurons induce apnea that can be reversed by

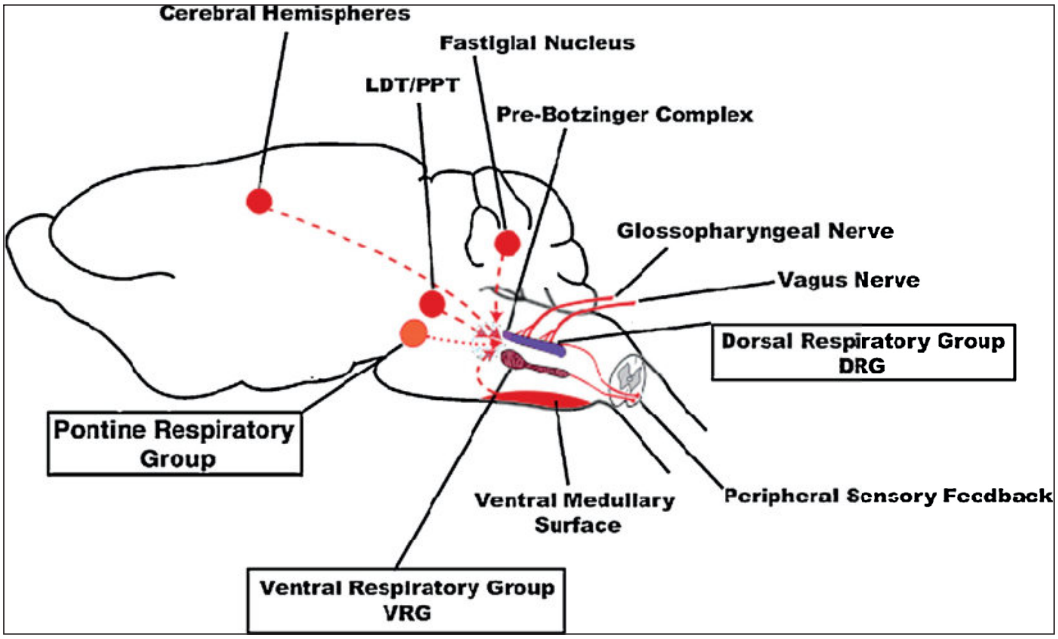

Figure 2. Distribution of neuron groups important for respiration in rat brain and their projections.

atropine (25). According to other authors, ventral respiratory group of neurons consists of five separate subgroups: caudate, intermediate, rostral, pre-Bötzinger and Bötzinger (24). Pontine group is known as pneumotaxic centre. It consists of two groups of neurons – medial parabrachial nucleus and Kölliker-Fuse nucleus (24). Its main function is to switch from inspiration to expiration (25). It is believed that pre-Bötzinger and Bötzinger complexes and rostral ventrolateral medulla contain the neuronal circuits of the respiratory central pattern generator (26). The special details of the distribution of these groups of neurons are shown in Figure 2.

CENTRAL COMPONENT OF ANTICHOLINESTERASE-INDUCED RESPIRATORY DEPRESSION

Nerve agents have tendency to produce the highest levels of AChE inhibition in the ponto-medullar region where these nuclei are located (27). It automatically means that in these discrete brain regions acetylcholine builds up the most. Indeed, anticholinesterases, depending on the dose injected, first stimulate respiration (21, 22).

Higher doses of anticholinesterases induce respiratory depression. It can be manifested as bradypnoea, prolonged pause between inspirations and expirations and, in most severe cases, as total desynchronisation of the central respiratory stimuli, resulting in chaotic contractions of respiratory muscles that make the respiration inefficient and lead to hypoxaemia and hypercapnia (28).

Local administration of soman into the intermediate part of the ventral surface of medulla oblongata profoundly affected both the respiratory and cardiovascular functions. All these effects were reproduced after replacing soman with muscarinic receptor agonist oxotremorine and reversed by atropine, implying the involvement of muscarinic mechanisms (29).

At the same time, nicotine does not only act as a powerful poison of respiratory centres, since mecamylamine, a centrally-acting nicotinic receptor antagonist can restore the OPC-induced respiration and exerts significant protection of mice poisoned with soman (30). These and other findings suggest the involvement of both muscarinic and nicotinic receptors in the anticholinesterase-induced central respiratory failure (31).

What is probably more important than the elucidation of the primary receptor pathway of the central anticholinesterase-induced respiratory failure is the fact that most of the authors ascertained that the central respiratory component was endangered even by lower doses of organophosphates and carbamates. For example, in the anaesthetized cat, only 1 LD50 of soman was needed to cause a central respiratory paralysis, in comparison with 14 LD50 of soman needed for obtaining the neuromuscular blockade (32). These cats were instrumented in such a way that gastrocnemius muscle was electrically stimulated in situ, allowing thus the peripheral neuromuscular function to be checked irrespectively of the discharges from the CNS, which were at the same time monitored in the proximal part of the phrenic nerve. In this experiment, Rickett et al (32) found that a 14-fold higher dose of soman was needed to block the sciatic nerve-gastrocnemius preparation in situ, than to cause a central cessation of phrenic nerve discharges.

This central versus peripheral ratio varied, depending on both the animal species and the anticholinesterase used. Under the same conditions, dose capable of causing central and peripheral respiratory blockade in guinea pigs

were 38 and 400 mcg/kg IV for soman (ratio 1:10) and 82 and 650 mcg/kg IV for sarin (ratio 1:8) (33). It was shown that VX that has the slowest onset of action, has roughly the equal potential for central and peripheral AChE inhibition and respiratory impairment (18, 34). The same applies to tetraethyl pyrophosphate (TEPP) poisoning in cats (22). As already mentioned, soman is a typical example of predominantly centrally acting anticholinesterase nerve agents, where the activity of respiratory centre is impaired first, followed by the neuromuscular transmission and pulmonary muscarinic syndrome, which is of definitely least clinical significance (14). At the same time, TEPP and sarin in rabbits first affect the neuromuscular transmission and then cause a respiratory arrest (21).

THERAPEUTIC REGIMENS FOR ANTICHOLINESTERASE-INDUCED RESPIRATORY DEPRESSION

From the therapeutic point of view, pulmonary muscarinic syndrome can be easily treated with muscarinic receptor antagonist atropine (35), which remains without any effect on nicotinic receptors of the neuromuscular junction even when applied in very high doses (21, 36). Additional proof that bronchoconstriction, bronchorrhoea and bradycardia are of a purely peripheral nature consists in a finding that they can be effectively treated even with N-methyl atropine, a quaternary derivative of atropine that cannot pass the BBB (33).

Use of a ganglionic blocker pentamethonium (C5) or a classical neuromuscular nicotinic receptor antagonist d-tubocurarine assures protection against endogenous acetylcholine-induced toxicity resulting from poisonings with nerve agents (22, 37). However, since in the clinical settings it is not easy to find the right dose of nicotinic receptor antagonists that would not be paralyzing per se, peripheral nicotinic receptors are usually treated with oximes, as AChE reactivators (2, 36). They act as chelators that remove the inhibitor from the active centre of AChE and thus restore the enzyme's activity (11, 38).

Therapeutic implications of centrally acting anticholinesterases include use of atropine or even more lipophilic antimuscarinic agent scopolamine. In a vast range of doses (0.5-10 mg/kg) and routes of administration (IV, IM, SC), atropine eliminates signs of central respiratory depression induced by nerve agents (7, 14, 23), while N-methylatropine remains without any effect even after administration of the 100-fold equimolar doses proving thus the central site of the atropine therapeutic action (7). Atropine 2 mg/kg IV was able to counteract the respiratory arrest and bradycardia induced by microinjections of sarin into the lateral reticular nucleus of the rabbit medulla (39). At the same time, there are some limits to the effectiveness of atropine only regimens against soman-induced respiratory depression in guinea pigs – dose of 10 mg/kg IV is efficacious after administration of 2 LD50, partially effective after 5 LD50 and totally ineffective after 10 LD50 of soman (40).

Part of the treatment protocols for patients poisoned with anticholinesterases is treatment with atropine and oxime, in order to manage both the central muscarinic and peripheral muscarinic and nicotinic signs of intoxication, respectively (11, 41). Although not efficient as a reactivator of tabun- or soman-inhibited AChE, pralidoxime, in the form of chloride (2-PAM) or methanesulphonate (P2S) has been the most widely used oxime, effective against sarin and VX, but also against many anticholinesterase insecticides (11). It has been determined that the maintenance of pralidoxime minimum plasma concentration of 4 mg/l is crucial for its therapeutic effect (42). Since it falls bellow this level after 1.5-2 h after the IV bolus administration of 1 g of 2-PAM, it is recommended that this oxime is administered as a continuous IV infusion at a rate of 0.5 g/h (43). In the open field situation, however, IV route of administration might be too demanding for the health personnel trying to treat a number of seriously poisoned individuals and IM route therefore should be preferred, when a single dose of 500 mg of pralidoxime should be injected. Such application, up to three times and separated with 20-min intervals, should not produce any adverse effects, while the same therapeutic regimen in individuals already taking pralidoxime PO as prophylaxis may induce a reversible visual impairment (44).

It is generally accepted that soman and other centrally-acting AChE inhibitors via cholinoceptor hyperstimulation start a vicious circle that ends up with glutamatergic excitatory discharges that clinically manifest themselves as seizures and leave the survivors with serious brain damage (45, 46). It is not clear what the relation between the onset of seizures and the occurrence of respiratory depression is in soman-poisoned animals, since most of the cited experiments have been performed under urethane anaesthesia (47). In non-anaesthetised animals, loss of consciousness coincided with seizures and respiratory depression occurred immediately thereafter (33). In epilepsy, a considerable number of patients with seizures developed a central apnoea and hypoxaemia (48) and it is therefore logical to assume that then same applies to the seizures induced by acetylcholinesterase inhibitors. For the same reason, it seems plausible to conclude that administration of diazepam, as anticonvulsant, is beneficial for the treatment of both conditions (11).

Although the efficacy of atropine in preventing the central respiratory depression induced by anticholinesterases suggests the obvious role of muscarinic receptors, nicotinic receptors are likely to be involved, too and this mechanism probably includes some non-cholinergic transmitters, such as endogenous opioids. Indeed, it seems that, in presence of soman and atropine, it is the stimulation of nicotinic presynaptic receptors that induces the liberation of glutamate (49). There is some evidence that this pathway can also represent a link between cholinergic and glutamatergic neurons that in turn exerts control over release of endogenous opioids in the CNS (50).

CONCLUSION

Only peripherally-acting anticholinesterases induce death by overstimulating with excess of acetylcholine the nicotinic receptors at the neuromuscular junction, while muscarinic pulmonary syndrome is of minor importance for survival. Centrally-acting anticholinesterases inhibit AChE in the pontomedullary region and impair the functioning of the respiratory centre neurons, since they receive numerous cholinergic inputs. Phrenic nerve discharges cease in these cases at doses of anticholinesterases much lower than those needed for inducing peripheral neuromuscular block. The recommended therapy includes atropine that blocks central and peripheral muscarinic receptors and oximes, which reactivate the inhibited AChE mainly outside the CNS, but also diazepam, in order to control seizures. Possible involvement of central nicotinic receptors as well as the other neurotransmitter systems – glutamatergic, opioidergic – necessitates further research of additional antidotes.

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SPONTANEOUS SUBCAPSULAR RENAL HEMATOMA AS A COMPLICATION OF ACUTE PYELONEPHRITIS: A CASE REPORT

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SPONTANI SUBKAPSULARNI HEMATOM BUBREGA KAO KOMPLIKACIJA AKUTNOG PIJELONEFRITISA: PRIKAZ SLUČAJA

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ABSTRACT

Spontaneous subcapsular renal hematoma (SSRH) emerged as a complication of acute pyelonephritis (APN) is an extremely rare condition. We showed a patient aged 63 years hospitalized due to languor, febrility, abdominal pain accompanied by nausea and vomiting. Ultrasound (US) examination of the abdomen and multidetector computed tomography (MDCT) showed the presence of subcapsular hematoma of the left kidney with calculus in the initial part of the left ureter. Laboratory tests registered a positive inflammation syndrome, anemia and an increase in nitrogen compounds. Based on laboratory results, clinical presentation and imaging techniques it has been found that it is APN with spontaneous subcapsular hematoma. After application of non-surgical treatment which included antibiotic therapy with percutaneous drainage of hematoma, a good clinical response with regression of subjective symptoms and hematoma was obtained. On repeated US and MDCT after 18 months no pathological changes in the kidneys were registered. Spontaneous subcapsular hematoma extremely rare occurs as a result of APN associated with calculosis. The use of non-surgical treatment, which includes appropriate antibiotic therapy with percutaneous drainage of hematoma would represent a method of first choice.

Keywords: *spontaneous subcapsular renal hematoma, pyelonephritis, percutaneous drainage*

SAŽETAK

Spontani subkapsularni hematom bubrega (SSHB) nastao kao komplikacija akutnog pijelonefritisa (APN) je izuzetno retko stanje. Prikazali smo pacijentkinju starosti 63 godine hospitalizovanu zbog malaksalosti, febrilnosti, bola u trbuhu praćenim mučninom i povraćanjem. Ultrazvukom (UZ) i multidetektorskom kompjuterizovanom tomografijom (MDCT) abdomena dokazano je prisustvo subkapsularnog hematoma levog bubrega sa kalkulusom u početnom delu levog uretera. Laboratorijskim pretragama registrovani su pozitivan zapaljenjski sindrom, anemija kao i porast azotnih materija. Na osnovu pomenutih laboratorijskih nalaza, kliničke slike kao i vizualizacionih tehnika utvrđeno je da se radi o APN sa spontanim subkapsularnim hematomom. Nakon primene nehirurškog tretmana koji je podrazumevao antibiotsku terapiju sa perkutanom drenažom hematoma dobijen je dobar klinički odgovor sa regresijom subjektivnih tegoba kao i hematoma. Na ponovljenom UZ i MDCT pregledu nakon 18 meseci nisu registrovane patološke promene na bubrezima. Spontani subkapsularni hematom izuzetno retko nastaje kao posledica APN udruženog sa kalkulozom. Primena nehirurškog tretmana koji podrazumeva odgovarajuću antibiotsku terapiju sa perkutanom drenažom hematoma bi predstavljala metodu prvog izbora.

Ključne reči: *spontani subkapsularni hematom bubrega, pijelonefritis, perkutana drenaža*

ABBREVIATIONS

SSRH- Spontaneous subcapsular renal hematoma
APN - Acute pyelonephritis
MDCT - Multidetector computed tomography
US - Ultrasound

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INTRODUCTION

Spontaneous subcapsular renal hematoma (SSRH) emerged as a complication of acute pyelonephritis (APN) is an extremely rare condition. In recent years, thanks to advances in imaging procedures like US and MDCT, diagnosis of disease was significantly facilitated. A decision about the treatment of disease represents a dilemma, in the sense that the surgical treatment that involves radical nephrectomy should be implemented or apply another approach to treatment. Previously it was considered that the most common cause of SSRH was a kidney tumor with no obvious etiology, therefore a radical nehrectomy was always advised.

We have presented a case of a woman with APN and ureteral calculosis with the creation of a SSRH as a complication. By applying non-surgical treatment, which includes antibiotic therapy with percutaneous drainage of hematoma (intervention radiological procedure), optimal therapeutic response and complete regression of hematoma and clinical symptoms were obtained.

CASE REPORT

A woman aged 63 years was admitted to the Center for Emergency Medicine with the complaints in the form of fatigue, fever, vomiting and abdominal pain, predominantly in the left lumbar flunk. These symptoms last for two days backward. On admission she denied the existence of any injury. She did not give information about the existence of other diseases, nor was treated. Laboratory analyzes indicated the positive inflammatory syndrome: Sed Rate 86 mm/h, CRP 186 mg/L, Le 12.1 x 10^{9}. Other laboratory analysis indicated the presence of anemia Er 2.81 x 10^{12}, Hgb 68 g/L, HCT 0.213 and also the presence of asothaemia (urea 24.8 mmol/L, creatinine 375 μ/L) ionogram and hepatogram were within the normal range. The finding in the urine is indicated for bacteriuria with piuria.

By US examination of the abdomen, it can be seen, together with the lateral contour of the left kidney, hypoechogenic, ovoid area, diameter 155 x 47 mm, which surrounds the kidney (figure 1). Doppler examination did not show any arteriovenous fistula as a cause of the collection. MDCT confirms the existence of SSRH, with the inclusions of gas (figure 2). Compression of parenchyma and collecting system of the kidney is noticeable, with no signs of intrarenal hematoma, and also at the beginning of the left uretere there is a calculus 20 x 13 mm in size, with minimal urinary retention.

Based on the findings of MDCT a decision was taken on further non-surgical treatment of SSRH. With the empirical use of antibiotics a percutaneous drainage of collection by "pigtail" 9F catheter was performed. About 200 ml of non-homogeneous, hemorrhagic content was drained from whom the bacterium Escherichia coli was isolated and also confirmed in the urine culture.

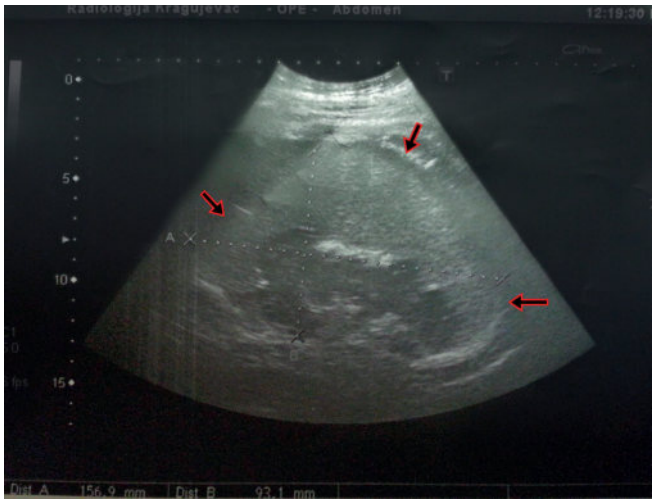

Figure 1. US of left kidney shows hypoechogenic, ovoid area which surrounds the kidney

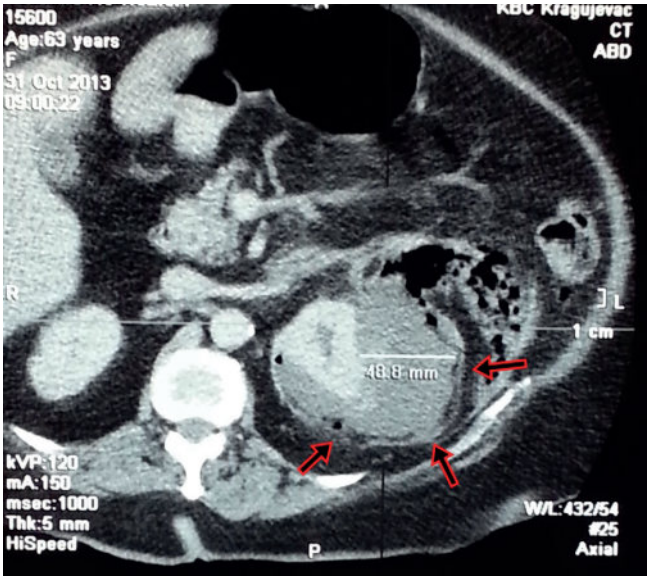

Figure 2. MDCT confirms the existence of subcapsular haemathoma, with the inclusions of gas. Arrows show the presents of hematoma.

After drainage and antibiotic therapy acording to the antibiogram the patient feels better, laboratory analyses show regression of inflammatory syndrome. Control US and MDCT confirms the regression of changes (figure 3). After 12 days, the patient was discharged from hospital. By subsequent MDCT control after 18 months there were no new renal changes registered (figure 4).

DISCUSSION

Spontaneous subcapsular hematoma of the kidney can be divided into three types: intrarenal, perirenal and subcapsular hematoma. It generally occurs as a result of other pathological conditions, like tumors, vascular diseases, extremely rare as a result of infection, or other

causes, including the state of an unknown etiology (1, 2). Very rarely SSRH may occur as a complication of APN, and in the available literature, we found only three such cases (3-5). In the clinical picture dominates general symptomatology (fatigue, pain, fever), and a decrease in hemoglobin (6). Meta analysis shows that the most common causes of SSRH are tumors - 61.5%. Malignant tumors (most commonly renal cell carcinoma) are responsible for 31.5% and benign (most angiomyolipoma) to 29.7%. Vascular disease causes SSRH to 17%, infection in 2.4% and 6.7% of cases of idiopathic etiology (1). In the literature as a rare cause of SSRH are mentioned hypertension, anticoagulation, nimesulide, percutaneous angiography (6-9). US is useful for rapid identification of findings, but the diagnosis must be confirmed by MDCT examination, which is the method of choice. MDCT has a high sensitivity and specificity to confirm the existence of hematoma, as well as the cause of its occurrence (1, 9). Regarding the therapeutic approach, a number of authors in their work suggest that the best solution is non-surgical treatment, which involves percutaneous drainage of the hematoma (as a intervention radiological procedure), with the elimination of the basic causes of this disease (6-8).

This attitude is confirmed by the authors, which refer APN as a cause of SSRH (3-5). In the presented case we highlight the importance of timely and adequate diagnostic approach (US, MDCT), and then, in accordance with the diagnosis of the underlying cause, the implementation of appropriate antibiotic therapy (according to the antibiogram), as well as percutaneous drainage of SSRH. APN potentiated by the presence of kidney stone in the proximal part of the ipsilateral ureter, we claim, can give SSRH as a complication. Non-surgical treatment provides an adequate solution, not only because of the drainage of the hematoma, but also for the confirmation of bacterial pathogens, and therefore application of effective antibiotics.

In conclusion, an extremely rare SSRH occurs as a result of an APN associated with calculosis and the use of non-surgical treatment (antibiotics with percutaneous drainage) is the first line of choice.

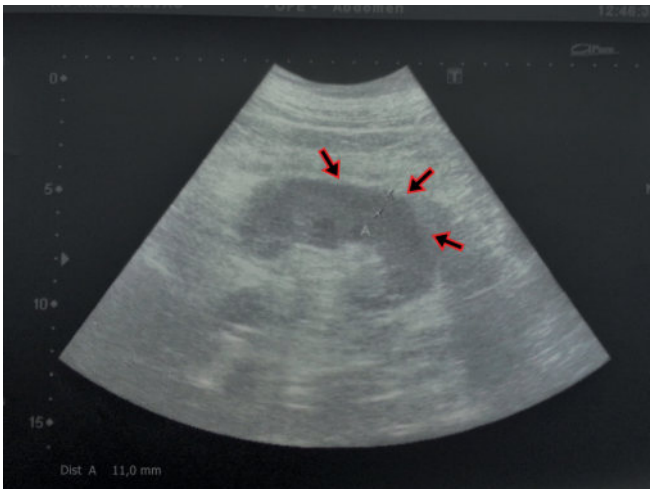

Figure 3. US shows the regresion of hematoma.

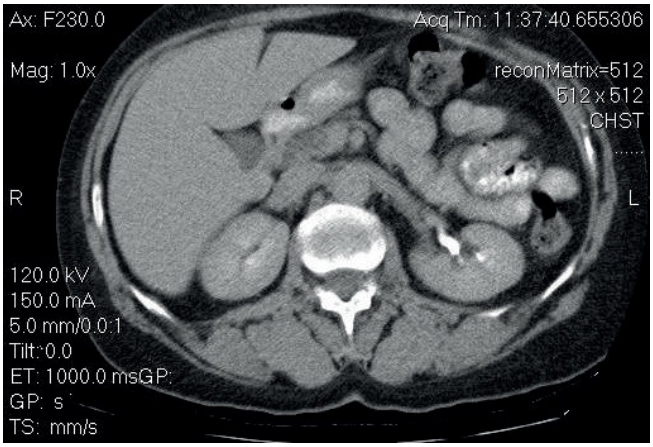

Figure 4. By subsequent MDCT control after 18 months there were no new renal changes registered.

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GENERALIZED PUSTULAR PSORIASIS IN ASSOCIATION WITH SQUAMOUS CELL CARCINOMA OF THE HYPOPHARYNX

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GENERALIZOVANA PUSTULOZNA PSORIJAZA UDRUŽENA SA SKVAMOCELULARNIM KARCINOMOM HIPOFARINKSA

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ABSTRACT

Pustular psoriasis is an uncommon form of psoriasis consisting of widespread pustules on an erythematous background. Very rarely pustular psoriasis represent a paraneoplastic dermatosis. In this report we describe a case of generalized pustular psoriasis (GPP) associated with advanced, inoperable, metastatic squamous cell carcinomaof the hypopharynx. We suggest that physicians should be alert for the worsening of existing psoriasis or formation of novel psoriasiform eruptions and should undertake clinical evaluation of possible neoplastic disease.

Keywords: *Pustular psoriasis, squamous cell carcinoma, paraneoplastic dermatosis*

SAŽETAK

Pustularna psorijaza je retka forma psorijaze koja se manifestuje diseminovanim pustulama na eritematoznoj osnovi. Veoma retko pustulozna psorijaza predstavlja paraneoplastičnu dermatozu. U ovom radu predstavljamo slučaj generalizovane pustulozne psorijaze udružene sa uznapredovalim, inoperabilnim skvamocelularnim karcinomom hipofarinksa. Smatramo da lekari treba da uoče pogoršanje postojeće psorijaze ili pojavu nove psorijaziformne erupcije sa ciljem da se klinički evaluira moguć neoplastički proces.

Ključne reči: *Pustularna psorijaza, skvamocelularni karcinom, paraneoplastična dermatoza*

ABBREVIATIONS

GPP-generalized pustular psoriasis

INTRODUCTION

Psoriasis is a chronic, relapsing skin disease presented in majority of cases with widespread erythematous papules and plaques covered with a scale. Vary rarely psoriasis can be manifested with widespread pustules on an erythematous ground localized on the whole body surface. These condition is known as a generalized pustular psoriasis and it is an uncommon form of psoriasis (1).

Paraneoplastic dermatoses represent a group of cutaneous disorders related to underlying internal malignancies. In available literature psoriasis, expecially it's pustular form is rarely described in association with malignancy (2-6).

In this report we describe a case of generalized pustular psoriasis (GPP) associated with advanced, inoperable, metastatic squamous cell carcinomaof the hypopharynx. To the best of our knowledge this is the first report of GPP accompanied by squamous cell carcinoma.

CASE REPORT

A 46-years old male with no personal and/or family history of psoriasis was referred to Department of Dermatology. He was febrile, malaise, in distress and dehydrated. Clinically, generalized bright erythema with individual and coalescent pustules localized on whole body surface was present (Fig.1a). The face and scalp were deep red in color and covered by thick yellowish scales, while on palms and soles lamellar thick scale was observed (Fig.1b,1c). No mucosal involvement was present. Physical examination revealed a tumorous formation fixed to skin and underlying structures on right side of the neck (Fig.1d). Patient appeared undernourished, with poor personal appearance. He stated that skin redness began ten days before referring to the dermatologist. He denied taking any drugs and medication but admitted that he is a heavy cigarette smoker and alcohol abuser.

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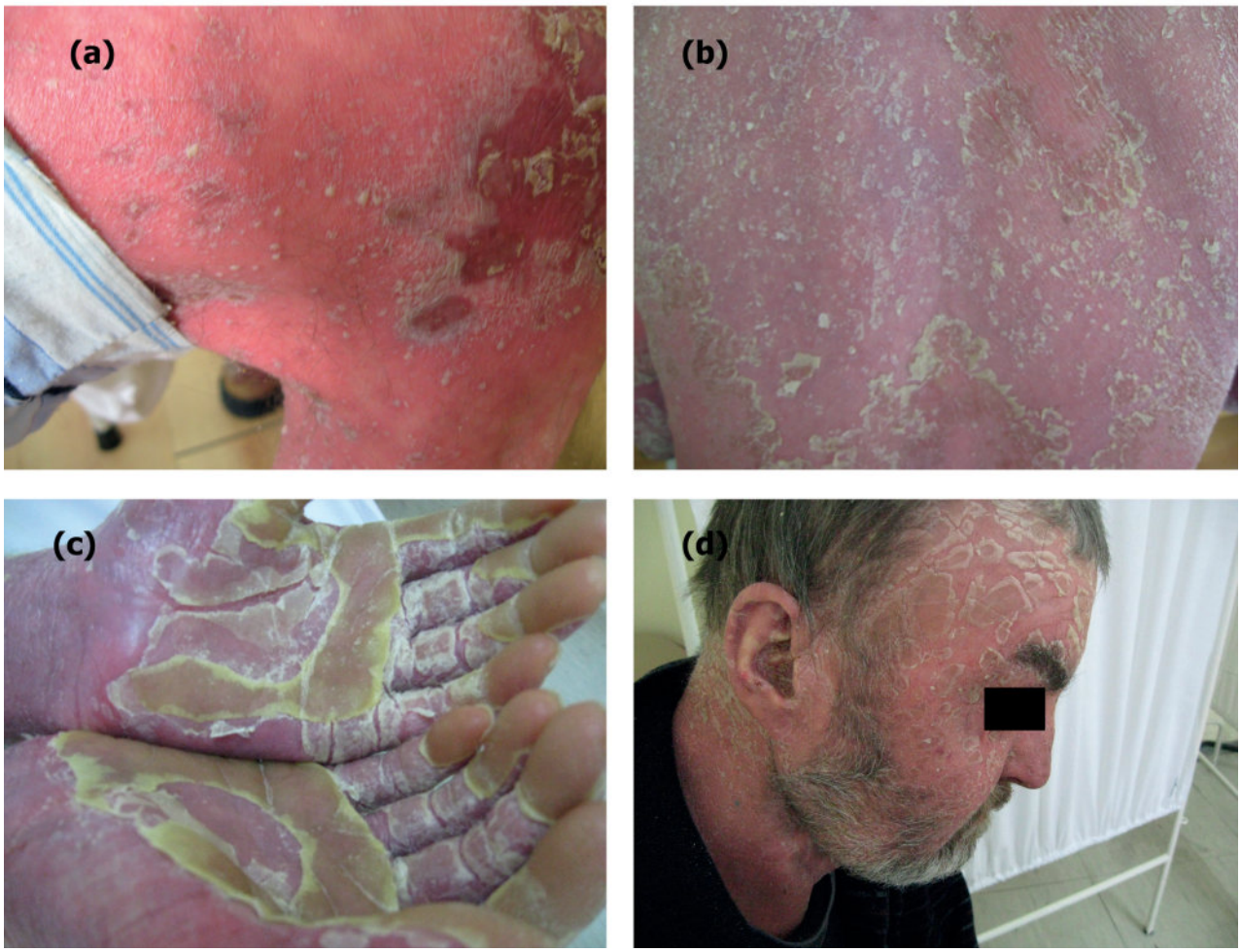

Figure 1. Clinical presentation at admission showing (a) Bright erythema with individual and coalescent pustules, (b) Generalized erythema on the back with scales, (c) Thick yellowish, laminar scales on palms, (d) Face and scalp covered by thick scales and tumor formation on right side of the neck.

We have conducted various laboratory studies. Complete blood count has showed mild anaemia and leukocytosis with predominance of neutrophils. Sedimentation rate was elevated, as well as C-reactive protein, while procalcitonin was within normal values. The peripheral smear showed no abnormalities. Biochemical analyses showed elevated urea and creatinine levels, hypoproteinemia and hypoalbuminemia, elevated hepatic enzymes, and decrease iron serum levels. Other biochemical parameters as well as urine analyses were normal. Fecal occult blood tests were negative. His blood cultures were negative. Cultures for bacteria and fungi from the pustules did not reveal any organisms. Carcinoembryonic antigen (CEA) and CA 19-9 were within normal levels. The abdominal ultrasound and chest radiography showed no abnormalities.

Since tumorous formation fixed to skin and underlying structures on right side of the neck was present otolaryngologist was consulted. Otorhinolaryngologic examination revealed infiltrating tumorous outgrowth of the right lateral wall of the hypopharynx which also invades tongue and oral cavity base. Ultrasound of the neck showed metastatic lymph nodes on both sides of the neck and solid hypo and hyperechoic formation on the right side which is in continuity with the endopharynx.

Biopsies were obtained from the tumorous tissue and skin. Skin biopsy showed epidermal hyperkeratosis, parakeratosis, elongation of rete ridges and subcorneal macropustula with numerous neutrophils, dilated capillaries and perivascular mononuclear infiltrate in the edematous superficial dermis (Fig. 2a). Histopathological examination of hypopharynx tumor disclosed invasive squamous cell carcinoma, moderate differentiated, with incomplete keratinization (Fig. 2b).

After the admission, patient was treated with antibiotics (consecutively amoxicillin, garamycin andamoxicillin/clavulanic acid), corticosteroids (methylprednisolone and prednisolone), supportive therapy (antipyretics, gastroprotective agents, prevention of alcohol withdrawal syndrome, intravenous fluid infusion), topical emollients. Ten days after admission patient's clinical state was improved and retinoid therapy (40 mg of acitretin daily) was started. Three weeks later he was transferred from our department to Oncology department in order to start with cancer treatment (radical radiotherapy, 66Gy in 33 fractions with chemotherapy with cisplatin). Dermatological checkup, two weeks after, showed generalized erythema with less prominent scales then on admission and no pustules flares. He was released from Oncology department with advice

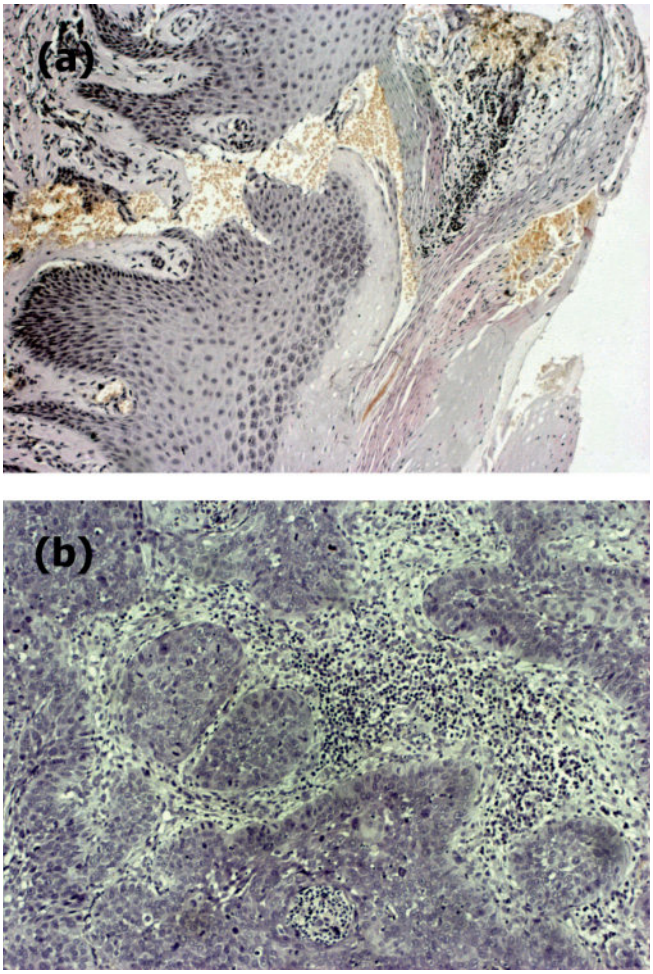

Figure 2. Histopathologic findings (a) Pustular psoriasis, epidermal hyperkeratosis, parakeratosis, elongation of rete ridges and subcorneal macropustula with numerous neutrophils. Dilated capillaries and perivascular mononuclear infiltrate in the edematous superficial dermis. (Hematoxylin& eosin stain, original magnification, × 200), (b) Invasive squamous cell carcinoma, moderate differentiated, with incomplete keratinization (Hematoxylin & eosin stain, original magnification, × 200).

to continue his radiotherapy and chemotherapy in Oncology Daily Center. But he never again returns to oncologist, otolaryngologist or dermatologist. His outcome remains unknown to all specialist involved in his treatment.

DISCUSSION

Paraneoplastic dermatoses represent a group of cutaneous disorders related to underlying internal malignancies. Their clinical appearance and course may range from specific dermatosis characteristic of a particular cancer to entirely atypical eruption (7, 8). Some paraneoplastic dermatosis could respond to non-causal therapy options, such as topical and systemic corticosteroids but usually cancer treatment is required for skin disorders remission (7).

Psoriasis, especially generalized pustular psoriasis is uncommon as a paraneoplastic dermatosis (6) but there are reports in literature which suggest that psoriasis can develop at the onset of a tumor, improved after tumor treatment or exacerbated when tumor relapsed or metastasized (2-5). Development of psoriasis and psoriasiform eruption associated with malignant tumorous is not entirely clarified but possible mechanisms could be growth factors and cytokines produced by malignant cells (8-10).

Our patient denied any history of psoriasis. He came to our department for the first time, had no medical records of previous examination nor treatment, which psoriatic patients usually have. One can argue that heavy alcohol abuse and cigarette smoking could work as a trigger in this case. However, it seems appropriate to hypothesize that in our case generalized pustular psoriasis was skin manifestation of underlying malignancy. Late onset of the disease at the age of 46, rapid course of the disease (confirmed by patient's relative who also stated that he had no skin related problems before) and slight improvement of skin lesions after radiotherapy and chemotherapy were commenced suggest our hypothesize.

We present this case in order to emphasize that appearance and behavior of psoriasis and pustular psoriasis could be linked to malignancy, so dermatologist should undertake clinical evaluation of possible neoplastic disease.

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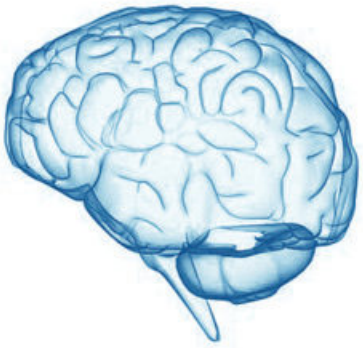

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