ISSN 1820-8665

Vol. 14•No2 • JUNE 2013.

Serbian Journal of Experimental and Clinical Research

2000

Serbian Journal of Experimental and Clinical Research

2000

Indexed in
EMBASE/Excerpta Medica, Index Copernicus, BioMedWorld, KoBSON, SCIndeks

Address:
Serbian Journal of Experimental and Clinical Research, Faculty of Medical Sciences, University of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac, PO Box 124
Serbia
http://www.medf.kg.ac.rs/sjecr/index.php

SJECR is a member of WAME and COPE. SJECR is published four times circulation 250 issues
The Journal is financially supported by Ministry for Science and Technological Development, Republic of Serbia
ISSN 1820 – 8665

Table Of Contents

CELL VOLUME - ROLE IN OBESITY AND ITS MAINTENANCE MINIREVIEW

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ZAPREMINA ĆELIJE I NJENA ULOGA U NASTANKU I ODRŽAVANJU GOJAZNOSTI

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Received / Primljen: 5.07.2013. Accepted / Prihvaćen: 5.07.2013.

ABSTRACT

Keeping volume within certain limits is a prerequisite for cell integrity and proper function. The defense against excess cell swelling is accomplished also by a reduction of the intracellular osmolarity by synthesis of osmotically less active macromolecules from their specific subunits (proteosynthesis and lipogenesis). At the same time proteolysis and lipolysis is inhibited. Conversely, cell shrinkage stimulates lipolysis, degradation of proteins to amino acids, degradation of glycogen to glucosephosphate and simultaneously protein, lipid and glycogen synthesis is inibited. The degradation products are osmotically more active than the macromolecules and their breakdown generates cellular osmolarity resulting in water entry into cells. Recently new interesting view of ***chronic*** *changes of the cell volume and its role in model of body weight loss and regain in obesity is considered; secretion profile of adipocytes is related to their size. Decrease of body weight is constituted by the drop of fat in adipocytes, they consequently shrink. Surrounding extracellular matrix has to adjust to traction forces. To ascertain a sufficient supply of glucose and fat for re-storage, adipocytes change their pattern of secreted adipokines altering the total body metabolism and promoting energy intake. Shrunken adipocytes show insulin resistance, whereas glucose uptake is facilitated in osmotically swollen adipocytes. Compensating mechanism in patients on very-low calorie diet during weight maintenance period includes increase in water content of adipose tissue correlating with increase of insulin sensitivity. Consideration of participation these mechanisms might bring new insight into understanding of obesity pathophysiology and treatment.*

Key words: chronic change of cell volume, obesity, adipokines, insulin

SAŽETAK

Održavanje volumena ćelije u okviru određenih granica je preduslov za očuvanje njenog integriteta i adekvatno funkcionisanje. Sprečavanje preteranog bubrenja ćelije se, između ostalog, postiže smanjenjem intracelularne osmolarnosti, sintezom osmotski manje aktivnih makromolekula iz njihovih specifičnih subjedinica (proteosinteza i lipogeneza). Istovremeno, procesi lipolize i proteolize su inhibisani. Nasuprot tome, smežuravanje ćelije podstiče lipolizu, razgradnju proteina do amino-kiselina, i razgradnju glikogena u glukozofosfate, dok su sinteza proteina, lipida i glikogena inhibisani. Produkti razgradnje su osmotski aktivniji u odnosu na makromolekule, a njihova dalja degradacija povećava ćelijsku osmolarnost, što rezultuje ulaskom vode u ćeliju. Nedavno je razmotreno novo, zanimljivo gledište na hroničnе promene ćelijskog volumena i njenu ulogu u modelu gubitka telesne težine i ponovne gojaznosti - sekretorni potencijal adipocita je povezan sa njihovom veličinom. Smanjenje telesne težine je bazirano na redukciji količine masti u adipocitima i njihovom sledstvenom smežuravanju. Okolni ekstracelularni matriks mora da se prilagodi trakcionim silama. Da bi obezbedili dovoljne količine glukoze i masti za ponovno skladištenje, adipociti menjaju način sekrecije adipokina, a time i ukupan metabolizam organizma, stimulišući unos energije. Smežurani adipociti pokazuju insulinsku rezistenciju, dok osmotski nabubreli lakše preuzimaju glukozu. Kompenzacijski mehanizam kod pacijenata sa veoma niskim kalorijskim unosom, tokom perioda održavanja telesne težine, obuhvata porast sadržaja vode u masnom tkivu, što je povezano sa povećanjem osetljivosti na insulin. Uzimanje u obzir ovih mehanizama može doneti novi uvid u razumevanju patofiziologije i lečenja gojaznosti.

Ključne reči: *hronične promene ćelijskog volumena, gojaznost, adipokini, insulin*

ABBREVIATION

ECM – extracellular matrix

UDK: 616-008.9-092.18 ; 576.385-026.52 / Ser J Exp Clin Res 2013; 14 (2): 45-48
DOI: 10.5937/SJECR14-4141

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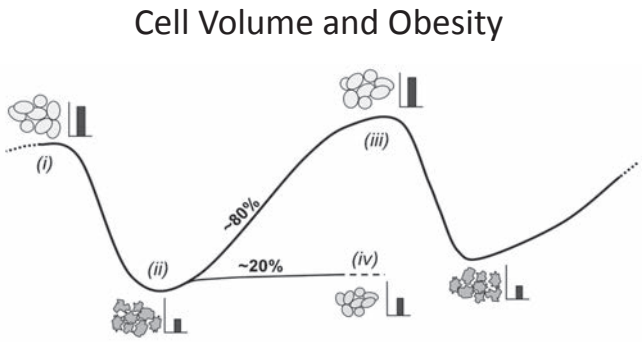
Keeping volume within certain limits is a prerequisite for cell integrity and proper function. Protective mechanisms should have to develop in early forms of life. A variety of organisms and cell types spanning all taxonomic groups are exposed to osmotic stresses. Phylogenetically divergent organisms employ uniquely adapted mechanisms of cell volume regulation proceeding by highly conserved physiological processes[1]. Most of the acute cell reactions to volume changes are aiming at the reestablishment of initial volume status. To drive water flux, which finally accomplishes cell volume adjustment, cells produce an osmotic difference between the intra- and extracellular spaces by inducing osmolyte transport. The defense against excess cell swelling is accomplished by a reduction of the intracellular osmolarity by release of inorganic and organic osmolytes from the cell and by synthesis of osmotically less active macromolecules from their specific subunits (proteosynthesis and lipogenesis[2-6]). At the same time proteolysis and lipolysis is inhibited. Consequently water leaves the cells, their size and shape return to regular values. Conversely, acute cell shrinkage stimulates the degradation of lipids, proteins to amino acids and glycogen to glucosephosphate and inhibits lipid, protein and glycogen synthesis[6]. The degradation products are osmotically more active than the macromolecules and their breakdown generates cellular osmolarity resulting in water entry.

In higher organisms cell volume response to osmotic challenge is integrated into a signal transduction network regulating various cell functions including gene expression, cell proliferation, apoptosis, migration, metabolism, secretion of hormones and mechanism of their effect [6-10]. Very interesting is powerful effect of cell swelling to induce exocytosis of material in intracellular secretory vesicles[8-10]. This mechanism induces secretion of peptide and protein hormones and enzymes. Considering that endocrine secretion is normally under delicate control leading to the release of appropriate amount of hormone in response to endogenous secretagogues or specific stimuli, swelling-induced exocytosis represents stimulus relatively independent from hormonal regulation.

CELL VOLUME AND OBESITY.

Cell volume returns to original values after moderate osmotic challenge within minutes[10]. While acute changes of cell volume are well known to be integrated into a signal transduction network affecting various functions, impact of chronic changes of cell volume on cellular functions have been rarely investigated. Recent article of Edwin Mariman[11] brings new interesting view on the mechanism involved in body weight loss and regain in obese patients: in this biological model long lasting cellular volume changes are considered. Chronic changes of the volume[11,12] and lipid content of adipocytes and their impact on secretion of adipokines (Fig. 1) play a pivotal role. Obese people have large adipocytes; a decrease of body weight after calory restriction is constituted by the release of fat from adipocytes, they consequently shrink. According to model of Mariman[11, 13] their surrounding extracellular matrix (ECM) has to be adjusted and reconstructed as well (Fig. 1). For the construction of new collagen fibers, collagen proteins have to be synthesized and modified, which is an energy-demanding process. Under conditions of calorie restriction during weight loss, such energy is not available. As a consequence, the adaptation of the ECM cannot keep up with that of the cell and stress will build up between the ECM and the cell due to traction forces inducing a cut down on the further release of fat - cellular stress results in increased resistance against releasing fat. Secretion of adipokines changes. The adipocytes under normal conditions secrete numerous peptide hormones that regulate the metabolic activity of other peripheral tissues but also influence the energy intake[14]. By changing the adipokine profile, adipocytes can manipulate the eating behavior of their host. In fact, Skurk et al showed that the secretion profile is related to adipocyte size[15] suggesting that during weight loss and shrinkage of cells, adipocytes will automatically change their adipokine secretion profile. Leptin is one of those hormones that play a role in signaling the fat content of the body to the brain. Its plasma levels are associated to the amount of fat mass[16]. If the stored fat content is sufficient, it is signaled by leptin to the brain and as a consequence appetite is repressed. The production of leptin[16] is directly and adiponectin undirectly proportional to adipocytes size. Consequently during weight loss their levels change allowing the feeling of hunger. Interestingly, during weight loss plasma leptin levels drop far beyond what would be expected from the amount of lost fat mass. Typically, a 15-20% loss of fat mass is accom-



E.C.M. Mariman: An adipobiological model for weight regain after weight loss. Adipobiology, 3; 9-16, 2011

Figure 1. Adipobiology of weight cycling. The weight cycling is shown by the solid line. Obese people have large adipocytes and high plasma levels of leptin as shown by the bar (i). Upon weight loss adipocytes shrink and accumulate cell stress. Leptin levels are reduced by 40-60% (ii). The changed adipokine profile induces eating behavior and up to 80% of people return to the original weight or go beyond it. Leptin levels rise again dramatically (iii). Twenty percentage of people or more succeed in maintaining a lower weight with a reduced leptin level (iv). The others start on their next round of weight loss and attempt of weight maintenance (iii). From Mariman, Adipobiology 2011;3:9-16 with permission.

panied by a reduction of 40-60% in leptin[11]. This signals a state of leptin deficiency in the brain[17]. Eventual increase of energy intake by the host supplying sufficient glucose and fat promotes storage of triglycerides and return to the original volume with relieve from cell stress. In addition to leptin, also other adipokines may help promote energy flux towards the stressed adipocytes. For the host this would mean that after weight loss there is increased risks for weight regain originating from the cellular stress of the adipocytes. A biological model for weight regain after weight loss[11, 18] is based on the behavior of shrinken adipocytes, accumulation of structural stress and change of adipokines secretion.

INSULIN RESISTANCE

As recently reviewed, acute cell volume changes induced in adipocytes by an application of a hypertonic or a hypotonic solution alter some of their fundamental physiological functions: glucose uptake and metabolism[19]. Osmotically shrunken adipocytes show insulin resistance, which is a characteristic of type 2 diabetes mellitus and metabolic syndrome, whereas glucose uptake is facilitated in osmotically swollen adipocytes[19]. Cell swelling induced by insulin mediates some of its effects in hepatocytes[20] including proteosynthesis. Uptake of water by the shrunken cells to compensate in part for the lost adipocyte volume[18] seems to be very effective solution. Laaksonen et al.[21] observed that the water content of adipose tissue of obese men and women significantly increased after 9 weeks on a very-low-calorie diet and further increased during a 1 year weight maintenance period. Moreover, at various time points during the follow up, increase in subcutaneous adipose tissue water content correlated with increase in insulin sensitivity. Although the authors assume that the increased water content is due to increase in blood flow and blood volume, improved glucose uptake suggests that adipocytes accumulated water to compensate decreased volume. Indeed, one of the consistent metabolic feature of adipocytes in obese/overweight subjects after weight loss and a short period of weight maintenance is an improved glucose uptake capacity[22].

CONCLUSION

Release of fat from adipocytes results in the decrease of their volume. This is connected with the change of spectrum of secreted adipokines, traction stress between extracellular matrix and shrunken adipocytes and insulin resistance. Successful maintenance of lower body weight is accompanying by increase of water in adipose tissue connected with increase of insulin sensitivity. Consideration of the role of cell volume changes in the obesity may contribute to better understanding of its pathophysiology and bring novel approach in treatment.

ACKNOWLEDGEMENTS

This research was supported by APVV 0235-06, APVV-0253-10, APVV 0486-10, Chicago Diabetes Project and VEGA 2/0132/12.

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PERIODONTAL DISEASE RELATED TO OXIDATIVE STRESS

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POVEZANOST PARODONTOPATIJE I OKSIDATIVNOG STRESA

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Received / Primljen: 19.04.2013. Accepted / Prihvaćen: 12.08.2013.

ABSTRACT

The study evaluates oxidative stress (OS) in patients with different stages of periodontal disease (PD) and the influence of their smoking habits on OS. PD is related to connective tissue inflammation, which leads to deterioration of the supporting structures of the tooth. OS is a condition characterised by overproduction of free radicals (FR), which may be involved in PD, causing periodontal tissue damage and other related diseases. Study patients' were grouped as I-non smokers (n=45) and II-smokers (n=45); and consisted of patients with 3 PD stages: mild (1), moderate (2) and severe (3). As a control group, 30 healthy subjects (all non smokers) with no signs of PD were selected. FR levels were determined by the D-Roms test, total antioxidant capacity (AOC) was determined by the OXY-adsorbent test, (Diacron, Italy) and lipid peroxidation (LP) was determined by the fluorometric method with thiobarbituric acid and its end product, malonyldialdehyde (MDA). OS was found in the periodontal tissue and serum of PD patients, with the highest level of FR in the severe stage (3) in non smokers ($p<0.05$) as well as smokers ($p<0.01$); AOC showed decreasing values from mild (1) to severe stage (3) of PD for smokers ($p<0.05$). LP in serum showed the highest level in severe stage (3) in both groups i.e., non smokers and smokers ($p<0.05$) compared to controls.

Based on the obtained results, we may conclude that PD is related to OS and may either be a cause or a trigger for more accelerated OS. Cigarette smoking increased FR production and is a serious factor exacerbating further tissue damage in PD. These findings may contribute to possible use of efficient antioxidant agents as a preventive measure for PD and as a therapy for better disease outcome.

Key words: *periodontal disease, oxidative stress, smoking.*

SAŽETAK

Ova studija se bavi procenom oksidativnog stresa (OS) kod pacijenata sa različitim stadijumima oboljenja paradoncijuma - paradontopatija (PD), i uticaja pušačkih navika pacijenata. PD je povezana sa zapaljenjem vezivnih tkiva koje uzrokuje oštećenje tkivnog oslonca zuba. OS je stanje koje nastaje usled prevelike produkcije slobodnih radikala (SR) koji mogu biti uključeni u patogenezu PD, uzrokujući oštećenje paradontalnih tkiva i drugih bliskih tkiva. Pacijenti su bili podeljeni u sledeće grupe: I – nepušači (n=45), II - pušači (n=45), koji su razvrstani na osnovu stadijuma PD na: blag (1), umeren (2), težak (3) i kontrolnu grupu (n=30). SR su određivani pomoću D-Roms testa, ukupni antioksidativni kapacitet (UAK) pomoću OXY-adsorbent testa (Diacron, Italija), a lipidna peroksidacija (LP) fluorometrijskom metodom pomoću tiobarbiturne kiseline i njenog završnog metabolita, malonildialdehida (MDA). OS je detektovan u tkivu i serumu pacijenata sa PD: SR su pokazali najviši nivo kod pacijenata sa teškim stadijumom PD u grupi nepušača ($p<0.05$) i pušača ($p<0.01$); vrednosti UAK su se smanjivale od blagog (1) do teškog stadijuma (3) PD u grupi pušača ($p<0.05$). LP u serumu je imala najviši nivo u teškom stadijumu PD, i u grupi pušača i u grupi nepušača ($p<0.05$).

Na osnovu dobijenih rezultata možemo da zaključimo da je PD povezana sa OS, i može biti jedan od uzroka ili pokretačkih mehanizama za dinamičnije povećanje OS. Pušenje cigareta povećava nastajanje SR, i predstavlja značajan faktor daljeg oštećenja tkiva u PD. Ovi rezultati mogu da doprinesu mogućoj upotrebi antioksidativnih preparata u prevenciji PD, kao i terapiji, u cilju postizanja boljeg ishoda bolesti.

Ključne reči: *paradontopatija, oksidativni stres, pušenje.*

UDK: 616-008.9:577.334:546.21 ; 616.314.7 / Ser J Exp Clin Res 2013; 14 (2): 49-53
DOI: 10.5937/SJECR14-3787

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INTRODUCTION

The periodontal complex comprises alveolar bone, periodontal ligament, root cementum and the gingival tissue, all of which are crucial supporting structures of the tooth. Periodontal disease (PD) is characteriszed by chronic inflammation of the connective tissue leading to bone damage, which can be attributed to an impaired immune system. PD has also been linked to oral cancer, heart disease, stroke, osteoporosis, pre-term births, diabetes, and respiratory infections. Neutrophils that have been sequestered in the connective tissue and gingival sulcus attack bacteria and release enzymes that cause cell destruction, triggering oxidative stress (OS) by free radical (FR) production (1, 2). Internally generated FRs are from sources such as mitochondria, phagocytes, cytochrome P-450 reactions, peroxisomal fatty acid metabolism, xanthine oxidase and inflammation, where sources of externally generated FRs are cigarette smoking, air pollution, radiation, ultraviolet lights, chemicals, toxins, pathogenic microorganisms, etc. (3). Oxygen uptake in neutrophils is based on the flavoprotein cytochrome oxidase system, which increases NADPH production via hexose monophosphate shunt. Thus, it generates FRs such as superoxide anions, hydrogen peroxide, hydroxyl and hypochlorous acid. Due to unpaired electrons in the FR structure, they are highly reactive substances. Consequently, they are able to attack and damage cells by affecting lipids, proteins, carbohydrates and DNA molecules. They are capable of damaging either the cell membrane or certain bio-molecules, which leads to lipid peroxidation (LP), protein and DNA damage, enzyme oxidation and cytokine release (4). If the antioxidant capacity (AOC) is not sufficient to prevent and treat the cell impairment with the help of antioxidant enzymes, antioxidant molecules and some repair enzymes (5, 6), irreversible pathological processes may occur. This process may be accelerated by cigarette smoking, which has a likely role in periodontal disease progression. Severity of the disease may be proportional to the intensity of smoking (7). In addition to PD (8, 9, 10), OS has been implicated in the pathogenesis of a number of diseases, such as diabetes mellitus, cardiovascular (11) and neurodegenerative diseases (12) and malignancies (13). The aim of this study was to evaluate OS in patients with different stages of PD and the influence of their smoking habits on OS.

MATERIAL AND METHODS

Ninety patients with PD aged 37±15 years were divided into 2 groups: group I (n=45) included patients who did not smoke cigarettes and group II (n=45) included patients who smoked cigarettes. As a control group, 30 healthy subjects (all non smokers) with no signs of PD were selected. Both patient groups were divided into 3 subgroups of 15 patients each, representing the 3 different disease stages, i.e., mild, moderate and severe PD, classified on the basis of anamnesis, clinical and roentgenographic examination. The clinical examination was performed using the dental plaque index based on the scale of Loe & Silness, 1963 (14) to score the disease stage from 0 to 3 as follows: stage 0 - normal periodontal tissue, without plaque, which constituted the control group; stage 1 - mild inflammation with slight colour changes in periodontal tissue, slight edema and no bleeding on probing; stage 2 - moderate inflammation with redness, edema, glazing, bleeding on probing and presence of plaque in periodontal pocket, evident by visual examination; and stage 3 - severe inflammation with marked redness and edema, ulceration and tendency to spontaneous bleeding.

None of the patients had any detectable chronic disease, such as renal and liver failure or cardiovascular disease, or any other infection, neither were they given any medications. Roentgenographic examination confirmed the determined stage of PD and together with clinical examination and anamnesis contributed to the precise disease stage distinction. For laboratory testing, periodontal tissue and serum were used. The samples were taken during periodontal surgery in the course of normal treatment. After withdrawal, periodontal tissue was first measured, followed by addition of 1 ml phosphate buffer and storage at a temperature of -80°C. The samples were homogenised for 5 minutes in Microson ultrasonic cell disruptor and centrifuged for 5 minutes at 5000 rpm. For serum, blood sample from cubital vein was centrifuged for 10 minutes at 3000 rpm and stored at -4°C.

FRs were measured using the spectrophotometric method based ony the D-Roms test, AOC was measured using the OXY-adsorbent test (Diacron, Italy), and LP was determined by its end product malonyldialdehyde (MDA) using the modified fluorometric method with thiobarbituric acid (Ohkawa at al, 1978) (15). The degree of LP in serum and periodontal tissue was estimated by the modified fluorometric method, measuring thiobarbituric acid reactive substances (TBARS) using 1 % TBA (Thiobarbituric acid) in 0.05 NaOH incubated with serum and periodontal tissue at 100 °C for 15 min and read at 530 nm. Krebs-Henseleit solution was used as a blank probe.

For the statistical analysis, a Student t-test was used, and statistical significance was considered for $p<0.05$.

RESULTS

In the control group, FRs showed higher values in serum (293±68 UCarr) than in periodontal tissue (220±53 UCarr) ($p<0.05$). However, no difference was found in AOC between periodontal tissue (338±63 μmolHCLO/ml) and serum (342±67 μmolHCLO/ml) in the control group. The level of LP in serum was 3.5±0.9 μmol/l in the control group. All obtained values in control group were considered as reference values (Table 1, 2, 3).

OS was found in both the periodontal tissue and the serum of PD patients. In the periodontal tissue, production

Subject groups	Periodontal disease Intensity	Number of cases	Free radicals UCarr	Antioxidant capacity µmol HCLO/ml
Healthy subjects (control group)	/	n=30	220±53	338±63
PD patients (non smokers)	Stage 1	n=15	323±75	329±77
	Stage 2	n=15	400±71*	298±69
	Stage 3	n=15	440±88*	290±85
PD patients (smokers)	Stage 1	n=15	396±67*	297±79
	Stage 2	n=15	439±72**	288±53*
	Stage 3	n=15	528±90**	267±55*

Table 1: OS markers in periodontal tissue in non smokers and smokers with different PD stages *, p<0.05; **, p<0.01

of FR progressively increased from mild, i.e., stage 1, to severe, i.e., stage 3 of PD. It showed the highest level in stage 3 in both patient groups: non smokers (440±88 UCarr) (p<0.05) and smokers (528±90 UCarr) (p<0.01). The AOC of periodontal tissue progressively decreased from stage 1 to stage 3 of PD with statistical significance in smokers (267±55 µmolHCLO/ml) (p<0.05) (Table 1).

In serum, FR production was progressively increased from stage 1 to stage 3 of PD. It showed the highest level in stage 3 in both groups, non smokers (371±75 UCarr) (p<0.05) and smokers (410±79 UCarr) (p<0.01). In serum, AOC showed decreasing values from stage 1 to stage 3 of PD, with statistical significance in smokers (292±61 µmolHCLO/ml) (p<0.05) (Table 2).

Subject groups	Periodontal inflammation Intensity	Number of cases	Free radicals UCarr	Antioxidant capacity µmol HCLO/ml
Healthy subjects (control group)	/	n=30	293±68	342±67
PD patients (non smokers)	Stage 1	n=15	295±50	337±73
	Stage 2	n=15	332±63	340±79
	Stage 3	n=15	371±75*	327±64
PD patients (smokers)	Stage 1	n=15	334±61	330±77
	Stage 2	n=15	385±65*	300±63
	Stage 3	n=15	410±79**	292±61*

Table 2: OS markers in serum of non smokers and smokers with different PD stages *, p<0.05; **, p<0.01

FRs from periodontal tissue in stage 3 of PD were greater in both non smokers (440±88 UCarr) and smokers (528±90 UCarr) compared with reference values of the control group (220±53 UCarr). FRs showed similar trends, albeit of a lesser magnitude, when serum samples of stage 3 PD in both non smokers (371±75 UCarr) as well as smokers (410±79 UCarr) were compared with controls (293±68 UCarr) (Figure 1).

Compared with the reference values from the control group (338±63 µmolHCLO/ml), AOC of periodontal tissue showed greater reduction in values in stage 3 of PD in both non smokers (290±85 µmolHCLO/ml) and smokers (267±55 µmolHCLO/ml). Compared to controls (342±67 µmolHCLO/ml), though AOC showed similar decrements in serum from stage 3 PD in both non smokers (327±64 µmolHCLO/ml) and smokers (292±61 µmolHCLO/ml), the magnitude of change was lesser than that in periodontal tissue (Figure 2).

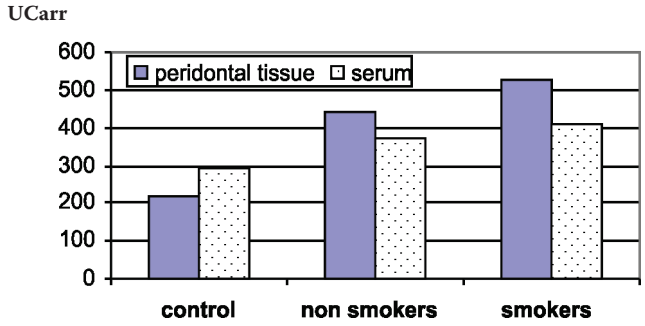

Figure 1: Free radicals in stage 3 of non smokers and smokers with PD

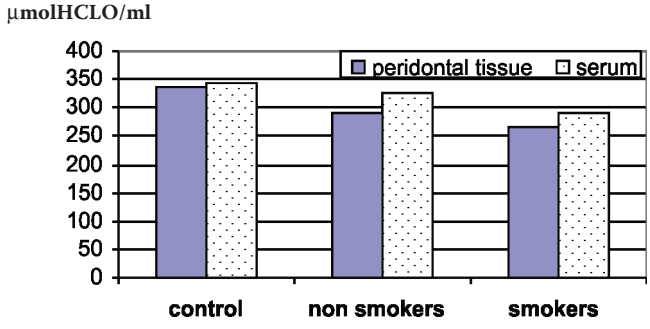

Figure 2: Antioxidant capacity in stage 3 of non smokers and smokers with PD

Subject groups	Periodontal inflammation intensity	Number of cases	Lipid peroxidation (MDA) µmol/l
Healthy subjects (control group)	/	n=30	3.5±0.9
PD patients (non smokers)	Stage 1	n=15	3.4±1.0
	Stage 2	n=15	3.5±1.2
	Stage 3	n=15	3.9±0.9*
PD patients (smokers)	Stage 1	n=15	4.0±1.3*
	Stage 2	n=15	4.2±1.2*
	Stage 3	n=15	4.1±1.1*

Table 3: OS markers in serum of non smokers and smokers with different PD stages *, p<0.05;

LP in serum of PD patients demonstrated progressively increasing values from stage 1 to stage 3 of PD. The highest level of LP observed in stage 3 was significantly greater than controls in both non smokers (3.9±0.9 µmol/l) ($p<0.05$) and smokers (4.1±1.1 µmol/l) ($p<0.05$) (Table 3).

DISCUSSION

Although FR showed higher values in serum than in periodontal tissue in healthy subjects, increasing FR production towards the severe stage of PD was much more evident in periodontal tissue in both non smokers and smokers. This is most likely because intravascular fluid is more frequently replenished with antioxidant agents such as albumin, bilirubin, uric acid, glutathione, ascorbic acid, and ubiquinol, which do not allow rapid FR increase in serum compared to periodontal tissue.

Concerning disease severity, higher the stage of PD, the more severe OS observed. This relationship was even more evident in the group of smokers suffering from PD. Thus, OS was demonstrated by increased FR and decreased AOC in periodontal tissue and serum of the patient groups, and also by increased serum LP process. The imbalance between the antioxidant defence and repair system and pro-oxidant mechanism of cell damage leads to tissue destruction either by increased free radical production or by a lowered AOC defence (16).

FRs such as superoxide anion, hydrogen peroxide and hydroxyl radicals, produced by neutrophils, can attack biological molecules. As an initial product, superoxide anion, generated by the molecular oxygen reduction under NADPH oxidase increases cell oxygen consumption, activates several cell surface G proteins and provokes a cascade of events, resulting in cell damage (17). Hydroxyl radicals damage important molecules, such as DNA, proteins and lipids; hydrogen peroxide is known to cross nuclear membrane and damages DNA; and superoxide anion is involved in bone reabsorption. Moreover, hydrogen peroxide stimulates phosphorylation of nuclear factor kappa β (NFkβ) complex and facilitates nuclear translocation and causes production of proinflammatory cytokines including interleukin-2 (Il-2), interleukin-6 (Il-6), interleukin-8 (Il-8), β-interferon and tumour necrosis factor-α (TNF-α). All of these agents are well known as very important factors in the PD pathogenesis (18). Cigarette smoking boosts FR production in patients with PD, which is most evident in the severe stage of disease. This might be due to increased antioxidant consumption in smokers which impairs antioxidant body defence and causes OS progression. Systemic and local MDA as an end product of LP is increased by smoking and has a strong relation with the inflammation of periodontal tissue. Concerning low AOC, decreased activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase might be caused by smoking. Inflammation is greater in periodontal tissue of smokers, which can be a cause of increased metallothionein as a free radical scavenger (19, 20). In the study of Garg N. et al, 2006, in smokers, the analysed parameters such as LP, superoxide dismutase, catalase, glutathione and total thiol showed increased OS proportionally related to the number of cigarettes smoked per day (21). Nicotine affects gingival blood flow, cytokine production, neutrophil and other immune cell function, as well as connective tissue turnover, all of which can be responsible for overall effects on periodontal tissues (22, 23). Furthermore, involvement of salivary differed histamine and increased salivary calcium in smokers exacerbates PD (24, 25). Antioxidant agents may overcome this impairment and may attenuate disease progression by down regulating glutathione detoxification / redox buffering system and by inhibiting key transcription factors, which lead to bone reabsorption. The factors such as forkhead box (FoxOs) family members induce the expression of genes controlling defence against OS and promote cell survival by inhibiting cyclin in cell cycle. The stage of PD, linked to smoking, may be a critical marker of a susceptible immune system, also initiating cancer risk by a pro-oxidant inflammatory profile (26, 27, 28).

CONCLUSION

Based on our results, we may conclude that OS has a great influence on PD pathogenesis, which may also be a trigger mechanism leading to a further periodontal damage. This process may be further accelerated by cigarette smoking, thus revealing the link between nicotine presence

and increased FR production. More studies are required to clarify the therapeutic effects of efficient antioxidant agents for PD to arrest further periodontal tissue damage and contribute to better disease outcome.

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THE IMPORTANCE OF POTASSIUM CHANNELS IN THE MECHANISM OF THE RELAXING EFFECT OF PENTOXIFYLLINE ON ISOLATED RAT UTERI

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VAŽNOST RASTUĆIH KONCENTRACIJA PENTOKSIFILINA NA KONTRAKTILNOST IZOLOVANOG UTERUSA PACOVA U PRISUSTVU BLOKATORA KALIJUMSKIH KANALA

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Received / Primljen: 4.06.2012. Accepted / Prihvaćen: 19.06.2013.

ABSTRACT

Background. *Pentoxifylline is a methylxanthine derivative that is used to treat peripheral vascular disease. One of the mechanisms of action of pentoxifylline is the vasodilatation of blood vessels. This study examined the effect of increasing pentoxifylline concentrations on the contractility of isolated rat uteri in the presence of a potassium channel antagonist.*

Methods. *The uteri were isolated from virgin Wistar rats (180-220 g) and suspended in an isolated organ bath chamber containing De Jalon's solution and aerated with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C. Isometric contractions were recorded using an isometric force transducer (Ugo Basile). The preload of the preparation was approximately 1 g. The uteri were allowed to contract spontaneously or in the presence of Ca^{2+} (0.018 and 0.36 mM) and acetylcholine (ACh) and were treated with pentoxifylline.*

Results. *Pentoxifylline caused concentration-dependent inhibition of spontaneous rhythmic uterine activity and uterine activity caused by calcium Ca^{2+} (0.018 mM and 0,36 mM). We showed that the inhibitory effects of pentoxifylline depend on the type of muscle contractions activated and that the inhibitory effect is significantly stronger for spontaneous rhythmic activity and forin Ca^2 -induced contractions of isolated rat uteri$^+$. The relaxing effect of pentoxifylline depends on the calcium concentration in the medium. Pentoxifylline exerted the weakest relaxant effects on contractions induced by acetylcholine. In contrast to methylene blue, tetraethylammonium, or 4-aminopyridine, glibenclamide did not antagonise the relaxing effect of pentoxifylline on isolated rat uteri..*

Conclusion. *The results obtained suggest that the mechanism of action of pentoxifylline does not lead to the opening of K_{ATP} channels. However, the opening of BKCa and voltage-dependent Ca^{2+} channels had some role, but to varying degrees, in the mechanism of the relaxing effect of pentoxifylline on the spontaneous rhythmic activity and calcium-induced con-*

SAŽETAK

Cilj. *Pentoksifilin, koji se koristi za lečenje perifernih vaskularnih oboljenja, je derivat metilksantina. Jedan od načina delovanja pentoksifilina je prouzrokovanje vazodilatacije krvnih sudova. U ovom radu ispitivali smo efekat rastućih koncentracija pentoksifilina na kontraktilnost izolovanog uterusa pacova, u prisustvu blokatora kalijumskih kanala.*

Metode. *Uterusi, koji su izolovani od neparenih ženki pacova Wistar soja (180-220 g), držani su u kupatilu za izolovane organe na temperaturi od 37°C, u De Jalon-ovom rastvoru kroz koji je propuštana smeša gasova od 95% kiseonika i 5% ugljendioksida. Izometrijske kontrakcije su registrovane korišćenjem izometrijskog transdjusera Ugo Basile, pri opterećenju preparata od 1 g. Ispitivan je efekat pentoksifilina na kontrakcije za vreme spontane ritmičke aktivnosti i u prisustvu kalcijuma, Ca^{2+} (0.018 and 0.36 mM)) i acetilholina (ACh).*

Rezultati. *Pentoksifilin je prouzrokovao koncentracijski zavisnu inhibciju spontane ritmičke aktivnosti, kao i fazne aktivnosti prouzrokovane kalcijumom. Inhibicijski efekt pentoksifilina zavisio je od tipa aktivacije glatkog mišića uterusa. On je ispoljio značajno jači relaksirajući efekt na spontanu ritmičku aktivnost i kontrakcije prouzrokovane sa 0.018 mM kalcijuma. Njegov relaksirajući efekt zavisi i od koncentracije Ca^{2+} u medijumu. Najslabiji relaksirajući efekat pentoksifilina je zabeležen na acetilholinskom tipu aktivacije. Nasuprot metilenskom plavilu, 4-aminopiridinu i tetraetilamonijumu, glibenklamid ne antagonizuje relaksirajući efekat pentoksifilina na izolovanom uterusu pacova.*

Zaključak. *Dobijeni rezultati sugerišu da u mehanizmu relaksantnog delovanja pentoksifilina nije zastupljeno otvaranje ATP kalijumskih kanala. Međutim, otvaranje BKCa i voltažno zavisnih Ca^{2+} kalijumskih kanala ima izvestan značaj, ali u različitom stepenu, u mehanizmu relaksirajućeg delovanja pentoksifilina na spontanu ritmičku*

UDK: 615.256 / Ser J Exp Clin Res 2013; 14 (2): 55-64
DOI: 10.5937/SJECR14-3972

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tractions of isolated rat uteri. Our results provide additional confirmation of the dominance of the NO/cGMP signalling pathway in the mechanism of the relaxing effect of pentoxifylline (because the presence of methylene blue significantly antagonised this effect) in relation to the opening of potassium channels, especially K_{ATP} channels. These results indicate that pentoxifylline could be a potential tocolytic drug.

Keywords: *pentoxifylline, rat uterus, potassium channel blockers, glibenclamide (GLB), tetraethylammonium (TEA), 4-aminopyridine (4-A), methylene blue (MB)*

Running title: *Potassium Channels in the Mechanism of Action of Pentoxifylline*

aktivnost i kontrakcije prouzrokovane kalcijumom. Naši rezultati su dodana potvrda o dominaciji NO/cGMP signalinih puteva kojima pentoksifilin prouzrokuje relaksaciju glatkih mišičnih ćelija uterusa (jer metilensko plavilo značajno anntagonizuje njegov efekt), u odnosu na otvaranje kalijumskih kanala, posebno ATP zavisnih kalijumskih kanala. Rezultati ukazuju da bi pentoksifiin mogao da bude potencijalni tokolitički lek.

Ključne reči: *pentoksifilin, uterus pacova, blokatori kalijumskih kanala, glibenklamid (GLB), tetratilamonijum (TEA), 4-aminopiridin (4-AP) i metilensko plavilo*

Kratki naslov: *Kalijumski kanali u delovanju pentoksifilina*

INTRODUCTION

Potassium channels are abundantly represented in the smooth muscle of the uterus[1,2,3]. Recent results show that the mechanisms of action of well-known powerful vasodilators such as sodium nitroprusside (NO donor), protamine sulphate and minoxidil involve the opening of potassium channelss of action.[4,5,6,7]

Pretreatment with tetraethylammonium (TEA, 6 mmol/l), an inhibitor of BKCa channels and a suppressor of two types of voltage-gated K^+ channel currents, decreased the protamine sulphate-mediated relaxation of both spontaneous and Ca^{2+}-induced contractions. An inhibitor of voltage-gated K-channels, 4-aminopyridine (4-AP, 1 mmol/l), and a selective ATP-sensitive potassium channel blocker, glibenclamide (2 μmol/l), decreased the protamine sulphate-mediated relaxation of Ca^{2+}-induced contractions, without effects on the spontaneous contractions. Protamine sulphate acts downstream of cAMP- and cGMP-mediated phosphorylation at the level of BKCa and Kv-channels in spontaneous contractions and at the level of all three K-channel types in Ca^{2+}-induced contractions.[7]

Various types of tocolytics (most frequently $beta_2$-adrenergic agonists) are used to prevent premature miscarriage and birth, but they have not yet solved these problems due to insufficient effectiveness and multiple side effects.[8] For this reason, it is important to find new drugs with potential tocolytic characteristics, including calcium agonists, potassium channel openers and other vasodilators.[9] It has been shown that even otomolar concentrations of nicardipine inhibit the spontaneous rhythmic activity of isolated uteri.[10] Nitric oxide (NO), a mediator and modulator of numerous processes in the body under physiological and pathological conditions, plays a significant role in the mechanism of the relaxing effects of protamine sulphate and other vasodilators on smooth muscles.[11] In cells, NO is created due to NO synthesis.[12] High doses of L-arginine increase blood flow through the blood vessels of the heart, mesentery, lungs and liver, without effecting the total peripheral resistance or blood pressure.[13] L-arginine, however, can cause significant hypotension in normotensive rats, but only in animals pretreated with physostigmine.[14] Experiments on animals have shown the presence of increased NO synthesis during normal pregnancy. In humans, a lack of NO causes vasoconstriction and preeclampsia. NO is characterised by extreme reactivity to intracellular enzymes. Additionally, NO affects the activity of guanylate cyclase (GC). The reaction between NO and GC can be inhibited with methylene blue.[15]

Pentoxifylline, a drug used to treat peripheral vascular disease, is a methyl xanthine derivative. Potential new indications for the application of this drug are under intensive study, as are its drug's mechanism of its action, which has not been sufficiently investigated on the molecular level.[16]

In our previous studies, we have shown that the endothelium plays a significant role in the pentoxifylline-induced relaxing of isolated rat mesenteric arteries. In the present experiment, we studied the effects of increasing pentoxifylline concentrations on various types of activation of isolated rat uteri (spontaneous rhythmic activity and contractions induced by calcium chloride at concentrations of 0.018 mM and 0.36 mM and by acetylcholine-ACh).

To elucidate the mechanism of action of pentoxifylline on the smooth muscles of the uterus, we studied the effects of this compound in the presence of: methylene blue, a guanylate cyclase blocker; tetraethylammonium (TEA, 6 mM), a potassium channel antagonist, an inhibitor of BKCa channels and a suppressor of two types of voltage-gated K^+ channel currents; 4-aminopyridine (4-AP, 1 mM), an inhibitor of voltage-gated K-channels; and glibenclamide (2 μM), a selective ATP-sensitive potassium channel blocker.

MATERIALS AND METHODS

All protocols for handling rats were approved by the local Ethical Committee for Animal Experiments, which strictly follows international regulations. Isolated uteri of virgin Wistar rats (200–250 g) in oestrus, as determined by daily vaginal lavage, were used in this study. The uterus was

suspended in an isolated organ bath chamber (Ugo Basile) containing De Jalon's solution (g/l: NaCl 9.0, KCl 0.42, NaHCO3 0.5, CaCl2 0.06, glucose 0.5) and aerated with 95% O_2 and 5% CO_2. The temperature was maintained at 37°C. Isometric contractions were recorded using an isometric force transducer (Ugo Basile). The uteri, either spontaneously active or treated with Ca^{2+} (6 mM), were allowed to equilibrate at 1 g tension before the experimental drugs were added. After stable spontaneous contractions had been established (approx. 20 min), the uteri were treated with increasing concentrations of pentoxifylline (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM) until total cessation of contractions. To explore the mechanism of action of pentoxifylline on the smooth muscle of the uterus, we studied the effects of this compound in the presence of tetraethylammonium (TEA, 6 mM), 4-aminopyridine (4-AP, 1 mM), glibenclamide (2 μM) and methylene blue (0.9 μM). Each of the substances was added to De Jalon's solution 10 min before pentoxifylline.

The effects of the treatments on uterine contractions were calculated as the percentage of the control or untreated contractions. All data are expressed as the mean ± SD. Differences between groups were evaluated by two-way ANOVA with treatment and dose as factors and were considered statistically significant when $p<0.05$.

Pentoxifylline, methylene blue, tetraethylammonium (TEA), 4-aminopyridine (4-AP) and glibenclamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The salts for De Jalon's solution were obtained from ZORKA Pharma (Sabac, Serbia) and Merck (Darmstadt, Germany). All drugs were dissolved in distilled water, except for glibenclamide, which was dissolved in polyethylene glycol.

RESULTS

Influence of different types of uterine activation (spontaneous rhythmic activity and contractions induced by 0.018 and 0.36 mM Ca^{2+} and acetylcholine) on the relaxant effects of pentoxifylline

Increasing pentoxifylline concentrations (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM) caused the concentration-dependent inhibition of spontaneous rhythmic activity and contractions of isolated rat uteri induced by different concentrations of calcium chloride (Ca^{2+}, 0.018 and 0.36 mM) and by acetylcholine (ACh) (ANOVA, statistically significant effect of the concentration of pentoxifylline, $p<0.001$, Figure 1). However, the degree of relaxation depended on the type of activation (ANOVA, effect of type of activation, $p<0.001$ and

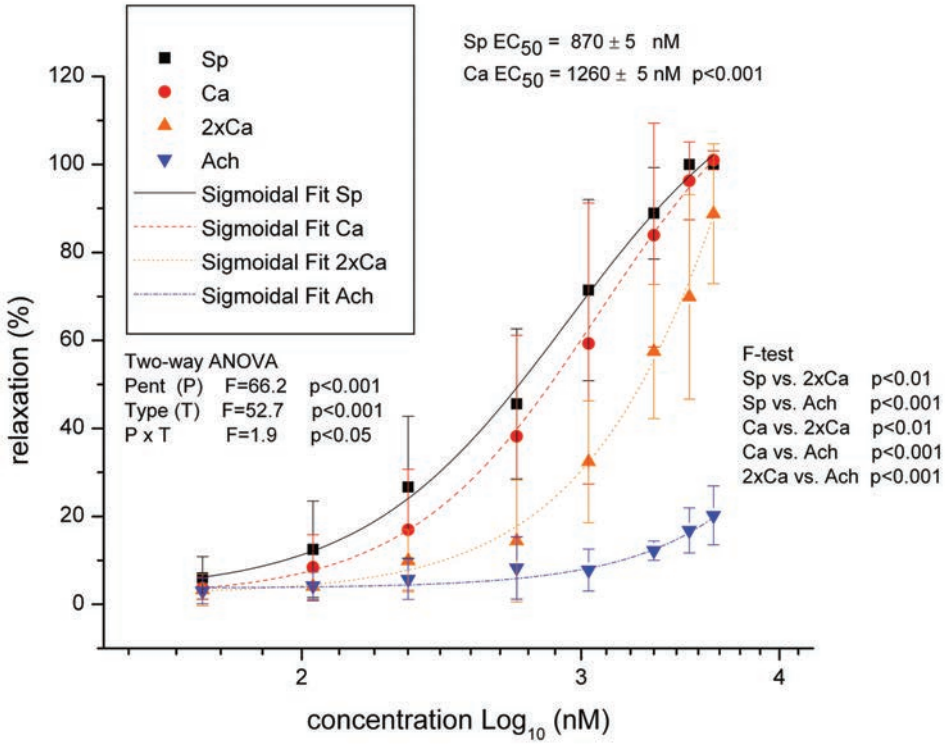

Fig. 1. Relaxation of *spontaneous rhythmic activity* and of Ca^{2+}- (0.018 and 0.36 mM) and ACh-induced *activity of isolated rat uteri* treated with pentoxifylline (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM). The black curve indicates the relaxant effect of pentoxifylline *on spontaneous activity (Sp)*. The red curve indicates the relaxant effect of pentoxifylline on calcium (Ca^{2+})-induced activity (0.018 mM). The red curve with triangles indicates the relaxant effect of pentoxifylline with a double concentration of calcium in the medium (2 x Ca, 0.36 mM). The blue curve indicates the relaxant effect of pentoxifylline on acetylcholine-induced activity (ACh). The results are presented as the mean ± standard deviation (n = 8-12 experiments). The data were analysed using two-way ANOVA with concentration (C) and activation type (T) as factors ($p<0.05$ was considered significant). Pentoxifylline significantly relaxed spontaneous and Ca-activated rat uteri (ANOVA significant effect of concentration (C), $p<0.001$). The effect of pentoxifylline on ACh-induced uterine contractions was not significantly different from that on the other types of activation (significant ANOVA type effect (T), and C x T interactions). Sigmoidal fit curves were constructed and compared with the F-test. EC_{50} values were extracted from the curves and compared with the t-test. The statistical significance of the differences is shown. The sigmoidal fit of the ACh-induced curve was not statistically significant ($Chi^2/DoF< 0.05$).

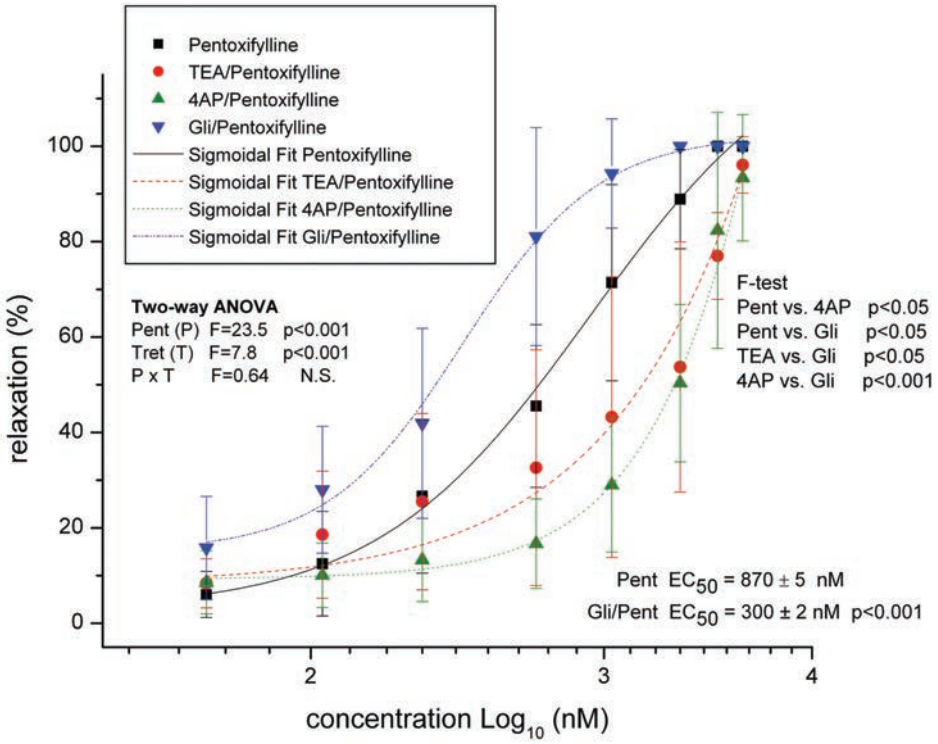

Fig. 2. Relaxation of spontaneous active rat uteri treated with pentoxifylline (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM) and pretreated with TEA, 4-AP or glibenclamide. The data are expressed as the mean ± SD (n = 8-12). The red curve indicates the relaxant effect of pentoxifylline in the presence of tetraethylammonium (TEA/Pent), the blue curve indicates the relaxant effect of pentoxifylline in the presence of glibenclamide (GLB/ pentoxifylline), the green curve indicates the relaxant effect of pentoxifylline in the presence of 4-aminopyridine (4-AP/ pentoxifylline), and the black curve shows the relaxant effect of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri(control curve). The data were analysed using two-way ANOVA with concentration (C) and pretreatment (T) as factors ($p<0.05$ was considered significant; NS – not significant). Sigmoidal fit curves were constructed and compared using the F-test. EC_{50} values were extracted from the curves and compared with the t-test. The statistical significance of the differences is shown. The sigmoidal fit of the curve for TEA-pretreated uteri was not statistically significant ($Chi^2/df=0.24$).

interaction P x T $p<0.05$). Pentoxifylline exhibited stronger relaxing effects on the spontaneous rhythmic activity and the contraction of the uterus induced by 0.018 mM Ca^{2+} (lowest EC_{50}). Pentoxifylline exerted the weakest relaxant effects on the contractions induced by acetylcholine.

The relaxing effect of pentoxifylline was lower for the Ca^{2+}-stimulated and activity compared to the spontaneous rhythmic activity, which is reflected by the significantly higher EC_{50} for this type of active uterus. The relaxation curves for the spontaneously contracting and Ca^{2+}-activated uteri were statistically modelled using the sigmoid model (Chi^2/DoF <0.05), whereas in the activated uteri, the calcium double (0.36 mM Ca^{2+}) sigmoid model was not significant due to the lack of a dose that would cause the maximum relaxation effect.

For uterine contractions induced by acetylcholine (ACh), the pentoxifylline concentrations used were too low to cause significant concentration-dependent relaxation, and thus, the effects could not be modelled in a sigmoid manner. A comparison of the F-test curves showed that the curves for active and spontaneous Ca^{2+}-stimulated active uteri have similar slopes and are significantly different compared to the other two types.

Influence of the potassium channel blockers glibenclamide (GLB), 4-aminopyridine (4-AP) and tetraethylammonium (TEA) on the relaxant effect of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri.

To elucidate the mechanism of pentoxifylline's relaxant effect on the smooth muscles of the uterus and the possible role of potassium channels, that which are abundantly present in the smooth muscle of the uterus, we studied the effects of this compound in the presence of potassium channel blockers including TEA (an inhibitor of BKCa channels and a suppressor of two types of voltage-gated K^+ channel currents), 4-AP (an inhibitor of voltage-gated K-channels) and GLB (a selective ATP-sensitive potassium channel blocker) on the spontaneous rhythmic activity of isolated rat uteri. In a second series of experiments, we completed the same studies infor calcium- induced contractions.

When a uterus with spontaneous rhythmic activity was pretreated with TEA, 4-AP or glibenclamide, the activity level depended on the concentration of the reserves of the relaxant pentoxifylline (ANOVA, statistically significant effect of the concentration of pentoxifylline, $p<0.001$, Figure 2), but this does not significantly change the degree of relaxation, depending on the pretreatment (ANOVA, statistically significant effect of pretreatment $p<0.001$; the differences are reflected in the EC_{50} values and the effects of the sigmoid comparisons of the modelled fault). Pretreatment with glibenclamide resulted in a statistically

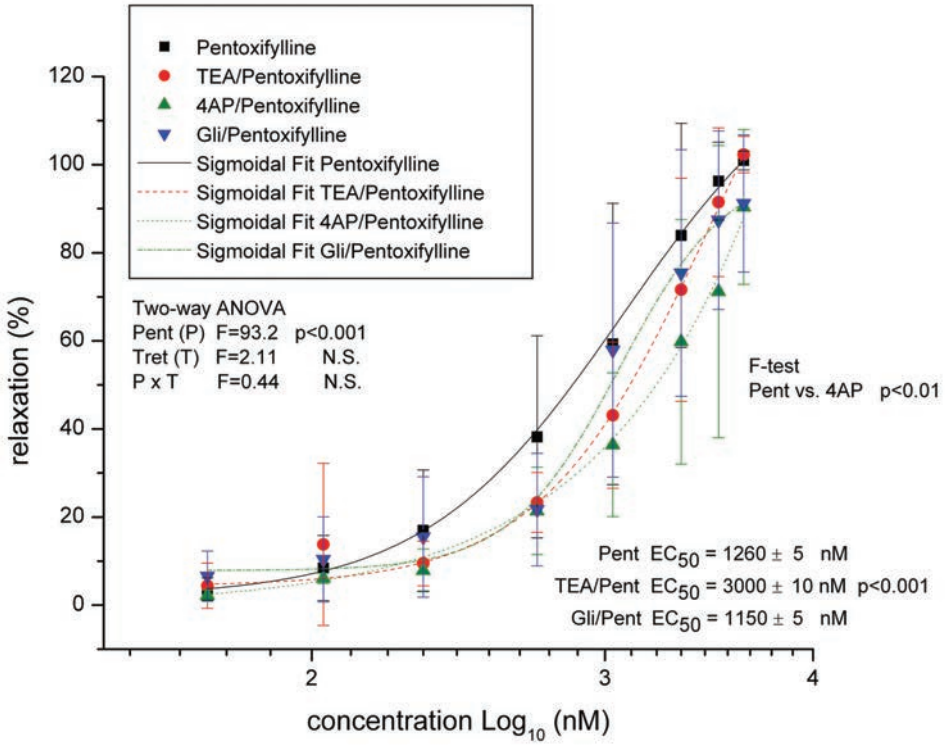

Fig. 3. Relaxation of Ca^{2+}-activated rat uteri treated with pentoxifylline (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM) and pretreated with TEA, 4-AP or glibenclamide. The data are expressed as the mean ± SD (n = 8-12). The red curve indicates the relaxant effect of pentoxifylline in the presence of tetraethylammonium (TEA/ pentoxifylline), the blue curve indicates the relaxant effect of pentoxifylline in the presence of glibenclamide (GLB/ pentoxifylline), the green curve indicates the relaxant effect of pentoxifylline in the presence of 4-aminopyridine (4-AP/ pentoxifylline), and the black curve represents the relaxant effect of pentoxifylline on the calcium-induced activity of isolated rat uteri (control curve). The data were analysed by two-way ANOVA with concentration (C) and pretreatment (T) as factors ($p<0.05$ was considered significant; NS – not significant). Sigmoidal fit curves were constructed and compared with the F-test. EC_{50} values were extracted from the curves and compared with the t-test. The statistical significance of the differences is shown. The sigmoidal fits of the curves for the uteri pre-treated with 4-AP and glibenclamide were not statistically significant (Chi^2/ df=0.2 and 0.09, respectively).

significant increase in the relaxing effect of pentoxifylline (significantly lower EC_{50} and a different slope expressed by the F-test, Figure 2), whereas pretreatment with 4-AP significantly decreased the relaxation effect, especially in the lower concentration range (statistically expressed by the F-test; 4-AP pretreatment did not change the relaxant effect of the highest dose of pentoxifylline).

In this series of experiments, we showed that the presence of glibenclamide (2 mM) **stimulated** the relaxant effect of increasing concentrations of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri. In control experiments without glibenclamide, pentoxifylline caused significant inhibition of the spontaneous rhythmic activity at a concentration of 19.2 mM. However, in most experiments, a pentoxifylline concentration of 6.6 mM was sufficient for the total inhibition of spontaneous rhythmic contractions in the presence of glibenclamide (Figure 2).

Influence of potassium channel blockers (glibenclamide, 4-aminopyridine and tetraethylammonium) on the relaxant effect of pentoxifylline on the contractions of isolated uteri induced by calcium

According to the analysis of variance, pretreatment of a Ca^{2+}-induced active uterus with TEA, 4-AP or glibenclamide did not affect the degree of relaxation induced by pentoxifylline (ANOVA, no statistically significant effect of pretreatment; Figure 3). However, a comparison of the EC_{50} values and the comparison of the F-test curves are-indicate that pretreatment with TEA reduced the relaxant effect of pentoxifylline (statistically significant difference in the EC_{50} values relative to pentoxifylline alone), mainly due to its efficacy at lower concentrations. Pretreatment with 4-AP decreased the relaxing effect of higher concentrations of pentoxifylline, and thus, the pentoxifylline applied after pretreatment with 4-AP could achieve the maximum relaxation effect, which led to statistically significant differences in the slope of the sigmoid curves (F-test, $p<0.05$).

Influence of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri in the presence of methylene blue

In these experiments, the effects of increasing concentrations of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri were studied in the presence of methylene blue (0.9 mM). Methylene blue (a guanylate cyclase blocker) statistically significantly ($p<0.001$) antagonised the relaxing effect of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri. For example, in the presence of methylene blue, even the highest concentration of pentoxifylline did not cause complete inhibition of uterine contractions (Figure 4.).

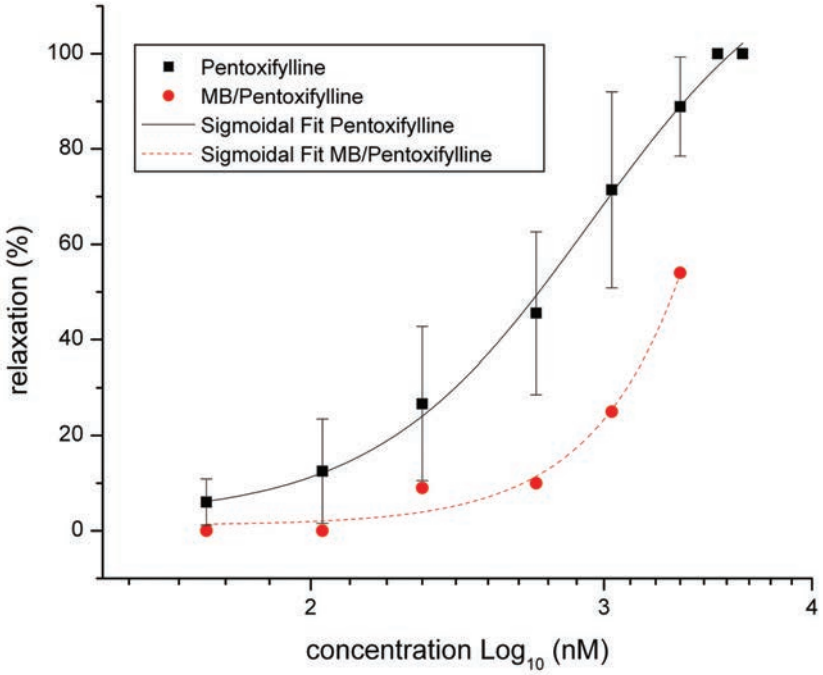

Fig. 4. The relaxing effect of increasing concentrations of pentoxifylline (0.05, 0.11, 0.22, 0.54, 1.08, 2.16, 3.23 and 4.31 mM) on the spontaneous rhythmic activity of isolated rat uteri and on the spontaneous activity of isolated rat uteri pretreated with methylene blue (MB). The red curve indicates the relaxant effect of pentoxifylline in the presence of MB/ pentoxifylline. The blue curve is the control experiment with pentoxifylline without MB. The results are presented as the mean ± SD (n = 8-12 experiments). The statistical significance of the effects was tested by two-factor ANOVA, with the concentration of pentoxifylline (P) and the type of pretreatment (T) as factors. Curves were modelled using the sigmoid model and compared by the F-test. The F-test comparison showed a statistically significant difference between the modelled sigmoid curves. Pretreatment with methylene blue significantly antagonised the relaxant effect of increasing concentrations of pentoxifylline ($p<0.001$).

DISCUSSION

Pentoxifylline induced the concentration-dependent inhibition of spontaneous rhythmic activity and contractions in isolated rat uteri induced by calcium (0.018 mM and 0.16 mM) and acetylcholine (ACh). We showed that the degree of the inhibitory effect of pentoxifylline depends on the type of muscle activation and the effect of this compound is significantly stronger on spontaneous rhythmic activity (lowest EC_{50}) and contractions caused by 0.018 mM calcium. In contrast, this effect is very weak when the uterus is stimulated by ACh. The activation of isolated uteri with double the amount of calcium reduced the relaxant effect of pentoxifylline so that even at the highest concentrations, there was no complete relaxation. Compared to the results obtained from the spontaneous rhythmic activity, the relaxant effect of pentoxifylline was weaker for calcium-induced contractions of the isolated uteri. These results show that pentoxifylline may be a potential drug that could be used to prevent premature births and miscarriages.

Our results show that the degree of pentoxifylline's inhibitory effect depends on the muscle activation type are in accordance with the data in the literature regarding calcium antagonist's effects on the contraction of the smooth muscle of the uterus. However, differences in the inhibitory effects of vasodilatory substances are most pronounced in experiments with calcium antagonists. For instance, nicardipine and nitrendipine have the most potent inhibitory effects on the contractions of isolated rat uteri caused by electrical stimulation (inhibited by otomolar concentrations), with weaker effects on spontaneous rhythmic activity and acetylcholine-induced activity. The weakest effect is on oxytocin-induced activities (inhibited by micromolar concentrations).[10, 31]

Our experiments and those of other authors also showed that pentoxifylline has a weaker relaxing effect on muscle activity in the presence of added extracellular calcium than on spontaneous rhythmic activity. This result suggests that spontaneous rhythmic muscle activity could depend on the calcium concentration present in the cell to a greater extent than previously expected.[5] Protamine sulphate also caused concentration-dependent relaxation infor both types of contractions.[6]

Spontaneous rhythmic muscle activity is achieved by calcium influx into the cells from extracellular spaces through calcium channels located in the cellular membrane. This signifies that spontaneous rhythmic activity depends primarily on the calcium concentrations present outside the cell. Other types of muscle activation (caused by acetylcholine or oxytocin) depend more on intracellular calcium or calcium that enters the cell via the activation of muscarinic and oxytocin receptors.[17, 18]

Potassium channels are present in numerous types of smooth muscle, including the uterus.[3,9,19,30,31] Recent results show that the mechanisms of action of some known potent

vasodilators, such as sodium nitroprusside (NO donor) and minoxidil, involve the opening of potassium channelss of action.[3,4,7,20]

One of the most studied types, which is dominant in the smooth muscles of the uterus, is the large calcium dependent potassium channel, BK_{ca} or maxi K. During gestation, its importance is even greater, especially during delivery because its inhibition provides an increased level of intracellular Ca^{2+}, which is necessary for birth contractions.[21] Additionally, there is the important role of ATP-dependent potassium channels (K_{ATP}) in the smooth muscles of the uterus. They form the connection between the metabolic state of the cell and cell excitation, i.e., contractility.[21,22,27,28,29,31]

Changes in the expression and activity of potassium channels are very important for the control of uterine contractility.[9] For example, H_2O_2 induces concentration-dependent relaxation of isolated rat uteri via potassium "voltage-dependent channels".[23,31] Based on these findings, studying the influence of potassium channel modulators and their interactions with NO on uterine tissue is important for both uterine physiology and pathophysiology studies and for finding new therapeutic concepts in the treatment of uterine contractility disturbances.[6,9,10]

H_2O_2 oxidises intracellular thiol groups in the potassium voltage-related channel and activates the channel, inducing relaxation. The cell's antioxidative defence system quickly activates the glutathione peroxidase (GSHPx) defence mechanism, but not the catalase (CAT) defence mechanism. Intracellular redox mechanisms repair the oxidised sites and again establish the deactivated state of *KV* channels, recuperating contractility. In conclusion, this activity demonstrates that *KV* channels can be altered in a time-dependent manner by reversible redox-dependent intracellular alterations.[31]

Together with detailed information about K^+ channels, there is a pronounced pharmaceutical interest in the synthesis and development of selective K^+ channel modulators. Scientists are also reevaluating the spectrum of existing drugs and substances that influence the permeability of smooth muscle cell membranes for K^+ ions, especially those vasodilators with-direct effects: minoxidil, diazoxide, and dimethyl sulfoxide (DMSO), which is a widely used solvent.[2,5,6,10,30]

Because there is insufficient data demonstrating whether, in addition to the NO/cGMP[5] system, potassium channels (channel type and role) also participate in the mechanism of the relaxant effect of pentoxifylline, we investigated the effect of this compound in the presence of various potassium channel blockers: glibenclamide (K_{ATP} channel blocker), 4-aminopyridine (BK_{Ca} blocker) and tetraethylammonium (voltage dependent Ca^{2+} channel blocker).

In these experiments, we showed that the opening of potassium channels does not play as important a role in the mechanism of action of pentoxifylline as in the case of pinacidil or protamine sulphate. Generally, in both types of activation, the opening of K_{ATP} channels is not important for the relaxant effect of pentoxifylline.

In our experiments, we found that the relaxing effect of pentoxifylline was higher in the presence of glibenclamide, a selective blocker of K_{ATP} channels. The complete inhibition of contractions in the presence of glibenclamide was achieved by lower pentoxifylline concentrations.

Pretreatment with glibenclamide, a K_{ATP} channel blocker, even led to a statistically significant increase in the relaxing effect of pentoxifylline. Glibenclamide, an antagonist of this type of potassium channel, led to prolonged depolarisation and prevented the output current of potassium that is responsible for the repolarisation of the cell membrane.

However, it has been shown that glibenclamide leads to the inhibition of contractions of arterial muscle elements, most likely by interacting with and blocking voltage-dependent calcium channels.[24,25] Our results could be interpreted in the same way. It is possible that glibenclamide stimulates the relaxing effect of pentoxifylline by blocking voltage-dependent calcium channels, thus decreasing the flux of calcium into the cell and causing relaxation of the muscles.[5]

However, pretreatment with 4-AP (BK_{Ca} blocker) significantly reduced the relaxant effect of pentoxifylline on spontaneous rhythmic activity but did not change the relaxant effect of the highest concentrations of pentoxifylline. For calcium-induced activation, TEA (a voltage-dependent Ca^{2+} channel blocker) reduced the relaxant effect of pentoxifylline, mostly because of its efficiency at lower concentrations. This result is dissimilar to the pretreatment with 4-AP, which decreased the relaxant effect of higher concentrations of pentoxifylline; in the presence of 4-AP, pentoxifylline could not reach its maximum relaxant effect (F-test, $p<0.05$). These findings indicate that the mechanism of action of pentoxifylline is not related to the opening of K_{ATP} channels, as is the case for other vasodilators, such as minoxidil and pinacidil. However, the opening of BK_{Ca} and voltage-dependent Ca^{2+} channels has some role, but to varying degrees, in the mechanism of the relaxing effect of pentoxifylline on spontaneous rhythmic activity and contractions of isolated rat uteri induced by calcium.

Our results are an additional confirmation of the dominance of NO/cGMP in the mechanism of the relaxing effect of pentoxifylline in relation to the opening of potassium channels, especially K_{ATP} channels. Given that the relaxant effect of pentoxifylline depends on the calcium concentration (it is weaker if the contractile activity is induced with 0.16 mM than with 0.018 mM), we can say that pentoxifylline, to some extent, acts as a calcium antagonist. Our results also suggest that pentoxifylline may be a potential tocolytic agent. However, to reach a definitive conclusion, it is necessary to test the relaxing effect of pentoxifylline on the uterus in vivo in order to monitor for possible side effects.

In this series of experiments, we found that all three K^+ channel blockers used in the experiments antagonised the inhibitory effect of pentoxifylline on spontaneous rhythmic activity and phase activity caused by calcium to some extent. However, unlike our results obtained with protamine and pinacidil, glibenclamide exerted a poor (statistically

insignificant) antagonistic effect at a concentration of 3 μM, while for 4-AP and TEA, only concentrations of 3 mM proved more effective. However, only 4-AP had a somewhat significant effect on pentoxifylline inhibition.

Our results show that the opening of potassium channels (especially of the K_{ATP} type) does not have any significant role in the relaxant mechanism of pentoxifylline, except to a certain extent for BKCa calcium channels. In contrast to these results with pentoxifylline, our results and the results of other authors with the relaxant pinacidil (a calcium channel opener) show that calcium channel opening, especially that of the K_{ATP} type, plays a significant role in this compound's mechanism of action[3,5,6,9]. The role of potassium channel opening in the relaxant effect of vasodilators seems to depend on the type of tissue.[24] Likely, potassium channel opening is more important for the relaxant effect of certain vasodilatory substances in the case of blood vessels than in the case of the uterus.

Methylene blue inhibits the production of cGMP by preventing the interaction of NO with guanylate cyclase. The results of our earlier series of experiments[5] and the present experiments (no= 12) with new statistical analyses of these results also showed that methylene blue decreased the relaxing effect of pentoxifylline on the spontaneous rhythmic activity of isolated rat uteri. Not even the highest applied concentration of pentoxifylline achieved complete discontinuation of contractions.

The data indicating that methylene blue antagonises the relaxing effect of Na-nitroprusside[12] and pentoxifylline[5] are in accordance with both our earlier[5] and present results. Methylene blue makes the activation of guanylate cyclases impossible and thus prevents muscle relaxation. cGMP activates cGMP-dependent protein kinase, which blocks the entrance of calcium ions, activates potassium channels and decreases the IP3 level, finally leading to vasodilatation. However, it has been shown that methylene blue cannot antagonise the relaxing effects of sodium azide and nitroglycerol.[25] Additionally, pretreatment with methylene blue (0.3 μg/ml) had no effect on protamine-induced ation, despite the use of N(G)-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M) and propranolol (5 μg/ml), which potentiated the protamine's relaxing effect on the spontaneous contractile activity of isolated rat uteri and had no effect on Ca^{2+}-induced contractions.[2] L-arginine in the cell is used as the substance for the synthesis of NO and can raise NO concentrations, leading to smooth muscle cell relaxation. In human and animal models not able to produce NO due to endothelial dysfunction, it has been shown that L-arginine restores the lost ability of endothelium-dependent vasodilatation.

Unlike methylene blue (a guanylate cyclase blocker), L-arginine (an NO precursor) does not antagonise the relaxation effect of pentoxifylline on the contraction of isolated rat uteri, except partially in spontaneous rhythmic activity (without changing its maximum inhibitory effect).[5] These results suggest that pentoxifylline most likely achieves its relaxing effect on the uterus regardless of the presence of nitric oxide.

In contrast to our results, in experiments on isolated renal and mesenteric arteries taken from normotensive and hypertensive rats, it has been found that L-arginine even antagonised the relaxation caused by sodium nitroprusside.[18] The authors found a possible explanation for the noted phenomenon in the ability of sodium nitroprusside to achieve its action through peroxynitrate and not through S-nitrosothiol.

The results of our earlier series of experiments with L-arginine (0.3 μmol) and pentoxifylline[5] and the results of our present experiments with methylene blue suggest that NO is not significantly involved in pentoxifylline's effects. There are data regarding the existence of signalling pathways in the cell, including NO-independent cGMP creation, that do not lead to relaxation.[5]

In many papers, pentoxifylline is referred to as a phosphodiesterase blocker.[26] By blocking phosphodiesterase, pentoxifylline directly increases the cAMP levels in muscle cells, leading to relaxation. Because methylene blue (a guanylate cyclase blocker) decreased the inhibitory effects of pentoxifylline, it is possible that even cGMP is involved in muscle relaxation via signalling pathways not dependent on NO.

CONCLUSIONS

Pentoxifylline, which is used to treat peripheral vascular disease, is a derivative of methylxanthines. One of the mechanisms of action of pentoxifylline is through the vasodilatation of blood vessels. This study examined the effect of increasing concentrations of pentoxifylline on the contractility of isolated rat uterus in the presence of a potassium channel antagonist and methylene blue. Uteri were allowed to contract spontaneously or in the presence of Ca^{2+} (0.018 and 0.36 mM) and acetylcholine and were then treated with pentoxifylline.

Our results suggest that the mechanism of action of pentoxifylline does not include the opening of K_{ATP} channels. However, the opening of BKCa and voltage dependent Ca^{2+} channels has some role, but to varying degrees, in the mechanism of the relaxing effect of pentoxifylline on spontaneous rhythmic activity and the contraction of isolated rat uteri induced by calcium. Pretreatment with glibenclamide, a K_{ATP} channel blocker, led to a statistically significant increase in the relaxing effect of pentoxifylline. Glibenclamide, an antagonist of this type of potassium channel, led to prolonged depolarisation, preventing the output current of potassium that is responsible for the repolarisation of the cell membrane.

The degree of the inhibitory effect of pentoxifylline depended on the type of muscle activation; it was significantly stronger against spontaneous rhythmic activity (lowest EC_{50}) and contractions caused by a 0.018 mM calcium and was very weak when the uterus was stimulated by ACh. The activation of isolated uteri with double the amount of calcium reduced the relaxant effect of pentoxifylline so that even at the highest concentrations used there was no complete relaxation.

Compared to the effect on spontaneous rhythmic activity, the relaxing effect of pentoxifylline was lower for the contraction of isolated rat uteri induced by Ca^{2+}.

Our results provide additional confirmation of the dominance of the NO/cGMP signalling pathway in the mechanism of the relaxing effect of pentoxifylline (because the presence of methylene blue significantly antagonised this effect) in relation to the opening of potassium channels, especially K_{ATP} channels. These results indicate that pentoxifylline could be a potential tocolytic drug.

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EVALUATION OF THE IMPACT OF ETHANOL ON THE NERVOUS CONTROL OF MICTURITION - THE ANALYSIS OF THE LINK BETWEEN THE CONCENTRATION OF ALCOHOL IN THE BLOOD AND THE VOLUME OF URINE IN THE BLADDER

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EVALUACIJA UTICAJA ETANOLA NA NERVNU KONTROLI MOKRENJA –ANALIZA VEZE IZMEĐU KONCENTRACIJE ALKOHOLA U KRVI I ZAPREMINE MOKRAĆE U MOKRAĆNOJ BEŠICI

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Received / Primljen: 22.06.2012. Accepted / Prihvaćen: 12.08.2013.

ABSTRACT

Numerous biochemical and electrophysiological studies have determined that the functions of certain receptors and ion channels for which the are in some way affected by ethanol. Some of these are involved in the neural control of micturition, and it is therefore possible that ethanol induces urinary retention. The aim of this study was to analyse the relationship between the concentration of alcohol in the blood and the volume of urine in the bladder measured during autopsy. The study was based on autopsy protocol data from 702 people. The inclusion criteria were age not exceeding 50 years and a blood alcohol level of at least 0.5 parts per thousand. A correlation between the blood alcohol concentration and a bladder urine volume over 200 ml can be portrayed as part of a range limited by curves asymptotically converging at a value of 3 per mil. This phenomenon may be explained by the effect that alcohol has on receptors involved in the micturition reflex, which should typically be engaged at approximately 200 ml of urine in the bladder. The lower limit of this range can be explained by GABA-A stimulation as well as the inhibition of NMDA receptors or by decreasing influx through calcium channels. Both of these effects inhibit the micturition reflex. However, the upper limit, which indicates reduced urine retention, may be caused by the action of high concentrations of ethanol at the ends of the adrenergic fibres, causing their dysfunction, or by changes in the intracellular calcium ion concentration.

Key words: *Alcohols, Bladder, Ethanol, Urine*

SAŽETAK

Brojne biohemijske i elektrofiziološke studije su utvrdile da su funkcije određenih receptora i jonskih kanala, na neki način, pogođene dejstvom etanola. Neki od njih su uključeni u nervnu kontrolu mokrenja, i zato je moguće da etanol izaziva retenciju urina. Cilj ove studije bio je da analizira odnos između koncentracije alkohola u krvi i zapremine urina u mokraćnoj bešici, merenih prilikom obdukcije. Studija je zasnovana na podacima iz obducionih protokola 702 ljudi. Kriterijumi za uključivanje su bili starost ispod 50 godina i količina alkohola u krvi najmanje 0,5 promila. Korelacija između koncentracije alkohola u krvi i zapremine urina u mokraćnoj bešici preko 200 ml može biti predstavljena kao deo opsega ograničenog krivama koje asimptotski konvergiraju ka vrednosti 3 promila. Ovaj fenomen se može objasniti efektom koji alkohol ima na receptore koji su uključeni u refleks mokrenja, koji se obično aktiviraju pri zapremini od oko 200 ml mokraće u mokraćnoj bešici. Donja granica tog opsega se može objasniti stimulacijom GABA-A receptora kao i inhibicijom NMDA receptora, ili smanjenjem influksa kroz kanale za kalcijum. Oba ova efekta inhibišu refleks mokrenja. Međutim, gornja granica, koja ukazuje na smanjenu retenciju urina, nastaje dejstvom visokih koncentracija etanola na završetke adrenergičkih vlakana, izazivajući njihovu disfunkciju, ili promene u intracelularnoj koncentraciji jona kalcijuma.

Ključne reči: *alkoholi, mokraćna bešika, etanol, urin*

UDK: 612.464:547.26 / Ser J Exp Clin Res 2013; 14 (2): 65-70
DOI: 10.5937/SJECR14-4072

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INTRODUCTION

Several case reports linking ethanol and urinary bladder dysfunction with resulting urinary retention have been published. [1,2] Animal studies using the urinary bladders of rabbits have demonstrated that ethanol increases bladder capacity and decreases detrusor muscle contraction activity. However, the mechanism regulating this phenomenon is unclear. [16] Opinions differ as to whether this is controlled by the effect of an increased intracellular calcium level, nitric oxide synthesis inhibition or the regulation of receptor function. [3, 4, 6]

Ethanol influences the functioning of receptors involved in the regulation of reflex micturition. GABA-A receptors inhibit the voiding reflex, whereas the NMDA receptors activate it. The influence of the autonomic nervous system is also important. Parasympathetic postganglionic axons in the pelvic nerve release acetylcholine (Ach), which results in bladder contraction by stimulating M3 muscarinic receptors in the bladder smooth muscle. However, sympathetic postganglionic neurons release noradrenaline (NA), which activates beta-3 adrenergic receptors to relax the bladder smooth muscle and alfa-1 adrenergic receptors to contract the urethral smooth muscle. [3]

MATERIALS AND METHODS

The study was performed via a retrospective analysis of autopsy protocols archived at the Forensic Medicine Department of Jagiellonian University Medical College from 2006 to 2010. The study group comprised 702 people younger than 50 years of age with a blood ethanol concentration at or above 0.5 per mill. We limited the age of the decease to a maximum of 50 years to exclude the influence of benign prostatic hyperplasia, which can cause urinary retention. The exclusion criteria also included the presence of drugs and toxic substances such as diazepam, carbamazepine, non-steroidal anti-inflammatory drugs, chlorpromazine, morphine and bencyclane in the blood sample. This action eliminated their influence on the results by causing urinary retention. [1] The statistical analysis was performed using Statistica (Statsoft, v8.0) software.

The statistical analysis was performed for all cases and a group without a history of chronic ethanol abuse. Urine volume was measured in millilitres (ml), and the ethanol blood concentration was per mills (ppm). Descriptive statistical analysis was performed for all cases. Ninety-five per cent confidence intervals (95% CI) were also calculated on the basis of the Poisson distribution. The distributions of the blood alcohol concentration and bladder urine volume were checked for normality with the use of the Shapiro-Wilk test. If P values were less than 0.01, there was no normality. However, when a data set is larger than 100 cases, parametric methods are the most appropriate. Simple linear correlations and linear multiple regression were used for the assessments.

RESULTS

Table 1. The results of the linear correlation analysis of urine volume in the urinary bladder and blood ethanol concentration in the two groups.

Data analysis was performed in the following groups:	Pearson's correlation coefficient	The coefficient of determination (R^2)	Level of significance (p-value)
All cases (n=702)	0.20	0.04	0.00001
Cases without alcoholics (n=473)	0.18	0.03	0.00007

The coefficient of determination showed the degree of fit of our model to a model with linear correlations between variables. According to the low level of correlation, which was 3-4%, there was no statistically significant linear correlation.

Analysed relationship to the variable: volume of urine in the bladder	All cases (n=702)	Cases without alcoholics (n=473)
blood alcohol level^2	Beta = 0.19 R^2 = 0.03 p = 0.00002	Beta = 0.16 R^2 = 0.02 p = 0.0001
blood alcohol level^3	Beta = 0.17 R^2 = 0.03 p = 0.00007	Beta = 0.12 R^2 = 0.01 p = 0.004
sqrt(blood alcohol level)	Beta = 0.2 R^2 = 0.04 p = 0.00001	Beta = 0.18 R^2 = 0.03 p = 0.00005
log(blood alcohol level)	Beta = 0.19 R^2 = 0.04 p =0.00001	Beta = 0.2 R^2 = 0.04 p = 0.00002
$e^{\text{blood alcohol level}}$	Beta = -0.01 R^2 = 0.006 p = 0.9	Beta = 0.002 R^2 = 0.0006 p = 0.8

Table 2. The results of a linearised linear regression of urine volume in the urinary bladder and blood ethanol concentration with the analysed factors.

When we considered the coefficients of determination, no nonlinear model could be described by any function that could explain this correlation.

A scatterplot of correlations between blood ethanol concentration and urine volume in the urinary bladder was made to investigate more advanced correlations.

In cases with a urine volume above 200 ml, we observed that dispersion of the blood ethanol concentration decreased. The scatterplot of correlations between blood ethanol concentration and urine volume in the urinary bladder could be limited by asymptotic curves convergent at a blood ethanol concentration of 3 per mills. This correlation was more noticeable in cases without a history of chronic alcohol abuse.

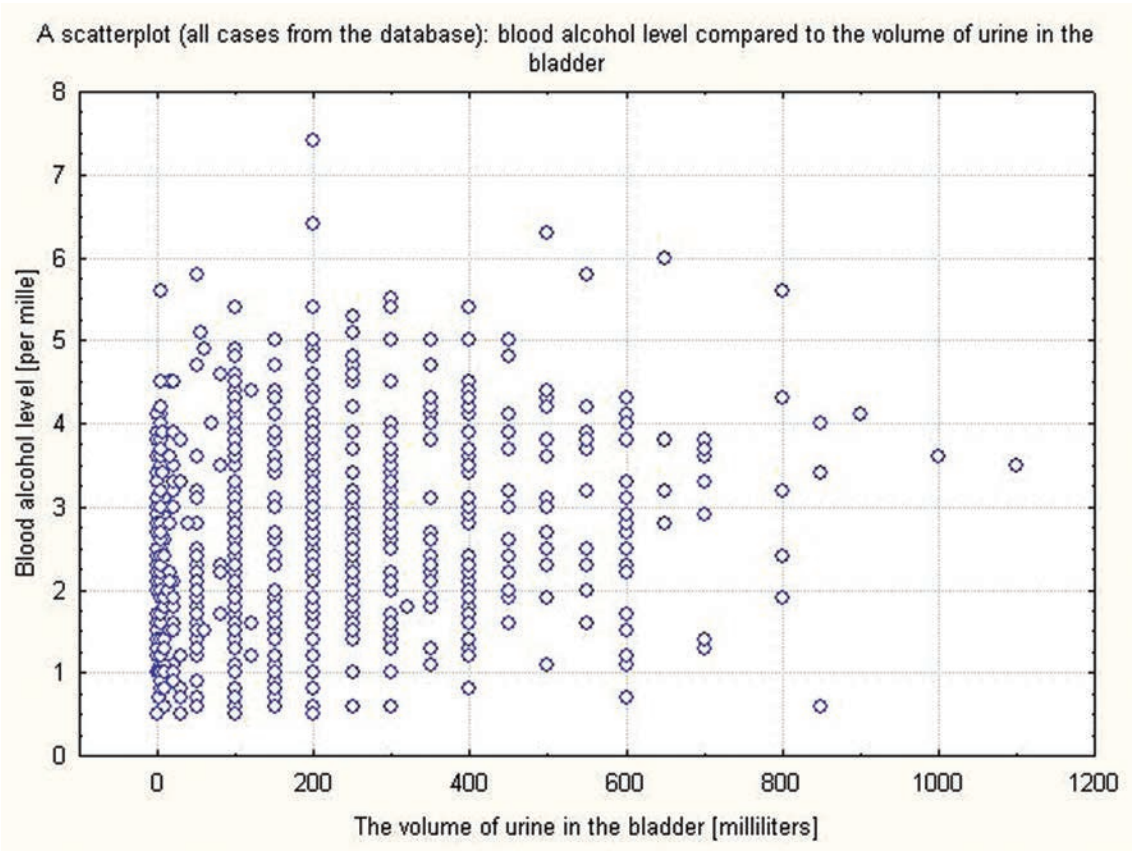

Figure 1. Figure showing the relation between urine volume in the urinary bladder and blood ethanol

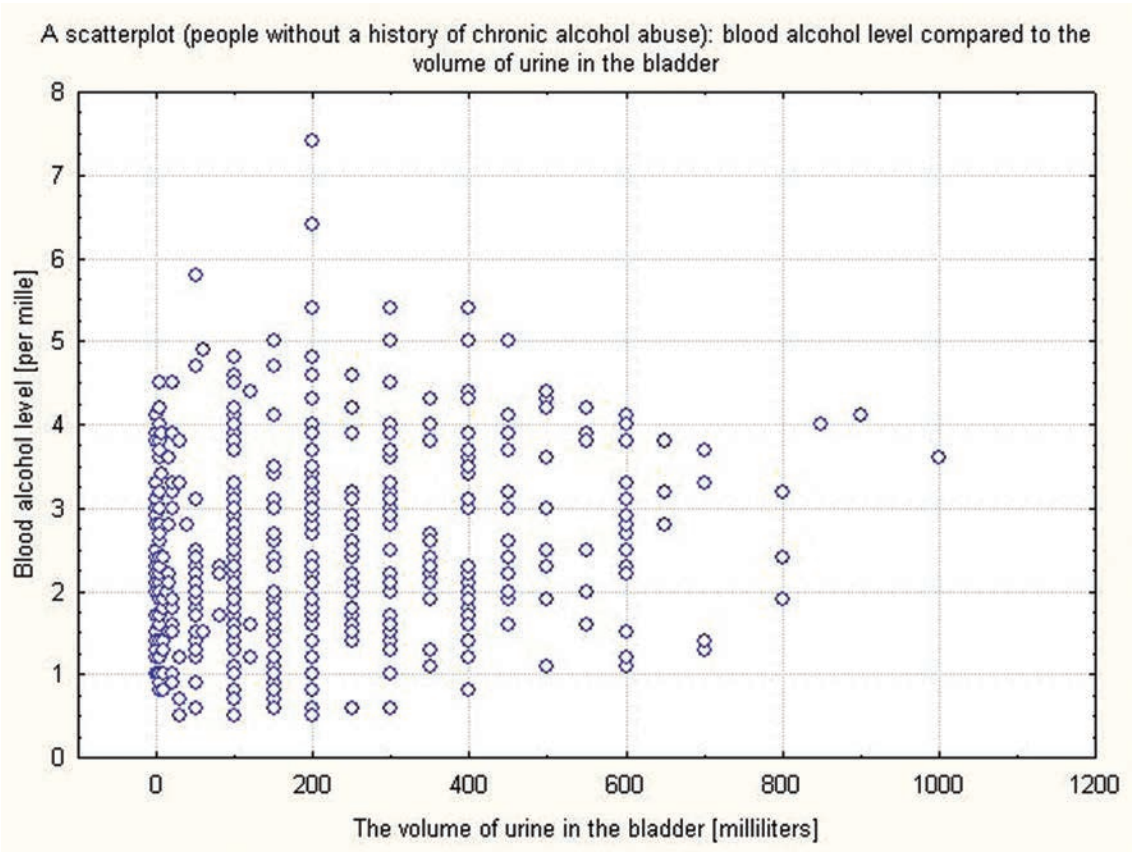

Figure 2. Relation of urine volume in the urinary bladder and blood ethanol concentration in the group without a history of chronic ethanol abuse.

Figure 3. A scatterplot of urine volume in the urinary bladder and blood ethanol concentration with asymptotic limiting curves (cases without a history of chronic ethanol abuse).

As shown in figure 3, limitation curves were created to gain a better visual understanding of our argument.

DISCUSSION

Molecular pharmacology studies have disclosed a few primary targets for ethanol such as N-methyl-D-aspartate (NMDA), γ-aminobutyric acid A (GABA-A), glycine, 5-hydroxytryptamine-3 (5-HT3), neuronal nicotinic acetylcholine (nACh) receptors and L-type Ca^{2+} channels. [5,7,8,9] Some of these neurotransmitter systems are responsible for the effects of ethanol on urine retention described in our study. Experiments performed on rats by Matsumoto have shown that the intravenous administration of an NMDA receptor antagonist blocks the bladder contractions evoked by electrostimulation of the PMC (pontine micturition centre), supporting the role of NMDA receptors in mediating excitatory transmission in the descending limb of the spinobulbospinal micturition reflex pathway. [18] In the next section, we describe the results of Lovinger's experiment, which confirmed the inhibitory effect of ethanol on NMDA receptors and may explain our findings. In Pehrson's experiment, the blockade of GABA-A and GABA-B receptors in the spinal cord and brain stimulated rat micturition, which confirmed the assumption that activation of GABA-A and GABA-B receptors may be responsible for the continuous inhibition of the micturition reflex within the CNS. [19] The results of studies confirming the stimulating effect of ethanol on GABA receptors and the implications of these findings with respect to our results are described in the next section. The action of ethanol on serotonin receptors cannot be considered because it affects subtype 5-HT3, whereas subtypes 5-HT1 and 5-HT7 are involved in controlling the micturition reflex. Additionally, the impact of ethanol on acetylcholine receptors cannot be considered because although several studies have confirmed its action on nicotinic receptors, its effect on muscarinic receptors remains unclear, and research has focused primarily on chronic abuse. [20,21] Göthert's experiment suggested a relation between the ethanol concentration and noradrenaline output. The noradrenaline output from peripheral sympathetic nerves was inhibited only by lethal concentrations of alcohol. Many studies have shown that noradrenaline causes detrusor muscle relaxation and inhibits adrenergic fibres, resulting in detrusor contraction. [22] The influence of ethanol on ion channels, particularly calcium channels, which subsequently increases the intracellular calcium concentration and may facilitate detrusor muscle constriction, might also be important. [16]

We separately analysed a group with a history of chronic alcohol abuse to investigate neuroadaptation of receptor systems caused by the effects of long-term ethanol use on neurotransmitters. Ethanol tolerance was caused by a decreased number of GABA-A receptors (down-regulation); changes in receptor structure and a decreased response by neurotransmitters; or an increased number of NMDA receptors (up-regulation). [5, 7-12] This adaptation of the receptor system to chronic activation/inhibition could have interfered with our outcomes if the receptor response participates in the effect of ethanol on micturition.

In our analysis, the described correlation in the group with a urine volume above 200 ml could be explained by the receptor response because micturition is activated by this amount of urine. [4] Moreover, after the exclusion of alcoholics, the scatterplot was more restricted, which could be explained by adaptation of the receptor system.

As shown in Figure 3, the lower limiting curve demonstrated increased urine retention. This could be the effect of micturition reflex inhibition or an inability to contract the detrusor muscle. The research of Lovinger on NMDA receptors in rat ganglions showed that ethanol inhibition was dependent on the ethanol concentration. Increasing inhibition potential was observed from a blood ethanol concentration of 0.12 per mills (2.5 mM) to 2.3 per mills (50 mM); a further concentration increase had no influence on the strength of receptor inhibition. The IC50 (the concentration that inhibits 50% of receptors) was 0.46 per mills (10 mM) [8-10]. Stimulated NMDA receptors activate the micturition reflex. Inhibition of NMDA receptors explains the increasing urinary retention observed at a low blood ethanol concentration. We hypothesise that the lower limitation curve at a blood ethanol concentration of 0.5 per mills to 3 per mills was caused by this mechanism. Our research indicates that ethanol activates GABA-A receptors at a blood concentration above 2.76 per mills (60 mM), but receptors containing the delta (δ) subunit were activated by a blood ethanol concentration of at least 0.14 per mills (3 mM). [5, 12] The GABA-A receptors that participated in the micturition reflex consisted of $\alpha_2\delta$ subunits, and their activation inhibited the micturition reflex and influenced the lower limitation curve. [11] Another explanation of the lower limitation curve could be the influence of ethanol on smooth muscle tension, which is regulated by calcium channels. This leads to decreased intracellular calcium inflow and limits detrusor muscle contraction. A study using PC12 cells showed this effect at ethanol concentrations of 1.15-1.38 per mills (25-30 mM). However, an ethanol concentration above 4.6 per mills (100 mM) increased the free intracellular calcium level. This effect could explain the upper limiting curve, when decreased urinary retention was caused by detrusor muscle contraction. [10, 15] The mechanism through which the calcium level was increased is unclear, but calcium can be released from intracellular depots. [6, 16] Ethanol may also cause adrenergic nerve fibres to malfunction, which could influence the upper limiting curve. Laboratory experiments on isolated rat detrusor muscle have shown that ethanol can weaken the beta-adrenergic response upon stimulation. [13] Research on noradrenaline nerve excretion in the presence of ethanol revealed 50% decreased excretion (IC50) in response to chemical stimulation at an ethanol concentration of 5.6 per mills (129 mM). [14] According to cited research, the inhibition of urine retention as an effect of a high ethanol concentration could be explained by adrenergic system inhibition and dominance of the cholinergic system. [17]

CONCLUSION

It has been shown that ethyl alcohol affects the micturition reflex and can cause urine retention. Ethanol inhibited the micturition reflex at a blood concentration below 3.0 per mills. Higher ethanol concentrations inhibited urinary retention via inhibition of the adrenergic system and activated the micturition reflex.

A low ethanol concentration affected NMDA receptors, GABA-A receptors and the intracellular calcium level, inhibiting the micturition reflex and leading to urinary retention. A higher ethanol concentration inhibited the adrenergic system and affected the intracellular calcium level, inhibiting urinary retention and facilitating detrusor muscle contraction.

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GRADATION CRITERIA FOR EXPERIMENTALLY INDUCED PERIAPICAL LESIONS IN MICE

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GRADACIONI KRITERIJUMI EKSPERIMENTALNIH PERIAPEKSNIH LEZIJA KOD MIŠEVA

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Received / Primljen: 20.05.2012. Accepted / Prihvaćen: 20.08.2013.

ABSTRACT

A periapical lesion occurs as periapical tissue reacts to a dental pulp anaerobic infection. This phenomenon may result from carious lesions, tooth fractures, or iatrogenic and other circumstances that allow for bacteria to penetrate into the pulpal tissues.

Objectives: *This study histologically evaluated experimentally induced periapical lesions using the Autodesk AutoCAD 2010 software. Additionally, based on the amounts of alveolar bone destruction sizes in the mouse model regions, a proposed experimental periapical lesion gradation criterion was created.*

Methods: *Twelve BALB/c mice were utilizised in a periapical model, whereby their mandibular right first molars were coronally opened, allowing for pulp exposure to host oral bacterial flora. These mice were sacrificed two and four weeks following pulp exposure. Following each sacrifice, hemi-mandibles were fixed in 4% paraformaldehyde, decalcified in 3% formic acid, embedded in paraffin and cut into 4-µm-thick sections. The sections were stained with haematoxylin-eosin and examined with light microscopy (40x). Section images that included the mandibular first molar distal roots and passed through the apical foramens were selected for analysis. The periodontal ligament sizes were measured using the Autodesk AutoCAD 2010 software.*

Results: *There was a highly significant lesgion size difference at the two different time points following the lesion induction (p=0.002). The periapical lesions were classified according to periapical bone resorption sizes. We determined the values of quartiles (25% and 75%) and median areas (50%) of the mean lesion values at both experimental periods. These data enabled for scoring of the lesions with grades from 1 to 4. The area of the normal periodontal ligament space was assigned with a grade of 0.*

Conclusion: *This newly designed gradation criteria represent a significant advantage compared with the previous descriptive methods used for determining periapical tissue bone destruction levels. It The criteria achieves this advantage by excluding subjectivity, facilitating a numerical presentation of the data and reducing the possibility of making errors by using the highly availably Autodesk AutoCAD 2010 software.*

Keywords: *Periapical lesion, inflammation, gradation*

SAŽETAK

Periapiksna lezija predstavlja reakciju periapeksnog tkiva na anaerobnu infekciju pulpe, koja najčešće nastaje kao posledica karijesa, frakture zuba, dejstva jatrogenih i drugih faktora koji omogućavaju prodor mikroorganizama u komoru puple.

Ciljevi: *Izračunati površine eksperimentalno indukovanih periapeksnih lezija korišćenjem programa Autodesk AutoCAD 2010, i na osnovu veličine prostora resorbovane alveolarne kosti u periapeksnom regionu predložiti kriterijume za gradaciju ovih lezija.*

Metode: *BALB/c miševima otvorena je pulpa prvog mandibularnog molara (n=12). Miševi su žrtvovani dve i četiri nedelje nakon indukcije lezija, izolovane su desne hemi-mandibule, fiksirane u 4% paraformaldehidu, dekalcifikovane u 3% mravljoj kiselini i ukalupljene u parafin. Zatim su napravljeni preparati debljine 4 µm. Nakon bojenja hematoksilin-eozinom, preparati su posmatrani svetlosnim mikroskopom (40x), fotografisani, i analizirani korišćenjem programa Autodesk AutoCAD 2010. Za analizu su izabrani preparati koji obuhvataju distalni koren prvog mandibularnog molara i prolaze kroz apikalni foramen.*

Rezultati: *Postojala je visoka signifikantna razlika između površina periapeksnih lezija izmerenih u dva različita vremenska termina nakon indukcije lezija (p=0,002). Određivanje vrednosti kvartila (25% i 75%) i medijane (50%) od prosečne vrednosti svih lezija omogućilo je gradiranje periapeksnih lezija brojevima od 1 do 4. Prostoru normalnog periodontalnog ligamenta dodeljen je gradus 0.*

Zaključci: *Novo-dizajnirani gradacioni kriterijumi predstavljaju značajnu prednost u odnosu na do sada opisane deskriptivne metode za određivanje stepena destrukcije kosti u periapeksnom regionu, jer isključuju subjektivnost, redukuju mogućnost pravljenja greški i olakšavaju numeričku prezentaciju podataka.*

Ključne reči: *Periapeksna lezija, inflamacija, gradacija*

UDK: 616.314-002 / Ser J Exp Clin Res 2013; 14 (2): 71-76
DOI: 10.5937/SJECR14-4340

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INTRODUCTION

A dental periapical lesion occurs as the periapical tissue reacts to anaerobic infections in the dental pulp [1]. The dental pulp is protected from oral cavity microorganisms by enamel and dentine. The exposure of the dental pulp to microorganisms and their products, as a consequence of carious lesions, tooth fractures, traumatic operative dental procedures, or iatrogenic and other circumstances, can trigger local inflammatory responses [2]. The progression of such infections and inflammation may result in pulp necrosis. Subsequently, the periapical tissues may be affected following the egress of the microorganisms, usually from an infected root canal [3]. The host's response to these infections appears to be similar to the response to bacterial infections elsewhere in the body, with the additional feature that the alveolar bone surrounding the dental root apex often becomes resorbed [4]. This response is characterised by the persistent migration of inflammatory cells to the infected sites [5]. Together with the resident connective tissue cells, the migrated cells release several mediators, which contribute to infection progression curtailment. This inflammatory process, however, might also mediate the development of surrounding tissue lesions as well as bone resorption [6].

Although many studies have been conducted aiming to investigate experimental periapical lesion kinetics, a precise gradation criterion of these lesions has yet to be established. Periapical lesions are known to expand most rapidly two weeks following pulp exposure to pathogens (active phase), with lesion enlargement occurring at a slower rate thereafter (chronic phase) [7]. Additionally, the periapical bone destruction levels were described as small, increased or extensive, without any explanation for these categories [8, 9].

The aims of this study were to evaluate any associations between changes in periapical tissue alveolar bone resorption levels and different lesion grades, as well as to evaluate the prevalence of these grades. For that purpose, the mandibular first molar dental pulps were exposed in BALB/c mice. As has been observed in previous studies using this mouse strain, periapical lesions increase to their maximum size 2-4 weeks following pulp exposure [3]. Herein, we evaluated the periapical bone resorption amounts at these time points using the AutoDesk AutoCAD 2010 software. Autodesk AutoCAD is a computer-aided design program that is traditionally used for calculations and design in architecture and engineering [10]. The concept of using AutoCAD to precisely calculate surface areas and volumes is a relatively new method used in dental studies.

MATERIALS AND METHODS

Animals

We used 6- to 8-week-old female wild type (WT) BALB/c mice for the induction of periapical lesions. WT BALB/c mice were kindly provided by the Military Medical Academy (MMA, Belgrade, Serbia). All animals were maintained in our animal facilities (Vivariums of the Faculty of Medical Sciences, University of Kragujevac). Mice were housed in a temperature-controlled environment with a 12 h light/12 h dark cycle and were administered standard laboratory chow and water *ad libitum.* All animals received humane care, and all experiments were approved by the Animal Ethics Committee of the Faculty of Medicine, University of Kragujevac, Serbia.

Periapical lesion induction

Periapical lesions were induced by exposing the right-sided mandibular first molar pulp to the host oral bacterial flora, as previously described [11]. Mice were anesthetised with ketamine hydrochloride (60 mg/kg of body weight) and xylazine (10 mg/kg of body weight) in sterile phosphate-buffered saline (PBS) by intraperitoneal injection, and mounted on a jaw-retraction board. The mandibular molar pulps were exposed using a high-speed electric dental handpiece (W&H Dentalwerk, Bürmoos, Austria) with a no. 1/4 round carbide bur. The exposure size was approximately equivalent to the bur diameter. The pulp chambers were opened until the entrances of the canals could be visualised and probed with a no. 08 endodontic file. Exposed teeth were left open to the oral environment for 14 (n=6) or 28 days (n=6). The non-exposed teeth served as negative controls.

Histology evaluation

The mice were sacrificed by diet hylether asphyxiation, and the mandibles were then isolated and fixed in 4% paraformaldehyde for 24 h. The fixed mandibles were decalcified in 3% formic acid for 24 h and then embedded in paraffin. Tissue blocks were cut in 4 μm longitudinal serial sections. Every fourth sample was mounted and stained with haematoxylin and eosin (H&E).

Semi-quantitative measurements of the periapical lesions

The sections that included the mandibular first molar distal roots and passed through the apical foramen (or close to it) were selected for semi-quantitative lesion size measurements. The distal roots were used for the analyses because these roots were better preserved and because the sectioning was more parallel to the long axes of these roots than of the mesial roots. The area of the periapical region was traced surrounding the apical third of the roots were traced with the Paint program (Fig. 1A). Measurements were made using the Autodesk AutoCAD 2010 software. Each photo was converted into a newly created Autodesk AutoCAD 2010.dxf file using the Img2CAD program, which is recognized by the Autodesk AutoCAD 2010 software. As this conversion is not always perfect, we had to make minor corrections

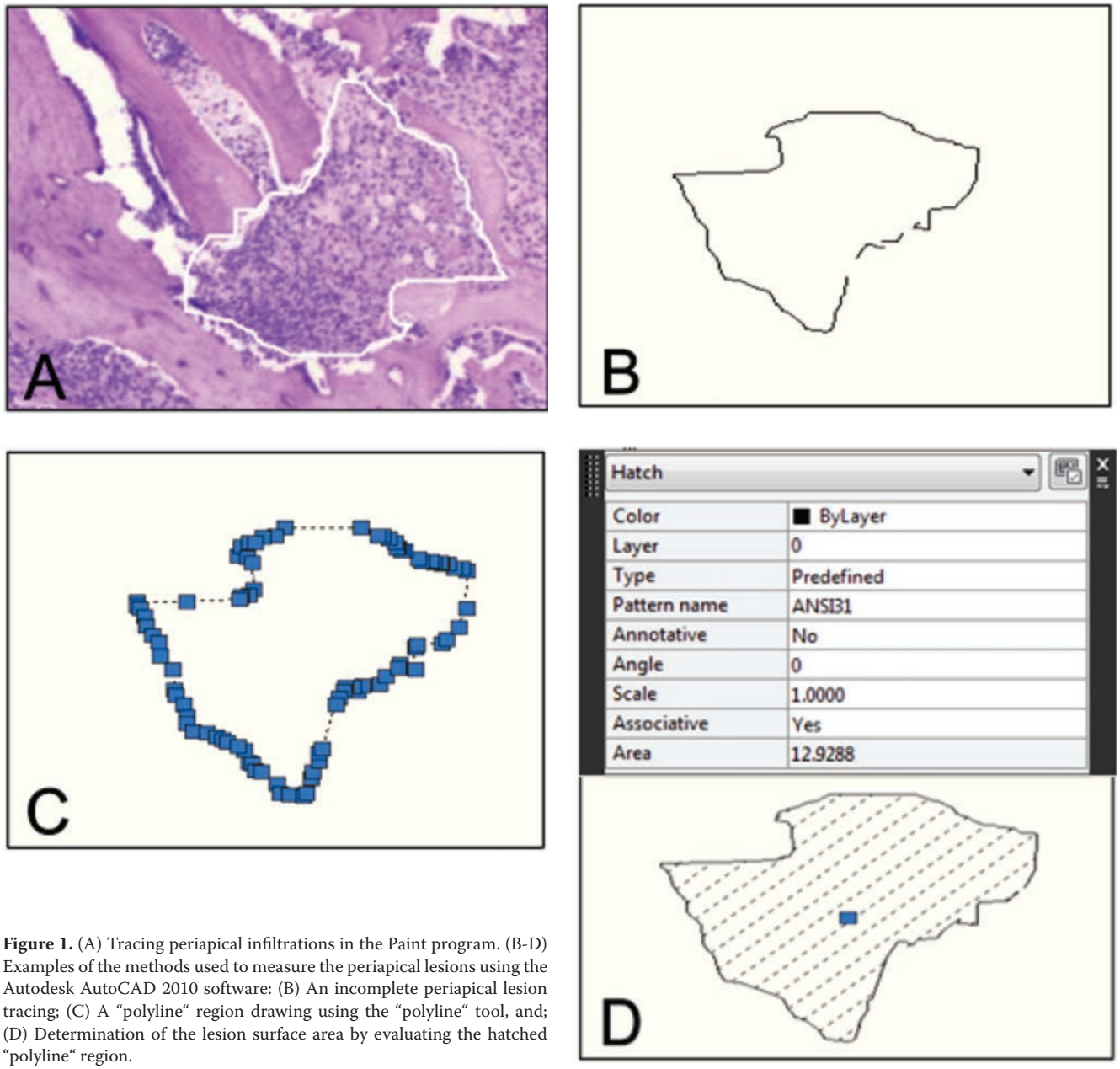

Figure 1. (A) Tracing periapical infiltrations in the Paint program. (B-D) Examples of the methods used to measure the periapical lesions using the Autodesk AutoCAD 2010 software: (B) An incomplete periapical lesion tracing; (C) A "polyline" region drawing using the "polyline" tool, and; (D) Determination of the lesion surface area by evaluating the hatched "polyline" region.

to the traced lesions after opening the files in AutoCAD to obtain a closed area whose surface could be calculated. Additionally, by using the "polyline" tool, we were able to draw the parts of the lesions that were not recognized by the program. Thus, we were able to connect the open ends of the lesions (Fig. 1B) and obtain "polyline" regions representing the periapical lesions (Fig. 1C). Then, we used the "Hatch" tool and within it selected the "Add: Select objects" option to mark these "polyline" regions. We determined the values of the drawn regions (Fig. 1D) by selecting the hatch area (by double clicking on the ENTER key). Prior to the determination of these areas, we established the scale bar values in the Autodesk AutoCAD 2010 software because the real surface of the periapical lesions was actually a ratio of these drawn regions, whereby the scale bar was multiplied by the square of the real value of the scale bar (scale bar = 100 μm). The stock value of the Autodesk AutoCAD 2010 scale bar was 1 (Scale = 1; Fig. 1D); therefore, it was not difficult to determine this ratio.

Statistical analyses

All measurements were presented as mean values ± SEM. Data were analysed using the SPSS statistical program, version 13. The distribution normality was assessed with the Shapiro-Wilk test. The study population did not follow a normal distribution; therefore, we determined significant differences in periapical lesion areas at two different time points using the Wilcoxon Rank Sum test. The results were considered significantly different if $p<0.05$ and highly significantly different if $p<0.01$.

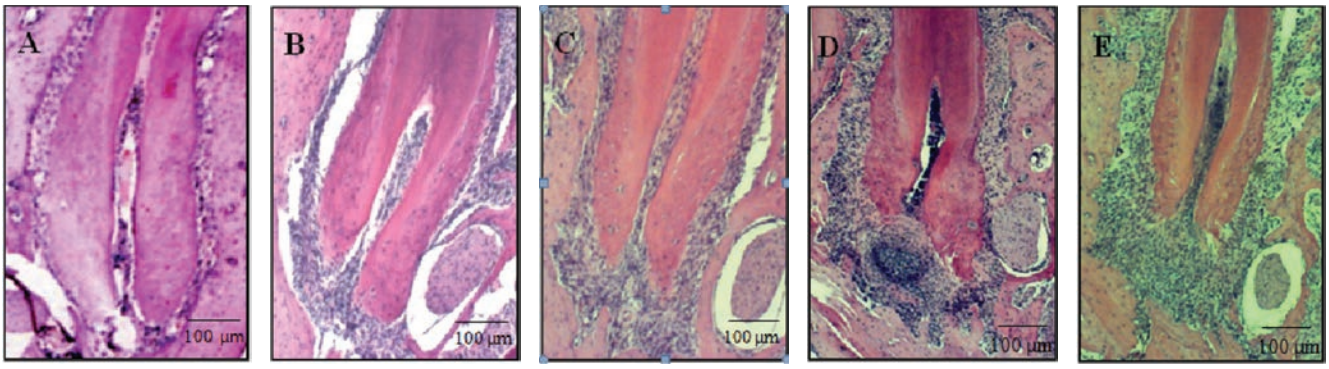

Figure 2. Histological findings. Representative haematoxylin-eosin stained histological sections: (A) Grade 0, (B) grade 1, (C) grade 2, (D) grade 3 and (E) grade 4 (Slides were evaluated with an Olympus BX51 microscope at 40X original magnification, scale bar = 100 µm).

RESULTS

Histological findings

Two weeks after pulp exposures, necrosis was observed in the upper half of the pulpal tissue. Additionally, slight or moderate inflammatory cell infiltration was observed in the lower half of the pulpal and periapical tissues (Fig. 2B and 2C). Inflammatory cell infiltrates were mostly in the form of polymorphonuclear cell sheets. Occasionally, round mononuclear cells with large condensed nuclei and low amounts of cytoplasm were observed, as well as large kidney-shaped cells with abundant cytoplasm. These cells were morphologically consistent with lymphocytes and macrophages, respectively. At this time, periapical alveolar bone resorption was also evident and was accompanied by multiple resorption lacunae containing multinucleated osteoclasts (Fig. 3). It was also possible to observe cementum resorption in the periapical regions.

Four weeks after pulp exposure, the pulpal tissue became completely necrotic. Moreover, the periapical regions exhibited an increased amount of bone resorption (Fig. 2D and 2E). Additionally, abscesses were found around the root apexes, and severe inflammatory cell infiltration was observed in the periapical tissues. Fibrous granulomatous tissue was also observed peripheral to the infiltrates, with fibroblasts detected among the many neutrophils and other inflammatory cells. Cementum resorption was also noted.

No abnormalities were observed in the untreated mouse pulpal and periapical tissues (Fig. 2A).

Although there was some variability in lesion size and inflammatory cell content among the specimens, differences were observed between periapical tissue alveolar bone resorption levels and inflammatory cell infiltration levels between two and four weeks following pulp exposure.

Histometric findings

The extent of the periapical bone destruction was quantified by histomorphometry. Using the Autodesk AutoCAD 2010 software-aided graphical evaluation, we detected and calculated the surfaces of each periapical region in the mandibular first molar histological tissue sections. As shown in Fig. 4, a highly significant lesion size difference was observed at two different time points following lesion induction (p=0.002). The apical periodontal ligament area was significantly increased at 4 weeks (the periapical lesion "chronic phase") following pulp exposure when compared with the 2-week (the periapical lesion "active phase") time point.

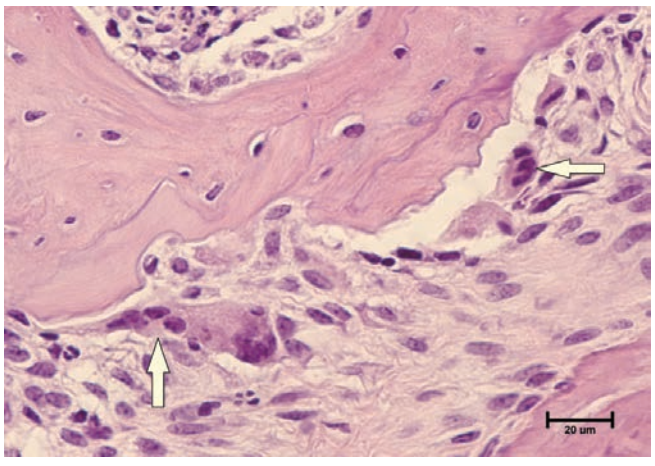

Figure 3. Periapical alveolar bone resorption was accompanied by multiple resorption lacunae containing multinucleated osteoclasts (haematoxylin-eosin stain; Nikon eclipse 50i; 600X original magnification; scale bar = 20 µm).

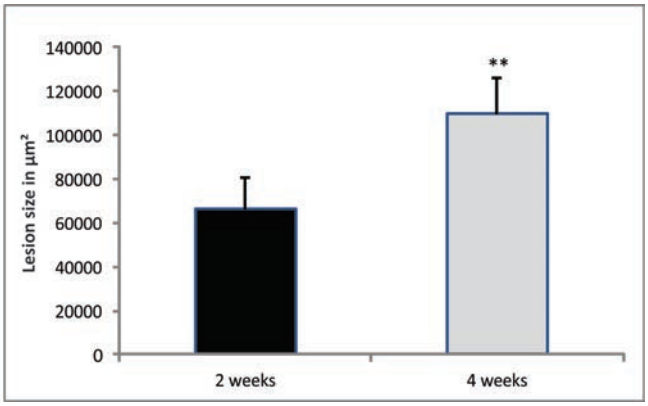

Figure 4. Histometric findings. Data are presented as the means ± SEM of the mouse mandibular first molar lesion areas used in the study (n=6 at each time point; *p<0.05; **p<0.01).

Gradation of the experimentally induced mouse periapical lesions

The periapical lesions were classified according to the periapical bone resorption sizes. We determined the quartile (25% and 75%) and median area (50%) values of the lesion mean values at both experimental time points. These data enabled for scoring of the lesions with a grading system of 1 to 4. Note that indicated control resorption areas represent the normal periodontal ligament space; therefore, this area was assigned a grade of 0. Finally, based on the periapical region alveolar bone resorption sizes, we proposed the following gradation criteria for the observed periapical lesions in these experimental mice:

Grade 0: *Normal histology.* The area of the normal periodontal ligament space in uninfected mice. In these mice, the apical region size occupied less than 20,000 μm^2 (Figure 1A).

Grade 1: *Small periapical bone resorption*. The apical region occupied less than 25 percent of the mean lesion value. The size of alveolar bone destruction in the periapical region was between 20,000- 42,672 μm^2 in these mice (Figure 1B).

Grade 2: *Moderate periapical bone resorption*. The apical region occupied between 25-50 percent of the mean lesion value. The alveolar bone destruction size in the periapical region was between 42,672-85,344 μm^2 in these mice (Figure 1C).

Grade 3: *Increased periapical bone resorption*. The apical region occupied between 50-75 percent of the mean lesion value. In these mice, the alveolar bone destruction size in the periapical region was between 85,344 – 128,016 μm^2 (Figure 1D).

Grade 4: *Extensive periapical bone resorption*. The apical region occupied more than 75 percent of the mean lesion value. The alveolar bone destruction size in the periapical region was more than 128,016 μm^2 in these mice (Figure 1E).

Grade 1 was not a common finding, and comprised only 8.33% of the observed samples. The most prevalent findings, however, were the grades 2 and 3 lesions, which comprised 66.66% of the samples (Table 1).

Periapical lesion grade	n	% of the samples
Grade 1	1	8.33
Grade 2	4	33.33
Grade 3	4	33.33
Grade 4	3	25

Table 1. The prevalence of different experimentally induced periapical lesion grades.

DISCUSSION

The induction of periapical lesions in mice by the exposure of the right-sided mandibular first molar pulps to the host root canal bacterial flora is a useful method to study the pathogenesis of this condition [1, 12].

Although many studies have examined the histomorphometry of periapical lesions, there is no standardised and generally accepted method for the quantification of experimental periapical lesions in tissue sections [13, 14]. Because of this lack, we have presented a new histological method for the quantification of periapical bone destruction in tissue sections using the Autodesk AutoCAD 2010 software. AutoCAD is a computer-aided design (CAD) program, which maximises productivity by using powerful tools for creating, documenting, and sharing drawings [15, 16]. With this technology, we measured the amount of periapical bone destruction by using the "polyline" method with the AutoCAD software. The results were highly specific and relevant to this histological analysis, confirming the reliability and accuracy of the Autodesk AutoCAD 2010 software for quantifying periapical bone destruction in this model.

The Autodesk AutoCAD software has been used in previous dental studies, including for root canal apical transportation evaluations, dentinal sealer penetration depth analyses and the determination of cemental and periodontal ligament thicknesses during various orthodontic tooth movements; however, there have been no reported studies regarding its use for experimental periapical lesion detection and quantification [17-20]. In line with our results, previous studies showed that the Autodesk AutoCAD software is reliable and can precisely calculate periapical region surface areas. Furthermore, the AutoCAD commands are very user friendly. These commands decrease the amount of time needed to conduct a task while also diminishing the amount of errors that occur. All of these findings confirm the validity of our semi-quantitative method for evaluating periapical lesions. Additionally, an important advantage of this method is the high availability of this software; therefore, this method could replace others that are more expensive and involve health risks, such as computed tomography and radiography [7, 21].

Until today, precise gradation criteria for evaluating experimentally induced periapical lesions had not been established. Previous studies used descriptive methods for determining periapical bone destruction levels (small, increased or extensive); therefore, the possibility of making an error was high [8, 9]. To grade periapical lesions, we determined the quartile (25% and 75%) and median area (50%) values of the mean lesion values. These data enabled for scoring of the lesions with grades from 1 to 4. Subsequently, we summarised our histometric results and descriptive histological analyses to assign rational explanations for these grades. Because a normal periodontal ligament space represents ordinary histology, it was assigned with a grade of 0. By using these gradation criteria, we obtained precise results, suggesting that the procedure described herein is more accurate at determining periapical region bone resorption levels than previous descriptive methods. The potential reasons for this improvement are exclusion of subjectivity, reduction of the possibility to make errors, facilitation of a numerical presentation of the data and provides an opportunity for researchers with an opportunity with an opportunity to reliably and precisely evaluate experimental periapical lesions.

ACKNOWLEDGEMENTS

We thank Nikola Miljković, Miloš Milosavljević and Aleksandar Ilić for their excellent technical assistance. This work was supported by the grants from the Serbian Ministry of Science and Technological Development (OP 175071), Serbia.

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ENDOVENOUS LASER ABLATION OF THE GREAT SAPHENOUS VEIN IN PATIENTS WITH VON WILLEBRAND DISEASE

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ENDOVENSKA LASERSKA ABLACIJA VELIKE SAFENSKE VENE KOD PACIJENTA SA "VON WILLEBRAND"-OVOM BOLEŠĆU

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Received / Primljen: 2.05.2013. Accepted / Prihvaćen: 6.06.2013.

ABSTRACT

Introduction

Patients with von Willebrand disease have a high risk of bleeding during the surgical treatment of varicose veins. Endovenous laser ablation (EVLA) of the great saphenous vein (GSV) is a minimally invasive treatment for saphenofemoral junction incompetence. The number of complications and relapse rate of EVLA are lower than in those of conventional surgical treatment.

Case presentation

A patient with great saphenous vein insufficiency, von Willebrand disease and hypofibrinogenemia successfully underwent endovenous laser ablation. Replacement therapy with cryoprecipitate was administered preoperatively and postoperatively. The surgical procedure and postoperative course were unremarkable.

Conclusion

In patients with von Willebrand disease and other coagulation disorders, endovenous laser ablation is a safe and effective treatment. The use of minimally invasive treatment, the adequate correction of haemostasis, and close cooperation between the haematologist, anaesthesiologist and endovascular surgeon reduce the risk of bleeding.

Key words: *Von Willebrand disease; varicose veins; surgery; endovenous laser ablation; great saphenous vein;*

SAŽETAK

Uvod

Kod pacijenata sa von Willebrand-ovom bolešću pri operativnom lečenju varikoznih vena postoji povišen rizik od krvavljenja. Endovaskularna laserska ablacija velike vene safene je minimalno invazivna metoda lečenja insuficijencije safenofemoralnog ušća. U odnosu na klasičan hirurški tretman manji je broj komplikacija i recidiva.

Prikaz slučaja

Pacijentkinja sa insuficijencijom velike vene safene, von Willbrand-ovom bolešću i hipofibrinogenemijom uspešno je operisana laserskom ablacijom vene. Preoperativno i postoperativno ordinirana je supstituciona terapija krioprecipitatom. Operativni zahvat i postoperativni tok protekao je uredno.

Zaključak

Kod pacijenata sa von Willebrand-ovom bolešću i drugim poremećajima koagulacije, endovaskularna laserska ablacija vene je sigurna i efikasna metoda lečenja. Primenom minimalno invazivnih metoda lečenja, adekvatnom korekcijom hemostaze, saradnjom hematologa, anesteziologa i endovaskularnog hirurga smanjuje se rizik za nastanak krvavljenja.

Ključne reči: *Von Willebrand-ova bolest; varikozne vene; hirurgija; endovaskularna laserska ablacija; velika vena safena*

INTRODUCTION

Von Willebrand disease (vWD) is the most common congenital haemorrhagic syndrome, characterised by a variety of quantitative and/or qualitative abnormalities in von Willebrand factor (vWF)[1,2]. Mutations, deletions and vWF gene polymorphisms cause vWD[3]. The inheritance is usually autosomal dominant, while severe forms are inherited recessively. There are three main subtypes; type 1 refers to a quantitative deficiency of vWF, while vWF and F VIII are qualitatively normal in approximately 80% of cases[4]; type 2 includes qualitative defects, and type 3 is defined as a complete lack of vWF[5]. vWF is a protein that mediates platelet adhesion and aggregation at the site of vascular injury and serves as a carrier for factor VIII, extending its half-life from approximately 2 hours (in the absence of vWF) to 8-12 hours. An abnormality of vWF can manifest as a primary haemostasis disorder[1,6] with a prevalence of 0.86%

UDK: 616.14-089/ Ser J Exp Clin Res 2013; 14 (2): 77-80
DOI: 10.5937/SJECR14-3851

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to 1.6% [7-10]. The prevalence is twice as high in women as in men, likely because menorrhagia contributes to a more frequent diagnosis, particularly of milder forms[8]. The likelihood of bleeding depends on the vWD subtype and the level of vWF deficiency[11].

In patients with vWD who require surgery or other invasive procedures, it is necessary to adequately maintain haemostasis preoperatively and postoperatively to prevent bleeding[12,13]. Three main therapeutic modalities are used for the treatment of vWD: desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) acetate, plasma derivatives (complex concentrate FVIII/vWF; cryoprecipitate) and antifibrinolytics[2,5,12-20]. If bleeding occurs, it is important to determine whether it resulted from surgery or inadequate haemostasis.

Early treatment of great saphenous vein insufficiency (GSV) is recommended[21] in saphenofemoral reflux. Classical surgical techniques and endovascular treatment are applied in CEAP class C primary chronic venous insufficiency (CVI) [22]. Clinical relapses were not significantly different between EVLA or endovenous radiofrequency ablation and surgery [23]. Endovenous ablation has advantages in decreasing the incidence of pain, haematoma, and wound infection and ain permitting a more rapid return to work [23]. Endovenous laser ablation (EVLA) is applied in the treatment of superficial and perforating venous reflux. EVLA is a safe and effective treatment for GSV insufficiency[24-27]. Relapse after EVLA is 3-5%, whereas that in conventional surgery is 20-40% within 5 years [26].

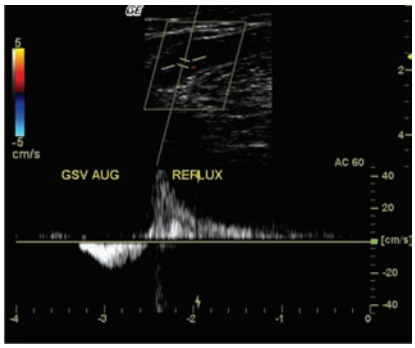

Figure 1.
GSV insufficiency

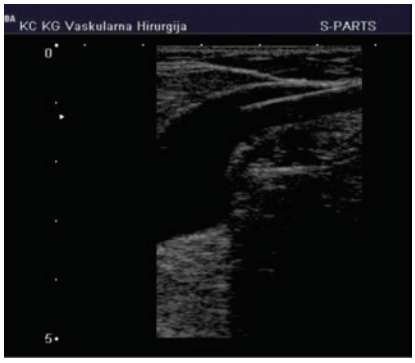

Figure 2.
Biolitec catheter positioned at GSV junction

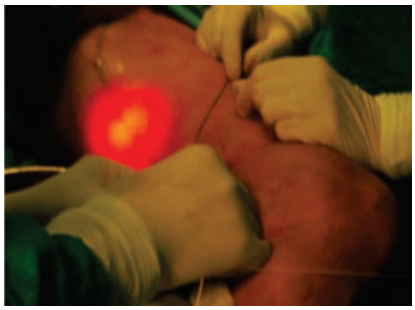

Figure 3.
GSV laser ablation

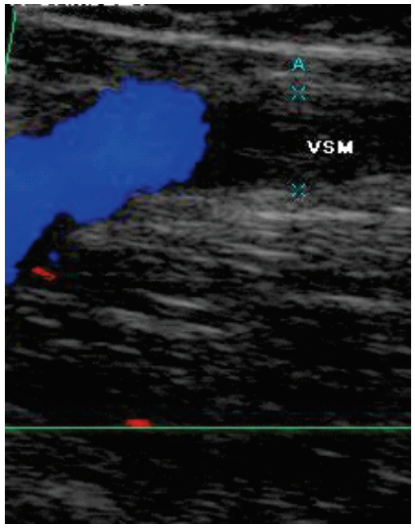

Figure 4.
Occlusion of the GSV junction after EVLA

CASE PRESENTATION

We investigated a female patient, 44 years of age, with vWD, hypofibrinogenemia and popliteal and thigh varicose veins. Stage C3 GSV insufficiency was determined using preoperative Doppler colour flow mapping (CDS).

In her personal history, the patient had excessive menstruation and postpartum bleeding. After the second birth, accompanied by heavy and delayed postpartum bleeding, the diagnosis of type I von Willebrand disease was established, along with hypofibrinogenemia of unknown origin. She reported no other diseases. There was no information about hereditary diseases or a tendency to haemorrhage among immediate family members.

Slightly prolonged bleeding time, as measured by the Duke method (4 min 30 sec), slightly lower levels of vWF (49.2%) and lower fibrinogen levels (1.706 g/l) were detected preoperatively. The concentrations of coagulation factors, complete blood count and hepatogram were within the normal range. A haematologist and anaesthesiologist were consulted preoperatively because of the patient's risk of bleeding. Preoperative and postoperative cryoprecipitate replacement therapy was given due to the simultaneous lack of fibrinogen and type 1 von Willebrand disease. After adequate preoperative preparation, the corrected haemostasis parameters were within the reference range.

The patient underwent EVLA treatment of the GSV using 1470 nm (Biolitec) 6F catheter and CDS monitoring. During the operation, CDS-guided vein catheterisation was performed along with catheter positioning, tumescent infiltration, laser energy administration and postoperative control. Total intravenous anaesthesia was applied with tu-

mescent local anaesthesia and intraoperative monitoring. The operative course was uncomplicated. The patient was mobile 2 h after the intervention.

On the first and second postoperative days, the fibrinogen values were 1.910 g/l, 2.214 g/l and 2.880 g/l. The bleeding time measurements and haematological parameters remained within the normal range.

The patient was discharged from hospital in good general condition with no local complications 48 h after the intervention. She was monitored from postoperative days 7-30. Postoperatively, graduated elastic compression stockings (23-32 mmHg) were applied, and sclerosant foam was injected into the varicose veins (in an outpatient setting) as sclerotherapy for GSV varicose tributaries and reticular veins.

DISCUSSION

In patients with vWD, the aim of surgical treatment is to apply sufficiently effective therapy with minimal risk of bleeding. Erik von Willebrand contributed to the discovery and treatment of vWD when he first described the haemorrhagic syndrome in a 5-year-old girl and her family members in 1926. Zimmerman identified the antigen associated with F VIII and called it vWF[1].

The surgical treatment of patients with von Willebrand disease is complex. In our patient, although in its mild form, type 1 von Willebrand disease was further complicated by hypofibrinogenemia of unknown aetiology. The hereditary haemorrhagic diathesis in these patients increases the risk of bleeding during surgical intervention, and follow-up care is more complicated. Through adequate preoperative and postoperative replacement, the coagulation factors were corrected, and the risk of bleeding was minimised [12-20].

If venous reflux is not treated appropriately, it can lead to disease progression, the development of superficial thrombophlebitis, vein rupture and bleeding, and the occurrence of venous ulcers and skin lesions. The extensive classical surgical ligation and stripping of varicose veins due to GSV insufficiency has a high risk of bleeding associated with a number of intraoperative and postoperative complications compared with endovenous ablation [22-34]. The application of minimally invasive EVLA or GSV with adequate correction of haemostasis according to the type of vWD minimizises the risk to the patient, with satisfactory healing of CVI. EVLA exceeds traditional surgical treatment for fewer complications, shorter hospitalisation, reduced treatment costs and more rapid return to daily life activities. The patient subjectively feels better, with less postoperative pain, and the aesthetic outcome is more favourable [27-34].

CONCLUSIONS

In patients with von Willebrand disease and other coagulation disorders, endovenous laser ablation is the method of choice when surgical treatment of the GSV insufficiency is necessary. The use of minimally invasive methods, appropriate prophylaxis, and intraoperative and perioperative monitoring reduces the risk of bleeding. Through adequate cooperation among haematologists, anaesthesiologists and endovascular surgeons, laser ablation of the GSV in patients with coagulation disorders can be performed safely and effectively.

ACKNOWLEDGEMENTS

This study was partially financed by the Grant No 175007 from Serbian Ministry of Education and by the Grant No 01-404 from the Ministry of Science, Montenegro.

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ERRATUM TO: SERUM DEPRIVATION INDUCES APOPTOTIC CELL DEATH IN THESC CELL LINE

Ana Petrovic, Ivana Nikolic, Milan Zaric, Ivanka Zelen, Danijela Jovanovic, Zoran Milosavljevic, Tatjana Kastratovic, Maja Čolić, Marija Andjelkovic, Marina Mitrovic

Erratum to: Serbian Journal of Experimental and Clinical Research 14 (1): 23-28

In the original article the name of one of the authors has erroneously been erased from the list of authors. The name of this author is Marija Andjelkovic. Everything else in the paper remains correct.

The online version of the original article can be found under
doi:10.5937/sjecr14-1912
UDK: 618.14-006.36 / Ser J Exp Clin Res 2013; 14 (1): 23-28

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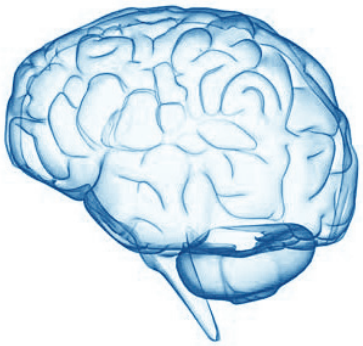

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SERBIAN Journal of Experimental and Clinical Research
editor-in-chief Vladimir Jakovljević.
- Vol. 9, N° 1 (April 2008) -
- Kragujevac (Svetozara Markovića 69) :
Medical Faculty, 2008 - (Kragujevac : Medical Faculty). - 29 cm

Je nastavak: Medicus (Kragujevac) = ISSN 1450-7994
ISSN 1820-8665 = Serbian Journal of
Experimental and Clinical Research
COBISS.SR-ID 149695244

Serbian Journal of Experimental and Clinical Research
2000

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