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Protective effects of osajin in ischemia-reperfusion of laboratory rat kidney

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The aim of this study was to analyze the antioxidative effect of osajin during prophylactic administration. The pathological model for *in vivo* experiment was the unilateral ischemia-reperfusion of kidney of the laboratory rat. The animals were randomly divided into five groups. Osajin was administrated orally in doses of 5, 10 and 20 mg/kg once a day to three premedicated groups. Placebo – 0.5% solution of Avicel – was given to the fourth group and the fifth group was completely intact. The premedication lasted 15 days and subsequently the ischemia of the left kidney was incited in general anaesthesia for 60 min. The reperfusion lasted 10 min and it was finished by blood collection from the left ventricle and the reperfused kidney was recovered. Selected biochemical markers were assessed in blood: superoxide dismutase, glutathion peroxidase, total antioxidative capacity and malondialdehyde. The kidney tissue samples were used for histopathological examination. Laboratory and histopathological results confirmed supposed effects of osajine. The dependence between the effect and the applied dose of osajin was linear. The best biochemical results were reached after administration of osajin at the dose of 5 mg/kg. The best histopathological results were reached after administration of osajin at the dose of 10 mg/kg.

1. Introduction

The results of intensive research in the last decade confirm that the pathological increase of free radicals significantly contributes to the emergence and development of many diseases, e.g. inflammatory, cardiovascular, degenerative syndromes, etc. If the balance between the action of free radicals and protective mechanisms of the organism is disturbed, it results in the development of a condition known as oxidative stress (Sies 1991; Bartosova et al. 2003). To regulate the physiological quantity of free radicals, the organism has developed antioxidative mechanisms formed by natural antioxidants and enzymes (e.g. superoxide dismutase, catalase, glutathion redox system, compounds chelating iron and copper ions, etc.) (Rotilio 1994). However, the remedy of the oxidative injury to the organism is difficult. Much more effective is the way of prevention which consists in minimizing the sources of formation of free radicals and reinforcing the natural antioxidative mechanism by the administration of compounds which act as antioxidants or so called free radical scavengers (Necas et al. 1997; Bartosikova et al. 1998; Petrikova et al. 2002; Pecivova et al. 2004).

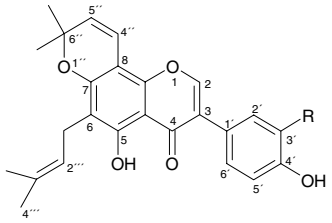
The study of biological activity and mechanism of the effect of flavonoids has been the subject of research for many years. Flavonoids form one of the largest groups of natural phenols. They occur as a rule as glycosides in plants, being also contained in fruits and vegetables. In particular aglycones are pharmacologically effective. Many of them manifest hepatoprotective, diuretic, vasodilatative, antibacterial, chemoprotective effects; anti-inflammatory, antidiabetic, antiallergic and other effects were described as well (Read 1995; Calomme et al. 1996; Perez et al. 1998; Yamamura et al. 1998). Recently, an increased attention has been given to the study of their antioxidative activity and their capacity to scavenge or take up free radicals (Jovanovic et al. 1994; Rice-Evans et al. 1995; Catapano 1997; Kubinova and Suchy 1999).

Osajin was extracted from *Maclura pomifera*, Moraceae. The methods, chemistry and properties of osajin are described elsewhere (Liskova et al. 2005).

Preliminary *in vivo* studies also proved its antioxidative activity (Bartosikova et al. 2002). This fact became a stimulus for further studies. The objective of this study was to analyse the antioxidative effect of the applied dose of osajin during prophylactic administration in the conditions of ischemia-reperfusion of kidney in the laboratory rat.

The study and its experimental protocol were approved and monitored by the Ethics Committee of the University of Veterinary and Pharmaceutical Sciences in Brno. The state of health of all animals was inspected regularly several times a day both during the acclimation of the ani-

mals and in the course of the whole experiment performed by the work group whose members are holders of the Eligibility Certificate issued by the Central Commission for Animal Protection pursuant to Section 17 of the Czech National Council Act No 246/1992 Coll. on animal protection against maltreatment.



VII. osajin R = H

$C_{25}H_{24}O_5$, Mr = 402

2. Investigations and results

2.1. Laboratory analysis

The results of laboratory analysis are given in Table 1.
A statistically highly significant increase of SOD values ($p \leq 0.01$) was detected in the groups treated with osajin the doses of 5, 10, and 20 mg/kg, compared with the placebo group. Further, a statistically highly significant increase of SOD values ($p \leq 0.01$) was found in the groups treated with osajin at doses of 5, 10, and 20 mg/kg, compared with the intact animal group. Mutual comparison of the SOD values received from the animal groups treated with doses of 5 and 10 mg/kg of osajin showed a significant difference ($p \leq 0.05$).
A statistically significant decrease in GSHPx values ($p \leq 0.01$) was detected in the group treated with osajin at the dose of 5 mg/kg, compared with the placebo group.
Further, a statistically significant difference in GSHPx values ($p \leq 0.01$) was found in the group treated with osajin at the dose of 20 mg/kg in comparison with the intact animal group. Mutual comparison of the GSHPx values received from the animal groups treated with doses of 5 and 10 mg/kg and 10 and 20 mg/kg of osajin showed a significant difference ($p \leq 0.05$). Mutual comparison of the GSHPx values received from the animal groups treated with doses of 5 and 20 mg/kg of osajin showed a significant difference ($p \leq 0.01$) too.
A statistically highly significant increase of AOC values ($p \leq 0.01$) was detected in the groups treated with osajin at the dose of 5 mg/kg and significant increase of AOC values ($p \leq 0.05$) in the groups treated with osajin at doses of 10 and 20 mg/kg, compared with the placebo group. Further, a statistically significant increase of AOC values ($p \leq 0.01$; $p \leq 0.05$) was found in the groups treated with osajin at the doses of 5, 10, and 20 mg/kg, compared with the intact animal group. Mutual comparison of the AOC values received from the animal groups treated with doses of 5 and 10 mg/kg and 5 and 20 mg/kg of osajin showed a significant difference ($p \leq 0.01$).
A statistically highly significant decrease of MDA values ($p \leq 0.01$) was detected in the groups treated with osajin at the doses of 5, 10, and 20 mg/kg, compared with the placebo group. Further, a statistically highly significant difference of MDA values ($p \leq 0.01$) was found in the groups treated with osajin at doses of 5, 10, and 20 mg/kg in comparison with the intact animal group. Mutual comparison of the MDA values received from the animal groups treated with different doses of osajin showed no significant difference.
Comparison of the values obtained from the placebo group and intact animal group showed a significant increase ($p \leq 0.01$) of the MDA value in the placebo group, which is the result of the pathological state induction.

2.2. Histopathological examination

2.2.1. Treated groups

The optimal protective effect appeared at the 10 mg/kg dose of osajin, both in the protection of medulla and in the protection of cortex with the glomerules (average score per one sample: 1.55 and 1.10). Osajin proved to be the most effective protective agent of the channel epithelia at the dose of 10 mg/kg (average score per one sample: 1.10).

2.2.2. Placebo group

In all samples massive hemorrhage in the interstitium was observed, especially on the cortex and medulla boundaries, hemorrhage in the glomerule area (Bowman's capsule and capillary convolusion) as well as in the medulla. The channels have regressively changed epithelia from the simple edema to epithelia necrosis with all the above described features. In the lumina there is mostly a proteinic content with hyaline cylinder formation. The accompanying edema and the generally increased cellularity of the glomerule are inflammatory reactive. The more marked inflammatory infiltrate is smaller than in the treated groups in the form of sporadic lymphocytes with rare polynuclears. The total average score was oscillating from 5 to 7.

2.2.3. Intact control group

Hemorrhage occurred only accidentally, most probably caused by contusion.

Table: Values of laboratory parameters studied (X ± SD)

Group of animals (n = 10)	SOD (U/ml)	GSHPx (μkat/l)	AOC (mmol/l)	MDA (mmol/l)
Treated (5 mg/kg of osajin)	$190.14 \pm 12.24^{2,4}$	1675.00 ± 189.80^{2}	$0.52 \pm 0.04^{2,4}$	$2.99 \pm 0.98^{2,4}$
Treated (10 mg/kg of osajin)	$179.32 \pm 5.75^{2,4}$	1927.22 ± 230.15	$0.44 \pm 0.04^{1,3}$	$2.42 \pm 0.75^{2,4}$
Treated (20 mg/kg of osajin)	$184.84 \pm 13.19^{2,4}$	2200.30 ± 266.66^{4}	$0.44 \pm 0.03^{1,3}$	$2.44 \pm 1.12^{2,4}$
Placebo group	63.24 ± 3.57	2180.40 ± 376.02	0.39 ± 0.05	11.95 ± 4.55^{6}
Intact group	58.82 ± 2.76	1821.60 ± 205.07	0.40 ± 0.06	1.01 ± 0.52

Explanations:
[1] $p \leq 0.05$ treated vs placebo group [2] $p \leq 0.01$ treated vs placebo placebo
[3] $p \leq 0.05$ treated vs intact group [4] $p \leq 0.01$ treated vs intact group
[5] $p \leq 0.05$ placebo vs intact group [6] $p \leq 0.01$ placebo vs intact group

3. Discussion

Free radicals play an important role in the pathogenesis of kidney ischemia-reperfusion injury (Lien et al. 2003; Devinder and Kanwaljit 2004; Gurel et al. 2004). During the ischemic period only the anaerobic glycolysis, whose ATP production is insufficient, proceeds in the tissue. There is not enough energy to maintain the membrane actions, and Na^+ enters the cells. In the reperfusion period, these ions are replaced by Ca^{2+} which causes the incapacity of mitochondria to produce ATP and activate intracellular proteases and phospholipases (Lien et al. 2003; Bosetti et al. 2004). In addition to cellular membrane degradation, arachidonic acid is released, in the metabolism of which free radicals and other cytotoxic products arise. Other sources of free radicals are activated neutrophilic granulocytes, mitochondrial oxidation chain at a very low partial oxygen pressure and metabolism of catecholamines released in a stress situation. Hypoxia also leads to the transformation of xanthindehydrogenase into xanthinoxidase which gives origin to two superoxide radicals during the biosynthesis of uric acid. The quantity of the released radicals, moreover very different, leads to the organ injury (Racek et al. 1997).

Osajin is an original isolated substance which so far has been rarely used for *in vivo* testing on pathological biomodels. In our study, the antioxidative action of osajin used for prophylactic administration in the conditions of kidney ischemia-reperfusion in the laboratory rat was analysed. A significant increase of SOD values was observed in the groups treated with osajin at the respective doses (see Table), compared with the placebo group and also the intact animal group. For GSHPx, decrease in values was found in the groups treated with osajin at the dose of 5 mg/kg, compared with the placebo group.

The statistically significant higher SOD levels found in the treated groups confirm the readiness to the destruction of superoxide, disposal of hydrogen peroxide and other free radicals causing injury to the reperfusion sustained kidney tissue. It is supposed that this is a result of the previous preventive supplementation of the animal group with a compound having the *in vitro* evidenced antioxidative effect. In SOD (and in GSHPx either) the dependence of the examined parameter value on the administered dose was established. The analysed enzymes act intracellularly and their activity mostly follows one after another. It may be supposed that their activity can change in accordance with the state of organism, or in relation to the on-going pathological processes. The test results demonstrate that the mutual compensation mechanisms formed by the effect coordination of more enzymes can be potentiated by the administered antioxidants presence.

The detection of the lower SOD level during the artificially induced kidney ischemia-reperfusion without the previous prophylactic administration of the antioxidants, which was found in the placebo group, has also been described by other authors, in some cases as early as the ischemic phase (Racek et al. 1997).

Individual authors greatly differ in their views on the change of SOD and GSHPx activity caused by the declining function of kidneys. Literature references include both the increasing SOD activity (Mimic-Oka et al. 1995) dependent on the declining function of kidneys and the detection of the reduced SOD and GSHPx activity (Racek et al. 1995) as well as the normal one (Durak et al. 1994). For better assessment of the existing problem it would be more suitable to determine the erythrocyte catalase activity in experimental animals, which is sometimes reduced in patients with declining function of kidneys (Durak et al. 1994), and the level of selenium, which has antioxidative effects, forms a part of GSHPx, and the deficit of which is frequently diagnosed in patients with renal failure (Bonomini and Albertazzi 1995).

Statistically highly significant increase of AOC values in the treated animal groups, without a dependence on the administered dose of osajin, was recorded in comparison with the values of the placebo group and also of the intact animal group. It is a significant difference and it may be supposed that this is another logical result of the previous supplementation with a compound with antioxidative effect which was first induced by the lowest dose of osajin. On the other hand, also an increased level of uric acid may cause an AOC increase (Racek et al. 1997). The uric acid should not only be considered a nitrogenous metabolite of purine compounds but it also has significant antioxidative effects. The authors differ in their views on the AOC changes due to possibly declining function of kidneys (Toborek et al. 1992; Jackson et al. 1995).

The results of the statistical comparison of the MDA values demonstrate significant changes at the statistical significance level ($p \leq 0.01$); in the treated animal groups, a statistically significantly lower average value of this toxic by-product of lipid peroxidation was found, compared with the control placebo group. Comparing the results of the performed studies, many authors agree on the increased MDA concentration in plasma or in erythrocytes (Racek et al. 1995) in patients with renal failure; however, the cause can be not only its increased formation from lipid peroxides but also its reduced renal elimination (Racek et al. 1997). MDA can subsequently modify the proteins and lead to similar changes which can be observed during their glycation (Roselaar et al. 1995; Racek et al. 1997).

The positive effect of the antioxidant administration in the conditions connected with ischemia and subsequent reperfusion of kidney tissue in relation to the improvement of the values of the antioxidative system indicators is also discussed in other studies (Lee et al. 1992; Zurovski et al. 1995).

In our study, the potential protective effect of osajin is demonstrated in the prophylaxis of ischemic-reperfusion injury to kidney in the laboratory rat. The assumption is supported by the results of the evaluation of histopathological findings in the kidney samples examined.

4. Experimental

4.1. Antioxidative activity of osajin in vitro

The antioxidative activity of osajin was confirmed *in vitro* by the assessment of lipid peroxidation in liver microsomes of the laboratory rat using butylhydroxytoluen (BHT) as a reference standard (Vesela et al. 2004).

4.2. Materials and methods

The experiments were performed in male Wistar SPF (AnLab, SRN) laboratory rats of the same age and comparable body mass (250 ± 10 g). The animals were housed in a room with a standard controlled temperature, fed a standard M1 diet for small laboratory animals, and had access to water *ad libitum*. After 10 days of acclimation, the animals were randomly divided into 5 groups. The test compound were administered to three groups of treated animals ($n = 10$) at concentrations of 5 mg/kg, 10 mg/kg, and 20 mg/kg in 0.5% Avicel solution perorally once a day. The fourth group ($n = 10$) – the placebo group – was given only 0.5% Avicel solution in the quantity and by the mode of administration used in the treated groups. The fifth group of animals, the intact one ($n = 10$), was left without any medication. After the discontinuation of medication on day 15, laparotomy in general anaesthesia (2% Rometar 0.5 ml + 1% Narkamon 10 ml, dose 0.5 ml solution/100 g of the rat body mass) was performed, renal ischemia was induced by clamping the left renal artery with

a vascular clamp for 60 min with subsequent 10 min renal reperfusion. After the termination of reperfusion, the animals were exsanguinated by blood collection from the left ventricle and selected laboratory parameters were analysed – superoxide dismutase (SOD), glutathion peroxidase (GSHPx), total antioxidative capacity (AOC) using RANDOX testing kits (Dublin, Ireland), in COBAS MIRA S automatic analyser, and malondialdehyde (MDA) was analysed spectrophotometrically using the TBARs method (Uchiama and Mihara 1978). The obtained values of the studied laboratory parameters were processed by the Microsoft Excel table processor and statistically interpreted using a non-pair T-test. A value $p \leq 0.05$ was considered significant.

The samples of the reperfused kidney tissue were employed for histopathological examination. The material was fixed in 10% formaldehyde and processed manually. Two blocks were made of each sample, the sections being stained with hematoxylin-eosin. All evaluated samples were of outstanding quality, the evaluation being performed by a histopathologist without knowledge of the experimental protocol.

Evaluation principle: all samples in the material were evaluated and scored separately in three kidney topicalities, the result was added up and in the end the average score of each medicated group was stated.

Scoring schedule:

First topicality – kidney medulla – the grade of tissue destruction through bleeding (according to the extent) and presence of inflammatory infiltrate (max. $+++$) were evaluated.

Second topicality – cortex and glomerules – both extraglomerular ($+$) presence of hemorrhages and increased cellularity and extravasates in the glomerule (max. $++$) were evaluated.

Third topicality – kidney channels – presence of regressive changes of epithelia from edema to necrosis was evaluated ($+$ in the case of necrosis and $\pm$ in the case of regression not reaching the grade of necrosis). In addition, the channel content was evaluated (protein and hyaline cylinders $+$). Maximum $++$.

The highest possible (worst) result per one sample was 7 (7 times $+$).

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References

Bartosikova L, Necas J, Pavlicek V, Kuchtickova S, Frana P, Zavadilova R, Husek K (1998) Study of the effect of the sympatolytic carvedilol in the conditions of experimental alloxan diabetes in the laboratory rat. Cs Slov Farm 47: 151–154.

Bartosikova L, Necas J, Kotolova H, Placek D, Florian T, Frydrych M (2002) Monitoring of antioxidative effect of osajin in the preclinical experiment. Skripta Med Brno 75: 343.

Bartosova L, Frydrych M, Mokry P, Brunclik V, Kotolova H (2003) Changes in heart rate after application of newly developed ultrashort acting beta-adrenergic blockers. Pharmazie 58: 841–842.

Bonomini M, Albertazzi A (1995) Selenium in uremia. Artif Organs 19: 443–448.

Bosetti F, Baracca A, Lenaz G, Solaini G (2004) Increased state 4 mitochondrial respiration and swelling in early post-ischemic reperfusion of rat heart. FEBS Letters 563: 161–164.

Calomme M, Pieters L, Vlietinck A, Vanden Berghe D (1996) Inhibition of bacterial mutagenesis by Citrus flavonoids. Planta Med 62: 222–226.

Catapano AL (1997) Antioxidant effect of flavonoids. Angiology 48: 39–44.

Devinder S, Kanwaljit Ch (2004) The effect of naringin, a bioflavonoid on ischemia-reperfusion induced renal injury in rats. Pharmacol Res 50: 187–193.

Durak I, Kacmaz M, Elgun S, Ozturk HS (1994) Oxidative stress in patients with chronic renal failure: Effect of hemodialysis. Med Princ Pract 13: 84–87.

Gurel A, Armutcu F, Sahin S, Sogut S, Ozyurt H, Gulec M, Kutlu NU, Skyll O (2004) Protective role of α-tocopherol and caffeic acid phenethyl ester on ischemia-reperfusion injury via nitric oxide and myeloperoxidase in rat kidneys. Clin Chim Acta 339: 33–41.

Jackson P, Loughrey CM, Lightbody JH, McNamee PT, Young IS (1995) Effect of hemodialysis on total antioxidant capacity and serum antioxidants in patients with chronic renal failure. Clin Chem 41: 1135–1138.

Jovanovic SV, Steenken S, Tosic M, Marjanovic B, Simic MG (1994) Flavonoids as antioxidants. J Am Chem Soc 116: 4846–4851.

Kubinova R, Suchy V (1999) Antioxidants of biogenic origin. Cs Slov Farm 48: 9–14.

Lee PH, Chung YC, Hu RH, Huang MT, Lee CS (1992) Protective effect of superoxide dismutase and allopurinol on oxygen free radical-induced damage to the kidney. Transpl Proc 24: 1353–1354.

Lien YH, Lai L, Silva AL (2003) Pathogenesis of renal ischemia/reperfusion injury: lessons from knockout mice. Life Sci. 74: 543–552.

Liskova M, Marek J, Jankovska D, Sukupova L, Zemlicka M, Vanco J (2005) Osajin. Acta Crystal 61: 01848–01850.

Mimic-Oka J, Simic T, Ekmescic V, Dragicevic P (1995) Erythrocyte glutathione peroxidase and superoxide dismutase activities in different stages of chronic renal failure. Clin Nephrol 1: 44–48.

Necas J, Bartosikova L, Drapelova L, Husek K, Pavlicek V, Kuchtickova S (1997) Experimental investigation of the action of the beta-blocker carvedilol in ischemic-reperfusion renal damage. Vnitr Lek 43: 707–711.

Pecivova J, Macickova T, Ciz M, Nosal R, Lojek A (2004) Effect of Stobadine on Opsonized Zymosan Stimulated Generation of Reactive Oxygen Species in Human Blood. Cells Physiol Res 53: 97–102.

Perez RM, Zaval MA, Perez S, Perez C (1998) Antidiabetec effect of compounds isolated from plants. Phytomed 5: 55–57.

Petrikova M, Jancinova V, Nosal R, Majekova M, Danihelova E (2002) Antiplateled activity of carvedilol in comparison to propranolol. Platelets 13: 479–485.

Racek J, Eiselt J, Holecek V, Vesela E, Krejcova I, Treska V, Opatrny K, Valenta J (1997) Free radicals and kidney disease. Klin Biochem Metab 26: 92–97.

Racek J, Vesela E, Holecek V, Treska V (1995) The significance of free radicals in patients with renal failure and during kidney transplantation. Klin Biochem Metab 24 Suppl: 4–6.

Read MA (1995) Flavonoids: Naturally occuring anti-inflamatory agents. Am J Pathol 147: 235–237.

Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB (1995) The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Rad Res 22: 375–383.

Roselaar SE, Naznat NB, Winyard PG, Jones P, Cunningham J, Blake DR (1995) Detection of oxidants in uremic plasma by electron spin resonance spectroscopy. Kidney Int 48: 199–206.

Rotilio G (1994) Enzymes and enzyme cofactors as terapeutic agents: a challenge for modern biotechnology. In: Alberghina L, Frontali L, Sensi P (ed.) Proceedings of the 6th European Congress on Biotechnology, Elsevier Science p. 763.

Sies H (1991) Natürliche und syntetische antioxidanten. Atemw Lungenkrkh 17 Suppl: 16.

Toborek M, Wasik T, Drozdz M, Klein M, Magnerwrobel K, Kopiecznagrzebieniak E (1992) Effect of hemodialysis on lipid peroxidation and antioxidant system in patients with chronic renal failure. Metabolism 41: 1299–1232.

Uchiyama M, Mihara M (1978) Determination of malondialdehyd precursor in tissues by thiobarbituric acid test. Anal Biochem 3: 271–278.

Vesela D, Kubinova R et al (2004) Antioxidative and EROD activities of osajin and pomiferin. Fitoterapia 75: 209–211.

Yamamura S, Ozawa K, Ohtani K, Kasai R, Yamaski K (1998) Antihistaminic flavones and aliphatic glycosides from Mentha spicata. Phytochem 48: 131–136.

Zurovsky Y, Eligal Z, Grossman S (1995) Unilateral renal ischemia reperfusion in the rat: Effect of blood volume trapped in the kidney, sucrose infusion, and antioxidant treatments. Exp Toxic Path 47: 471–478.