Original Article

Pentoxifylline reduces biochemical markers of ischemia-reperfusion induced spinal cord injury in rabbits

S Savaş^{*1}, N Delibaş², Ç Savaş³, R Sütçü² and A Cindaş¹

¹Department of Physical Medicine and Rehabilitation, Suleyman Demirel University Medical School, Isparta, Turkey;

²Department of Biochemistry, Suleyman Demirel University Medical School, Isparta, Turkey; ³Department of Pediatric Surgery, Suleyman Demirel University Medical School, Isparta, Turkey

Study design: Occlusion of the infrarenal abdominal aorta with administration of pentoxifylline was applied to adult rabbits, followed by removal of aortic clamp and reperfusion. Tissue levels of cytokines, lipid peroxides, and antioxidant enzymes were assayed and compared within groups.

Objectives: To examine the effect of pentoxifylline (PTX) on cytokine levels, lipid peroxidation, and antioxidant enzymes in a rabbit model of spinal cord ischemia-reperfusion injury induced by aortic occlusion.

Setting: Isparta, Turkey.

Methods: Rabbits were randomly allocated into four groups of sham laparotomy (SHAM), sham laparotomy with PTX administration (SHAM + PTX), aortic occlusion and reperfusion (AOR), aortic occlusion and reperfusion with PTX administration (AOR + PTX). An intravenous bolus of 50 mg/kg PTX was given just before aortic cross clamping. An atraumatic microvascular clamp was then placed on the abdominal aorta immediately distal to the left renal artery for 30 min. PTX was infused at a rate of 0.5 mg/kg/min during the aortic occlusion. Animals were subjected to 120 min of reperfusion after removal of the aortic clamp. All animals were sacrificed at the end of reperfusion. The lumbosacral segments of spinal cords were quickly harvested and stored at -78°C for biochemical assays of IL-6, TNF- α , MDA, SOD, and CAT levels. Differences among groups were analyzed by one-way analysis of variance followed by a *post hoc* Tukey's honestly significant difference test.

Results: No differences in mean levels of IL-6, TNF- α , MDA, SOD, and CAT were noted between SHAM and SHAM + PTX groups ($P > 0.05$). There was a significant increase in all biochemical parameters in the AOR group ($P < 0.05$). Administration of PTX significantly attenuated the levels of all biochemical parameters in the AOR + PTX group ($P < 0.05$).

Conclusion: PTX pretreatment attenuated ischemia-reperfusion induced spinal cord injury in a rabbit model, in terms of biochemical parameters of ischemia and reperfusion.

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Keywords: pentoxifylline; spinal cord ischemia; free radicals; cytokines; antioxidant enzymes

Introduction

Paraplegia, with an incidence between 0.9 to 40%, is a serious complication of aortic surgery.¹ Spinal cord injury due to surgical procedures involving the thoraco-abdominal aorta is partly the result of a cascade of events mediated by the deleterious effects of oxygen-derived free radicals.² Neutrophils have also been suggested to contribute and aggravate this so-called 'ischemia-reperfusion (IR) injury' to the spinal cord.^{3,4} Many agents such as iloprost,² nitroglycerin,⁵

phenytoin,⁶ steroids,⁷ memantine,⁸ trimetazidine,⁹ riluzole,¹⁰ aprotinin¹¹ and various treatment modalities such as ischemic pretreatment^{12,13} and hypothermia^{14,15} have been suggested to prevent this complication. Another pharmacological agent with the potential for altering the ischemia-reperfusion injury is the methylxanthine derivate, Pentoxifylline (PTX); this has been used in many types of cerebrovascular disease including transient ischemic attacks, sequelae of cerebral thrombosis and hemorrhage, and chronic ischemic disorders beside the experiments on IR injury.^{16–18}

*Correspondence: S Savas, Posta Kutusu-76, 32000, Isparta, Turkey

This novel drug has been shown to modulate neutrophil and endothelial cell function, and to decrease activated neutrophil adherence and aggregation, as well as to decrease free radical production through a number of different mechanisms.^{19,20} To the best of our knowledge, there have been limited reports in the English literature investigating the role of PTX on spinal cord histology in IR injury.^{21,22} Therefore, this experimental study was designed to examine the effect of PTX on cytokine levels, lipid peroxidation, and antioxidant enzymes in a rabbit model of spinal cord IR injury induced by aortic occlusion.

Methods

We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during the course of this research.

This study was carried out on 32 adult male albino New Zealand rabbits weighing between 2.5–3 kg. The animals were acquired from the university vivarium sources and were housed in individual cages in a temperature and light–dark cycle controlled environment with free access to food and water. All animals received humane care, in compliance with the ‘Principles of Laboratory Animal Care’ formulated by the National Society for Medical Research and the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Experimental design

Animals were randomly assigned to experimental groups and were operated on in random order. Investigators blinded to the experimental groups made outcome assessments. Animals were divided into four groups each consisting of eight rabbits: sham laparotomy (SHAM), sham laparotomy with PTX administration (SHAM+PTX), aortic occlusion and reperfusion (AOR), aortic occlusion and reperfusion with PTX administration (AOR+PTX).

Surgical procedure

Animals fasted for 12 h and were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg). Rabbits were allowed to breathe spontaneously, and general anesthesia was maintained with a mixture of 1.5% halothane and oxygen administered by means of a face cone at a rate of 6 L/min. A catheter (24 gauge) was placed in a marginal ear vein for administration of fluids and drugs. Normal saline solution was infused at a rate of 4 mL/kg/h throughout the experiment. No animals received hemodynamic or ventilatory support. A midline laparotomy was performed after skin shaving and preparation with 10% povidone-iodine solution. The intestines were reflected to the right and the infrarenal abdominal

aorta was exposed. An intravenous bolus of 50 mg/kg PTX (Trental, Hoechst, Germany) was given just before aortic cross clamping. An atraumatic microvascular clamp (vascu-stats II, midi straight 1001-532; Scanlan Int, St Paul, MN, USA) was then placed to the abdominal aorta immediately distal to the left renal artery for 30 min. PTX was infused at a rate of 0.5 mg/kg/min during the aortic cross clamping. Aortic clamps were removed after 30 min warm ischemic insult and animals were allowed 120 min of reperfusion. Aortic occlusion and reperfusion were confirmed by the loss and reappearance of satisfactory pulsation in the distal aorta. In sham-operated animals, the same surgical procedure was applied without cross-clamping the abdominal aorta. All animals were sacrificed by a lethal dose of pentobarbital (200 mg/kg) at the end of the reperfusion period. The lumbosacral segments of spinal cords were quickly harvested and stored at –78°C for biochemical assays.

Biochemical procedure

Briefly, the frozen sample of rabbit spinal cord was weighed and homogenized (Ultra Turrax T25, Germany) (1:10, w/v) in 100 mmol/L phosphate buffer (pH 7.4) containing 0.05% sodium azide in an ice bath. The homogenate was sonicated (Bandelin, Germany) for 30 s and centrifuged (5000 g for 10 min). The supernatant was frozen at –78°C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the Lowry method.²³

Cytokine assays

The concentrations of tumor necrosis factor- α (TNF- α) and interleukine-6 (IL-6) in the supernatant were determined using enzyme-linked immunosorbent assay kits for TNF- α (Immunotech, Marseille, France) and for IL-6 (Cytimmune, Maryland, USA) according to the manufacturer’s specifications. Results are expressed as picograms per milligram of tissue protein (pg/mg protein).

Malondialdehyde assay

Malondialdehyde (MDA) was estimated by the double heating method of Draper and Hadley.²⁴ The principle of the method is the spectrophotometric measurement of the color generated by the reaction of thiobarbituric acid (TBA) with MDA. For this purpose 2.5 mL of 100 g/L trichloroacetic acid solution was added to 0.5 mL supernatant in each centrifuge tube and the tubes placed in a boiling water bath for 15 min. After cooling in tap water, the tubes were centrifuged at 1000 g for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and the tube placed in a boiling water bath for 15 min. The solution was then cooled in tap water and its absorbance was measured using a spectrophotometer (Shimadzu UV-1601, Japan) at 532 nm. The concen-

tration of MDA was calculated by the absorbance coefficient of the MDA-TBA complex (absorbance coefficient $\varepsilon = 1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and is expressed as nanomoles per milligram of protein (nmol/mg protein).

Antioxidant enzyme assays

Superoxide dismutase (SOD) activity was measured according to the method of Sun.²⁵ The determination of SOD activity was based on the reaction of xanthine with xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride to form a red formazan dye. The SOD activity is then determined as the degree of inhibition of this reaction. Results are expressed as units per milligram of protein (U/mg protein).

Catalase (CAT) activity was measured according to the method of Aebi.²⁶ The assay is based on the determination of the rate constant (s^{-1} , k) of hydrogen peroxide decomposition. The rate constant was calculated using the formula $k = (2.3/\Delta t)(a/b)\log(A_1/A_2)$, where A_1 and A_2 are the absorbance values of hydrogen peroxide at times t_1 (0th second) and t_2 (15th second), ('a' is a dilution factor, and 'b' is the protein content of the supernatant). Results are expressed as catalytic activity per milligram protein (cat/mg protein).

Statistical analysis

All statistical analyses were performed using SPSS 9.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean $\pm$ SEM. Differences among groups were analyzed by one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's honestly significant difference test. A probability value less than 0.05 was considered significant ($P < 0.05$).

Results

IL-6 levels were measured in SHAM and SHAM + PTX groups as 11.46 ± 1.09 and 11.13 ± 1.04 pg/mg protein, respectively ($P > 0.05$). The level of IL-6 was significantly increased to 87.40 ± 5.86 pg/mg protein in the AOR group, and PTX pretreatment significantly attenuated the level of IL-6 to 43.43 ± 2.73 pg/mg protein in the AOR + PTX group ($P < 0.05$) (Figure 1).

TNF- α levels were measured in SHAM and SHAM + PTX groups as 25.34 ± 1.03 and 25.12 ± 1.01 pg/mg protein, respectively ($P > 0.05$). The level of TNF- α was significantly increased to 120.44 ± 8.95 pg/mg protein in the AOR group, and PTX pretreatment significantly attenuated the level of TNF- α to 59.04 ± 2.09 pg/mg protein in the AOR + PTX group ($P < 0.05$) (Figure 2).

In SHAM and SHAM + PTX groups, MDA levels were 35.24 ± 2.73 and 33.69 ± 2.52 nmol/mg protein, respectively ($P > 0.05$). MDA level was significantly

Figure 1 Boxplot of the IL-6 levels of each group of animals. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles

Figure 2 Boxplot of the TNF- α levels of each group of animals. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles

increased to 95.51 ± 5.87 nmol/mg protein in the AOR group ($P < 0.05$). With the administration PTX, MDA level was significantly decreased to 59.04 ± 2.09 nmol/mg protein in the AOR + PTX group ($P < 0.05$) (Figure 3).

Mean SOD levels were 8.47 ± 0.66 and 7.88 ± 0.81 U/mg protein in SHAM and SHAM + PTX groups, respectively ($P > 0.05$). The level of SOD was significantly increased to 18.30 ± 0.87 U/mg protein in the AOR group ($P < 0.05$). SOD level was significantly decreased to 12.63 ± 0.37 in the AOR + PTX group with PTX pretreatment ($P < 0.05$) (Figure 4).

Mean CAT levels were measured in SHAM and SHAM + PTX groups as 9.40 ± 0.39 and 9.25 ± 0.40 U/mg protein, respectively ($P > 0.05$). CAT level was significantly increased to 20.24 ± 1.4 U/mg protein in the AOR group ($P < 0.05$). With the administration of PTX in the AOR + PTX group, the level of CAT was significantly decreased to 13.39 ± 0.43 U/mg protein ($P < 0.05$) (Figure 5).

Figure 3 Boxplot of the MDA levels of each group of animals. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles

Figure 4 Boxplot of the SOD levels of each group of animals. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles

Figure 5 Boxplot of the CAT levels of each group of animals. Horizontal bars represent the 90th, 75th, 50th (median), 25th, and 10th percentiles

Discussion

This study documents the effects of PTX on modulating levels of cytokines, lipid peroxidation and antioxidant enzymes in a rabbit model of spinal cord IR injury induced by aortic occlusion. Briefly, no differences in mean levels of IL-6, TNF- α , MDA, SOD, and CAT were noted between SHAM and SHAM+PTX groups. There was an increase in all biochemical parameters of IR injury in the AOR group. Administration of PTX decreased the levels of all biochemical parameters in the AOR+PTX group. In the present study we hypothesized that the free radical scavenger, neutrophil stabilizer, and blood flow regulator agent PTX would attenuate IR induced spinal cord injury in a rabbit model. Our results support our hypothesis. PTX decreased free radical production, antioxidant enzymes, and cytokine release from the activated neutrophils in this study.

The present study models thoracoabdominal aortic surgery leading to spinal cord ischemia. The rabbit spinal cord is a reliable model for systematically and rapidly observing the protective effects of agents on IR injury.⁹ The studies on the vascularization of rabbit spinal cords show that there are few differences between rabbits and humans.²

A decrease in the blood flow to an organ results in ischemic damage. When blood flow is restored further damage, so called reperfusion injury, occurs. Two mechanisms have been suggested in the development of IR injury. These are the enhanced generation of oxygen radicals and the activation of phospholipase A₂.²⁷ Lipid based cell membranes are the primary target of oxygen derived free radicals. They react directly with polyunsaturated fatty acids, leading to lipid peroxidation within the cell membranes followed by disintegration of the cells and ultimately to cell death. End products of lipid peroxidation include malondialdehyde (MDA), other aldehydes, hydrocarbon gases, and conjugated dienes.¹⁷

Beside their direct damage to the tissue, free radicals seem to trigger the accumulation of neutrophils in the tissue.²⁸ Free radicals stimulate monocytes to produce various cytokines such as IL-1, and TNF- α .²⁹ These cytokines act on endothelial cells to promote the production of interleukins (IL-2, IL-4, IL-6 and IL-8). IL-6 is a major stimulus of acute-phase response and is also responsible for the respiratory burst of neutrophils and release of free radicals.^{19,28} This cascade might explain a preventive role of PTX in IR injury. It has been documented that PTX is a free radical scavenger, inhibits cytokine production (mainly TNF- α), and reduces leukocyte-endothelial cell interactions.¹⁹ In the present study PTX significantly reduced cytokine levels and free radical production as well as antioxidant enzymes. Previous studies have shown that antagonism of neutrophil adherence to endothelium by means of monoclonal antibodies or platelet activating factor antagonist reduced spinal cord injury after transient ischemia in animal models.^{3,22,29} PTX is a

potent drug for neutrophil stabilization.¹⁷ If one considers the decrease in TNF- α and IL-6 levels, our results suggest that PTX inhibits neutrophil activation due to free radical generation.

Endogenous free radical scavenger enzymes, such as SOD and CAT have been found to reduce the degree of damage in IR models.²⁷ SOD is a protective enzyme that efficiently and specifically scavenges the superoxide radicals by catalyzing their dismutation to hydrogen peroxide and oxygen. On the other hand, catalase is an enzyme that enhances the degradation of hydrogen peroxide to oxygen and water.²⁷ Hence, determining the concentrations of these enzymes in tissue will indirectly give some idea about free radical formation following an ischemic insult. Our data also support this idea, and the levels of SOD and CAT in all groups correlated well with MDA levels.

PTX is a methyl xanthine derivate that has been used for its regulatory effects on blood flow for the treatment of peripheral vascular disease, cerebrovascular disease and a number of other conditions involving a defective regional microcirculation.¹⁶ Pentoxifylline increases the flexibility of red and white blood cells, reduces the blood viscosity by decreasing plasma fibrinogen concentrations and decrease platelet aggregation and thrombus formation.^{16–22} Polymorphonuclear leukocyte-mediated effects, such as superoxide production, chemotaxis, phagocytosis and tumor necrosis factor (TNF) production are inhibited by pentoxifylline.^{16–22} PTX has also been shown to attenuate IR injury in organs other than spinal cord.¹⁷

In a previous study, Turkoz *et al*²² evaluated the effects of PTX on spinal cord ischemia in a rabbit model. They used 20–24 min of ischemia and 72 h of reperfusion. They found that PTX administration did not change the outcome. They evaluated motor function of the lower limbs and routine histopathology of the spinal cord. However, in the present study cytokine levels, free radical generation, and antioxidant enzymes were assayed; PTX was also administered in a larger dose than their experiment.

In another study, Thompson and co-workers²¹ revealed that PTX treatment significantly increased spinal cord blood flow. In the PTX-treated group, spinal cord blood flow was significantly higher from 120 to 240 min post injury compared to the control group. They concluded that via its multiple physiologic effects, PTX significantly improves spinal cord blood flow in experimental spinal cord injury.

Fifteen minutes has been shown to be the minimal duration of aortic occlusion after which the spinal cord becomes ischemic.² The duration of aortic cross-clamping was 30 min in this study and, it is compatible with previous works on spinal cord ischemia in rabbits in which the duration of aortic occlusion was between 20 to 45 min.^{2,3,6–10,12–14,22}

For several reasons, we opted to investigate the effect of PTX after short periods of reperfusion. We preferred 120 min of reperfusion instead of longer periods. As far as we know, this is the shortest period

of reperfusion studied in a rabbit spinal cord IR injury model. It is documented that free radicals are generated within the first minutes of reperfusion when oxygen reaches the ischemic organ.²⁷ In a rabbit model of spinal cord IR injury, it has been shown that lipid peroxidation reaches its peak level by 4 h of reperfusion.² Leukocyte accumulation has also been shown to occur in a few hours after the onset of ischemia and to be coincident with the development of postischemic hypoperfusion and progressive neuronal death.³ As we have focused on early assay of biochemical markers of IR injury, 120 min of reperfusion should be sufficient to see those biochemical changes. But, in order to evaluate neurological deficit of lower limbs, one must apply longer periods of reperfusion such as 24 or 48 h.

Conclusion

Our data suggest that PTX reduces biochemical markers of IR induced spinal cord injury in a rabbit model. However, the increase in biochemical parameters does not necessarily mean irreversible IR injury to the spinal cord. Further studies are needed to correlate our findings with histopathological evidence of irreversible spinal cord damage and clinical outcome.

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