

Preventive Effect of Intrathecal Paracetamol on Spinal Cord Injury in Rats

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Background: Ischemic injury of the spinal cord during the surgical repair of thoracoabdominal aortic aneurysms might lead to paraplegia. Although a number of different mechanisms have been proposed, the exact cause of paraplegia has remained unknown, hampering the development of effective pharmacologic or other strategies for prevention of this condition. A number of studies suggested that cyclooxygenases (COX) contribute to neural breakdown; thus, COX inhibitors might reduce injury.

Objectives: We aimed to assess the preventive effect of intrathecal (IT) pretreatment with paracetamol on spinal cord injury in a rat model.

Materials and Methods: This experimental study was performed in Ataturk University Animal Research Laboratory Center, Erzurum, Turkey. Adult male Wistar rats were randomly allocated to three experimental groups (n = 6) to receive IT physiologic saline (controls), 50 µg of paracetamol, or 100 µg paracetamol one hour before induction of spinal cord ischemia. Six other rats were considered as the sham group. For the assessment of ischemic injury, motor functions of the hind limbs and histopathologic changes of the lumbar spinal cord were evaluated. Additional 20 rats were divided into two equal groups for the second part of the study where the survival rates were recorded in controls and in animals receiving 100 µg of paracetamol during the 28-day observation period.

Results: Pretreatment with 100 µg of paracetamol resulted in a significant improvement in motor functions and histopathologic findings (P < 0.05). Despite a higher rate of survival in 100 µg of paracetamol group (70%) at day 28, the difference was not statistically significant in comparison with controls.

Conclusions: Our results suggest a protective effect of pretreatment with IT paracetamol on ischemic spinal cord injury during thoracolumbar aortic aneurysm surgery.

Keywords: Spinal Cord; Ischemia; Paraplegia

1. Background

Paraplegia is an occasional complication of thoracoabdominal aortic aneurysm surgery and its occurrence is explained on the basis of temporary or permanent ischemia of the spinal cord due to interrupted blood flow during aortic cross-clamp (1). The reported incidence varies ranges from 4% to 33% (2, 3). Despite a number of proposed explanatory factors including microcirculatory disturbance, inflammatory factors, cellular necrosis and apoptosis, or biochemical autodestructive factors such as calcium ion overload, free radicals, and stimulatory amino acids, the exact mechanisms has remained largely unknown (4) and the responsible pathophysiological processes for the development of ischemic/hypoxic injury of the spinal cord are still obscure (5). Lumbar drains, intercostal artery reimplantation, left heart bypass, and hypothermic circulatory arrest can be protective against the development of paraparesis or paraplegia following aortic surgery (6-9); however, their complex and invasive nature is inevitably associated with additional complications, which limit their

widespread prophylactic utility (7). Paracetamol (N-acetyl-p-aminophenol) is a widely used ant nociceptive and antipyretic medication (10). Although it was first synthesized more than 100 years ago, the mechanisms of its analgesic effects have not been fully understood yet (11-15). Paracetamol inhibits cyclooxygenase (COX) 1 and 2 through blocking their peroxidase function. Contrary to nonselective nonsteroidal anti-inflammatory drugs (NSAID) and selective COX-2 inhibitors, paracetamol inhibits other peroxidase enzymes such as myeloperoxidase (16). The discovery of the role of end cannabinoids in pain modulation has provided a new point of view. Anandamide and 2-arachidonoylglycerol (two endogenous CB1 and CB2 receptor ligands) are mainly metabolized by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase and both of them produce ant nociceptive effects separately. It has been reported in various studies that cerebral injection of cannabinoids produces ant nociception in periaqueductal grey or rostroventral medulla. Acetaminophen is metabolized to

N-arachidonoyl phenol amine (AM404) in the brain and AM404 inhibits anandamide reuptake by CB1 stimulation through FAAH. Therefore, ant nociceptive activity of acetaminophen is based on its interaction with end cannabinoid system (17). Mallet et al. have demonstrated that acetaminophen is metabolized to AM404 by FAAH in rodent nervous system. In addition, *in vivo* studies have shown that AM404 is the potent activator of capsaicin receptor/transient receptor potential vanilloid 1 (TRPV1) and is an inhibitor of COX (18). Paracetamol does not directly bind to cannabinoid receptors but one of its metabolites exhibits cannabinoid-like activity. Accordingly, paracetamol, as a prodrug, can activate the end cannabinoid system (19). Intrathecal (IT) drug administration, as an alternative route for analgesia and anesthesia and acceptable adverse effects, is mainly used when sufficient efficacy cannot be achieved with high-dose oral or parenteral administration (20).

2. Objectives

IT administration of paracetamol, at a dose ranging from 50 to 200 µg, has been previously reported to provide significant ant nociceptive effects in rats (21). However, our literature search did not reveal any studies examining the protective effect of IT administration of paracetamol against reperfusion injury in spinal cord ischemia. In view of potential effects of paracetamol on central COX systems, we decided to examine such an effect.

3. Materials and Methods

The study protocol was approved by Institutional Animal Care Committee of Kahramanmaraş Sutcu Imam University, Kahramanmaraş, Turkey. All procedures were performed in accordance with the standards for animal care, approved by the Ethical Committee of Kahramanmaraş Sutcu Imam University (Protocol No: 2013/03-8). Animals were provided and maintained in Atatürk University Animal Research Laboratory Center, Erzurum, Turkey. Our study design was based on a previous model reported by Hsieh et al. (1). All groups were maintained under the same condition (temperature of 22°C ± 2°C and 12-hour dark-light cycle) and supplied with food and water *ad libitum*. We use simple random allocation.

3.1. Intrathecal Catheterization Procedure

A total of 44 male Wistar rats, weighing between 400 and 425 g, were included. Rats were catheterized for at least three days before the induction of spinal cord ischemia (22). Rats were placed in a plastic box and anesthetized by 1.5% to 2% isoflurane. After shaving the head and posterior neck, the head was fixed anteriorly to allow maintenance of isoflurane anesthesia with facemask. A skin incision on the posterior nuchal area was made and occipital muscles were separated from the base of the

skull. A polyethylene catheter (PE-10; BD Intramedic Polyethylene Tubing) was advanced to the lumbar expansion area along the cisternal membrane and was externalized at the posterior head. Rats experiencing a motor injury during the procedure were excluded from the study and immediately killed.

3.2. Ischemia-Reperfusion Procedure

Anesthesia maintenance in rats that were anesthetized within the plastic boxes was done by administration of 1.5% to 2% isoflurane via facemask (Anesthesia WorkStation AWS). Heating pads were placed under the rats to maintain normal body temperature. The tail artery was cannulated with a 22G catheter for intra-arterial heparin infusion. Proximal artery pressure (PAP) of the left carotid artery was measured after cannulation with a 20G catheter. For spinal cord ischemia, the left femoral artery was exposed and a 2F Fogarty catheter (Edward Lifesciences, USA) was advanced through the thoracic aorta. The tip of the catheter was localized at the junction of the left subclavian artery to aorta, which corresponds to a catheter length of 10.8 to 11.4 cm as reported in other studies. Immediately after the placement of the arterial catheter, 200 U (0.2 mL) of heparin (Nevparin Mustafa Nevzat Ilac, Istanbul, Turkey) was injected through the tail artery. The balloon was filled with 0.05 mL of physiologic saline for 11 minutes to induce spinal cord ischemia. The diastolic arterial pressure (DAP) was measured at the tail artery to assess the effectiveness of occlusion. PAP was measured from the left carotid artery and was maintained around 40 mm Hg. DAP, PAP, and body temperature were monitored before ischemia and during ischemia/reperfusion using the Philips Intelli value MP30 (Philips, USA). After inducing ischemia, the balloon was deflated. After all procedures were completed, catheters were removed and wounds were closed. Thereafter, 4 mg of protamine sulphate (Protamin HCl, Onko and Kocsel, Istanbul, Turkey) was injected subcutaneously to counteract the anticoagulant effect of heparin.

3.3. Drug Administration

In the first part of the study, rats were randomly allocated to four groups (n = 6) to assess the effect of IT paracetamol on neurological signs and histopathologic changes: control group (Group C), paracetamol groups (Group A1, Group A2), and the sham group (Group S). One hour before the induction of ischemia, 10 µL of physiologic saline was injected to control rats while rats in group A1 received 50 µg and rats in group A2 received 100 µg of paracetamol (Sigma-Aldrich, USA) in 10 µL of physiologic saline (19). A 50-µL Hamilton syringe was used for drug administration. In the Group S, a Fogarty catheter was placed but the balloon was not inflated and PAP was decreased to 40 mm Hg for 11 minutes. No medication was given to rats of Group S.

3.4. Neurologic Evaluation

After spinal ischemia, rats were transferred to their cages for recovery and their neurologic functions were assessed in the first postoperative 24 hours. For the assessment of motor functions, hind limb placing/stepping reflex was recorded. Ambulation in the hind limbs was graded as follows: 0, normal (symmetrical and coordinated hind limbs movements); 1, the first toe is immobile while walking, but ataxia is present; 2, knuckle walking; 3, absence of knuckling, but some mobility exists in the hind limbs; and 4, no movement in the hind limbs. The placing/stepping reflex was assessed by dragging the dorsum of the hind paw along the edge of a surface, which normally requires a coordinated pulling-up and placing response and was graded as follows: 0, normal; 1, weak; and 2, no stepping. A motor deficit index (MDI) was calculated for each rat as the sum of both scores (maximum, 6 points; a score of 4 for movements and 2 for placing/stepping reflex). MDI was calculated by an observer who was blinded to the assigned treatments.

3.5. Tissue Preparation and Histopathologic Changes

After observing the motor behavior for 24 hours, rats were anesthetized by intraperitoneal ketamine injection (10 mg/kg), which was followed by the trans cardiac perfusion of 100 ml of heparinized physiologic saline. Then 150 mL of 4% paraformaldehyde with phosphate buffer was given immediately. The lumbar expansion of the spinal cord at L3 or L4 was removed and kept in the same fixative at 40°C overnight. The samples were embedded in paraffin and the transverse cross-sections of 5-μm thickness were prepared and stained with hematoxylin and eosin. The samples were assessed by a pathologist who was blinded to treatment groups. The acute grey matter injury was calculated on the basis of the proportion of death or abnormal cells in the ventral horn as follows: 0, no neuronal injury or death; 1, mild injury (<10%); 2, moderate injury (10-50%); and 3, severe injury (> 50%). For each rat, the score corresponded to the findings in the right and left hemi cords in three consecutive sections.

3.6. Survival Study

In the second part of the study, the survival rate during the 28-day follow-up period was assessed in Group C-2 (physiologic saline group, n = 10) and in Group A2-2 (100 μg of paracetamol, n = 10).

3.7. Statistical Analyses

For statistical analysis, SPSS17.0 (SPSS Inc., Chicago, IL, USA) was used. Differences in motor and histopathologic scores were evaluated using the nonparametric Mann-Whitney U test (Monte Carlo significance test). Fisher's exact probability test was used for the comparisons in survival rates. A p value < 0.05 was considered statistically significant.

4. Results

The groups were similar in body weight. A significant difference in MDI and histopathologic scores was observed 24 hours after the spinal cord ischemia among the groups (Tables 1 and 2). After 11 minutes of the aortic occlusion by proximal controlled hypotension (40 mm Hg), Group S had normal motor functions while acute flaccid paraplegia developed in Group C. Moreover, spastic paraplegia occurred 24 hours after reperfusion in Group C. Three of the six rats in Group A1 had slight decrease in the mobility of the hind limbs (MDI of 4 and 5) 24 hours after spinal cord ischemia, while no rats in Group A2 had severe paraplegia (MDI of 0 and 1).

There were significant differences in staying/stepping reflex, MDI, and histopathologic scoring between Group S and Group A2, ($P < 0.05$). There was no difference in weight among all study groups ($P > 0.05$). In histopathologic evaluation, two rats in Group S had mild neuronal injury, whereas four had no neuronal injury (Figure 1 A). Two of the rats in the Group C had severe neuronal injury of the lumbar spinal cord while the remaining four rats had moderate neuronal injury 24 hours after intervention (Figure 1 B). In Group A1, three rats had moderate neuronal injury, with mild injury in three (Figure 1 C). In Group A2, three rats had mild neuronal injury with no injury in three (Figure 1 D). A significantly better motor improvement was seen in Group A2 in comparison to Group A1; therefore, the second part of our study involved rats in Group A2. In Group C-2 (n = 10), only four rats were alive after 28 days. In Group A2-2 (n = 10), seven rats were still alive at day 28 with preservation of good motor functions. The survival rate of the rats in Group A2-2, (70%) was higher in comparison with that of the rats in Group C-2 (30%); however, this difference did not reach statistical significance with Fisher's exact probability test ($P > 0.05$) (Figure 2). This study had 88% power to detect a difference of one unit difference between treatment and either of the sham or control groups with assuming a standard deviation of 2.5 units

Table 1. Weight, Motor Deficit Index, and Histopathologic Scores of the Four Study Groups ^{a,b}

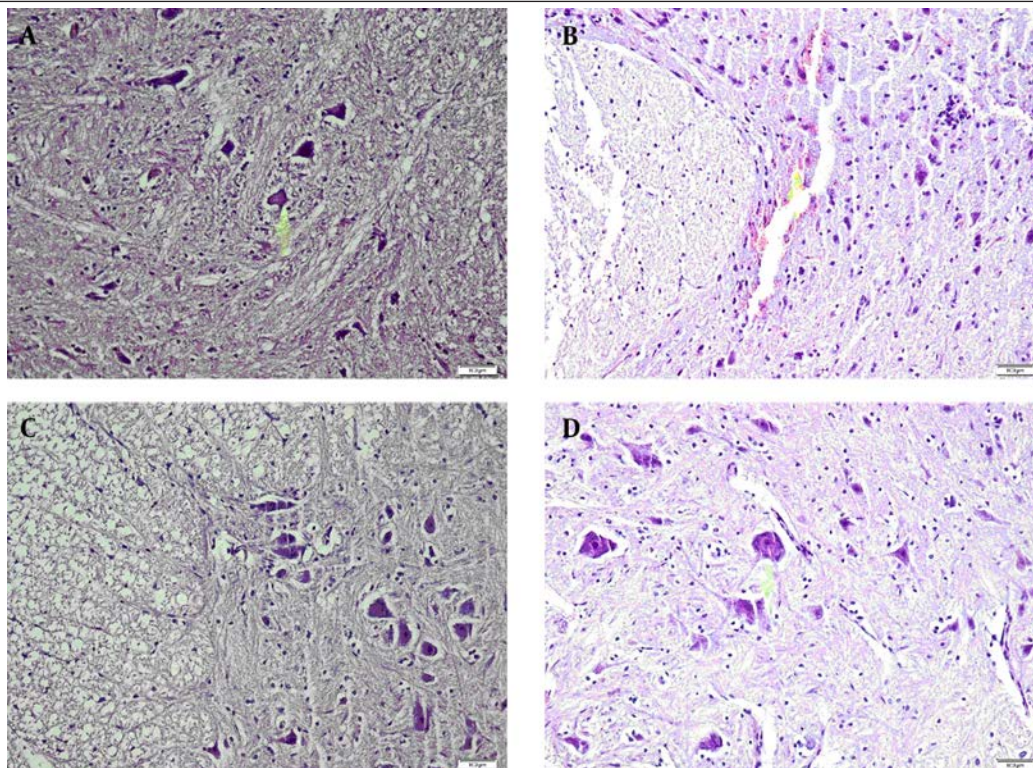
	Group S (n = 6)	Group C (n = 6)	Group A1 (n = 6)	Group A2 (n = 6)
MDI	0.0 (0.0-1.25)	5.0 (3.75-6.0)	3.5 (2.75-4.25)	0.5 (0.0-1.0)
Histopathologic Scoring	0.0 (0.0-1.0)	2.0 (2.0-3.0)	1.5 (1.0-2.0)	0.5 (0.0-1.0)
Weight	412.0 ± 6.9	415.3 ± 7.5	412.1 ± 10.2	414.3 ± 4.5

^a Abbreviation: MDI, motor deficit index.

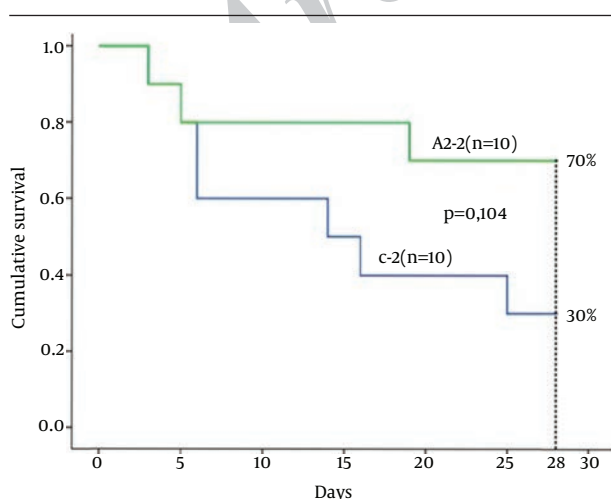
^b Data are presented as median (Q1-Q3) or mean ± SD.

Table 2. Comparisons of Experimental Groups With the Control Group ^a

Comparisons With the Control Group	Group S		Group A1		Group A2	
	Z	p ^b	Z	p ^b	Z	p ^b
MDI	-2.945	0.002 ^c	-1.803	0.116	-2.934	0.002 ^c
Histopathologic Scoring	-3.000	0.002 ^c	-2.166	0.078	-2.983	0.002 ^c
Weight	-0.723	0.524	-0.566	0.616	-0.405	0.732

^a Abbreviation: MDI, motor deficit index.^b Monte Carlo Sig (2-tailed).^c P < 0.05 (Mann-Whitney U test).**Figure 1.** Examples of histopathological images obtained from the lumbar expansion of rat spinal cord 24 hours after ischemia/reperfusion

A), control group; B), Sham group; C), A1 group; and D) A2 group (x200, hematoxylin and eosin staining).

**Figure 2.** Cumulative Survival Curve for the 28-Day Period After Spinal Cord Ischemia

5. Discussion

Our results show that pretreatment with IT paracetamol (100 µg) was associated with a significant decrease in hind limb motor dysfunction due to ischemic spinal cord injury 24 hours after ischemia/reperfusion in rats. In addition, pretreatment with 100 µg of paracetamol could prevent histopathologic changes in the spinal cord 24 hours after ischemia, while lower dose of paracetamol (50 µg) was not associated with a similar effect.

Neuroprotective effects of paracetamol are not well defined. Although weak, its anti-inflammatory properties might be partly responsible for the observed effects (12, 23). Previously, the ant nociceptive effect of paracetamol has been proposed to be mediated through spinal serotonin 5-HT receptors (13, 21, 24, 25). However, our literature search did not reveal any studies on the neuroprotective effects of paracetamol. Multiple mechanisms play role in spinal cord ischemia-reperfusion injury including short-

age of blood supply and energy, hypoxic endothelial cell activation, excitotoxicity, and oxidative stress. Oxidative stress results in the production of free oxygen radicals, which in turn lead to inflammatory events such as lipid peroxidation and protein and DNA breakdown (26). Graham and Scott reported two important findings regarding the effect of paracetamol on peroxidase function. First, it reduces glutathione through its effect on COX-1 and COX-2. Glutathione is a cofactor for many enzymes including PGE synthase, a membrane related enzyme. Thus, glutathione deficiency results in a decrease in PGE2 production. Second, two active metabolites of paracetamol directly interact and inhibit the enzymes that have role in prostaglandin synthesis (27). Smith speculated that paracetamol competitively inhibits COX-1, which in turn inactivates peroxidase function (17). These mechanisms might be responsible for the effect of paracetamol on ischemia reperfusion. The other proposed mechanisms were inhibiting nitrous oxide formation and N-methyl-D-aspartate or substance P mediated hyperalgesia. One of the recently used approaches is the structural similarity between paracetamol and AM404. AM404 is associated with the group of bioactive N-acylamines including endogenous lipid anandamide. AM404 is the potent activator of TRPV1 and inhibitor of anandamide cellular uptake, which lead in the reduction of endogenous cannabinoid levels. Another study on mice demonstrated the deacetylation of paracetamol in the brain and spinal cord, conjugation with arachidonic acid, and development of AM404; they stated that FAAH was the enzyme in that process. These new findings explain the mechanism of action of paracetamol and probably the inhibition of prostaglandin in the brain (28).

Botting and Ayoub proposed that the most likely explanation for the reduced PGE2 levels in the central nervous system (CNS) upon paracetamol administration was COX-3 inhibition, which led to analgesia and hypothermia. In addition, others have purported that the inhibited COX isoenzyme by paracetamol actually represents another isoenzyme, referred to as COX-4, which has been identified in isolated cells only (29). Probably, paracetamol exerts its preventive effect against ischemia-reperfusion injury through its interaction with these enzymes. Again, in a recent study by Graham et al., paracetamol has been reported to inhibit peroxidase enzymes including myeloperoxidase (16). IT administration of a drug has several advantages including rapidly achieved high drug levels and need for lower doses. On the other hand, increase in intracranial pressure after injection or infusion, slow distribution of the drug within cerebrospinal fluid, and risks of hemorrhage, neurotoxicity, and central nervous system infections are its disadvantages (30). DeLeo et al. pointed out the risks of inflammatory events and tissue reactions in response to chronic IT catheterization. They emphasized the association of IT catheterization with a strong neuroimmune activation resulting in the production of glial markers and specific cytokines, thereby in-

dicating the need for alternative methods (31). The effect of spinal cord ischemia on drug bioavailability is another disadvantage of IT route. Garcia-Lopez et al. evaluated the oral bioavailability of paracetamol in their study on an experimental model of spinal cord injury in rats. They observed a significant decrease in oral paracetamol bioavailability in the acute phase of spinal cord injury. This decrease was transient as paracetamol concentrations showed a partial recovery in the subacute phase and were similar to that of controls at the chronic phase (32). As for our study, rats were pretreated with IT paracetamol before inducing neurologic injury, its preventive effects were evaluated, and the drug was administered directly to the site of injury.

In conclusion, our results have shown that IT paracetamol administered at a dose of 100 µg could reduce the ischemic injury of the spinal cord in rats. Again, a significantly better improvements in motor functions and histopathologic changes were observed in comparison with the controls. These findings suggest that pre-surgical administration of IT paracetamol might have a place in the reduction of ischemic complications. However, the responsible mechanisms for the observed effect are not clear. Different proposed central and peripheral mechanisms for paracetamol paved the way for the studies on IT administration; however, due to its risks, IT approach is only used for local anesthetics and for opioids to achieve analgesia and anesthesia and its use for neuroprotection is still experimental.

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Authors' Contributions

Murat Sahin, main author, conduction of anesthetic procedures, and conception, design, administration, and critical revision of the manuscript; Ilyas Sayar, data collection and analysis; Kemal Peker and Huriye Gullu, data collection, analysis, and interpretation and critical revision of manuscript; and Huseyin Yildiz, data collection and critical revision of manuscript.

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