ORIGINAL ARTICLE

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Effect of Pretreatment with Cilostazol on Spinal Cord Ischemia-reperfusion Injury in Rats

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Abstract

Objective: Following the aortic aneurysm repair surgery, ischemic spinal cord injury is a substantial complication which may lead to paraplegia. This study aims to explore the protective effect of cilostazol, which is a phosphodiesterase type-3 inhibitor, against ischemic/reperfusion-induced spinal cord injury that is experimentally forged in medulla spinalis of rats.

Methods: A total of 24 rats were separated into three workgroups. The control group (n=8); the ischemic group (n=8), in which aortic clamping was performed without cilostazol administration; and finally the cilostazol-adminsistered group (n=8). Each mouse was subjected to induced ischemia for 45 min by clamping of the abdominal aorta. Afterwards, blood build up was provided by de-clamping. Serial assessments of motor and sensory functions of all rats were performed prior to the operation and, at 24 and 48 h of reperfusion, using the Tarlov and LeMay scores. Later on, spinal cord tissues were collected for histopathologic examination.

Results: Tarlov scores at postoperative hours 24 and 48 tend to be significantly higher in the cilostazol-treated group than in the non-treated ischemia group (3.13±0.64 versus 1.25±0.71, p=0.0029 for the 24th hour; 2.75±0.71 versus 0.38±0.52, p=0.0016 for the 48th hour). LeMay scores at postoperative hours 24 and 48 were as well significantly higher in the cilostazol-treated group than in the non-treated ischemia group (9.13±1.13 versus 4.50±0.76, p=0.0018 for the 24th hour; 9.00±1.20 versus 3.75±0.89, p=0.0018 for the 48th hour). Histologic outcomes were strongly correlated to the neurologic outcomes.

Conclusion: These results suggest that pre-ischemia cilostazol treatment has a protective effect against ischemia/reperfusion-induced spinal cord injury.

Keywords: Ischemia/reperfusion, spinal cord injury, cilostazol, rat, animal model

INTRODUCTION

Due to the medulla spinalis's exposure of temporary or permanent ischemia during the surgery, paraplegia is undoubtedly one of the most important emerging and undesirable complications that might result after thoracoabdominal aneurysm repair surgeries (1). Lintott et al. (2) reported the frequency of paraplegia occurrence due to extended clamp durations, dissection, and rupture. Eventhough every procedure has been performed during surgery to ensure continuous perfusion of the medulla spinalis, paraplegia could be inevitable (3,4). The damage mechanism caused by the reperfusion after ischemia is not clearly known. Increase in lipid peroxidation after reperfusion and neuronal damage secondary to fiber degeneration and results in loss of motor functions.

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Cilostazol is known as a selective inhibitor of cyclic nucleotide phosphodiesterase 3 (PDE3) (5). Intracellular cyclic adenosine monophosphate (cAMP) levels increase due to the inhibition of PDE3 activity and the decrease in cAMP degradation which results in diminished thrombocyte aggregation and vasodilatation. Besides, the pleiotropic effects of cilostazol have been used for the prevention of clinical disorders like recurrent stroke, coronary artery disease, and peripheral occlusive disease (6,7). The pre-clinical studies where vasodilator and antiplatelet effects of cilostazol were presented are the determinants of these indications (5-7). This study explores the prophylactic properties of cilostazol on neurobehavioral disorders and histopathological changes observed due to experimentally induced ischemic/reperfusion in spinal cord injury on rats.

METHODS

Istanbul University Animal Experiments Local Ethics Committee approval were obtained during the study (decision no: 145, date: 09.11.2009). The rats were exposed to 12 h of daylight as well as 12 h of darkness cycle. The shelter environment temperature was (20 °C-22 °C) and humidity was (50-60%) where standard rat feed and enough water was provided as well.

Experimental Design

5 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey) and 60 mg/kg ketamine (Ketalar, Parke-Davis Eczacıbasi¸ Istanbul, Turkey) were both used simultaneously for anesthesia. No mechanical ventilator was needed to support the animals' respiration during the experiment. A single 15 mg/kg dose of cefazolin (Cefamezin, Eczacibasi, Istanbul, Turkey) was administered in the postoperative period. During the experiment, these mice were administered 0.9% NaCl intravenously for volume replacement. After sterilization of the operation site, the abdominal aorta was attained via a transperitoneal approach through a 10-cm incision from the midline.

The cross clamp was placed after 100 U/kg systemic heparinization for anticoagulation. The aorta was cross-clamped by the use of aneurysm clips. During the procedure a surgical microscope was used. These clips were placed below the renal artery and above the iliac bifurcation. After 45 min, follows removal of the cross clamp. With the help of 4-Fr indwelling catheters placed beneath and above the clamp, distal and proximal aortic pressures were monitored. The incision was closed in layers. The control group was subjected to the exact surgical procedure except for aortic cross clamping. Before being placed in their cages.

The rodents were placed in a plastic box at 28 °C for 3 h to recover after the surgery.

Study groups: Twenty-four wistar-Albino male rats (weight 370-480 g) were divided into three different groups, as follows:

1. Sham group (n=8): The operation was performed with similar conditions except for aortic clamping.

2. Ischemia group (n=8): The operation was performed with similar conditions including aortic clamping for 45 min.

3. Cilostazol group (n=8): Cilostazol (100 mg/kg), dissolved in dimethyl sulfoxide, was injected intraperitoneally 2 h prior to operation. The surgery was performed in similar conditions including aortic clamping for 45 min.

Evaluation of the Neurobehavioral Outcome

Evaluations of the motor and sensory functions in the hind limbs of the rats was performed prior to surgery and after 24th and 48th hours of reperfusion. While measuring, it was assessed using the LeMay score and Tarlov scale (8,9). The Tarlov motor scale is read as follows: 0, complete paraplegia; 1, slight movement in the joint; 2, enough mobility in the joint but an inability to stand; 3, able to stand and able to walk; and 4, complete recovery. The LeMay score was calculated using a 15-point spinal cord performance scale. Motor-sensory deficits of the animals are evaluated using an index for each animal at each point in time (Appendix 1). The maximum deficit calculated by the LeMay score was 15. The rats (n=8 per group) were assigned to be killed after the second neurobehavioral assessment (48th hour). The rodents were killed by a high dose injection of sodium pentothal (200 mg/kg). The rapidly collected spinal tissues were placed in 10% formaldehyde at 4 °C for 48 h.

Histopathological Analysis

Spinal cord samples were taken out from the 10% formaldehyde after 48 h fixation period. The specimens were dehydrated by placing them in 95% alcohol for 30 min, then four changes were applied for 1 h each in 100% alcohol and five changes of toluene for 1 h each in a vacuum at 37 °C. After the spinal cords were infiltrated with paraffin, they were embedded in paraffin at 60 °C under vacuum and pressure. Transverse sections have been examined with a microtome. Five-micrometer sections were obtained through the spinal cord. Sections were deparaffinized and stained with cresyl violet, hematoxylin & eosin, Luxol Fast Blue staining (to check for the integrity of the myelin structure) and studied using light microscopy. Histopathologic changes of the ventral motor horn cells in medulla spinalis were scored on a 3-point scale for motor deficits, myelin injury, edema,

ependymal cell injury, vasocongestion as follows: 0, no damage; 1, mild lesion (<10%) observed; 2, a moderate lesion (10% to 50%) observed; 3, a severe lesion (>50%) observed. A blind study was done with the neuropathologist who was unaware of the experimental conditions.

Statistical Analysis

The results obtained were reported as means ± standard deviation. Data analysis was performed using the Statistical Package for Siocial Sciences version 14.0. Non-parametric tests such as Mann-Whitney U tests, Kruskal-Wallis tests, Spearman's correlation analyses, linear regression analysis, and paired Wilcoxon tests were carried out. Bonferroni correction was used where appropriate. P values of less than 0.05 were considered statistically significant.

RESULTS

The surgery was well tolerated by every mice. The mean proximal arterial pressure and mean distal arterial pressure values revealed no difference among study groups (p=0.840, and p=0.982, respectively) (Table 1).

Table 1. Hemodynamic differences with respect to groups

Arterial pressure	Sham group	Ischemia group	Cilostazol group	p value
Mean proximal arterial pressure, mmHg	79.38±1.06	79.13±0.83	79.25±1.04	p=0.840
Mean distal arterial pressure, mmHg	10.88±0.83	10.75±1.04	10.75±1.04	p=0.982

For each group, neurological examinations were performed during the 24th and 48th hours. For each group, Tarlov scores (Table 2) and LeMay scores (Table 3) revealed no difference between two-time points (24th and 48th hours of reperfusion) (p=0.07368 and p=0.160, respectively).

Histopathological analysis revealed a significant difference among study groups (p<0.05) (Table 4). While no significant damage was observed in the neurons in the sham-operated animal group, neuronal damage was detected in the control group rodents.

On the contrary, pretreatment with cilostazol was found to significantly reduce the histologic changes. Motor deficits, myelin injury, ependymal cell injury, and vasocongestion were found to be significantly lower in the cilostazol-treated group than in the non-treated ischemic group (p=0.0079, p=0.0023, p=0.0200, and p=0.0104, respectively). Regarding edema, both groups did not differ from each other significantly (p=0.1268) (Table 4) (Figure 1-3).

DISCUSSION

In this study, the transient ischemia-induced spinal cord ischemia (SCI) was significantly attenuated in rats that received cilostazol, (a type III phosphodiesterase inhibitor) compared with control animals. Cilostazol also prevented histologic changes induced by the transient ischemia, such as motor deficits, myelin injury, ependymal cell injury, and vasocongestion, both 24 and 48 h after the ischemia.

Ischemic spinal cord injury secondary to clamping the aorta may occur during thoracoabdominal aortic aneurysm and

Table 2. Tarlov scores

Groups	Tarlov score 24 h	[a]p value	Tarlov score 48 h	[a]p value
Sham	3.75±0.46	Group 1 vs. 2, p=0.014	3.88±0.35	Group 1 vs. 2, p=0.0010
Ischemia	1.25±0.71	Group 2 vs. 3, p=0.0029	0.38±0.52	Group 2 vs. 3, p=0.0016
Cilostazol	3.13±0.64	Group 1 vs. 3, p=0.1354	2.75±0.71	Group 1 vs. 3, p=0.0105
	**p<0.001		**p<0.001	

**p value obtained from the Kruskal-Wallis test, [a]p value obtainedafter performing the Bonferonni-adjusted Mann-Whitney U-test

Table 3. Lemay scores

Groups	Lemay score 24 h	[a]p value	Lemay score 48 h	[a]p value
Sham	13.00±1.31	Group 1 vs. 2, p=0.0019	12.75±1.49	Group 1 vs. 2, p=0.0018
Ischemia	4.50±0.76	Group 2 vs. 3, p=0.0018	3.75±0.89	Group 2 vs. 3, p=0.0018
Cilostazol	9.13±1.13	Group 1 vs. 3, p=0.0026	9.00±1.20	Group 1 vs. 3, p=0.0036
	**p<0.001		**p<0.001	

**p value obtained through the Kruskal-Wallis test, [a]p value obtained after conducting the Bonferonni-adjusted Mann-Whitney U-test

dissecting operations. As a result, paraplegia may develop. In experimentally induced SCI, while oxidative stress does not permit antioxidant activity, local antioxidants protect the neural tissue from oxidative stress. Reperfusion occurs 1-2 days after SCI, exacerbating the neural damage (8-10).

It is well known that, oxidative stress triggers the lipid peroxidation cascade resulting in cell membrane damage after a couple of days following SCI (11,12). Treatments which decrease oxidative stress might provide benefit for neurological diseases (13). The central nervous system which is well recognized for its rich lipid composition l is more prone to damage as a result of

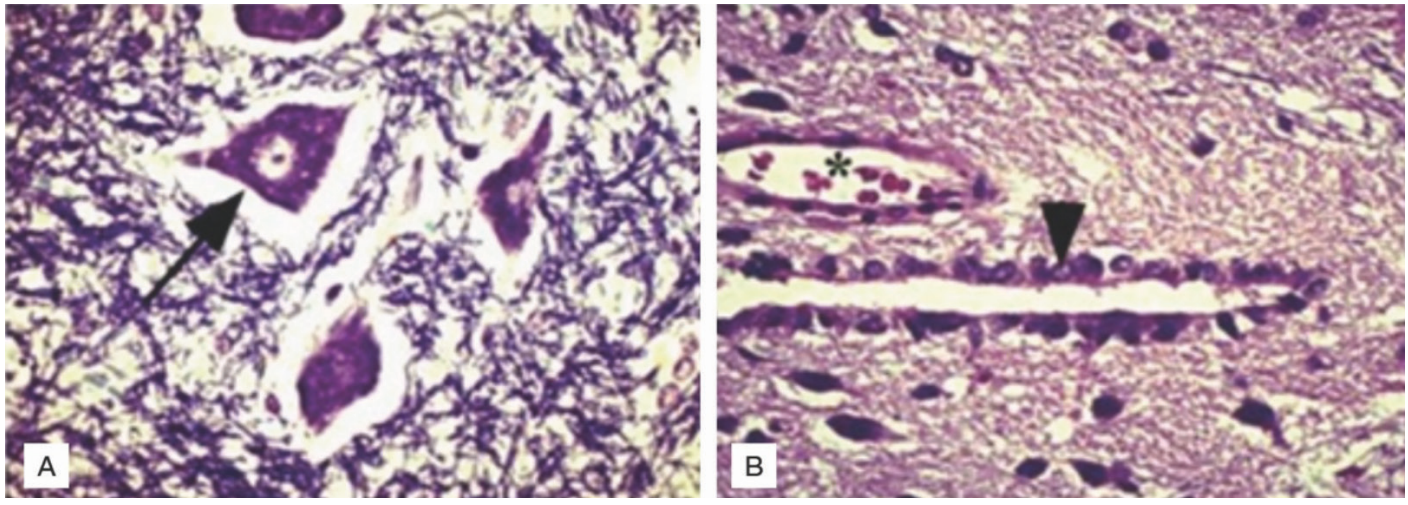

Figure 1. Group 1 (control group); normal morphology (A), nerve cells (arrow) (B), ependymal cells (arrowhead) and vascular structure (*) are observed. (A) Luxol fast blue (Kluver Berrare) stain x100; (B) hematoxylin & eosin stain x100; insert: x400

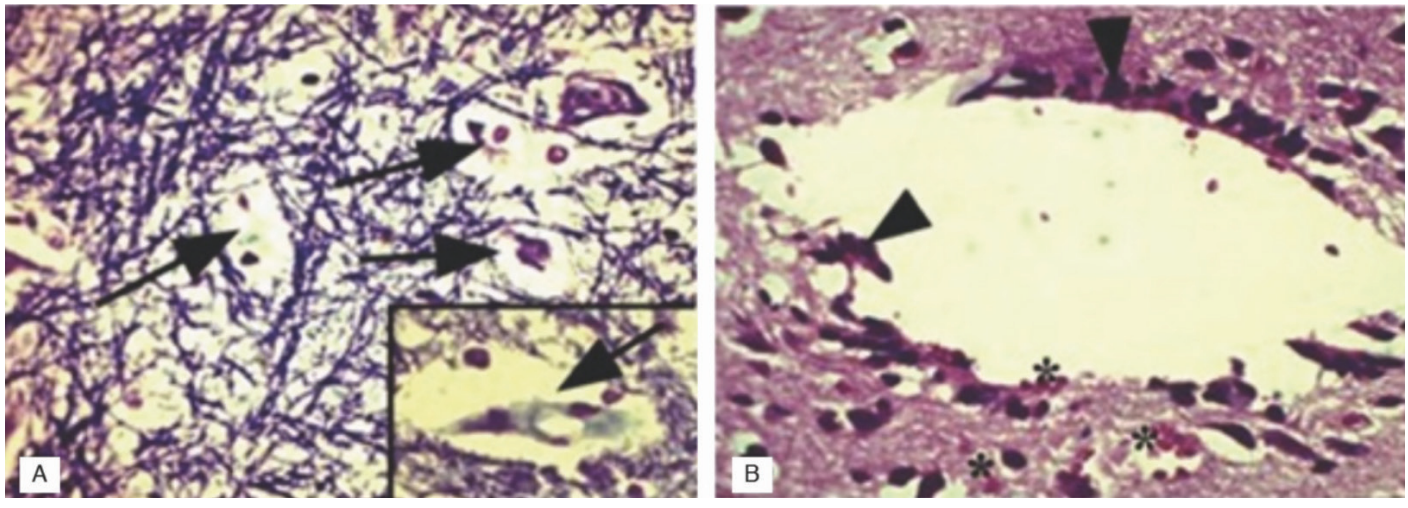

Figure 2. In group 2 (ischemia group); (A) damaged nerve cells (arrow), diminished myelination, (A) damaged ependymal cells (arrowhead) and vasocongestion (*) are seen. (A) Luxol fast blue (Kluver Berrare) stain x100; (B) hematoxylin & eosin stain x100; insert: x400

Table 4. Comparison of histopathological score among groups

Groups	Motor deficits	Myelin injury	Edema	Ependimal cell injury	Vasocongestion
Group 1	0.00±0.00	0.13±0.35	0.13±0.35	0.13±0.35	0.00±0.00
Group 2	2.25±0.46	2.13±0.35	1.75±0.71	2.63±0.52	2.50±0.53
Group 3	1.25±0.46	1.13±0.35	1.13±0.35	1.75±0.46	1.38±0.52
Group 1 vs. 2	p=0.0006	p=0.0007	p=0.0020	p=0.0010	p=0.0008
Group 2 vs. 3	p=0.0079	p=0.0023	p=0.1268	p=0.0200	p=0.0104
Group 1 vs. 3	p=0.0006	p=0.0023	p=0.0023	p=0.0013	p=0.0007

lipid peroxidation resulting from free radicals. The purpose of neuroprotection is to prevent neurons from lipid peroxidation occurring after SCI (14).

This study discloses a neuroprotective effect of cilostazol in an *in vivo* SCI model. To protect the spinal cord from the ischemic damage due to distal aortic perfusion, drainage of the cerebrospinal fluid, reimplantation of the intercostal arteries, and pharmacological treatments have been used. Many pharmacological agents like; magnesium, calcium channel blockers, opioid receptor antagonists, corticosteroids, free radical cleaners, sodium channel blockers, cyclosporin A, N-methyl-d-aspartate receptor antagonists, and thyrotropin-releasing hormone are used in the prophylaxis of SCI (15). Reperfusion occurs in 1-2 days following SCI. While the oxygen provided by the reperfusion ensures neural revival, catalysis some enzymatic oxidative reactions at the same time. Reactive oxygens resulted from oxidation reaction, causes DNA fragmentation by starting apoptosis (16). In one of their studies, Lee et al. (17) had applied cilostazol after they had occluded the middle cerebral artery for 2 h. From the samples obtained after 24-48 h of reperfusion, they realized that the DNA fragmentation has been significantly suppressed. It has been declared that, DNA chain breakdown is elicited by excessive poly(ADP-ribose) polymerase (PARP) activity which is a nuclear protein, resulting from ischemia/reperfusion. Thus, leading to necrosis (18). In another study, it is been determined by an enzyme analysis performed that with a low IC_{50} value of cilostazol, PARP is inhibited. Besides, cilostazol reduced the PARP activity in the rat's cerebral cortex exposed to ischemic-reperfusion damage and improved the product of activated PARP (19). Matsumoto et al. (20) reported that cilostazol inhibits the procoagulant activity caused by thrombin and this inhibition is dependent on cilostazol concentration. With various studies, it has been disclosed that cilostazol has a protective effect against damages caused by transient or chronic cerebral ischemia. It is been revealed by the studies performed in rats that, cilostazol inhibited apoptotic and oxidative cell death, decreased gray and white matter damage thus substantially decreased ischemic brain infarction after 24 h from focal cerebral ischemia (21,22). With their studies performed in rats, Lee et al. (23) have scanned with magnetic resonance imaging that cilostazol had decreased the brain edema caused by ischemic infarction. Cilostazol prevented cognitive disorder devisal in rats where chronic cerebral hypoperfusion had been created with common carotid artery ligation and protected rats from the formation of white matter lesions (24).

The rat model used in our study was inspired gy the rat model of LeMay et al. (9). The rat model involving aortic clamping is well established and has been previously used for testing the potential neuroprotective effect of drugs (25). In all rats where aortic cross clamp have been applied under normothermia, the observed paraplegia paced quite heavily. Thus, the study has a high repeatability ratio. The arterial vascularization of the spinal cord is very similar in rats. Both have heterosegmental aorta and some anterior radicular arteries (26). Recent experiments revealed that 45 min of aortic occlusion resulted in complete loss of evoked motor potentials and paraplegia (27). Thus, it is likely that the marked reduction in neuronal damage was affiliated to improved spinal cord function. The histopathological evaluation includes neuronal and axonal damage as well as microglial infiltration. The control group had no spinal injury. Interestingly, the cilostazol-treated group has significantly better histopathological results comparted to the

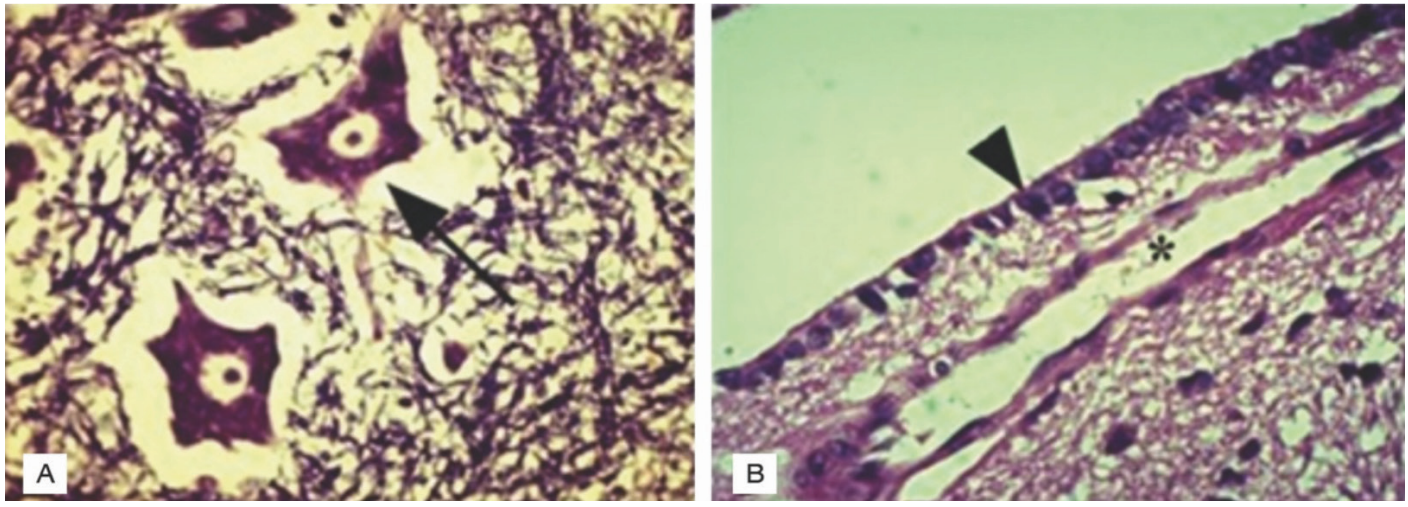

Figure 3. In group 3 (cilostazol group); (A) normal nerve cells (arrow), (B) almost normal ependymal cells (arroehead) and vascular structure (*) are observed. (E) Luxol fast blue (Kluver Berrare) stain x100; (B): hematoxylin & eosin stain x100; insert: x400

sham-operated group. These results suggest that cilostazol may also have beneficial effects in protecting the intact and fully healthy spinal cords. It is believed that this effect is achieved by reducing oxidative stress. This study also evaluated the motor and sensory functions in the hind limbs of rats during the 24th and 48th hour of reperfusion, using the Tarlov scale and the LeMay score. Regarding postoperative 24th and 48th hour measurements, both LeMay and Tarlov scores have been ascertained as high in the group treated with cilostazol with respect to the ischemia group. However, when the control group is compared with the cilostazol treatment group, it is been ascertained that there is no difference in Tarlov scores obtained in 24 h.

As a result of this study, it could be assumed that; the motor functions of hind limbs of rats which had received cilostazol treatment, are healed with respect to the neurological examination done on rats after ischemic-reperfusion. With reference to these results, our hypothesis has been verified. As neurological scores of the mice in the control group were higher with respect to the cilostazol group, we think that cilostazol alone is not sufficient for the treatment of motor function disorders caused by the SCI-reperfusion damage. The beneficial effect of cilostazol was also confirmed by a histopathological study.

Study Limitations

In this study, only functional outcomes and histopathological parameters were evaluated. The lack of biochemical and immunohistochemical assessment is the major pitfall of our study.

CONCLUSION

In a clinically relevant rat model of aortic cross-clamping, cilostazol given before ischemia markedly reduced morphological spinal cord injury. It could be said that cilostazol might have a healing effect on the motor functions in rats caused by the spinal cord damage as a result of ischemic-reperfusion, though relying on the literature evidence bespoken. However, more scientific research is needed on this subject.

Ethics

Ethics Committee Approval: Istanbul University Animal Experiments Local Ethics Committee approval were obtained during the study (decision no: 145, date: 09.11.2009).

Informed Consent: There is no need.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ü.K.K., G.E.Ş., Concept: Y.K., Design: Y.K., Data Collection or Processing: Ü.K.K., Analysis or Interpretation: G.E.Ş., Literature Search: Ü.K.K., Writing: Ü.K.K., G.E.Ş.

Conflict of Interest: No conflict of interest was declared by the authors.

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Appendix 1. Spinal performance scale

Variable		Score
Walking with lower extremities	Normal (symmetrical and coordinated ambulation)	4
	Toes flat under body when walking. but ataxia present	3
	Knuckle walking	2
	Movement in lower extremities but inability to knuckle walk	1
	No movement drags lower extremities	0
Horizontal rope	Grasps rope and pulls up with lower extremity	3
	Grasps rope without pulling	2
	Unable grasp rope	1
	Does not raise lower extremity	0
45 ºC Bar	Grasps bar for >10 s	3
	Grasps bar for 5-10 s	2
	Grasps bar for <5 s	1
	No attempt to grasp bar	0
Pain sensation	Normal. withdrawal to toe pinch	2
	Squeals to toe pinch but does not withdraw	1
	No reaction to toe pinch	0
Rotating screen	Grasps screen to 180° for >5 s	3
	Grasps screen to 180° for <5 s	2
	Grasps screen past 270° but not to 180°	1
	Falls from vertical screen	0
Total score		15