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

Kulaçoğlu et al. Effect of Cilostazol on Ischemia-reperfusion Injury

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Abstract
Objective: Following the aortic aneurysm repair surgery, ischemic spinal cord injury is a substantial pathologic outcome which may lead to paraplegia. This study aims the research of 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 divided into 3 workgroups. the control group (n=8); the ischemia group (n=8), which aortic clamping applied without cilostazol administration ; and the cilostazol-adminsistered group (n=8). All animals were exposed to ischemia for 45 minutes by clamping the abdominal aorta. Afterwards, blood build up was provided by de-clamping. Serial assessments of motor and sensory functions of all rats were performed before the operation and, at 24 and 48 hours of reperfusion, using Tarlov scores and the score of LeMay. Spinal cords were harvested for histopathologic examination.
Results: Tarlov scores at postoperative hours 24 and 48 tended to be higher in the cilostazol-treated group than in the nontreated 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 tended to be higher in the cilostazol-treated group than in the nontreated 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 correlated well with the neurologic outcomes.
Conclusion: The results of this research have proven to us 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 complication that might result after thoracoabdominal aneurysm repair surgeries. [1]. Lintot et al. have reported that regarding the frequency of paraplegia occurrence due to extended clamp durations, dissection, and rupture. [2]. Eventhough all the procedures have been exercised during the surgery to provide the continuation of medulla spinalis perfusion, paraplegia could be inevitable. [3,4]. The damage mechanism resulted by the reperfusion after ischemia has not been clarified yet. Increase in lipid peroxidation after reperfusion and loss of neurons following the fiber degeneration resulting in loss of motor functions.

Cilostazol is known as a selective inhibitor of cyclic nucleotide phosphodiesterase 3 (PDE3).[5]. Intracellular cAMP levels increase due to the inhibition of PD3 activity and the decrease of cAMP degradation which results in degradation of thrombocyte aggregation and vasodilatation. Besides, pleiotropic effects of Cilostazol have been defined to be used for the prevention of clinical issues i.e. 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 targets the research of the preventive effects of Cilostazol upon neurobehavioral disorders and histopathological changes observed due to experimentally created ischemic/reperfusion-induced spinal cord injury on rats.

METHODS

Animal care: Institutional Animal Care and Use Committees approval were obtained during the study. (145-09.11.2009). Rats have been exposed to 12 hrs of daylight and 12 hrs of darkness cycle. The shelter environment temperature was (20-22°C) and humidity was (%50-60) where standard rat feed and enough water have been provided as well **Experimental Design:** 5 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey) and 60 mg/kg ketamine (Ketalar, Parke-Davis Eczacıbasi¸ Istanbul, Turkey) in combination were used for anesthesia. No mechanical ventilator was needed to support the animals' respiration during the experiment. A single dose 15 mg/kg cefazolin (Cefamezin, Eczacibasi, Istanbul, Turkey) was administered in the postoperative period. During the experiment, the animals were given for volume replacement with 0.9% NaCl intravenously. After the surgery site was sterilized, the abdominal aorta was reached with a transperitoneal approach with 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 using aneurysm clips. During the procedure a surgical microscope was used.The clips were placed under the renal artery and above the iliac bifurcation. After 45 minutes, blood stream is provided with the 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 exactly the same to the surgical procedure except for aortic cross clamping. Before placed in their cages

animals were placed in a plastic box at 28°C for 3 hours 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 (100mg/kg), dissolved in dimethyl sulfoxide (DMSO), was injected intraperitoneally 2 hours before the operation. Operation was performed with 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 animals were made before the operation and after 24th and 48th hours of reperfusion. While measuring, it was made using the score of le May and Tarlov scale [8,9]. The Tarlov motor scale is 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 time point. (Appendix). The maximum deficit calculated by the score of LeMay was 15. The animals (n=8 per group) were assigned to be killed after the second neurobehavioral assessment (48th hour). Animals were killed with an injection of high dose sodium pentothal (200mg/kg). The rapidly harvested spinal cords were placed in 10% formaldehyde at 4°C for 48 hours.

Histopathological analysis: Spinal cords were removed from 10% formaldehyde after 48 hours of fixation. Specimens were dehydrated by keeping them in 95% alcohol for 30 minutes, then four changes were applied for 1 hour each in 100% alcohol and five changes of toluene for 1 hour each under 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 (HE), 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, the moderate lesion (10% to 50%) observed; 3, the severe lesion (>50%) observed. A blind study was done with the neuropathologist who was unaware of the experimental conditions.

Statistic analysis

The results obtained are given as means ±SD. SPSS version 14.0 was used when analyzing the data. We used nonparametric tests such as Mann-Whitney U tests, Kruskal-Wallis tests, Spearman's correlation analyses, linear regression analysis, and paired Wilcoxon tests. Bonferroni correction was used where appropriate. P values of less than 0.05 were considered statistically significant.

RESULTS

All animals tolerated the operation well. 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).

For each group, neurological examinations were performed at 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 animals.

On the contrary, pretreatment with Cilostazol was found to be significantly reduced these 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 nontreated ischemia group (p=0.0079, p=0.0023, p=0.0200, and p=0.0104, respectively). As for edema,

another histologic parameter, both groups did not differ from each other significantly (p=0,1268) (Table 4) (Figure 1-3).

DISCUSSION

In the present study, the transient ischemia-induced 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 hours after the ischemia.

Ischemic spinal cord injury secondary to clamping the aorta may occur during thoracoabdominal aortic aneurysm and dissection operations. As a result, paraplegia may develop. In experimentally created spinal cord ischemia, while oxidative stress does not allow antioxidant activity to work, local antioxidants protect the neural tissue from oxidative stress. Reperfusion occurs 1-2 days after SCI, exacerbating the neural damage.

[8-10]. It's been informed that, oxidative stress trigger the lipid peroxidation cascade which causes cell membranes damage after a couple of days following SCI [11,12]. Treatments which decreases oxidative stress might provide benefit for neurological diseases [13]. Central nervous system highly formed by lipids is more likely prone to damaging as a result of lipid peroxidation caused by free radicals. The purpose of neuroprotection is to prevent neurons from lipid peroxidation which occurs after SCI [14].

The present study shows a neuroprotective effect of cilostazol in an in vivo spinal cord ischemia model. To protect the spinal cord from the ischaemic 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 have been used to prevent SCI, like; magnesium, calcium channel blockers, opioid receptor antagonists, corticosteroids, free radical cleaners, sodium channel blockers, cyclosporin A, NMDA receptor antagonists, and thyrotropin-releasing hormone [15]. Reperfusion takes place in 1-2 days following SCI. While the oxygen provided by the reperfusion ensures neural revival, catalysis some enzyatic oxidative reactions at the same time. Reactive oxygens resulted by oxidative reaction, causes DNA fragmentation by starting apoptosis [16]. In one of their studies, Lee et al. had applied Cilostazol after they had occluded the middle cerebral artery for 2 hours. From the samples obtained after 24-48 hours of reperfusion, they had observed that the DNA fragmentation has been significantly suppressed. [17]. It has been declared that, DNA chain breakdowns have been elicited by over-action of PARP which is a nuclear protein, resulting from ischemia/reperfusion. As a result of this fact, necrosis is taking place [18]. In another study, it's been determined by an enzyme analysis performed that with a low IC50 value of Cilostazol, PARP is inhibited. Besides, Cilostazol had decreased the PARP activity in rat's cerebral cortex exposed to ischemic reperfusion damage and had increased the product of activated PARP [19]. In their study, Matsumoto, Y et al. have shown that Cilostazol inhibits the procoagulant activity caused by thrombin and this inhibition is dependent to Cilostazol concentration [20]. With various studies, it has been presented Cilostazol's having protective effect against damages caused by transient or chronic cerebral ischemia. It's been showed by the studies performed with rats that, Cilostazol had inhibited apoptotic and oxidative cell death, decreased grey and white matter damage thus substantially decreased ischemic brain infarction after 24 hours from focal cerebral ischemia [21,22]. With their studies performed with rats, Lee, JH. et al. have scanned with MRI that cilostazol had decreased the brain edema caused by ischemic infarction [23]. Cilostazol had 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 has been inspired from 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 spinal cord is very similar in rats. Both have heterosegmental aorta and some anterior radicular arteries[26]. Recent experiments showed 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 with improved spinal cord function. The histopathological evaluation includes neuronal and axonal damage and microglial infiltration. The control group had no spinal cord injury. Interestingly, the cilostazol treated group has significantly better histopathological results than the sham-operated group. These results show that cilostazol may also have beneficial effects in protecting the intact and fully healthy spinal cords.We think that this effect is achieved by reducing oxidative stress. The present study also evaluated the motor and sensory functions in the hind limbs of rats at 24 and 48 hours of reperfusion, using the Tarlov scale and the score of LeMay. Regarding postoperative 24th and 48th hours 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's been ascertained that there is no difference in Tarlov scores obtained in 24 hours.

As a result of this study, we can say that; the motor functions of hinder limbs of the animals which had received Cilostazol treatment, have cured with respect to the neurological examination done on rats after ischemic-reperfusion. Relying on these results, our hypothesis has been verified. As neurological scores of the animals in the control group have been found 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 spinal cord ischemia-reperfusion damage. The beneficial effect of cilostazol was also confirmed by a histopathological study. In the presented study, only functional outcomes and histopathological parameters were analyzed. 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 the morphological spinal cord injury. We can say that Cilostazol might have a curing 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.

Conflict of interests

None

Authors' contributions

UK designed the study, conducted the animal experiments in the animal laboratory, and performed the statistical analysis of the study.

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None

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Table 1. Hemodynamic differences according to groups

Arterial pressure	**Sham Group**	**Ischemia Group**	**Cilostazol Group**	**p Value**
Mean proximal arterial pressure, mm Hg	79.38±1.06	79.13±0.83	79.25±1.04	p=0.840
Mean distal arterial pressure, mm Hg	10.88±0.83	10.75±1.04	10.75±1.04	p=0.982

Table 2. Tarlov scores

Groups	**Tarlov score 24h**	**p-value[a]**	**Tarlov score 48h**	**p-value[a]**
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 with Kruskal-Wallis test.
[a] p-value obtained with Bonferonni-adjusted Mann-Whitney U-test.

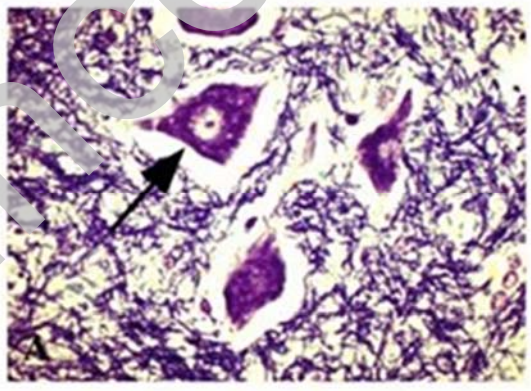
Table 3. Le-May scores

Groups	**Le-May score 24 h**	**p-value[a]**	**Le-May score 48 h**	**p-value[a]**
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 with Kruskal-Wallis test.
[a] p-value obtained with Bonferonni-adjusted Mann-Whitney U-test.

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



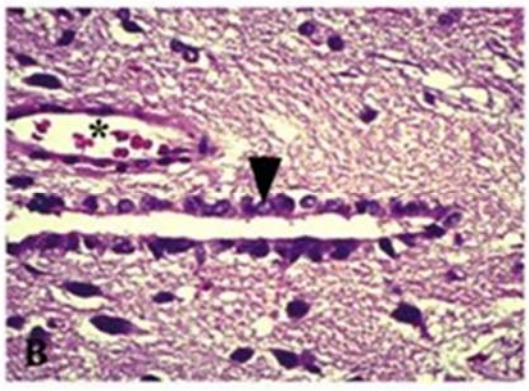


Figure 1: In Group 1 (Control group); normal morphology **A)** nerve cells (arrow) **B)** ependymal cells (arrowhead) and vascular structure (*) are seen. A:Luxol fast blue (Kluver Berrare) stain x 100; B: Hematoxylin-Eosin stain x 100; insert:x400

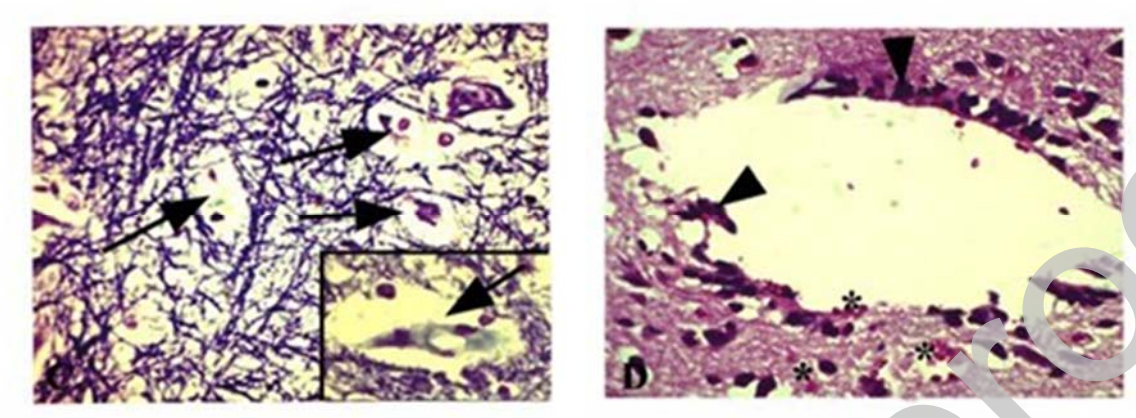


Figure 2: In Group 2 (Ischemia group); **C)** damaged nerve cells (arrow), reduced myelination, **D)** damaged ependymal cells (arrowhead) and vasocongestion(*) are seen. C: Luxol fast blue (Kluver Berrare) stain x 100; D: Hematoxylin-Eosin stain x 100; insert:x400

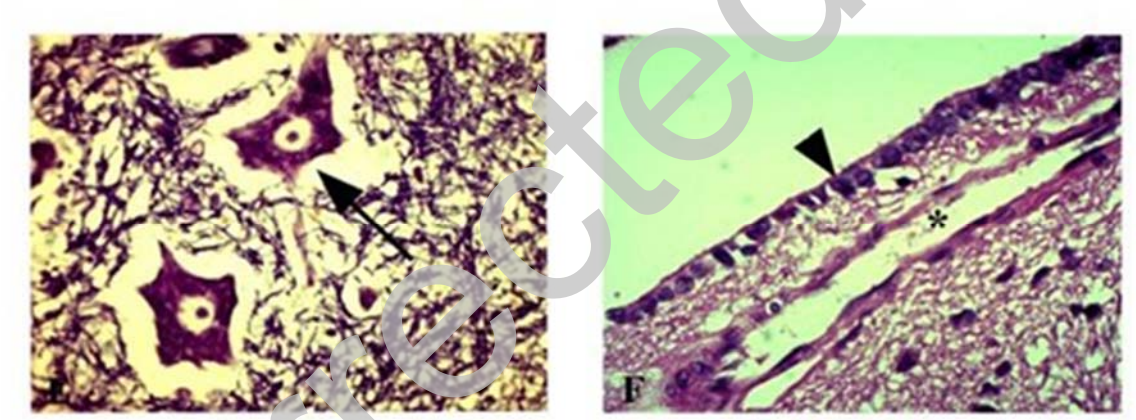


Figure 3: In Group 3 (Cilostazol group); **E)** nerve cells in normal morphology (arrow), **F)** almost normal ependymal cells (arroehead) and vascular structure (*) are seen. E:Luxol fast blue (Kluver Berrare) stain x 100; F: Hematoxylin-Eosin stain x 100; insert:x400