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Neuroprotective effects of FK-506, L-carnitine and azathioprine on spinal cord ischemia-reperfusion injury

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Abstract

Objective: In our experimental study, we aimed to test the effect of FK506, azathioprine and L-carnitine on protection of spinal cord injury due to ischemia-reperfusion. **Methods**: Twenty-seven Spraque–Dawley male rats were randomly divided into five groups. They were subjected to spinal cord ischemia by clamping the abdominal aorta for 45 min. Thirty minutes before the aortic clamping, group I received 0.5 mg/kg FK506, group II received 100 mg/kg L-carnitine, group III received 4 mg/kg azathioprine, the fourth group was the control group and received only normal saline injection intravenously and the last group was the sham group. Neurological status was scored by using the Tarlov scoring system. Sections of the lumbar cord were harvested for histopathological grades (1–4), having regard to percentage of the apoptotic cells. **Results**: Hind-limb motor function had recovered normally 48 h after the operation in all rats which received FK506, azathioprine and L-carnitine prophylactically. In contrast, all rats in the control group had deteriorated to paraplegia by 48 h after the operation ($P < 0.05$). Histopathologic sections in the involved spinal cord segment showed that a greater number of motor neuron cells were preserved and there were less apoptotic cells in the rats that received FK506, azathioprine and L-carnitine than those in control group. **Conclusions**: These results suggest that prophylactic use of FK506, azathioprine and L-carnitine protects motor neuron cells from ischemic spinal cord injury.

Keywords: Ischemia/reperfusion; Spinal cord

1. Introduction

The most sensitive organ to ischemia is the spinal cord in aortic surgery. Following aortic surgery, the most important complication is the development of paraplegia or paraparesis [1–3]. These complications tend to decrease due to increasing surgical and anesthesiological technology, however, their incidence is still 5–40% [4]. Because identification of the Adamkiewicz artery and its selective reconstruction are difficult in the majority of cases, mechanical and pharmacological interventions have been attempted to reduce this incidence. These interventions include hypothermia, vascular shunting, left heart bypass, drainage of cerebrospinal fluid, monitoring of somatosensory evoked potentials, single clamp technique and reimplantation of major intercostal arteries [5–11]. Also, there are experimental studies like ischemic preconditioning and adjunctive medications for reducing the incidence of this complication [12]. It is known that in ischemia and reperfusion injury, there is a possible role of oxygen free radicals. These oxygen free radicals produce lipid peroxidation, protein and DNA damage. Neutrophil activation also plays an important role in ischemia and reperfusion injury, since their activation leads to oxygen free radical production. FK506 (Tacrolimus; Fujisawa Pharmaceuticals, Osaka, Japan) is a macrolide antibiotic with major immunosuppressive properties, and is used in transplantation for the prevention of rejection [13]. Several studies have reported a neuroprotective effect of FK506 in experimental models of cerebral ischemia or spinal cord

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injury [14,15]. The neuroprotective effect of FK506 is thought to be related mainly to the inhibition of the protein phosphate calcineurin, but FK506 might also be neuroprotective through a reduction of peroxynitrite radical production and through an inhibition of calcium triggered apoptosis [16].

L-Carnitine is an essential cofactor for the transportation of fatty acyl groups into the mitochondrial matrix where they undergo beta-oxidation, resulting in the production of ATP. It also plays an important role in the regulation of glucose oxidation. The protective effect of carnitine on ischemia reperfusion injury has been proven on various tissues as brain, myocardium, and splanchnic [17,18]. There is also one study showing the neuroprotective effect of regional carnitine on spinal cord ischemia reperfusion injury [19].

Azathioprine (Imuran; Glaxo-Wellcome) is another immunosuppressive agent used mostly after transplantations. The protective effect of azathioprine on ischemia reperfusion injury has been shown on various studies [20], but there is no study attempting to describe the protective effect of azathioprine on spinal cord ischemia reperfusion injury. We designed this study to determine whether FK506, L-carnitine and azathioprine could improve neurological outcome, whether there are any differences between these three drugs and whether they reduce histologic evidence of spinal cord injury in a rat model of transient spinal cord ischemia.

2. Materials and methods

After approval of the study by the ethical committee, experiments were done on 27 Spraque–Dawley male rats weighing 350–375 g. All animals were allowed free access to laboratory chow and tap water in day–night regulated quarters at 25 °C. All animals received care in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health (NIH publication 85-23, revised 1985). The animals were randomly divided into five groups. Rats were initially anesthetized with intramuscular Ketamine (50 mg/kg) and followed by a half dose as required during the procedure. Animals did not receive ventilatory support. An intravenous catheter was placed in tail vein, and fluid of 0.9% NaCl was infused during the procedure. Rectal temperature was continuously monitored with a flexible probe inserted 5 cm into the rectum in all animals, and it was maintained at 37.5 ± 0.5 °C by a thermal pad and a heating lamp. Cefazolin was injected intravenously with a single dose at 10 mg/kg. To monitor proximal and distal aorta blood pressures, we placed two catheters into the aorta and femoral arteries. The abdominal aorta was reached through midline laparotomy. Animals in sham group (group V, $n = 3$) were anesthetized and subjected to laparotomy without aortic occlusion. In other groups, animals were subjected to 45 min of crossclamping. Vascular clamps were placed under the left renal vein and above the bifurcation in the aorta. Each rat received 150 IU/kg of heparin before aortic occlusion and protamin was not administered after the procedure. In group I ($n = 6$) the animals received 0.5 mg/kg of FK506 intravenously 30 min before the crossclamping, group II ($n = 6$) animals received 100 mg/kg of L-carnitine 30 min before crossclamping; group III ($n = 6$) animals received 4 mg/kg of azathioprine and group IV, which is the control group received normal saline solution as vector 30 min before the crossclamping. The aortic clamps were removed after 45 min and the abdomen was closed appropriately. Animals were allowed to recover in a plastic box at 28 °C for 3 h and were then placed in their cages with free access to food and water. The Crede maneuver was used twice daily to empty the urinary bladders of paraplegic animals.

Neurologic status was scored by assessment of hindlimb neurologic function at 24 and 48 h after the procedure using the Tarlov Scoring System [21] (Table 1). A score of 0–5 was assigned to each animal as follows: 0, no voluntary hindlimb movement; 1, movement of joints perceptible; 2, active movement but unable to sit without assistance; 3, able to sit but unable to hop; 4, weak hop; 5, complete recovery of hindlimb function. All rats were killed using lethal dose of sodium pentobarbital injection. Sections of the lumbar cord were harvested for histologic examination immediately after lethal injection. The samples were fixed in 10% formalin solution for histopathological examination.

2.1. Histopathologic examination

Tissue samples were fixed in 10% formalin and embedded in paraffin with routine follow-up procedure; 4 μm were cut from paraffin blocks and stained with hemotoxylin and eosin for light microscopic examination. Apoptosis was monitored by an immunohistochemical marker; anti-poly (ADP-ribose) polymerase (PARP) p85 fragment pAb; which is a polyclonal antibody directed against the 85-kDa caspase-cleaved fragment (p85) of human PARP. Paraffin-embedded blocks were cut at 4-μm sections, deparaffinized, and rehydrated. Anti-PARP p85 fragment pAb was used as the primary antibody (anti-PARP p85 fragment pAb; Promega Corporation, 50 μl, Cat #

Table 1
Determination of hindlimb motor function by Tarlov score

Tarlov scale score	Description
0	No voluntary hindlimb movement
1	Movement of joints perceptible
2	Active movement but unable to sit without assistance
3	Able to sit but unable to hop
4	Weak hop
5	Complete recovery of hindlimb function

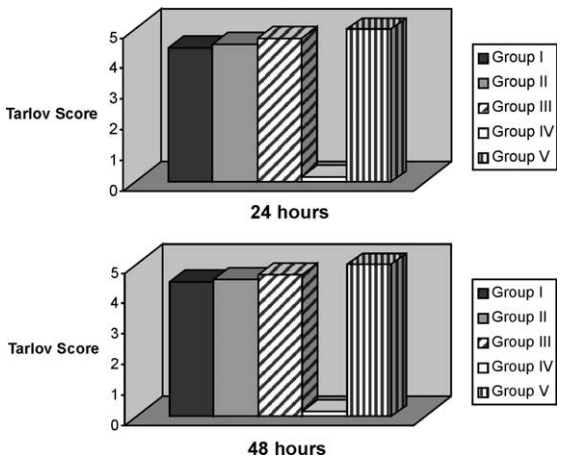


Fig. 1. Mean neurologic scores of animals showed a significant difference between control and treatment groups ($P = 0.0019$) both at 24 and 48 h.

G7341). Streptavidin-horseradish peroxidase was performed followed by 3-3′-diaminobenzidine. All slides were counterstained with hematoxylin. Appropriate slides were used as positive and negative controls. Histopathologic grade of cellular apoptosis was evaluated from 1 to 4 by a neuropathologist who was blinded to groups. A grade of 1–4 was assigned to each section as follows: grade 1, number of apoptotic cells $<25\%$; grade 2, number of apoptotic cells between 25 and 50%; grade 3 number of apoptotic cells between 50 and 75%; and grade 4 number of apoptotic cells $>75\%$.

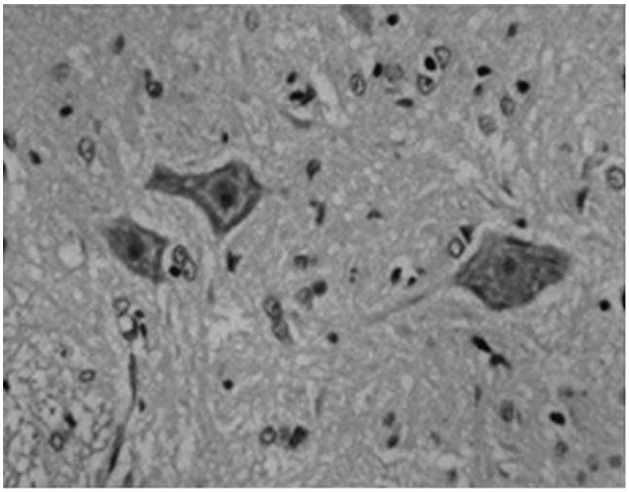
2.2. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). Statistical analysis of the neurologic scores was performed with the Kruskal–Wallis test and Dunn's multiple comparison test. The test used for the comparison with baseline is Friedman test and Dunn's multiple comparison test. Differences were considered statistically significant at the $P < 0.05$ level.

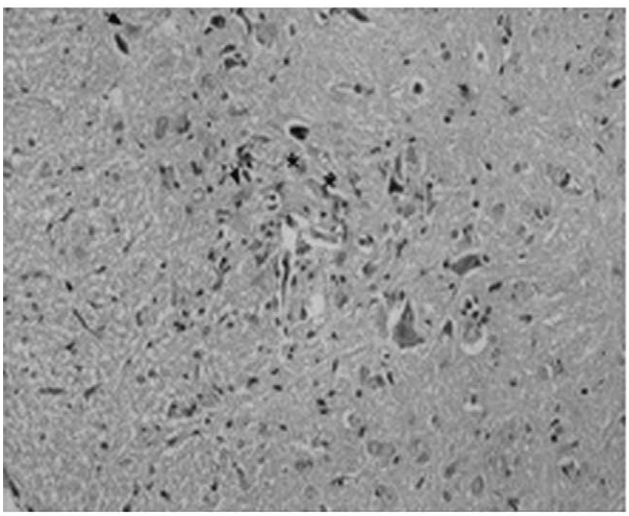
3. Results

Table 2 presents the physiologic variables recorded in animals during the experiment. All animals in which an aortic clamp had been applied group (I–IV) demonstrated significant differences between baseline and post-occlusion pressures in the femoral artery ($P = 0.0001$).

All animals survived and were evaluated 24 and 48 h after reperfusion, according to the modified Tarlov's scores. The ratio of the specific grades of each group is shown in Fig. 1. All sham-operated rats had a normal postoperative neurologic outcome, whereas all control rats had a spastic paraplegia at 24 h and remained severely paraplegic throughout the observation period. The neurologic status of FK506, carnitine and azathioprine groups



(a)



(b)

Fig. 2. Light microscopical photomicrograph of sham group (group V) with normal histologic appearance, anti-PARP staining, 400×. Light microscopical photomicrograph of control group, shows the disappearance of Nissl bodies and nuclei and shows the apoptotic bodies, anti-PARP staining, 200×.

were significantly superior to the control group but there was no statistically significant difference between these three study groups.

Hematoxylene and eosin (H and E) and anti-PARP staining were used to analyze neuronal cell death in gray matter and apoptosis. To objectively quantify the amount of neuronal damage seen in these histologic sections, we devised a grading system described where grade 1, number of apoptotic cells $<25\%$; grade 2, number of apoptotic cells between 25 and 50%; grade 3, number of apoptotic cells between 50 and 75%; and grade 4, number of apoptotic cells $>75\%$.

A neuropathologist blinded to the groups, graded all samples. Light microscopical changes were considered to be consistent with ischemia reperfusion injury and included

Table 2
Physiologic variables

Variable	Groups				
	FK506 ($n = 6$)	L-Carnitine ($n = 6$)	Azathioprine ($n = 6$)	Control ($n = 6$)	Sham ($n = 3$)
MABP (mmHg)					
Baseline	88.0 ± 3	90.0 ± 2	91.0 ± 3	92.0 ± 4	97.0 ± 6
Occlusion	14.0 ± 4*	13.0 ± 2*	14.0 ± 4*	15.0 ± 4*	–
Reperfusion	70.0 ± 3	68.0 ± 2	71.0 ± 2	67.0 ± 4	86.0 ± 3
Rectal temperature (°C)					
Baseline	37.6 ± 0.4	37.6 ± 0.3	37.5 ± 0.5	37.5 ± 0.3	37.6 ± 0.4
Occlusion	37.5 ± 0.3	37.4 ± 0.5	37.5 ± 0.5	37.6 ± 0.4	–
Reperfusion	37.4 ± 0.5	37.6 ± 0.3	37.5 ± 0.3	37.5 ± 0.3	37.6 ± 0.5

All animals in which an aortic clamp had been applied (groups I–IV) demonstrated significant differences between baseline and postocclusion pressures in the femoral artery ($P = 0.0001$). *$P = 0.0001$.

shrunken, necrotic neurons and axonal swelling. A significant increase in neutrophils was not observed in any of the specimens. No sign of spinal cord damage was observed in sections in sham operated rats (Fig. 2a). However, in the control rats, the segment that had been subjected to spinal cord ischemia showed neuronal damage, as evidenced by disappearance of Nissl bodies and nuclei in the motor neuron cells and in anti-PARP study, there was apoptotic neurons, staining of vessel endothelium and central canal was observed indicating ischemic damage (Fig. 2b). Although there appeared to be a difference between groups in histopathological evaluation, no statistical significance was noted (Table 3). Fig. 3a–c show the histologic changes in groups pretreated with FK506, carnitine and azathioprine. In these study groups less histologic damage was exhibited and the number of apoptotic cells were also less than the control group.

4. Conclusions

The results of this series of experiments demonstrate that FK506, carnitine and azathioprine reduces neurologic injury in this rat model of spinal cord ischemia. This protection was induced by administration of these three drugs 30 min before a 45-min ischemic insult and resulted in improved function at 24 and 48 h.

Table 3
Histopathologic grade of cellular apoptosis

Groups	Histopathological grades				
	Grade 1	Grade 2	Grade 3	Grade 4	
I (FK506)	6	2	2	2	0
II (L-Carnitine)	6	2	2	1	1
III (Azathioprine)	6	1	3	2	0
IV (Control)	6	0	0	0	6
V (Sham)	3	3	0	0	0

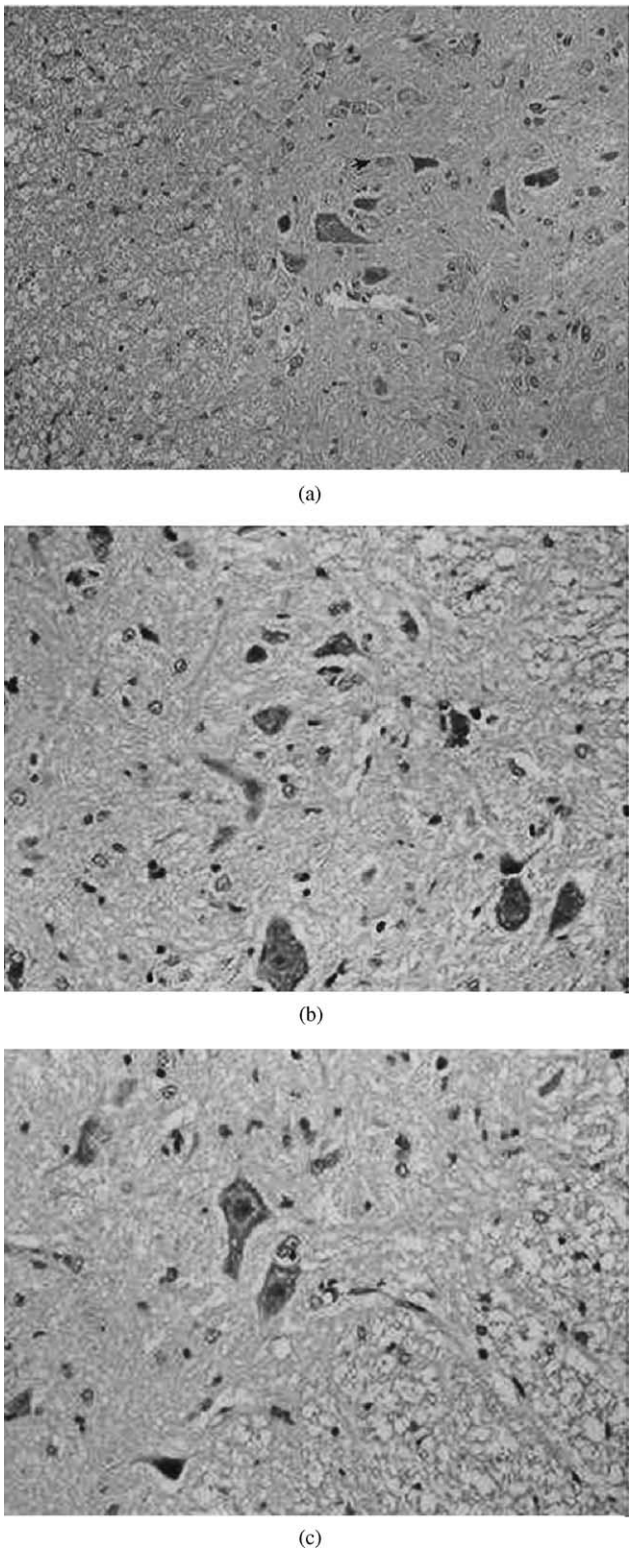


Fig. 3. (a) Light microscopical photomicrograph of group I that received FK506, shows an apoptotic neuron among the normal neurons, anti-PARP staining, 200 × . (b) Light microscopical photomicrograph of group II that received L-carnitine, shows the normal appearance of neurons, anti-PARP staining, 200 × . (c) Light microscopical photomicrograph of group III that received azathioprine, shows the normal appearance of neurons, anti-PARP staining, 200 × .

The histology of the spinal cords confirms the clinical observations. Qualitatively, evidence of both necrosis and apoptosis (anti-PARP) was apparent in lumbar sections of control group. Animals pretreated with FK506, carnitine and azathioprine, on the other hand, not only exhibited preserved neurologic function but also demonstrated less histologic damage. The attempt to quantify the differences seen on histologic exam revealed no statistical significance. This could be due to the small number of the groups.

There is growing enthusiasm regarding the neuroprotective effects of FK506 and carnitine. FK506 has an excellent diffusion across the blood–brain barrier and is neuroprotective at doses of 0.1–1 mg/kg [14,15]. The precise mechanism of action of this drug is not completely understood, and its neuroprotective action is probably multifactorial. FK506 complexes the 12-kDa immunophilin FKBP12 (FK binding protein 12), resulting in inhibition of the calcium/calmodulin dependent protein phosphatase calcineurin [14]. It has also been reported to be neurotrophic, with a potency comparable to those of well known neurotrophic proteins such as BDNF, NT3, GDNF and NGF, and it improves functional recovery after spinal cord injury [22]. In this study, pretreatment of rats with FK506 significantly improved neurologic outcome but incompletely prevented gray matter injury. However, this study demonstrates that a single injection of FK506 before aortic clamping can attenuate significantly the apoptotic phenomena in the gray matter of most treated animals. Major adverse effects have been observed with the chronic use of FK506. These include drug-induced neurotoxicity varying from 5 to 30% of patients and consisting in severe organic brain syndrome [13]. However, it seems improbable to observe such side effects with a single dose injection of FK506.

L-propionyl carnitine (LPC), an endogenous ester that plays an important role in cellular fatty acid oxidation and metabolism, has been shown to exert a protective effect in myocardial ischemia reperfusion injury [23,24]. The administration of propionyl carnitine leads to less hydrogen peroxide formation and less available free iron, resulting in an attenuation of free radical mediated damage [25]. In our study, group II animals, which received carnitine, had a full recovery. This improvement of neurologic function rates and the histopathological findings reveal the neuroprotective effect of carnitine.

In our study one of the other drugs studied was azathioprine, which is frequently used in transplantations for immunosuppression. It is known to be protective against ischemia reperfusion injury in liver and kidney. This experimental study was the first report of azathioprine for the protection on spinal cord ischemia-reperfusion. However, no study attempting to describe the protective effect of administration of this agent on spinal cord ischemia-reperfusion has yet appeared. Our results suggest that azathioprine pretreatment has a protective effect in ischemia reperfusion injury of spinal cord. The animals that received azathioprine showed a full recovery in neurologic function and minimal damage histologically.

In conclusion, the results suggest that FK506, L-carnitine and azathioprine reduces ischemia reperfusion damage in the spinal cord and provides a better neurologic outcome. The analysis of the three pharmacological agents did not have enough power to achieve superiority for neuroprotection on spinal cord ischemia-reperfusion injury. But further studies are warranted to confirm their clinical effects.

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