Neuroprotective effect of regional carnitine on spinal cord ischemia–reperfusion injury

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Abstract

Objective: The purpose of this study was to investigate the effect of regional infusion of carnitine on spinal cord ischemia–reperfusion (I–R) in rabbits. Methods: The 36 rabbits were divided into four equal groups, group I (sham operated, no I–R injury), group II (control, only I–R), group III (I–R + intraaortic lactated Ringer’s, LR, during aortic occlusion), group IV (I–R + LR plus 100 mg/kg carnitine). Spinal cord ischemia was induced by clamping the aorta both below the left renal artery and above the aortic bifurcation. The spinal cord function of all animals was assessed clinically 24 h after aortic declamping. Spinal cord samples were taken to measure the levels of tissue malondialdehyde (MDA) and to evaluate the histopathological changes. Results: We found significant increases in the levels of MDA in groups II and III compared with group I P, 0.01; and elevation of MDA in group IV was insignifi- cant. In group II, all animals (100%) were paraplegic with Tarlov’s score of 0 and in group III, eight animals (88%) were paraplegic with Tarlov’s score of 0 or 1. None of the animals (0%) from group IV was paraplegic. Histologic examination of spinal cords from group IV animals revealed that the appearance of the spinal cord was relatively preserved, whereas spinal cords from groups II and III had evidence of acute neuronal injury. Conclusion: The results suggest that regional infusion of carnitine during aortic clamping reduces spinal cord injury and prevents neurologic damage in rabbit spinal cord I–R model.

Keywords: Carnitine; Spinal cord; Ischemia–reperfusion injury

1. Introduction

The most sensitive organ is the spinal cord, and ischemic injury can produce paraparesis or paraplegia after operations on the thoracic aorta [1–3]. Over the past decades, numerous methods to protect the spinal cord against severe ischemic or reperfusion injury and to reduce the incidence of paraplegia in experimental and clinical situations [4] have been tried. These methods, which include the use of hypothermia [5], vascular shunting [6], left heart bypass [7], drainage of cerebrospinal fluid [8], monitoring of somatosensory evoked potentials [9], single clamp technique [10], reimplantation of major intercostal arteries [11] and adjunctive medications [8,12] are presently used in several clinical settings. Despite their use, paraplegia remains a persistent complication. Results of former experimentation confirm the possible role of oxygen free radicals in the pathophysiological development of spinal cord injury induced by aortic crossclamping. Oxygen free radicals are known to produce lipid peroxidation in cellular components. Prevention of lipid peroxidation appears to be the most important mechanism in protecting the spinal cord from ischemia–reperfusion (I–R) injury [2–4]. Carnitine is an essential co-factor for the transportation of fatty acyl groups into the mitochondrial matrix where they undergo β-oxidation, resulting in the production of ATP. Carnitine also has an important role in the regulation of glucose oxidation. Carnitine has been used successfully in the treatment of a variety of diseases. The protective effect of carnitine on I–R injury has been proven on various tissues such as brain, myocardium and splanchnic [13,14]. However, no study attempting to describe the protective effect of regional administration of this agent on spinal cord I–R has yet appeared. In this study, we examined the effect of carnitine on histopathological changes, lipid peroxidation production and neurologic recovery in rabbits subjected to 40 min spinal cord ischemia followed by reperfusion. We used a rabbit model for spinal cord ischemia...
because of the unique segmental arterial blood supply to the spinal cord from the infrarenal aorta in this animal.

2. Materials and methods

2.1. Experimental protocol

After approval of the study by the ethical committee, experiments were done on 36 New Zealand white rabbits of both sexes weighing from 2.2 to 3.5 kg (mean 2.8 kg). Animal care and all procedures were performed in compliance with the European Convention on Animal Care. The animals were randomly divided into four groups of nine animals each. Rabbits were initially anaesthetised with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg), followed by a half-dose as required during the procedure. Animals did not receive blood pressure or ventilatory support. A rectal probe and warming blanket were used to record and support core temperature, respectively. The animals were placed in a nose cone to breathe oxygen at a rate of 3 l/min. An intravenous catheter (24 gauge) was placed in an ear vein, and fluid of 0.9% NaCl was infused at a rate of 3 l/min. An intravenous catheter was attached to a pump (Abbott LC 5000, USA) and two different solutions at room temperature were infused for 4 min through the pump at a constant rate of 5 ml/min. We administered 20 ml of lactated Ringer’s (LR) solution to group III and 20 ml LR solution plus 100 mg/kg of carnitine (Carneten, Sigma-Tau, Italy) to group IV. The catheter was withdrawn after this procedure, and the insertion point of the catheter into the aorta was sutured with 7/0 propylene. The aortic clamps were removed after 40 min and the abdomen was closed appropriately.

Heart rate, proximal and distal aortic blood pressure were monitored (model SC 6000, Siemens) before crossclamping and after clamping and removal of the crossclamps. Blood samples were collected, blood gases analysed and blood glucose levels determined. In case of metabolic acidosis, sodium bicarbonate was administered.

After the neurologic examination at 24 h post-operation, all animals were put to death by intracardiac formalin (10%) injection. Sections of the lower thoracic and lumbar cord were harvested for biochemical and histologic examination immediately after lethal injection. The samples were divided into two equal parts after washing in saline solution. The first halves were wrapped in aluminium foil and stored at −70°C until the time of biochemical examination. The remaining halves were fixed in 10% formalin solution for histopathological examination.

2.2. Neurologic examination

The neurologic status was assessed at 24 h post-operation by an independent observer, who had no prior knowledge of the experimental protocol, using Tarlov’s scores [5]. Subjects were graded as below:

- T 0, spastic paraplegia with no movement of the hind limb;
- T 1, spastic paraplegia with slight movement of the hind limbs;
- T 2, good movement of the hind limbs but unable to stand;
- T 3, able to stand, but unable to walk normally;
- T 4, complete recovery.

2.3. Malondialdehyde (MDA) level analysis

MDA levels were measured in spinal cord tissue according to the method of Ohkawa et al. [15]. Nine millilitres of 1.15% KCl was added to 1 g of wet tissue (1:10) and homogenisation was ensured with an Ultra-Turrax homogeniser according to the following procedure: 0.2 ml 8.1% sodium dodecyl sulphate (SDS) and 1.5 ml 20% acetic acid solution were added to 0.2 ml 10% (w/v) tissue homogenate. The pH was adjusted to 3.5 with NaOH and 1.5 ml of 0.8% aqueous thiobarbutyric acid solution was then added and the final volume was made up to 4 ml with distilled water. Then the samples were incubated for 60 min in boiling water at 95°C. After cooling with tap water, a mixture of 1 ml distilled water and 5 ml n-butanol–pyridin (15:1 v/v) was added and the mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm measured. Tissue protein levels were measured according to the Lowry method [16]. Results were shown in U/mg protein.

2.4. Histopathologic examination

Tissue samples were fixed in 10% formalin and embedded in paraffin with routine follow-up procedure. Sections with a width of 4 μm were cut from paraffin blocks, and stained with hematoxylin eosin and cresyl violet stains for light microscopic examination. Morphologic evaluation of spinal cords in groups was done blindly.
Histopathological findings were graded 1, 2 and 3 [17]. Grade 1 indicates the appearance of a normal spinal cord. Grades 2 and 3 mean swollen axons with occasional necrotic neurons and swollen axons with many necrotic neurons, respectively. The specimens in groups II–IV were compared with sections in group I (sham operated) not suffering from an ischemic episode.

2.5. Statistical analysis

Data were expressed as mean ± standard deviation. For comparison of the haemodynamic parameters between different (baseline and occlusion) periods within each group, the Wilcoxon test was utilised. In post-operative neurologic status and histopathological gradings, we used the chi-square (Fischer exact-$\chi^2$) to determine significant differences among the groups. The MDA levels were evaluated using one-way analysis of variance (ANOVA) and the differences among the groups were analysed with Tukey-B and Sheffe tests. Differences were considered statistically significant at the $P < 0.05$ level.

3. Results

There was no difference in heart rate, proximal and distal pressure values among groups (Table 1). Heart rates were not statistically different in three intervals for all groups. All animals in which an aortic clamp had been applied (groups II–IV) demonstrated significant differences between baseline and post-occlusion pressures both in the proximal aorta and in the femoral artery ($P < 0.05$ and $P < 0.001$, respectively). No difference was observed between different groups in terms of sodium bicarbonate requirements to correct the metabolic acidosis and blood glucose levels.

All animals survived and were evaluated 24 h after reperfusion, according to the modified Tarlov’s scores. The ratio of the specific grades of each group is shown in Fig. 1. Spastic paraplegia was represented as grade 0 or 1 in Tarlov’s scores. Spastic paraplegia occurred in 100% (9/9) of group II and 88% (8/9) of group III. None of the rabbits in groups I and IV showed paraplegia. The rates of complete recovery were 100% (9/9), 0 (0/9), 0 (0/9) and 66.6% (6/9) in groups I–IV, respectively. The neurologic status of group I was significantly superior to that of group II and to group III ($P < 0.001$). There was no significant difference in Tarlov’s scores between group I and group IV, which received carnitine.

The mean MDA levels were 11.74 ± 1.80, 19.24 ± 2.42, 17.91 ± 2.60 and 12.42 ± 1.62 nmol/mg protein in groups I–IV, respectively (Fig. 2). The difference between groups I and IV was not significant, but MDA levels in groups II and III were found to be significantly elevated when compared with group I ($P < 0.01$).

The results of histopathological evaluation were indicated in Table 2. Histopathological changes were considered to be consistent with I–R injury and included shrunken, necrotic neurons and axonal swelling. A significant increase in neutrophils was not observed in any of the specimens and no signs of spinal vessel thrombosis were found either.

Table 1
Haemodynamic parameters

|                     | Baseline | Occlusion | Reperfusion |
|---------------------|----------|-----------|-------------|
|                     | I        | II        | III         | IV          | I          | II        | III         | IV          |
| Heart rate (bpm)    | 188 ± 10 | 186 ± 7   | 190 ± 7     | 192 ± 6     | 195 ± 7    | 194 ± 5   | 196 ± 8     | 195 ± 5     |
| Mean proximal pressure (mmHg) | 56 ± 7   | 54 ± 7   | 58 ± 4      | 57 ± 3      | 55 ± 6     | 66 ± 7*   | 70 ± 6*     | 69 ± 5*     |
| Mean distal pressure (mmHg) | 55 ± 6   | 52 ± 8   | 57 ± 5      | 55 ± 4      | 54 ± 4     | 14 ± 5**  | 15 ± 4**    | 14 ± 4**    |

* $P < 0.05$, baseline versus occlusion for groups II–IV.

** $P < 0.001$, baseline versus occlusion for groups II–IV.
Comparison of group I with groups II and III revealed significant differences \( P \leq 0.01 \). There was no significant difference between groups I and IV.

The histopathological findings were correlated with the neurologic status. While the anterior horn motor neurons from animals described with Tarlov’s score 4 were indistinguishable from those with normal spinal cords and nerve cells were preserved (Fig. 3), animals with Tarlov’s score 3 (three rabbits) exhibited only swollen axons. In animals with Tarlov’s score 1 and 0, which included all the rabbits in group II and most of the animals in group III, destruction of the motor neurons (grade 2 or 3) were observed in the anterior horns of thoracic spinal cord after I–R (Fig. 4).

### 4. Discussion

Spinal cord injury after thoracic aortic crossclamping is a persistent clinical problem, occurring in about 5–10% of cases. The causes of spinal cord ischemia during operations on the descending thoracic and thoracoabdominal aorta are complex [2]. This experimental model was designed only for evaluating the effect of crossclamping time and did not deal with other etiologic factors of spinal cord injury caused by surgical treatment of the large thoracic and thoracoabdominal aortic aneurysm resection.

There is increasing evidence that free radicals are generated by ischemia and they contribute to tissue injury [3]. During the ischemic episode, degradation of adenosine triphosphate and the formation of oxygen free radicals via the hypoxanthine to xanthine reaction are believed to be important in I–R injury [2]. Also, reperfusion of ischemic organs may aggravate tissue injury via leucocyte activation and free radical production [18]. The oxygen free radicals as products of cellular metabolism present in small amounts in normal perfusion states accumulate after the onset of ischemia, but are produced in abundance in the phenomenon referred to as the oxidative burst when perfusion is reestablished. Those unstable radicals are potent initiators of protein degradation and lipid peroxidation, which in turn lead to cell membrane dysfunction [19]. These radicals also directly induce endothelial damage and coordinate with cytokines to enhance neutrophil chemotactic mechanisms. Consequently, oxygen radical mediated lipid peroxidation is a self-perpetuating process that can spread to the circumferential undamaged neuronal tissue, leading to further collapse of microcirculation and to irreversible damage to myelin and axons [4]. Several pharmacologic methods used to treat traumatic spinal cord injury might be applied to reduce ischemic or reperfusion injury of the spinal cord and to prevent post-operative paraplegia. MDA is main product of lipid peroxidation in spinal myelin, glial

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### Table 2

Results of histopathologic examination.

| Groups     | n | Histopathological grade |
|------------|---|-------------------------|
|            |   | Grade 1 | Grade 2 | Grade 3 |
| Group I    | 9 | 9        | 0      | 0       |
| Group II†  | 9 | 0        | 0      | 9       |
| Group III† | 9 | 1        | 2      | 6       |
| Group IV   | 9 | 6        | 3      | 0       |

* † \( P < 0.05 \), group I versus groups II and III.

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Fig. 3. The appearance of normal motor neurons in group IV, which received carnitine (cresyl violet × 400).
and neuronal membranes and other cellular elements [20]. In our study, we observed that the MDA content increased after spinal ischemia, and that the increase was diminished by carnitine administration during aortic occlusion.

In man, carnitine is synthesised from proteic trimethylsine in liver, brain and kidney. Carnitine is present in biological materials as free carnitine and as acyl carnitines, which are metabolic products of reactions utilising acyl CoA catalysed by carnitine acyl transferases. Acyl CoA esters do enhance the lipid peroxidation more than acyl carnitine. The addition of carnitine increases the conversion of acyl CoA to acyl carnitine, which also decreases the substrate supply in the cytosol for peroxisomal oxidation [21]. The antioxidant action may play an important role in membrane stabilisation, as well. The stabilisation of the mitochondrial membranes by carnitine probably represents the removal of disruptive acyl CoA esters from the membranes as acyl carnitines.

L-propionil carnitine (LPC), an endogenous ester that plays a crucial role in cellular fatty acid oxidation and metabolism, has been shown to exert a protective effect in myocardial I–R injury [22–24]. The administration of propionyl carnitine leads to less hydrogen peroxide formation and less available free iron, resulting in an attenuation of the free radical mediated damage [25]. In a splanchnic artery occlusion model of I–R injury, LPC afforded significant protection that may be achieved through inhibiting leucocyte infiltration into intestinal tissue and preserving endothelial function, thereby decreasing microvascular permeability and maintaining tissue perfusion [14]. Calvani et al. [13] demonstrated that acyl carnitine can have significant clinical neuroprotective effects when administered shortly after the onset of focal global cerebral ischemia. They suggested that the transfer of the acetyl group to coenzyme A to form acyl CoA as the primary source of energy is a plausible mechanism of action of acyl carnitine.

In our study, group IV animals, which received carnitine, had a 66.6% full recovery at 24 h after the 40 min ischemic interval and 24 h of reperfusion. These animals were standing and walking with no difficulty and had intact sensory and motor reflexes. In contrast, all animals in the I–R and I–R + LR groups were paraplegic with no sensory or motor reflexes. This improvement of neurologic function rates and the histopathological findings reveal the protective effect of carnitine on spinal tissue against I–R injury, which also agrees with the results of the biochemical data.

In conclusion, the results suggest that carnitine reduces I–R damage in the spinal cord and provides a better neurologic outcome. Our study includes two features: the use of carnitine as a neuroprotective drug and the regional administration of the agent. We believe that the protective action of carnitine against I–R injury includes an increased rate of cellular transport stimulation of fatty acid oxidation and a reduction of free radical formation. Although carnitine has been used successfully in many clinical conditions, no report on the utilisation of carnitine in the prevention of reperfusion injury on the human spinal cord exists. Conclusions reached in experimental models such as the present report may not be readily applicable to humans. Thus, further study is needed to define the biochemical aspects of these events and to determine the correct dose necessary for maximal benefits.

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**Fig. 4.** Abnormal motor neurons of group II (only ischemia–reperfusion). Thin arrows indicate severe neuronal degeneration exhibited by shrunken and triangular-shaped cell and large arrows indicate total cell destruction (cresyl violet × 400).
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