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Aprotinin reduces injury of the spinal cord in transient ischemia

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Abstract

Objective: The protective effect of aprotinin, which is a protease inhibitor, was assessed in a rabbit spinal cord ischemia model. **Design:** Randomized, controlled, prospective study. **Setting:** University research laboratory. **Subjects:** New Zealand white rabbits (36) of both sexes. **Methods:** In 24 animals, ischemia was induced with midline laparotomy and clamping the aorta just distal to left renal artery and proximal to aortic bifurcation for 20 min. Aprotinin was given 30 000 KIU as a short intravenous injection after anesthesia, and was followed by 10 000 KIU/h by continuous infusion in group 1 ($n = 12$). Similar volume of saline solution was used in control group of animals (group 2, $n = 12$). Group 3 of animals (sham group, $n = 12$) were anesthetized and subjected to laparotomy without aortic occlusion. Physiological parameters and somatosensory evoked-potentials (SEP) were monitored in animals before ischemia, during ischemia and in the first 60 min of reperfusion. Their neurological outcome was clinically evaluated up to 48 h postischemia. Their motor function was scored, and the intergroup differences were compared. The animals were sacrificed after two days of postischemia. Their spinal cord, abdominal aorta, and its branches were processed for histopathological examination. **Results:** In group 3, SEP amplitudes did not change during the procedures, and all animals recovered without neurologic deficits. At the end of ischemic period, the average amplitude was reduced to $53 \pm 7\%$ of the baseline in all ischemic animals. This was followed by a gradual return to 89 ± 8 and $81 \pm 13\%$ of the initial amplitude after 60 min of reperfusion in group 1 and group 2 correspondingly ($P > 0.05$). The average motor function score was significantly higher in group 1 than group 2 at 24 and 48 h after the ischemic insult ($P < 0.05$). Histological observations were clearly correlated with the neurological findings. **Conclusion:** The results suggest that aprotinin reduces spinal cord injury and preserves neurologic function in transient spinal cord ischemia in rabbits. © 1997 Elsevier Science B.V.

Keywords: Aprotinin; Spinal cord; Ischemia-reperfusion injury; Somatosensory evoked potentials

1. Introduction

Ischemic spinal cord injury represents the main complication in surgical repair of thoracic and thoracoabdominal aneurisms and remains a persistent clinical problem. Temporary aortic occlusion may produce a critical reduction in spinal cord perfusion with a risk of irreversible ischemic injury. Reperfusion may also cause numerous further negative effect in the case of ischemia is severe and prolonged.

To date, numerous clinical and laboratory studies in attempt to decrease the risk of this devastating complication have been reported. Some of these techniques have met with varying success and presently used in several clinical settings, such as regional or systemic hypothermia [1], cerebrospinal fluid drainage [2], shunts [3], monitoring of somatosensory evoked potentials and

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reimplantation of major intercostal or lumbar arteries [4]. Numerous adjunctive medications have also been used for preserving spinal cord function [5,6]. Despite their use, paraplegia remains a persistent complication of the operations on descending aorta. The reported prevalence of neurologic injury for these operations ranges up to %40 [7].

In the mechanisms of the pathogenesis of ischemia-reperfusion injury, activated neutrophils are thought to be important in terms of their ability to produce various kinds of proteinases, which can degrade various proteins constructing human tissues [8]. Similarly, activation of blood protease cascades may contribute to cellular injury under this condition [9,10].

Aprotinin is a serine-protease inhibitor and has been shown to inhibit activation of kallikrein-kinin system, plasmin fibrinolysis system, preserve platelet function and partially inhibit neutrophil activation [11,12].

Since the discovery of aprotinin in 1930, its clinical application has been sought for a number of situations, including cardiopulmonary bypass and ischemia-reperfusion, in which activation of blood protease cascades may play an important role [12,13]. The protective effect of aprotinin on ischemia-reperfusion injury has been proven on various tissues such as brain and myocardium [9,10,13,14]. However, no study attempting to describe the protective effect of this agent on spinal cord ischemia appeared. In this study, we examined the effects of aprotinin on histopathological changes and neurologic recovery in temporary induced spinal cord ischemia in rabbits.

2. Material and methods

2.1. Animal preparation

After approval of the study by the local Institutional Committee, experiments were performed on 36 New Zealand white rabbits of both sexes, each weighing 2.4–3.3 kg (mean 2.9 kg). Rabbits were anesthetized with intramuscular ketamine with an initial dose of 50 mg/kg, followed by 25 mg/kg fractionally as needed during the procedure. No animals received haemodynamic or ventilatory support. The animals were placed in a nose cone to breath oxygen at a rate of 3 l/min. Body temperature monitored with a thermistor probe in rectum and maintained close to 38°C using heating pads. An intravenous catheter (24 gauge) was placed in an ear vein, and preoperatively cefazolin 10 mg/kg was given as a single dose. Maintenance fluid of 0.9% NaCl was infused at a rate of 20 ml/h during the procedure. A femoral artery was exposed for arterial line with a catheter (24 gauge) and this catheter was connected to a blood pressure/heart rate transducer and monitor (Hewlett-Packard 1495C). Determinations of blood glucose, hemoglobin level, blood gases-electrolytes and pH were also accomplished with this line (Stat Profile 5 autoanalyser).

2.2. Experimental groups and surgical procedure

Rabbits were randomly allocated into three groups each consisting of 12 rabbits. In group 1, aprotinin (10 000 KIU/ml) was injected 30 000 KIU/kg as an initial bolus via the ear vein immediately after anesthesia and perfusion was continued at a rate of 10 000 KIU/h. A similar volume of saline solution was given in the control groups correspondingly. After the surgical preparation, using aseptic techniques, a midline laparotomy was performed. The abdominal aorta was exposed and mobilized from just inferior to the left renal vein down to aortic bifurcation. Each rabbit was anticoagulated with heparine 150 U/kg. In group 1 and group-2, spinal cord ischemia was induced with clamping the aorta just below the left renal vein with a bulldog clamp (FB328). A second similar clamp was placed above the aortic bifurcation for occluding iliac collateral circulation. Animals were subjected to 20 min of crossclamp time. After the ischemic period, the clamps were removed and restoration of the blood flow was verified visually. The abdomen was then closed. In group 3 of animals (sham operated), laparotomy incision was left open for 20 min corresponding to spinal ischemia period but the aorta was not occluded. This group of animals were used for eliciting the effects of anesthesia and operation on results.

Blood samples were obtained before ischemia in basal condition, at 5th and 60th minute of reperfusion. The systemic acidosis determined by measurement of arterial blood gases was corrected by intravenous administration of sodium bicarbonate.

2.3. SEP recording

To asses the acute neurologic recovery, somatosensory evoked potentials (SEP) were recorded before ischemia, during ischemia and first 60 min of reperfusion with 5 min intervals. SEP responses were evaluated for recovery to the percentages of their baseline control values. Nihon Kohden Neuropack II plus (MEB 5000) was used for the recordings. A teflon-coated silver wire active electrode was inserted over the parietal region 2 mm lateral to midline and 2 mm caudal from the coronal suture. A similar reference electrode was inserted to midline over nasion. The sciatic nerve contralaterale to the scull electrode was stimulated by surface electrodes. The stimulus rate was 5/s with a duration of 0.2 ms and the stimulus intensity was 3–4 mA. 256 responses were averaged. Bandpass filter was set at 20 Hz-1 Kz.

2.4. Postoperative care and assessment

Arterial and venous lines were removed and all medications were stopped at the 60th min of reperfusion. When the animals awakened from anesthesia, they were returned to their cages. The Crade maneuver was used to empty the bladders of the paraplegic animals at least twice daily. Six animals (three rabbits originally assigned to the group 1, 2 to group 2, and 1 to group 3) died in the early postoperative period (within first 8 h) and they were excluded from the study. Others survived without any clinical sign of pathology except paraplegia.

The animals were examined neurologically 24 and 48 h after the operation and spinal cord function was assessed by an independent observer. Bladder function was evaluated, and the motor function of hindlimbs was scored by the following scale:

0, no movement;
1, trace movement;
2, able to sit but only with assistance;
3, able to sit without assistance but cannot hop;
4, able to hop but without normal strength;
5, normal motor function.

2.5. Histopathology

After the last neurological examination at 48 h postoperation, the animals were anaesthetized with intramuscular ketamine 50 mg/kg in dose and killed with transcardially perfused 10% neutral formol. The retroperitoneal region including abdominal aorta and it's branches were removed extensively and fixed in 10% formol solution. The spinal cord was than removed, immersed in the same fixative, and postfixed for about 14 days before being set in paraffin blocks for sectioning. Sections of the thoracic and lumbar cord were stained with hemotoxylin and eosin. Abdominal aorta and its branches were examined for revealing possible thrombosis or embolic occlusion.

2.6. Statistical analysis

All data are expressed as means $\pm$ S.D. Control variables such as blood pressures, blood gases, and pH were compared among groups with one-way analysis of variance. Nonparametric analyses with Wilcoxon–Mann–Whitney U-test using the Bonferroni correction were performed on the data of the hindlimb motor scores. Somatosensory evoked potential data are expressed as percent of control. Recovery of SEP amplitude in aprotinin treated animals was compared with that value of ischemia control animals using t-tests and Bonferroni corrections. Fisher exact probability test was used in the analysis of bladder function. A P value less than 0.05 was considered significant.

3. Results

3.1. Physiological parameters

No differences in mean levels of blood glucose, in arterial blood gases, in pH, in arterial pressures or body temperatures were noted among the control groups and the group treated with aprotinin before ischemia. These physiological parameters stayed quite similar during the procedure in group 3 of animals. The femoral artery blood pressure fell and remained near zero in the animals subjected to ischemia during the ischemic period and became gradually normal within the first few min of reperfusion. In these groups of animals, a slight decrease in arterial pH observed at the 3rd min of reperfusion were corrected with sodium bicarbonate injection. There was no difference in any of the three groups in the amount of bicarbonate required to correct the acidosis (0.6 ± 1.3 mEq in group 1; 0.8 ± 1.6 mEq in group 2; and 0.0 ± 0.0 mEq in group 3).

3.2. SEP recordings

SEP recordings consistently showed a large negative peak (N1) with an average latency of 11 ± 2 ms, and a positive peak (P1) with an average latency of 19 ± 3 ms and two other negative following peaks. The peaks were stable in group 3 control animals during the procedure. In ischemic groups, N1 and P1 peaks progressively declining with an average of 11 ± 3 min after aortic occusion while other waves were resistant to ischemia. At the end of ischemic period N1-P1 amplitude decreased 42–68% of preischemic baseline level in all ischemic animals. The average amplitude was reduced to $54 \pm 7\%$ and $51 \pm 8\%$ of the baseline in group 1 and group 2 correspondingly ($P > 0.05$). This was followed by a gradual return to $89 \pm 8\%$ of the initial amplitude after 60 min of reperfusion in group 1. This recovery was $81 \pm 13\%$ in group 2 of animals ($P > 0.05$). The typical changes in N1–P1 amplitudes are represented in Fig. 1.

3.3. Neurological status

Group 3 control animals exhibited full neurologic recovery after the procedure. The evaluations of the motor function score after 24 and 48 h postischemia in group 1 and group 2 are shown in table. Most of animals (95%) exhibited score-4 or score-3 motor function at 24 h and full neurologic recovery was not seen in any of these animals. Additional deterioration in neurologic score was observed in 2 rabbits in group 1 (1 rabbit from 4 to 3 and 1 rabbit from 4 to 2) and in 3 rabbits in group 2 (1 rabbit from 4 to 3, 1 rabbit from 3 to 2, and 1 rabbit from 3 to 1) within 24 to 48 h. The average motor score of group 1 were significantly better

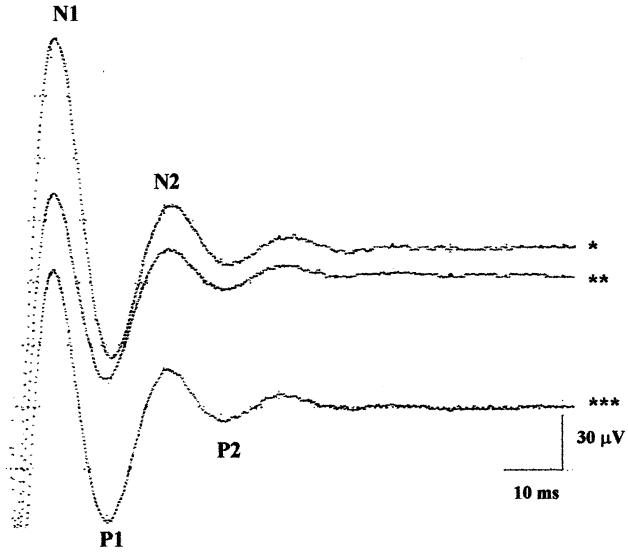

Fig. 1. The typical changes in SEP recordings. The traces of before ischemia, at the end of ischemia and 60th min of reperfusion. (*) Preischemia; (**) at the end of ischemia; (***) at 60th min of reperfusion.

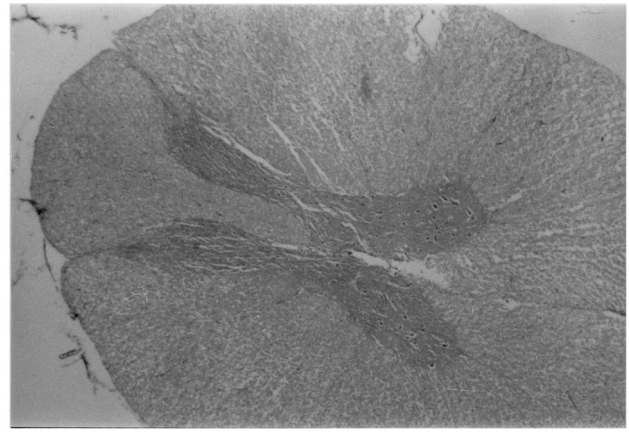

Fig. 2. Normal morphological appearance of unaltered structure of spinal cord and central gray matter (Group 1, score-4 of motor neurological function, lumbosacral segment, × 40 hemotoxylin and eosin).

than group 2 at both after 24 and 48 h postischemia ($P < 0.05$) Table 1.

Bladder function was retained to 48 h postischemia in 7 of 9 animals treated with aprotinin (group 1) compared with 6 of 10 group 2 control animals ($P > 0.05$)

3.4. Histopathological findings

Histologic examination of abdominal aorta and its branches were normal and reveal no thrombus formation in all animals.

The histopathological findings of spinal cord were correlating with the neurological status. The animals with high motor score exhibited unaltered structure of gray matter or minimal necrosis in small patchy areas (Fig. 2). In contrast, animals with low motor score had typical infarctions of variable size in central gray matter which were detected primarily in the medial zone, with predominant localization in lumbosacral segments (Figs. 3 and 4).

4. Discussion

Aprotinin inhibits a wide range of serine protease with varying efficacy. It has been shown in vitro that it directly inhibits trypsin, plasmin, and kallikrein of human origin and weakly inhibits the neutral lysosomal elastase and cathepsin G from human neutrophils [13]. It has been also shown that aprotinin reduces activation of neutrophils possibly by kallikrein inhibition during cardiopulmonary bypass [15] and inflammation [16].

There is evidence from models of cerebral ischemia that aprotinin has some beneficial effects on recovery. Kamiya demonstrated that ischemic brain edema is closely related to plasma and brain tissue bradikinin levels and pretreatment with aprotinin reduces cerebral water content after ischemia in a rat model [9]. Bradykinin is generated by activation of kallikrein–kinin system and is known to be an important chemical

Table 1
Scores of hindlimb motor functions examined in the groups 24 and 48 h after the operation

Animal groups	*n*	Motor score						Average motor score
		0	1	2	3	4	5	
Group 1								
24th h	9	—	—	—	2	7	—	3.78 ± 0.44*
48th h	9	—	—	1	3	5	—	3.44 ± 0.72*
Group 2								
24th h	10	—	—	1	7	2	—	3.10 ± 0.56
48th h	10	—	1	3	5	1	—	2.60 ± 0.84

* Significantly different from group 2 ($P < 0.05$).

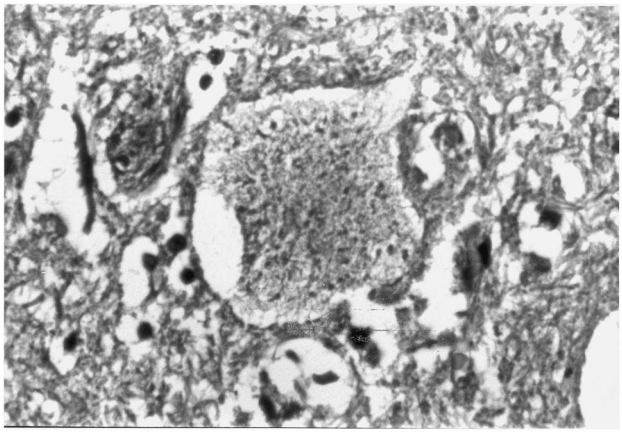

Fig. 3. Neuronal death characterized by blured sytoplasmic border, degranulation of nissl substances and karyolysis (Group 2, score-3 of motor neurological function, lumbosacral segment, ∗400 hemotoxylin and eosin).

mediator of local inflammation. It also effects blood vessels and vascular smooth muscle and increase vasodilatation and vascular permeability resulting with tissue odema [9,15]. Aoki demonstrated that aprotinin enhances acute recovery of cerebral energy methabolism after hypothermic circulatory arrest in piglets through mechanisms involving preservation of vascular integrity [10]. The present study extends these observations to the spinal cord.

The rabbit spinal cord is a reliable model for systematically and rapidly observing the protective effects of investigated agents on ischemia and reperfusion injury. However, the rabbit abdominal aorta ligation does not produce complete ischemia in the spinal cord. There are considerable individual variations in the residual collateral blood flow, and in most animals, about 2% of normal blood flow remains after 20 min of ligation [17]. In the present study, we used a second clamp to distal abdominal aorta for occluding the iliac collateral circulation and we monitored the spinal cord ischemia with SEP. Monitoring of SEP during aortic occlusion has been used for identification and exclusion of animals that had sufficient collateral blood flow to permit the spinal cord to survive long periods of aortic occlusion [5]. In the present study, despite of minimal individual variations, SEP amplitudes decreased to about 50% of their preischemic values in most animals at 20th min of ischemia. Previous studies have shown that a decrease of 15–20% in amplitude can be taken as a criteria of significant SEP changes [18]. In the present study, monitoring of SEP in this model has permitted observation of similar collateral blood flow properties in studied groups of animals.

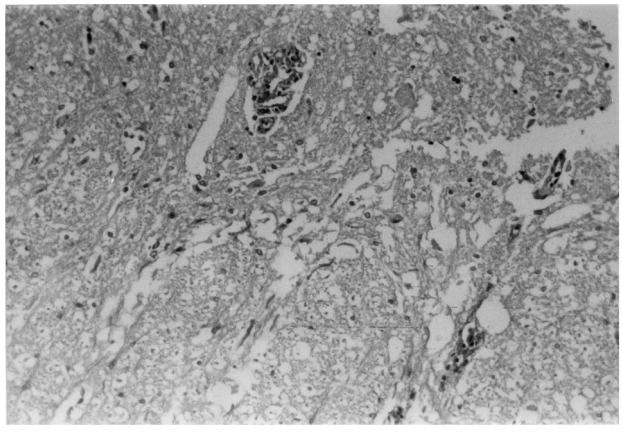

Fig. 4. Large necrosis in central gray matter and capiller proliferation (Group 2, score-2 of motor neurological function, lumbosacral segment, ∗100 hemotoxylin and eosin.

While some authors attempt to define the SEP recovery during early reperfusion as a predictor of neurological outcome [5], some articles have documented some examples where SEP did not accurately predict postoperative deficits, particularly motor deficits [19,20]. In the present study, although the difference in recovery of SEP amplitudes did not attain statistical significance ($P > 0.05$), we observed significantly better neurologic outcomes ($P < 0.05$) in aprotinin treated group.

Spinal cord monitoring can be based on noninvasive recording of cortical SEP to lower limb stimulation, or to more invasive recordings involving electrodes in spinal bony spinous processes, intervertebral ligaments or spinal epidural space. Cortical SEP recently have been criticized because of occasional false correlations with neurological outcome. However, most authors still believe that the cortical SEP reliably reflect the spinal cord function [21]. Thus, we preferred cortical SEP monitoring because of it is safe, easy and it does not prolong the operative procedure. The possible effects of used anesthesia and operation on SEP recordings was ruled out with the evaluation of group 3 in the present study.

It is known that deterioration in neurological function after transient spinal cord ischemia of rabbits usually worsens 24–48 h after injury [22,23]. In this study, 2 rabbits in group 1 and 3 rabbits in group 2 showed additional motor deterioration within 24–48 h after the ischemic insult (26%). Although the mechanisms to account for this progressive deterioration remain uncertain, some investigators have suggested that cytotoxic substances such as free radicals, excitotoxins, proteases, or arachidonic acid metabolites found in damaged neural tissue are responsible [22,23].

Aprotinin is a potentially antigenic agent and may cause allergic reactions [24]. Its effects on postoperative thrombosis formation have also been discussed extensively [13,25]. We have no observation suggesting these adverse effects of aprotinin in the rabbits in the present study. Histopathological examination of abdominal aorta and its branches revealed no thrombus formation in both aprotinin treated and control groups.

In conclusion, the results suggest that aprotinin reduces ischemic and reperfusion damage in transient spinal cord ischemia and provide better neurologic outcome. Aprotinin does not cross the blood-brain barrier; therefore its main site of action must be in the microcirculation. We believe that it decreases ischemic injury in endothelium by reducing bradikinin generation and provides better microcirculatory environment during early reperfusion by preventing endothelial cell lysis by proteases from activated neutrophils. There are considerable species differences in specific protease affinity to aprotinin, so that the results of an animal study such as this may not be fully reproducible in humans. Further studies are needed to define biochemical aspects of these events and to determine the correct dose necessary for maximal benefit.

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