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ORIGINAL ARTICLE

Pretreatment with erythropoietin attenuates the neurological injury after spinal cord ischemia

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Study design: Experimental study.
Objectives: To investigate the effect of erythropoietin (EPO) pretreatment on spinal cord ischemic injury.
Setting: Experimental Research Center at Seoul National University Bundang Hospital, Korea.
Methods: Rats were treated with either 1000 IU kg^{-1} of EPO (EPO group, n=8) or saline (control group, n=8) 24 h before ischemia. Spinal cord ischemia was induced using a balloon-tipped catheter placed on the proximal descending aorta in the control group and the EPO group, but not in the sham group (n=8). Neurological function was assessed using the motor deficit index (MDI; 0=normal, 6=complete paralysis) until 7 days after reperfusion, and histological examination of spinal cord was performed.
Results: At the first day after reperfusion, the EPO group demonstrated a significantly lower MDI compared with the control group (2.0 (0.3–2.0) vs 4.0 (3.0–4.8), median (interquartile range); EPO group vs control group, respectively; P=0.002). This trend was sustained until 7 days after reperfusion (1.0 (1.0–1.8) vs 4.5 (3.3–5.0); EPO group vs control group, respectively; P=0.001), and more normal motor neurons (29.9 ± 3.1 vs 21.4 ± 3.4, mean ± s.d.; EPO group vs control group, respectively; P<0.001) were observed. However, compared with the sham group, the EPO group displayed a significantly higher MDI (0.0, sham group) and fewer intact motor neurons (37.8 ± 5.5, sham group; P<0.001, sham vs control group).
Conclusion: Pretreatment with EPO significantly attenuates neurological injury following spinal cord ischemia, although it cannot completely abolish the ischemic injury.
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Keywords: erythropoietin; spinal cord ischemia; thoracoabdominal aortic surgery

INTRODUCTION

Paraplegia is a catastrophic complication of thoracoabdominal aortic aneurysm repair,[1] and its prevention is one of the major concerns of thoracoabdominal aortic surgery. The mechanism of paraplegia following thoracoabdominal aortic surgery involves an ischemia-reperfusion injury secondary to aortic clamping and declamping during the operation. Erythropoietin (EPO), traditionally used as a hematopoietic hormone,[2] is demonstrated to protect against ischemic injury.[3–7] Recent studies revealed that EPO has a major role in ischemic preconditioning,[4] and the administration of exogenous EPO before an ischemic event offers protection against the following ischemic insult similar to ischemic preconditioning.[8] The protective effect of pretreatment with EPO has been reported for cerebral[3–6] and cardiac ischemia.[7]

The effect of EPO in spinal cord ischemic injury has been studied in only a limited number of reports, and a group of investigators has reported that EPO protects against spinal cord ischemic injury when it is administered following the ischemic insult.[9] EPO is an important mediator of the preconditioning effect[3,4] and pretreatment with EPO offers a protective effect against ischemic injury in various organs.[3–5,7] Thus, we hypothesized that pretreatment with EPO could offer a protective effect against spinal cord ischemia. This study was designed to evaluate the preconditioning effect of EPO on spinal cord ischemic injury. EPO was administered 24 h before ischemic insult as a preconditioning agent, and the neurological recovery and histopathological changes were studied.

MATERIALS AND METHODS

Animal care and experimental protocol

The experimental protocol was approved by the Institutional Animal Care and Use Committee of our university. All animals used received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health.

Before the surgery, male Sprague-Dawley rats (weight=250–300 g) were randomly assigned to one of the three groups: (1) control group (n=8), where saline was administered intraperitoneally 24 h before the surgery (2) EPO preconditioning group (EPO group, n=8), where recombinant human erythropoietin (Epokine, CJ Pharma, Seoul, Korea) 1000 IU kg^{-1} was administered intraperitoneally 24 h before ischemia and (3) sham surgery group (sham group, n=8), where surgical preparation was performed in the same manner, but spinal cord ischemia was not induced.

Anesthesia was induced in a chamber with 5 vol% isoflurane in 100% oxygen. After anesthetic induction, the anesthesia was maintained with 1.5–2.5 vol% isoflurane via mask. Spinal cord ischemia was induced by Taira and Marsala's method.[10] The tail artery was cannulated with a polyethylene catheter

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(PE-50) for heparin injection, collection of blood specimens and distal arterial pressure monitoring. The right carotid artery was cannulated with a 20-gauge catheter (BD Insyte, Becton Dickinson, Sandy, UT, USA) and was connected to a saline-filled external blood reservoir to allow blood drainage during the aortic occlusion period. A 2 Fr Fogarty catheter (Fogarty Arterial Embolectomy Catheter, Edwards Lifesciences, Irvine, CA, USA) was inserted via the left femoral artery into the descending thoracic aorta so that the tip of the catheter reached the left subclavian artery. After the completion of all cannulation procedures, heparin (150 units) was injected into the tail artery and the balloon of the Fogarty catheter was inflated with 0.05 ml of saline. At the same time, the blood flow from the carotid artery was drained into the external reservoir to prevent proximal hypertension. The success of aortic occlusion was confirmed by an immediate drop and sustained loss of distal arterial pressure. After 10 min and 30 s of aortic occlusion, the balloon of Fogarty was deflated, and the drained blood was re-infused. During the surgery, the body temperature was monitored with a rectal probe and maintained at 37.5 °C by using a circulating water pad and heating lamp. After finishing the surgery, all catheters were removed and incisions were closed. The rats were then allowed to recover from anesthesia and were returned to their cages.

Physiological parameters

Mean arterial pressure, heart rate and body temperature were continuously monitored. Blood pressure and heart rat were directly monitored on the tail artery and body temperature was monitored with a rectal temperature probe. The measured values were recorded at the following time points: immediately before aortic occlusion, during occlusion and 10 min after reperfusion. The 'during occlusion' values were the averaged value of the corresponding parameter measured every 2 min throughout the aortic occlusion period. Arterial blood gases and Hct were measured immediately before the placement of the Fogarty catheter and 5 min after reperfusion.

Assessment of neurological function

Neurological function was assessed at 1, 3, 5 and 7 day after reperfusion. Hind limb motor function was evaluated by assessment of ambulation and placing/stepping reflex according to Taira and Marsala's method.[10] Ambulation was graded as follows: 0, normal (symmetrical and coordinated ambulation); 1, toes flat under body when walking but ataxia present; 2, knuckle-walking; 3, movement in the lower extremities but unable to knuckle-walk and 4, no movement and drags lower extremities. The placing/stepping reflex was assessed by dragging the dorsum of the hind paw over the edge of a surface. Normally this evokes a coordinating lifting and placing (stepping) response. The placing/stepping reflex was graded as follows: 0, normal; 1, weak and 2, no stepping. The sum of each score at the observed period was considered as a motor deficit score. The motor deficit index (MDI) was defined as the sum of the scores (ambulation with lower extremities plus the placing/stepping reflex), and the MDI was evaluated by two observers who were blinded to the group assignment.

Histopathological analysis

After the last neurological assessment, the rats were deeply anesthetized with isoflurane. The heart was exposed and transcardially perfused with 100 ml of heparinized saline. The spinal cord was removed and fixed in 10% buffered formalin for 24 h. Spinal cord segments from L3 to L5 were embedded in paraffin. Transverse sections were cut and stained with hematoxylin and eosin. Neuronal injury was evaluated by an investigator blinded to the group assignment. The number of normal motor neurons in the anterior horn of spinal cord was counted in three sections for each animal and averaged.

Statistical analysis

The SPSS software (version 15) was used for statistical analysis. Data were expressed as mean ± s.d. or median (interquartile range). The physiological data from each time point and the number of normal motor neurons were compared among the groups using one-way ANOVA. Dunnett *post-hoc* test was used when appropriate. At each time point, the hind-limb motor function of the three groups was compared by the Kruskal–Wallis test, followed by the Mann–Whitney *U* test. The change in MDI within each group was compared with the Friedman test followed by the Wilcoxon rank sum test. A *P*-value of less than 0.05 was considered significant.

RESULTS

Mean arterial pressure, heart rate and rectal temperature were not significantly different among the three groups at each time point (Table 1). The blood gas and Hct values are listed on Table 2. The arterial pH, PaO_2 and $PaCO_2$ were similar between the groups at each time point. No significant difference in Hct values was observed between the groups before the aortic occlusion. After reperfusion, both the control and EPO groups presented significantly decreased Hct values compared with the sham group ($P<0.01$, each group vs sham group), but there was no significant difference between the control group and the EPO group.

All animals survived until the final neurological assessment at 7 days after reperfusion. The sham group presented an MDI of 0 throughout the experimental period. The MDI in the control group and the EPO group is presented in Figure 1. During the post-reperfusion observation period, the EPO group showed a significantly

Table 1 Hemodynamic variables and temperature during the study

	MAP	*HR*	*Temperature (°C)*
Before aortic occlusion			
Sham	96.8 ± 11.2	338.6 ± 14.8	37.3 ± 0.5
Control	97.5 ± 8.3	339.3 ± 15.0	37.3 ± 0.5
EPO	92.1 ± 8.3	345.9 ± 8.4	37.6 ± 0.4
During aortic occlusion			
Sham	No occlusion	No occlusion	No occlusion
Control	5.8 ± 1.1	303.1 ± 28.2	37.1 ± 0.5
EPO	5.5 ± 1.0	336.1 ± 15.3	37.2 ± 0.5
After reperfusion			
Sham	96.8 ± 11.2	334.8 ± 15.2	37.6 ± 0.6
Control	95.9 ± 6.5	305.4 ± 24.2	37.3 ± 0.7
EPO	92.1 ± 8.3	306.4 ± 17.8	37.4 ± 0.4

Abbreviations: EPO, erythropoietin; HR, heart rate; MAP, mean arterial pressure.
Sham, aortic occlusion was not performed; EPO, recombinant human erythropoietin 1000 IU kg^{-1} i.p. 24 h before aortic occlusion; and control, saline i.p. 24 h before aortic occlusion.
Values are presented as means ± s.d.
The groups did not show any difference in the measured hemodynamic variables at each time point.

Table 2 Hematological parameters during the study

	pH	*PaO_2*	*$PaCO_2$*	*Hct*
Before aortic occlusion				
Sham	7.40 ± 0.05	289.8 ± 42.8	33.8 ± 4.4	38.9 ± 2.4
Control	7.39 ± 0.02	257.0 ± 26.3	35.7 ± 4.5	39.0 ± 2.1
EPO	7.40 ± 0.02	250.4 ± 23.3	36.0 ± 3.6	39.4 ± 2.9
After reperfusion				
Sham	7.40 ± 0.03	287.4 ± 52.7	33.5 ± 2.8	38.7 ± 2.5
Control	7.38 ± 0.04	264.4 ± 32.9	31.5 ± 4.8	35.1 ± 2.2*
EPO	7.38 ± 0.03	272.0 ± 17.3	33.6 ± 4.4	35.5 ± 1.8*

Abbreviation: EPO, erythropoietin
Sham, aortic occlusion was not performed; EPO, recombinant human erythropoietin 1000 IU/kg i.p. 24 h before aortic occlusion; and control, saline i.p. 24 h before aortic occlusion.
Values are presented as means ± s.d. *$P<0.05$ vs Sham.
The groups did not show any difference in blood gas data at each time point. Hct did not differ among the three groups before aortic occlusion. After reperfusion, the control group and EPO group did not show any significant difference in Hct value, but the sham group showed significant higher value when compared with the other two groups.

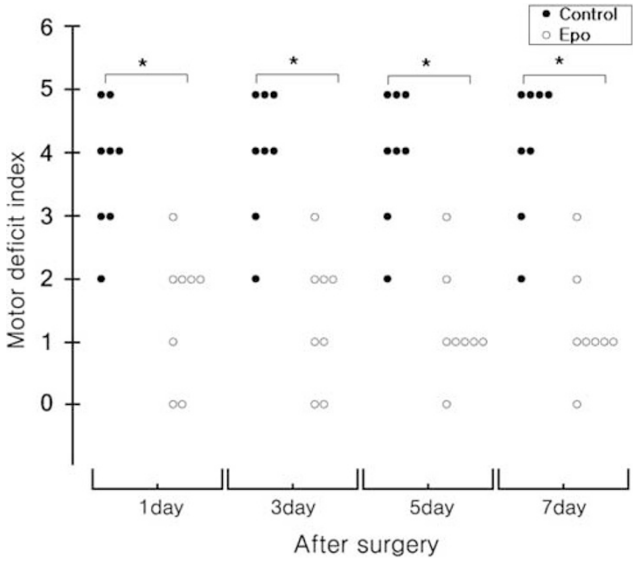

Figure 1 Hind limb motor function assessed with motor deficit index (MDI). Control: saline i.p. 24 h before aortic occlusion. EPO: recombinant human erythropoietin 1000 IU kg^{-1} i.p. 24 h before aortic occlusion. Each dot represents the MDI for the individual rat at each corresponding time point. The EPO group demonstrated significantly lower MDI compared with the control group at each time point. *$P<0.05$ between the control group and the EPO group at each time point.

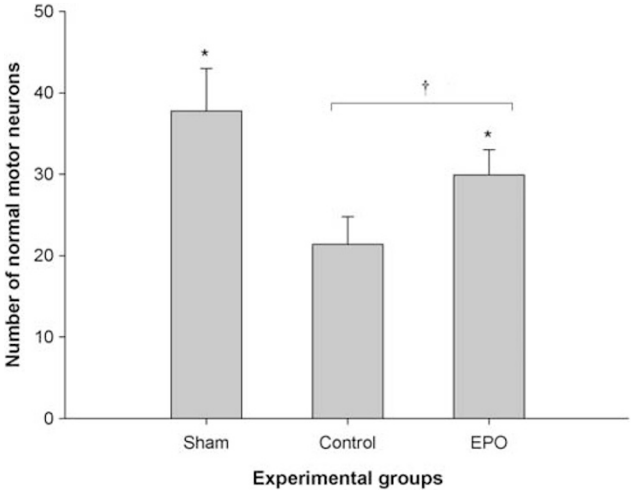

Figure 2 The number of normal motor neurons in the anterior horn of the spinal cord. Sham: aortic occlusion was not performed. Control: saline i.p. 24 h before aortic occlusion. EPO: recombinant human erythropoietin 1000 IU kg^{-1} i.p. 24 h before aortic occlusion. Data are presented as means ± s.d. The number of normal motor neuron was significantly higher in the sham group, compared with the other groups, where aortic occlusion was performed. When comparing the two groups for which aortic occlusion was performed, the EPO group had a higher number of motor neurons compared with the control group. *$P<0.05$ compared with sham group. †$P<0.05$ between the control group and the EPO group.

lower MDI compared with the control group. Within each group, there was no change in MDI during the study period.

Figure 2 presents the number of normal motor neurons in the anterior spinal cord. Compared with the control group, the EPO group displayed a higher number of normal motor neurons, but both the control and EPO groups presented significantly fewer intact motor neurons compared with the sham group. Representative photos from each group are presented in Figure 3.

DISCUSSION

The present study indicates that pretreatment with EPO offers a sustained neuroprotective effect against spinal cord ischemic injury. Specifically, 1000 IU kg^{-1} of recombinant human EPO was administered 24 h before ischemic insult. A neurological assessment was performed until post-reperfusion 7 days, and the number of normal motor neurons was compared among groups. In a rat model of spinal cord ischemia, the animals pretreated with EPO presented a significantly improved neurological outcome. Based on the long post-reperfusion observation period, the results of this study indicate a robust neuroprotective effect of EPO against spinal cord ischemia.

EPO, a member of the type 1 cytokine superfamily, was first identified as a hormone that stimulates the transformation of erythroid progenitors to mature erythrocytes. EPO has traditionally been used as a hematopoietic agent in patients with chronic renal failure.[2] Currently, however, EPO is considered to be a tissue-protective cytokine that regulates the local stress response, including the inflammatory reaction[7] and apoptosis.[3,9] Various tissue injuries caused by ischemia,[3–7] excitotoxin[3,11] or mechanical trauma[12] are attenuated by EPO. Recently, EPO has been reported to have an essential role in ischemic preconditioning. The mechanism of ischemic preconditioning depends on EPO upregulation within the affected tissue, and the blockade of EPO significantly reduces or totally abolishes the protective effect of hypoxic preconditioning.[4] Also, the exogenous administration of EPO mimics ischemic preconditioning.[8] The protective effect of EPO preconditioning has been reported from *in vivo* ischemia models of the brain[5] and heart.[7] Pretreatment with EPO significantly improves neuronal survival in an *in vitro* model of cerebral ischemia.[4] This protective effect was maximized when EPO was applied 24–48 h before the ischemic insult. In an *in vivo* model of focal cerebral ischemia, the mice treated with EPO 24 h before the ischemia exhibited a 47% reduction in the volume of the cortical infarct, as compared with the vehicle-treated control group.[5] The effect of pretreatment with EPO has also been reported in traumatic spinal cord injury.[12] Our results, consistent with these previous reports, indicate that pretreatment with EPO attenuates neurological injury after spinal cord ischemia.

There are only a few investigations about the effect of EPO on spinal cord ischemia. Simon *et al.*[13] reported that EPO, administered immediately before ischemia and additionally after reperfusion, did not improve neurological recovery following spinal cord ischemia. Forty-five minutes of aortic occlusion was applied to the swine for this study, and both EPO-treated and saline-treated animals presented totally paralyzed lower extremities with marked histological injury. However, 30 min of aortic cross-clamping usually induces completely irreversible paraplegia in the swine model.[14] The significantly longer aortic cross-clamping period, 45 min of aortic occlusion instead of the standard 30 min of aortic occlusion in swine model, would have caused an excessive spinal cord injury. Thus, the results from Simon *et al.* could be interpreted in the way that pretreatment with EPO cannot offer protection against extensive spinal cord in injury. Celik *et al.*[9] have reported the protective effect of EPO against spinal cord ischemia using a rabbit model. In this study, EPO was administered immediately after the reperfusion, and the rabbits treated with EPO demonstrated better neurological recovery and less inflammation when compared with the control group. The difference between our study and the Celik *et al.*'s study is the timing of EPO treatment: after

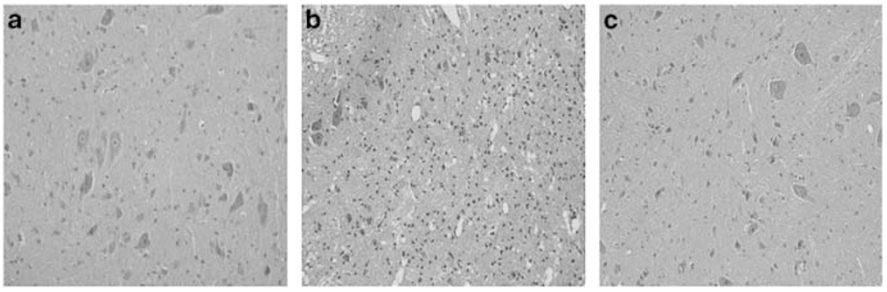

Figure 3 Representative histological findings of spinal cord sections stained with hematoxylin and eosin. There is no specific histological change in the sham group (**a**) The control group presented a marked vacuolization and significant loss of motor neuron cells (**b**), whereas the EPO group presented a better preservation of motor neurons (**c**).

reperfusion and pretreatment 24 h before ischemia. As the used animal model and the method of neurological assessment are different, it is impossible to discuss which administration timing is better in terms of neuroprotection. Taking into consideration that the protection mediated by EPO requires a certain time window of 24–48 h for the expression of multiple genes and the activation of particular proteins,[3,4] our regimen, with pretreatment 24 h before ischemic insult, has a theoretical advantage. Our results are more or less consistent with a recent report from Smith *et al.*[15] that reported on the protective effect of mouse recombinant EPO administered 4 h before the ischemic insult. In this study, spinal cord ischemia was induced by applying 5 min of aortic occlusion, and the mice were observed until post-reperfusion 48 h. Taking into consideration that 11 min of aortic occlusion is usually necessary for inducing spinal cord ischemia in the mice,[16] the 5 min of occlusion might have been too brief. In addition, this study observed the animals only for 48 h, and thus could not conclude if EPO can reduce the neurological deficit following cord ischemia or if EPO can merely delay its onset. However, the finding from this study, the superior neurological scores in EPO-treated animals during the first 48 hour-post-reperfusion period, suggests the possibility of the protective effect of EPO pretreatment in spinal cord ischemia. Our study assessed neurological outcomes until 7 days post-reperfusion using a well-characterized rat spinal cord ischemia model. Considering that most neurological deficit following thoracoabdominal aortic surgery occurs within 4 days post-surgery,[17] our study with a week of observation period can clearly demonstrate that the pretreatment with EPO can reduce the neurological deficit following spinal cord ischemia.

The feasibility of EPO use in patients suffering from ischemic injury, such as myocardial infarction[18] or stroke,[19] has been documented in clinical trials. At present, the preoperative use of EPO is approved as a transfusion-reduction strategy.[2] Moreover, EPO can penetrate the blood–brain barrier,[11] and thus regional administration, such as intrathecal injection, which imposes the risk of spinal cord hematoma, is not required. In this regard, our results that indicate the protective effect of systemically administered EPO against spinal cord ischemic injury can be applicable in clinical practice for patients undergoing thoracoabdominal aortic surgery.

Our study has some limitations. First, we applied 1000 IU kg^{-1} of EPO in our study. Both 5000 IU kg^{-1} and 1000 IU kg^{-1} have been widely accepted for hypoxic injury models,[3,6,9,11] but to lessen side effect, such as thrombosis,[2,20] we choose 1000 IU kg^{-1}. The effect of EPO, however, has been reported to be more or less dose dependent.[3,4] Therefore, our study was not able to determine the optimal dosing regimen, and further investigation will be necessary to identify the optimal preconditioning EPO dose. Second, as was described above, our current study cannot determine the optimal timing of administration. Although a pretreatment regimen has several theoretical advantages,[4] further study may be necessary to determine the best timing of administration. Third, in our study, we tried to investigate the neuroprotective effect of EPO given 24 h before ischemic insult, not elucidate the preconditioning mechanism of the protective effect. Further studies are needed to determine the mechanism underlining the neuroprotective effect of EPO.

In summary, we investigated the effect of EPO pretreatment by using a rat model of spinal cord ischemia. The rats that were administered 1000 IU kg^{-1} of EPO 24 h before the ischemic insult presented significant improvement in neurological results and histopathological outcomes during the post-reperfuison 7-day observational period. In conclusion, pretreatement with EPO significantly attenuates neurological injury following spinal cord ischemia.

DATA ARCHIVING

There were no data to deposit.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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