

# Ethyl Pyruvate Attenuates Spinal Cord Ischemic Injury with a Wide Therapeutic Window through Inhibiting High-mobility Group Box 1 Release in Rabbits

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**Background:** Ethyl pyruvate (EP) has been reported to offer a protective effect against ischemic injury through its anti-inflammatory action. The nuclear protein high-mobility group box 1 (HMGB1) can activate inflammatory pathways when released from ischemic cells. This study was designed to investigate the neuroprotective effect of EP against spinal cord ischemic injury and the potential role of HMGB1 in this process.

**Methods:** EP was administered at various time points before or after 20 min of spinal cord ischemia in male New Zealand rabbits. All animals were sacrificed at 72 h after reperfusion with modified Tarlov criteria, and the spinal cord segment (L4) was harvested for histopathological examination and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining. The HMGB1 levels in serum and spinal cord tissue were analyzed by enzyme-linked immunosorbent assay.

**Results:** The treatment of EP at 30 min before ischemia or at 6 h after reperfusion significantly improved the hind-limb motor function scores and increased the numbers of normal motor neurons, which was accompanied with reduction of the number of apoptotic neurons and levels of HMGB1 in serum and spinal cord tissue. The HMGB1 contents of spinal cord tissue correlated well with the numbers of apoptotic motor neurons in the anterior spinal cord at 72 h after reperfusion.

**Conclusion:** These results suggest that EP affords a strong protection against the transient spinal cord ischemic injury with a wide therapeutic window through inhibition of HMGB1 release.

ISCHEMIC spinal cord injury represents the main complication in surgical repair of thoracic and thoracoabdominal aneurisms and remains a persistent clinical problem. The development of paraparesis or paraplegia after aortic surgery can be classed as immediate or delayed. A primary cause of immediate spinal cord dysfunction is ischemia induced by temporary interruption of blood flow to the spinal cord during aortic cross-clamping. Delayed neurologic complications can develop during the period of reperfusion.<sup>1</sup> Of the many pathophysiological events that may contribute to this delayed

injury, postischemic inflammation and apoptosis have been suggested to contribute to the late stages of the reperfusion injury and to result in a worsening of neurologic outcome.<sup>2–4</sup>

Ethyl pyruvate (EP), a stable and lipophilic derivative of pyruvate, protects various tissue injuries, such as lethal sepsis and systemic inflammation.<sup>5</sup> Recently, the protective effect of EP on ischemia-reperfusion (I/R) injury to brain and myocardium has been reported.<sup>6–9</sup> So far, there are no studies attempting to describe the protective effect of this agent on spinal cord ischemia.

In addition, high-mobility group box 1 (HMGB1) was originally identified as a DNA-binding protein that functions as a structural cofactor critical for proper transcriptional regulation in somatic cells. There is a wealth of evidence to indicate that HMGB1 is massively released during the excitotoxicity-induced acute damaging process in the postischemic brain. The extracellular HMGB1 triggers inflammatory processes, suggesting that HMGB1 acts as a novel mediator that links excitotoxicity-induced acute damage and subsequent inflammatory processes in the postischemic brain.<sup>10–12</sup> However, the contribution of HMGB1 to pathogenesis after spinal cord I/R are unknown.

Therefore, the objective of this study was to test the neuroprotective effect of EP in a rabbit spinal cord ischemia model of infrarenal aortic occlusion and to investigate the underlying mechanisms responsible for HMGB1 in terms of effective therapeutic windows. We hypothesized that EP could reduce spinal cord ischemic injury with a wide therapeutic window through inhibition of HMGB1 release. This hypothesis was based on previous observations that extracellular HMGB1 serves as a late cytokine-like mediator of systemic inflammation that provides a wider time frame for clinical intervention against progressive inflammatory disorders.<sup>13</sup> This hypothesis was also based on the knowledge that EP inhibits HMGB1 secretion from human macrophages in a concentration-dependent fashion with an estimated effective concentration clinically achievable.<sup>14</sup>

## Materials and Methods

### Instrumentation of Animals

All experiments were performed on male New Zealand White rabbits weighing 2.2 to 2.5 kg. The animals were provided by the Experimental Animal Center of the Fourth Military Medical University, Xi'an, China. The

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animals were studied at Xijing Hospital, Fourth Military Medical University (Xi'an, China). The experimental protocol was approved by the Ethics Committee for Animal Experimentation, and all animals received humane care in compliance with the guidelines for Animal Experimentation of the Fourth Military Medical University (Xi'an, China), which was in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). All efforts were made to minimize animal suffering and the number of animals used for this study.

#### *Experimental Protocols*

The present study consisted of two experiments. In experiment I, 40 rabbits were randomly divided into 5 groups ( $n = 8$  each group): control, EP, EP-6 h, EP-12 h and EP-24h groups. The rabbits in the control group underwent only I/R. The rabbits in EP, EP-6 h, EP-12 h, and EP-24h groups intravenously received a dose of 40 mg/kg EP at 30 min before ischemia or at 6 h, 12 h, or 24 h after reperfusion, respectively. In experiment II, 24 rabbits were randomly divided into 3 groups ( $n = 8$  each group): sham, control, and EP groups. The animals in sham group only underwent the surgical procedure, but the aorta was not occluded. The rabbits in control and EP groups received Ringer's solution (IV, 1 ml) and a dose of 40 mg/kg EP at 30 min before ischemia, respectively. EP (98% pure) was purchased from Sigma-Aldrich Co. (St. Louis, MO) and was prepared and injected in the form of Ringer's ethyl pyruvate solution. The injection volume was 1 ml per dose. For the sake of minimizing the number of animals used for this study, we designed the experimental protocol by consulting previous studies on assessment of neuroprotections in the rabbit spinal cord ischemia model of infrarenal aortic occlusion. The same eight animals per group were chosen in our experimental protocol as other studies.<sup>15,16</sup> Moreover, the expected statistical differences were obtained according to this experimental protocol.

#### *Animals and Surgical Preparation*

After an overnight fast with unrestricted access to water, the rabbits were anesthetized with pentobarbital sodium (30 mg/kg, IV) and allowed to breathe spontaneously and inhaled oxygen by face mask at a flow rate of 2 l/min. The lactated Ringer's solution ( $4 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) was infused intravenously. A 22-gauge catheter was inserted into the ear artery to measure the proximal blood pressure. Another catheter was inserted into the left femoral artery to measure the distal blood pressure. Blood pressure was monitored continuously by using a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Medical Inc., Redmond, WA). Rectal temperature was maintained between 38°C and 39°C by an overhead lamp during the experiments.

Arterial blood was sampled at preischemia, 10 min after ischemia, and 10 min after reperfusion, respectively, for the determination of arterial oxygen tension ( $\text{PaO}_2$ ), arterial carbon dioxide tension ( $\text{PaCO}_2$ ), pH, and plasma glucose. Arterial blood gases were measured by means of the OMNI Modular System (AVL List GmbH Medizintechnik, Kleiststrabe, Graz, Austria).

#### *Spinal Cord Ischemia*

Spinal cord ischemia was induced by infrarenal aortic occlusion in the rabbits as described in our previous studies.<sup>15,16</sup> Briefly, animals were placed in supine position. The abdominal aorta was exposed at the level of the left renal artery through a 3- to 4-cm-long medial incision. Spinal cord ischemia was induced with the aorta clamping with a bulldog clamp just below the renal artery, and 400 units of heparin was administered 5 min before the aortic occlusion. Obstruction of the blood flow lasted for 20 min. Then the bulldog clamp was removed, and the abdominal wall was closed with wound clips. Local anesthetic infiltration with 0.25% bupivacaine hydrochloride was applied around the wound for postoperative analgesia. Animals were placed on a heating pad to maintain body temperature until recovery from anesthesia. The animals were then returned to their home cage, received prophylactic antibiotic (gentamicin; 40,000 IU once a day for 3 days) and saline (5 ml, twice a day for 5 days) to prevent dehydration. Bladders were voided manually twice a day until normal function returned. Over the course of postsurgery, all animals were monitored carefully for any distress, pain, and discomfort until they were alert, mobile, and could freely access food and water. The animals were then euthanized if they were in extremes.

#### *Neurologic Assessment*

In experiment 1, the motor function of the hind limb of animals was assessed at 24 h, 48 h and 72 h after reperfusion by an independent observer, who had no prior knowledge of the experimental protocol and was unaware of the grouping. A modified Tarlov criteria was used: 0, no voluntary hind-limb function; 1, only perceptible joint movement; 2, active movement but unable to stand; 3, able to stand but unable to walk; 4, complete normal hind-limb motor function.

#### *Hematoxylin and Eosin Staining*

In experiment I, a histopathologic evaluation was performed in the spinal cord at 72 h after reperfusion. After all animals were reanesthetized with 40 mg/kg sodium pentobarbiturate, transcardiac perfusion and fixation was performed with 1000 ml heparinized saline followed by 500 ml of 10% buffered formalin. The lumbar spinal cord (L4-6 segments) was removed and refrigerated in 10% phosphate-buffered formalin for 48 h. After dehydration in the graded ethanol and butanol, the spi-

nal cord was embedded in the paraffin. Coronal sections of the spinal cord (L4 segment) were cut at a thickness of 6  $\mu\text{m}$  and stained with hematoxylin and eosin. Neuronal injury was evaluated with a light microscope at a magnification of  $\times 100$  by an observer who was unaware of grouping. Ischemic feature changes of motor neurons were identified by shrunken cellular bodies, a disappearance of Nissl granules, an intensely eosinophilic cytoplasm, and triangular and pyknotic nuclei. The remaining normal neurons in the ischemic ventral spinal cord in each animal, judged by their morphological appearance, were counted in three sections selected randomly from the rostral, middle, and caudal levels of the L4 segment and then averaged. The numbers of normal motor neurons per section in the anterior spinal cord (anterior to an imaginary line drawn through the central canal perpendicular to the vertical axis) were compared among five groups.

#### *TUNEL Staining and Quantification of Apoptosis*

To detect DNA fragmentation in the nuclei of the cells, we applied a kit for immunohistochemical detection and quantification of apoptosis, based on labeling of DNA strand breaks, according to instruction manual (Roche Diagnostics GmbH, Mannheim, Germany) and protocol described in our previous study.<sup>17</sup> After deparaffinization, the nuclei of the tissue sections were stripped of proteins by incubation with 20  $\mu\text{g}/\text{ml}$  proteinase K for 10 min. After being treated with 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in distilled water for 5 min, they were incubated with 50  $\mu\text{l}$  of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) reaction mixture (enzyme solution:label solution = 1:9) for 60 min at 37°C in a humidified atmosphere in the dark. The tissue sections were washed three times with 0.01 M phosphate-buffered saline and were analyzed under a BX-60 fluorescence microscope (Olympus Corporation, Tokyo, Japan). To determine the number of motor neurons that underwent apoptosis, a blinded researcher counted the motor neurons that were positive or negative in the TUNEL staining.

#### *Serum Concentrations of HMGB1*

In experiment 2, blood samples (1 ml) were collected from the femoral vein at preischemia, at the onset of reperfusion, and at 2 h, 6 h, 24 h, 48 h, and 72 h after reperfusion, respectively. Serum was isolated after centrifugation at 2,000g for 20 min at 4°C. After centrifugation, serum was frozen at  $-80^\circ\text{C}$  until enzyme-linked immunosorbent assay (ELISA) analysis was performed. The serum HMGB1 concentrations were measured using a commercial ELISA kit specific for rabbit HMGB1 (Central Institute, Shino-Test, Kanagawa, Japan) as described in previous study.<sup>18</sup>

#### *Spinal Cord HMGB1 Contents*

At 72 h after reperfusion, the lumbar spinal cord (L4–6 segments) of all animals were removed under anesthesia with 40 mg/kg sodium pentobarbiturate. Spinal cord samples were snap frozen in liquid nitrogen immediately after harvest and stored at  $-80^\circ\text{C}$  pending protein extraction. Tissue samples were homogenized in a test tube containing 1 ml of immunoprecipitation assay buffer with 10  $\mu\text{l}$  of protease inhibitor cocktail and placed on ice for 30 min. The samples were then sonicated for 20 s and incubated on ice for an additional 30 min. These samples were then centrifuged at 10,000 g for 10 min. The supernatants were then decanted, aliquoted, and frozen ( $-80^\circ\text{C}$ ) until analysis with ELISA. ELISA kits (Central Institute, Shino-Test) using the quantitative sandwich enzyme immunoassay technique were used to determine levels of HMGB1 in the protein extracts. The ELISA plates were read on a Model 550-microplate reader (Bio-Rad Laboratories, Hercules, CA). The protein content values were extrapolated off the standard curve and normalized to the total protein concentration, which was determined with a bicinchoninic acid Protein Assay Reagent Kit (Pierce Biotechnology Inc., Rockford, IL).

#### *Statistical Analysis*

Spinal cord sections were examined by two independent and blinded investigators. SPSS 11.0 for Windows (SPSS Inc., Chicago, IL) was used to conduct statistical analyses. All values, except for neurologic scores, are presented as mean  $\pm$  SEM. The physiologic data and the serum HMGB concentration were analyzed using two-way repeated-measures (time and group) analysis of variance followed by the *post hoc* Student-Newman-Keuls test. The spinal cord HMGB1 content were analyzed by one-way analysis of variance, and between-group differences were detected with Independent-Samples *t* test. The scores of hind-limb motor function were expressed as median (minimum-maximum value). The score of hind-limb motor function, the number of normal neurons, and the number of TUNEL-positive motor neurons in the anterior spinal cord were analyzed with Kruskal-Wallis test followed by the Mann-Whitney *U* test with Bonferroni correction. Correlations of the spinal cord HMGB1 levels and the numbers of apoptotic motor neurons in anterior horns of spinal cords were analyzed using Spearman's rank correlation test. A *P* value of less than 0.05 was considered to be statistically significant.

## **Results**

#### *Physiologic Parameters*

The hemodynamics, rectal temperature, arterial pH,  $\text{Paco}_2$ ,  $\text{PaO}_2$ , and blood glucose concentrations were similar in all groups at any time point. The distal blood

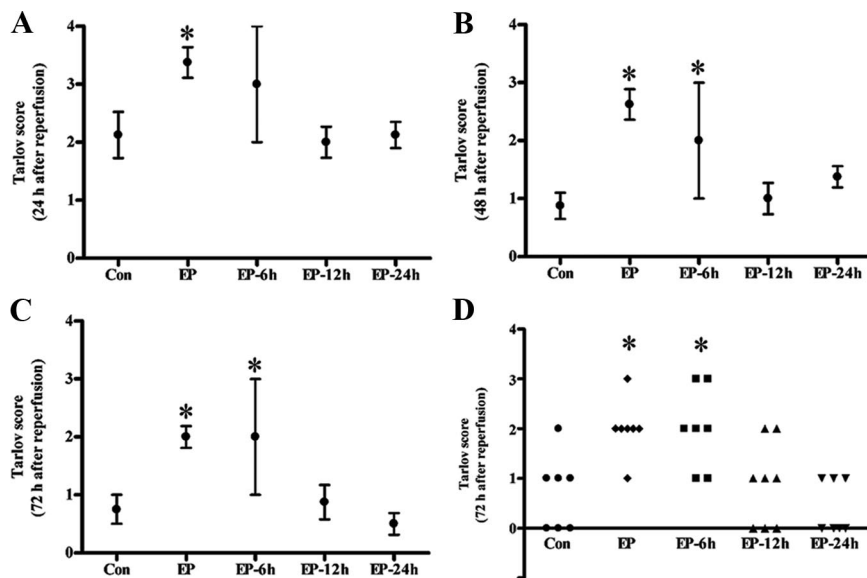


Fig. 1. The hind-limb motor function scores of rats at 24 h, 48 h, and 72 h after reperfusion ( $n = 8$ ). (A–C) The Tarlov scores of rats in five groups at 24 h, 48 h, and 72 h after reperfusion, respectively. Data are expressed as median (range) of eight rats in each group. (D) The Tarlov scores of each animal from five groups at 72 h after reperfusion. \*  $P < 0.01$  versus Control group. Con = control; EP = ethyl pyruvate.

pressure was approximately 75 to 83 mmHg (10 to 11 kPa) before blocking the abdominal aorta and decreased to 7 to 12 mmHg (1.0 to 1.1 kPa) during the time artery was clamped. Ten minutes after the beginning of reperfusion, the value of the distal blood pressure was recovered to 90–93% preischemic level (data not shown).

#### Treatment with EP within 6 h after Reperfusion Improves Neurologic Outcomes

All animals survived until the final neurologic behavior assessment at 24 h, 48 h, and 72 h after reperfusion. The hind-limb motor function scores of five groups at 24 h, 48 h, and 72 h after reperfusion are shown in figure 1. In control group, all animals developed complete paraplegia of the hindlimbs (grade 1) at 72 h after reperfusion. The neurologic status of the EP group was significantly improved compared to that of control group at 24 h, 48 h, and 72 h after reperfusion ( $P = 0.029$ ,  $0.001$ ,  $0.004$ , respectively). The neurologic outcome of EP-6 h group was better than that of control group at 48 h and 72 h after reperfusion ( $P = 0.009$  and  $0.008$ , respectively). However, the hind-limb motor function scores in EP-12 h and EP-24 h groups were similar to the control group at 24 h, 48 h, and 72 h after reperfusion. Moreover, there were no difference in the Tarlov scores between the EP group and EP-6 h group at 48 h and 72 h after reperfusion.

#### Treatment with EP within 6 h after Reperfusion Increases the Numbers of Normal Motor Neurons

The representative micrographs of hematoxylin and eosin staining in the ventral horn of L4 spinal cord segments 72 h after reperfusion are shown in figures 2A–C. The numbers of normal motor neurons in the anterior horn of spinal cord are shown at 72 h after

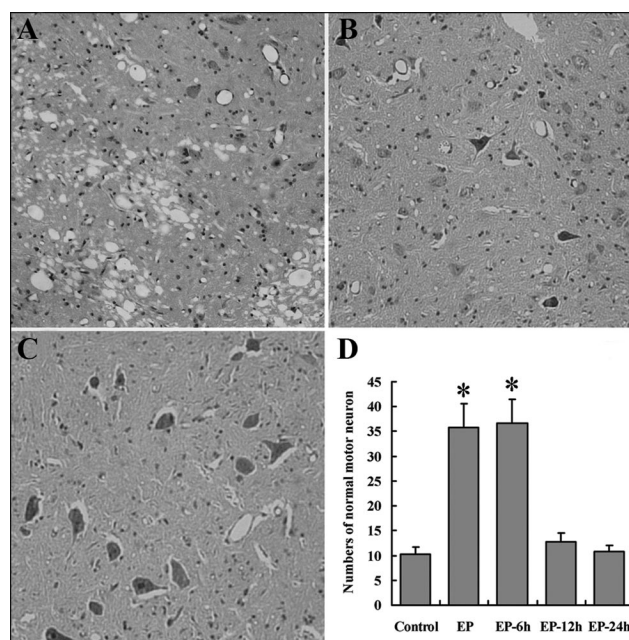
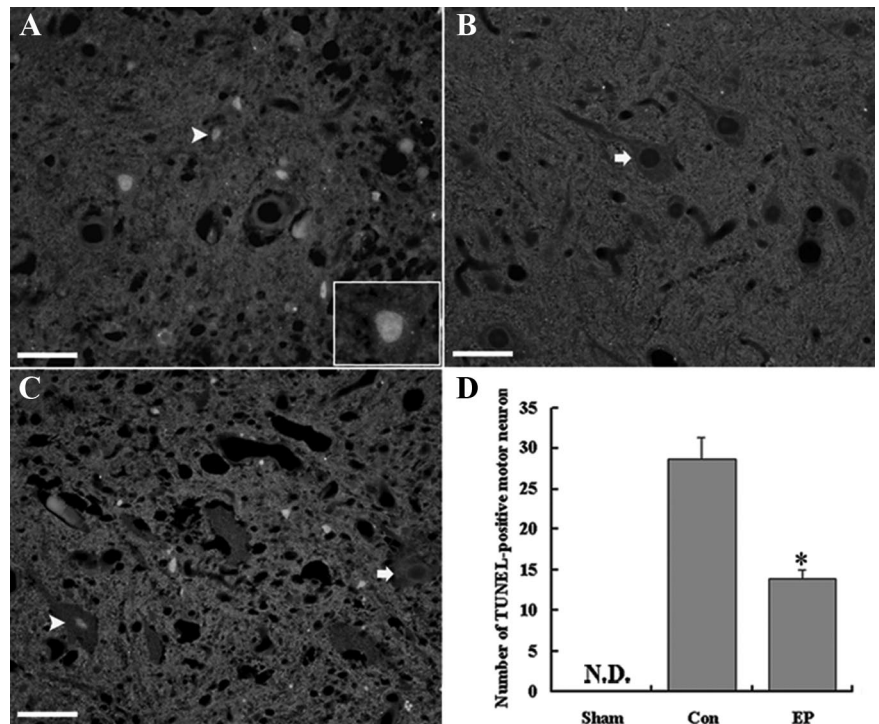


Fig. 2. Representative micrographs of hematoxylin and eosin staining and quantification of normal motor neurons ( $n = 8$ ). (A–C) Representative micrographs of hematoxylin and eosin staining in the ventral horn of spinal cord of L4 segments 72 h after reperfusion (original magnification,  $\times 200$ ). (A) The histopathological findings from an animal with Tarlov score 0 in the control group revealed that there was little normal neuron and karyopyknosis vacuolization around cells in the anterior spinal cord after spinal cord ischemia injury. (B) The histopathological findings from an animal with Tarlov score 4 in the EP group revealed that the more normal neuron had a polygonal structure; on the nucleus structure, there were Nissl bodies in the endochylema. (C) The histopathological findings from an animal with Tarlov score 2 in the EP-6 h group exhibited more motor neurons, but the cellular membrane exhibits no integrity. (D) The bar graph showing the quantitative analysis of the number of normal motor neurons in the anterior horn of spinal cord of L4 segments in five groups. Data are expressed as means  $\pm$  SEM of 8 rats in each group. \*  $P < 0.01$  versus Control group. EP = ethyl pyruvate.

**Fig. 3.** Representative fluorescence micrographs of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining and quantification of apoptotic motor neurons ( $n = 8$ ). (A–C) Representative fluorescence micrographs of TUNEL staining in the ventral horn of spinal cord of L4 segments from animals in the sham, control (Con), and ethyl pyruvate (EP) groups at 72 h after reperfusion, respectively. Few fluorescent motor neurons were visible in the sham group (A). A large number of fluorescent motor neurons were visible in the control group (B), whereas only a small number of fluorescent motor neurons were observed in the EP-treated group (C). The arrow indicates normal motor neurons, and the arrowhead indicates TUNEL-positive motor neurons. Scale bars = 50  $\mu\text{m}$ . (D) Quantitative analysis of the number of TUNEL-positive cells in the anterior horn of spinal cord of L4 segments in three groups. Data are expressed as means  $\pm$  SEM of eight rats in each group. \* $P < 0.01$  versus control group. N.D. = not detected.



reperfusion in figure 2D. The numbers of normal neurons in EP and EP-6 h groups were more than that in control group ( $P = 0.001$  and  $0.001$ , respectively), whereas the numbers of normal neurons in EP-12 h and EP-24h groups were similar to that in control group ( $P = 0.344$  and  $0.874$ , respectively). However, there were no significant differences in the numbers of normal neurons between EP group and EP-6 h group ( $P = 0.833$ ).

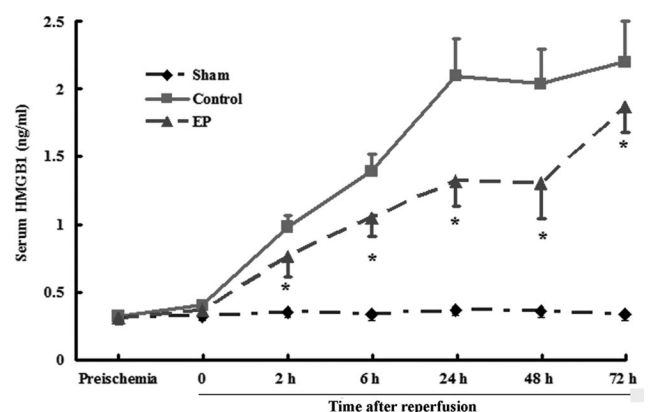
#### Treatment with EP within 6 h after Reperfusion Alleviates Motor Neuron Apoptosis

Photographs of TUNEL staining of the spinal cords are shown in figure 3. TUNEL staining identified no apoptotic cells in the cord sections of the sham-operated animals (fig. 3A). In the spinal cords from the control group, numerous motor neurons were strongly positive for TUNEL staining (fig. 3B). In the samples from the EP group, however, a few of the motor neurons were positive for TUNEL staining (fig. 3C).

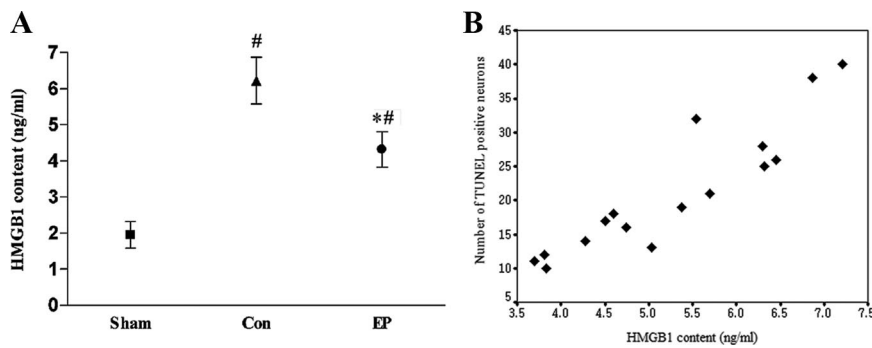
For quantitative measurement, the number of motor neurons that were positive or negative for TUNEL was recorded in each specimen in a blind fashion. Administration of EP at 30 min before ischemia or at 6 h after reperfusion significantly reduced the total number of apoptotic motor neurons compared with that in the control group ( $P = 0.001$  and  $0.001$ , respectively). No significant difference in the total number of apoptotic motor neurons was found between the EP and EP-6 h groups ( $P = 0.628$ ). The number of TUNEL-positive motor neurons at 72 h after reperfusion in the EP-12 h and EP-24h groups was similar to that in C group (fig. 3D).

#### Serum HMGB1 Concentrations

As shown in figure 4, the serum HMGB1 concentrations of the sham animals were unchanged in the period of the experimental procedure. However, the concentration of serum HMGB1 in both control group and EP group significantly increased at 2 h after reperfusion compared with that of preischemia and remained at higher levels thereafter ( $P < 0.05$ ). Serum HMGB1 concentrations of the animals treated with EP were significantly lower than that of control animals from 2 h to 72 h after reperfusion ( $P < 0.05$ ).



**Fig. 4.** Time course of serum high-mobility group box 1 (HMGB1) concentrations ( $n = 8$ ). The serum HMGB1 levels were significantly increased after spinal cord ischemia-reperfusion compared with that of preischemia ( $P < 0.05$ ), whereas they were significantly decreased in the animals treated by ethyl pyruvate (EP) compared with that of control animals ( $P < 0.05$ ). Data are shown as mean  $\pm$  SEM of eight rats in each group. \* $P < 0.05$  versus control group.



**Fig. 5.** Correlations between the high-mobility group box 1 (HMGB1) contents of spinal cord tissue and the numbers of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL)-positive neurons in the anterior spinal cord at 72 h after reperfusion. (A) The HMGB1 contents of spinal cord tissue in the animals from three groups. Data are shown as means  $\pm$  SEM of eight rats in each group. \*  $P < 0.01$  versus control (Con) group, #  $P < 0.01$  versus sham group. (B) The HMGB1 contents of spinal cord tissue correlated well with the numbers of TUNEL-positive motor neurons in

the anterior spinal cord at 72 h after reperfusion ( $n = 16$  pairs,  $r = 0.915$ ,  $P = 0.000$ ). EP = ethyl pyruvate.

### The Contents of HMGB1 in Spinal Cord Tissue at 72 h after Reperfusion

At 72 h after reperfusion, the HMGB1 contents of spinal cord tissue in animals treated with EP were significantly lower than those of control group ( $P = 0.000$ ) (fig. 5A). The HMGB1 contents of spinal cord tissue correlated well with the numbers of apoptotic motor neurons in the anterior spinal cord at 72 h after reperfusion ( $r = 0.915$ ,  $P = 0.000$ , fig. 5B).

## Discussion

There is a wealth of evidence to suggest that the systemic inflammatory response associated with I/R injury contributes to the morbidity and mortality associated with repair of thoracoabdominal aortic aneurysms.<sup>19</sup> Administration of high-dose methylprednisolone, a glucocorticoid steroid, after spinal cord injuries has been considered the only widely accepted pharmacological therapy currently in use in humans. The principal mechanism by which methylprednisolone confers neuroprotection is likely through its ability to inhibit posttraumatic lipid peroxidation and inflammatory responses. Although clinical results were initially promising, there have been growing concerns that the modest neurologic improvements seen with high-dose methylprednisolone treatment in injured patients are not worth the associated risks.<sup>20</sup> Therefore, there is a critical need to develop new pharmacologic therapies for treatment of spinal cord injuries through inhibiting inflammatory responses.

EP has been shown to exert an effective antiinflammatory effect through inhibiting the expression of various proinflammatory mediators as well as circulating levels of HMGB1 in a variety of *in vitro* and *in vivo* models.<sup>21,22</sup> Recently, EP has been reported to provide a neuroprotective effect on cerebral ischemia, in which it was found to be effective when administered as late as 24 h after I/R. The significant neuroprotection was accompanied by the suppression of microglial activation and proinflammatory cytokines. These results suggest that EP has a strong protective effect with a wide therapeutic window related to its antiinflammatory action.<sup>9</sup> In the

current study, we show that EP significantly attenuated spinal cord I/R injury when administered at 30 min before ischemia or at 6 h after the onset of reperfusion. The extended therapeutic window implies that the EP affords neuroprotection *via* a mechanism that modulates the delayed damaging processes in the postischemic spinal cord.

HMGB1 is a nonhistone nuclear protein with dual function. Inside the cells, HMGB1 binds DNA and plays a role in transcriptional regulation. Outside the cell, HMGB1 serves as a late cytokine-like mediator of systemic inflammation.<sup>13</sup> HMGB1 can activate inflammatory pathways when released from ischemic cells. Studies indicate that HMGB1 acts as an early mediator of inflammation and organ damage in hepatic I/R injury. HMGB1 levels were increased during liver I/R as early as 1 h after reperfusion and then increased in a time-dependent manner up to 24 h. Inhibition of HMGB1 activity with neutralizing antibody significantly decreased liver damage after I/R, whereas administration of recombinant HMGB1 worsened I/R injury.<sup>23,24</sup> Moreover, HMGB1 is massively released extracellularly and plays a cytokine-like function in the postischemic brain.<sup>10-12</sup> HMGB1, as a mediator of postischemic brain damage, plays a critical role in the development of brain infarction through the amplification of plural inflammatory responses in the ischemic region and could be an outstandingly suitable target for the treatment.<sup>25</sup> Intravenous injection of neutralizing anti-HMGB1 monoclonal antibody provides a novel therapeutic strategy for ischemic stroke.<sup>26</sup> In addition, serum HMGB1 levels were significantly elevated in patients with myocardial ischemia and cerebral ischemia, suggesting that systemic HMGB1 levels are elevated in human ischemic disease.<sup>27</sup> In this study, serum HMGB1 concentrations were increased during spinal cord I/R as early as 2 h after reperfusion and then increased in a time-dependent manner up to 72 h. Moreover, spinal cord HMGB1 contents were increased at 72 h after reperfusion. Those results indicate that HMGB1 was involved in the proinflammatory stress response to I/R injuries of spinal cord manifested as a time-dependent production of HMGB1 after spinal cord I/R in rabbits.

Obviously, inhibition of HMGB1 secretion or release represents a novel and promising strategy for the therapy of I/R injuries.<sup>25</sup> EP was the first described pharmacological inhibitor for HMGB1 secretion.<sup>5</sup> In the current study, EP attenuated serum HMGB1 levels after spinal cord I/R injury when administered at 30 min before ischemia. To assess the efficacy of treatment with EP, we performed an analysis with the “last observation carried forward” method. The HMGB1 contents of spinal cord tissue in the animals treated with EP at 72 h after reperfusion were found to be significantly lower than those of control ones. In line with this, EP provided a strong protective effect with a wide therapeutic window related to its inhibitory effect on HMGB1 release.

On the other hand, apoptosis has been demonstrated to be an important mode of neuron death in the ischemic spinal cord and to play an important role in delayed paraplegia in the rabbit model of aortic occlusion.<sup>3,28</sup> In this study, apoptotic cells were detected on the basis of positive TUNEL staining, with the fluorescent nucleus in a granular pattern. We used this method because it is a highly sensitive and specific means of identifying DNA fragmentation. As noted, numerous apoptotic motor neurons were observed in the spinal cords of the control animals. The total number of TUNEL-positive motor neurons was reduced significantly after EP treatment. The results showed that EP alleviated neuron apoptosis induced by spinal cord I/R. In line with this, the animals treated with EP had better neurologic outcomes than those in the control ones. Moreover, at 72 h after reperfusion, the HMGB1 levels of spinal cord tissue in animals treated with EP were significantly lower than those of control group. The HMGB1 levels of spinal cord tissue correlated well with the numbers of apoptotic motor neurons in the anterior spinal cord. Together, these results indicate that inhibition of HMGB1 release by EP resulted in less apoptosis and better functional recovery. In accordance with our experimental results, the relationship between apoptosis and HMGB1 release in macrophages and other cells was investigated in *in vitro* studies, and those results indicated that HMGB1 release from macrophages was correlated with the occurrence of apoptosis and suggested that these processes reflect common mechanisms and can occur concomitantly.<sup>29</sup> However, other studies have shown that HMGB1 production was downstream of apoptosis on the final common pathway to organ damage in severe sepsis.<sup>30</sup> Thus, the crosstalks between HMGB1 and apoptosis need to be further explored.

Several limitations of this study merit comment. First, we cannot conclude whether neuronal apoptosis was inhibited or simply delayed by EP intervention during the 72 h recovery interval in our study. On the basis of the data from previous studies, neuronal apoptosis begins as early as 4 h and lasts for a couple of weeks after spinal cord injury.<sup>28</sup> Our results showed that administra-

tion of EP significantly reduced the total number of apoptotic motor neurons at 72 h after reperfusion, which indicated that EP alleviated spinal cord I/R through, at least, delayed neuronal apoptosis. A longer recovery period might be necessary for the assessments of the inhibiting effects of EP on apoptosis. Second, it is not known whether earlier treatment with EP affords a better neuroprotective effect against spinal cord I/R than 6 h after reperfusion or multiple administration (for example, once a day for 3 days). Those issues would be addressed in the future study.

In conclusion, to our best knowledge, our study demonstrates for the first time that HMGB1 release plays an important role in spinal cord I/R damage and is involved in motor neuronal apoptosis. EP affords strong protection against transient spinal cord I/R injury with a wide therapeutic window. This protective effect of EP is related to the inhibition of HMGB1 release induced by spinal cord I/R. These data suggest a new therapeutic possibility for treatment of postischemic injury with EP. Future research should be directed toward developing a better understanding of the cross-talk between HMGB1 and apoptosis, as this ultimately might lead to therapeutic strategies for humans.

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