RESEARCH ARTICLE

Delayed xenon post-conditioning mitigates spinal cord ischemia/reperfusion injury in rabbits by regulating microglial activation and inflammatory factors

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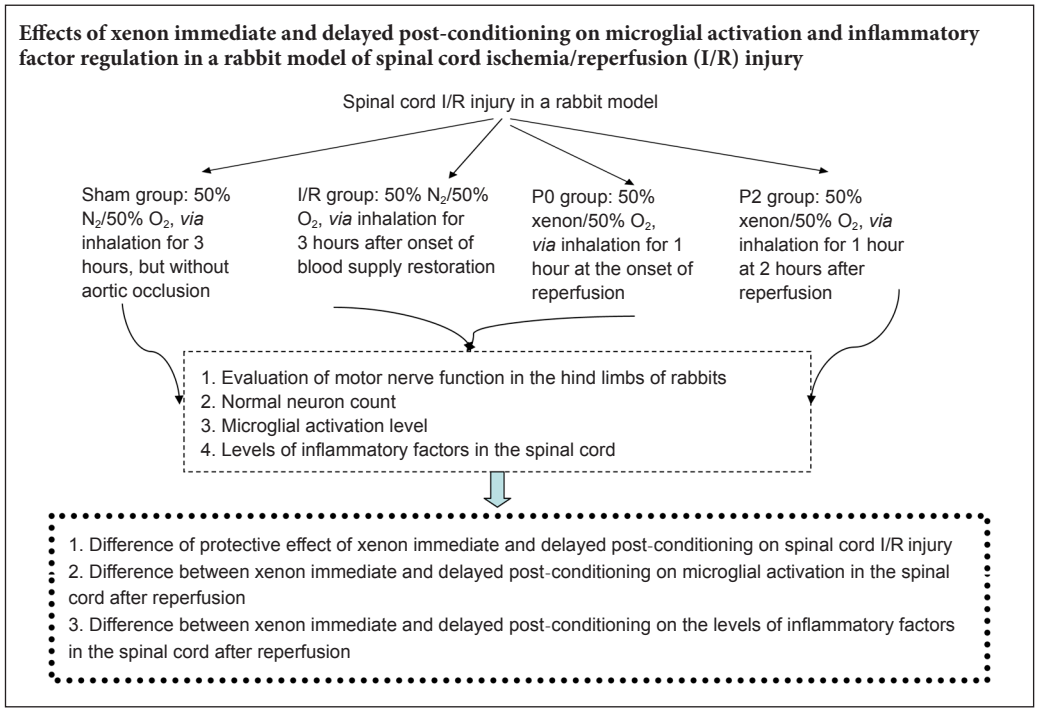
Graphical Abstract



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Abstract

The neuroprotective effect against spinal cord ischemia/reperfusion injury in rats exerted by delayed xenon post-conditioning is stronger than that produced by immediate xenon post-conditioning. However, the mechanisms underlying this process remain unclear. Activated microglia are the main inflammatory cell type in the nervous system. The release of pro-inflammatory factors following microglial activation can lead to spinal cord damage, and inhibition of microglial activation can relieve spinal cord ischemia/reperfusion injury. To investigate how xenon regulates microglial activation and the release of inflammatory factors, a rabbit model of spinal cord ischemia/reperfusion injury was induced by balloon occlusion of the infrarenal aorta. After establishment of the model, two interventions were given: (1) immediate xenon post-conditioning—after reperfusion, inhalation of 50% xenon for 1 hour, 50% N_2/50%O_2 for 2 hours; (2) delayed xenon post-conditioning—after reperfusion, inhalation of 50% N_2/50%O_2 for 2 hours, 50% xenon for 1 hour. At 4, 8, 24, 48 and 72 hours after reperfusion, hindlimb locomotor function was scored using the Jacobs locomotor scale. At 72 hours after reperfusion, interleukin 6 and interleukin 10 levels in the spinal cord of each group were measured using western blot assays. Iba1 levels were determined using immunohistochemistry and a western blot assay. The number of normal neurons at the injury site was quantified using hematoxylin-eosin staining. At 72 hours after reperfusion, delayed xenon post-conditioning remarkably enhanced hindlimb motor function, increased the number of normal neurons at the injury site, decreased Iba1 levels, and inhibited interleukin-6 and interleukin-10 levels in the spinal cord. Immediate xenon post-conditioning did not noticeably affect the above-mentioned indexes. These findings indicate that delayed xenon post-conditioning after spinal cord injury improves the recovery of neurological function by reducing microglial activation and the release of interleukin-6 and interleukin-10.

Key Words: *nerve regeneration; spinal cord injury; xenon; immediate post-conditioning; delayed post-conditioning; ischemia/reperfusion; microglia; interleukin-6; interleukin-10; ionized calcium binding adaptor molecule 1; inflammatory reaction; neural regeneration*

Introduction

Spinal cord ischemia/reperfusion injury (SCIRI) is a major cause of immediate and/or delayed neurological morbidity in people undergoing surgery to treat aortic aneurysm or aortic dissection (Cambria et al., 2002; Tan et al., 2010). Many studies have confirmed that the inflammatory reaction induced by ischemia is a major cause of SCIRI with extensive inflammatory cell infiltration (Savas et al., 2002; Papakostas et al., 2006; Tsuruta et al., 2006; Oz Oyar et al., 2008; Hasturk et al., 2009; Paulson et al., 2013; Temiz et al., 2013). Microglia are important inflammatory cell types that are activated in early stages after SCIRI and are associated with increased inflammatory chemokine release (Tikka et al., 2001; Matsumoto et al., 2003; Olson, 2010). Interleukin-6 (IL-6), a proinflammatory cytokine, is released following SCIRI, and its levels are significantly increased in the days following SCIRI (Smith et al., 2012). Levels of interleukin-10 (IL-10), a potent anti-inflammatory cytokine, are also increased following SCIRI and decrease spinal cord inflammation (Plunkett et al., 2001; Fan et al., 2011).

Studies in many animal models have shown immediate or delayed xenon post-conditioning to be effective at protecting the spinal cord after spinal cord injury (SCI) because it suppresses apoptosis (Yang et al., 2014). Interestingly, the optimal time for administration of 50% xenon to rats was 1 hour after reperfusion and not at the onset of reperfusion. Similarly, Fries et al. (2008, 2009) found that xenon administered 1 hour after resuscitation improved short-term neurocognitive function and histology compared with xenon administered sooner (10 minutes after resuscitation). Furthermore, the protective effects of xenon were most pronounced when administered soon after ischemic insult to the brain, preferably within 8 hours (Ma et al., 2005; David et al., 2008; Peng et al., 2013). Previous studies have focused on the neuroprotective effects of xenon, but have not explored the reasons for its poor neuroprotective effect when used early in the reperfusion period. Therefore, the underlying mechanisms of xenon's effects remain unknown.

Inflammation is an important element in the pathobiology of SCI (David and Kroner, 2011; Karalija et al., 2014; Ren et al., 2016; Shultz and Zhong, 2017). However, whether inflammation plays a harmful or beneficial role following central nervous system injury has yet not to be determined. Different diseases can trigger microglia to become pro- or anti-inflammatory, which can be harmful or protective, respectively (David and Kroner, 2011; Nguyen et al., 2017). Xenon can regulate the inflammatory response and microglial activation in both humans and rats (Clark et al., 2005; Fahlenkamp et al., 2011; Breuer et al., 2015). However, to the best of our knowledge, the effect of xenon on microglial activation and the inflammatory response after spinal cord ischemia reperfusion in rabbits has not been explored.

It is not yet clear whether the length of time between transient ischemia and xenon administration affects microglial activation and/or if microglial polarization influences the pro- or anti-inflammatory effect. This study examined the effects of immediate and delayed xenon post-conditioning on the activation of microglial cells and inflammatory responses after SCI in rabbits.

Materials and Methods

Animals

Twenty-four male New Zealand rabbits aged 8–12 months and weighing 2–2.5 kg were provided by the Experimental Animal Center of Beijing Institute of Heart, Lung and Blood Vessel Diseases, China [license No. SYXK (Jing) 2005-0026]. Rabbits were housed under standard conditions in the Animal Research Laboratory at Capital Medical University and received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

Rabbits were randomly assigned into an ischemia/reperfusion group (I/R, $n = 6$), an ischemia/reperfusion + immediate xenon post-conditioning group (P0, $n = 6$), an ischemia/reperfusion + delayed xenon post-conditioning group (P2, $n = 6$), and a sham operation group (S, $n = 6$).

Spinal cord ischemia model

After fasting for 12 hours, rabbits were anesthetized *via* intravenous injection of urethane (1,000 mg/kg) *via* a 22 G catheter (B. Braun Medical Inc., Bethlehem, PA, USA) inserted into the left ear vein. Heparin was injected at a rate of 6 mg/kg per hour during the surgery. A 24-gauge catheter (B. Braun Medical Inc.) was inserted into the right ear artery for continuous monitoring of mean arterial pressure and arterial blood gases. A 24-gauge catheter was inserted into the left femoral artery to measure mean distal aortic pressure. Mean proximal aortic pressure, mean distal arterial pressure, and temperature were recorded using a Powerlab/8SP Polygraph (AD Instruments, Sydney, Australia). Rabbits were placed under heat lamps to maintain body temperature at 38.5°C until recovered from the anesthesia. With rabbits placed in a supine position, spinal cord ischemia was induced by imbedding a 2F balloon catheter (Edwards Life sciences, Irvine, CA, USA), *via* the right femoral artery (Watanabe et al., 2005). The end of the catheter was located 0.5–1 cm below the renal artery (approximately 11 cm caudal to the insertion point). The aortic occlusion was confirmed by an immediate and sustained loss of detectable pulse pressure and a decrease in mean distal arterial pressure. Each rabbit received an injection of 200 U protamine and then the catheter was removed and blood flow was restored to the spinal cord. The balloon was deflated after the artery was occluded for 22 minutes. The surgical incisions were then sutured and the rabbits were monitored in their cages until full recovery. A clamping time of 22 minutes was chosen based on previous clamping tests (15–25 minutes). Rabbits in the S group underwent the same surgical procedure as described above, but without aortic catheter occlusion. Arterial blood samples were taken from the left femoral artery at pre-ischemia, 5 minutes after aortic clamping and 15 minutes after reperfusion. Arterial blood gases were detected by a blood gas and pH analyzer (Ciba-Corning Diagnostics, East Walpole, MA, USA).

Rabbits of all groups were euthanized 72 hours after reperfusion for histological examination. Neurological as-

sessments were performed in all rabbits at 4, 8, 24, 48 and 72 hours after reperfusion.

Experimental protocols

Rabbits were randomly divided into four groups (n = 6 rabbits/group) as follows. The I/R group underwent 22 minutes of ischemia followed by inhalation of 50 vol% nitrogen, 50 vol% O_2 for 3 hours after onset of reperfusion. The P0 group underwent 22 minutes of ischemia followed by a xenon post-conditioning protocol in which rabbits inhaled 50 vol% xenon, 50 vol% O_2 for 1 hour at the onset of reperfusion then 50 vol% nitrogen, 50 vol% O_2 (xenon concentration monitor, Empaer Technology Company, Shenzhen, China) for 2 hours, beginning 1 hour after onset of reperfusion. The P2 group underwent 22 minutes of ischemia followed by a xenon post-conditioning protocol in which they inhaled 50 vol% nitrogen, 50 vol% O_2 for 2 hours at the beginning of reperfusion and then a 50 vol% xenon, 50 vol% O_2 for 1 hour beginning 2 hours after the onset of reperfusion. Rabbits in the S group did not undergo aortic occlusion, but inhaled 50 vol% nitrogen and 50 vol% O_2 for 3 hours.

Assessment of neurological function

Hindlimb locomotor function was scored using the Jacobs locomotor scale (Jacobs et al., 1987), which ranged from 0 (no detectable hindlimb movement) to 5 (normal hindlimb locomotion). Jacobs scores were recorded at 4, 8, 24, 48 and 72 hours after reperfusion by two people who were blind to the group identity of the animals.

Sample collection

Rabbits were euthanized *via* intraperitoneal injection of 3% (30–50 mg/kg) sodium pentobarbital (Sigma-Aldrich Shanghai Trading, Shanghai, China) 72 hours after reperfusion and after a final neurological functional assessment. Lumbar (L_{3-5}) spinal cord segments were rapidly removed after euthanasia. A portion of spinal cord was post-fixed in 4% (w/v) paraformaldehyde at 4°C for 3 days and serial sections (5 μm thick) were then cut for hematoxylin-eosin staining and immunohistochemistry. The remaining spinal cord segments were stored at −80°C for subsequent western blot assays.

Hematoxylin-eosin staining and neuron counts

Spinal cord segments were fixed in 4% paraformaldehyde at 4°C and then transferred to 30% sucrose for 3 days at 4°C. The samples were then frozen and 5-μm sections cut with a microtome (Leica). Sections were then stained with hematoxylin-eosin (Roche Diagnostics, Mannheim, Germany) and neuropathological changes were examined by two pathologists who were blind to the group identity of the animals. The total number of normal motor neurons in half of the anterior horn of each section was counted in five random fields (× 200) using a light microscope (Olympus BX60, Tokyo, Japan).

Immunohistochemistry

After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 minutes. Sections were permeabilized with 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 20 minutes. Non-specific adsorption was minimized by incubating sections in 2% (v/v) normal goat serum in PBS for 20 minutes. To reduce non-specific staining, endogenous biotin-binding or avidin-binding sites were blocked by sequential incubation for 15 minutes with biotin and avidin. Sections were then incubated overnight at 4°C with anti-ionized calcium binding adaptor molecule 1 (Iba1) polyclonal antibody (rabbit McAb, 1:500; Abcam, Cambridge, UK). After washing in PBS (3 × 5 minutes), sections were incubated with a biotinylated sheep anti-rabbit secondary antibody at 37°C for 30 minutes (1:500; Abcam) and then with streptavidin-biotin complex. Reactions were developed with diaminobenzidine and sections counterstained with hematoxylin. Images were analyzed using NIS-ELEMENTS (Nikon, Tokyo, Japan) with ImagePro Plus 3.0 software (ECLIPSE80i 90i; Nikon).

Western blot assay

Seventy-two hours after reperfusion, spinal cord samples were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, and 0.1% sodium dodecyl sulfate with phenylmethyl sulfonylfluoride]. Forty milligrams of protein per lane were resolved by 10% sodium dodecyl sulfate-polyacrylamide electrophoresis and then transferred to polyvinylidene difluoride membranes. Membranes were then blocked with 5% nonfat milk and incubated with primary antibodies targeting Iba1, IL6 and IL10 (Rabbit McAb, 1:1,000; Abcam) and GAPDH (1:1,000; Beijing Biosynthesis Biotechnology, Beijing, China) at 4°C overnight. Membranes were then washed and incubated with appropriate secondary antibodies (sheep anti-rabbit or sheep anti-mouse 1:10,000; Beijing Biosynthesis Biotechnology) at room temperature for 1 hour. The optical density of each band was quantified using Image J software (Scion Corporation, San Francisco, CA, USA) and normalized to that of GAPDH.

Statistical analysis

All data, presented as the mean ± SEM, were analyzed using SPSS 20.0 software (IBM, Armonk, NY, USA). All data were tested using one-way analysis of variance followed by Tukey's *post hoc* test. A value of $P < 0.05$ was considered statistically significant.

Results

General condition of rabbits

Blood gases, temperature, and proximal and distal blood pressure were stable and not significantly different among the four groups at any of the three time points (before, during, or after ischemia) (**Table 1**).

Effects of delayed xenon post-conditioning on neurological function in rabbits with SCIRI

Functional data were collected according to the Jacobs locomotor scale at 4, 8, 24, 48 and 72 hours after reperfusion (**Table 2**). Rabbits in the I/R, P0 and P2 groups had significant motor dysfunction following SCI at 4, 8, 24, 48 and 72 hours after reperfusion. However, rabbits in the S group

Table 1 Physiological parameters before, during and after spinal cord ischemia and reperfusion

Variable	I/R	P0	P2	S
Rectal temperature (°C)				
Preischemia	37.8±0.5	37.6±0.3	37.7±0.3	37.4±0.6
5 minutes after ischemia	37.6±0.4	37.8±0.5	37.5±0.4	37.8±0.5
15 minutes after reperfusion	37.6±0.6	37.6±0.4	37.6±0.5	37.8±0.5
Distal MAP (mmHg)				
Preischemia	90.00±17.30	83.83±5.56	80.00±15.52	81.83±3.06
5 minutes after ischemia	10.00±2.00†	10.67±1.86†	8.33±3.38†	82.83±3.66
15 minutes after reperfusion	88.50±14.30	82.83±3.92	77.00±14.04	80.50±5.28
pH				
10 minutes before ischemia	7.37±0.02	7.40±0.05	7.36±0.05	7.42±0.03
15 minutes after reperfusion	7.39±0.09	7.41±0.04	7.37±0.02	7.42±0.05
PaO_2 (kPa)				
10 minutes before ischemia	92±4	89±9	93±6	96±3
15 minutes after reperfusion	93±2	92±1	92±4	92±6
$PaCO_2$ (kPa)				
10 minutes before ischemia	32±3	31±7	36±3	35±2
15 minutes after reperfusion	34±2	30±6	32±6	32±4

Data are expressed as the mean ± SEM (n = 6, one-way analysis of variance followed by Tukey's *post hoc* test). †P < 0.05, *vs.* preischemia. Arterial blood gases and pH were measured 10 minutes before ischemia and 15 minutes after reperfusion. The I/R group was given 50% N_2/50% O_2, *via* inhalation for 3 hours at the onset of blood supply restoration. The P0 and P2 groups were given 50% xenon/50% O_2, *via* inhalation for 1 hour at the onset of reperfusion or 2 hours after reperfusion, respectively. The S group was given 50% N_2/50% O_2, *via* inhalation for 3 hours, as above, but without aortic occlusion. S: Sham operation group; I/R: ischemia/reperfusion group; P0: ischemia reperfusion + immediate xenon post-conditioning group; P2: ischemia reperfusion + delayed xenon post-conditioning group. I/R: Ischemia/reperfusion.

maintained normal locomotor function. The Jacobs score was significantly higher in the P2 group compared with the P0 and I/R groups 72 hours after reperfusion. However, there was no significant difference in Jacobs score between the P0 and I/R groups at 4, 8, 24, 48 and 72 hours after reperfusion.

Effects of delayed xenon post-conditioning on the number of normal neurons at the injury site of rabbits with SCIRI

At 72 hours after reperfusion, spinal cord tissues and neurons were normal and well maintained in the S group as shown by histological examination and hematoxylin-eosin staining. However, neurons from rabbits in the I/R, P0 and P2 groups were morphologically damaged to different degrees and fewer normal neurons were found in the I/R, P0 and P2 groups compared with the S group. The P2 group had more normal neurons and fewer damaged neurons compared with the I/R and P0 groups (**Figure 1**).

Effects of delayed xenon post-conditioning on IL-6 and IL-10 protein levels and Iba1 immunoreactivity at the injury site of rabbits with SCIRI

IL-6 and IL-10 levels were detected in all groups by western blot assays. IL-6 and IL-10 levels were lowest in the S group and highest in the P0 group 72 hours after reperfusion (P < 0.05; **Figure 2**). However, IL-6 and IL-10 levels were significantly lower in the P2 group compared with the I/R group after reperfusion (P < 0.05).

Iba1 immunoreactivity was detected in all groups by western blot assay. Iba1 immunoreactivity was lowest in the S group and highest in the P0 group 72 hours after reperfusion (P < 0.05; **Figure 3**). However, Iba1 immunoreactivity was significantly lower in the P2 group compared with the I/R group after reperfusion (P < 0.05).

Table 2 Hindlimb locomotor function changes at 4, 8, 24, 48, and 72 hours after reperfusion

Group	Time after reperfusion (hours)				
	4	8	24	48	72
S	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0	5.0±0.0
I/R	2.6±0.2*	2.5±0.2*	1.5 ±0.4*&	1.3±0.3*&	0.8±0.3*&
P0	2.5±0.2*	2.4±0.2*	1.7±0.3*	1.3±0.3*&	1±0.3*
P2	3±0.3*#	3.2±0.2*#	2.7 ±0.4*#	2.7 ±0.4*#	2.5 ±0.4*#

Data are expressed as the mean ± SEM (n = 6, one-way analysis of variance followed by Tukey's *post hoc* test). *P < 0.05, *vs.* S group; &P < 0.05, *vs.* 4 hours after reperfusion in each group; #P < 0.05, *vs.* I/R group. The Jacobs scale ranged from 0 (no detectable hindlimb movement) to 5 (normal hindlimb locomotion). The I/R group was given 50% N_2/50% O_2, *via* inhalation for 3 hours at the onset of blood supply restoration. The P0 and P2 groups were given 50% xenon/50% O_2, *via* inhalation for 1 hour at the onset of reperfusion or 2 hours after reperfusion, respectively. The S group was given 50% N_2/50% O_2 *via* inhalation for 3 hours, as above, but without aortic occlusion. S: Sham operation group; I/R: ischemia/reperfusion group; P0: ischemia reperfusion + immediate xenon post-conditioning group; P2: ischemia reperfusion + delayed xenon post-conditioning group. I/R: Ischemia/reperfusion.

Discussion

To the best of our knowledge, this is the first study to examine the effects of immediate and delayed xenon post-conditioning on the release of inflammatory factors and microg-

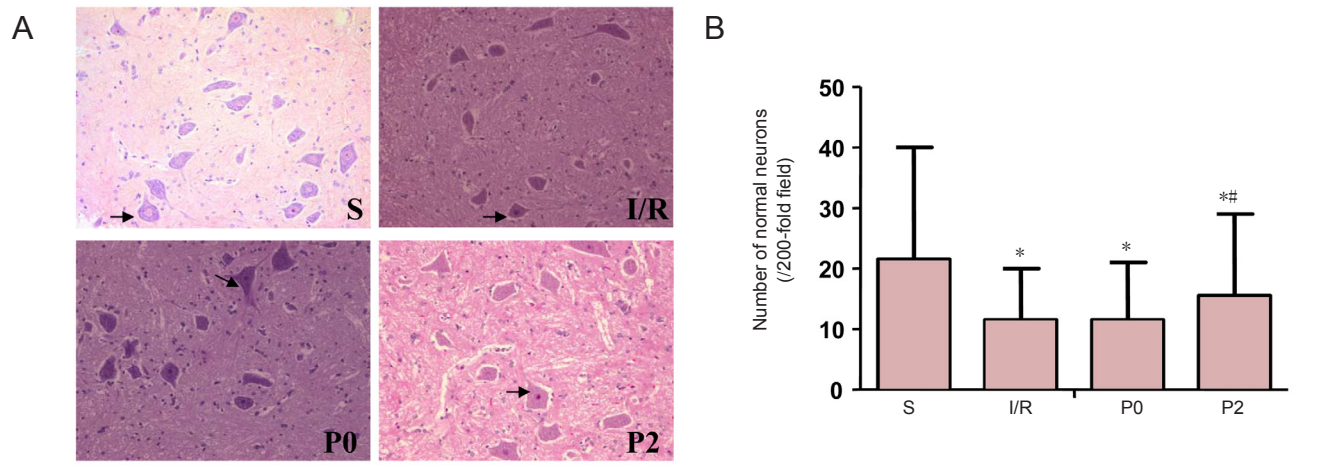

Figure 1 Effects of delayed xenon post-conditioning on the number of normal neurons at the injury site.
(A) Hematoxylin-eosin staining of spinal cord sections from rabbits in the S, I/R P0 and P2 groups at 72 hours after reperfusion (original magnification, 200×). Black arrows indicate normal neuronal cells. (B) Number of normal neurons in the anterior horn of the spinal cord. ∗$P < 0.05$, *vs.* S group; #$P < 0.05$, *vs.* I/R group. Data are expressed as the mean ± SEM ($n = 6$, one-way analysis of variance followed by Tukey's *post hoc* test). The I/R group was given 50% N_2/50% O_2, *via* inhalation for 3 hours at the onset of blood supply restoration. The P0 and P2 groups were given 50% xenon/50% O_2 *via* inhalation for 1 hour at the onset of reperfusion or 2 hours after reperfusion, respectively. The S group was given 50% N_2/50% O_2 *via* inhalation for 3 hours, as above, but without aortic occlusion. S: Sham operation group; I/R: ischemia/reperfusion group; P0: ischemia reperfusion + immediate xenon post-conditioning group; P2: ischemia reperfusion + delayed xenon post-conditioning group. I/R: Ischemia/reperfusion.

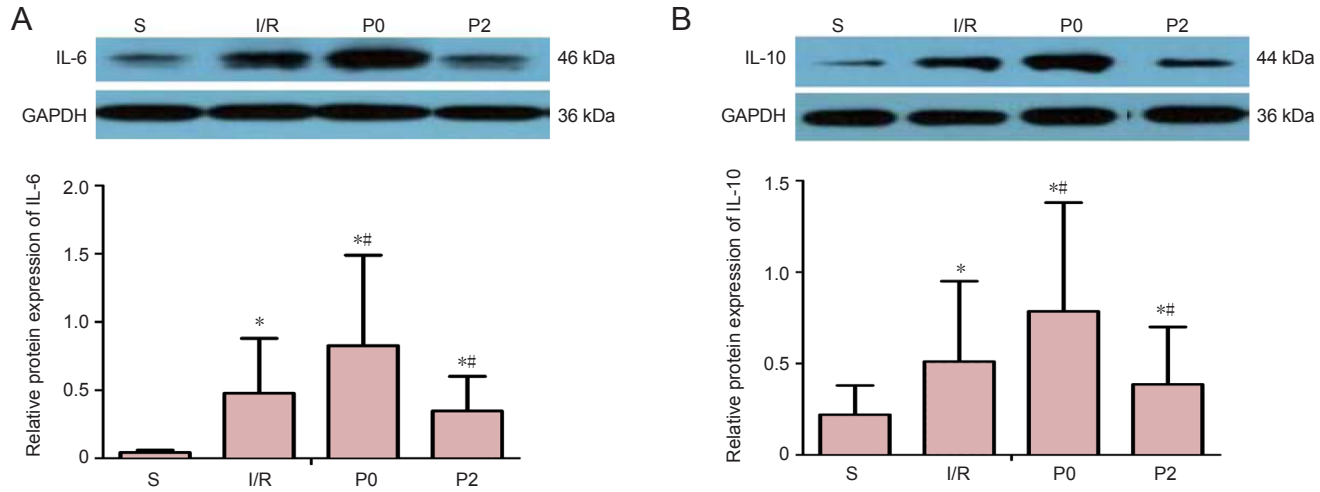

Figure 2 Effect of delayed xenon post-conditioning on IL-6 and IL-10 protein levels in rabbits with spinal cord I/R injury 72 hours after reperfusion.
(A) IL-6 levels 72 hours after reperfusion. (B) IL-10 levels 72 hours after reperfusion. Protein levels were normalized to GAPDH as the internal reference. Data are expressed as the mean ± SEM ($n = 6$, one-way analysis of variance followed by Tukey's *post hoc* test). ∗$P < 0.05$, *vs.* S group; #$P < 0.05$, *vs.* I/R group. The I/R group was given 50% N_2/50% O_2, *via* inhalation for 3 hours at the onset of blood supply restoration. The P0 and P2 groups were given 50% xenon/50% O_2, *via* inhalation for 1 hour at the onset of reperfusion or 2 hours after reperfusion, respectively. The S group was given 50% N_2/50% O_2 *via* inhalation for 3 hours, as above, but without aortic occlusion. S: Sham operation group; I/R: ischemia/reperfusion group; P0: ischemia reperfusion + immediate xenon post-conditioning group; P2: ischemia reperfusion + delayed xenon post-conditioning group. I/R: Ischemia/reperfusion; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IL: interleukin.

lial activation in a rabbit model of SCI. The major findings of this study are summarized as follows. (1) Delayed xenon post-conditioning significantly improved neurological function in rabbits and attenuated the microglia-mediated inflammatory response. (2) Immediate xenon post-conditioning amplified the microglia-mediated inflammatory response.

The neuroprotective effects exerted by xenon post-conditioning in animal models of cerebral injury are different according to the timing of administration; the optimal time was delayed rather than immediate administration (David et al., 2003, 2008, 2010; Ma et al., 2005; Dingley et al., 2006; Fries et al., 2008, 2009; Hobbs et al., 2008; Thoresen et al., 2009; Britton et al., 2010; Sheng et al., 2012; Yang et al., 2012; Zhuang et al., 2012; Peng et al., 2013; Metaxa et al., 2014). In the current study, delayed xenon administration following SCI had a neuroprotective effect, which is consistent with our previous study (Yang et al., 2014). Interesting-

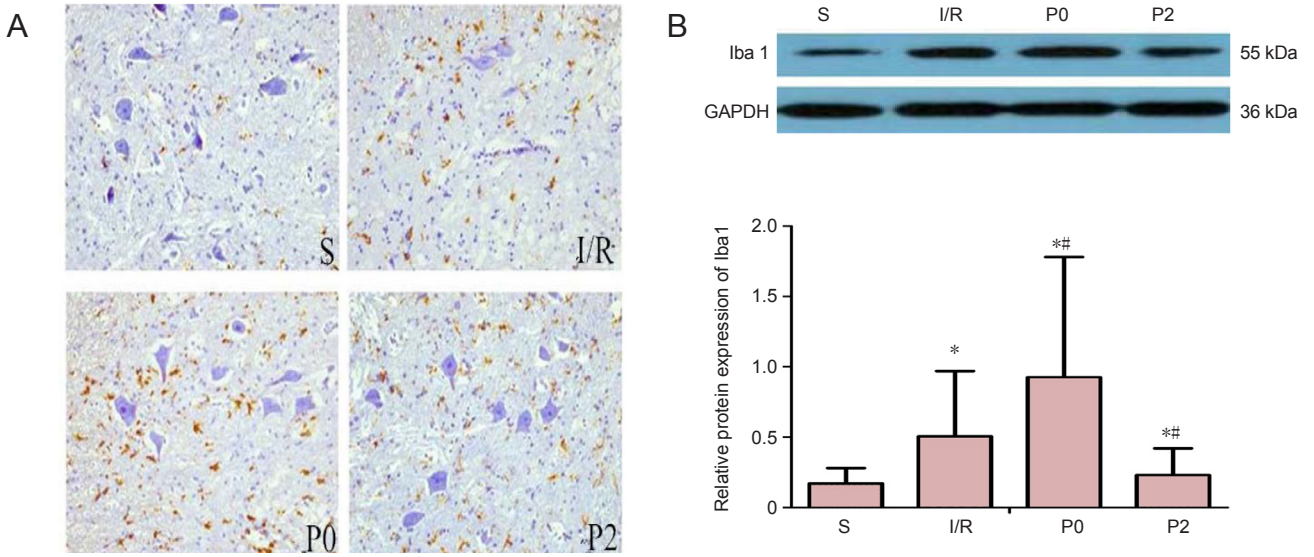

Figure 3 Effect of delayed xenon post-conditioning on microglial activation in rabbits with spinal cord I/R injury.
(A) Immunohistochemical staining of Iba1 in rabbits 72 hours after reperfusion in the S, I/R, P0 and P2 groups (original magnification, 200×). Iba1-immunoreactive cells were damaged. (B) Western blot assay of Iba1 in spinal cord sections at 72 hours after reperfusion. Protein levels (gray values) were normalized to GAPDH as the internal reference. Data are expressed as the mean ± SEM (n = 6, one-way analysis of variance followed by Tukey's *post hoc* test). ∗P < 0.05, *vs*. S group; #P < 0.05, *vs*. I/R group. The I/R group was given 50% N_2/50% O_2, *via* inhalation for 3 hours at the onset of blood supply restoration. The P0 and P2 groups were given 50% xenon/50% O_2 *via* inhalation for 1 hour at the onset of reperfusion or 2 hours after reperfusion, respectively. I/R group. The S group was given 50% N_2/50% O_2 *via* inhalation for 3 hours, as above, but without aortic occlusion. S: Sham operation group; I/R: ischemia/reperfusion group; P0: ischemia reperfusion + immediate xenon post-conditioning group; P2: ischemia reperfusion + delayed xenon post-conditioning group. I/R: Ischemia/reperfusion; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Iba1: ionized calcium binding adaptor molecule 1.

ly, no neuroprotective effect was observed with immediate xenon post-conditioning and the underlying mechanism for this phenomenon remains unknown.

The microglia-mediated inflammatory response was stronger in the P0 group than in the other groups and had no neuroprotective effect. Previous studies have shown that pro-inflammatory cytokines, such as tumor necrosis factor and IL6, are the major cause of secondary pathological injury following spinal cord ischemia (Nesic et al., 2001; Pineau and Lacroix, 2007; Jia et al., 2012). In addition, tumor necrosis factor and IL1 are also implicated in microglial activation (Bartholdi and Schwab, 1997; Kwon et al., 2004; Wang et al., 2005). However, exactly how xenon post-conditioning regulates the secondary inflammatory response after reperfusion in SCI is unclear and only a few studies have explored the relationship between xenon and the inflammatory response. de Rossi et al. (2004) demonstrated anti-inflammatory properties of xenon. However, Saravanan et al. (2009) found that xenon aggravated the inflammatory response by activating leucocytes and platelets. It is well known that necrosis and apoptosis can coexist in SCI (Karin and Ben-Neriah, 2000). The inflammatory profiles induced by immediate and delayed xenon post-conditioning were examined in the current study. IL-6 and IL-10 levels were significantly increased in the P0 group compared with the P2 and I/R groups. A previous study had reported a pro-inflammatory effect of xenon in patients receiving cardiac surgery (Breuer et al., 2015); thus, it is possible that the significantly increased inflammatory response in the P0 group may have increased necrosis of spinal cord cells, resulting in the production of large amounts of necrotic cell debris. Therefore, aggravated microglia-mediated inflammatory responses may have resulted in ineffective neuroprotection.

Because there is a relationship between the inflammatory response and microglia, the activation of microglia was also investigated in this study. Levels of Iba1 towards the spinal cord tended to be higher in the P0 group compared to the P2 and I/R groups. Microglia respond within minutes after SCI and activated microglia produce high levels of pro-inflammatory cytokines. However, Yong and Rivest (2009) reasoned that this may be attributed to the beneficial effect of activated microglia in protecting neurons. Microglia are the first cells to respond to injury in the spinal cord and central nervous system and produce inflammatory cytokines (Probert et al., 2000; Ma et al., 2005). In view of these different inflammatory responses, the number of activated microglia was also examined in the current study. The number of activated microglia tended to be higher in the P0 group compared with the P2 and I/R groups. Therefore, the higher number of activated microglia in the P0 group significantly affected the essential xenon pro-inflammatory mechanisms after spinal cord ischemia injury.

The polarization of microglia may underlie different functional properties of microglia. IL6 is up-regulated by microglia and macrophages during the early stage of SCI (Ma et al., 2005; Longbrake et al., 2007). A recent study characterized macrophage/microglial polarization in a mouse SCI model and reported that most activated microglia were type M1 and not type M2 (Kigerl et al., 2009). It is thought that the low number of anti-inflammatory M2 macrophages

may aggravate the pro-inflammatory effect after SCI. In addition, we recently found that M1- and M2-type cells were rapidly up-regulated by immediate xenon post-conditioning in a cultured microglia oxygen-glucose deprivation model, whereas only the M2 markers were up-regulated by 2-hour delayed xenon post-conditioning. Therefore, we speculate that xenon may affect the spinal cord environment after ischemia, which affects the timing of polarization of different subsets of microglia.

This study has several limitations. First, it was performed in a rabbit model of spinal cord ischemia and needs to be validated in other animal models. Second, the analysis only focused on microglia and did not assess astrocytes, which are probably involved in the systemic inflammatory response. Due to the limited number of animals, only a few important cytokines could be detected. Other inflammatory cytokines should be examined in *in vivo* and *in vitro* studies. Third, the polarization of microglia lasted for a long time after SCI. The effects of xenon on inflammatory responses and microglial polarization should be evaluated over a longer period.

In summary, delayed xenon post-conditioning provided better protective effects against SCI in the rabbit model *in vivo* compared with immediate xenon post-conditioning. This was likely due to suppression of microglial activation. Immediate xenon post-conditioning triggered microglial activation and amplified both pro- and anti-inflammatory responses, which could offset neuroprotective effects when used early in the reperfusion period. Microglial polarization may be modulated by xenon depending on the timing of administration after reperfusion. The authors will extend their study to determine which subsets of microglia are affected and whether astrocytes are affected.

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