Isoflurane Produces Delayed Preconditioning against Spinal Cord Ischemic Injury via Release of Free Radicals in Rabbits

Hanfei Sang, M.D., Ph.D.,* Lin Cao, M.D.,† Pengxin Qiu, M.D.,‡ Lize Xiong, M.D., Ph.D.,§ Rongrong Wang, M.D.,† Guangmei Yan, M.D., Ph.D.||

Background: Whether isoflurane preconditioning produces delayed neuroprotection in the spinal cord is unclear. The authors tested the hypothesis that isoflurane produces delayed preconditioning against spinal cord ischemic injury and, further, that the beneficial effect is dependent on free radicals.

Methods: In experiment 1, 63 rabbits were randomly assigned to seven groups (n = 9 each): Animals in the control group only underwent spinal cord ischemia without pretreatment; animals in the Iso24h, Iso48h, and Iso72h groups received 40 min of 1.0% minimum alveolar concentration isoflurane in 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia; animals in the O224h, O248h, and O272h groups received 40 min of 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia. In experiment 2, 48 rabbits were randomly assigned into four groups (n = 12 each): Animals in the O2 and Iso groups received 3 ml/kg saline intraperitoneally 1 h before each session of oxygen pretreatment and isoflurane pretreatment, respectively. In the DMTU+Iso and DMTU+O2 groups, 10% dimethylthiourea (DMTU, a potent free radical scavenger) dissolved in saline (3 ml/kg) was administered at the same time point. Twenty-four hours after the last pretreatment, animals were subjected to spinal cord ischemia. Spinal cord ischemia was induced by an infrarenal aorta clamping for 20 min. Forty-eight hours after reperfusion, neurologic function and histopathology of the spinal cord were examined.

Results: In experiment 1, the neurologic and histopathologic outcomes in the Iso24h and Iso48h groups were better than those in the control group (P < 0.005 for each comparison); the neurologic and histopathologic outcomes in the control group showed no significant differences in comparison with the O224h, O248h, O272h, and Iso72h groups (P > 0.05 for each comparison). In experiment 2, the neurologic and histopathologic outcomes in the Iso group were better than those in the DMTU+Iso, O2, and DMTU+O2 groups (P < 0.01 for each comparison); there were no significant differences in the neurologic and histopathologic outcomes among the DMTU+Iso, O2, and DMTU+O2 groups (P > 0.05 for each comparison).

Conclusions: Isoflurane produces delayed preconditioning against spinal cord ischemic injury, and the beneficial effect may be dependent on the release of free radicals.

Operations that require aortic occlusion result in ischemia to the distal organs. The most vulnerable of these organs is the spinal cord. Neurologic injury is a result of aortic occlusion in the absence of adequate collateral flow and increases with the duration of occlusion. The reported prevalence rate of neurologic injury (paraplegia and paraparesis) from such operations ranges from 5% to 15%. Spinal cord ischemic injury is even more frequent among patients who need extent II thoracoabdominal aortic aneurysm repair, with paraplegia rates ranging from 8% to 32%. Optimal protection of the spinal cord from ischemia–reperfusion injury is the cornerstone for success with thoracoabdominal aortic surgery. Therefore, different operative and nonoperative strategies have been developed to increase spinal cord tolerance to ischemia and minimize the incidence of neurologic complications after aortic surgery.

Ischemic preconditioning is a phenomenon whereby a brief period of nonlethal ischemia increases the tolerance of the tissue to a subsequent lethal ischemia. Two phases of ischemic preconditioning have been described: The early phase is observed within minutes and disappears approximately 2–3 h later, and the delayed phase develops hours after the preconditioning stimulus and lasts for several days. Although these two types of ischemic preconditioning have been documented for spinal cord protection after aortic occlusion, its safety margins and potential for eliciting injury remains controversial in the clinical setting. Therefore, it is still important to search for effective pharmacologic preconditioning with greater safety margins and established potential for clinical implementation.

Isoflurane is a clinically widely used volatile anesthetic. Multiple studies have shown that preconditioning with isoflurane mimicked the protective effects of early and delayed ischemic preconditioning in the heart and brain. A recent investigation provided evidence that isoflurane produced early preconditioning against spinal cord ischemic injury. However, whether isoflurane produces delayed preconditioning in the spinal cord remains unclear.

Although the precise mechanism of isoflurane-induced preconditioning is not fully elucidated, adenosine triphosphate–regulated potassium (K\textsubscript{ATP}) channels have been implicated in delayed preconditioning with isoflu-
rane in the heart and brain. The mitochondrial K\textsubscript{ATP} channels were initially thought to be the end effector of ischemic preconditioning, but evidence suggested that the opening of this channel may actually trigger the ischemic preconditioning by generating free radicals.

In myocardium, studies also indicated that isoflurane-induced preconditioning depended on the release of free radicals and suggested mitochondrial K\textsubscript{ATP} channel opening acted as a trigger for isoflurane-induced preconditioning by generating free radicals. Recently, a study also suggested that mitochondrial K\textsubscript{ATP} channels involved in isoflurane-induced preconditioning against spinal cord ischemic injury. In addition, free radicals have been reported to contribute to ischemic tolerance in the brain and spinal cord. Therefore, we hypothesize that free radical are also critically important for isoflurane-induced preconditioning in the spinal cord.

The current study was undertaken to determine whether isoflurane produces delayed preconditioning against spinal cord ischemic injury and, if so, whether release of free radicals is involved in the process.

Materials and Methods

The experimental protocol used in this study was approved by the Ethics Committee for Animal Experimentation and was conducted according to the Guidelines for Animal Experimentation of Sun Yat-Sen University (Guangzhou, China). The animals were studied at Zhongshan Medical College of Sun Yat-Sen University (Guangzhou, Guangdong, China).

Experimental Protocol

All experiments were performed on male New Zealand White rabbits weighing 2.2–2.5 kg. This study consisted of two experiments. Experiment 1 was designed to determine whether delayed preconditioning with isoflurane could induce ischemic tolerance in the spinal cord. Experiment 2 was undertaken to elucidate whether release of free radicals could be involved in the delayed preconditioning with isoflurane against spinal cord ischemic injury.

Experiment 1. The design of experiment 1 is illustrated in figure 1. A total of 63 male New Zealand White rabbits were randomly assigned to seven groups (n = 9 in each). The animals in the Iso24h, Iso48h, and Iso72h groups received 40 min of 1.0 minimum alveolar concentration (MAC) isoflurane (2.1%) in 100% oxygen during spontaneous ventilation each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia. The animals in the control group only underwent spinal cord ischemia without pretreatment. Each rabbit was then allowed to recover in room air (21% oxygen) and housed until the acute experiments. In five additional rabbits, middle ear arterial blood pressure, arterial oxygen tension (PaO\textsubscript{2}), arterial carbon dioxide tension (PaCO\textsubscript{2}), and pH were measured through an arterial catheter placed in carotid artery at the onset and end of 40 min of exposure to 2.1% isoflurane.

Experiment 2. The design of experiment 2 is illustrated in figure 2. A total of 48 male New Zealand White rabbits were randomly assigned to four groups (n = 12 each): the O\textsubscript{2}4h, O\textsubscript{2}48h, and O\textsubscript{2}72h groups received 40 min of 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia. During preconditioning, the rabbits were placed on a temperature-controlled heating pad for maintenance of rectal temperature at approximately 38°–38.5°C. The animals in the control group only underwent spinal cord ischemia without pretreatment. Each rabbit was then allowed to recover in room air (21% oxygen) and housed until the acute experiments. Five additional rabbits, middle ear arterial blood pressure, arterial oxygen tension (PaO\textsubscript{2}), arterial carbon dioxide tension (PaCO\textsubscript{2}), and pH were measured through an arterial catheter placed in carotid artery at the onset and end of 40 min of exposure to 2.1% isoflurane.
Experiment two

<table>
<thead>
<tr>
<th></th>
<th>saline once/day for 5 days</th>
<th>1h</th>
<th>24h</th>
<th>100% oxygen once/day for 5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>O2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iso</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMTU+Iso</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMTU+O2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Schematic illustration of protocol for experiment 2. O2 and Iso: animals received saline intraperitoneally 1 h before each session of oxygen pretreatment and isoflurane pretreatment, respectively; DMTU+Iso and DMTU+O2: animals received the same volume of dimethylthiourea (DMTU, a free radical scavenger) intraperitoneally 1 h before each session of oxygen pretreatment and isoflurane pretreatment, respectively. All animals were subjected to spinal cord ischemia 24 h after the last pretreatment. MAC = minimum alveolar concentration.

Animal and Surgical Preparation

After an overnight fast with unrestricted access to water, the rabbits were anesthetized with pentobarbital sodium (30 mg/kg intravenously). Lactated Ringer's solution (4 ml · kg⁻¹ · h⁻¹) was infused intravenously. A 22-gauge catheter was inserted into the ear artery to measure proximal blood pressure. Another catheter was inserted to the left femoral artery to measure distal blood pressure. Blood pressure was monitored continuously by using a calibrated pressure transducer connected to an invasive pressure monitor (Spacelabs Med. Inc., Redmond, WA). Rectal temperature was maintained between 38° and 39°C by an overhead lamp during the experiments. Arterial blood was sampled at preischemic state, 10 min after ischemia, and 10 min after reperfusion for the determination of $\text{PaO}_2$, $\text{Paco}_2$, pH, and plasma glucose. Arterial blood gases were measured by means of the OMNI Modular System (AVL List GmbH Medizintechnik, Graz, Austria).

Spinal Cord Ischemia

The induction of spinal cord ischemia was performed as previously described by Johnson et al.²⁶ 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 U 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. The local infiltration around the wound with 0.25% bupivacaine hydrochloride was applied for postoperative analgesia. An antibiotic (40,000 U gentamicin) was administered intramuscularly immediately after the operation. The animals were then returned to their home cage and survived for 2 days. Bladder content was compressed manually as required.

Neurologic and Histopathologic Evaluations

The animal's behavior was neurologically assessed at 48 h after reperfusion by an observer who was unaware of the grouping. A modified Tarlov criteria²⁷ scoring system 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. A histopathologic evaluation was performed in the spinal cord at 48 h after reperfusion. Transcardiac perfusion and fixation were performed with 1,000 ml heparinized saline followed by 500 ml buffered formalin, 10%. The lumbar spinal cord was removed and refrigerated in 10% phosphate-buffered formalin for 48 h. After dehydration in the graded ethanol and butanol, the spinal cord was embedded in the paraffin. Coronal sections of the spinal cord (L5 segment) were cut at a thickness of 6 µm and stained with hematoxylin and eosin. Neuronal injury was evaluated at a magnification of ×400 by an observer who was unaware of the grouping. Injured neurons were identified by intensely eosinophilic cytoplasm with loss of Nissl substance and by the presence of pyknotic homogenous nuclei. The remaining normal neurons in the anterior spinal cord (anterior to a line drawn through the central canal perpendicular to the vertebral axis), judged by their morphologic appearance, were counted in three sections selected randomly from the rostral, middle, and caudal levels of the L5 segment and then averaged.

Statistical Analysis

Mean arterial pressure, heart rate, rectal temperature, blood gases, and blood glucose were expressed as mean ± SD. Physiologic values at the onset and end of isoflurane pretreatment were compared by using a paired-samples t test. Physiologic variables during surgical operation were compared by using one-way analysis of variance followed by Student-Newman-Keuls test. The scores of hind-limb motor function and the numbers of normal neurons in the anterior spinal cord were analyzed by using a nonparametric method (Kruskal-Wallis test) followed by the Mann–Whitney U test. A P value of less than 0.05 was considered to be statistically significant.

Results

Experiment 1

Physiologic Variables. Physiologic values at the onset and end of isoflurane pretreatment are presented in
Table 1. Physiologic Variables at the Onset and End of Isoflurane Preconditioning (n = 5)

<table>
<thead>
<tr>
<th></th>
<th>MAP, mmHg</th>
<th>PaCO₂, mmHg</th>
<th>PaO₂, mmHg</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset of IP</td>
<td>88 ± 7</td>
<td>99 ± 4</td>
<td>36 ± 3</td>
<td>7.40 ± 0.03</td>
</tr>
<tr>
<td>End of IP</td>
<td>84 ± 5</td>
<td>98 ± 3</td>
<td>39 ± 3</td>
<td>7.39 ± 0.02</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.
IP = isoflurane preconditioning; MAP = mean arterial pressure; PaCO₂ = arterial carbon dioxide tension; PaO₂ = arterial oxygen tension.

Table 2. Physiologic Variables during Preischemic and Postsischemic State (n = 9)

<table>
<thead>
<tr>
<th></th>
<th>Proximal MAP, mmHg</th>
<th>Distal MAP, mmHg</th>
<th>HR, beats/min</th>
<th>T, °C</th>
<th>PaCO₂, mmHg</th>
<th>PaO₂, mmHg</th>
<th>pH</th>
<th>Glucose, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85 ± 10</td>
<td>86 ± 10</td>
<td>259 ± 20</td>
<td>38.4 ± 0.3</td>
<td>107 ± 9</td>
<td>36 ± 3</td>
<td>7.40 ± 0.04</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td>O₂24h</td>
<td>86 ± 12</td>
<td>86 ± 11</td>
<td>257 ± 21</td>
<td>38.5 ± 0.3</td>
<td>104 ± 9</td>
<td>36 ± 3</td>
<td>7.38 ± 0.06</td>
<td>6.4 ± 1.6</td>
</tr>
<tr>
<td>O₂48h</td>
<td>85 ± 9</td>
<td>84 ± 8</td>
<td>260 ± 19</td>
<td>38.3 ± 0.3</td>
<td>103 ± 11</td>
<td>38 ± 5</td>
<td>7.37 ± 0.06</td>
<td>6.3 ± 1.7</td>
</tr>
<tr>
<td>O₂72h</td>
<td>83 ± 9</td>
<td>83 ± 10</td>
<td>264 ± 20</td>
<td>38.3 ± 0.3</td>
<td>104 ± 10</td>
<td>37 ± 4</td>
<td>7.37 ± 0.03</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td>Iso24h</td>
<td>84 ± 10</td>
<td>85 ± 9</td>
<td>262 ± 16</td>
<td>38.4 ± 0.2</td>
<td>104 ± 7</td>
<td>37 ± 3</td>
<td>7.38 ± 0.05</td>
<td>6.2 ± 1.4</td>
</tr>
<tr>
<td>Iso48h</td>
<td>83 ± 9</td>
<td>84 ± 8</td>
<td>261 ± 22</td>
<td>38.5 ± 0.4</td>
<td>105 ± 11</td>
<td>36 ± 5</td>
<td>7.39 ± 0.06</td>
<td>6.6 ± 1.5</td>
</tr>
<tr>
<td>Iso72h</td>
<td>82 ± 10</td>
<td>83 ± 10</td>
<td>265 ± 17</td>
<td>38.3 ± 0.2</td>
<td>107 ± 12</td>
<td>36 ± 5</td>
<td>7.38 ± 0.05</td>
<td>5.9 ± 1.3</td>
</tr>
<tr>
<td>Ischemia 10 min</td>
<td>86 ± 11</td>
<td>8 ± 2</td>
<td>254 ± 20</td>
<td>38.3 ± 0.3</td>
<td>108 ± 8</td>
<td>35 ± 3</td>
<td>7.41 ± 0.05</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>O₂24h</td>
<td>87 ± 11</td>
<td>7 ± 2</td>
<td>253 ± 16</td>
<td>38.4 ± 0.2</td>
<td>106 ± 8</td>
<td>35 ± 3</td>
<td>7.40 ± 0.05</td>
<td>6.8 ± 1.5</td>
</tr>
<tr>
<td>O₂48h</td>
<td>87 ± 9</td>
<td>8 ± 2</td>
<td>254 ± 18</td>
<td>38.4 ± 0.2</td>
<td>107 ± 10</td>
<td>36 ± 4</td>
<td>7.38 ± 0.06</td>
<td>6.3 ± 1.4</td>
</tr>
<tr>
<td>O₂72h</td>
<td>84 ± 9</td>
<td>8 ± 2</td>
<td>259 ± 15</td>
<td>38.3 ± 0.2</td>
<td>107 ± 11</td>
<td>35 ± 4</td>
<td>7.38 ± 0.03</td>
<td>6.7 ± 1.3</td>
</tr>
<tr>
<td>Iso24h</td>
<td>85 ± 10</td>
<td>8 ± 1</td>
<td>251 ± 24</td>
<td>38.4 ± 0.3</td>
<td>106 ± 8</td>
<td>37 ± 3</td>
<td>7.38 ± 0.04</td>
<td>6.8 ± 1.6</td>
</tr>
<tr>
<td>Iso48h</td>
<td>84 ± 10</td>
<td>8 ± 1</td>
<td>255 ± 16</td>
<td>38.4 ± 0.2</td>
<td>107 ± 11</td>
<td>35 ± 4</td>
<td>7.41 ± 0.06</td>
<td>6.6 ± 1.4</td>
</tr>
<tr>
<td>Iso72h</td>
<td>84 ± 10</td>
<td>8 ± 2</td>
<td>256 ± 21</td>
<td>38.3 ± 0.3</td>
<td>107 ± 12</td>
<td>35 ± 5</td>
<td>7.39 ± 0.05</td>
<td>7.2 ± 1.6</td>
</tr>
<tr>
<td>Reperfusion 10 min</td>
<td>79 ± 12</td>
<td>79 ± 12</td>
<td>262 ± 23</td>
<td>38.4 ± 0.3</td>
<td>109 ± 9</td>
<td>34 ± 3</td>
<td>7.38 ± 0.04</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td>O₂24h</td>
<td>82 ± 13</td>
<td>81 ± 14</td>
<td>260 ± 19</td>
<td>38.3 ± 0.3</td>
<td>107 ± 8</td>
<td>35 ± 3</td>
<td>7.39 ± 0.06</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>O₂48h</td>
<td>83 ± 11</td>
<td>80 ± 10</td>
<td>259 ± 16</td>
<td>38.4 ± 0.4</td>
<td>109 ± 10</td>
<td>35 ± 4</td>
<td>7.38 ± 0.05</td>
<td>7.0 ± 1.3</td>
</tr>
<tr>
<td>O₂72h</td>
<td>80 ± 11</td>
<td>80 ± 11</td>
<td>263 ± 15</td>
<td>38.3 ± 0.2</td>
<td>110 ± 10</td>
<td>34 ± 3</td>
<td>7.37 ± 0.03</td>
<td>6.8 ± 1.4</td>
</tr>
<tr>
<td>Iso24h</td>
<td>77 ± 9</td>
<td>76 ± 9</td>
<td>266 ± 20</td>
<td>38.4 ± 0.2</td>
<td>108 ± 7</td>
<td>35 ± 2</td>
<td>7.38 ± 0.05</td>
<td>6.7 ± 1.5</td>
</tr>
<tr>
<td>Iso48h</td>
<td>78 ± 10</td>
<td>76 ± 10</td>
<td>261 ± 17</td>
<td>38.4 ± 0.2</td>
<td>110 ± 10</td>
<td>34 ± 3</td>
<td>7.40 ± 0.06</td>
<td>6.9 ± 1.4</td>
</tr>
<tr>
<td>Iso72h</td>
<td>78 ± 11</td>
<td>76 ± 12</td>
<td>264 ± 18</td>
<td>38.3 ± 0.3</td>
<td>109 ± 12</td>
<td>34 ± 4</td>
<td>7.37 ± 0.04</td>
<td>6.5 ± 1.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.
Control: animals that only underwent spinal cord ischemia without pretreatment; O₂24h, O₂48h, and O₂72h: animals that received 40 min of 100% oxygen each day for 5 days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia; Iso24h, Iso48h, and Iso72h: animals that received 40 min of 2.1% isoflurane in 100% oxygen each day for 5 days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia.
HR = heart rate; MAP = mean arterial pressure; PaCO₂ = arterial carbon dioxide tension; PaO₂ = arterial oxygen tension; T = rectal temperature.

Histopathologic Outcome. The histopathologic results are shown in figure 4. Forty-eight hours after reperfusion, the normal neurons in the anterior spinal cord of the Iso24h and Iso48h groups were more than those of the control group (P = 0.00078 and P = 0.0012, respectively; fig. 4). However, the number of normal neurons at the anterior spinal cord in the control group revealed no difference compared with the O₂24h, O₂48h, O₂72h, and Iso72h groups (P > 0.05 for each comparison; fig. 4). In the control group, a rabbit rated as Tarlov score 0 showed severe neuronal damage, as evidenced by disappearance of Nissl bodies and nuclei, and vacuolation of gray matter (fig. 5A). In contrast, in the Iso24h group, a rabbit rated as Tarlov score 4 showed normal motor neurons (fig. 5B).

Anesthesiology, V 105, No 5, Nov 2006
Physiologic Variables. Hemodynamics, rectal temperature, blood gases, and blood glucose concentrations were similar in all groups, regardless of the treatment patterns (data not shown). The distal blood pressure was approximately 75–90 mmHg before blocking the abdominal aorta and decreased to 5–10 mmHg during the artery was clamped. Ten minutes after the beginning of reperfusion, the value of the distal blood pressure was recovered to nearly preischemic level.

Neurologic Outcome. The neurologic results are summarized in figure 6. All animals survived until the final neurologic assessment at 48 h after reperfusion. Animals in the Iso group showed higher hind-limb motor function scores than those in the DMTU/H11001 Iso group (P < 0.008), O2 group (P < 0.001), and DMTU/H11001 O2 group (P < 0.001) (fig. 6). However, there were no significant differences in hind-limb motor function scores of animals among the DMTU/H11001, O2, and DMTU/H11001 O2 groups (P > 0.05 for each comparison; fig. 6).

Histopathologic Outcome. The histopathologic results are summarized in figure 7. Forty-eight hours after reperfusion, more normal motor neurons in the anterior spinal cord were seen in the Iso group compared with the DMTU+Iso group (P = 0.008), O2 group (P < 0.001), and DMTU+O2 group (P < 0.001) (fig. 6). However, there were no significant differences in hind-limb motor function scores of animals among the DMTU+Iso, O2, and DMTU+O2 groups (P > 0.05 for each comparison; fig. 6).

Fig. 3. Experiment 1: Neurologic outcome in each animal of seven groups 48 h after reperfusion (** P < 0.005 vs. control). ▲, □, ○, ●, △, and ◆ represent animals in the control, O2/4h, O2/8h, O2/72h, Iso/4h, Iso/8h, and Iso/72h groups, respectively. Control: animals that only underwent spinal cord ischemia without pretreatment; O2/4h, O2/8h, and O2/72h: animals that received 40 min of 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia; Iso/4h, Iso/8h, and Iso/72h: animals that received 40 min of 2.1% isoflurane in 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia.

Fig. 4. Experiment 1: Histopathologic outcome in each animal of seven groups 48 h after reperfusion (** P < 0.005 vs. control). ▲, □, ○, ●, △, and ◆ represent animals in the control, O2/4h, O2/8h, O2/72h, Iso/4h, Iso/8h, and Iso/72h groups, respectively. Control: animals that only underwent spinal cord ischemia without pretreatment; O2/4h, O2/8h, and O2/72h: animals that received 40 min of 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia; Iso/4h, Iso/8h, and Iso/72h: animals that received 40 min of 2.1% isoflurane in 100% oxygen each day for 5 consecutive days, with the last pretreatment at 24, 48, and 72 h, respectively, before spinal cord ischemia.

Fig. 5. Representative photomicrographs of lumbar spinal cord sections (L5) stained with hematoxylin and eosin 48 h after reperfusion. (A) Section from a rabbit rated as Tarlov score 0 in the control group showed severe neuronal damage, as evidenced by disappearance of Nissl bodies and nuclei, and extensive vacuolation of gray matter. (B) Section from a rabbit rated as Tarlov score 4 in the Iso24h group showed numerous normal motoneurons (arrows) and several eosinophilic neurons (arrowheads). Note the darkly stained cytoplasm of dead neurons (arrowheads) compared with the fine granular cytoplasm and Nissl substance of the viable cells (arrows). Eosinophilic neurites are clearly visible (open arrowheads). Scale bars represent 150 μm.
0.001), and DMTU+O₂ group (P < 0.001) (fig. 7). However, there were no significant differences in the number of normal neurons at the anterior spinal cord among the DMTU/Iso, O₂, and DMTU+O₂ groups (P > 0.05 for each comparison; fig. 7). Some normal motor neurons remained in the rabbit rated as Tarlov score 3 in the Iso group (fig. 8A); however, few normal motor neurons were observed, and vacuolation was noted in the rabbit rated as Tarlov score 1 in the DMTU/Iso group (fig. 8B).

Discussion

The current study demonstrated for the first time that repeated inhalation with isoflurane induced a delayed phase of ischemic tolerance to transient spinal cord ischemia in rabbits after 24 and 48 h but not 72 h. The administration of dimethylthiourea, a free radical scavenger, before each isoflurane pretreatment abolished the ischemic tolerance induced by isoflurane. Kersten et al.¹⁰ first described early phase of ischemic tolerance induced by isoflurane in the heart. Subsequent studies also demonstrated the delayed phase of ischemic tolerance induced by isoflurane in the heart¹³,¹⁴ and brain.¹⁶,¹⁷ Recently, Park et al.¹⁹ found that early preconditioning induced by isoflurane also existed in the
spinal cord. In their experiment, preischemic isoflurane exposure reduced the spinal cord ischemic injury and improved neurologic outcome within a 45-min interval between isoflurane pretreatment and the ischemic injury in rabbits. Our study further demonstrated that 2.1% isoflurane pretreatment (40 min each day for 5 consecutive days) induced a delayed phase of ischemic tolerance in the spinal cord as manifested by more normal neurons and higher neurologic scores. In previous studies, the time window of delayed neuroprotection induced by isoflurane has not been investigated. The current study demonstrated that isoflurane induced delayed neuroprotection 24 and 48 h after repeated inhalation of isoflurane, but the delayed neuroprotection was dissipated 72 h after isoflurane pretreatment.

However, three important factors must be considered in this regard. First, during the 40 min of isoflurane pretreatment, we did not intubate and mechanically ventilate the rabbits because, compared with anesthesia alone in spontaneously breathing animals, oral intubation and mechanical ventilation yield a higher level of stress. Because isoflurane can inhibit respiration, rabbits may have carbon dioxide retention and hypoxemia during the pretreatment. For this reason, we measured the arterial blood gases at the onset and end of 40 min of exposure to 2.1% isoflurane, which should represent the worst situation. Our results showed that there were no significant differences in pH, PaCO2, or PaO2 between the onset and end of isoflurane pretreatment. In addition, no rabbits had hypoxemia (PaO2 < 60 mmHg) during 40 min of exposure to 2.1% isoflurane. Therefore, it is unlikely that the mild carbon dioxide retention, acidemia, or hypoxemia during the isoflurane pretreatment contributed to the final isoflurane preconditioning effects. The second important factor that deserves attention is blood pressure. It is possible that isoflurane pretreatment caused hypotension that induced preconditioning effects. However, no hypotension was found during the 40 min of exposure to 2.1% isoflurane in our study. Third, a previous study demonstrated that neuroprotection induced by isoflurane in 100% oxygen for 5 consecutive days may be more neuroprotective than that induced by isoflurane. Zheng et al. demonstrated that exposure to isoflurane for 30 min at 24 h before focal brain ischemia reduced brain infarct sizes and improved neurologic deficit scores. Kapinya et al. found that pretreatment with isoflurane for 3 h induced tolerance against ischemic neuronal injury. The optimal duration of isoflurane exposure for the induction of delayed neuroprotection has not been defined. In our study, 2.1% isoflurane during the preconditioning was applied, because 2.1% isoflurane for New Zealand White rabbits is approximately equal to 1 MAC, which is often achieved in clinical practice.

Previous studies indicated KATP channels play a pivotal role in isoflurane-induced preconditioning in the heart10,12,13 and brain. However, accumulated evidence revealed opening of mitochondrial KATP channels may not be the final step in the preconditioning cascade. Pain et al. suggested that opening of mitochondrial KATP channels during ischemic preconditioning of the heart triggered the preconditioned state by generating free radicals, resulting in activation of several protein kinases. McPherson et al. provided evidence that morphine-induced preconditioning in cultured cardiomyocytes led to activation of mitochondrial KATP channels, resulting in an increase of intracellular free radicals. Similarly, Tanaka et al. also demonstrated that mitochondrial KATP channels are not the end effecter in the preconditioning induced by isoflurane in myocardium but rather act as a trigger for the preconditioned protection through the generation of free radicals. Recently, Park et al. suggested that the neuroprotection induced by isoflurane preconditioning in the spinal cord depended on the activation of mitochondrial KATP. Therefore, we postulated that neuroprotection induced by isoflurane preconditioning in the spinal cord also depended on the release of free radicals.

Dimethylthiourea is a potent free radical scavenger that is highly diffusible and scavenges not only ·OH but also O2− and peroxynitrite. This dose of dimethylthiourea in our experiment was chosen based on the previous study. Our results indicated that dimethylthiourea could abolish the neuroprotection induced by isoflurane preconditioning if administered before each isoflurane pretreatment. While given alone, dimethylthiourea did not influence the neurologic and histopathologic outcomes. This study did indicate that dimethylthiourea, a potent free radical scavenger, reversed the neuroprotection induced by isoflurane preconditioning in the spinal cord and that the preconditioning effect may be related to free radicals.

Because it has been shown that mitochondrial KATP channel blocker inhibited free radical production induced by isoflurane in myocardium, it is possible that
mitochondrial K<sub>ATP</sub> channel opening may be an upstream event of free radical production in isoflurane preconditioning-induced neuroprotection. However, the relation between mitochondrial K<sub>ATP</sub> channel opening and free radical production during isoflurane preconditioning in the spinal cord merits further investigation.

A recent study demonstrated that isoflurane preconditioning induced neuroprotection against ischemia via activation of P38 mitogen-activated protein kinases. Moreover, free radicals could activate P38 mitogen-activated protein kinase. These findings suggested that free radical production induced by isoflurane preconditioning may be linked to subsequent activation of kinases implicated in the signal transduction responsible for protection against ischemic injury. However, the current investigation did not examine the downstream events in response to free radical generation during the preconditioning induced by isoflurane. These objectives represent important goals of future research.

Previous studies have proved the worsening of neurologic function at 14–48 h after spinal cord ischemia in the rabbit model. Therefore, the final assessments sent important goals of future research.

Investigation did not examine the downstream events in response to free radical generation during the preconditioning induced by isoflurane. These objectives represent important goals of future research.

In summary, the current study demonstrated that repeated exposure to isoflurane induced delayed preconditioning against spinal cord ischemic injury in rabbits after 24 and 48 h but not 72 h, and that the delayed neuroprotection induced by isoflurane preconditioning may be related to the production of free radicals.

References