

Isoflurane Preconditioning Improves Long-term Neurologic Outcome after Hypoxic–Ischemic Brain Injury in Neonatal Rats

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Background: Preconditioning the brain with relatively safe drugs seems to be a viable option to reduce ischemic brain injury. The authors and others have shown that the volatile anesthetic isoflurane can precondition the brain against ischemia. Here, the authors determine whether isoflurane preconditioning improves long-term neurologic outcome after brain ischemia.

Methods: Six-day-old rats were exposed to 1.5% isoflurane for 30 min at 24 h before the brain hypoxia–ischemia that was induced by left common carotid arterial ligation and then exposure to 8% oxygen for 2 h. The neuropathology, motor coordination, and learning and memory functions were assayed 1 month after the brain ischemia. Western analysis was performed to quantify the expression of the heat shock protein 70, Bcl-2, and survivin 24 h after isoflurane exposure.

Results: The mortality was 45% after brain hypoxia–ischemia. Isoflurane preconditioning did not affect this mortality. However, isoflurane preconditioning attenuated ischemia-induced loss of neurons and brain tissues, such as cerebral cortex and hippocampus in the survivors. Isoflurane also improved the motor coordination of rats at 1 month after ischemia. The learning and memory functions as measured by performance of Y-maze and social recognition tasks in the survivors were not affected by the brain hypoxia–ischemia or isoflurane preconditioning. The expression of Bcl-2, a well-known antiapoptotic protein, in the hippocampus is increased after isoflurane exposure. This increase was reduced by the inhibitors of inducible nitric oxide synthase. Inducible nitric oxide synthase inhibition also abolished isoflurane preconditioning–induced neuroprotection.

Conclusions: Isoflurane preconditioning improved the long-term neurologic outcome after brain ischemia. Inducible nitric oxide synthase may be involved in this neuroprotection.

PERINATAL hypoxic–ischemic brain injury is estimated to occur in 1 of 4,000 births.¹ Most of the survivors (approximately 60%) have long-term neurologic or cognitive disability.^{1–3} Because of the huge impact on human health and financial burden on our society, finding methods to reduce ischemic brain injury has been a

focus of medical research. Many interventions have been explored for potential neuroprotection. However, clinically practical methods to reduce ischemic brain injury have not been well established yet.

One of the important advances on ischemic brain injury research in the recent years is the recognition that ischemic injury is a dynamic process characterized by ongoing neuronal loss for a long period of time after ischemia (for weeks in rodents).^{4,5} Various methods or approaches have been shown to be neuroprotective in animal studies. However, few of them are effective in improving neurologic outcome in clinical studies. One of the possible reasons for this phenomenon is that previous animal studies often examined the neurologic outcome a few days after the brain ischemia and that human studies frequently evaluated neurologic outcome a few months later. It is now a well-known phenomenon that some of the protective methods may just delay cell death after brain ischemia.^{6–8} Therefore, it is important to evaluate the long-term neuroprotective effects of a method in preclinical studies.

Pretreatment of various organs, including brain, with brief episodes of ischemia has been shown to reduce injury after a prolonged episode of ischemia.⁹ This phenomenon is called *ischemic preconditioning*. Various other stimuli, such as hypoxia and hypothermia, have been shown to induce preconditioning effects.^{10–13} However, the utility of the preconditioning effects induced by these stimuli in clinical practice is questionable because of the danger or the complex biologic effects of the stimuli. We and others have shown that isoflurane can induce preconditioning effects in the brain.^{14–16} Isoflurane is a commonly used volatile anesthetic and has been safely used in clinical practice for decades. We designed this study to test the hypothesis that isoflurane preconditioning can improve long-term neurologic outcome after brain ischemia.

Materials and Methods

The animal protocol was approved by the institutional Animal Care and Use Committee of the University of Virginia, Charlottesville, Virginia. All animal experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 80-23) revised in 1996. All reagents unless specified

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below were obtained from Sigma Chemical (St. Louis, MO).

Neonatal Cerebral Hypoxia-Ischemia Model

Cerebral hypoxia-ischemia was induced as we previously described.¹⁷ Briefly, 7-day-old male and female Sprague-Dawley rats were anesthetized by isoflurane in 30% O₂-70% N₂, and their left common carotid arteries were permanently ligated with a double 7-0 surgical silk. The rats were allowed to awake and were returned to their cages with the mothers for 3 h. The neonates were then placed in a chamber containing humidified 8% O₂-92% N₂ for 2 h at 37°C. The air temperature in the chamber was continuously monitored and maintained at 37°C. The chamber was then opened to room air for 15 min, and the animals were returned to their cages.

Isoflurane Preconditioning and Study Groups

Six-day-old rats were placed in a chamber containing 1.5% isoflurane carried by 30% O₂-70% N₂ for 30 min at 24 h before the cerebral hypoxia-ischemia. The neonates usually started to feed within 30 min after the isoflurane application. In the first set of experiments, six groups of neonates were studied: (1) control, (2) 1.5% isoflurane treatment only, (3) cerebral hypoxia-ischemia, (4) 1.5% isoflurane pretreatment and then cerebral hypoxia-ischemia, (5) 1 mg/kg *N*-(3-(aminomethyl)benzyl)acetamide (1400 W; BIOMOL Research Laboratories Inc., Plymouth Meeting, PA) injected intraperitoneally 24 h before cerebral hypoxia-ischemia, and (6) 1 mg/kg 1400 W injected intraperitoneally 30 min before the isoflurane pretreatment and then cerebral hypoxia-ischemia. Neonates from the same mother were assigned to these six experimental conditions. Neonates in groups 2, 4, and 6 were pretreated with isoflurane, whereas the others from the same mother were placed in a chamber containing 30% O₂-70% N₂ but no isoflurane for 30 min and were assigned to groups 1, 3, and 5.

In the second set of experiments, four groups of rats were studied: (1) control, (2) 1.5% isoflurane treatment, (3) 200 mg/kg aminoguanidine administered intraperitoneally 30 min before the isoflurane treatment, and (4) 1 mg/kg 1400 W injected intraperitoneally 30 min before the isoflurane treatment. Aminoguanidine and 1400 W were dissolved in normal saline, and the injected volume was from 0.16 to 0.2 ml per rat. Rats in the control group and isoflurane treatment only group received 0.2 ml normal saline at the corresponding times. Aminoguanidine and 1400 W are inducible nitric oxide synthase (iNOS) inhibitors that have been shown to inhibit iNOS activity in rat brain¹⁸ and iNOS-mediated neuroprotection induced by isoflurane and prenatal hypoxic preconditioning at the regimen used in this study.^{11,17} The rat brains were harvested 24 h after isoflurane treatment for Western analysis.

Mortality and Body Weight Monitoring

Death during the period from the onset of cerebral hypoxia-ischemia to 1 month afterward was recorded, and the mortality rate was calculated. Rat body weights were measured just before and 1 month after the cerebral hypoxia-ischemia.

Brain Histopathology

Brain histopathologic evaluation was performed in rats in the first set of experiments. One month (30 days) after the cerebral hypoxia-ischemia, rats were euthanized by isoflurane and transcardially perfused with 30 ml saline. Brains were removed and stored in 4% phosphate-buffered paraformaldehyde for 4 h at room temperature. Eight-micrometer-thick cryostat coronal sections at approximately 3.3 mm caudal to bregma were obtained and subjected to Nissl staining. These sections were examined by an observer blinded to the group assignment of the sections. The cerebral cortical and hippocampal areas in each of the hemispheres were measured by using National Institutes of Health Image 1.60 (Bethesda, MD). The area ratio of the cerebral cortex and hippocampus in the left hemisphere to those in the right hemisphere was calculated and used to reflect brain tissue loss in the left hemisphere after brain hypoxia-ischemia. Neuronal density in the perirhinal cortex was determined as follows. A reticle (approximately 0.034 mm²) was used to count cells in the same size area. Nissl staining-positive cells were counted in the area. Three determinations, each on different locations in the left perirhinal cortex, were performed and averaged to yield a single number (density of the neurons) for the brain region of each individual rat. The neuronal density in the right perirhinal cortex was determined in the same way. The neuronal density ratio in the left/right perirhinal cortex was then calculated to measure the neuronal loss after brain hypoxia-ischemia.

Motor Coordination Evaluation

This evaluation was performed just before the rats were killed for brain histopathology. Rats were placed on a rotarod whose speed increased from 4 to 40 rpm in 5 min. The latency and the speed of rats' falling off the rod were recorded. Each rat was tested three times, and the speed-latency index (latency in seconds × speed in rpm) for each trial was calculated. The mean index value of the three trials was used to reflect the motor coordination functions of each rat.

Y Maze and Social Recognition

The Y-maze and social recognition tests were performed as described previously¹⁹ at 1 day before the rats were killed for brain histopathology. During Y-maze test, rats were placed in the center of a symmetrical Y maze and were allowed to explore freely in the maze for 8 min. The total number and sequence of arms entered

were recorded. An arm was entered if the hind paws of the rat were completely in the arm. The percentage alternation that was the percentage of the number of entry into all three arms in the maximum possible alternations (the total number of arms entered divided by 3) was calculated for each rat.

The social recognition task was tested by placing a test rat in a clean acrylic cage. A male juvenile (3- to 4-week-old) rat was placed into the cage with the test rat for 2 min. The two rats were separated for 3 h and were placed together again for 2 min. The duration of social investigation of the juvenile rat by the test rat during the two 2-min periods was recorded. Social investigation behaviors include direct contact with the juvenile for inspection and close following (< 1 cm) of the juvenile. If there was any aggressive encounter between the rats, the experiments were terminated and the data were excluded from analysis. The ratio of duration of the social investigation during the second 2-min period in the duration of the first 2-min period was calculated to measure the social recognition memory.

Western Blot Analysis

Cerebral cortex and hippocampus were dissected from the rats in the second set of experiments and were sonicated in ice-cold 20 mM Tris-HCl (pH 7.5) containing 5 mM Mg Cl₂, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin, 1 mM DL-dithiothreitol, and 2 mM sodium orthovanadate. The sample was centrifuged at 1,000g at 4°C for 10 min. The protein concentrations in the supernatants were determined by the Lowry assay using a protein assay kit. Equal protein samples (50 µg per lane) were separated by 12% sodium dodecyl sulfate-polyacrylamide gels and then electrotransferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). The primary antibodies were rabbit polyclonal anti-heat shock protein 70 (HSP70) antibody (1:1,500 dilution, catalog No. SPA-812; Stressgen, Victoria, British Columbia, Canada), antisurvivin antibody (1:500 dilution, catalog No. S8191), antiactin antibody (1:2,000 dilution, catalog No. A2066), and mouse monoclonal anti-Bcl-2 antibody (1:2,000 dilution, catalog No. sc-509; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Protein bands were visualized by the enhanced chemiluminescence detection method with reagents from Amersham Pharmacia Biotech (Piscataway, NJ). The protein band volumes were quantified by a densitometry with ImageQuant 5.0 Windows NT software (Molecular Dynamics, Sunnyvale, CA). The volumes of Bcl-2, HSP70, and survivin protein bands were normalized to those of actin to control for errors in protein sample loading and transferring during the Western blot analysis. The results in the groups after isoflurane exposure were then normalized to those of control animals.

Statistical Analysis

Our previous study showed that a 2-h left hemisphere hypoxia-ischemia reduced the weight of left brain hemisphere by approximately 30% and isoflurane preconditioning decreased this brain loss to approximately 10% with an SD of approximately 12% when the brains were examined at 7 days after the brain hypoxia-ischemia.¹⁷ Based on these results, it was estimated that 7 rats per group would be needed to detect the protective effects (brain loss reduction/brain pathology) of isoflurane preconditioning with a desired power of 80% at an α level of 0.05 by *t* test. However, this sample estimate was used only as a reference in the experimental design of this study because of the obvious differences in the duration of observation after brain hypoxia-ischemia (1 week *vs.* 1 month) and outcome parameters between this study and our previous study.¹⁷

Data are presented as mean \pm SD. Results of hippocampal and cortical area ratio, neuronal density ratio, speed-latency index, percentage of alternation, and the ratio of the investigation times of the different study groups were compared by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) method or by one-way ANOVA on ranks followed by the Dunn method as appropriate. The Western blot data were analyzed by one-way ANOVA on ranks followed by the Dunn method. The mortality rates among groups were analyzed by *Z* test. The comparison of body weight among groups was performed by ANOVA for repeated measures followed by the SNK method. $P < 0.05$ was considered significant. All statistical analyses were performed with SigmaStat (Systat Software, Inc., Point Richmond, CA).

Results

General characteristics of various study groups in the first set of experiments are presented in table 1. The mortality was 45% in rats that had brain hypoxia-ischemia for 2 h. This mortality was not significantly altered by isoflurane preconditioning or application of 1400 W. The body weight of 7-day-old neonates (just before the brain hypoxia-ischemia) and 37-day-old neonates (30 days after the brain hypoxia-ischemia) among all study groups including control rats was not different (table 1).

There were significant differences among the various groups of rats in the left brain loss/damage as assessed grossly (fig. 1) or by the ratio of left/right cerebral cortical area ($F(5, 45) = 13.82, P < 0.001$), hippocampal area ($F(5, 45) = 10.98, P < 0.001$), and neuronal density in the perirhinal cortex ($F(5, 45) = 15.61, P < 0.001$) (fig. 2). Brain hypoxia-ischemia caused significant brain loss/damage in the left hemisphere assessed at 30 days after the injury (compared with control group by SNK method, $q = 7.75, P < 0.001$ for cortical area; $q = 8.36,$

Table 1. General Characteristics

	Fate after Brain Hypoxia-Ischemia			Body Weight of the Survivors, g	
	Dead	Survived	Mortality, %	7 days old	37 days old
Control	0	24	0	13.7 ± 1.9	143 ± 30
1.5% Iso	0	23	0	13.7 ± 1.4	150 ± 35
HI	25	30	45*	14.3 ± 2.0	161 ± 23
1.5% Iso + HI	38	30	56*	14.4 ± 2.0	144 ± 35
1400 W + HI	9	9	50*	14.9 ± 1.7	156 ± 13
1400 W + 1.5% Iso + HI	9	9	50*	14.9 ± 1.2	153 ± 5

Values (body weight) are mean ± SD.

* $P < 0.05$ compared with control.

1400 W = 1 mg/kg *N*-(3-(aminomethyl)benzyl)acetamide injected intraperitoneally; HI = brain hypoxia-ischemia that was induced by left common carotid artery ligation plus hypoxia with 8% O₂ for 2 h at 37°C to 7-day-old rats; Iso = isoflurane for 30 min.

$P < 0.001$ for hippocampal area; and $q = 9.49$, $P < 0.001$ for neuronal density in the perirhinal cortex) (figs. 1 and 2). This hypoxia-ischemia-induced brain loss/damage was significantly attenuated by preconditioning with 1.5% isoflurane (comparison between hypoxia-ischemia and isoflurane preconditioning plus hypoxia-ischemia by SNK method, $q = 3.69$, $P = 0.033$ for cortical area; $q = 3.87$, $P = 0.043$ for hippocampal area; and $q = 4.40$,

$P = 0.016$ for neuronal density in the perirhinal cortex) (fig. 2). These results indicated that isoflurane preconditioning improved neuropathology even at 1 month after brain hypoxia-ischemia. This isoflurane preconditioning-induced improvement was attenuated by the iNOS inhibitor 1400 W (fig. 1 and 2), suggesting a role of iNOS in this protection.

The motor coordination functions as reflected by the speed-latency index in the rotarod test among the rats in various groups were statistically different ($F(5, 112) = 8.66$, $P < 0.001$) (fig. 3). The brain hypoxia-ischemia impaired motor coordination functions (compared with control group by SNK method, $q = 4.53$, $P = 0.005$). This impairment was significantly attenuated by isoflurane preconditioning (comparison between hypoxia-ischemia and isoflurane preconditioning plus hypoxia-ischemia by SNK method, $q = 3.26$, $P = 0.023$) (fig. 3), suggesting that isoflurane preconditioning improved motor functions after brain ischemia. The iNOS inhibitor 1400 W abolished this improvement caused by isoflurane preconditioning (comparison between isoflurane preconditioning plus hypoxia-ischemia and 1400 W plus isoflurane preconditioning plus hypoxia-ischemia by SNK method, $q = 5.28$, $P = 0.002$), indicating the role of iNOS in the isoflurane preconditioning-induced motor coordination improvement. There was no difference among the rats from various groups in the performance of Y maze (by one-way ANOVA on ranks, $H^5 = 7.82$, $P = 0.166$) or the social recognition tasks (by one-way ANOVA on ranks, $H^5 = 4.29$, $P = 0.509$) (fig. 3), suggesting that brain hypoxia-ischemia or isoflurane treatment did not affect the performance of rats in the Y-maze and social recognition tasks. Of note, the total numbers of arms entered by control rats and rats with cerebral hypoxia-ischemia only were 15 ± 9 and 16 ± 12 , respectively ($P > 0.05$), suggesting that cerebral hypoxia-ischemia did not impair the motor functions severely enough to affect the performance of rats in the Y-maze test.

Western analysis showed that there was significant difference in Bcl-2 expression in the hippocampus

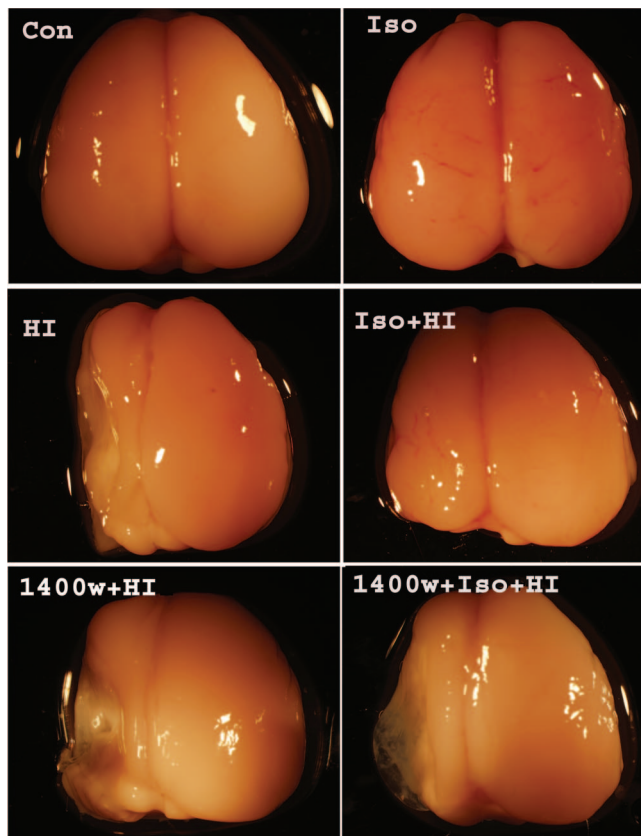
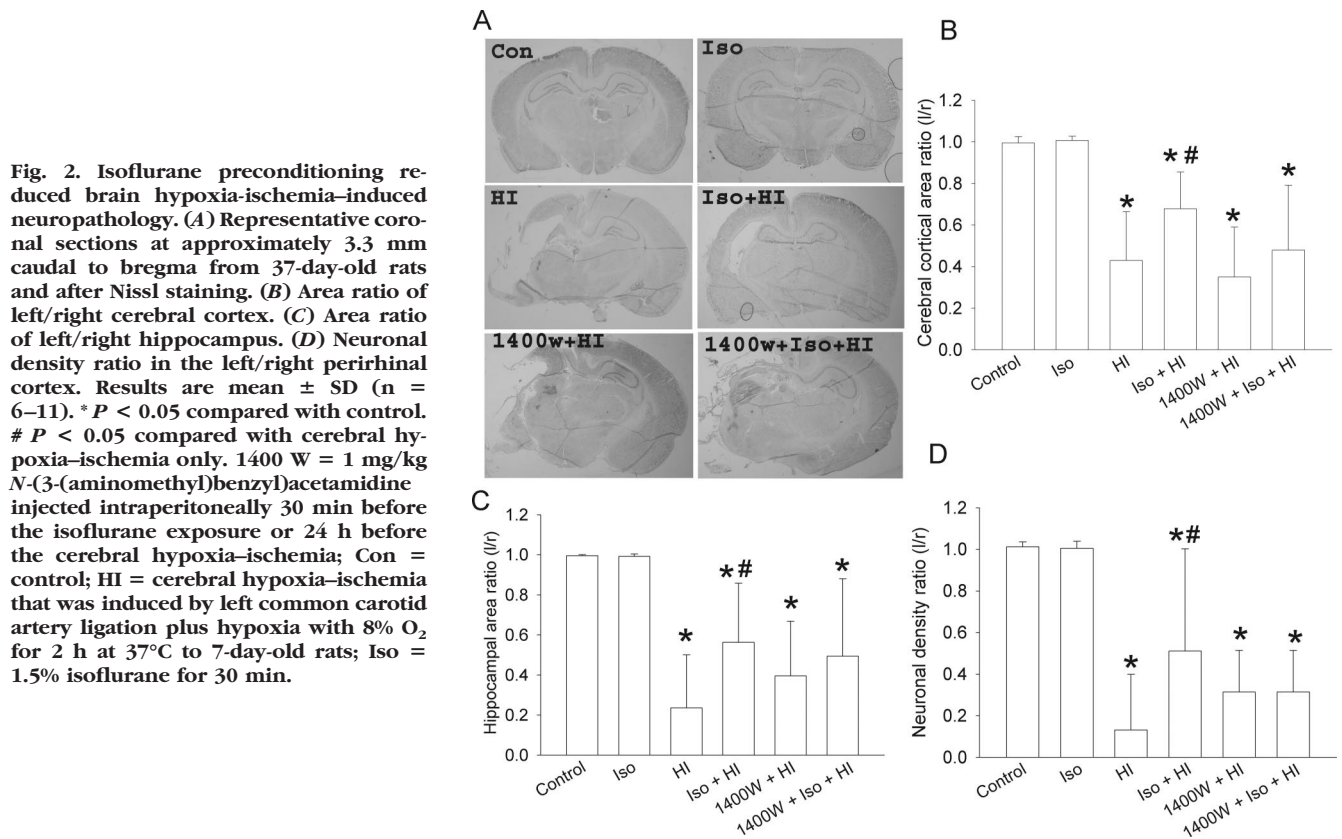


Fig. 1. Brain hypoxia-ischemia-induced brain tissue loss. Representative brains of 37-day-old rats with various treatments. 1400 W = 1 mg/kg *N*-(3-(aminomethyl)benzyl)acetamide injected intraperitoneally 30 min before the isoflurane exposure or 24 h before the cerebral hypoxia-ischemia; Con = control; HI = cerebral hypoxia-ischemia that was induced by left common carotid artery ligation plus hypoxia with 8% O₂ for 2 h at 37°C to 7-day-old rats; Iso = 1.5% isoflurane for 30 min.



among the various groups (by one-way ANOVA on ranks, $H^3 = 13.18$, $P = 0.004$). Isoflurane significantly increased the expression of Bcl-2 in the hippocampus (comparison between control and isoflurane exposure groups by Dunn method, $Q = 2.98$, $P < 0.05$). This increased expression was decreased by aminoguanidine and 1400 W, two iNOS inhibitors, suggesting that isoflurane-induced Bcl-2 expression was iNOS dependent (fig. 4). Isoflurane exposure did not change the expression of survivin and HSP70 in the hippocampus or cerebral cortex (fig. 4).

Discussion

Early studies showed infarct maturation after 2 days in rat models of stroke.^{20,21} Studies in the recent years have shown that ischemic injury is a dynamic process characterized by ongoing neuronal loss for at least 14 days after ischemia in rodents.^{4,5} Protective methods, such as postinjury mild hypothermia, reduced brain injury evaluated a few days after the ischemia but did not show protection when the evaluation was performed 1 month after the brain ischemia.^{7,8} These studies underscore the importance of examining the long-term neurologic outcome of any protective methods. Our study showed that isoflurane preconditioning reduced cerebral cortical and hippocampal loss and improved motor coordination functions at 1 month after the brain hypoxia-ischemia.

These results suggest that isoflurane preconditioning improves the long-term neurologic outcome after brain ischemia.

We used 1.5% isoflurane in this study. This concentration was the highest concentration that did not significantly affect the blood gases and pH but induced preconditioning effects on the brain in our previous study using the same animal model.¹⁷ One minimum alveolar concentration (the concentration to inhibit 50% of subjects to respond to surgical stimuli) of isoflurane is 1.12% and 1.15%, respectively, for adult rats and humans^{22,23} and 1.6% for human neonates.²⁴ Therefore, the isoflurane concentration used in this study is clinically relevant.

We ligated one common carotid artery and then exposed 7-day-old neonates to 8% oxygen to induce hypoxic-ischemic brain injury. This is a widely used animal model to simulate human perinatal brain ischemia.²⁵ The maturation of the brain in the 7-day-old rat is similar to that of human newborn brain.^{25,26} Perinatal brain ischemia in human is often caused by brain ischemia superimposed on severe systemic hypoxia.²⁷ Therefore, the brain injury in the animal model used in our study shares many features of the human perinatal brain injury.

We monitored the mortality and body weight gain to measure the general well being of the rats in each study group. The hypoxic-ischemic injury caused 45% mortality and isoflurane preconditioning did not affect this

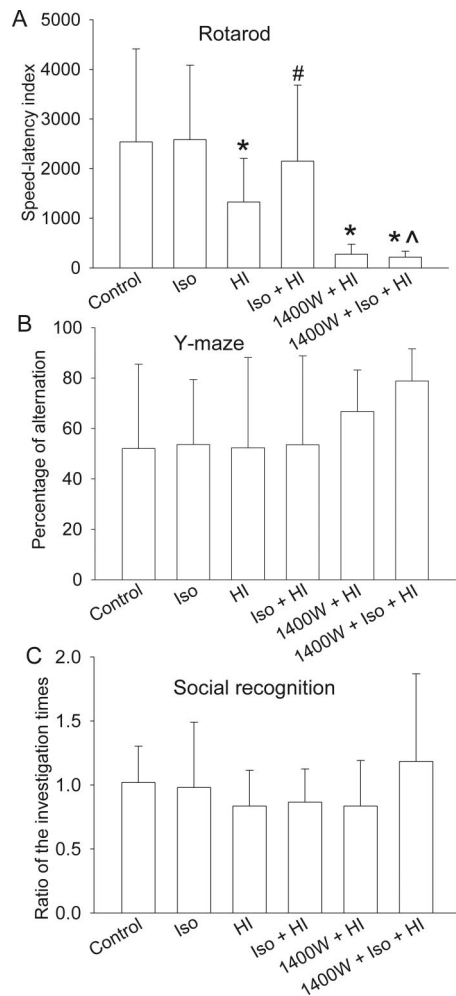


Fig. 3. Isoflurane preconditioning improved motor coordination after brain hypoxia–ischemia. (A) Speed–latency index of 37-day-old rats with various treatments in the rotarod test. (B) Percentage of alternation of 37-day-old rats with various treatments in the Y-maze test. (C) Ratio of the investigation times in the second trial over those in the first trial. Results are mean \pm SD ($n = 9$ – 30). * $P < 0.05$ compared with control. # $P < 0.05$ compared with cerebral hypoxia–ischemia only. ^ $P < 0.05$ compared with isoflurane preconditioning plus cerebral hypoxia–ischemia. 1400 W = 1 mg/kg *N*-(3-(aminomethyl)benzyl)acetamide injected intraperitoneally 30 min before the isoflurane exposure or 24 h before the cerebral hypoxia–ischemia; HI = cerebral hypoxia–ischemia that was induced by left common carotid artery ligation plus hypoxia with 8% O_2 for 2 h at 37°C to 7-day-old rats; Iso = 1.5% isoflurane for 30 min.

mortality rate. These results are consistent with those of our previous report.¹⁷ The body weights of the survivors at the end of the study in all four groups are not significantly different, suggesting the body weight gain is not a sensitive parameter to reflect the degree of brain injury in this animal model. These results are also similar to our previous data.¹⁷

We quantified hypoxia–ischemia–induced brain loss by the cortical and hippocampal area and neuronal density in the perirhinal cortex at approximately 3.3 mm caudal to bregma. The section at this level was used to reflex the brain injury because this section contains hippocam-

pus and the degree of brain injury was similar in sections from 1 mm rostral to 5 mm caudal to bregma in this brain hypoxia–ischemia model.²⁸ We chose to count neuronal density in the perirhinal cortex because this brain region is easily recognized and localization of this region in brain sections can be very accurate. Blood supply of the perirhinal cortex is mainly from the ipsilateral internal carotid artery. Therefore, significant neuronal injury in the perirhinal cortex was anticipated in our newborn rats after the cerebral hypoxia–ischemia. Our study showed that the cortical and hippocampal area and the neuronal density in the perirhinal cortex of the ischemic hemisphere were significantly decreased by the hypoxia–ischemia and this decrease was attenuated by isoflurane preconditioning. These results are strong evidence that isoflurane preconditioning improves neuropathological outcome at 1 month after brain hypoxia–ischemia.

Two types of neurologic functions were monitored in our study. The motor coordination functions of the rats were assayed by the rotarod test. Hypoxic–ischemic brain injury significantly reduced the duration and speed that the rats could stay on the rotarod compared with control rats, suggesting that these rats had impaired motor coordination functions. Rats preconditioned by isoflurane before the hypoxic–ischemic injury performed better than rats subjected to hypoxic–ischemic injury only. Therefore, isoflurane preconditioning improves not only the neuropathologic outcome but also neurologic functions after brain ischemia.

The learning and memory functions were evaluated by the Y-maze and social recognition tasks. These two tasks are very sensitive for measuring early learning and memory deficits.¹⁹ The social recognition tests rats to identify and remember con-specifics, whereas the spontaneous alternation Y maze assesses spatial working memory. Because these two tasks measure hippocampus-dependent learning and memory functions¹⁹ and the hippocampus in the ischemic hemisphere in our model was obviously injured, one would expect that the rats after hypoxic–ischemic brain injury would have had worse performance than did control rats in the Y-maze and social recognition tasks. To our surprise, there was no significant difference in the performance of these two tasks among the six groups of rats. Poor performance on water maze tasks that examine long-term spatial learning and reference memory was found with the neonatal rats after hypoxic–ischemic brain injury.²⁹ However, the performance of those rats on eight-arm maze tasks that test long-term reference memory and short-term working memory was not significantly different from the control rats in the same study.²⁹ The reasons for the apparent discrepancy of the findings from water maze and eight-arm maze tasks in this previous study and the obvious brain structure injury and the maintained learning and memory functions assayed by the Y-maze and social

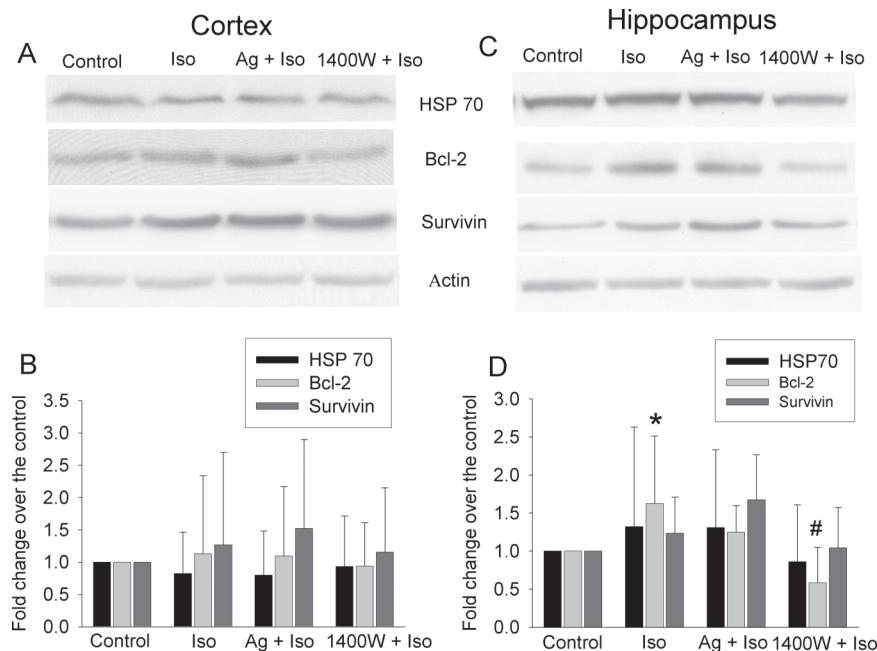


Fig. 4. The effects of isoflurane on the expression of heat shock protein 70 (HSP70), Bcl-2, and survivin proteins in the cerebral cortex and hippocampus of 7-day-old rats. Six-day-old rats were exposed to 1.5% isoflurane for 30 min, and the cerebral cortex and hippocampus were removed for Western analysis at 24 h after the isoflurane exposure. (A and C) A representative film image of the bands. (B and D) The graphic presentation of HSP70, Bcl-2, and survivin protein abundance quantified by integrating the volume of bands from 5–10 rats for each experimental condition and normalizing the data by those of actin. Values in graphs are mean \pm SD of the fold changes over the controls, with the controls being set as 1. * $P < 0.05$ compared with controls. # $P < 0.05$ compared with isoflurane only. 1400 W + Iso = 1 mg/kg *N*-(3-(aminomethyl)benzyl)acetamide (1400 W) injected intraperitoneally 30 min before the isoflurane exposure; Ag + Iso = 200 mg/kg aminoguanidine injected intraperitoneally at 30 min before the isoflurane exposure; Iso = 1.5% isoflurane for 30 min.

recognition tasks in our study are not known. Y-maze, social recognition, and eight-arm tasks are nonstressful, and the water maze test is stressful. One explanation for the discrepancy is that rats after hypoxic-ischemic brain injury can compensate well with various mechanisms, such as through the functions of the nonischemic hemisphere, in performing nonstressful tasks but not perform well in the stressful tasks that measure the learning and memory functions.

It has been proposed that delayed neuroprotection that occurs a few hours after the application of preconditioning stimuli involves synthesis of protective proteins.³⁰ Bcl-2 can reduce ischemia-induced increase of mitochondrial membrane permeability and cytochrome *c* release from the mitochondrion.³¹ The released cytochrome *c* will bind with protease-activating factor 1 to form the apoptosome that will activate caspase 9. This process ultimately will activate caspase 3 to induce cell apoptosis. Bcl-2 can bind with the C terminus of protease-activating factor 1 to inhibit the association of caspase 9 with protease-activating factor 1.^{32–34} Thus, Bcl-2, *via* acting on various steps, inhibits apoptosis and is a protective protein. Our results showed that rats exposed to isoflurane had an increased Bcl-2 in the hippocampus and this increase was inhibited by aminoguanidine and 1400 W, two iNOS inhibitors. Isoflurane preconditioning-induced neuroprotection observed 1 week after the brain ischemia was shown to be iNOS

dependent in our previous study using the same animal model.¹⁷ In this study, 1400 W abolished the isoflurane preconditioning-induced neuroprotection. Therefore, our results suggest that the increased Bcl-2 expression contributes to the neuroprotection induced by isoflurane preconditioning. Consistent with this idea, it is known that brain injury in the neonatal brain hypoxia-ischemia model is caused, at least partly, by apoptosis.^{35,36} Our results also suggest a link between iNOS and Bcl-2. Nitric oxide can induce Bcl-2 expression.³⁷ Nitric oxide produced by iNOS can activate signal transducer and activator of transcription 3,³⁸ a transcription factor that increases Bcl-2 expression.³⁹ These previous studies, along with the results presented here, suggest that iNOS is a signaling molecule upstream of Bcl-2 to mediate isoflurane preconditioning-induced neuroprotection.

Survivin is a member of the inhibitor of apoptosis gene family and may be through its association with caspases to inhibit apoptosis.^{40,41} Our results showed that isoflurane exposure did not alter the expression of survivin in the cerebral cortex and hippocampus of rats, suggesting that isoflurane preconditioning-induced neuroprotection does not involve survivin. Isoflurane exposure also did not change the expression of HSP70. HSP70 is molecular chaperons for damaged proteins and can be induced by various stress stimuli to provide protection.^{42,43} Our results indicate that HSP70 is not involved

in the isoflurane preconditioning-induced neuroprotection. However, caution should be exercised regarding the suggestion of noninvolvement of survivin and HSP70 in the isoflurane preconditioning-induced neuroprotection because the expression change of these two proteins may occur at other time points after isoflurane preconditioning. We chose to quantify protein expression at 24 h after isoflurane exposure in this study because we subjected the rats to the brain hypoxic-ischemic injury at this time point and reasoned that the involved protective proteins should be expressed at this time to reduce brain injury.

In summary, we have shown that preconditioning with isoflurane at a clinically relevant concentration can induce a long-lasting neuroprotection in rats. This effect may involve an increased expression of Bcl-2. Because isoflurane is a commonly used and relatively safe drug, our finding may have implications in clinical situations that brain ischemia occurs as a planned or anticipated event, such as perceived difficult labor with potential newborn brain ischemia, newborns for open heart surgery and newborns with high risks of intraventricular or periventricular hemorrhage.

References

- Lynch JK, Nelson KB: Epidemiology of perinatal stroke. *Curr Opin Pediatr* 2001; 13:499-505
- Sran SK, Baumann RJ: Outcome of neonatal strokes. *Am J Dis Child* 1988; 142:1086-8
- Sreenan C, Bhargava R, Robertson CM: Cerebral infarction in the term newborn: Clinical presentation and long-term outcome. *J Pediatr* 2000; 137:351-5
- Li Y, Chopp M, Jiang N, Yao F, Zaloga C: Temporal profile of *in situ* DNA fragmentation after transient middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 1995; 15:389-97
- Du C, Hu R, Csernansky CA, Hsu CY, Choi D: Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? *J Cereb Blood Flow Metab* 1996; 16:195-201
- Kawaguchi M, Kimbro JR, Drummond JC, Cole DJ, Kelly PJ, Patel PM: Isoflurane delays but does not prevent cerebral infarction in rats subjected to focal ischemia. *ANESTHESIOLOGY* 2000; 92:1335-42
- Dietrich WD, Busto R, Alonso O, Globus MY, Ginsberg MD: Intraischemic but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. *J Cereb Blood Flow Metab* 1993; 13:541-9
- Trescher WH, Ishiwa S, Johnston MV: Brief post-hypoxic-ischemic hypothermia markedly delays neonatal brain injury. *Brain Dev* 1997; 19:326-38
- Nandagopal K, Dawson TM, Dawson VL: Critical role for nitric oxide signaling in cardiac and neuronal ischemic preconditioning and tolerance. *JPET* 2001; 297:474-8
- Gidday JM, Shah AR, Maceren RG, Wang Q, Pelligrino DA, Holtzman DM, Park TS: Nitric oxide mediates cerebral ischemic tolerance in a neonatal rat model of hypoxic preconditioning. *J Cereb Blood Flow Metab* 1999; 19:331-40
- Zhao P, Zuo Z: Prenatal hypoxia-induced adaptation and neuroprotection that is inducible nitric oxide synthase-dependent. *Neurobiol Dis* 2005; 20:871-80
- Yuan H-B, Huang Y, Zheng S, Zuo Z: Hypothermic preconditioning increases survival of Purkinje neurons in rat cerebellar slices after an *in vitro* simulated ischemia. *ANESTHESIOLOGY* 2004; 100:331-7
- Yuan HB, Huang Y, Zheng S, Zuo Z: Hypothermic preconditioning reduces Purkinje cell death possibly by preventing the over-expression of inducible nitric oxide synthase in rat cerebellar slices after an *in vitro* simulated ischemia. *Neuroscience* 2006; 142:381-9
- Zheng S, Zuo Z: Isoflurane preconditioning reduces Purkinje cell death in an *in vitro* model of rat cerebellar ischemia. *Neuroscience* 2003; 118:99-106
- Zheng S, Zuo Z: Isoflurane preconditioning induces neuroprotection against ischemia *via* activation of p38 mitogen-activated protein kinase. *Mol Pharmacol* 2004; 65:1172-80
- Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke K, Isaev NK, Dirnagl U: Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible NO synthase dependent. *Stroke* 2002; 33:1889-98
- Zhao P, Zuo Z: Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase-dependent in the neonatal rats. *ANESTHESIOLOGY* 2004; 101:695-702
- Nagayama M, Zhang F, Iadecola C: Delayed treatment with aminoguanidine decreases focal cerebral ischemic damage and enhances neurologic recovery in rats. *J Cereb Blood Flow Metab* 1998; 18:1107-13
- Ohno M, Sametsky EA, Younkin LH, Oakley H, Younkin SG, Citron M, Vassar R, Disterhoft JF: BACE1 deficiency rescues memory deficits and cholinergic dysfunction in a mouse model of Alzheimer's disease. *Neuron* 2004; 41:27-33
- Garcia J, Yoshida Y, Chen H, Li Y, Zhang Z, Lian J, Chen S, Chopp M: Progression from ischemic injury to infarct following middle cerebral artery occlusion in the rat. *Am J Pathol* 1993; 142:623-35
- Lin T, He Y, Wu G, Khan M, Hsu C: Effect of brain edema on infarct volume in a focal cerebral ischemia model in rats. *Stroke* 1993; 24:117-21
- Orliaguette G, Vivien B, Langeron O, Bouhemad B, Coriat P, Riou B: Minimum alveolar concentration of volatile anesthetics in rats during postnatal maturation. *ANESTHESIOLOGY* 2001; 95:734-9
- Eger EI: Uptake and distribution, Anesthesia. Edited by Miller RD. Philadelphia, Churchill Livingstone, 2000, pp 74-95
- LeDez KM, Lerman J: The minimum alveolar concentration (MAC) of isoflurane in preterm neonates. *ANESTHESIOLOGY* 1987; 67:301-7
- Hagberg H, Bona E, Gilland E, Puka-Sundvall M: Hypoxia-ischaemia model in the 7-day-old rat: Possibilities and shortcomings. *Acta Paediatr Suppl* 1997; 422:85-8
- Dobbing J, Sands J: The brain growth spurt in various mammalian species. *Early Hum Dev* 1979; 3:79-84
- Johnston MV: Neonatal hypoxic-ischemic brain insults and their mechanisms, *New Concepts in Cerebral Ischemia*. Edited by Simon SA, Nicoletti MAL. Boca Raton, CRC Press, 2002, pp 31-61
- Ma D, Hossain M, Chow A, Arshad M, Battson RM, Sanders RD, Mehmet H, Edwards AD, Franks NP, Maze M: Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. *Ann. Neurol* 2005; 58:182-93
- Ikeda T, Mishima K, Yoshikawa T, Iwasaki K, Fujiwara M, Xia YX, Ikenoue T: Selective and long-term learning impairment following neonatal hypoxic-ischemic brain insult in rats. *Behav Brain Res* 2001; 118:17-25
- Dirnagl U, Simon RP, Hallenbeck JM: Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci* 2003; 26:248-54
- Kluck RM, Bossy-Wetzell E, Green DR, Newmeyer DD: The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275:1132-6
- Hu Y, Benedict MA, Wu D, Inohara N, Nunez G: Bcl-XL interacts with Apaf-1 and inhibits Apaf-1-dependent caspase-9 activation. *Proc Natl Acad Sci U S A* 1998; 95:4386-91
- Huang DC, Adams JM, Cory S: The conserved N-terminal BH4 domain of Bcl-2 homologues is essential for inhibition of apoptosis and interaction with CED-4. *EMBO J* 1998; 17:1029-39
- Pan G, O'Rourke K, Dixit VM: Caspase-9, Bcl-XL, and Apaf-1 form a ternary complex. *J Biol Chem* 1998; 273:5841-5
- Nakajima W, Ishida A, Lange MS, Gabrielson KL, Wilson MA, Martin LJ, Blue ME, Johnston MV: Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. *J. Neurosci* 2000; 20:7994-8004
- Gill R, Soriano M, Blomgren K, Hagberg H, Wybrecht R, Miss MT, Hoefler S, Adam G, Niederhauser O, Kemp JA, Loetscher H: Role of caspase-3 activation in cerebral ischemia-induced neurodegeneration in adult and neonatal brain. *J Cereb Blood Flow Metab* 2002; 22:420-30
- Nishikawa M, Takeda K, Sato EF, Kuroki T, Inoue M: Nitric oxide regulates energy metabolism and Bcl-2 expression in intestinal epithelial cells. *Am J Physiol* 1998; 274:G797-801
- Hierholzer C, Kalf J, Billiar TR, Bauer AJ, Tweardy DJ, Harbrecht BG: Induced nitric oxide promotes intestinal inflammation following hemorrhagic shock. *Am J Physiol Gastrointest Liver Physiol* 2004; 286:G225-33
- Alas S, Emmanouilides C, Bonavida B: Inhibition of interleukin 10 by rituximab results in down-regulation of bcl-2 and sensitization of B-cell non-Hodgkin's lymphoma to apoptosis. *Clin Cancer Res* 2001; 7:709-23
- Altieri DC: Survivin in apoptosis control and cell cycle regulation in cancer. *Prog Cell Cycle Res* 2003; 5:447-52
- Kobayashi K, Hatano M, Otaki M, Ogasawara T, Tokuhisa T: Expression of a murine homologue of the inhibitor of apoptosis protein is related to cell proliferation. *Proc Natl Acad Sci U S A* 1999; 96:1457-62
- Kelly S, Yenari MA: Neuroprotection: Heat shock proteins. *Curr Med Res Opin* 2002; 18 (suppl 2):55-60
- Giffard RG, Yenari MA: Many mechanisms for hsp70 protection from cerebral ischemia. *J Neurosurg Anesthesiol* 2004; 16:53-61