

# Sevoflurane Preconditioning against Focal Cerebral Ischemia

## Inhibition of Apoptosis in the Face of Transient Improvement of Neurological Outcome

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**Background:** Preconditioning the brain with volatile anesthetics seems to be a viable option for reducing ischemic cerebral injury. However, it is uncertain whether this preconditioning effect extends over a longer period of time. The purpose of this study was to determine if sevoflurane preconditioning offers durable neuroprotection against cerebral ischemia.

**Methods:** Rats (Sprague-Dawley) were randomly allocated to two groups: nonpreconditioned control group ( $n = 44$ ) and preconditioned group ( $n = 45$ ) exposed to 2.7 vol% sevoflurane (45 min) 60 min before surgery. Animals in both groups were anesthetized with 3.0 vol% sevoflurane and subjected to transient middle cerebral artery occlusion. After 60 min of awake focal ischemia, the filament was removed. Functional neurologic outcome (range 0–18; 0 = no deficit), cerebral infarct size (Nissl staining), and apoptosis (Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling; cleaved caspase-3 staining) were evaluated at 3, 7, and 14 days after ischemia.

**Results:** Sevoflurane preconditioning significantly improved functional outcome and reduced infarct volume ( $109 \pm 43$  vs.  $148 \pm 56$  mm<sup>3</sup>) 3 days after ischemia compared to the control group. However, after 7- and 14-day recovery periods, no significant differences were observed between groups. The number of apoptotic cells was significantly lower in the preconditioned group than in the control group after 3- and 7-day recovery periods. Fourteen days after ischemia, no differences were observed between groups.

**Conclusion:** In this model of transient focal cerebral ischemia, sevoflurane preconditioning induced effective but transient neuroprotective effects. Sevoflurane preconditioning also decreased ischemia-induced apoptosis in a more sustained way because it was observed up to 7 days after injury.

VOLATILE anesthetics exert direct neuroprotective effects when they are present during ischemia *in vitro*<sup>1</sup> and *in vivo*<sup>2</sup> and also when they are administered before an ischemic insult<sup>3</sup> (anesthetic preconditioning [APC]). APC was first described in the heart<sup>4</sup> and more recently in both *in vitro*<sup>5,6</sup> and *in vivo*<sup>6-11</sup> models of cerebral ischemia. In particular, sevoflurane preconditioning has

been shown to reduce *in vitro* hippocampal neuronal damage after hypoxia<sup>5</sup> and *in vivo* after global cerebral ischemia.<sup>12</sup> Most of these studies on cerebral APC have assessed histopathological and neurologic outcomes for a period of less than 7 days after injury. It is therefore uncertain whether this preconditioning effect extends over a longer period of time. As Kawaguchi *et al.*<sup>13</sup> reported in their study on the direct neuroprotection afforded by volatile anesthetics, the role of neuronal apoptosis is central in the pathogenesis of cerebral ischemia. This study shows that volatile anesthetics delayed but did not prevent neuronal apoptosis after focal cerebral ischemia. The effect of APC on neuronal apoptosis is still poorly explored. Only indirect effects have been reported by Zhao *et al.*<sup>14</sup> who showed during APC against neonatal hypoxic-ischemic brain injury an increase in expression of the antiapoptotic protein B-cell lymphoma-2.

In this context, we first studied the time-course of neuroprotection induced by sevoflurane preconditioning by using an *in vivo* model of transient focal cerebral ischemia in the rat. Neuroprotection was assessed by neurologic and histopathological evaluations for 14 days after ischemia. Second, we explored whether sevoflurane preconditioning interfered with ischemia-induced apoptosis. Our hypothesis was that sevoflurane administered before the ischemic insult (APC) would induce a greater neuroprotective effect than administration during the ischemic time alone (direct neuroprotection).

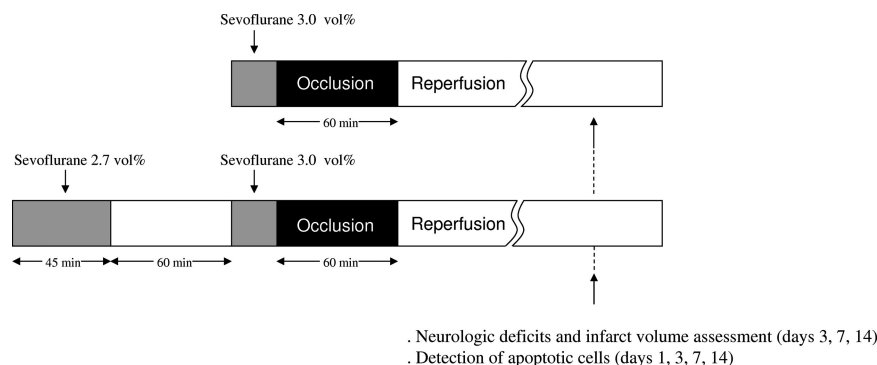
## Materials and Methods

This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Helsinki Declaration. The experiments were performed after approval of the protocol by the Institution's Animal Care and Use Committee (Université de la Méditerranée, Marseille, France), in an authorized laboratory, and under the supervision of an authorized researcher (Dr. Pisano; authorization N°: 13-59). Sprague-Dawley rats (200–250 g) fasted overnight were randomly allocated to the control group ( $n = 44$ ) or preconditioned group ( $n = 45$ ).

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**Fig. 1. Schematic illustration depicting the experimental protocol.**

### Transient Middle Cerebral Artery Occlusion

In the preconditioned group, animals were intubated and ventilated mechanically (Microvent 1; Hallowell EMC, TEM sega, Lormont, France) with 2.7 vol% sevoflurane for 45 min and allowed to wake up for a 1-h wash out period before anesthesia for surgery (fig. 1). In both groups, after the trachea was intubated and the lungs ventilated mechanically with 3.0 vol% sevoflurane in a gas mixture of 30% oxygen and 70% nitrogen, focal cerebral ischemia was produced according to the technique of Longa *et al.*<sup>15</sup> by occlusion of the right middle cerebral artery with slight modifications. Briefly, a middle line incision was made on the ventral side of the neck, the right common carotid artery was exposed, and the vagus and sympathetic nerves were carefully separated from the artery. The superior and inferior thyroid arteries were coagulated, and the external carotid artery was permanently ligated 2 mm distal to the bifurcation of the common carotid artery. The internal and common carotid arteries were temporarily clamped. A 0.38-mm diameter nylon monofilament (Nacrita 20  $\mu$ m; La Tortue, La Soie, Boulogne Billancourt, France) previously coated with silicone was inserted into the external carotid artery and advanced *via* the internal carotid artery up to the origin of the middle cerebral artery, to a distance of 18–20 mm from the carotid artery bifurcation until slight resistance was felt. The pterygopalatine artery was permanently ligated. At the end of surgery, the surgical wounds were closed and infiltrated with 0.5% bupivacaine, anesthesia was discontinued, and animals were allowed to awaken in a heated chamber. After a 60-min ischemic interval, the filament was withdrawn, allowing reperfusion. During preconditioning and surgery, inspired and expired fraction of sevoflurane, oxygen, and carbon dioxide were continuously monitored (Capnomac Ultima; Datex-Ohmeda, Helsinki, Finland). The tail artery was catheterized to monitor mean arterial pressure (M1166A; Hewlett-Packard, Evry, France). Plasma glucose concentration (Accu-chek Sensor; Roche Diagnostic, Meylan, France), blood gases and acid-base status (ABL5; Radiometer Copenhagen, Neuilly-Plaisance, France) were assessed at three timepoints; before surgery, at the beginning of ischemia, and at the onset of reperfusion. Temperature sensors were inserted into the rectum and

between the right temporal muscle and temporal bone (Mon-a-therm, subcutaneous sensor; Mallinckrodt, Tyco, Plaisir, France). Pericranial temperature was maintained constant at 37.5°C using a servo-controlled heating blanket (Harvard Apparatus®, Les Ulis, France). Adequacy of middle cerebral artery occlusion (MCAO) and reperfusion was confirmed by laser Doppler flowmetry (PF5010; Perimed, Stockholm, Sweden) using a flexible optical fiber probe (probe 418; Perimed) attached to the skull over the ipsilateral parietal cortex (6-mm lateral and 1-mm posterior of bregma). The surgical procedure was considered technically adequate if regional cerebral blood flow decreased more than 70% after placement of the intraluminal filament and if a cerebral blood flow signal was rapidly restored during reperfusion.

### Measurement of Neurologic Outcome

Neurologic deficits were evaluated at 3, 7, and 14 days after MCAO by an investigator blinded to the treatment conditions and using the modified Neurologic Severity Score graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18).<sup>16</sup>

### Tissue Preparation

Animals were randomly assigned to one of four different postischemic observation periods of 1, 3, 7, and 14 days after MCAO. At the end of the observation period, animals were anesthetized with sodium pentobarbital (Clin Midy, Paris, France) and transcardially perfused with 4% paraformaldehyde (Sigma, Saint-Quentin Fallavier, France) in 0.1 M phosphate buffer (pH 7.4). After decapitation, the brain was carefully removed from the skull and postfixed for 24 h in 4% phosphate-buffered paraformaldehyde at 4°C. Brains were successively cryopreserved in sucrose 10% and 30% for 6 and 72 h, respectively, snap-frozen, and stored at –80°C.

### Infarct Volume Assessment

Detection of infarct tissue was performed on 20 consecutive sections cut into 40- $\mu$ m-thick coronal sections at 400- $\mu$ m intervals (from olfactory bulb to cerebellum) using cresyl violet staining (Nissl staining). Tissue sections were fixed in a formol-acetic acid-methanol mix-

ture for 10 min and washed twice in water. Sections were stained for 2 min in filtered 0.5% cresyl violet (Sigma-Aldrich) solution in 20% ethanol and then rinsed in distilled water. They were then washed and dehydrated in graded ethanol solutions (70%, 95%, 100%) for 1 min each and coverslipped. Infarction volume was measured from 20 consecutive Nissl-stained sections scanned using a flatbed scanner, and the images were digitally stored in the tagged image file format. The infarct areas were measured using SigmaScan Pro 5.0 software (SPSS Inc, San Rafael, CA). Infarct volume was calculated by multiplying the infarct area of each slice by the distance (400  $\mu\text{m}$ ) between successive slices. To eliminate the contribution of postischemic edema to the volume of injury, values were corrected for swelling according to the method of Lin *et al.*<sup>17</sup>

#### Detection of Apoptotic Cells

Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) and cleaved caspase-3 labeling on sections cut into 6- $\mu\text{m}$ -thick coronal sections approximately 1.3 mm rostral to the bregma. DNA fragmentation labeling was performed with In Situ Cell Death Kit, POD (Roche, Mannheim, Germany). Briefly, endogenous peroxidase activity was quenched with 3%  $\text{H}_2\text{O}_2$ , and brain sections were permeabilized with 0.3% Triton X-100 and 0.1% sodium citrate (pH 6.0; Sigma-Aldrich) at 4°C. Sections were incubated for 90 min with terminal deoxynucleotidyl transferase and then for 30 min at 37°C with peroxidase-conjugated antiluorescein antibody. After rinsing, immunocomplexes were visualized by exposure to  $\text{H}_2\text{O}_2$  and diaminobenzidine (Sigma-Aldrich). Positive (DNaseI pretreatment, Sigma-Aldrich) and negative (terminal deoxynucleotidyl transferase incubation omitted) controls were used to check efficiency of immunostaining. Only cells containing apoptotic bodies are referred to as apoptotic cells.<sup>18</sup> Cleaved caspase-3 labeling was performed using primary rabbit anti-cleaved caspase-3 (Cell Signaling, Danvers, MA). In brief, sections were permeabilized with 0.3% Triton X-100 and 0.1% sodium citrate (pH 6.0; Sigma-Aldrich) at 4°C, and nonspecific binding activity was blocked by 10% goat serum (Sigma-Aldrich) for 1 h. Sections were then incubated overnight at 4°C with primary antibody diluted to 1:100. After rinsing, sections were incubated with Alexa Fluor 546, goat anti-rabbit IgG (Molecular Probes, Interchim, Montluçon, France) diluted to 1:500 for 1 h at room temperature. Sections were then rinsed and mounted. TUNEL and cleaved caspase-3-positive cell numbers were counted in five to ten fields in the inner boundary zone (defined as a part of the penumbra directly in contact with ischemic core)<sup>19</sup> at high-power microscopic magnification ( $\times 400$ ) and expressed as number of positive cells per square millimeter section.

#### Statistical Analyses

Values were reported as mean  $\pm$  SD unless otherwise indicated. Physiologic variables were analyzed by repeated-measures analysis of variance. Cerebral infarct volumes, TUNEL, and cleaved caspase-3-positive cell density were compared with unpaired *t* tests with Bonferroni corrections for *post hoc* intergroup comparisons. Neurologic scores were analyzed by the Kruskal-Wallis test. The same test was used for the comparison of time periods, which were considered independent groups because the animals were tested only once. For between-groups comparison, this was followed by the Mann-Whitney U test with Bonferroni correction. Statistical analyses were performed with SigmaStat<sup>®</sup> 2.03, (SPSS Inc). A value of  $P < 0.05$  was considered statistically significant.

#### Results

Of 89 animals instrumented, 7 died (4 controls, 3 preconditioned) before infarct volume determination and were excluded from the analysis. Physiologic variables before, during, and after MCAO are summarized in table 1. There

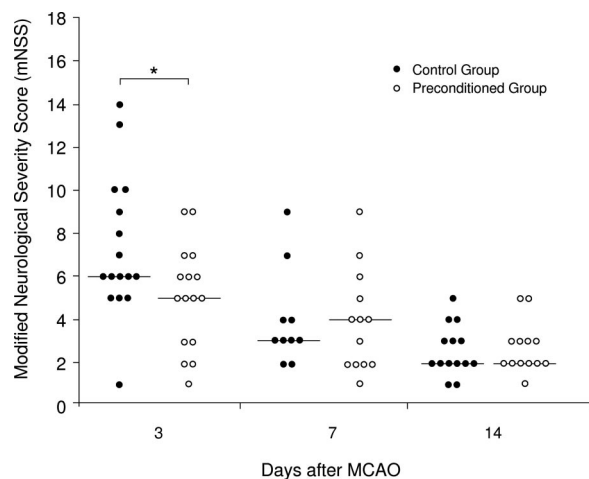
**Table 1. Physiologic Variables in Experimental Groups**

	Control Group (n = 40)	Preconditioned Group (n = 42)
Weight, g	241 $\pm$ 21	242 $\pm$ 22
Temperature, °C		
Before MCAO	37.4 $\pm$ 0.5	37.3 $\pm$ 0.6
During MCAO	37.2 $\pm$ 0.4	37.1 $\pm$ 0.6
Reperfusion	37.3 $\pm$ 0.3	37.2 $\pm$ 0.6
pH		
Before MCAO	7.41 $\pm$ 0.03	7.42 $\pm$ 0.04
During MCAO	7.41 $\pm$ 0.04	7.44 $\pm$ 0.02
Reperfusion	7.39 $\pm$ 0.03	7.41 $\pm$ 0.01
Paco <sub>2</sub> , mmHg		
Before MCAO	44 $\pm$ 6	43 $\pm$ 5
During MCAO	39 $\pm$ 4	36 $\pm$ 5
Reperfusion	42 $\pm$ 4	39 $\pm$ 3
PaO <sub>2</sub> , mmHg		
Before MCAO	131 $\pm$ 19	133 $\pm$ 18
During MCAO	135 $\pm$ 17	138 $\pm$ 9
Reperfusion	136 $\pm$ 18	141 $\pm$ 13
MAP, mmHg		
Before MCAO	82 $\pm$ 26	81 $\pm$ 19
During MCAO	99 $\pm$ 11	86 $\pm$ 15
Reperfusion	98 $\pm$ 12	96 $\pm$ 12
Glucose, mmol/l		
Before MCAO	8.2 $\pm$ 1.9	7.9 $\pm$ 2.1
During MCAO	6.0 $\pm$ 2.2	8.4 $\pm$ 1.6*
Reperfusion	7.3 $\pm$ 2.7	8.9 $\pm$ 2.4
Hemoglobin, g/dl		
Before MCAO	14.3 $\pm$ 0.2	14.5 $\pm$ 0.1
During MCAO	14.1 $\pm$ 0.1	14.0 $\pm$ 0.1
Reperfusion	14.4 $\pm$ 0.1	14.4 $\pm$ 0.1

Data are mean  $\pm$  SD. Before MCAO designates 5 min after induction of anesthesia; during MCAO designates the beginning of MCAO before waking; reperfusion designates 3 min after reperfusion.

\*  $P < 0.05$  vs. control group.

MAP = mean arterial pressure; MCAO = middle cerebral artery occlusion; PaO<sub>2</sub> = arterial oxygen tension; Pco<sub>2</sub> = arterial carbon dioxide tension.



**Fig. 2.** Neurologic outcome was evaluated 3, 7, and 14 days after middle cerebral artery occlusion (MCAO) by using the Modified Neurologic Severity Score<sup>16</sup> graded on a scale from 0 to 18 (0, no neurologic deficit; 18, maximal deficit). Three days after ischemia, neurologic scores were significantly lower in the preconditioned group compared to the control group. After 7- and 14-day recovery periods, there were no significant differences in neurologic scores between groups. Data are expressed as median (horizontal bars) with the individual values of control animals (filled circles) and sevoflurane-preconditioned animals (open circles). \*  $P < 0.05$  versus control group.

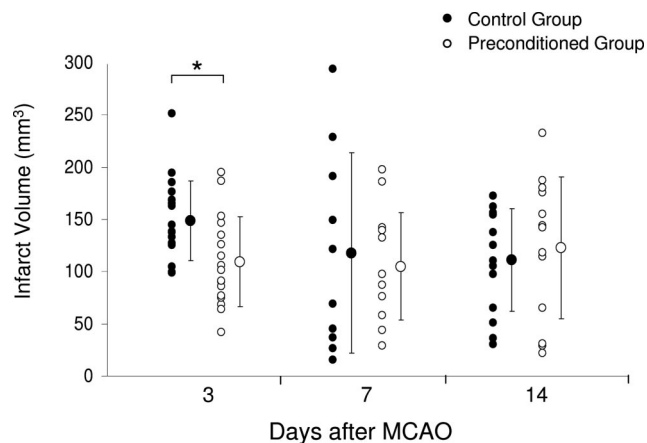
were no significant differences in mean arterial pressure, pH,  $P_{CO_2}$ ,  $P_{O_2}$ , and hemoglobin concentration between the control and sevoflurane-preconditioned groups at each timepoint. There were no differences among groups assigned to different postischemic observation periods (data not shown). Therefore, these parameters are presented together for each treatment. Plasma glucose concentration was significantly higher in the sevoflurane-preconditioned group during MCAO. Mean duration of 3.0 vol% sevoflurane exposure during surgery for control and preconditioned group was  $35.5 \pm 10.4$  and  $34.3 \pm 8.2$  min, respectively.

#### Neurologic Assessment

Results of the modified Neurologic Severity Score test are reported in figure 2. Three days after ischemia, neurologic scores were significantly lower in the sevoflurane-preconditioned group compared to the control group (median [interquartile range], 5 [3-6.5] vs. 6 [5.5-9.5];  $n = 16$  per group;  $P = 0.003$ ). After 7- and 14-day recovery periods, there were no significant differences in neurologic scores between groups. In both groups, a significant improvement in neurologic scores was observed ( $P < 0.05$  for time periods).

#### Histologic Analysis

The values of infarct volume in the control and the sevoflurane-preconditioned group are presented in figure 3. Sevoflurane preconditioning significantly reduced infarct volume at day 3 compared to the control group ( $109 \pm 43$  mm<sup>3</sup> vs.  $148 \pm 56$  mm<sup>3</sup>; control group:  $n =$



**Fig. 3.** Volumes of cerebral infarction 3, 7, and 14 days after 60 min of middle cerebral artery occlusion (MCAO) in control group (filled circles) and sevoflurane-preconditioned group (open circles). Three days after ischemia, the values of infarct volumes were significantly lower in the sevoflurane-preconditioned group than in the control group. No significant difference was observed at remote time points. Data are presented as mean  $\pm$  SD with the individual values. \*  $P < 0.05$  versus control group.

14, preconditioned group:  $n = 16$ ;  $P = 0.042$ ). At 7 and 14 days after ischemia, there were no significant differences in infarct volumes between groups.

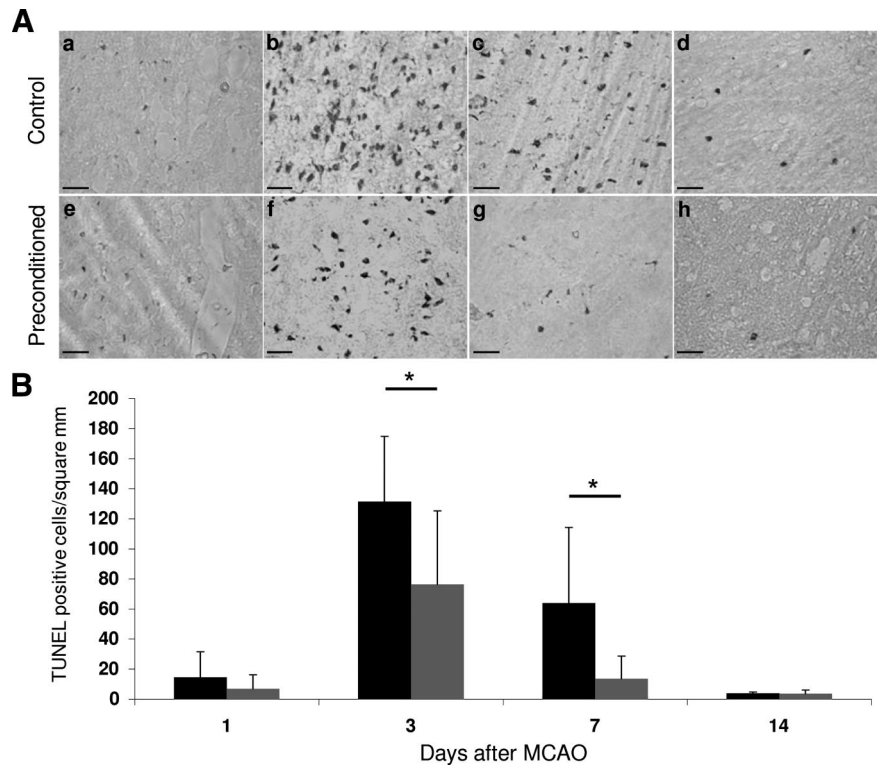
#### Apoptosis Detection

Figures 4 and 5, respectively, show TUNEL ( $n = 6$  per day and group) and cleaved caspase-3 ( $n = 8$  per day and group) staining within the inner boundary zone of the evolving infarct. Compared to the contralateral hemisphere, the number of apoptotic cells increased at day 1, peaked at day 3, and persisted at day 14 after MCAO. The number of apoptotic cells was significantly lower in the sevoflurane-preconditioned group than in the control group 3 days (TUNEL-positive cells/mm<sup>2</sup>:  $78.3 \pm 48.9$  vs.  $131.4 \pm 43.5$ ,  $P = 0.038$ ; cleaved caspase-3-positive cells/mm<sup>2</sup>:  $22.7 \pm 12.6$  vs.  $52.6 \pm 30.1$ ,  $P = 0.018$ ) and 7 days (TUNEL-positive cells/mm<sup>2</sup>:  $15.6 \pm 16.8$  vs.  $63.8 \pm 51.9$ ,  $P = 0.030$ ; cleaved caspase-3-positive cells/mm<sup>2</sup>:  $21.0 \pm 10.2$  vs.  $40.5 \pm 6.5$ ,  $P = 0.004$ ) after ischemia. At day 14, the number of TUNEL-positive and cleaved caspase-3-positive cells was not significantly different between groups. Neither TUNEL nor cleaved caspase-3 staining were detected in the contralateral nonischemic hemisphere.

#### Discussion

This study uses an *in vivo* model of transient focal cerebral ischemia in rats to demonstrate that preconditioning with sevoflurane induces effective but transient neuroprotective effects in terms of neurologic scores and infarct volumes. Sevoflurane preconditioning also decreased ischemia-induced apoptosis. This antiapoptotic effect was observed up to 7 days after injury.

**Fig. 4. (A)** Representative sections of nuclear DNA fragmentation assays performed by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining in the inner boundary zone of the infarct in control (*a–d*) and sevoflurane-preconditioned (*e–h*) rats 1 (*a, e*), 3 (*b, c*), 7 (*c, g*) and 14 (*d, h*) days after middle cerebral artery occlusion (MCAO). Scale bar = 40  $\mu\text{m}$ . **(B)** Number of TUNEL-positive cells/ $\text{mm}^2$  in the inner boundary zone of the infarct in control (black bars) and sevoflurane-preconditioned groups (gray bars) 1, 3, 7, and 14 days after MCAO. Preconditioning with sevoflurane induced a significant decrease in the number of TUNEL-positive cells 3 and 7 days after ischemia in the sevoflurane-preconditioned group compared to the control group.  $n = 6$  per day and group. Data are presented as mean  $\pm$  SD. \*  $P < 0.05$  versus control group.

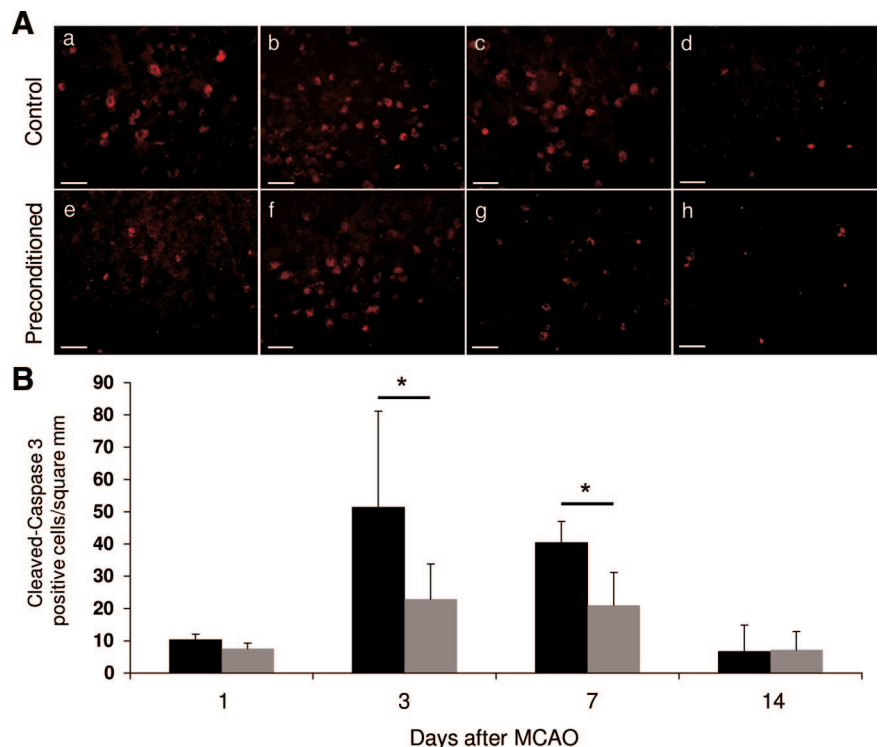


We used 2.7–3.0 vol% sevoflurane. One minimal alveolar concentration of sevoflurane is 2.3 vol% in adult rats.<sup>20</sup> Therefore, the sevoflurane concentration used in this study was relevant.

Sevoflurane preconditioning afforded transient neuroprotection against focal cerebral ischemia in rats as assessed by neurologic scores and infarct volumes. This

neuroprotection was effective on day 3, disappearing 7 and 14 days after ischemia. Investigators have recently begun examining whether sevoflurane-induced protection can occur through preconditioning before ischemic brain injury. In *in vitro* models, sevoflurane preconditioning of rat hippocampal slices 15 min before hypoxia and reoxygenation increased recovery of neuronal func-

**Fig. 5. (A)** Representative sections of cleaved caspase-3-positive cells in the inner boundary zone of the infarct in control (*a–d*) and sevoflurane-preconditioned (*e–h*) rats 1 (*a, e*), 3 (*b, f*), 7 (*c, g*), and 14 (*d, h*) days after middle cerebral artery occlusion (MCAO). Scale bar = 40  $\mu\text{m}$ . **(B)** Number of cleaved caspase-3-positive cells/ $\text{mm}^2$  in the inner boundary zone of the infarct in control (black bars) and sevoflurane-preconditioned (gray bars) groups 1, 3, 7, and 14 days after MCAO. The number of cleaved caspase-3-positive cells was significantly decreased 3 and 7 days after ischemia compared to the control group.  $n = 8$  per day and group. Data are presented as mean  $\pm$  SD. \*  $P < 0.05$  versus control group.



tion 24 h after hypoxia in a dose-dependent manner.<sup>5</sup> *In vivo*, Payne *et al.*<sup>12</sup> showed that sevoflurane-induced preconditioning against global cerebral ischemia reduced neuronal damage in rats 7 days after injury, but they did not evaluate this neuroprotection at remote timepoints. Some studies of APC on focal cerebral ischemia<sup>7,8,10,11,21</sup> demonstrated APC-induced neuroprotection, but they assessed neurologic injury only 24 to 96 h after ischemia and did not evaluate the long-term effect of preconditioning. Other studies of APC against hypoxia/ischemia in neonatal rats demonstrated long-term neuroprotection.<sup>9,14</sup> However, neonatal hypoxia/ischemia induced slowly progressive brain damage mainly related to apoptotic mechanisms,<sup>22</sup> whereas short-term necrotic cell death predominates after MCAO. It is therefore difficult to compare the protection afforded by APC between these two models. Volatile anesthetic-induced direct neuroprotection has been demonstrated when drugs were administered during the ischemic injury *in vivo*<sup>23–25</sup> and *in vitro*.<sup>2,26</sup> Results varied depending on the severity of injury. During incomplete cerebral ischemia<sup>23</sup> or mild focal ischemic injury induced by transient MCAO without permanent ligation of the common carotid artery,<sup>2</sup> volatile anesthetics provided 4 to 8 weeks of protection. Conversely, during severe ischemic injury, as described by Kawaguchi *et al.*<sup>13,27</sup> after transient MCAO with permanent ligation of the common carotid artery in rats, isoflurane reduced cerebral infarction rates at 2 and 4 days, but not 7 and 14 days, after ischemia. In a similar way, these authors reported a sevoflurane-induced improvement in neurologic outcome 2 days after MCAO, which disappeared 14 days after ischemia.<sup>27</sup>

It seems that this preconditioning effect is a shared feature among volatile anesthetics and may be mediated by common and interdigitated pathways activated by volatile anesthetics.<sup>28</sup> Possible candidate pathways have been reviewed at the myocardial<sup>29</sup> and cerebral<sup>3,30</sup> level. For example, activation of adenosine A1 receptors<sup>31</sup> and adenosine triphosphate-sensitive potassium channels,<sup>32</sup> through reactive oxygen species generation,<sup>33</sup> have been involved in myocardium and brain preconditioning by isoflurane<sup>7,21</sup> as well as by sevoflurane.<sup>5,34</sup> Recently, brain Akt activation, which controls the balance between survival and death signaling, has also been involved in APC.<sup>30</sup> These molecular mechanisms may, at least in part, explain that inhibition of ischemia-induced apoptosis also appears to play an important role in APC at the level of the myocardium<sup>35</sup> and the brain.<sup>14,36–37</sup> In this study, we present a timecourse for the antiapoptotic effect of sevoflurane preconditioning against cerebral ischemia. In the control group, apoptotic cells assessed by TUNEL and cleaved caspase-3 staining were observed from 1 to 14 days after MCAO and peaked 3 days after MCAO. Similar apoptosis timecourse results obtained with the same MCAO model were reported by Xu *et al.*<sup>38</sup> We show that sevoflurane preconditioning induced a

decrease in the number of apoptotic cells 3 days after ischemia that extended for 7 days after ischemia. Very few studies have produced evidence that APC can induce protection against ischemia-induced apoptosis. Wise-Faberowski *et al.*<sup>39</sup> showed *in vitro* that isoflurane preconditioning of rat neuronal cell cultures subjected to oxygen glucose deprivation led to a concentration-dependent reduction of oxygen glucose deprivation-induced neuronal apoptosis. *In vivo*, antiapoptotic effects of APC in the brain have been reported, either after permanent MCAO or on neonatal hypoxia/ischemia models, that an increase in the anti-apoptotic B-cell lymphoma-2 expression blocked cytochrome c release from mitochondria.<sup>14,36</sup> These authors moreover proposed a link between this increase in B-cell lymphoma-2 and an APC-induced activation of P38 mitogen-activated protein kinases. The *in vivo* effects of volatile anesthetics on ischemia-induced apoptosis were also studied when drugs were administered during ischemic injury and not before. Contradictory results were reported depending on severity of injury. During mild ischemic injury, volatile anesthetics induced a sustained decrease in key apoptotic protein expression,<sup>23</sup> whereas these drugs delayed but did not prevent ischemia-induced apoptosis during severe ischemic injury<sup>13</sup> and had to be administered with caspase inhibitors to obtain a sustained improvement of neurologic outcome.<sup>39–40</sup> Our results showed that sevoflurane-induced reduction in cerebral apoptosis was sustained longer than improvement in functional outcome. The discrepancy between functional outcome and apoptosis assessment may be related to a weakness in the neurologic scores, which did not allow subtle differentiation of impairment severity. It may also be due to a rapid recovery of neurologic function in rats, with no rat having a score above 10 after day 3. There are several limitations in this study. First, the study was limited to 14 days after ischemia. As suggested by Sakai *et al.*<sup>2</sup> longer periods of time may be necessary to assess the permanent neuroprotective effect of anesthetics. However, maintaining rats alive for such a long period of time after cerebral ischemia is not feasible in every laboratory and requires special skills and methods. This is clearly a subject for another specifically designed study. Second, the optimal dose and time lapse between administration of sevoflurane and ischemia to maximize neuroprotection are not clearly established, varying widely in the literature. We chose 45-min administration of approximately 1.2 minimum alveolar concentration sevoflurane for 1 h before the surgical procedure. A longer interval between preconditioning and ischemia might enhance neuroprotection through long-lasting changes in gene expression<sup>41</sup> or protein kinase activity.<sup>8</sup> In the study by Kapinya *et al.*<sup>10</sup> the interval between APC and ischemia up to 24 h did not change the neuroprotective effect. A larger dose of sevoflurane might provide better neuroprotection, but in one study with isoflurane,

minimum alveolar concentration values less than 1.5 provided better neurologic outcomes than higher concentrations.<sup>42</sup> Third, intras ischemic plasma glucose concentrations were higher in sevoflurane-preconditioned rats than in control animals. This may represent a confounding variable because hyperglycemia may worsen the outcome from cerebral ischemia.<sup>43</sup> Nevertheless, sevoflurane-preconditioned rats showed less cerebral necrosis and apoptosis. It is therefore unlikely that differences in plasma glucose concentration contributed to differences in outcome between the two groups. Finally, the control condition we chose here (anesthesia with sevoflurane during the surgical procedure) requires explanation. It seems likely that sevoflurane administration during the surgical preparation may have an impact on neurologic outcome. Indeed, in a study of sevoflurane preconditioning, we would have used an intravenous anesthetic for surgical preparation, except that this would be somewhat artificial in our opinion, because inhalational anesthesia is the most common anesthetic technique used in humans, and our study design reflects clinical practice. Moreover, most of the intravenous anesthetics also have an impact on neurologic outcome<sup>44</sup>; no matter which anesthetic had been used for surgery, the same criticism could have been formulated.

In conclusion, this study used an *in vivo* model of transient focal cerebral ischemia in rats to demonstrate that sevoflurane preconditioning induced effective but transient neuroprotective effects compared to sevoflurane administered immediately before ischemia. Sevoflurane preconditioning also decreased ischemia-induced apoptosis up to 7 days after the ischemic insult. Additional work is needed to evaluate the role of apoptosis reduction in APC and to search for the mechanisms associated with this effect. Clinically, there is much interest in determining the feasibility of anesthetic preconditioning of the brain before exposure to ischemic or mechanical injury from surgery or other interventional procedures and its impact on neurologic complications like perioperative stroke. It seems likely that this kind of neuroprotection cannot be achieved by a single agent but more probably by multiple approaches targeting different mechanisms. In light of this, sevoflurane preconditioning may offer a time window to limit the extent of cerebral damage, but future research is needed that compares the long-term histologic and functional consequences of a multi-targeting preconditioning approach.

## References

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