Fetal exposure to high isoflurane concentration induces postnatal memory and learning deficits in rats

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ABSTRACT

We developed a maternal fetal rat model to study the effects of isoflurane-induced neurotoxicity on the fetuses of pregnant rats exposed in utero. Pregnant rats at gestational day 14 were exposed to 1.3 or 3% isoflurane for 1 h. At postnatal day 28, spatial learning and memory of the offspring were examined using the Morris Water Maze. The apoptosis was evaluated by caspase-3 immunohistochemistry in the hippocampal CA1 region. Simultaneously, the ultrastructure changes of synapse in the hippocampal CA1 and dentate gyrus region were observed by transmission electron microscopy (TEM). The 3% isoflurane treatment group showed significantly longer escape latency, less time spent in the third quadrant and fewer original platform crossings in the Morris Water Maze test, significantly increased number and optical densities of caspase-3 neurons. This treatment also produced remarkable changes in synaptic ultrastructure compared with the control and the 1.3% isoflurane groups. There were no differences in the Morris Water Maze test, densities of caspase-3 positive cells, or synaptic ultrastructure between the control and 1.3% isoflurane groups. High isoflurane concentration (3%) exposure during pregnancy caused spatial memory and learning impairments and more neurodegeneration in the offspring rats compared with control or lower isoflurane concentrations.

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1. Introduction

Inhalation anesthetics such as isoflurane have been widely used in recent years in clinical and research practices. A growing body of evidence both in animals [1–3], and humans [4–6] supports the view that exposure to anesthetics early in life causes neurohistopathologic changes and long-term cognitive impairments. Our recent study also demonstrated gestational exposure to a clinically relevant concentration of isoflurane could cause neuron apoptosis, changes of synaptic structure, and postnatal spatial memory and learning impairments in the offspring rats [7,8]. Anesthesia given to immature rodents causes cognitive dysfunction, raising the possibility that the same might be true for millions of human fetuses, neonates and infants undergoing surgical procedures under general anesthesia each year. Nevertheless, the majority of prior neurodevelopmental studies focused on postnatal subjects rather than on the fetuses.

Many pregnant women, fetuses, and infants are exposed to a variety of anesthetic agents for surgical or diagnostic procedures each year. Pregnant women sometimes undergo general anesthesia during their pregnancy for surgeries unrelated to the delivery, such as fetal and non-obstetric surgeries, especially during mid-gestation [9,10]. It is estimated that some 1–2% of pregnant women in developed countries undergo anesthesia during their pregnancy for surgery unrelated to the delivery [11].

Since most general anesthetic agents are lipophilic and cross the placenta easily [12], the developing fetal brains will be exposed to anesthetics as well. We have previously shown that 1.3% isoflurane administered during pregnancy produced detectable effects to the rat pups [7,8]. Furthermore, in some cases, such as fetal surgery to correct various congenital malformations during mid-gestation, the fetal brain can be exposed to 2–3 times (2.5–3 Minimal Alveolar Concentration (MAC)) higher than clinically relevant concentrations of inhalation anesthetics to relax uterine smooth muscle and provide adequate anesthesia [10,13]. Fetal surgery is relatively new and rare, however, it is a rapidly growing and evolving area, and may become standard therapy for most disabling malformations that are currently treated in young infants [13,14]. Given the dose-dependent neurodegenerative properties
of anesthetics, we hypothesized that high isoflurane concentrations normally used during fetal surgery causes spatial memory and learning impairments and more neurodegeneration in offspring.

Here, we studied the potential effects of different isoflurane concentrations on neuroapoptosis and cognitive function on the offspring of pregnant rats exposed to anesthesia at gestational day 14. In addition, we investigated the changes of synaptic ultrastructure in the hippocampal area used transmission electron microscopy (TEM).

2. Materials and methods

2.1. Animals

All of the animals were treated according to the guidelines of the Guide for the Care and Use of Laboratory Animals (China Ministry of Health). The Laboratory Animal Care Committee of Zhejiang University approved all experimental procedures and protocols. All efforts were made to minimize the number of animals used and their suffering. The dams were housed in polypropylene cages, and the room temperature was maintained at 22 °C, with a 12-h light–dark cycle. The dams at gestational day 14 were used for all experiments, because this time corresponds approximately to mid-gestation in humans [15,16]; the period when most non-obstetric surgeries and fetal interventions are performed [9,10].

2.2. Anesthesia exposure

The dams were randomly divided into three groups: control, low concentration of isoflurane (1.3%), and high concentration of isoflurane (3%) treatment groups (n = 8). The dams were placed in plastic containers resting in water baths with a constant temperature of 38 °C. In these boxes, pregnant rats in isoflurane treatment groups were exposed to 1.3 or 3% isoflurane (Lot 826005U, Abbott Laboratories Limited, USA) in a humidified 30% oxygen carrier gas for 1 h; the control group was exposed to simply humidified 30% oxygen without any inhalational anesthetic for 1 h. We chose 1.3% because it represents 1 MAC in the pregnant rats [17], and 3% is equal to ~2 MAC. The determination of anesthetic duration based on our preliminary study which indicated that maternal physiological states remained stable throughout a 1-h isoflurane exposure. The isoflurane concentration, oxygen and carbon dioxide levels in the box were monitored with an agent gas monitor (Vamos, Drager Medical AG & Co. KgaA, Germany). Otherwise, control and experimental animals were under the same treatment and environment. Arterial blood gases (ABG) and blood glucose were measured at the end of the 1-h anesthetic exposure. The rectal temperature was maintained at 37 ± 0.5 °C. After exposure, all the dams were returned to their cages and allowed to deliver naturally. The postnatal body weights of the rat pups were monitored.

2.3. Memory and learning studies

Four rat pups (2 females and 2 males) from each dam were selected to determine cognitive function at postnatal day 28 with a Morris Water Maze test with minor modifications [1]. A round pool (diameter, 150 cm; depth, 50 cm) was filled with warm (24 °C) opaque water to a height of 1.5 cm above the top of the movable clear 15-cm-diameter platform in the third quadrant. A video tracking system recorded the swimming motions of animals, and the data were analyzed using motion-detection software for the Morris Water Maze (Actimetrics Software, Evanston, IL, USA). After every trial, each rat was wiped before returning to its regular cage, kept warm and allowed free access to food.

2.3.1. Place trials

The place trials were performed at postnatal day 29 for 4 days to determine the rats' ability to obtain spatial information. At postnatal day 28, rats were tested for their ability to swim to a visible platform through a 30-s swimming training. A dark black curtain surrounded the pool to prevent confounding visual cues. All rats received 4 trials per day in each of the four quadrants of the swimming pool. On each trial, rats were placed in a fixed position into the swimming pool facing the wall. They were allotted 120 s to find the platform upon which they sat for 20 s before being removed from the pool. If a rat did not find the platform within 120 s, the rat was gently guided to the platform and allowed to remain there for 20 s. For all training trials, swim speed and the time to reach the platform (escape latency) were recorded. The less time it took a rat to reach the platform, the better the learning ability. We took the average of four trials as the escape latency each day.

2.3.2. Probe trials

Probe trials were conducted immediately after the four-day period to evaluate memory retention capabilities. The probe trials involved the submerged platform of the third quadrant from the pool and allowing the rats to swim for 120 s in any of the four quadrants of the swimming pool. Time spent in the third quadrant and the number of original platform crossing in the third quadrant was recorded.

2.4. Transmission electron microscopy

After the Morris Water Maze test, six pups per group were anesthetized with a lethal dose of Nembutal. The thoracic cavities were opened and perfused intracardially with 100 mL of normal saline. Then the hippocampus, including CA1 and dentate gyrus area, of each rat was taken out immediately. Immersion fixation was completed on tissues about 1 mm³ from the hippocampus. Samples were rinsed in cold phosphate-buffered saline (PBS) and placed in 2.5% glutaraldehyde at 4 °C for 4 h. The tissue was rinsed in buffer and post-fixed with 1% osmium tetroxide for 1 h. Then, the tissue was rinsed with distilled water before undergoing a graded ethanol dehydration series and was infiltrated using a mixture of half propylene oxide and half resin overnight. Twenty-four hours later, the tissue was embedded in resin. 120 nm sections were cut and stained with 4% uranyl acetate for 20 min and 0.5% lead citrate for 5 min. Ultrastructure changes of synapse in the hippocampus were observed under a transmission electron microscope (Philips Tecnai 10, Holland).

2.5. Tissue section preparation

After the Morris Water Maze test, two pups from each dam were anesthetized by intraperitoneal injection of a lethal dose of Nembutal. The aorta was cannulated and the animal was firstly perfused with 200 mL of normal saline, then with 250 mL of 4% formaldehyde (freshly made from paraformaldehyde) for 20–30 min. The fixed brain was then removed from the cranial cavity and post-fixed overnight in the same fixative at 4 °C. The tissues were embedded in paraffin, and transverse paraffin sections containing the hippocampal area were mounted on silane-coated slides. Sections were deparaffinized and rehydrated. Then the sections were treated for antigen retrieval with 10.2 mmol/L sodium citrate buffer, pH 6.1, for 20 min at 95 °C for immunohistochemistry.

2.6. Immunohistochemistry for caspase-3

Caspase-3 positive cells were measured in the hippocampal CA1 region, using immunohistochemical methods described...
previously [7,8]. The brain region was chosen because it is particularly vulnerable to anesthesia-induced neurodegeneration [1] and is important to memory and learning. Briefly, the sections mentioned above were washed in 0.01 M PBS containing 0.3% Triton X-100 (pH 7.4, PBS-T), followed by blocking in 5% normal goat serum in 0.01 M PBS. The sections were then incubated in the primary antibodies rabbit polyclonal against anti-caspase-3 (1:200, Santa Cruz Biotechnology, USA) overnight at 4 °C. After a thorough wash in PBS, sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:200, Wuhan Boster Biological Technology, Ltd., China) for 2 h at room temperature, followed by avidin–biotin–peroxidase complex solution (ABC, 1:100, Wuhan Boster Biological Technology, Ltd., China) for 2 h at room temperature. Immunolabeling was visualized with 0.05% diaminobenzidine (DAB, Wuhan Boster Biological Technology, Ltd., China) plus 0.3% H2O2 in PBS and the reaction was stopped by rinsing the slides with 0.2 M Tris–HCl. Sections were mounted onto 0.02% poly-L-lysinecoated slides and allowed to dry at room temperature. Then the sections were dehydrated through a graded series of alcohols, cleared in xylene and finally coverslipped. Rat Immunoglobulin IgG (1:200, Biomeda Corporation, USA) was used instead of primary antibody as a negative control. Other chemicals used in this study were provided by Cell Signaling Technology (Beverly, MA). Three sections from hippocampal CA1 region of each animal were randomly selected and images were photographed under 400× magnification in 3 visual fields/per section, the caspase-3 positive neurons were counted in the same area. The optical densities of caspase-3 positive neurons were measured quantitatively using Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Silver Spring, USA). The optical density of caspase-3 positive cells in a particular brain region was calculated by dividing the integrated optical density of caspase-3 positive cells by the area of that brain region.

2.7. Statistical analysis

All data were presented as mean ± S.E.M. Results of weight of postnatal rat pups and place trials of postnatal rats were analyzed using 2-way ANOVA for repeated measurements. Other data were analyzed using one-way ANOVA, followed by Tukey post hoc multiple comparison tests. A P value of <0.05 was considered statistically significant. All statistical tests and graphs were performed or generated, respectively, using Graph-Pad Prism Version 4.0 (GraphPad Prism Software, Inc., CA, USA).

3. Results

3.1. Physiologic parameters

As shown in Table 1, ABG values and blood glucose levels were within the normal physiologic range. There were no significant differences between the low and high concentrations of isoflurane treatment groups on any measured variables for ABG values and blood glucose levels. Taking these measures reduces the possibility that isoflurane-induced neurodegeneration in the fetal brains was caused by physiologic side effects (e.g., hypoglycemia, hypoxia and hypercapnia). All pups were viable and there were no significant differences in growth rate of the rat pups among the three groups (P0, 7.23 ± 0.55, 7.24 ± 0.49 and 7.18 ± 0.67 g; P28, 102.26 ± 3.45, 103.19 ± 4.15 and 101.78 ± 4.15 g, in control, 1.3% and 3% isoflurane-exposed pups, respectively).

<table>
<thead>
<tr>
<th>Maternal physiological parameters during isoflurane exposure.</th>
<th>0h</th>
<th>1h</th>
<th>0.3% Iso</th>
<th>3% Iso</th>
<th>0.3% Iso</th>
<th>3% Iso</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.45 ± 0.02</td>
<td>7.41 ± 0.02</td>
<td>7.44 ± 0.02</td>
<td>7.36 ± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaCO2 (mmHg)</td>
<td>40.8 ± 2.01</td>
<td>40.6 ± 1.64</td>
<td>43.6 ± 2.65</td>
<td>43.9 ± 3.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PaO2 (mmHg)</td>
<td>159 ± 5.46</td>
<td>162 ± 4.25</td>
<td>163 ± 6.98</td>
<td>166 ± 5.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SaO2 (%)</td>
<td>96.7 ± 1.3</td>
<td>95.3 ± 1.1</td>
<td>96.1 ± 0.9</td>
<td>95.4 ± 1.2</td>
<td></td>
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</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>114 ± 16</td>
<td>116 ± 20</td>
<td>115 ± 21</td>
<td>114 ± 16</td>
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</table>

Values are mean ± S.E.M. n = 8 for each group.

Iso = isoflurane; PaCO2 = arterial carbon dioxide tension; PaO2 = arterial oxygen tension; SaO2 = arterial oxygen saturation.
3.2. Morris Water Maze test

Pups whose mother anesthetized with 3% isoflurane showed a significantly worse performance in the water maze. As shown in Fig. 1A, pups in all groups showed a rapid decrease in latency. While the pups of the 3% isoflurane group spent more time to find the platform than those of 1.3% isoflurane group and control group in the place trial \( F(2,63.969) = 4.715, P < 0.05 \). Swimming speeds were also analyzed during place trials, and no differences were observed among three groups. In the probe test, the number of crossing over the former platform location in 3% isoflurane-treated pups was fewer than the others \( F(2,29) = 5.265, P < 0.05 \); Fig. 1B), as well as the time spent in the third quadrant where the platform located \( F(2,29) = 4.417, P < 0.05 \); Fig. 1C). There were no significantly differences either in the place trial or in the probe test between the control and 1.3% isoflurane groups.

![Figures](image1.png)

**Fig. 2.** Rats exposed to isoflurane in utero at high concentration (3%) increased apoptosis in the hippocampus CA1 region. (Aa) Caspase-3 immunohistochemical staining in control pups \( \times 400 \). (Ab) Caspase-3 immunohistochemical staining in 1.3% isoflurane-exposed pups \( \times 400 \). (Ac) Caspase-3 immunohistochemical staining in 3% isoflurane-exposed pups \( \times 400 \). (B) The number (Ba) and optical density (Bb) of caspase-3 positive neurons in each group. Iso, isoflurane. Data represent mean ± S.E.M. of 48 sections of 16 postnatal rats from 8 pregnant mothers \( (n = 8) \) in each group. **P < 0.01 compared to control, ***P < 0.001 compared with both control and 1.3% Iso, ### P < 0.001 compared to 1.3% Iso. Scale bar = 50 μm.

![Figures](image2.png)

**Fig. 3.** Ultrastructural changes of synapse in the CA1 and dentate gyrus area of hippocampus under TEM. (A) TEM showed that 3% isoflurane (c) significantly decreased the number of synapses in pups compared to control (a) and 1.3% isoflurane (b) group (magnification, \( \times 6200 \)). Scale bar = 2 μm. (B) Higher magnification image (magnification, \( \times 24,000 \)) showed widened synaptic cleft and disintegration of postsynaptic densities in pups after 3% isoflurane exposure in utero. Arrows = synaptic cleft; arrowheads = postsynaptic densities. Scale bar = 0.5 μm.
3.3. Immunoreactivity assay

The 1.3% isoflurane for 1 h did not significantly affect the number and optical densities of caspase-3 positive neurons in the hippocampal CA1 region of the pups when compared with the control (Fig. 2Aa and b). However, 3% isoflurane for 1 h significantly increased caspase-3 number (222% increase over the control, F(2,27) = 13.55, P < 0.001; Fig. 2Ac and Ba) and optical densities in the CA1 region of the hippocampus (129% increase over the control, F(2,27) = 8.353, P < 0.01; Fig. 2Ac and Bb).

3.4. Ultrastructure changes in synapse of hippocampus

Synapses with postsynaptic densities, an inerratic synaptic cleft and a presynaptic ves were clearly visible in the control pups (Fig. 3Aa and Ba). The structure of synapse in the hippocampal CA1 and dentate gyrus region of pups whose mothers received 1.3% isoflurane treatment for 1 h was not significantly impaired (Fig. 3Ab and Bb). However, in the 3% isoflurane-treated pups, the number of synapses decreased in the dentate gyrus and CA1 area, while a widened synaptic cleft, thinned postsynaptic densities and loss of a presynaptic ves were observed (F(2,26) = 5.406, P < 0.05; Fig. 3Ac and Bc, Table 2).

4. Discussion

In the present study, we employed a new model, a maternal fetal rat model, to study the behavioral and neurotoxic effects of exposure to different isoflurane concentrations. The outcome of our study shows that 1 h of isoflurane anesthesia at a high concentration (3%) in pregnant rats impaired postnatal spatial memory and learning in the offspring rats, whereas pups that received low (1.3%) concentrations behaved similarly to control pups. Moreover, there was a tendency of increased apoptosis observed at the hippocampal level in pups subject to high concentration of isoflurane anesthesia, as well as remarkable impairments of synaptic ultrastructure in the hippocampal CA1 and dentate gyrus region.

Learning and memory are important aspects of cognitive function. The Water Maze protocol evaluates long-term/reference memory that involves a sequence of specific molecular processes in the CA1 area of the hippocampus [18]. The place trials were performed to determine the rats’ ability to obtain spatial information and the probe trials were conducted to evaluate memory retention capabilities. Our results showed that prenatal exposure to isoflurane at a high concentration (3%) displayed deficits in postnatal spatial learning and memory capabilities in pups as manifested by the longer escape latency to reach the platform, the fewer times of original platform crossing and the less time spent in the target quadrant in the Morris Water Maze test. The lack of differences in swimming speeds of all groups excluded the possibility that sensorimotor disturbances in any of the groups could have influenced the learning and memory deficits observed in our study. These behavioral changes are unlikely to be associated with an indirect deleterious effect of isoflurane on pregnancy because maternal physiological parameters during isoflurane anesthesia were normal, and there were no differences in non-cognitive variables, such as litter size, viability, and weight among the three groups, further suggesting that the fetal rat brain was impaired by maternally administered 3% isoflurane.

The observation of impaired performance in a spatial learning and memory test after high concentration anesthesia is in agreement with previous studies focusing on the developing neonatal brains of rodents [1,19] and the fetal brains of guinea pigs [20]. Taken together, these findings clearly reinforce the idea that high concentrations of isoflurane anesthesia are capable of causing neurodegeneration, even cognitive deficits of their offspring. However, the effects of anesthesia used during the development of fetal brains on postnatal memory and learning ability are controversial, with transient improvement [21], no effects [22] and permanent impairment [1,7,8] all being reported. These discrepancies could be due to methodological differences, species differences (rats vs. mice), pharmacological differences (isoflurane vs. sevoflurane), differences in anesthetic concentrations (0.5–2 MAC), or differences in anesthetic durations (1–6 h). Last but not the least is the time of isoflurane exposure. Since different neurodevelopmental events are performed in their timing relative to gestational age, it is expected that the vulnerability of the brain to the adverse effects of the anesthetic agents would be different depending on the time of exposure. Correspondingly, behavioral outcome varies as a function of the neurodevelopmental events occurring at the time of exposure. Altered neurodevelopmental programming in utero, cognitive deficits, psychiatric disturbances, and other diseases may occur [23–25]. The time of isoflurane exposure in the current study corresponds approximately to mid-gestation in human, and studies in several animal species suggest that susceptibility is limited to a brain developmental stage corresponding to the human second trimester of pregnancy. Together with our previous study [7,8], these results suggest that whether isoflurane induces neurodegeneration in the fetal rat brain or subsequent cognitive impairments depending on the time of isoflurane exposure (mid-gestation), higher concentrations (3% or around 2 MAC for 1 h), and longer anesthetic durations (1.3% for 6 h). These results are consistent with the dose- and time-dependent toxic effects of isoflurane in tissue cultures [26,27] and newborn animals [1].

The tendency of increased apoptosis in the hippocampal CA1 region in pups exposed to 3% isoflurane is in agreement with the impaired performance of these rats in the water maze test. The hippocampus plays a major role in spatial learning and memory [28], and its synaptic plasticity is altered by the majority of agents used in general anesthesia [1,7,8]. It is widely recognized that there is a relationship between hippocampal synaptic plasticity and learning and memory [29–31]. In our study, 1.3% isoflurane treatment did not significantly affect normal structure of synapses. However, 3% isoflurane induced sharp changes of synaptic ultrastructure in the dentate gyrus and CA1 area characterized by the decreased synapse number, the widened synaptic cleft and the thinned postsynaptic densities. The synaptic cleft is a region of information transmission among neurons and plays an important role in the dynamics of synaptic activity. The postsynaptic density is the material basis of synaptic efficacy. The thickness of postsynaptic densities and the ability of learning and memory training and memory retention go hand in hand [30,32]. A decreased number of synapses, a widened synaptic cleft and thinned postsynaptic densities changed synaptic activity. Taken together, we speculate that 3% isoflurane for 1 h significantly increased apoptosis in the CA1 area of hippocampus, leading to impairments in synapse structure and function and consequent damage in synaptic plasticity, and finally to spatial learning and memory deficits.

### Table 2

The hippocampal synaptic structural parameters among groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>1.3% isoflurane</th>
<th>3% isoflurane</th>
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</thead>
<tbody>
<tr>
<td>Numerical density (N/μm²)</td>
<td>2.45 ± 0.18</td>
<td>2.41 ± 0.20</td>
<td>1.44 ± 0.17</td>
</tr>
<tr>
<td>Width of synaptic cleft (nm)</td>
<td>24.91 ± 2.01</td>
<td>24.09 ± 2.16</td>
<td>33.26 ± 2.65</td>
</tr>
<tr>
<td>Postsynaptic density (nm)</td>
<td>76.59 ± 7.41</td>
<td>75.52 ± 6.25</td>
<td>50.25 ± 6.98</td>
</tr>
</tbody>
</table>

Data represent mean ± S.E.M. of 6 postnatal rats from 8 pregnant mothers (n = 8) in each group. 
P < 0.05 compared with both control and 1.3% isoflurane.
The mechanisms of inhalation anesthetic-mediated neurodegeneration in the developing brain are still not clear. There is a hypothesis that inhalational anesthetics, such as isoflurane, might induce cell death processes through activation of γ-aminobutyric acid type A receptors and/or inhibition of N-methyl-D-aspartate (NMDA) receptors in the developing brain [1,3,33,34], although this view has not been established definitively. Our recent studies suggest that CHOP and caspase-12-mediated ER stress-induced cell death appear to be the major mediators of anesthesia-mediated apoptotic cellular death [7]. In addition, we also demonstrate that inhalational anesthetics induce spatial memory and learning impairments through the down-regulation of GAP-43 and NPY in the hippocampus [8], the up-regulation of C/EBP homologous transcription factor protein (CHOP) and caspase-12 [7], and consequent impairments in synaptic plasticity [7,8]. Given the rapid development of fetal surgery [13,14] and the recent concerns over the possible harmful implications of the various anesthetic drugs at various stages of neurodevelopment, it is in urgent need of a better understanding of how maternal general anesthesia affects the developing fetal brain. A better understanding of the mechanisms of clinically relevant anesthetic neurotoxicity will help us to define the scope of the problem in humans and develop strategies that will minimize the possible harmful effects of general anesthesia to patients.

Acknowledgments

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