



REVISTA BRASILEIRA DE ANESTESIOLOGIA

Publicação Oficial da Sociedade Brasileira de Anestesiologia
www.sba.com.br



SCIENTIFIC ARTICLE

Effects of repeated exposure to different concentrations of sevoflurane on the neonatal mouse hippocampus



Omid Azimaraghi^a, Maryam Nezhad Sistani^b, Mohammad-Amin Abdollahifar^b,
Ali Movafegh^a, Anahid Maleki^c, Ebrahim Soltani^c, Alireza Shahbazkhani^d,
Reza Atef-Yekta^{a,*}

^a Tehran University of Medical Sciences, Dr. Shariati Hospital, Department of Anesthesiology and Critical Care Medicine, Tehran, Iran

^b Shahid Beheshti University of Medical Sciences, Department of Anatomical Sciences, Tehran, Iran

^c Tehran University of Medical Sciences, Children Medical Center Hospital, Department of Anesthesiology, Tehran, Iran

^d Tehran University of Medical Sciences, Dr. Ali Shariati Hospital, Anesthesiology Research Development Center, Tehran, Iran

Received 14 November 2017; accepted 4 September 2018

Available online 25 September 2018

KEYWORDS

Sevoflurane;
Hippocampus;
Neonatal mice;
Stereology;
Neurotoxicity

Abstract

Background and objectives: Developing brain is more vulnerable to environmental risk than is the developed brain. We evaluated the effects of repeated exposure to different concentrations of sevoflurane on the neonatal mouse hippocampus using stereological methods.

Methods: Eighteen neonatal male mice were randomly divided into three groups. Group A, inhaled sevoflurane at a concentration of 1.5%; Group B, inhaled sevoflurane at a concentration of 3%; and Group C (control group), inhaled only 100% oxygen. Treatments were applied for 30 min a day for 7 consecutive days. The hippocampal volume, dendrite length, number of neurons, and number of glial cells were evaluated in each group using stereological estimations.

Results: We identified a ~2% reduction in the volume of the hippocampus in Group A compared to Group C. Mean hippocampal volume was ~11% smaller in Group B than it was in Group C. However, these differences in hippocampal volume between the groups were not statistically significant ($p > 0.05$ for all). As for the number of neurons, we found significantly fewer neurons in Group A (~29% less) and Group B (~43% less) than we did in Group C ($p < 0.05$ for both). The dendrite length was ~8% shorter in Group A and ~11% shorter in Group B than it was in Group C.

* Corresponding author.

E-mail: r.atefyekta@gmail.com (R. Atef-Yekta).

PALAVRAS-CHAVE

Sevoflurano;
Hipocampo;
Ratos neonatais;
Estereologia;
Neurotoxicidade

Conclusions: Repeated exposure to sevoflurane, regardless of the concentration, reduced the volume of the neonatal mouse hippocampus, as well as the number of neurons and dendrite length.

© 2018 Sociedade Brasileira de Anestesiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Efeitos da exposição repetida a diferentes concentrações de sevoflurano sobre o hipocampo de ratos neonatos**Resumo**

Justificativa e objetivos: O cérebro em desenvolvimento é mais vulnerável ao risco ambiental que o cérebro já desenvolvido. Avaliamos os efeitos da exposição repetida a diferentes concentrações de sevoflurano sobre o hipocampo de ratos neonatos usando métodos estereológicos.

Métodos: Dezoito ratos neonatos foram divididos aleatoriamente em três grupos. O Grupo A foi submetido à inalação de sevoflurano a uma concentração de 1,5%; o Grupo B foi submetido à inalação de sevoflurano a uma concentração de 3%; o Grupo C (controle) foi submetido à inalação de apenas oxigênio a 100%. Os tratamentos foram aplicados durante 30 minutos por dia, durante sete dias consecutivos. Volume do hipocampo, comprimento do dendrito, número de neurônios e número de células gliais foram avaliados em cada grupo usando estimativas estereológicas.

Resultados: Identificamos uma redução de ~2% no volume do hipocampo no Grupo A em comparação com o Grupo C. O volume médio do hipocampo foi ~11% menor no Grupo B que no Grupo C. Entretanto, essas diferenças no volume do hipocampo entre os grupos não foram estatisticamente significativas ($p > 0,05$ para todos). Quanto ao número de neurônios, encontramos um número significativamente menor de neurônios no Grupo A (~29% menos) e no Grupo B (~43% menos) que no Grupo C ($p < 0,05$ para ambos). O comprimento do dendrito foi ~8% menor no Grupo A e ~1% menor no Grupo B que no Grupo C.

Conclusões: A exposição repetida ao sevoflurano, independente da concentração, reduziu o volume do hipocampo neonatal de camundongos, bem como o número de neurônios e o comprimento dos dendritos.

© 2018 Sociedade Brasileira de Anestesiologia. Publicado por Elsevier Editora Ltda. Este é um artigo Open Access sob uma licença CC BY-NC-ND (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Normal brain development depends on the appropriate formation of neuronal synapses and on the proper flow of information between neurons during the process of synaptogenesis.¹ The Brain Growth Spurt (BGS) is a period of extensive brain growth and maturation that occurs during mammalian brain development. In humans, the BGS begins during the last trimester of pregnancy and extends throughout the first two years of life.² In rodents, the BGS occurs during the neonatal period.³ Research shows that neonatal exposure to xenobiotics during the BGS can negatively affect neonatal brain development, resulting in impaired cognitive function in the adult mouse.⁴ However, the effects of specific anesthetic agents, such as sevoflurane, on brain development are still unclear.

Sevoflurane is a sweet-smelling, nonflammable, highly fluorinated methyl isopropyl ether that is often used as an inhalational anesthetic agent for inducing anesthesia

through a facemask in children, owing to its fast onset and offset.⁵ The most important characteristic of sevoflurane, when compared with older anesthetics such as isoflurane or halothane, is its lower blood solubility, which allows the level of sevoflurane in the brain to increase faster during anesthesia induction.^{6,7} The Minimum Alveolar Concentration (MAC) of sevoflurane, i.e., the concentration of sevoflurane in the lungs that is needed to prevent movement (motor response) in 50% of subjects in response to a surgical (pain) stimulus, decreases as age increases, thus higher MACs are required when administering sevoflurane during the neonatal and childhood period.⁸ Unfortunately, the effects that repeated exposures to different sevoflurane concentrations may have on the developing brain remain unknown.

Therefore, the aim of the current study was to evaluate the effects that repeated exposures to different concentrations of sevoflurane have on the neonatal mouse hippocampus by using stereological estimations.

Methods

Animals and brain preparation

Eighteen neonatal male mice were obtained from I.R. Iran Pasteur Institute (Tehran, Iran). All experimental mice were the product of mating between adult male and female mice that were kept in an animal house under standard conditions at a temperature of 22–24°C and on a 12 h light–dark cycle with free access to food and water. After mating, the pregnancy process was followed until delivery.

Forty-eight hours after birth, neonatal mice were placed in plastic containers, which were connected to an anesthetic machine with a pipe. The animals were randomly divided into three groups, as follows: Group A, 6 male neonatal mice (48 h old) inhaled sevoflurane at a concentration of 1.5% for 30 min a day for 7 consecutive days; Group B, 6 male neonatal mice (48 h old) inhaled sevoflurane at a concentration of 3% for 30 min a day for 7 consecutive days; and Group C (control group), 6 male neonatal mice (48 h old) inhaled 100% oxygen for 30 min a day for 7 consecutive days. Twenty-four hours after the last sevoflurane treatment, the mice were decapitated and the brains were completely excised through a midline incision in the skull. Then, the right hemispheres were immersed in neutral buffered formalin. Afterwards, the tissues were processed, embedded in paraffin, serially coronally sectioned (26- μm thick), and stained using cresyl violet (1% cresyl violet acetate in distilled water). Further, the blocks of the left hemispheres were processed for the Golgi impregnation procedure. Necrotic neurons were studied through hematoxylin and eosin staining.

Hippocampal volume determination using stereology

The location of the hippocampus in various sections was identified using the Paxinos Atlas. Cavalieri's technique was used to determine the volume of the hippocampus. First, the tissue was serially sectioned. Next, 10 tissue sections were selected at random. A projecting microscope (Nikon®, Japan) at a final magnification of 100 \times and a point probe were used to determine the hippocampal volume (in μm^3). The point probe had a grid of points, which was randomly superimposed on the tissue sections in three regions (CA1, CA2, and CA3), and the volume was estimated using the following equation: $V = \Sigma p \times (a/p) \times d$, where Σp is the number of points hitting the hippocampal volume, a/p is every point surface, as calculated with the below equation and a caliper on the microscope, and d is the distance between the sampled sections $a/p = (\Delta x \times \Delta y) / M^2$.

In the above equation, Δx and Δy are the distances between points on the x and y axes and M^2 is the square of the microscope magnification.

Determination of the number of hippocampal neurons

The Optical Dissector technique was used to determine the number of cells in the hippocampus including the number of neurons, glial cells, and degenerative neurons. A counting

frame specifically designed for cell counting was used. The entire visual field of each section (25- μm thickness) was evaluated with a microscope using a 40 \times objective lens BX41TE; Olympus, Japan). According to the grid counting method, the cells with their nuclei either inside the grid or on the grid borderlines were counted, while those with nuclei outside the grid lines were not counted. Then, the numerical density of the various hippocampal cells was calculated with the following equation: $Nv = (\sum_{i=1}^n Q) / (h \times \sum_{i=1}^n P \times a/f)$, where $\sum_{i=1}^n Q$ is the sum of the counted cells, $\sum_{i=1}^n P$ is the sum of the points hitting the selection fields, h is the height from which counting was done, and a/f is the proportion of the frame area to real tissue.

Determination of the hippocampal neuron dendrite length

Tissue sections (26- μm thick) that were stained using the Golgi method were used to estimate the dendrite length of hippocampal neurons. Some regions of the hippocampus were projected on to the monitor using a microscope (E-200; Nikon) equipped with an objective lens (40 \times numerical aperture of 1.4). Then, the dendrite length was measured using a cycloid grid, which was placed on the hippocampal dendrites parallel to the vertical axis. To estimate the dendrite length, all of the cross-sections between the dendrites' axes and cycloids were measured, and then estimated using the following equation: $T_N = 2 \times (a/l) \times (1/asf) M^{-1} \Sigma l$, where a/l is the examination area along every cycloid, asf is an area related to the cycloid grid, M is the magnification at 4000 \times , and Σl is the sum of the cross-sections between the dendrites and cycloid grid.

Statistical analysis

The collected data were compared among the groups by using non-parametric statistical tests such as the Kruskal–Wallis and Mann–Whitney U tests, which were performed with SPSS software. Differences were considered significant at $p < 0.05$.

Results

Hippocampal volume

Our analyses revealed that the mean hippocampal volume was ~2% smaller in Group A (inhaled 1.5% sevoflurane) and 11% smaller in Group B (inhaled 3% sevoflurane) than it was in Group C (control group, inhaled oxygen) (Fig. 1). However, the differences in hippocampal volume between the groups were not statistically significant ($p > 0.05$ for all).

Dendrite length

The analyses revealed that the dendrites were ~8% shorter in Group A and ~11% shorter in Group B than they were in Group C (Fig. 2). The differences between the groups were not statistically significant ($p > 0.05$ for all).

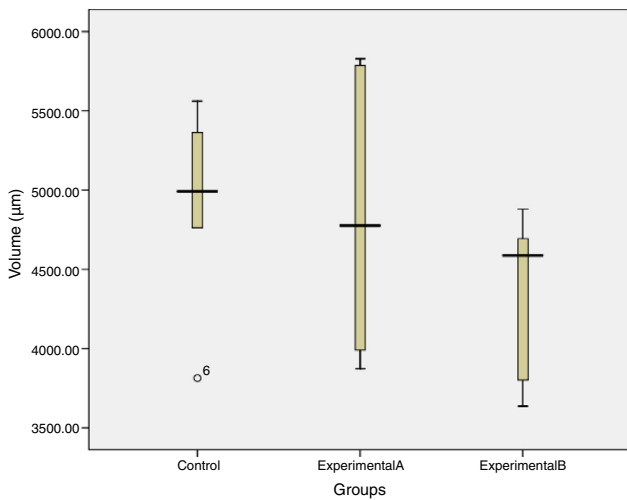


Figure 1 Hippocampal volume distributions of the study groups. Experimental Group A; neonatal mice whom inhaled sevoflurane at a concentration of 1.5%. Experimental Group B; neonatal mice whom inhaled sevoflurane at a concentration of 3% and Group C (control group), neonatal mice whom inhaled 100% oxygen. The figure shows the mean difference in hippocampal volume. The mean hippocampal volume was smaller in Group A and Group B compared to the control group ($p > 0.05$).

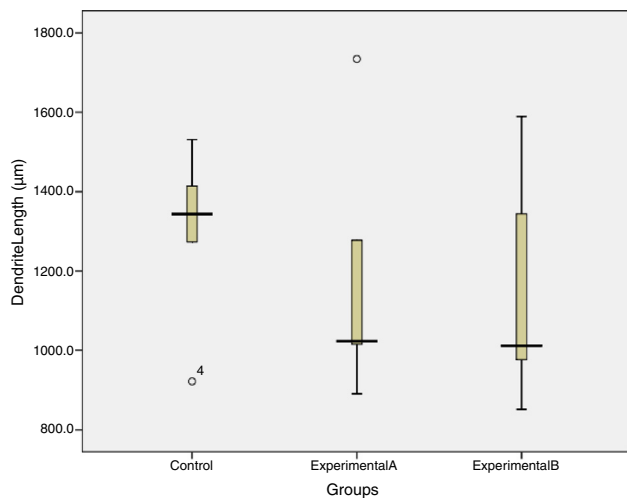


Figure 2 Distributions of the hippocampal neuron dendrite lengths in the study. Experimental Group A; neonatal mice whom inhaled sevoflurane at a concentration of 1.5%. Experimental Group B; neonatal mice whom inhaled sevoflurane at a concentration of 3% and Group C (control group), neonatal mice whom inhaled 100% oxygen. The figure shows the mean difference in Neuron Dendrite Lengths in the study groups. The analyses revealed that the dendrites were shorter in Group A and Group B compared to Group C. The differences between the groups were not statistically significant ($p > 0.05$).

Neuron and glial cell counts

Compared to Group C, ~29% and ~43% fewer neurons were found in Groups A and B, respectively, and these differences were statistically significant ($p < 0.05$ for both). However,

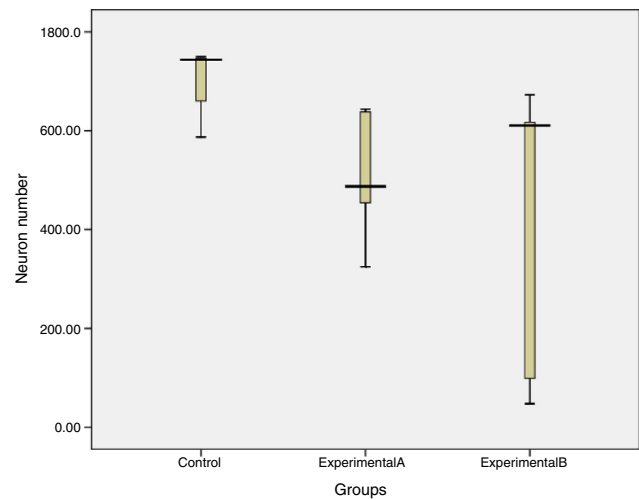


Figure 3 Hippocampus neuron number distribution of the study groups. Experimental Group A; neonatal mice whom inhaled sevoflurane at a concentration of 1.5%. Experimental Group B; neonatal mice whom inhaled sevoflurane at a concentration of 3% and Group C (control group), neonatal mice whom inhaled 100% oxygen. Fewer neurons were found in Groups A and B and these differences were statistically significant ($p < 0.05$).

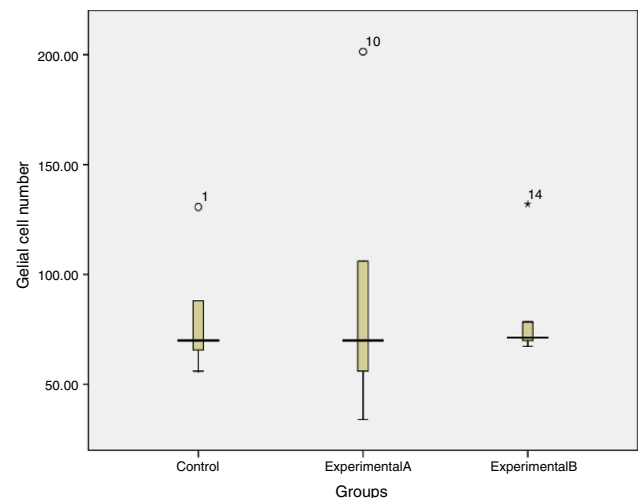


Figure 4 Hippocampus glial cell numbers of the study groups. Experimental Group A; neonatal mice whom inhaled sevoflurane at a concentration of 1.5%. Experimental Group B; neonatal mice whom inhaled sevoflurane at a concentration of 3% and Group C (control group), neonatal mice whom inhaled 100% oxygen. As for the glial cell counts, our analyses demonstrated more glial cells in Group A and in Group B compared to Group C. However, the differences between the groups were not statistically significant ($p > 0.05$).

the neuron counts of Groups A and B were not significantly different ($p > 0.05$) (Fig. 3).

As for the glial cell counts, our analyses demonstrated ~12% more glial cells in Group A and ~2% more glial cells in Group B compared to Group C. However, the differences between the groups were not statistically significant ($p > 0.05$ for all) (Fig. 4).

Discussion

The present study determined the effects that repeated exposure to different concentrations of sevoflurane had on the neonatal mouse hippocampus using stereological estimations. The findings suggested that repeated exposure to sevoflurane, regardless of the concentration, reduces the hippocampal volume, number of neurons, and dendrite lengths in neonatal mice. We also found that all of these reductions were slightly worse when a higher concentration of sevoflurane was used. Although the hippocampal volumes, neuron counts, and dendrite lengths were not significantly different between the two sevoflurane concentrations we tested, the differences may be important clinically and physiologically.

Many studies have discussed the short- and long-term effects of volatile anesthetics on neurogenesis and neurocognitive functions. Though various researchers have suggested that sevoflurane may have detrimental effects on the brain during childhood, discrepancies exist regarding what exposure dose, duration, and timing may cause the harmful effects.^{9–12} Previously, Zhou et al. studied the effects of administering different sevoflurane doses on the developing brain and showed that higher doses of sevoflurane lead to histopathological changes and apoptosis in the neonatal rat hippocampus, as well as temporal neurocognitive deficits.¹³ Our data are consistent with these previous findings, even though we used different sevoflurane concentrations and different methods of evaluating the hippocampal changes. In another study, Wang et al. found that 7-day-old rats exposed to 2.5% sevoflurane for 4 h showed significant spatial learning and memory impairments, as determined with the Morris water maze test.¹⁴ Additionally, Fang et al. found that multiple maternal sevoflurane exposures inhibited neurogenesis in the developing brains of mouse fetuses and revealed that this inhibition was regulated via the Pax6 pathway.¹⁵ In contrast, Chen et al. demonstrated that exposure to a low concentration of sevoflurane actually increases hippocampal neurogenesis in neonatal rats.¹⁶

Most of the abovementioned studies support that early life exposure to sevoflurane can induce long-term neurocognitive deficits, potentially through neuronal apoptosis. However, a study by Lu et al. suggested that neuronal apoptosis might not contribute to the long-term cognitive dysfunction that is observed after exposure to 2% sevoflurane, but rather that the sevoflurane-induced neurodevelopmental effects may depend on various factors.¹⁷

Here, we evaluated the sevoflurane-induced hippocampal changes using stereological estimations. This approach likely yielded more-accurate estimations of the hippocampal volume, neuron and glial cell counts, and dendrite lengths than those identified in previous reports.

From the clinical point of view, the most pronounced effects of early-life stress has been shown to have effects on the hippocampal anatomical structures which affect human health in later life.¹⁸ Previous studies have reported small hippocampal volumes before the manifestation of clinical symptoms of major depressive disorder.¹⁹ Hippocampal atrophy and its clinical correlates in subjects with Alzheimer's disease, mild cognitive impairment, and elderly controls have also been reported which bolsters the importance of

the anatomical structure of hippocampus on manifestation of various diseases.²⁰

Several limitations of our study should be noted. First, we only examined the hippocampus. Thus, additional studies on the changes that occur in response to sevoflurane in other regions of the brain are needed. Especially, further studies that focus on the possible short- and long-term effects of early life exposure to sevoflurane on the development of the motor and sensory systems should be performed. Second, we only studied male neonatal mice to reduce the possibility of sex-related biases in brain development. Hence, studies that use both sexes are required to help clarify the effects of sex on the sevoflurane-induced neuronal changes. Finally, we did not investigate the mechanisms underlying the sevoflurane-induced hippocampal changes we observed. While the evidence acquired regarding the changes that occur in developing animal models after sevoflurane exposure is certainly undeniable, continued studies into the mechanisms of anesthesia-induced apoptosis are essential.

Conclusion

Using stereological estimations, the present study revealed that repeated exposure to sevoflurane, regardless of concentration, induced reductions in the hippocampal volume, number of neurons, and dendrite lengths of neonatal mice. Given that sevoflurane is used on a daily basis for inducing and maintaining anesthesia in pediatric operating rooms and given the increasing number of premature babies undergoing a variety of surgeries, understanding the possible effects that anesthesia and surgery may have on infants and young children is essential for determining at what point the risks outweigh the benefits and surgery should not be performed. Our study provides data that adds to our knowledge of the effects that anesthetic agents have on the developing brain.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors would like to thank the staff at Dr. Shariati Hospital and the Tehran University of Medical Sciences Animal Lab who assisted us in this research. The authors are also grateful to the Pediatric Urology Research Center for supporting this study. This study was funded by Tehran University of Medical Sciences.

References

1. Jevtovic-Todorovic V. Developmental synaptogenesis and general anesthesia: a kiss of death? *Curr Pharm Des.* 2012;18:6225–31.
2. Mariussen E. Neurotoxic effects of perfluoroalkylated compounds: mechanisms of action and environmental relevance. *Arch Toxicol.* 2012;86:1349–67.
3. Viberg H, Fredriksson A, Jakobsson E, et al. Neurobehavioral derangements in adult mice receiving decabrominated diphenyl

- ether (PBDE 209) during a defined period of neonatal brain development. *Toxicol Sci.* 2003;76:112–20.
4. Buratovic S. Low-dose ionizing radiation induces neurotoxicity in the neonate: acute or fractionated doses and interaction with xenobiotics in mice. Doctoral dissertation, Acta Universitatis Upsaliensis; 2016.
 5. Sakai EM, Connolly LA, Klauck JA. Inhalation anesthesiology and volatile liquid anesthetics: focus on isoflurane, desflurane, and sevoflurane. *Pharmacotherapy.* 2005;25:1773–88.
 6. Lerman J, Sikich N, Kleinman S, et al. The pharmacology of sevoflurane in infants and children. *Anesthesiology.* 1994;80:814–24.
 7. Esper T, Wehner M, Meinecke CD, et al. Blood/gas partition coefficients for isoflurane, sevoflurane, and desflurane in a clinically relevant patient population. *Anesth Analg.* 2015;120:45–50.
 8. Tokuwaka J, Satsumae T, Mizutani T, et al. The relationship between age and minimum alveolar concentration of sevoflurane for maintaining bispectral index below 50 in children. *Anaesthesia.* 2015;70:318–22.
 9. Sikich N, Lerman J. Development and psychometric evaluation of the pediatric anesthesia emergence delirium scale. *Anesthesiology.* 2004;100:1138–45.
 10. Kalkman CJ, Peelen L, Moons KG, et al. Behavior and development in children and age at the time of first anesthetic exposure. *Anesthesiology.* 2009;110:805–12.
 11. Yip P, Middleton P, Cyna AM, et al. Cochrane review: non-pharmacological interventions for assisting the induction of anaesthesia in children. *Evid Based Child Health.* 2011;6:71–134.
 12. Ing C, DiMaggio C, Whitehouse A, et al. Long-term differences in language and cognitive function after childhood exposure to anesthesia. *Pediatrics.* 2012;130:476–85.
 13. Zhou X, Li W, Chen X, et al. Dose-dependent effects of sevoflurane exposure during early lifetime on apoptosis in hippocampus and neurocognitive outcomes in Sprague-Dawley rats. *Int J Physiol Pathophysiol Pharmacol.* 2016;8:111–9.
 14. Wang SQ, Fang F, Xue ZG, et al. Neonatal sevoflurane anesthesia induces long-term memory impairment and decreases hippocampal PSD-95 expression without neuronal loss. *Eur Rev Med Pharmacol Sci.* 2013;17:941–50.
 15. Fang F, Song R, Ling X, et al. Multiple sevoflurane anesthesia in pregnant mice inhibits neurogenesis of fetal hippocampus via repressing transcription factor Pax6. *Life Sci.* 2017;175:16–22.
 16. Chen C, Shen FY, Zhao X, et al. Low-dose sevoflurane promotes hippocampal neurogenesis and facilitates the development of dentate gyrus-dependent learning in neonatal rats. *ASN Neuro.* 2015;7:1759091415575845.
 17. Lu Y, Huang Y, Jiang J, et al. Neuronal apoptosis may not contribute to the long-term cognitive dysfunction induced by a brief exposure to 2% sevoflurane in developing rats. *Biomed Pharmacother.* 2016;78:322–8.
 18. Marečková K, Mareček R, Bencurova P, et al. Perinatal stress and human hippocampal volume: findings from typically developing young adults. *Sci Rep.* 2018;16:4696.
 19. Rao U, Chen LA, Bidesi AS, et al. Hippocampal changes associated with early-life adversity and vulnerability to depression. *Biol Psychiatry.* 2010;15:357–64.
 20. Morra JH, Tu Z, Apostolova LG, et al., Alzheimer's Disease Neuroimaging Initiative. Automated 3D mapping of hippocampal atrophy and its clinical correlates in 400 subjects with Alzheimer's disease, mild cognitive impairment, and elderly controls. *Hum Brain Mapp.* 2009;30:2766–88.