Original Article

The expanded effects of sevoflurane on the nervous system: the harmful effect of residual concentration of sevoflurane on the respiratory system through neurogenic inflammation

Feng-Lin Wang¹, Guang-Ting Zhang¹, Yan-Nan Zhou¹, Xin-Xin Yang¹, Lin Zhou¹, Jie Yuan¹, Xia Fei², Zhao-Qiong Zhu², De-Xing Liu¹*²

1. Soochow University Medical College, Suzhou, Jiangsu, China.
2. Department of Anesthesiology, Affiliated Hospital of Zunyi Medical University, Zunyi, Guizhou, China.

Abstract

Background: Neurogenic inflammation caused by sevoflurane may not only limit to the nervous system, but also expand to the respiratory system. The purpose of this study was to investigate the expression changes of transient receptor potential vanilloid 1 (TRPV1), neurokinin A (NKA), neurokinin B (NKB), calcitonin gene related peptide (CGRP) and substance P (SP) in 14, 21 and 42-day-old rats after inhaling 0.4% sevoflurane, in order to evaluate whether the residual sevoflurane be harmful to the respiratory system through neurogenic inflammation.

Methods: The anesthetic inhalation device was designed to allow 14, 21 and 42-day-old rats inhale 0.4% sevoflurane, while rats in the control group inhaled 40% O₂ for 1h. Rats in the antagonist group inhaled 0.4% sevoflurane or 40% O₂ for 1 h after Capsazepine (CPZ) pretreatment. The expression of TRPV1 in lung tissue was detected by western blot, and the expression of NKA, NKB, CGRP and SP in trachea was detected by immunohistochemistry.

Results: After inhaling 0.4% sevoflurane, the expression of TRPV1 in lung tissue of 14 and 21-day-old rats was significantly higher than that of the control group, as well as increased the expression of CGRP and SP in the trachea of 14-day-old rats and NKA, NKB, CGRP and SP in the trachea of 21-day-old rats. CPZ pretreatment could antagonize these effects.

Conclusion: Residual sevoflurane during resuscitation of inhalation anesthesia could induce neurogenic inflammation by activating TRPV1, which damaged to the developing respiratory system, but has no significant effect on the respiratory system in adulthood.

Key words: Residual sevoflurane; Development; Respiratory system; TRPV1; Neurogenic inflammation

Introduction

Sevoflurane has been widely used in clinic since it was officially approved for market in Japan in 1990s. Because of advantages such as stable anesthetic induction, less influence on lower esophageal sphincter and reducing reflux and aspiration in children, sevoflurane has gradually become the first choice for anesthesia in pediatric surgery (Li, et al., 2017). However, sevoflurane could also have noxious effects on the nervous system, which has been confirmed by research that sevoflurane causes postoperative cognitive dysfunction (POCD) through neuroinflammation (Yang, et al., 2019). Our previous studies have even discovered that the noxious effects of sevoflurane through neuroinflammation are not limited to the nervous system, but the respiratory system can still be observed, which is that low concentrations of sevoflurane (1.5%) could activate TRPV1 and cause harmful effects on the respiratory system, while high concentrations of sevoflurane (2.6%) could inhibit the activation of TRPV1 and have a protective effect on the respiratory system of developing rats (Liu, et al., 2020). Distributed widely in unmyelinated C-fibers, TRPV1 is an important nociceptive receptor in the lungs (McGarvey, et al., 2014; Melnick, et al., 2018), which is closely related to a variety of respiratory diseases (Kim, 2018). As a non-selective ligand gated cation channel, TRPV1 could be activated by a variety of chemical factors, resulting in the influx of Ca²⁺ and the generation of depolarizing current in sensory nerve cells (Benítez-Angeles, et al., 2020).
The depolarizing current is transmitted to the medulla oblongata and causes cough reflex, as well as retrograde conduction in the collateral nerve fibers (axon reflex), resulting in the local release of neurogenic inflammatory factors (NKA, NKB, CGRP and SP), causing a series of neurogenic inflammation such as airway contraction, mucus hypersecretion and plasma extravasation (Zhang, et al., 2018; Kim, et al., 2020) (Figure 1). The results of our previous experiments on the one hand explained the reasons for the harmful effect of low concentration sevoflurane on the developing respiratory system, on the other hand, it also caused us to ponder whether the nociceptive effect of sevoflurane is not limited to low concentration sevoflurane during surgical anesthesia, but extended to the very low concentration of sevoflurane residual in the lungs during anesthetic resuscitation. Although sevoflurane seldom accumulated in the body for a long time because of its low blood gas partition coefficient (Ikeda, et al., 2021). However, as the MACawake of sevoflurane in children is higher than that in adults, children could wake up when the concentration of sevoflurane drops to 0.8% (Davidson, et al., 2008). And it has been reported that even after children waking up, 0.6%-0.2% sevoflurane will remain in the body above 20 min (De, et al., 2019; Lu, et al., 2014). Therefore, children during the resuscitation of sevoflurane anesthesia will inevitably be affected by residual sevoflurane in the lungs. In clinic, the effect of residual sevoflurane on patients has been reported many times. Very low concentration of sevoflurane could not only cause irritability, delirium (Jung, et al., 2021) and epilepsy (Zheng, et al., 2021), but also affect the respiratory system and cause adverse reactions such as bronchospasm, atelectasis and even respiratory failure (Zucco, et al., 2021). Meanwhile, our clinical study found that children are more likely to have respiratory adverse events such as cough and dyspnea during the resuscitation of anesthesia, which may be related to sevoflurane residual in the lungs (Li, et al., 2013). Therefore, on the basis of discovering the nociceptive effect of low concentration sevoflurane on respiratory system during surgical anesthesia, it is necessary to further explore the effect of residual sevoflurane on respiratory system during resuscitation of inhalation anesthesia.

To sum up, we hypothesized that during the resuscitation of inhalation anesthesia, residual sevoflurane remaining in the lung was exposed to the respiratory system, which may activate TRPV1 and further affect the expression of downstream inflammatory factors such as NKA, NKB, SP and CGRP, resulting in neurogenic inflammation and airway adverse events such as cough, increased secretion and airway spasm. Therefore, on the basis of previous studies, 0.2 MAC (0.4%) sevoflurane was selected to simulate the residual sevoflurane in the lung during the resuscitation of inhalation anesthesia, 14, 21 and 42-day-old rats respectively simulate human respiratory system in infancy, puberty and adulthood (Picut, et al., 2015), the study aimed to observe whether the TRPV1 and neurogenic inflammatory factors in respiratory system of rats at different developmental stages were affected by residual sevoflurane and whether pretreatment with CPZ could antagonize this effect.

Materials and methods

Animals

Purchased from Changsha Tianqin Biotechnology Co., Ltd [SCXK (Xiang) 2014-0011], specific pathogen-free Sprague Dawley (SD) rats were divided into groups according to their age: 14 days (14 d) (n=32), 21 days (21 d) (n=32), and 42 days (42 d) (n=16). These animals are raised in accordance with rodent care guidelines, keeping the environment quiet, maintaining 22 ± 2°C and 50-60% relative humidity. The use and feeding of animals are approved by the Animal Management Committee of the Ethics Committee of Zunyi Medical University in accordance with the National Law on the use of Experimental Animals.

Sevoflurane inhalation unit

The sevoflurane inhalation device is composed of a transparent sealed inhalation anesthesia box, gas channel and monitoring instruments, which is designed and developed by ourselves (Figure 2). Before inhaling anesthesia, 2.5 cm thick sodium lime (Intersurgical Ltd., Wokingham, Berkshire, UK) was spread evenly at the bottom of the anesthetic box, and a mesh partition was placed on it to avoid direct contact burns with sodium lime. There are air inlet and outlet ports on both sides of the box, with a gas monitor (Vamos, Drager, Germany) continuously monitoring the sevoflurane concentration; they are connected to the inlet and outlet ports of the anesthesia machine (Fabius, Drager, Germany) through a thread tube. When the concentration of air inlet and outlet ports is equalized, it indicates that the concentration of sevoflurane in the anesthetic box is balanced.

Experimental grouping and intervention

In the first stage, 14 d, 21 d and 42 d rats were randomly divided into sevoflurane group and control group (n=8). The sevoflurane group inhaled 0.4% sevoflurane (Lunan Better Pharmaceutical Co., Ltd., Shandong, China) (4 L/min) for 1 h while the control group inhaled carrier gas (1 L/min O2+3 L/min air) for 1 h. In the second stage, 14 d and 21 d rats, whose results are positive in the first part, are randomly divided into sevoflurane + CPZ group and CPZ group. 30 min before inhalation of sevoflurane or carrier gas, capsazepine (CPZ, Med Chem Express, USA) was subcutaneously injected into the neck and back according to the 15 mg/kg standard (Cabral, et al., 2016). The rest of the grouping and intervention are the same as the first stage (Figure 3).
Measurement of weight and respiratory frequency
Rat weight was taken and recorded before inhalation gas or CPZ pretreatment. During the inhalation, 4 researchers monitored the respiratory frequency of the rats when the rats were transferred into the inhalation anesthesia box and 15 min after gas balance was achieved in the anesthesia box. Each researcher recorded the total number of breaths of 2 rats for 60 s. The colors of limb, tail and nasolabial of rats were continuously observed throughout the course to determine whether adverse reactions such as hypoxia and wheezing occurred.

Blood gas analysis and sampling
Before the blood gas analysis, the blood gas machine (GEM Premier 3000, USA) was calibrated using the standard solution. If the result range of pH, pO₂ and pCO₂ is less than 5%, the calibration is successful. After inhalation, 1% pentobarbital was intraperitoneally injected into rats according to the 30 mg/kg standard. The abdominal aorta, trachea and lung tissue were exposed through a median incision from the chest to the abdomen. The abdominal aorta was punctured with 1ml syringe, and the arterial blood gas was analyzed by blood gas machine. After the puncture, the lung tissue and trachea were removed in the hypothermic environment. The trachea was preserved in formalin and embedded in paraffin to make wax specimens, and the lung tissue was transferred to Eppendorf tube and preserved at -80℃.

Detection of TRPV1 in lung tissue with western blot
The lung tissue was homogenized with the lysate (10 ul RIPA and 0.1 ul PMSF per 1 mg lung tissue) at 4℃ and 1000 ×g for 10 min. The protein of the supernatant was quantified by bicinchoninic acid method. After denaturing at 95℃ for 5 min, Marker and the supernatant containing 20 μg total protein were added to 10% SDS-polyacrylamide gel, followed by transfer onto polyvinylidene fluoride membrane (Bio-Rad) via electrophoresis. then transferred to PVDF membrane by electroporation and incubated with 5% nonfat milk at room temperature for 1 h. The membrane was then incubated with TRPV1 antibody (1:1000, Abcam, Cambridge) and β-actin antibody (1:5000, Proteintech, China) overnight at 4℃. After washing with TBST for three times, the membranes were incubated in horseradish peroxidase coupled goat anti-rabbit second antibody (1:5000, Santa Cruz Biotechnology, Dallas, Texas, USA) for 1 hour at room temperature. Use TBST washing membrane for three times again. To develop the protein bands, the membranes were incubated in Hyperfilm-ECL Substrate (Amersham Pharmacia Biotech, Little Chalfont, UK) for 1 min. The intensities of protein bands were analyzed by Image J software, and the relative expression was expressed by the ratio of gray value of TRPV1 bands to β-actin bands.

Detection of neurogenic inflammatory mediators in tracheal tissue with immunohistochemistry
The trachea tissue embedded in paraffin was sliced, dewaxed, hydrated and sealed with hydrogen peroxide for 10 min, antigen was repaired with sodium citrate buffer (pH 6.0) at high pressure and 100℃ for 3 min. The sections were sealed with serum for 30 minutes, and then incubated overnight with antibodies against NKA (1:200, MyBioSource, USA), NKB (1:700, ThermoFisher Scientific, USA), CGRP (1:400, Bioss, China) and SP

Figure 1. Schematic illustration of the function of neurogenic inflammation. TRPV1 is activated by stimulation from C-fiber, resulting in the influx of nerve cell Ca²⁺ and the generation of depolarizing current. The depolarizing current is transmitted to the medulla oblongata and causes cough reflex, as well as retrograde conduction in the collateral nerve fibers (axon reflex), resulting in the local release of NKA, NKB, CGRP and SP. These inflammatory factors activate tachykinin receptors (NK1, NK2 and NK3) and CGRP receptors on effector cells such as airway smooth muscle cells, goblet cells and vascular endothelial cells, causing a series of neuroinflammatory responses such as airway contraction, mucus hypersecretion and plasma extravasation.
(1:400, Bioss, China) at 4°C respectively. The control group used the same amount of phosphate buffer solution instead. After rewarming 30 min at 37°C for 30 min, the sections were incubated incubator with horseradish peroxidase coupled rabbit anti-mouse secondary antibody (1:5000, Bioss, China) at 37°C for 30 min. Then horseradish peroxidase labeled streptomycin working solution was added to the sections for 15 min, and diaminobenzidine was used for coloration. The sections were re-stained with hematoxylin for 5 min, and then dehydrated in 70% ethanol, 80% ethanol, 90% ethanol and 100% ethanol for 2 min respectively. The sections were then immersed in xylene for 1 min, mounted with neutral resin, and photographed under a microscope (Olympus IX53, Japan) with a camera (Olympus DP73, Japan). The average optical density is calculated by Image-Pro Plus software (Media Cybernetics, USA).

Statistical analysis
Data were expressed as mean ± standard deviation (SD) of the mean and evaluated by Student’s T-test. All statistical analyses were carried out using SPSS 20.0 software (SPSS Inc, Chicago, Illinois, USA). Independent sample T-test was used to compare the two groups. One-way analysis of variance (ANOVA) was used to compare multiple groups. If the variance was homogeneous, the least-squares dating method was used to compare the variance. If the variance was not homogeneous, the Dunnett’s T3 method was used to compare the difference. p ≤ 0.05 was considered to be statistically significant.

Results
Weight measurement
The rats were weighted before inhaling carrier gas or sevoflurane in the first stage. The body weight of 42 d rats was significantly higher than that of 21 d and 14 d rats (Figure 4A, p < 0.01), and the body weight of 21 d rats was higher than that of 14d rats (Figure 4A, p < 0.01). The rats were weighed before CPZ pretreatment in the second stage. The body weight of 21 d rats was higher than that of 14 d rats, and the difference was statistically significant (Figure 4B, p < 0.01). There was no significant difference in weight of rats of the same age among the groups (Figure 4A, B).

Respiratory rate count
Because the rats in the control group were conscious and active in the process of inhalation, the reliability of manual counting of respiratory frequency was poor, and no counting was carried out. By counting the respiratory frequency of rats in sevoflurane group and sevoflurane + CPZ group, compared with the respiratory frequency of 14, 21 and 42-day-old rats during inhalation of sevoflurane for 15 min, there was no significant difference during inhalation of sevoflurane for 60 min (Figure 5A, B), and the respiratory frequency was in the normal range.
Effect of residual sevoflurane on arterial blood gas in rats

About 50% of 14 d rats failed to meet the requirements of arterial blood gas detection due to difficulties in collecting blood, so no statistical analysis was carried out. In 21 d rats, there was no significant difference in pH, pO2 and pCO2 between sevoflurane group and control group (Figure 6A, B, C). At the age of 42 days, there was no significant difference in pH and pO2 between the sevoflurane group and the control group (Figure 6A, B). The pCO2 was higher than that in the control group, and the difference was statistically significant (Figure 6C, p < 0.01). There was no significant difference in pH, pO2 and pCO2 between sevoflurane + CPZ group and CPZ group at the age of 21 days (Figure 6D, E, F). The blood pH, pO2 and pCO2 values of all blood samples were in the normal range, and no serious hypoxemia or CO2 accumulation was observed.

Effect of residual sevoflurane on the expression of TRPV1 in lung tissue

In order to explore whether TRPV1 is involved in the respiratory system response induced by residual sevoflurane, the effect of sevoflurane on the expression of TRPV1 in lung tissue was investigated. Compared with the control group, inhaling 0.4% sevoflurane increased the expression of TRPV1 in lung tissue of 14 d and 21 d rats, and the difference was statistically significant (Figure 7B, p 14 d < 0.05, p 21 d < 0.01). However, inhaled 0.4% sevoflurane had no effect on the expression of TRPV1 in the lung tissue of 42 d rats (Figure 7B). Compared with CPZ group, CPZ pretreatment before inhalation of sevoflurane did not cause significant changes in TRPV1 expression in lung tissue of 14 d and 21 d rats (Figure 8B). To sum up, these results suggest that sevoflurane could not up-regulate the expression of TRPV1 in the respiratory system of developing rats, and this effect could be antagonized by CPZ.

Effect of residual sevoflurane on the expression of neurogenic inflammatory mediators in tracheal tissue.

The activation of TRPV1 could induce the release of neurogenic inflammatory mediators NKA, NKB, CGRP and SP from the lateral process of the sensory nerve, and promote the airway neurogenic inflammatory response (Kichko, et al., 2015; Zhang et al., 2018). In order to explore whether NKA, NKB, CGRP and SP were...
involved in the airway response induced by residual sevoflurane, the expression of neurogenic inflammatory mediators in trachea was measured. As shown in Figure 9A and Figure 10A, the expression of NKA and NKB was found in the cytoplasm of tracheal smooth muscle cells and tracheal loop mucosal ciliated cells. Compared with the control group, after inhaling 0.4% sevoflurane, the expression of NKA and NKB in the trachea of 21d rats increased (Figure 9B, 10B, \( p_{21d} < 0.05 \)), but there was no significant difference in 14 d and 21 d rats (Figure 9B, 10B). Compared with the CPZ group, there was no significant difference in the expression of NKA and NKB in the trachea of 14 d and 21 d rats in the SEV+CPZ group (Figure 9C, 10C). CGRP and SP was mainly expressed in airway mucosal ciliated cells and airway smooth muscle cells (Figure 11A, 12A). Compared with the control group, inhaling 0.4% sevoflurane up-regulate the expression of CGRP and SP in the trachea of 14d and 21d rats (Figure 11B, 12B, \( p_{14d} < 0.01, p_{21d} < 0.05 \)). However, no significant difference was observed in CGRP and SP expression in the 42 d rat groups (Figure 11B, 12B). After 0.4% sevoflurane inhalation, CPZ pretreatment did not change the expression of CGRP and SP in the trachea of rats of all age groups (Figure 11C, 12C). Taken together, these results suggest that residual sevoflurane could increase the expression of neurogenic inflammatory mediators in the trachea of developing rats, especially in the trachea of late-developing rats. And this effect could be antagonized by CPZ. However, residual sevoflurane had no effect on the expression of neurogenic inflammatory mediators in the trachea of adult rats.

Figure 4. Comparison of body weight before sevoflurane inhalation and before CPZ pretreatment. (A) Histogram of body weight statistics in 42d, 21d and 14d rats before sevoflurane inhalation. (B) Histogram of body weight statistics in 21d and 14d rats before sevoflurane inhalation and CPZ pretreatment. The data is expressed as mean ± standard deviation (n = 8).

Figure 5. Respiratory rate of rats in each group during sevoflurane inhalation. (A) The respiratory rates of 14-, 21- and 42-day-old rats after inhalation of sevoflurane 15min and 60min, T-test. Comparison in each group, \( p > 0.05 \). (B) The respiratory rate of 14- and 21-day-old rats with CPZ pretreatment during sevoflurane inhaled 15min and 60 min, T-test. Comparison in each group, \( p > 0.05 \). The data is expressed as mean ± standard deviation (n=8).
Figure 6. Blood gas analysis of rats after sevoflurane inhalation. pH (A), pO2 (B) and pCO2 (C) measured in 21 d and 42 d rats after sevoflurane inhalation, T-test. pH (A) and pO2 (B) were no significant difference between the sevoflurane group and the control group (p > 0.05). The pCO2 (C) of 42 d rats in sevoflurane group was higher than that in control group (p < 0.01). pH (D), pO2 (E) and pCO2 (F) measured in 21 d rats pretreated with CPZ after sevoflurane inhalation, T-test. There was no significant difference between the sevoflurane + CPZ group and CPZ group (p > 0.05). The data is expressed as mean ± standard deviation (n=8).

Discussion
In this study, an independent designed inhalation anesthesia device was used to observe the effects of residual sevoflurane on rats at different developmental stages. The results showed that residual sevoflurane could up-regulate the expression of TRPV1 and sensory neuropeptides from downstream in the respiratory system of developing rats, and this effect could be antagonized by CPZ. However, no significant changes in the expression of TRPV1 and sensory neuropeptides were observed in the respiratory system of adult rats. These results confirm our hypothesis that the residual sevoflurane during the resuscitation of inhalation anesthesia could induce neurogenic inflammation by activating TRPV1, which is harmful to the developing respiratory system, but has no significant effect on the respiratory system in adults. There was no significant difference in body weight between the experimental group and the control group on 14 d, 21 d and 42 d, which eliminates the experimental error caused by abnormal growth of experimental animals. During the inhalation, in order to ensure that the inhaled gas was stably maintained at the preset concentration, to avoid the potential damage to the airway and lung caused by endotracheal intubation and mechanical ventilation (Iben, et al., 2006; Puyo, et al., 2019), and to accurately reflect the independent effect of sevoflurane on the respiratory system, we designed a set of non-invasive inhalation anesthesia device which could retain spontaneous breathing in rats. In this device, 40% O2 was used as a carrier gas to avoid the irritating effect of high concentration of O2 on the airway. Meanwhile, in order to avoid the accumulation of CO2, the sodium lime was laid on the bottom of the box to absorb CO2 from the exhaled gas, and the number of rats in the box was limited to 8 rats during inhalation. Some studies have shown that sevoflurane could affect the respiratory rate of SD rats and even lead to hypoxia and respiratory acidosis by inhibiting the respiratory center of medulla (Kuribayashi, et al., 2008). Chronic hypoxia could significantly up-regulate the expression of mRNA and protein of TRPV
l in pulmonary artery smooth muscle cells (Wang, et al., 2008). What’s more, H+ also directly activate TRPV1 channel (Zhao, et al., 2021). Therefore, the respiratory system, we designed a set of non-invasive inhalation anesthesia device which could retain spontaneous breathing in rats. In this device, 40% O2 was used as a carrier gas to avoid the irritating effect of high concentration of O2 on the airway. Meanwhile, in order to avoid the accumulation of CO2, the sodium lime was laid on the bottom of the box to absorb CO2 from the exhaled gas, and the number of rats in the box was limited to 8 rats during inhalation. Some studies have shown that sevoflurane could affect the respiratory rate of SD rats and even lead to hypoxia and respiratory acidosis by inhibiting the respiratory center of medulla (Kuribayashi, et al., 2008). Chronic hypoxia could significantly up-regulate the expression of mRNA and protein of TRPV1 in pulmonary artery smooth muscle cells (Wang, et al., 2008). What’s more, H+ also directly activate TRPV1 channel (Zhao, et al., 2021). Therefore, the respiratory frequency and the arterial blood gas were analyzed to observe whether there were hypoxia and acid-base imbalance caused by respiratory inhibition during sevoflurane anesthesia, so as to rule out their impact on TRPV1. In terms of respiratory frequency, because of the activity of rats, the respiratory rate of rats in the control group was not recorded. The respiratory rate in the sevoflurane group remained in the normal range (66-114 bpm / min), indicating that 0.4% sevoflurane did not significantly inhibit respiratory function. The results of blood gas showed that there were no adverse reactions such as hypoxia (pO2 < 60 mmHg), CO2 accumulation
(pCO₂ > 50 mmHg) and acid-base imbalance (pH < 7.3 or pH > 7.5) in sevoflurane groups (Yang, et al., 2016; Su, et al., 2019). Mild CO₂ accumulation occurred in rats in 42 d sevoflurane group, which may be related to the limitation of CO₂ excretion in adult rats placed in a closed anesthetic box. However, considering that the blood pH was in the normal range and the breathing was stable without cyanosis in the lips during inhalation, the effects of hypoxia and acidosis on TRPV1 protein were excluded.

As a synthetic inhalation drug developed for anesthesia, the harmful effect of sevoflurane on respiratory system during surgery may be masked by the protective effect of its anesthetic concentration, which may be one of the reasons why the nociceptive effect of sevoflurane is rarely discovered. However, our previous clinical observation found that airway adverse events such as holding breath and coughing were easy to occur during the resuscitation of sevoflurane anesthesia in children (Li et al., 2013). Similarly, a recent study also reported that the incidence of adverse airway events in premature infants under sevoflurane anesthesia was higher than that in normal infants, which may be related to airway immaturity (Lei, et al., 2019). The above studies suggest that in special conditions, such as very low concentrations of sevoflurane are not sufficient to produce protective effects or airway is immature, the nociceptive effect may return to the dominant role, resulting in a series of airway adverse events. As an important nociceptive receptor in the lung, TRPV1 plays an important role in a variety of pathophysiological processes of the respiratory system, which may be related to the nociceptive effect of sevoflurane. A study by Matta et.al just confirmed the relationship between them. By comparing the effects of

Figure 9. The expression of NKA in tracheal tissues after inhalation of sevoflurane. (A) Immunohistochemistry showed the expression of NKA in tracheal tissues, arrow indicates the position of NKA, d indicates days. Scale bar = 50 μm. (B) The OD of NKA in tracheal tissues after inhalation of sevoflurane. T-test. In 21 d rats, the expression of NKA in tracheal tissues of the sevoflurane group was higher than that of the control group (p < 0.01). (C) The OD of NKA in tracheal tissues received CPZ pretreatment, followed by sevoflurane inhalation. There was no significant difference between the sevoflurane + CPZ group and CPZ group (p > 0.05). The data is expressed as mean ± standard deviation (n=8).

Figure 10. The expression of NKB in tracheal tissues after inhalation of sevoflurane. (A) Immunohistochemistry showed the expression of NKB in tracheal tissues, arrow indicates the position of NKB, d indicates days. Scale bar = 50 μm. (B) The OD of NKB in tracheal tissues after inhalation of sevoflurane. T-test. In 21 d rats, the expression of NKB in tracheal tissues of the sevoflurane group was higher than that of the control group (p = 0.045). (C) The OD of NKB in tracheal tissues received CPZ pretreatment, followed by sevoflurane inhalation. There was no significant difference between the sevoflurane + CPZ group and CPZ group (p > 0.05). The data is expressed as mean ± standard deviation (n=8).
TRPV1 agonists and inhaled anesthetics on the structure of TRPV1 in nerve cells, it was found that inhaled anesthetics could directly bind to specific sites of TRPV1, activating the receptor and sensory nerve cells, causing neurogenic inflammation and cough reflex (Matta, et al., 2008). These above researches coincided with the results of our experiment. We found that the residual sevoflurane could up-regulate the expression of TRPV1 in lung tissue for 14 d and 21 d rats, this effect could be inhibited by TRPV1 antagonists. However, the effect on the respiratory system was not found in 42 d rats. In previous experiments, we have found that the effect on the respiratory system changes from protective to nociceptive with the decrease of sevoflurane concentration (Liu et al., 2020). This is because low concentration sevoflurane is not enough to play the role of relaxing tracheal smooth muscle which high concentration sevoflurane does, but acts as an irritating gas on the sensory nerve endings of airway epithelium to activate TRPV1 and inflammatory pathways. The results of our study not only filled the gap in the previous study on the effect of very low concentration of sevoflurane on respiratory system, which confirmed that sevoflurane at residual concentration during anesthetic resuscitation could also activate TRPV1, but also further found that this effect was age-dependent, which is that the effect of sevoflurane up-regulating TRPV1 in the respiratory system of developing rats (14 d and 21 d rats) was not found in adult rats (42 d rats). It is speculated that compared with the adult respiratory system, the sensory nerve endings of the developing airway epithelium have more TRPV1, but the airway epithelium is immature, so the nerve endings...
and TRPV1 are more likely to be exposed to the external environment and activated by sevoflurane (Korobkin, et al., 2013).

When TRPV1 channel is activated, triggered Ca\(^{2+}\) influx causes C-fiber to release sensory neuropeptides (NKA, NKB, CGRP, SP) through axonal reflex. These neuropeptides could activate tachykinin receptors and CGRP receptors on the airway epithelial cells, smooth muscle cells and vascular endothelial cells, which leads to neurogenic inflammatory (Achanta, et al., 2020). Therefore, by detecting the relative expression of NKA, NKB, CGRP and SP in tracheal tissue, this study further reveals the mechanism of sevoflurane leading to cough, excessive mucus secretion, tracheal spasm by activating TRPV1 and releasing tachykinin. The results showed that residual sevoflurane could increase the expression of NKA, NKB, CGRP and SP in the trachea of 21 d rats, and also promote the expression of CGRP and SP in the trachea of 14 d rats. These effects were inhibited after CPZ pretreatment, indicating that the effect of sevoflurane on the expression of neurogenic inflammatory factors in the respiratory system disappeared with the antagonism of TRPV1, but not found in the adult respiratory system. This was consistent with the change trend of the effect of sevoflurane on TRPV1, which further confirmed the close relationship among sevoflurane, TRPV1 and sensory neuropeptide in developing respiratory system. However, although the expression of NKA and NKB in the airway of rats increased after inhaling residual sevoflurane for 14 d, the difference was not statistically significant. It was speculated that neutral endopeptidase in airway epithelium and angiotensin converting enzyme in blood could rapidly degrade NKA and NKB (Lee, et al., 2004), which does not rule out the possibility that NKA and NKB have been partially degraded in the process of sampling and preservation of 14d rats. Besides, since 21 d rats are in puberty, gonadotropins produced at this developmental stage play a positive role in regulating the synthesis of NKA and NKB (Navarro, 2020), under the synergistic effect of gonadotropins, residual sevoflurane may be more likely to increase the expression of NKA and NKB in late developmental stage (puberty) rats. During resuscitation stage of inhalation anesthesia, very low concentration sevoflurane will inevitably remain in children's lungs. However, residual sevoflurane is not enough to relax the smooth muscle of trachea, but acts as an irritating gas in the development of respiratory system. Due to the incomplete development of airway epithelium, the TRPV1 of sensory nerve endings is easy to be activated, resulting in the release of downstream neurogenic inflammatory factors and causing a series of respiratory symptoms (Devys, et al., 2011). Therefore, it is suggested that residual sevoflurane in the lung during resuscitation of inhalation anesthesia is closely related to airway adverse events in children, and the potential risk should not be ignored. Sevoflurane may be actively discharged as soon as possible during the resuscitation of pediatric anesthesia to reduce the harmful effect. Since the harmful effects of sevoflurane on the developing respiratory system may change the current management of clinical anesthesia, further research is needed to draw a more reliable conclusion.

There are some limitations in this study. First of all, this study only found the effect of residual sevoflurane on the developing respiratory system through TRPV1 and sensory neuropeptides, but the specific mechanisms at the airway mechanics and cell molecular levels need to be further explored. It is also necessary to explore whether sevoflurane affects the respiratory system through other pathways besides TRPV1. Secondly, since this study only examined the effects of sevoflurane after inhalation of 60 min, the next study should investigate whether additional inhalation time will have a similar effect. Finally, as the connection of residual sevoflurane, TRPV1 and sensory neuropeptides is only observed in animal experiment, whether this connection will also exist in clinic needs to be verified by clinical research.

**Conclusion**

0.4% sevoflurane inhalation could up-regulate the expression of TRPV1 in the respiratory system of developing rats, causing changes in the expression of NKA, NKB, CGRP and SP, and CPZ could antagonize this change. However, no such effect was found in adult respiratory system, indicating that residual sevoflurane has a nociceptive effect on the developing respiratory system through TRPV1-neurogenic inflammation. Our results suggest the potential harm of residual sevoflurane to the developing respiratory system during resuscitation of inhalation anesthesia.

**Ethical statement**

All the operations on the involved participants were approved by the Animal Experiment Ethics Committee of Zunyi Medical University (Approval No. [ 2021] 2- 316). All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

**Acknowledgements**

Not applicable.

**Conflict of interest**

There is no conflict of interest in this study.

**Funding**

This work was supported by National Natural Science Foundation of China [grant numbers 81660021].

**Transparency statement**

All the authors affirms that this manuscript is an honest,
accurate, and transparent account of the study being reported; that no important aspects of the study have been omitted; and that any discrepancies from the study as planned (and, if relevant, registered) have been explained.

Authors' contribution
Feng-Lin Wang and De-Xing Liu contributed the central idea. Guang-Ting Zhang and Yan-Nan Zhou conceived and designed the experiments. Xin-Xin Yang and Lin Zhou analysed most of the data. Jie Yuan wrote the initial draft of the paper. Xia Fei and Zhao-Qiong Zhu contributed to refining the ideas, carrying out additional analyses and finalizing this paper.

References


