

Carbenoxolone does not Improve Sevoflurane-induced Neurotoxicity in the Developing Rat Hippocampus

Congjie Bi

Dalian Municipal Central Hospital

Dan Xie

Dalian Municipal Central Hospital

Donghai Yu

Dalian Municipal Central Hospital

Yang Wang (✉ wangy20202020@126.com)

Dalian Municipal Central Hospital

Research Article

Keywords: sevoflurane, Connexin43, Neurotoxicity , Carbenoxolone, Developing brain

DOI: <https://doi.org/10.21203/rs.3.rs-148165/v1>

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Abstract

Background: Connexin 43 is the most abundant connexin protein expressed in astrocytes. Our previous research found that sevoflurane-induced neurotoxicity was related to Cx43 via JNK/MAPK/Ap-1 signaling pathway. Cx43 functions through hemichannels (HCs) and gap junctions (GJs) and both of them could affect the homeostasis of central nervous system. Carbenoxolone (Cbx) is generally considered as a Cx43 hemichannel and gap junction inhibitor in traditional applications. Therefore, we hypothesized that preadministration of Cbx may attenuate sevoflurane-induced cognitive dysfunction.

Methods: Seven-day-old SD rats (P7) were exposed to 3% sevoflurane for 4 hours with or without Cbx pretreatment at a dose of 50 mg/kg. Levels of Bcl-2, Bax, Cx43 and caspase-3 positive cells in P8 rat's hippocampus were examined using Western blotting and immunohistochemistry. Morris water maze was performed from P28 to P33 to test the cognitive function.

Results: Cx43 levels and caspase-3 positive cells in P7 rat hippocampus were increased 1 day after exposure to 3% sevoflurane for 4 h compared with control rats. Sevoflurane anesthesia decreased the expression of Bcl-2 and increased the expression of Bax in P8 rat's hippocampus ($P < 0.05$, $n = 5$). Exposure to sevoflurane led to significant cognitive impairment from P28 to P33. All these defects could not be alleviated by pretreatment with Cbx.

Conclusions: These data suggested that Cbx could not improve sevoflurane-induced neurotoxicity by inhibiting Cx43 HCs in the developing brain.

Introduction

Beginning with William T. G. Morton's first successful public demonstration of general anesthesia with ether on October 16, 1846, volatile anesthetics have been widely used in clinical practice. Sevoflurane is the most popular anesthetic in pediatric and geriatric anesthesia due to its unique advantages. Unfortunately, increasing evidence suggests that sevoflurane exposure increases the risk of developing a learning disability, especially in the developing brain [1,2].

Previously, the main function of glial cells was to support the central nervous system and isolate neurons. However, in recent years, it has been found that glial cells, also play roles in synaptic transmission, buffering and releasing neurotransmitters, nutrition and the removal of harmful substances, immune regulation and other functions. As the most abundant glial cells, the role of astrocytes in the central nervous system has attracted increasing attention.

Connexins are transmembrane proteins that are part of a multigenic family present in almost every cell. A total of 20 and 21 connexins have been identified in mouse and human genomes respectively, and each connexin is named according to its respective molecular weight. Connexins are synthesized in the endoplasmic reticulum and then traffic to the plasma membrane through the Golgi apparatus. Connexin 43 (Cx43) is one of the most abundant connexin proteins in astrocytes [3]. Six connexins can form a

connexon, also known as hemichannel (HC), and two connexons or hemi-channels dock to form a gap junction (GJ) [4-6]. Many studies have shown that Cx43 is related to the development of the central nervous system, neuronal excitability, synaptic transmission, synaptic plasticity, learning, memory, and inflammation of the central nervous system. Our previous study found that sevoflurane-induced neurotoxicity in the developing brain was associated with the upregulation of Cx43, and the JNK/c-Jun/AP-1 signaling pathway was involved [7]. A single Cx43 protein does not play a role, but after oligomerization into HCs or GJs, Cx43 proteins could participate in many important roles.

GJs allow adjacent cells to exchange small molecules directly (<1.5 kDa), including ions, ATP, glutamate, glutathione, neurotransmitters and signaling molecules under normal conditions. HCs are not open or open only a small amount to maintain normal synaptic functions. When the central nervous system (CNS) suffers damage, a large number of HCs will open, leading to the destruction of cell membrane integrity and the release of cytotoxic substances, which will have adverse effects on the CNS [8-11]. Additionally, GJs can buffer toxic substances when a small amount of damage occurs. When sustained damage occurs, the toxic substances will spread distance, causing more cell damage.

Sevoflurane-induced neurotoxicity in the developing brain could increase the expression of the Cx43 protein, but the exact reason is still unclear. We have reason to believe that the opening of HCs can cause direct intracellular and extracellular communication and damage the CNS and that GJs can mitigate the damage from intracellular toxic substances between adjacent cells by diffusion, thus spreading harmful substances to distant places. Therefore, we hypothesize that sevoflurane-induced CNS damage is associated with the roles of HCs and GJs. We observed whether sevoflurane-induced neurotoxicity in the developing rat hippocampus could be reduced by intracerebroventricular injection of carbenoxolone (Cbx), a general HC and GJ inhibitor, to improve learning and memory ability in adolescence.

Materials And Methods

Our study was carried out in compliance with the ARRIVE guidelines 2.0 guidelines.

Experimental animals

All animal experimental procedures and protocols were approved by The Laboratory Animal Care Committee of China Medical University, Shenyang, China (NO. 2017PS019K) and were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, United States. Twenty pregnant Sprague – Dawley (SD) rats that were in their second trimester, weighing 250-300 g were purchased from Changsheng Shengwu (China). The pregnant rats were housed under a 14:10 constant light –dark cycle with free access to water and food for one week at room temperature (24 ± 1 °C). The day of birth was noted as postnatal day 0 (P0). Postnatal day 7 (P7) male or female rat pups weighing 14-18 g, were used in this study.

Sevoflurane exposure and drug administration

Seventy-two P7 pups from different litters were randomly allocated to four groups (18 pups were in each group): control (Ctrl), control+Cbx (Ctrl+Cbx), sevoflurane (Sevo) and sevoflurane+Cbx (Sevo+Cbx). Two hours before sevoflurane exposure, Ctrl+Cbx and Sevo+Cbx groups were injected Cx43 inhibitor Cbx intracerebroventricularly(i.c.v.) 50 mg/Kg. The Ctrl and Sevo groups were injected with the same amount of 0.9% saline intracerebroventricularly. All seventy-two P7 rat pups were separated from their mothers and placed in a glass chamber (40×24×20 cm) resting in a water bath to maintain a constant environmental temperature of 38°C. In the chamber, the rats were exposed to either 3% sevoflurane in 30% oxygen carrier gas (balanced with nitrogen) or a carrier gas without sevoflurane for 4 hours. All method descriptions are similar to our previously published study [7]. After sevoflurane exposure, all P7 pups remained in the chamber ventilated with the mixed gas (30% O₂/70% N₂) at a rate of 2 L/min until they were fully awakened. Therefore, the rats were transferred to their original cages.

Western blots

Five pups from each group were randomly selected and killed by decapitation 1 d after sevoflurane exposure and then the hippocampus was harvested and the protein was extracted (n = 5/group). All procedures are similar to our previously published study [7,12,13]. Fifty micrograms of each protein sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); using a semidry blotting apparatus (Bio-Rad Laboratories, Munich, Germany), the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, United States) and then incubated overnight at 4 °C with the appropriate primary antibodies: anti-Cx43 (1:1000; SAB4501175), anti-Bcl-2 (1:1000; 4695), anti-Bax (1:1000; 9252), and anti-GAPDH (1:1,000; A9169). Next, the membranes were washed in TBST and incubated with secondary antibodies (A9169, Sigma, United States) for 2 h at room temperature. The positive reactive bands were detected by Amersham enhanced chemiluminescence (ECL) reagents. The blots were scanned using an Amersham Image 600 scanner (GE Healthcare Life Sciences), and the protein band density was quantified using ImageJ software. Protein expression levels were evaluated by the GAPDH ratio.

Immunohistochemistry staining (IHC)

Neuronal apoptosis was detected by immunohistochemistry (IHC) staining of caspase-3 positive cells in the hippocampus. Five rats in each group were euthanized by transcardial perfusion with saline, followed immediately by 4% paraformaldehyde 1 day after sevoflurane exposure. The hippocampus of the rats was cut from the whole brain at a distance of 2 mm from the forehead, after which it was dehydrated in graded ethanol and was embedded in paraffin. Brain coronal slices of the paraffin-embedded tissue (2.5 um) were sectioned using a microtome. These tissue sections were then baked, deparaffinized, rehydrated, and quenched of endogenous peroxides. It was the same way that we've studied before [7].

Each slice was incubated with activated caspase-3 antibody (1:200 dilution, catalog no. 9662; Cell Signaling Technology) overnight at 4°C. The next day, the sections were exposed to peroxidase-conjugated goat antirabbit secondary antibody (Santa Cruz Biotechnology). The hippocampal CA1 region, was colorized with diaminobenzidine solution for 8 min and counterstained with hematoxylin. Finally, the sections were photographed by Nikon C1 microscope with 3 randomly chosen fields imaged per slide by an investigator who was blinded to the experimental intervention (n = 5/group). Caspase-3 positive cell accumulated optical density was calculated in each hippocampal slide vision.

Morris water maze (MWM)

Morris water maze experiment was conducted to evaluate spatial learning and memory ability of the rats. Thirty-two rats from four groups including Ctrl, Ctrl + Cbx, Sevo and Sevo + Cbx, were subjected to the MWM after 28 d (n = 8/group), as previously described [7]. Escape latency trials were conducted once per day for five consecutive days. The time spent finding the hidden platform and the swimming distance before reaching the platform were recorded. If the rat did not find the platform within 90 s, escape latency was recorded as 90 s. Then the rats were guided to stand on the concealed platform for 10 s. The spatial probe test was conducted on day 6 to test the ability of the rats to maintain their memory of the platform space after learning to find the platform. The platform was removed after the positioning navigation experiment was completed. After the platform was taken away from the maze,

the rats were put into the quadrant opposite the platform and permitted to swim freely for 90 s. The time in the target quadrant was noted. The entire behavioral test was recorded and analyzed using a Noldus Ethovision XT video analysis system (Netherland). After the MWM test, all 32 rats were sacrificed without biochemical analysis.

Statistical analysis

The statistical analysis was conducted using Graph Pad Prism 7.0 software (version 7.0; Graphpad Software, Inc.). Statistical significance was determined by One-way ANOVA followed by Tukey's test. Interaction between time and group factors in a two-way ANOVA with repeated measurements was used to analyze the difference of learning curves (based on escape latency) between rats in the MWM [7]. At least five individual trials were performed for each experiment and data represented as mean ± SEM. Differences were considered statistically significant for $P < 0.05$.

Results

Cbx did not affect the expression of Cx43 protein in hippocampus of P7 rats after exposure to 3% sevoflurane for 4 hours.

We observed the expression of the Cx43 protein in the hippocampus of P7 rats exposed to 3% sevoflurane 1 day after Cbx (50 mg/kg) was injected i.c.v. Western blot experiments showed that Ctrl + Cbx had no effect on Cx43 protein expression in the hippocampus of rats after exposure to sevoflurane for 1 day ($n = 5, P > 0.05$). Cx43 protein expression was significantly increased in the hippocampus of P7 rats 1 day after sevoflurane compared with that in the Ctrl group ($n=5, P < 0.05$), but there was no significant difference between the Sevo and Sevo + Cbx group rats regarding the expression of Cx43 ($n=5, P > 0.05$). These results suggest that 50 mg/kg Cbx i.c.v. had no significant inhibitory effect on Cx43 protein expression in the hippocampus of P7 rats 1 day after inhalation of sevoflurane (Figure1).

Cbx did not affect the expression of Bcl-2 and Bax in hippocampus of P7 rats exposed to 3% sevoflurane for 4 hours.

Apoptosis is the main pathway of programmed cell death and can be triggered by multiple death stimuli. The triggers of apoptotic pathways include the mitochondrial-mediated (intrinsic) pathway and the cell surface death receptor (extrinsic) pathway. The intrinsic apoptotic pathway is mediated by Bcl-2 family members and the mitochondrial permeability transition pore (PT pore). Bcl-2 is a family of proteins with proapoptotic (e.g., Bax) or antiapoptotic (e.g., Bcl-2) properties. Western blot experiments showed that the expression of the Bax protein in the hippocampus of rats increased significantly, while Bcl-2 decreased 1 day after inhalation of sevoflurane compared with the Ctrl group ($n=5, P < 0.05$). There were no differences in Bax and Bcl-2 expression in the hippocampi of the Cbx group, and the Sevo + Cbx group did not exhibit changes in Bax and Bcl-2 expression compared with the Sevo group ($n = 5, P > 0.05$). It was concluded that 50 mg/kg Cbx injected i.c.v. did not decrease the expression of apoptosis-related proteins in the hippocampus of P7 rats after exposure to 3% sevoflurane for 4 hours (Figure 2).

Cbx did not decrease the number of cleaved caspase-3 positive cells in the hippocampus of rats exposed to 3% sevoflurane for 4 hours.

Cleaved caspase-3-positive cells are the ultimate indicator of apoptosis. In our study, the cumulative optical density of cleaved caspase-3-positive cells in the hippocampus of P7 rats was observed by IHC after inhalation of 3% sevoflurane for 4 hours. The results showed that i.c.v. injection of Cbx alone did not affect the number of positive caspase-3 cells in the hippocampus ($n = 5, P > 0.05$). However, the cumulative optical density of cleaved caspase-3-positive cells in the hippocampus of the Sevo group increased significantly 1 day after exposure to sevoflurane ($n=5, P<0.05$). Compared with the Sevo group, the cumulative optical density of cleaved caspase-3-positive cells in the hippocampus of the Sevo+Cbx group did not decrease significantly 1 day after exposure to sevoflurane, suggesting that Cbx did not

decrease the apoptosis of neurons in the hippocampus of P7 rats after exposure to sevoflurane (Figure 3).

Cbx did not improve learning and memory impairment in rats after inhalation of 3% sevoflurane for 4 hours.

The Morris water maze (MWM) experiment is a classical experimental method to study animal behavior that is widely used in the field of neurobiology and can well reflect the spatial learning and memory ability of animals. The results showed that there was no significant difference in the performance of the four groups in the first three days. On trial day 5, when the rats were 32 days old, the time to find the platform in the Sevo group was significantly longer than that in the Ctrl group ($P < 0.05$, $n = 8$). The time of finding the platform in the Sevo + Cbx group was significantly longer than that in the Ctrl group on trial days 4 and 5 ($P < 0.05$, $P < 0.01$, $n = 8$, respectively), indicating that inhalation of 3% sevoflurane for 4 hours had a significant effect on the long-term spatial learning and memory ability of rats. However, i.c.v. injection of the Cx43 HC inhibitor Cbx before sevoflurane exposure did not improve the long-term learning and memory ability of rats. Compared with the Ctrl group, there was no significant difference in finding the submerged platform in the Ctrl + Cbx group. Similarly, when the rats were 33 days old, the number of platform traversions in the Sevo group and Sevo+Cbx group were less than that in the Ctrl group, and the spatial memory ability significantly decreased ($P < 0.05$, $n=8$). The number of rats crossing the platform in the Ctrl + Cbx group was similar to that in the Ctrl group ($P > 0.05$, $n = 8$). Additionally, there were no significant differences in the average swimming speeds among the four groups (results not shown). These data suggested that sevoflurane could affect hippocampal function in adolescent P7 SD rats and that 50 mg/kg Cbx i.c.v. could not attenuate sevoflurane anesthesia-induced cognitive impairment in young SD rats (Figure 4).

Discussion

The current study demonstrated that anesthesia with 3% sevoflurane for 4 hours caused significant upregulation of Cx43 and Bax, decreased the level of the Bcl-2 protein and increased the expression of cleaved caspase-3 in the hippocampus after 1 day. Cbx [14], injected i.c.v. 2 hours before sevoflurane exposure could not decrease the upregulation of Cx43, Bax and cleaved caspase-3 or increase Bcl-2 expression in the hippocampus. Cbx also could not improve rat cognitive impairment after 30 days, and the role of Cx43 HCs and GJs in sevoflurane-induced development of neurotoxicity needs further investigation.

Cx43 protein HCs exhibit a low open probability under normal conditions to maintain normal synaptic function. HCs could be deleterious by releasing excitotoxins (e.g., ATP and glutamate), disturbing the concentration of intracellular Ca^{2+} or altering cytoplasmic ionic and osmotic balance. When the CNS is stimulated by various inflammatory mediators (cytokines, NO, ROS, etc.), it first enhances the HCs activity of astrocytes and microglia. For example, microglia stimulated by detrimental agents will elevate the

release of TNF- α and IL-1 β via microglial Cx32 hemichannels, leading to an increase in astrocyte Cx43 protein HC activity. Then, astrocyte Cx43 HCs opening mediated by microglia could increase Ca²⁺ influx and release glutamate, thus disrupting normal synaptic function.

Cx43 HCs opening under pathological conditions could release many glial transmitters, such as ATP and glutamate. When the extracellular Ca²⁺ concentration decreases or the synaptic glutamate concentration increases, Cx43 HCs open and ATP is released into the synaptic space. ATP activates the P2Y1 purine receptor of inhibitory intermediate neurons [15], thereby enhancing synaptic inhibition. At the same time, the Cx43 HCs-dependent release of ATP can also enhance the synaptic transmission of glutamatergic neurons, but this mechanism is still unclear. It has been speculated that ATP activates the AMPA receptor of the postsynaptic membrane through the P2X7 receptor, which increases excitotoxicity. D-serine is also released in a HC-dependent form intracellularly to extracellularly to enhance synaptic transmission and synaptic plasticity. D-serine is a coagonist of the NMDA receptor in the postsynaptic membrane of neurons. Studies have shown that D-serine could act on glutamatergic receptors in the postsynaptic membrane and play a synaptic role [16]. Data have also shown that Cx43 HCs of hippocampal astrocytes also control long-term potentiation (LTP) by releasing D-serine. These results indicate that Cx43 HCs play an important role in gliotransmitter release in the synaptic space [17].

Similarly, Cx43 GJs play an important role in the CNS [18-20]. GJs in astrocytes contribute to the formation of functional syncytial bodies, promote the removal of ions and neurotransmitters during neuronal activity, and allow the transmission of Ca²⁺ waves between astrocytes. Neurotransmitters released by neurons can bind to receptors in astrocyte membranes and produce metabolites such as inositol 1,4,5 triphosphate (IP3), ATP, glutamate and D-serine, which diffuse through GJs and affect nerve function. This intercellular communication based on Cx43 GJs enables astrocytes to perceive and integrate local and global synaptic activity and respond to glial transmitters affecting presynaptic and postsynaptic transmission. Therefore, Cx43 GJs and gliotransmitters are important participants in the regulation of the nerve activity, behavior and homeostasis of brain function.

An increase or decrease in Cx43 expression does not directly indicate whether its role is protective or damaging in the CNS, because Cx43 plays a role in the formation of HCs and GJs [21]. It is generally believed that under normal conditions, only a small number of Cx43 HCs are open in order to play some basic roles. HCs will open in large quantities enabling intracellular and extracellular communication directly when the CNS suffers various stimuli, such as ischemia, hypoxia, trauma and growth factors. Harmful toxic substances should be released from the cells to the extracellular environment, which produces adverse effects [22]. GJs have been shown to be both neuroprotective and neurodestructive in ischemic situations. When damage is limited to a small extent, GJs can buffer the toxic substances between adjacent cells and allow the cells to survive. When the damage increases, GJs can spread toxic substances a longer distance, causing more cell death. Many diseases or pathophysiological conditions seem to be associated with Cx43 HCs and GJs which have become new pharmacological targets in recent years [23].

To date, there have been many reagents and drugs that inhibit Cx43 HCs and GJs, including Cbx, quinine, mefloquine, connexin mimetic peptides GAP26, and GAP27 and so on [24,25]. Although the structures of Cx43 HCs and GJs have been studied for a long time, there is still no specific inhibitors within Cx43 HC and GJ. Cbx, a semisynthetic derivative of glycyrrhetic acid (GA), is widely used as a Cx43 HCs and GJs blocker in various pathological processes of the brain. It has been confirmed that Cbx displays different protective effects in a model of Parkinson's disease [26], ischemic brain injury [27], posttraumatic epilepsy [28], allergic airway inflammation [29] and fatty liver disease in obese mice [30]. Also, Cbx did not induce significant side effects even at the highest dose tested (50 mg/kg) [31]. Unfortunately, little is known about the clear mechanism of Cbx on connexin HCs and GJs [32]. Meanwhile, Cbx was previously shown to leave Cx43 expression unaffected [33]. Similar findings were reported in our study.

Why Cbx failed to attenuate sevoflurane-induced neurotoxicity in developing hippocampus in our study. First, Cbx, a more water-soluble derivative of GA, has different cellular targets and also acts as an inhibitor of 11 β -hydroxysteroid dehydrogenase [34]. Second, in addition to blocking Cx43 HCs and GJs, Cbx can block many other channels and receptors including voltage-gated Ca²⁺ currents, homomeric Panx1 and heteromeric Panx1/Panx2 channels, P2X7 receptors, Cx26 and Cx38 HCs [35-37]. Third, Cbx was widely used as a potent blocker of the major astroglia-to-astroglia and astroglia-to-neuron HCs and GJs in previous studies [38]. Future study is needed to determine which type of glial cell (microglia or astrocyte) is most affected by Cbx. Last but not least, because there are no specific inhibitors for HCs and GJs, it is necessary to develop Cx43 HCs and GJs specific inhibitors to clarify the role of Cx43 HCs and GJs in sevoflurane-induced CNS toxicity in the future.

Conclusions

Overall, for the first time, we demonstrated that 50 mg/kg Cbx i.c.v. 2 hours before 3% sevoflurane exposure could not decrease Cx43 upregulation, or the number of cleaved caspase-3 positive cells in the hippocampus of developing rats and also could not improve learning and memory impairment in adolescence. Both HC opening and GJ communication could affect normal CNS function, but which of them plays a pivotal role in sevoflurane-induced neurotoxicity in the developing rat hippocampus still needs further study.

Declarations

Author Contributions

Congjie Bi has contributed to conception, design, acquisition, analysis and drafted the manuscript. Congjie Bi, Dan Xie, Donghai Yu have contributed to data collection and analysis. Yang Wang has contributed editing and interpretation.

Funding

This research was funded by Natural Science Foundation of Liaoning Province 2020-MS-333.

Compliance with Ethical Standards

The present study was approved by the animal care and ethics committee of ShengJing Hospital of China Medical University (Shenyang, China) and was performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Figures

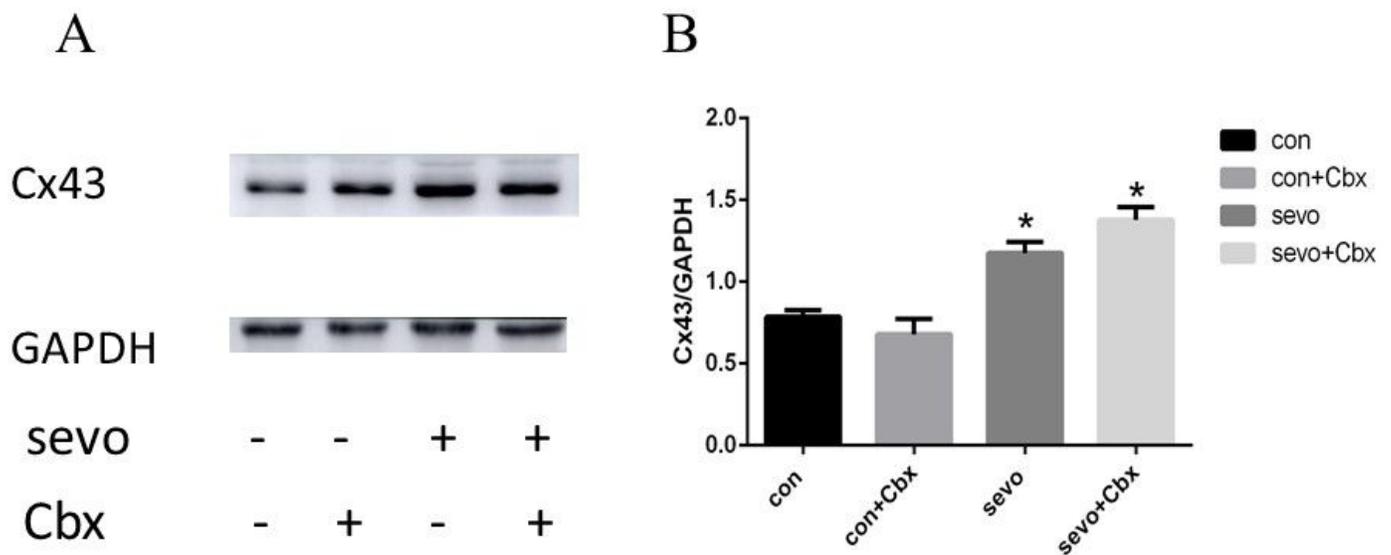


Figure 1

The effect of Cbx i.c.v. 2 hours before sevoflurane exposure on the expression of the Cx43 protein in the hippocampus of P7 rats. (A) Western blot results showed that Cbx i.c.v. 50 mg/Kg alone had no effect on the expression of Cx43 protein in the hippocampus of P7 rats 1 day after sevoflurane exposure compared with the Ctrl group. Sevoflurane could increase the expression of the Cx43 in the hippocampus. Cbx i.c.v. before sevoflurane did not reduce the up-regulation of Cx43 protein 1 day after sevoflurane exposure. (B) Quantification of the Western blotting results showed the differences of Cx43 between the Ctrl, Ctrl+Cbx, Sevo and Sevo+Cbx group 1 day after sevoflurane exposure. N=5 in each group. *P<0.05 compared with the Ctrl group.