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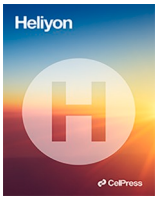

Research article

Sevoflurane-induced regulation of NKCC1/KCC2 phosphorylation through activation of Spak/OSR1 kinase and cognitive impairment in ischemia-reperfusion injury in rats

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ABSTRACT

The occurrence of excitotoxic damage caused by cerebral ischemia-reperfusion (I/R) injury is closely linked to a decrease in central inhibitory function, in which the concentration of chloride inside the cells ($[Cl-]_i$) plays a crucial role. The outflow and inflow of $[Cl-]_i$ are controlled by KCC2 and NKCC1, which are cellular cotransporters for K+/Cl− and Na+/K+/Cl−, respectively. NKCC1/KCC2 is regulated by upstream regulators such as SPAK and OSR1, whose activity is influenced by I/R. Sevoflurane is the most commonly used and controversial general anesthetic. To elucidate the impact of sevoflurane on cerebral ischemia-reperfusion (I/R) injury and its underlying mechanism, we investigated its influence on cognitive function and the mechanism of action utilizing a rat model of I/R. By activating the kinase Spak/OSR1, we discovered that I/R damage enhanced the function of NKCC1 and inhibited the function of KCC2, which triggered an imbalance of [Cl−]i concentration, leading to neurological dysfunction and cognitive dysfunction. At the beginning of reperfusion, administration of 1.3 MAC sevoflurane for 3 h increased activation of Spak/OSR1 kinases on day 7 post-perfusion, resulting in an additional dysregulation of NKCC1 and KCC2 activity, which disappeared on day 14. Administration of Closantel, a Spak/OSR1 kinase inhibitor, to animals treated with sevoflurane reverses the additional stimulation. The research revealed that sevoflurane modified the functioning of NKCC1 and KCC2, resulting in cognitive decline by activating Spak/OSR1 kinase. However, this issue could be resolved by inhibiting Spak/OSR1. The research revealed that sevoflurane transiently alters the function of NKCC1 and KCC2, resulting in exacerbating cognitive decline. However, this can be fixed by suppressing Spak/OSR1.

1. Introduction

To prevent primary and secondary strokes in patients with carotid artery stenosis, carotid endarterectomy has extensively been employed [1,2]. However, the removal of the blockage can potentially cause central ischemia and reperfusion (I/R) injury, which can lead to oxidative stress and neuronal harm [3]. It has been reported that the increased toxicity of excitatory amino acids contributes to

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cell death, and enhancing the effectiveness of central inhibition has neuroprotective benefits during the initial phase of injury [4]. The concentration of chloride inside the cell, known as intracellular chloride concentration ($[Cl\text{-}]_i$), is crucial for cell function and serves as the primary inhibitory ion within the cell. Hippocampal neurons [5] and slices [6] have demonstrated that Cl-imbalances can lead to neuronal death in hypoxia models, potentially as a result of the diminished inhibitory impact of γ-aminobutyric acid (GABA). Maintaining normal cognitive function and resisting excitatory toxicity in the nervous system requires the GABAergic system to regulate the balance between excitation and inhibition [7].

The inflow of Cl− ions is tightly regulated by the Na+/K+/Cl− cotransporter (NKCC1) and the outflow is regulated by the K+/Cl− cotransporter (KCC2), which ultimately controls the inhibition of the GABAergic system [8]. SPAK and OSR1, which are the primary regulators of NKCC1 and KCC2 cotransporters, frequently coexist in the same functional complex and control the concentration of $[Cl-]_i$ [9]. The occurrence of oxidative stress due to I/R injury can directly trigger the activation of STE20/SPS1-related proline/alanine-rich kinase-oxidative stress responsive 1 (Spak/OSR1), leading to controlled expression of target proteins downstream, activation of NKCC1, deactivation of KCC2, and consequent imbalance in the ratio of excitability to inhibition [10,11].The drug closantel, which specifically blocks Spak/OSR1 kinase, hinders the regulatory influence of this kinase on NKCC1/KCC2, as demonstrated by Gao et al. [12] and AlAmri et al. [13]. In bonecancerpain rat model, the closantel reduced pain behavior by blocking SPAK/OSR1 signal transduction and regulating the expression of downstream NKCC1 and KCC2 [14]. At the same time, increasing evidence suggests that targeting the WNK-SPAK/OSR1 pathway and the cation-chloride cotransporter (NKCC/KCC) to exert neuroprotective effects may be a new strategy for the treatment of stroke [15].

Cerebral I/R can lead to brain injury and cell death, and for anesthetic management, it is important to select the anesthetic that is most beneficial in terms of brain protection. Due to its low blood gas partition coefficient (0.69), rapid awakening induction, and minimal irritation to the respiratory tract, Sevoflurane is extensively utilized in clinical settings. According to Wang et al. [16], a single dose is highly effective, with a success rate of 99 %, in maintaining anesthesia in adult patients with a minimum alveolar concentration (MAC) of 1.3. The correlation between sevoflurane levels and postoperative neurocognitive impairment has recently attracted attention [17]. According to Kim et al. [18], the anti-apoptotic effect of sevoflurane posttreatment can enhance neuroprotection against cerebral I/R injury. On the other hand, other studies have shown that the use of sevoflurane increases cerebral I/R -induced apoptosis of neuronal cells [19]. At the molecular level, sevoflurane-induced apoptosis in neurons is thought to lead to cognitive dysfunction [20].

Exposure to isoflurane has been determined to be crucial in causing anesthesia neurotoxicity related to age in male rats. This exposure is strongly linked to disruptions in the levels of expression of NKCC1 and KCC2 [21]. A recent study found that 1.3 MAC sevoflurane exacerbated hypoxic brain injury in rats. However, the addition of an NKCC1 blocker [bumetanide] or a KCC2 activator [N-ethylmaleimide] could regulate intracellular chloride ion concentration, thereby reducing damage and improving neurocognitive outcomes [22]. At the beginning of ischemia/reperfusion (I/R), propofol after-treatment enhances the expression of KCC2 in the CA1 area of the hippocampus, thus enhancing neurobehavioral ability in rats [23].

In recent years, sevoflurane has been more widely used than isoflurane, although its impact on cognition has not been fully explored. Investigation is necessary to determine the specific impacts of sevoflurane on chloride transporter expression and their inherent mechanisms during the pathological progression of I/R. The objective of this research was to investigate the impact of sevoflurane on brain functionality and NKCC1/KCC2 activity following I/R injury.

2. Materials and methods

2.1. Ethics statement

Approval for the experimental procedure was granted by the Animal Ethics Committee of Wannan Medical College, with the reference code WNMC—AWE-2023072. Male Sprague-Dawley rats, classified as adults and weighing between 250 and 300 g, were obtained from the Chinese Academy of Military Medical Sciences. These rats, aged 3–4 months, were raised in accordance with the 'Guidelines for the Care and Use of Laboratory Animals'. The rodents were kept in a chamber with a temperature ranging from 26 to 28 °C, humidity levels between 40 and 60 %, and subjected to a 12-h cycle of light and darkness. The experimental procedures were conducted following the National Institutes of Health (NIH) Guidelines for the Protection and Use of Laboratory Animals (NIH Publication No., as per regulations 85–23, updated in 1996.

2.2. Experimental design

2.2.1. Experiment 1

Rats were randomly divided into three groups:

(1) The S group (consisting of 60 rats) underwent a sham procedure involving middle cerebral artery occlusion surgery without filament advancement.
(2) The I/R group consisted of 60 rats who experienced ischemia/reperfusion caused by the blockage of the middle cerebral artery for 60 min, followed by a period of reperfusion and inhalation of pure oxygen for 3 h.
(3) The SEV group consisted of 60 rats who underwent middle cerebral artery occlusion for 60 min, followed by reperfusion. During the 3-h reperfusion period, they inhaled sevoflurane at a concentration of 1.3 MAC. The sevoflurane used was obtained from Jiangsu Hengrui Pharmaceutical Co., Ltd. with the catalog number 2022S00823.

To determine the experimental concentrations of sevoflurane, oxygen, and carbon dioxide, the gas composition in the room was constantly monitored with an infrared analyzer (Datex Ohmeda, Madison, Wi, USA). To assess restoration of brain function, the Modified Nerve Severity Score (mNSS) and Morris Water Maze (MWM) were employed. To evaluate learning and memory, the mNSS test was performed on days 7 and 14 after operation, while the MWM space navigation test was performed on days 3–6 and 10–13. Additionally, space exploration tests were conducted on days 7 and 14 (Fig. 1). Samples of hippocampal CA1 were gathered on days 7 and 14 for assessing the presence of Spak/OSR1 enzyme and the phosphorylation of NKCC1 and KCC2 (Fig. 2).

2.2.2. Experiment 2

Randomly selected rats from Experiment 1 were divided into three additional subgroups:

(1) The Closantel + S group, consisting of 30 animals, received an intravenous injection of the Spak/OSR1 kinase inhibitor closantel (Cat #34093, Sigma Aldrich, St Louis, MO, USA) 30 min prior to the injection of normal saline, while the sham animals were not administered any treatment. Closantel (60 μg) was dissolved in 10 μL of dimethyl sulfoxide [24].
(2) The group treated with Closantel + I/R (n = 30) consisted of animals that received an intravenous injection of closantel (60 μg) 30 min prior to the administration of oxygen during reperfusion.
(3) In the closantel and SEV group (n = 30) SEV animals were administered closantel (60 μg) 30 min prior to reperfusion with sevoflurane.

Changes in $[Cl^-]_i$ were measured using N-(6-methoxyquinolineyl) acetylethyl ester (MQAE). The behavioral tests described in Section 2.2.1 were also used in this experiment (Figs. 3–5).

2.3. Experimental models

2.3.1. Middle cerebral artery occlusion (MCAO)

All rats were anesthetized intraperitoneally with Inactin (thiodine barbital, 100 mg/kg).

To administer closantel or saline and monitor mean arterial pressure, the heart rate, and the respiratory rate, a catheter made of polyethylene was inserted into the femoral vein on the right side. The vital signs were observed 30 min prior to the occurrence of ischemia, during the ischemic attack, 30 min following ischemia, at the initiation of reperfusion, and 30 min after reperfusion.

To cause ischemia, a minor cut was performed in the external carotid artery at the point at which the common carotid artery splits,

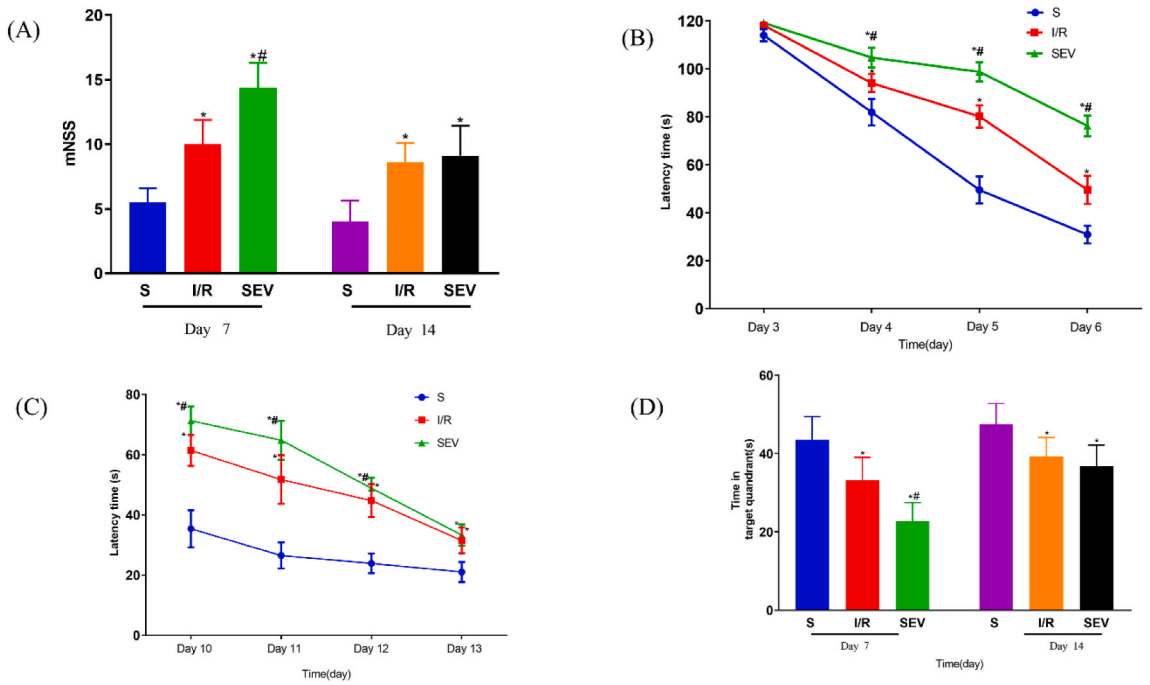

Fig. 1. Sevoflurane aggravates neurological and cognitive impairment caused by ischemic-reperfusion injury on day 7, but not on day 14. (A) Ischemia-reperfusion (I/R) injury caused neurological damage in rats on days 7 and 14, and the mNSS increased significantly. Sevoflurane aggravated this damage on day 7, but not on day 14. (B) I/R injury prolonged the seeking time in rats, and sevoflurane increased it further on days 3–6 and (C) day 13. (D) I/R injury reduced the time of residence in the target quadrant on both day 7 and day 14, which was exacerbated by sevoflurane on day 7, but not on day 14. Compared with the sham group, *P < 0.05; compared with the I/R group, #P < 0.05. Data are expressed as mean ± standard deviation (n = 10/group).

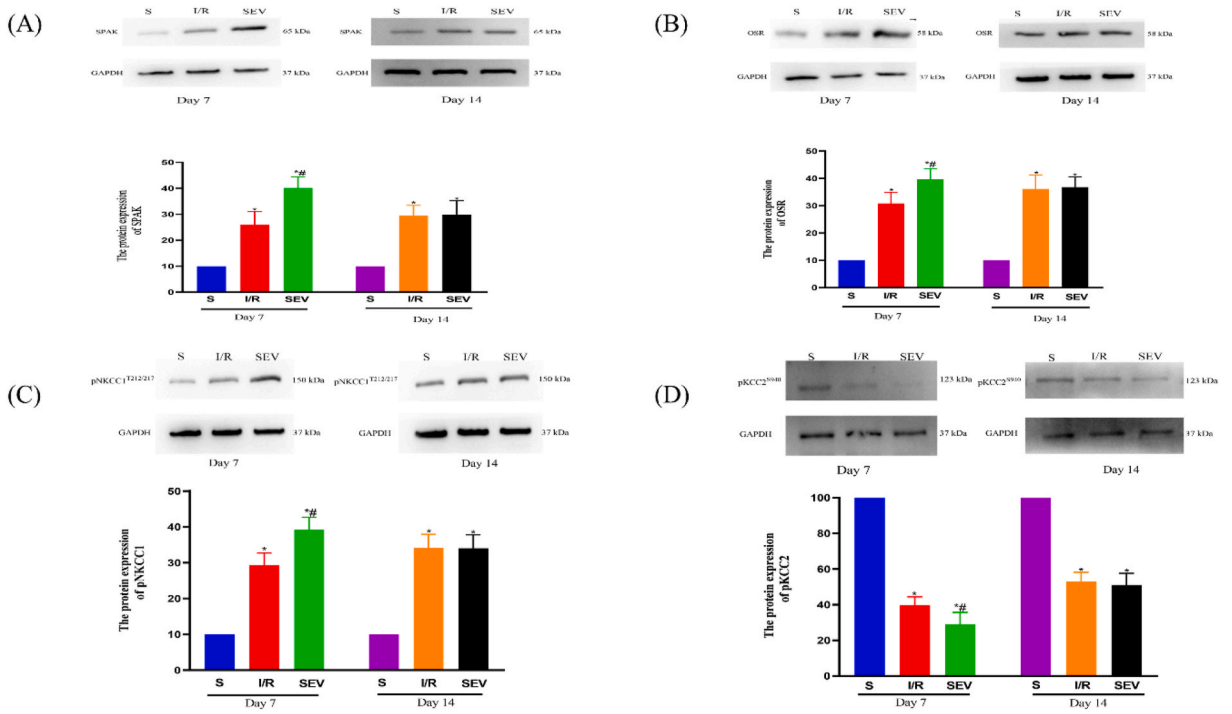

Fig. 2. Sevoflurane increases the expression level of STE20/SPS1-related proline/alanine-rich kinase-oxidative stress responsive 1 (Spak/OSR1) kinase, promotes phosphorylation of Na+/K+/Cl− cotransporter (NKCC1), and inhibits phosphorylation of potassium chloride cotransporter (KCC2) on day 7, but not on day 14. (A) I/R injury increased the expression level of SPAK protein on both days 7 and 14. Sevoflurane further promoted its expression on day 7, but not on day 14. (B) I/R injury increased the expression level of OSR protein on both day 7 and 14. Sevoflurane further promoted its expression on day 7, but not on day 14. Sevoflurane exacerbated this on day 7, but not on day 14. (C) Ischemia-reperfusion (I/R) injury upregulated the phosphorylation of NKCC1 protein on both day 7 and 14. (D) I/R injury inhibited phosphorylation of KCC2 protein on both day 7 and 14. Sevoflurane aggravated this on day 7, but not on day 14. Compared with the sham group, *P < 0.05; compared with the I/R group, #P < 0.05. Data are expressed as mean ± standard deviation (n = 10/group).

and a nylon monofilament (Ethicon, Raritan, NJ, USA) measuring 0.26 mm in diameter was inserted into the lumen of the internal carotid artery for a distance of 18–20 mm until a slight obstruction was sensed, indicating correct positioning at the bifurcation of the internal carotid and middle cerebral artery. Following 1 h of blockage, the thread was removed 10 mm away from the suture injury to allow for reperfusion. During the sham procedure, the filament was inserted into the internal carotid artery at a depth of less than 10 mm. The lamp irradiation method was used to maintain the body temperature at 37 ± 0.5 °C, as reported by Wang et al. [25].

2.3.2. *Primary hippocampal neuron culture and hypoxic injury model*

Extraction of primary hippocampal neurons [26]. The brains of newborn rats (Day 1) were collected, the vascular plexus was separated under a dissecting microscope, and hippocampal tissue was extracted. The cells were inoculated with L-polylysine coated plates and placed in a $37^0C/5\%CO_2$ constant temperature cell incubator after inoculation. After 4 h, the culture system was replaced by B27 (Gibco) and Neurobasal-A medium (Gibco, Thermo Fisher Science, Waltham,USA) of antibiotics (penicillin-streptomycin solution; HyClone). The density was 1×10^5 cell/cm^2, and then half the medium was changed every two days. Microtubule associated protein 2 staining confirmed the success of neuron culture (Fig. S1).

To induce hypoxia, neurons were placed in a hypoxic incubator at 37 °C with 5 % CO_2, 3 % O_2, and 92 % N_2 for 3 h [27]. Then the reagent was added according to the test requirements, and the indoor gas concentration was continuously monitored by infrared analyzer (DatexOhmeda, USA). After the drug treatment is completed, the corresponding culture dish is replaced with a fresh neuron-specific culture system and placed in a normal cell incubator to continue culturing for 24 h for subsequent experiments.

2.4. *mNSS test*

A blinded researcher conducted a neurological assessment on the animal group, as stated by Wang et al. [28]. The range of the score was between 0 and 18, comprising four elements: motion, feeling, reflexes, and equilibrium. A score of 0 indicates no deficits. A score of 1 signifies absence of assessed reflexes or incapability to execute tasks, scores varying from 1 to 6 suggest slight harm, scores varying from 7 to 12 suggest moderate harm, and scores varying from 13 to 18 suggest severe harm.

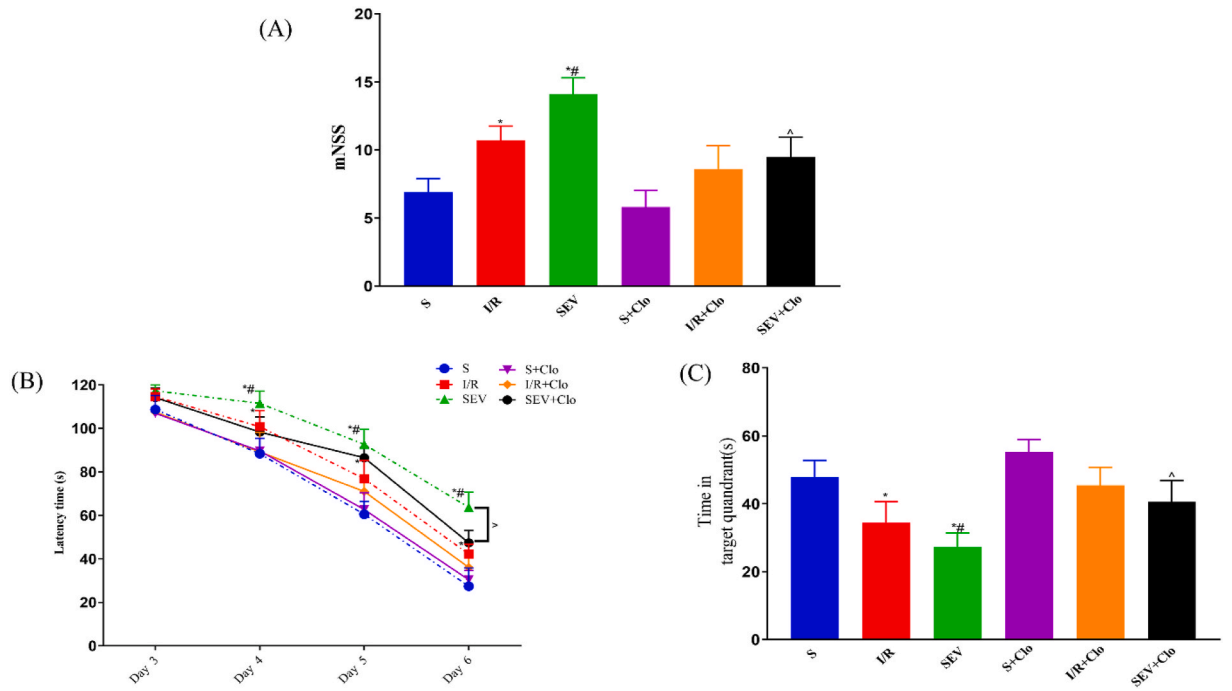

Fig. 3. Closantel reverses short-term damage to brain function caused by sevoflurane. (A) The ischemia-reperfusion (I/R) and sevoflurane (SEV) groups had higher Modified Nerve Severity Scores (mNSSs) on day 7 than the sham (S) group. The SEV group had significantly higher values than the I/R group. mNSSs were significantly reduced in the closantel + SEV group compared with those in the SEV group. (B) The I/R and SEV groups spent more time finding the platform than the S group. The SEV group spent more time finding the platform than the I/R group. The closantel + SEV group had a shorter search duration than the SEV group. (C) The I/R and SEV groups stayed in the target quadrant longer than the S group. The SEV group stayed for a shorter time compared to the I/R group. The closantel + SEV group had prolonged residence time compared to the SEV group. Compared with the S group, *P < 0.05; compared with the I/R group, #P < 0.05; compared with SEV group, ^P < 0.05. Data are expressed as mean ± standard deviation (n = 10/group).

2.5. MWM testing

The MWM test included space navigation and space sounding experiments [29]. The labyrinth (150 cm in diameter and 50 cm in height) contained water with a temperature ranging from 20 to 24 °C. Throughout the training session, the desired location was concealed beneath the water's surface, precisely 2 cm deep, and securely positioned within the southwestern section of the maze. During the experiment called 'Location Navigation', every rat underwent testing four times daily for a duration of four consecutive days. We directed rats that were unable to locate the platform within a time frame of 2 min. Throughout the trial, every rat was carefully placed into the water from a fixed starting point, followed by the discovery of a concealed platform within 60 s. In case the rat failed to locate the desired objective within the allocated time, we gently assisted it in reaching the platform for a duration of 30 s. Those rats who successfully located their target remained on the platform for 30 s before being taken away. Recorded was the duration of time spent searching for the platform, also known as escape latency. To conduct experiments related to space exploration, the concealed platform was eliminated, and the simulated rats were examined as a point of comparison. Each rat was given 60 s of free-swimming time. We recorded the amount of time spent within a circular region measuring 30 cm around the platform.

2.6. Western blot analysis

A radioimmunoprecipitation assay buffer (RIPA; Solarbio, R0010) supplemented with phenylmethanesulfonyl fluoride (PMSF; Solarbio, P0100) was used to lyse the hippocampal tissue in the rat brain. Centrifuge the mixture at 14，000×*g* for 5 min at 4 °C. The supernatant was collected and the protein concentration was quantified with the BCA assay kit. Then protein samples (20 μg) were separated using SDS-PAGE, followed by transfer to a polyvinylidene fluoride membrane. phosphor-NKCC1$^{T212/217}$(Thermo Fisher Scientific Cat# PA5-88206, RRID: AB_2804742), phosphor-KCC2^{S940} (Thermo Fisher Scientific Cat# PA5-95678, RRID: AB_2807480), SPAK (Cell Signaling Technology Cat# 2281, RRID AB_2196951), OSR1 (Proteintech Cat# 15611-1-AP, RRID: AB_2299030), and GAPDH (Bioworld Technology Cat# AP0063, RRID: AB_2651132) were used at a dilution of 1:1000 for an overnight incubation at 4 °C. After washing, the blot was treated with a secondary antibody (Thermo Fisher Scientific Cat# PA1-86141, RRID AB_931559) specific to rabbit IgG, obtained from goats. The signal was then detected using enhanced chemiluminescence. ImageJ (RRID SCR_003070) was utilized to analyze the band density, which was then normalized to β-actin. The Pierce® Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) was used to isolate cell surface proteins that express membranes (NKCC1/KCC2), according to the

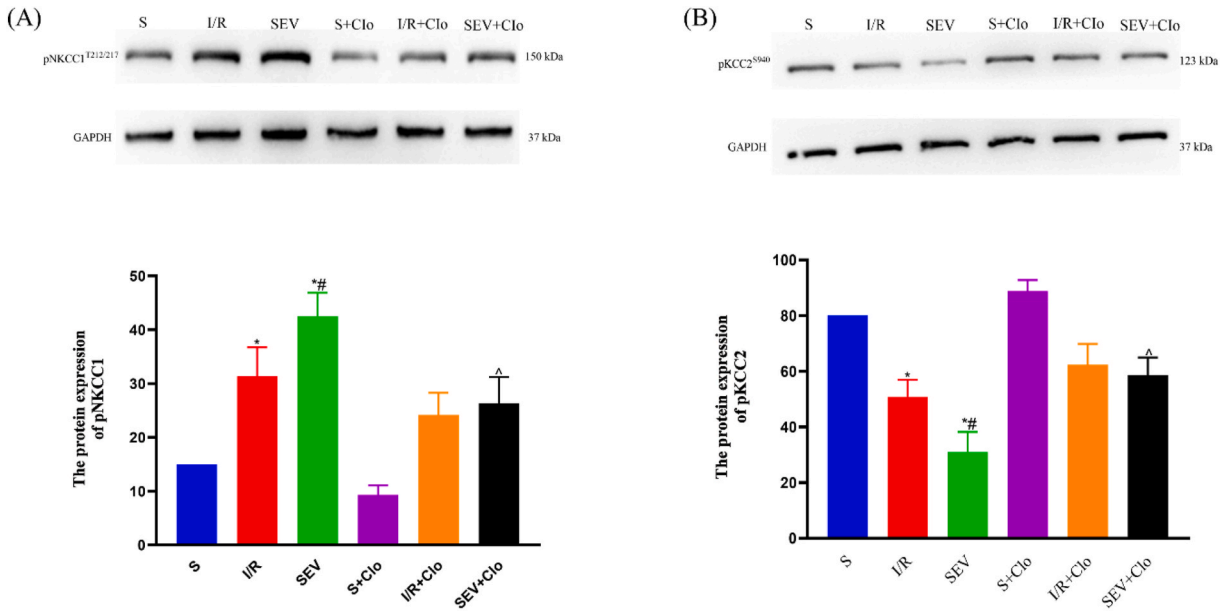

Fig. 4. Closantel alleviates the regulatory effect of sevoflurane on the phosphorylation level of $Na^+/K^+/Cl^-$ cotransporter/potassium chloride cotransporter (NKCC1/KCC2). (A) Compared with that in the sham (S) group, the phosphorylation level of NKCC1 in the CA1 region of the hippocampus was significantly increased in the ischemia-reperfusion (I/R) group and the sevoflurane (SEV) group. The SEV group had significantly higher phosphorylation levels compared to the I/R group. Compared with that in the SEV group, phosphorylation of NKCC1 in the CA1 region of the hippocampus was reduced in the closantel + SEV group. (B) Compared with that in the S group, the level of KCC2 phosphorylation in the CA1 region of the ischemic hippocampus group decreased significantly in the I/R and SEV groups. When compared with that in the I/R group, the phosphorylation of KCC2 was further reduced in the SEV group. Compared with that in the SEV group, KCC2 phosphorylation in the CA1 region of the ischemic hippocampus was significantly increased in the closantel + SEV group. Compared with the S group, *P < 0.05; Compared with the I/R group, #P < 0.05; Compared with the SEV group, ^P < 0.05. Data are expressed as mean ± standard deviation (n = 10/group).

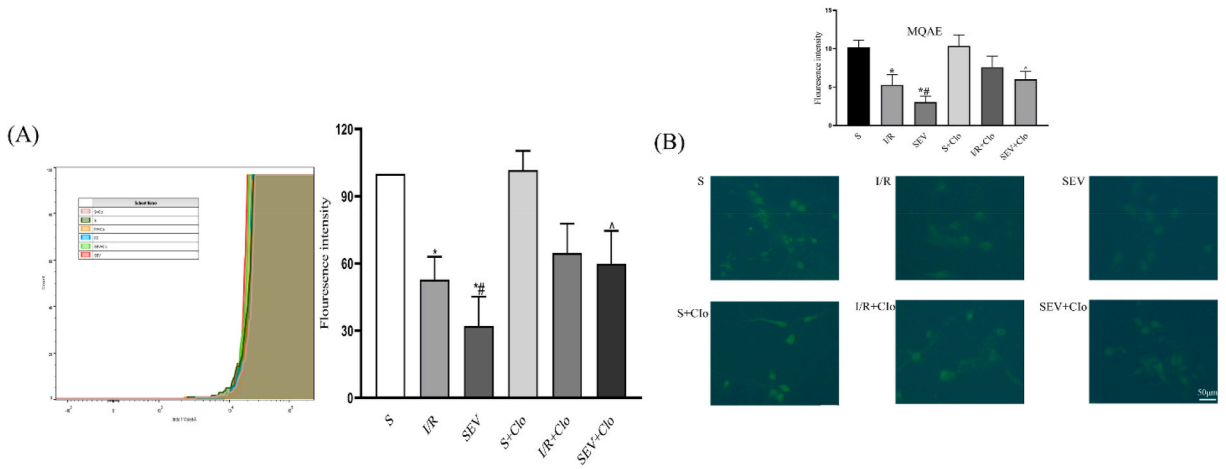

Fig. 5. Closantel mitigates the effect of sevoflurane on intracellular chloride concentration ($[Cl\text{-}]_i$). $[Cl^-]_i$ increased in the ischemia-reperfusion (I/R) and sevoflurane (SEV) groups. The increase was more significant in the SEV group than in the I/R group. $[Cl\text{-}]_i$ in the closantel + SEV group decreased significantly compared with that in the SEV group. (A): In vivo, (B): in vitro. Compared with the S group, *P < 0.05; compared with the I/R group, #P < 0.05; compared with the SEV group, ^P < 0.05. Data are expressed as mean ± standard deviation (n = 10/group).

instructions provided by the manufacturer. The experiment was repeated three times.

2.7. Intracellular chloride detection

In the cytosol, chloride ions are bound by the fluorescent probe MQAE, causing the fluorescence signal to decrease with higher cytosolic chloride levels and increase with lower chloride levels. Following a rinse in regular saline, the hippocampal tissue was

promptly transferred to a Petri dish filled with phosphate buffer saline (PBS) that had been pre-chilled to 4 °C. Ophthalmic scissors were used to crush the tissue, which was then washed with PBS. Pancreatic enzyme (0.25 %) was added for digestion at 37 °C, and then calf serum was added to terminate digestion. The tissue was pulverized using a 300-μm nylon mesh and then underwent filtration and centrifugation at 150*g* for a duration of 5 min. The supernatant was discarded, and the pellet was rinsed three times with PBS. To create a 1×10^6 cell/mL suspension, the cells were diluted using PBS. Then, 100 μL of this suspension was mixed with 10 mM MQAE (MedChemExpress Cat# HY-D0090) and incubated at 37 °C for 10 min, following the method described by Perry et al. [30]. Flow cytometry was used to measure the fluorescence intensity of MQAE (BD-FACSAria™ Fusion, Becton, Dickinson and Company, State of New Jersey). In another way of analysis, we used fluorescence microscope to observe the MQAE fluorescence in cultured hippocampal neurons, and the fluorescence intensity of the probe was inversely proportional to the concentration of $[Cl^-]_i$.

2.8. Statistical analysis

All data analyses were performed using the GraphPad Prism software (version 9.00; GraphPad Software, La Jolla, CA, USA; www.graphpad.com). To evaluate normality, the Shapiro-Wilk test was employed. Normally distributed data are presented as the mean plus the standard deviation (SD). We employed the two-way analysis of variance (ANOVA) to study animal behavior. One-way ANOVA was used to analyze all the remaining experiments. Tukey's post-hoc test was performed for all experiments, which were repeated in triplicate. A significance level of less than 0.05 was deemed statistically significant.

3. Results

Physiological parameters did not notably differ among the groups, as explained in the supplementary information (Table S1).

3.1. Sevoflurane aggravates neurological injury and causes cognitive impairment

The mNSSs of the I/R and SEV groups were significantly elevated compared to the S group on days 7 and 14. The SEV group had a considerably greater sore than the I/R group on day 7 (SEV vs I/R:14.7 ± 0.12vs11.5 ± 0.11, $P < 0.05$). but there was no notable disparity on day 14 (SEV vs I/R:8.7 ± 0.18vs.8.55 ± 0.11, $P > 0.05$). The results showed that the aggravating effect of sevoflurane disappeared (Fig. 1A).

During the space exploration experiment from days 3–6, both the I/R and SEV groups exhibited a considerably increased escape latency compared to the S group (S vs I/R：35.41 ± 4.28 vs 44.64 ± 2.25, $P < 0.05$; S vs SEV：5.41 ± 4.28 vs 79.25 ± 2.38, $P < 0.05$; Fig. 1B). In comparison to the I/R group, the SEV group exhibited a notably prolonged escape latency (SEV vs I/R：79.25 ± 2.38 vs 44.64 ± 2.25, $P < 0.05$, Fig. 1B). During days 10–13, both the I/R and SEV groups continued to exhibit a prolonged escape latency in comparison to the S group (S vs I/R：21.21 ± 2.05 vs 37.23 ± 3.07, $P < 0.05$; S vs SEV：21.21 ± 2.05 vs 39.08 ± 1.63, $P < 0.05$; Fig. 1C). However, the disparity between the SEV and I/R groups lost its statistical significance (SEV vs I/R：39.08 ± 1.63 vs 37.23 ± 3.07, $P > 0.05$; Fig. 1C).

Probe testing was performed on days 7 and 14 (Fig. 1D). The S group spent a considerably longer duration in the target quadrant compared to the I/R and SEV groups on day 7(S vs I/R：42.13 ± 1.34 vs 32.13 ± 2.71, $P < 0.05$; S vs SEV：42.13 ± 1.34 vs 21.23 ± 2.04, $P < 0.05$). Moreover, the SEV group spent considerably less time in the target quadrant compared to the I/R group (SEV vs I/R：21.23 ± 2.04 vs 32.13 ± 2.71, $P < 0.05$). By day 14, the S group continued to spend a considerably greater amount of time in the target quadrant compared to the I/R and SEV groups ((S vs I/R：52.38 ± 4.12 vs 39.15 ± 3.27, $P < 0.05$; S vs SEV：52.38 ± 4.12 vs 36.41 ± 2.97, $P < 0.05$). However, there was no longer a statistically significant distinction between the SEV and I/R groups (SEV vs I/R：36.41 ± 2.97 vs 39.15 ± 3.27, $P > 0.05$).

The expression level of Spak/OSR1 kinase Is enhanced by Sevoflurane, leading to the phosphorylation of NKCC1 and the inhibition of KCC2 phosphorylation.

On day 7, The levels of Spak/OSR1 protein in the I/R and SEV groups were significantly elevated compared to the S group on days 7 and 14. On day 7, levels in the SEV group were significantly higher compared to the I/R group, but this difference was not observed on day 14 (Fig. 2A/B).

the phosphorylation level of NKCC1 and KCC2 in the CA1 region of the hippocampus showed a significant increase and decrease, respectively, in the I/R and SEV groups compared to the S group ($P < 0.05$). The phosphorylation of NKCC1 and KCC2 significantly increased and decreased, respectively in the I/R as compared to the SEV group ($P < 0.05$). By day 14, the I/R and SEV groups exhibited no significant distinction anymore ($P > 0.05$; Fig. 2C/D).

3.2. Closantel reverses the short-term damage effect of sevoflurane induced by aggravating brain function

The mNSS of the closantel + SEV group was significantly reduced on day 7 in comparison to the SEV group (closantel + SEV vs SEV：8.42 ± 2.03 vs 12.43 ± 3.56, $P < 0.05$; Fig. 3A). During days 3–6 of the space exploration experiment, the closantel + SEV group exhibited a noticeably reduced escape latency in comparison to the SEV group (closantel + SEV vs SEV：49.34 ± 5.16 vs 71.62 ± 4.61, $P < 0.05$; Fig. 3B). Also, the closantel + SEV group was considerably longer in the target quadrant during the probe test on day 7 in comparison to the SEV group (closantel + SEV vs SEV：41.07 ± 3.42 vs 28.54 ± 2.79, $P < 0.05$; Fig. 3c).

3.3. Closantel prevents sevoflurane-induced change in phosphorylation of NKCC1/KCC2 and $[Cl^-]_i$

On the seventh day following administration of closantel, the group treated with closantel + SEV exhibited a notable decrease and increase in the phosphorylation of NKCC1 and KCC2, respectively, in comparison to the SEV group (as shown in Fig. 4A/B). On day 7, $[Cl\text{-}]_i$ was higher in the I/R and SEV groups compared to the S group. Additionally, a notable distinction existed between the I/R and SEV cohorts. In comparison to the SEV group, the closantel + SEV group had significantly reduced intracellular chloride concentrations in vivo and in vitro ($[Cl\text{-}]_i$; Fig. 5 A/B).

4. Discussion

After inhaling 1.3 MAC sevoflurane for a duration of 3 h, the CA1 area of the hippocampus notably increased in the presence of Spak/OSR1 kinase. This led to the promotion of NKCC1 phosphorylation, inhibition of KCC2 phosphorylation, and worsened cognitive impairment caused by I/R on the seventh day. Closantel, an inhibitor of Spak/OSR1 kinase, effectively reversed these effects, confirming that sevoflurane is involved in the regulation of chloride concentration through the enhancement of Spak/OSR1 kinase activity. Additionally, we verified the regulatory route of sevoflurane-Spak/OSR1-NKCC1/KCC2.

According to Song et al. [31], older men have a higher prevalence of carotid stenosis, with over one billion individuals worldwide having an abnormal carotid intimal-medial thickness of 1.0 mm or greater, and 1.5 % being affected. Carotid endarterectomy is a successful surgical technique for reducing the risk of stroke in individuals with carotid artery narrowing. Nevertheless, the prognosis can be significantly impacted by excitotoxicity resulting from cerebral ischemia-reperfusion injury [32]. The transmembrane $[Cl\text{-}]_i$ gradient, which is strictly controlled by NKCC1-mediated inflow, KCC2-mediated outflow, and SPAK/OSR1 regulation of the cotransporters, impacts excitatory toxicity after I/R. Consistent with the results of this research, Kaila et al. [33] stated that activation of SPAK/OSR1 by I/R leads to phosphorylation of NKCC1, inhibition of KCC2 phosphorylation, augmentation of cytotoxic edema, and deterioration of neurobehavioral function.

In surgical procedures that prioritize the protection of the central nervous system, the choice of anesthetic drugs plays a crucial role. In vitro [34] and in vivo [35] experiments, sevoflurane aggravated apoptosis, inflammatory response and oxidative stress in hippocampal neurons, worsened cognitive function, induced the occurrence of post operative cognitive dysfunction, inhibited sevoflurane-induced oxidative stress, and strengthened the protection of cognitive function. Following laboratory experiments, the adult rats treated with sevoflurane exhibited a MAC value of 2.3 ± 0.3 %. Additionally, when used solely for anesthesia maintenance, the 99 % effective dose was found to be 1.3 times the MAC value [14]. Hence, the target concentration for this experiment was set at 1.3 MAC, which is approximately 3.3 %. The CA1 region of the hippocampus is particularly sensitive to ischemic injury [36]. During this investigation, we discovered that sevoflurane worsened the neurocognitive impairment resulting from I/R injury within a brief period of 7 days. Additionally, it increased the expression of Spak/OSR1 kinase (Fig. 2A/B), leading to abnormal phosphorylation of NKCC1 and KCC2 in the CA1 area (Fig. 2C/D), and lead to the steady-state imbalance of $[Cl\text{-}]_I$ (Fig. 5). Our experimental results reveal that sevoflurane can aggravate the oxidative stress induced by I/R, further worsen the balance of Spak/OSR1 kinase expression, eventually lead to neurological dysfunction, and expand the cognitive impairment mechanism of sevoflurane. However, it was not clear at this time whether the activity of NKCC1/KCC2 depends on the regulation of Spak/OSR1 kinase. The results of the second part had showed that we introduced the Spak/OSR1 kinase inhibitor closantel and once again verified the phosphorylation level of NKCC1/KCC2 (Fig. 4). The results supported our previous hypothesis that the inhibitor closantel inhibits the effect of sevoflurane on Spak/OSR1 kinase, does not cause abnormal changes in NKCC1/KCC2 phosphorylation levels, and maintains chloride homeostasis in vivo and in vitro (Fig. 5), which supported the previous results in the opposite direction. Finally, it was clarified that sevoflurane enhanced the expression level of Spak/OSR1 kinase on the seventh day, leading to the phosphorylation of NKCC1 and inhibiting the phosphorylation of KCC2. In the rat model of bone cancer pain (BCP), Wang et al. demonstrated that activation of WNK1-SPAK/OSR1 signaling promotes BCP in rats by regulating NKCC1 and KCC2 expression, and closantel can alleviate pain levels [12]. On this basis, we expanded the research space of NKCC1/KCC2 and the application scenarios of closantel, and further revealed that the change of chloride ion concentration is the key to signal transduction.

Bumetanide is an FDA-approved NKCC1 inhibitor. Experiments have found that early treatment with bumetanide can alleviate hippocampal memory impairment caused by hypoxia-ischemia [37]. At the same time, inhibiting NKCC1 reduces white matter damage and cognitive impairment caused by chronic hypoperfusion [38]. Several neurological disorders, including multiple subtypes of epilepsy, neuropathy, pain, and schizophrenia, as well as cognitive impairment due to trauma and ischemia, are associated with a significant decrease in KCC2-mediated extrusion capacity of [Cl−]I [39]. Our first part of experiments has confirmed that sevoflurane can further worsen the expression balance of Spak/OSR1 kinase by aggravating the oxidative stress response induced by I/R, leading to enhanced NKCC1 activity and reduced KCC2 activity.The closantel is an antiparasitic drug that can reduce the activation of SPAK/OSR1 kinase, thereby inhibiting NKCC1 activity and increasing KCC2 activity. In the second part of the experiment, the combination of closantel and sevoflurane was found to have a significant positive effect on neurocognitive dysfunction in rats, suggesting that closantel can reverse the additional damage caused by sevoflurane in I/R damage (Fig. 3). Based on the accumulation of previous experiments, this experiment filled in the effect of unified regulation of NKCC1/KCC2 activity on neurological function, and provided a new mechanism and compensatory measures for the impact of sevoflurane on cognitive function. It must be emphasized that NKCC1/KCC2 chloride cotransporters are regulated by SPAK/OSR1 through phosphorylation, which in turn affects the concentration of chloride in neurons, and then the effects of different phosphorylation sites are opposite. Through literature review, we determined that SPAK/OSR1-mediated phosphorylation of NKCC1 T212、T217 resulted in enhanced NKCC1 activity [40], and phosphorylation of KCC2 at S940 sites, resulting in its functional activation [41,42]. Therefore, we took the T212 and T217 sites of NKCC1 and the S940

site of KCC2 as our research targets.

This study has some limitations. In clinical anesthesia, it is uncommon to use inhalation anesthetics as the sole method; instead, a combination of analgesics, muscle relaxants, sedatives, and vasoactive drugs is employed, leading to intricate drug interactions. Moreover, the amount of drugs given to rats and humans considerably differs, and evoflurane has multifaceted impacts on mouse memory at varying levels of inhalation concentrations and durations, with distinct underlying mechanisms. For example, Xie et al. [43] discovered that inhalation of sevoflurane at a concentration of 3 % for a duration of 2 h not only resulted in temporary cognitive impairment in mature rats but also caused cognitive dysfunction that could be reversed in the long term. On the other hand, research has indicated that minimal levels of sevoflurane have no impact on synaptic plasticity in the CA1 area of the hippocampus in rats. The findings of our study align with prior research [44], indicating that the cognitive decline in rats due to sevoflurane was temporary, and our experiment provided additional evidence of the potential for recovery from this decline. The results on day 14 showed that sevoflurane anesthesia does not worsen the neurocognitive function after tissue damage. Our experiments provide some suggestions for improving the clinical application of sevoflurane, especially for patients who are allergic to lipids and can only use inhalation anesthesia.

In summary, we discovered that during I/R injury, sevoflurane post-treatment temporarily modifies phosphorylation of NKCC1/KCC2 by enhancing the presence of SPAK/OSR1 kinase. This results in reduced $[Cl\text{-}]_i$ and neurocognitive impairment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding author.

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CRediT authorship contribution statement

Yuefeng Wang: Project administration, Methodology. **Yuanyu Zhang:** Writing – original draft, Visualization. **Wei Yu:** Methodology, Investigation. **Mengjuan Dong:** Software, Data curation. **Pingping Cheng:** Writing – review & editing. **Ye Wang:** Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32481.

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