



MiR-128-3p Attenuates the Neurotoxicity in Rats Induced by Isoflurane Anesthesia

Duo Qian¹ · Shijie Dai² · Yujing Sun³ · Yawei Yuan⁴ · Long Wang⁵

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Abstract

Isoflurane (ISO) has been widely used in clinical anesthesia, and exposure to ISO leads to cognitive dysfunction. Our paper aimed to investigate the effect of miR-128-3p on cognitive impairment, inflammation, and oxidative stress elicited by ISO anesthesia in Sprague–Dawley (SD) rats. The SD rats were treated with ISO to mimic the ISO-injured situation, and the concentration of miR-128-3p was quantified utilizing real-time PCR. The miR-128-3p's impacts in ISO-engendered rat models on the respects of inflammatory condition and oxidative activities were measured by the commercial kits. The Morris water maze test was adopted to measure the neuro-function regarding miR-128-3p. Additionally, the target was tested by the alternation of luciferase activity. The irritation of ISO suppressed miR-128-3p expression in rats, which was enhanced by the injection of miR-128-3p agomir. The adverse roles of ISO on inflammation, oxidative stress, and cognitive disorders were partially abrogated by an increment of miR-128-3p. A miR-128-3p's interconnection with specificity protein 1 (SP1) was pinpointed, and aggrandized mRNA levels of SP1 were found under ISO state. MiR-128 acted as a regulator in ISO damage in the respects of cognition, inflammation, and oxidative stress. The SP1's link of miR-128-3p was showcased.

Keywords Isoflurane · miR-128-3p · Inflammation · Oxidative stress · SP1

Introduction

Isoflurane (ISO) is a volatile anesthetic approved by the Federal Drug Administration for common anesthesia (Eger 1981). Like most volatile anesthetic agents, ISO is a halogenated ether compound. ISO is utilized in general anesthesia operations owing to its high safety profile, rapid onset, and rapid recovery (Isoflurane 2012). Recent studies have

shown that exposure to ISO anesthesia can aggravate the memory deficits of mice, leading to circadian rhythm disorders (Song et al. 2018). Studies in juvenile rodents and nonhuman primates have also shown that early exposure to general anesthesia will impair brain development, such as inhibition of neurogenesis, induction of extensive neuronal apoptosis, and long-term neurocognitive impairment (Song et al. 2018; Zhang et al. 2019). As a hub of learning and memory in the central nervous system, the hippocampus is considered as the neural circuit biomarker of cognitive impairment and cognitive improvement, and it is particularly susceptible to anesthesia in ISO-induced rat cognitive impairment models (Hu et al. 2017; Li et al. 2019). Given the prominent role of hippocampi in cognition, the roles of ISO on hippocampi were critical. It is currently believed that inhalation anesthesia can lead to cognitive impairment through various pathophysiological processes, including neuroinflammation, mitochondrial damage, and oxidative stress (Berger et al. 1528; Wang et al. 2018). Therefore, efforts at ISO-induced nerve damage and inflammation are necessary, and this may improve its application.

MicroRNA is a single-stranded non-coded small molecule RNA consisting of 21–25 nucleotides. A large amount of miRNAs has been proved to be dysregulated in nervous

Duo Qian and Shijie Dai contributed equally to this work.

✉ Long Wang
wacmo7692@163.com

¹ Department of Anesthesiology, Affiliated Hospital of North Sichuan Medical College, Nanchong 637000, Sichuan, China

² Department of Anesthesiology, Xuzhou No.1 People's Hospital, Xuzhou 221000, China

³ Department of Anesthesia Surgery, Dongying Hospital of Traditional Chinese Medicine, Dongying 257055, China

⁴ Department of Anesthesiology, Shanghai First Maternity and Infant Hospital, School of Medicine, Tongji University, Shanghai 200092, China

⁵ Department of Pain Medicine, First Medical Center, Chinese PLA General Hospital, Beijing 100853, China

development, such as learning and memory (Wang et al. 2020b; Maiese 2019). Several studies discover that specific miRNA associates with anesthesia and plays roles in the cognitive disorders caused by anesthesia (Yu et al. 2021; Zhao et al. 2021). For instance, miR-140-5p expression in diabetic rats treated with ISO is significantly increased, and this tendency is closely related to cognitive impairment caused by ISO (Fan et al. 2020). Another publication indicates that miR-142-5p is upregulated under management by ISO, and lack of miR-142-5p is beneficial to neurological function (Xie et al. 2020). Many papers prove that miR-128-3p plays role in nervous system, learning, and memory. In Huntington's disease, a chronic neurodegenerative impairment, the abnormal expression of miR-128-3p implicated in cognitive state (Aganzo et al. 2018). In addition, miR-128-3p is lowly expressed in the rat model of ischemia/reperfusion, and it participates in the regulation of neuroinflammation (Wang et al. 2020a). Consequently, the roles of miR-128-3p attract our concern. In addition, SP1 is a target of miR-128-3p in atrial fibrillation, and it is proved to be a regulator in postoperative cognitive dysfunction influenced by anesthesia (Cao et al. 2019; Lv et al. 2020).

Our paper established a rat model of ISO damage to corroborate the miR-128-3p's significance on ISO. For this reason, the quantification of miR-128-3p was certified and regulated in a rat model. The values of miR-128-3p on inflammation, oxidative stress, and cognition were further elucidated. Additionally, the potential mechanism behind miR-128-3p was reasoned.

Materials and Methods

Maintenance of Animals

A 7-day-old Sprague–Dawley (SD) rats (13 ± 3 g) were purchased from the CAVENS company (Changzhou, China). All the rats were placed in cages with a temperature of 21 ± 1 °C and kept in a 12-h day/night cycle. Rats could freely get food and water. All animal procedures were endorsed by the Animal Ethics Committee. Each group encompassed ten random rats.

Transfection and Anesthesia Administration

The reagents of miR-128-3p agomir and miR-NC were purchased from GenePharma (China). The SD rats in ISO group were anesthetized with 1.5% isoflurane anesthesia for 6 h, and rats in ISO + miR-agomir and ISO + miR-NC groups were maintained anesthesia for 6 h after stereotaxic injection of miR-128-3p agomir and its negative sequences into the left and right hippocampus, respectively. The rats in the miR-NC group and miR-agomir group were only injected corresponding reagent into bilateral hippocampus of rats without ISO treatment.

The protocols of anesthesia on SD rats were performed according to the previous study written by Lunardi (Lunardi et al. 2010). The Penlon Prima SP anesthesia machine (Penlon, UK) was used to connect the anesthesia chamber to form an anesthesia pipeline. The self-made sealed anesthesia chamber was placed in a 37 °C thermostat. The experimental newborn rats were placed in a self-made sealed anesthetic chamber and were continuously given a mixture of 1.5% isoflurane. Rats in the untreated group were given a gas mixture containing a mixed air of nitrogen and oxygen with a flow rate of 2 L/min. After this experiment, the rats were divided into two groups randomly. Rats in one group were used to detect the expression of miR-128-3p in hippocampus, and the other group of rats underwent MWM experiment.

Tissue Extraction and Real-Time PCR

Half rats were randomly selected from each group after inhaling ISO for 6 h. According to the dose of 350 mg/kg, an injection of 10% chloral hydrate was utilized. The brain was decapitated, and the hippocampal tissue was obtained.

The total RNA of the hippocampus of rats in each group was separated by TRIzol LS. The concentration of RNA was qualified by an ultraviolet spectrophotometer. For miR-128-3p, cDNA was synthesized from 500 ng RNA using miRNA 1st-strand cDNA synthesis kit (NovaBio, Shanghai, China). For SP1, a cDNA synthesis kit (TAKARA, Tokyo, Japan) was purchased to synthesize cDNA. PCR reaction was carried out on ABI fluorescence quantitative system using from SYBR-Green Supermix (Thermo Fisher Scientific, Inc.) (Wang et al. 2021). U6 and internal reference β -actin were amplified in the same reaction system. The relative levels were processed by the 2-delta Ct method. The primers utilized were as follows: miR-128-3p, forward 5'-AACGACATCACAGTGAACCG-3', reverse 5'-CAGAGCAGGGTCCGAGGTA-3'; U6, forward 5'-CGCTTCGGCAGCACATATAC-3', reverse 5'-TTCACGAATTTGCGTGTTCATC-3'; SP1, forward 5'-CTGCAAGGGTCTGATCTCTA-3', reverse 5'-AGCTTGTCCACCTTGAAC-3'; β -actin forward 5'-GCCACGGCTGCTTCCAGC-3' reverse 5'-AGGGTGTAACGCAACTAAGTC-3'.

Technical Evaluation

The oxidative stress situation was tested by the following four parameters: superoxide dismutase (SOD), reactive oxygen species (ROS), malondialdehyde (MDA), and lactate dehydrogenase (LDH) using the commercial kits from Jiancheng (China). The kits of TNF-alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 β) were purchased from BD Biosciences (USA). All the measure data were read from an enzyme labeling instrument.

Cognitive Deficits Were Examination

Morris water maze test was performed to test their learning and memory function after 7 days of anesthetization. The training experiment was conducted for 5 days, and an exploration assay was performed on the 6th day. The water maze was composed of a black pool with a diameter of 160 cm and a black platform placed in one quadrant. The diameter of the platform was 12 cm, and the platform was under the water surface. We placed the platform in the SW quadrant. Before starting the positioning navigation experiment, the animals were placed on the fixed platform for 10 s to get familiar with the environment. In the positioning navigation experiment, rats were educated four times a day. We put the rats into the water facing the pool wall and recorded the escape latency of each rat. If the rat could not arrive at the platform within 120 s, then it was guided to the platform, and its escape latency was recorded as 120 s. All rats were allowed to stay on the platform for 20 s. On the 6th day, the platform was removed. The percentage of trace length in the target quadrant to the total trace length was expressed as the percentage of distance in the target quadrant. The percentages of time and distance in the target quadrant within 120 s were recorded.

Luciferase Reporter Assay

To further confirm whether miR-128-3p could combine SP1, the 3'-UTR sequence of SP1 and mutated 3'-UTR reagent were cloned into psiCHECK-2 vectors to establish a wild-type and mutant SP1. The different vectors and miR-128-3p artificial sequences were cotransfected into HEK-293 cells correspondingly. After 48 h of routine culture, the fluorescence value was detected by a double fluorescence activity detection kit.

Statistical Processing

The data were analyzed by GraphPad statistical software. The results of behavioral data, SOD activity, inflammation,

and target certification were analyzed by ANOVA. The data were representative of three independent experiments, each with three biological replicates. $P < 0.05$ was regarded as the difference was statistically significant.

Results

Artificial miR-128-3p's Regulation and Expression in ISO

For the purpose of regulation of miR-128-3p expression, the agomir reagent was obtained and imported into experimental rats. As exhibited in Fig. 1A, miR-128-3p's content in miR-agomir group was elevated when compared with untreated rats ($P < 0.001$). Moreover, the real-time PCR results in the ISO-treated rats revealed lowly expressed miR-128-3p, whereas the agomir reagent reversed the downregulation of miR-128-3p (Fig. 1B, $P < 0.01$).

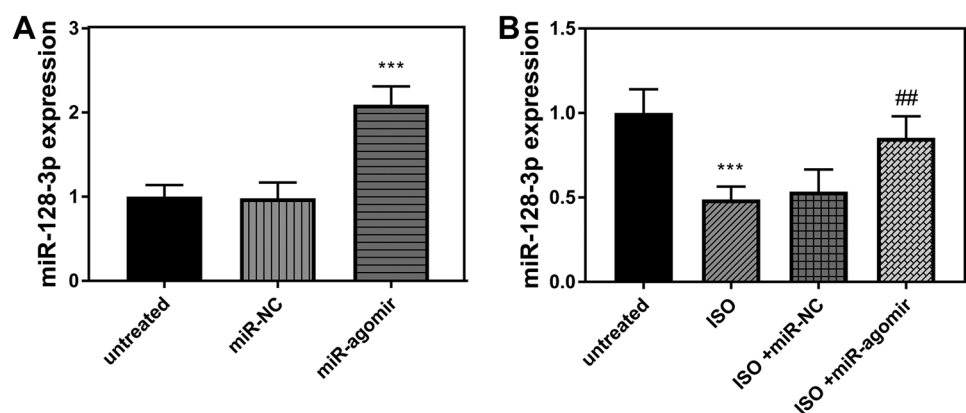
Roles of miR-128-3p on Inflammation

The changes of inflammation in rats with ISO were observed by enzyme labeling instrument. As shown in Fig. 2A–C, the contents of inflammatory indicators increased significantly immediately after ISO management and decreased significantly in the model group treated with ISO + miR-128-3p ($P < 0.001$).

MiR-128-3p Alleviated the Oxidative Stress

As a result of anesthesia, patients show temporal high oxidative stress markers and reactive oxygen species, which may lead to an abnormal increase of free radical level and decrease of antioxidant defense mechanism (Kundović et al. 2020). The treatment of ISO caused leakage of SOD with a decrease and the generation of ROS and MDA with an increase, while the overexpression of miR-128-3p

Fig. 1 The expression of miR-128-3p was detected in SD rats. **A** The regulation of miR-128-3p agomir. **B** The miR-128-3p agomir counteracted the inhibited expression of miR-128-3p in SD rats. *** $P < 0.001$, relative to untreated group; ## $P < 0.01$, relative to ISO rats



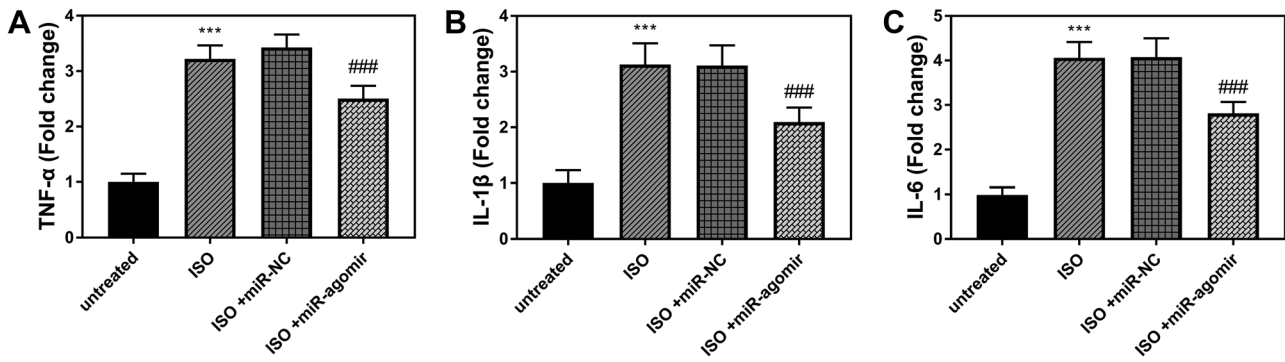


Fig. 2 The influence of miR-128-3p on inflammatory responses. The levels of **A** TNF- α , **B** IL-1 β , and **C** IL-6 were increased in the ISO group and partly reversed in the ISO + miR-agomir group. *** $P < 0.001$, relative to untreated group; ### $P < 0.001$, relative to ISO rats

mitigated these adverse roles of ISO (Fig. 3A–C, $P < 0.01$). In addition, the treatment of ISO accelerated the release of LDH, but the increased miR-128-3p inhibited the distinctive variation of LDH (Fig. 3D, $P < 0.001$).

The Neurotoxicity of ISO in Rats

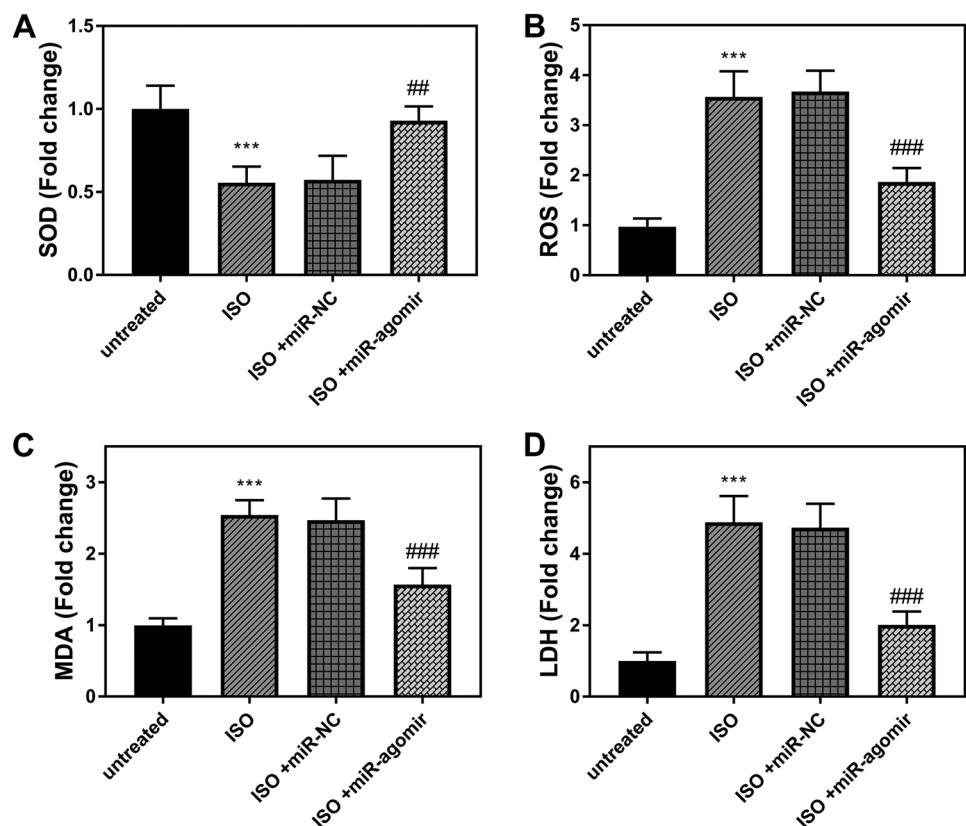
The Morris water maze test is one classical approach to test the spatial memory capacity in animals (Yi et al. 2018). Compared with the control group, the escape latency of the ISO group was prolonged, but the percentages of distance

and time in the target quadrant were decreased (Fig. 4A–C, $P < 0.01$). However, compared with the ISO group, the escape latency in the ISO + miR-agomir group was shorter, and percentages of distance and time in the target quadrant were increased in ISO + miR-agomir group (Fig. 4A–C, $P < 0.01$).

SP1 Was Connected to miR-128-3p

A complementary binding site between miR-128-3p and SP1 3'UTR was depicted (Fig. 5A). The luciferase activity was significantly increased after transfection of vectors with wild

Fig. 3 Roles of miR-128-3p on oxidative stress. The effects of ISO on **A** SOD, **B** ROS, **C** MDA, and **D** LDH were inhibited by the overexpression of miR-128-3p. *** $P < 0.001$, relative to untreated group; ### $P < 0.001$, relative to ISO rats



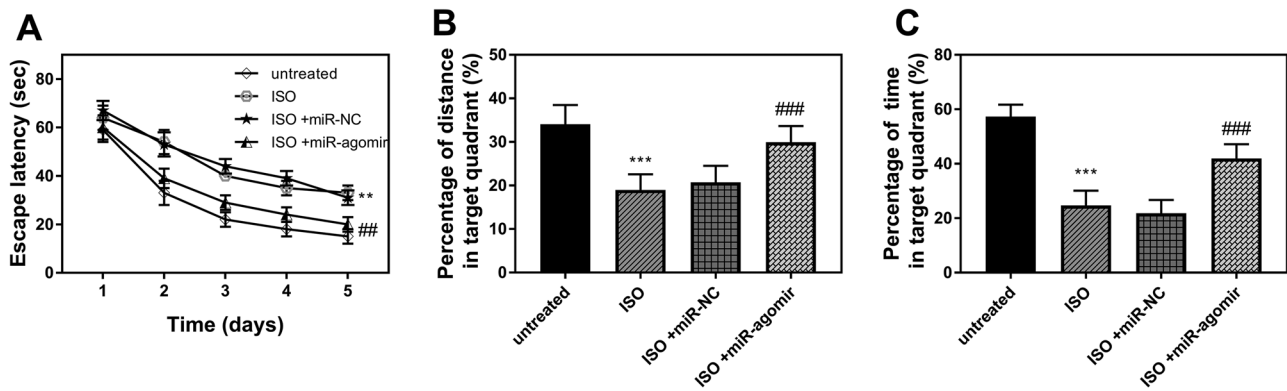


Fig. 4 The neurotoxicity of ISO and the neuroprotection of miR-128-3p. **A** The increased escape latency in rats treated by ISO was suppressed by the overexpression of miR-128-3p. The increased miR-128-3p expression recovered the decreased percentage of **B**

distance and **C** time in the target area induced by ISO. ** $P < 0.01$, *** $P < 0.001$, relative to untreated group; ### $P < 0.01$, ### $P < 0.001$, relative to ISO rats

SP1 expression and agomir reagent (Fig. 5B, $P < 0.001$), while activities were not promoted in mutant SP1 group (Fig. 5B, $P > 0.05$). What's more, the mRNA expression of

SP1 was also suppressed by importing miR-128-3p reagent (Fig. 5C, $P < 0.001$). The ISO circumstance led to an increment of mRNA level of SP1 (Fig. 5D, $P < 0.001$).

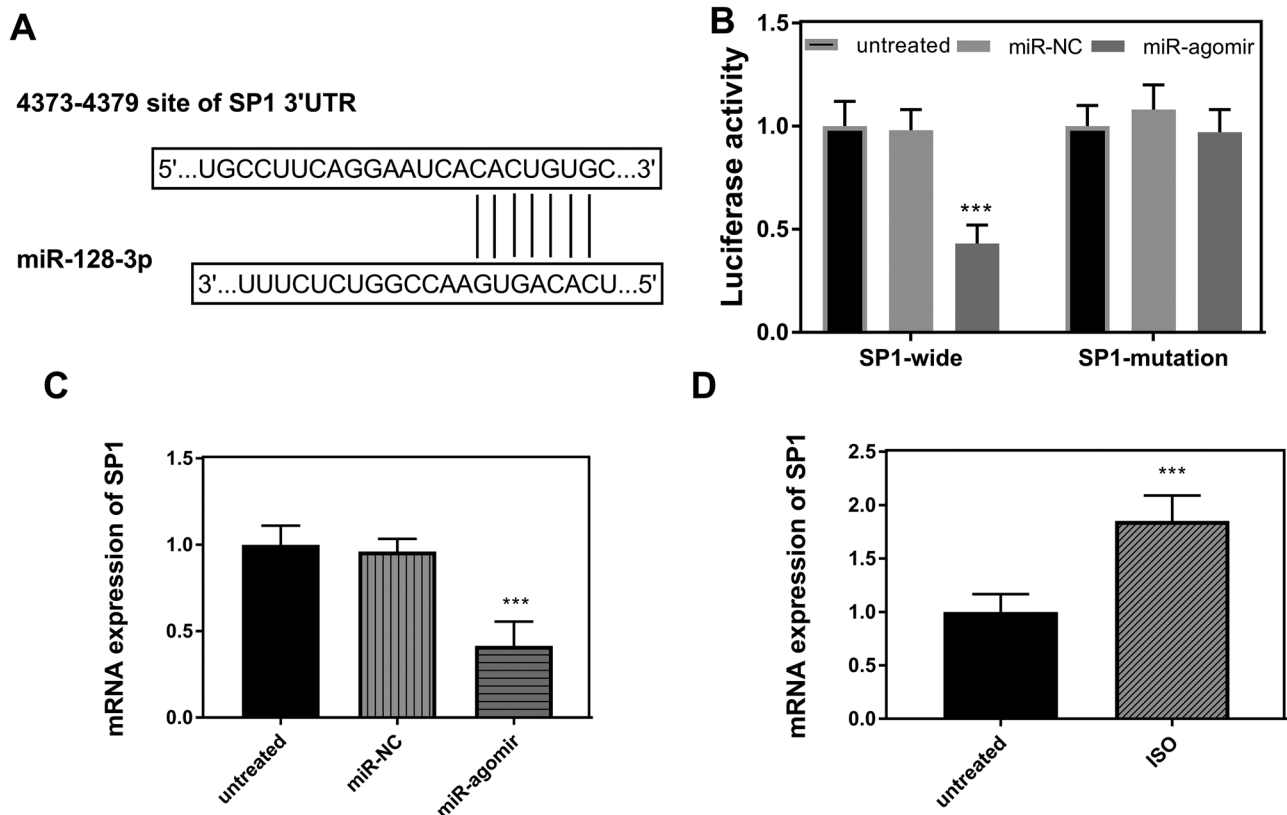


Fig. 5 The putative target of miR-128-3p. **A** The targeted site between SP1 and miR-128-3p. **B** The luciferase activity was decreased when SP1 was transfected with miR-agomir. **C** The

SP1 mRNA expression was decreased in the miR-agomir group. **D** The mRNA levels of SP1 were increased in ISO-stimulated rats. *** $P < 0.001$, relative to untreated group

Discussion

Inhaled anesthetics are proved to be secure and practical drugs for narcosis (Stachnik 2006). ISO is a widely utilized inhalation anesthetics in practice. ISO anesthesia can inhibit cardiopulmonary function and cause carbon dioxide accumulation and hypoxemia. Anesthesia is defined as the inhibition of peripheral nerve tissue activity or central nerve tissue activity by one or more drugs, resulting in a partial or total sensory loss. Cognitive lesion after operations is a complication of patients exposed to long-term anesthesia and surgery (Belrose and Noppens 2019; McCann and Soriano 2019). The cognitive disorder is broadly defined as the significant decrease of postoperative cognitive ability compared with baseline, and it is diagnosed as defects in many areas of neurocognition, including executive function, attention, language memory, and psychomotor speed. It is in line with a panel of negative results, such as variability in mood and personality, which may lead to a rapid deterioration of living standards or even death.

MiRNA can be used as a posttranscriptional regulator of gene expression and participates in neural cell development (Tang and Sun 2020). A variety of miRNAs are reported as an indicator in the ISO management and participate in the neurological impairment and neuroinflammation, such as miR-191, miR-150, and miR-133b (Li et al. 2021; Cui et al. 2020; Zhang et al. 2021). In the current publication, we found that ISO induced an increase of miR-128-3p expression, indicating that miR-128-3p might participate in the ISO treatment. Wang et al. report that miR-128-3p represses the neuroinflammation in the rat model of spinal cord ischemia/reperfusion (Wang et al. 2020a). In sepsis, the underexpression of miR-128-3p also shows an expansionary effect on the inflammatory situation (Yang et al. 2021). MiR-128-3p in this study was identified as a regulator in the inflammation and oxidative stress, which elucidated that miR-128-3p was pertinent to ISO by controlling the oxidative stress and inflammatory pattern. Zhao et al. also validate that miR-128-3p may modulate oxidative activity, which was an exemplification of our finding. The hippocampal region of the brain is related to the ability of memory, and the neurons in the hippocampus of rodents and humans play an important role in maintaining memory function. Morris water maze test is a spatially discriminative learning and memory model, which depends on the structure and function of the hippocampal region of the brain. Our results showed that in miR-128-3p, the escape latency of mice in the ISO group was longer, and the percentage of time and distance stay in target quadrant were decreased. It was suggested that ISO anesthesia can cause cognitive dysfunction in rat. Enhancement of content of miR-128-3p could improve the cognitive impairment, validating the beneficial roles of miR-128-3p.

Many experiments on the correlation between miR-128-3p and SP1 have been published to certify the targeted relationship between them (Wang et al. 2020a; Cao et al. 2019). Thus, SP1's interconnection with miR-128-3p was verified in our project, and it was found that there was a complementary binding site between miR-128-3p and 3'UTR of the SP1 gene. It was suggested that miR-128-3p could target SP1. Moreover, the assay of rats implied that miR-128-3p acted roles on expression patterns of SP1. A publication of postoperative cognitive dysfunction indicates that SP1 plays role in sevoflurane-induced cognitive disorders (Lv et al. 2020). Another example of Alzheimer's disease substantiates that SP1 might participate in the memory impairment via accommodating working memory and long-term memory deficits (Subaiea et al. 2013). However, the underlying of SP1 in the ISO-treatment rat needs to be investigated in further study.

Taken together, miR-128-3p was downregulated in the ISO rat models and alleviated the damage of ISO via attenuating inflammatory responses, repressing oxidative stress, and promoting the recovery of cognitive lesion triggering by ISO. SP1 was a target of miR-128-3p. ISO enforced the mRNA expression of SP1.

Author Contribution DQ and SD reviewed the references, completed the experiment, and revised the manuscript. YS designed the study. YY analyzed the data. LW wrote the manuscript. All authors have read the manuscript and agreed to the publication of this study.

Declarations

Conflict of Interest The authors declare no competing interests.

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