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Knockdown of lncRNA BDNF-AS alleviates isoflurane-induced neuro-inflammation and cognitive dysfunction through modulating miR-214-3p

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Abstract

Introduction: *As one of the most commonly used anesthetics, isoflurane has been demonstrated to possess a variety of protective effects. However, its' neurological impaired effect should be considered during clinical application. Roles of lncRNA BDNF-AS (BDNF-AS) and miR-214-3p in isoflurane-injured microglia and rats were investigated in this study, aiming to disclose the mechanism of isoflurane damage and to provide candidate therapeutic targets.*

Material and methods: *Isoflurane-induced microglia cells and rat models were established with 1.5% isoflurane. Inflammation and oxidative stress of microglia cells were evaluated with a level of pro-inflammation cytokines, malondialdehyde (MDA), superoxide dismutase (SOD), and nitrite. Cognitive and learning function of rats were assessed with Morris water maze task. Expressions of BDNF-AS and miR-214-3p and their function in the isoflurane-induced microglia cells and rats were estimated with PCR and corresponding transfection.*

Results: *Isoflurane induced significant neuro-inflammation and oxidative stress in the microglia cells. The increased BDNF-AS and the decreased miR-214-3p were noted, and BDNF-AS was found to negatively regulate miR-214-3p in the isoflurane-induced microglia cells. Isoflurane caused cognitive dysfunction in rats, and resulted in a significant inflammatory response. The knockdown of BDNF-AS significantly alleviated the neurological impairment induced by isoflurane, which was reversed by silencing miR-214-3p.*

Conclusions: *In isoflurane-induced neuro-inflammation and cognitive dysfunction, BDNF-AS showed a significant protective effect on the neurological impairment induced by isoflurane through modulating miR-214-3p.*

Key words: *isoflurane, lncRNA BDNF-AS, miR-214-3p, neuro-inflammation, cognitive and learning dysfunction.*

Introduction

Since great progress has been made in the development of anesthetic agents, anesthesia is an essential process during most surgeries involving a variety of anesthetics, including isoflurane. Isoflurane is a volatile and inhaled anesthetic, which has been applied in clinics for decades. Although isoflurane has been demonstrated to show neurological protective effect during treatment of some diseases, such as intra-cerebral

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hemorrhage, subarachnoid hemorrhage, and cerebral ischemia-reperfusion [4,13,29], it was also reported to possess neuro-toxicity damaging the recovery and neurological abilities of patients, which is closely correlated with its' dosage and timing of medication [22]. With the development of molecular biology, identification of effective biomarkers monitoring harmful effect of isoflurane has drawn special attention; it benefitted clinical application of isoflurane and prevented the damaging effect of isoflurane.

Long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are critical members of the non-coding RNA family, which could mediate a series of cellular processes, such as cell growth, differentiation, and metastasis, therefore regulate disease development [20,21]. Previously, some central nervous system-correlated lncRNAs leaked out, which was reported to regulate the progression of neuro-degenerative diseases and exert a protective effect on neurological impairment [25]. Among the dysregulated lncRNA BDNF-AS (BDNF-AS) was a dugout, which was illustrated to participate in the occurrence of Alzheimer's and Parkinson's diseases that were associated with neurological injury [8,9]. Although few data are available to evidence the specific function of BDNF-AS in isoflurane-induced neuro-toxicity, it was considered to possess great potential to regulate nerve damage caused by isoflurane. As the downstream target of BDNF-AS, miR-214-3p was suggested to regulate the autophagy and apoptosis pathways, and therefore mediate neurological disorders [2,30]. In addition, miR-214-3p was revealed to mediate the modulatory effect of lncRNA BACE1-AS1 in isoflurane-induced neuro-toxicity, implying its' potential in mediating the effect of BDNF-AS1 on isoflurane-induced neurological impairment [11].

This study aimed to investigate the role of BDNF-AS/miR-214-3p axis in isoflurane-induced neurological injury, and to provide a novel insight into the nerve damage resulting from anesthetic.

Material and methods

Cell culture and transfection

Mouse microglial cell line BV2 was obtained from CCTCC, and cultured in the DMEM culture medium supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. BV2 cells were proceeded with different treatments as follows: 1) Control group without isoflurane treatments or transfections; 2) Isoflurane group treated with 1.5% isoflurane for 6 hours; 3) si-BDNF-AS negative control (NC) group transfected with BDNF-AS siRNA NC (si-BDNF-AS NC, 5'-CCUCUCCACGCGCAGUACATT-3') before isoflurane treatment; 4) si-BDNF-AS group transfected with BDNF-AS siRNA (si-BDNF-AS 5'-GGCTCACCAGTTGTTTGTT-3') before treatment of isoflurane; 5) miR NC group co-transfected with si-BDNF-AS and miR-214-3p NC (5'-CAGUACUUUUGUGUAGUACAA-3') before treatment of isoflurane; 6) miR-214-3p inhibitor group co-transfected with si-BDNF-AS1 and miR-214-3p inhibitor (5'-ACUGCCUGUCUGUGCCUGCUGU-3') before treatment of isoflurane.

Transfections were obtained from invitrogen and conducted with lipofectamine 3000 (Invitrogen, USA) at room temperature. After 48 hours of transfection, the transfected cells were available for the following experiments.

Oxidative stress evaluation

The concentration of malondialdehyde (MDA) and nitrite (NO_2^-), and activity of superoxide dismutase (SOD) were used to indirectly evaluate oxidative stress of BV2 cells. Concentrations of MDA and nitrite were analyzed with lipid peroxidation assay kit (Cayman, USA) and Griess reagent kit (Sigma, USA), and SOD activity was estimated using superoxide dismutase assay kit (Cayman, USA), according to the manufacturer's instruction.

Dual-luciferase reporter assay

Binding sites between BDNF-AS and miR-214-3p predicted online (http://starbase.sysu.edu.cn) were cloned into pmirGLO vectors to establish a wild-type BDNF-AS (WT-BDNF-AS) vector, while mutant-type BDNF-AS (MT-BDNF-AS) vector was established by cloning mutant binding sites. The established vectors were co-transfected with miR-214-3p mimic (5'-ACAGCAGGCACAGACAGGCAGU-3'), miR-214-3p inhibitor, or miR NC into isoflurane-induced BV2 cells using lipofectamine 3000 (Invitrogen, USA). Luciferase activity of BDNF-AS was detected after 48 hours of transfection, and normalized to Renilla.

Grouping

The approval from the Guangzhou Red Cross Hospital had been obtained before the experiments. Male Sprague-Dawley rats were adopted in this study. All animals were maintained under controlled conditions at 23 ±2°C, with a humidity of 50 ±5%. Animals were allowed to adapt to the environment for 7 days with free access to food and drink.

Rats were randomly grouped with six rats of each group as follows: 1) Control group treated with an equal dose of stroke-physiological saline solution; 2) Isoflurane-induced group treated with 1.5% isoflurane for 6 hours with a flow rate of 2 l/min according to previous studies [17,24,28]; 3) BDNF-AS NC group injected with

small interference RNA negative control (siRNA-NC) of BDNF-AS before anesthetization with isoflurane; 4) si-BDNF-AS group injected with siRNA of BDNF-AS before anesthetization with isoflurane; 5) Antagomir NC group injected with antagomir NC of miR-214-3p based on BDNF NC group or si-BDNF-AS group; 6) miR-214-3p antagomir group was injected with antagomir of miR-214-3p based on BDNF NC group or si-BDNF-AS group. Hippocampus tissues were collected after 24 hours of anesthetization for analyses.

Morris water maze task

A circular pool with a diameter of 1.5 m and a height of 0.5 m was used. The test was conducted with a water depth of 0.3 m at 20 ±2°C. The pool was divided into four quadrants, and an invisible platform was placed in the center of Northeast quadrant. Rats were trained daily to find the platform. Specifically, rats were placed randomly in one of the four quadrants facing the wall of the pool, and were allowed to swim for up to 60 sec to find the hidden platform and rest on it for 1 min. On the final day, the platform was removed to the probe position, and rats were allowed to find the probe position in 1 min. Escape latency, percentage of the distance, and swimming time in the target quadrant were recorded to assess the cognitive function of rats. Behavior data were collected and analyzed by Ethovision XT8 software (Noldus, The Netherlands).

BDNF-AS and miR-214-3p expression evaluation

The expression of BDNF-AS and miR-214-3p was evaluated with real-time PCR after isolation of total RNA from the hippocampus and BV2 cells, using Trizol reagent. cDNA was further generated with high-performance cDNA reverse transcription kit (Applied Biosystem, USA) for BDNF-AS, and TaqMan MicroRNA reverse transcription kit was applied for miR-214-3p. The generated cDNA was amplified and detected on Applied Biosystem 7500 real-time PCR system with GAPDH (for BDNF-AS) and miR-39 (for miR-214-3p) as internal reference. Sequences of used primers were as follows: BDNF-AS F: 5'-CATCCGAGGACAAGGTGGCTTG-3', BDNF-AS R: 5'-GCCGAACTTTCTGGTCCTCATC-3'; miR-214-3p F: 5'-CAATACTGACAGCAGGCACA-3', miR-214-3p R: 5'-TATGGTTGTTCACGACTCCTTCAC-3'; GAPDH F: 5'-AAGCCTGCCGGTGACTAAC-3', GAPDH R: 5'-GCGCCCAATACGACCAAATC-3'; miR-39 F: 5'-UCACCGGGUGUAAAUCAGCUUG-3', miR-39 R: 5'-TCACCGGGTGTAAATCAGCTTG-3'. Reaction conditions of PCR included all samples that were pre-heated for 2 min at 94°C, followed by denaturation at 94°C for 10 sec. Then, annealing was conducted at 60°C for 45 sec and amplified at 72°C for 90 sec (35 cycles). Relative expression levels were calculated with $2^{-\Delta\Delta CT}$ method.

Pro-inflammation cytokines evaluation

Protein levels of pro-inflammation cytokines, including interleukin (IL)-1β, IL-6, IL-18, and tumor necrosis factor α (TNF-α) were evaluated with enzyme-linked immunosorbent assay. Tissues and cells were lysed and centrifugated at 5,000 g for 25 min, and supernatant was obtained. Protein levels were detected with enzyme-linked immunosorbent assay kits (Abcam, China) according to the manufacturer's instruction.

Statistical analysis

All experiments were performed in triplicate with three independent determinations. All data were expressed as mean value ±SD. Differences were assessed with Student's *t*-test or one-way ANOVA, followed by Turkey post-hoc test using SPSS version 26.0 software. Significance was marked with $p < 0.05$.

Results

Expression of BDNF-AS and miR-214-3p in isoflurane-induced BV2 cells

Compared with untreated BV2 cells, the treatment of isoflurane significantly improved the expression of BDNF-AS ($p < 0.001$, Fig. 1A), and suppressed the expression of miR-214-3p ($p < 0.001$, Fig. 1B). Meanwhile, the luciferase reporter results showed that over-expressing miR-214-3p dramatically inhibited the luciferase activity of BDNF-AS, and the opposite effect was observed in the BV2 cells with miR-214-3p knockdown ($p < 0.001$, Fig. 1C).

Effect of BDNF-AS and miR-214-3p on neurological inflammation and oxidative stress of BV2 cells

In the BV2 cells, the transfection of BDNF-AS siRNA dramatically reversed isoflurane-induced increase of BDNF-AS expression ($p < 0.01$, Fig. 2A). Similarly, the knockdown of BDNF-AS could prevent the miR-214-3p level from the inhibitory effect of isoflurane ($p < 0.001$), and co-transfection of its inhibitor was found to suppress the expression of miR-214-3p in BV2 cells ($p < 0.01$, Fig. 2B).

Isoflurane induced dramatic neurological inflammation in BV2 cells, behaving as the increasing levels of IL-1β, IL-6, IL-18, and TNF-α ($p < 0.001$, Fig. 3A). The knockdown of BDNF-AS was found to alleviate the neurological inflammation that was significantly reversed by the silencing of miR-214-3p ($p < 0.01$,

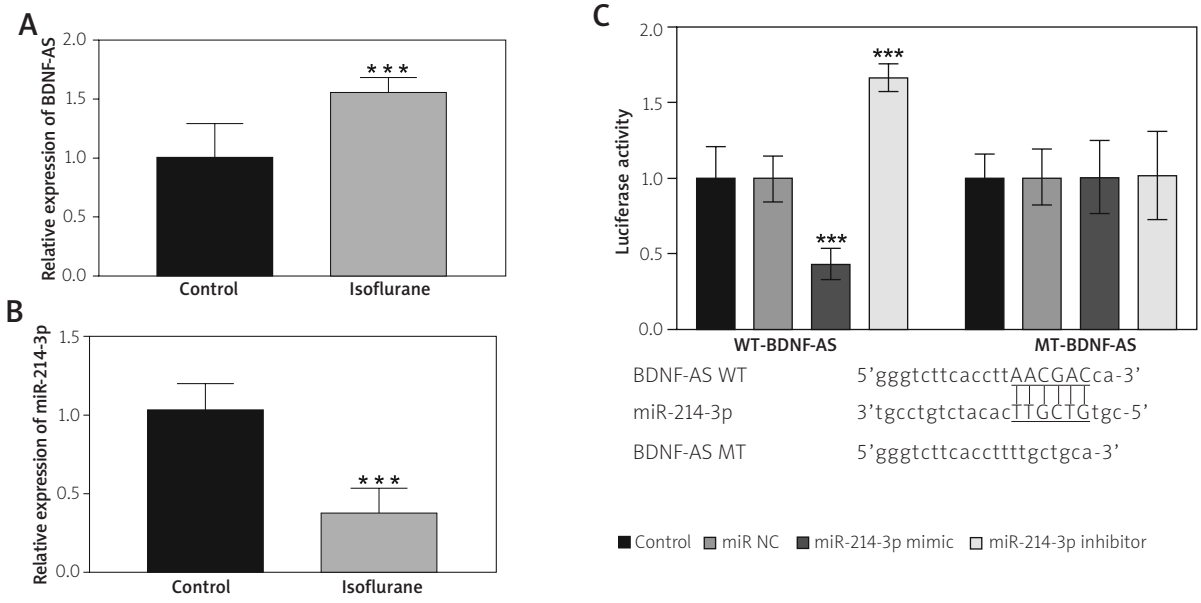

Fig. 1. Expression of lncRNA BDNF-AS and miR-214-3p in BV2 cells and their interaction. **A**, **B**) Upregulation of BDNF-AS (**A**) and downregulation of miR-214-3p (**B**) were observed in the isoflurane-induced BV2 cells compared with untreated cells. **C**) Overexpression of miR-214-3p significantly suppressed luciferase activity of BDNF-AS, and opposite effects were found in its' knockdown. ***$p < 0.001$.

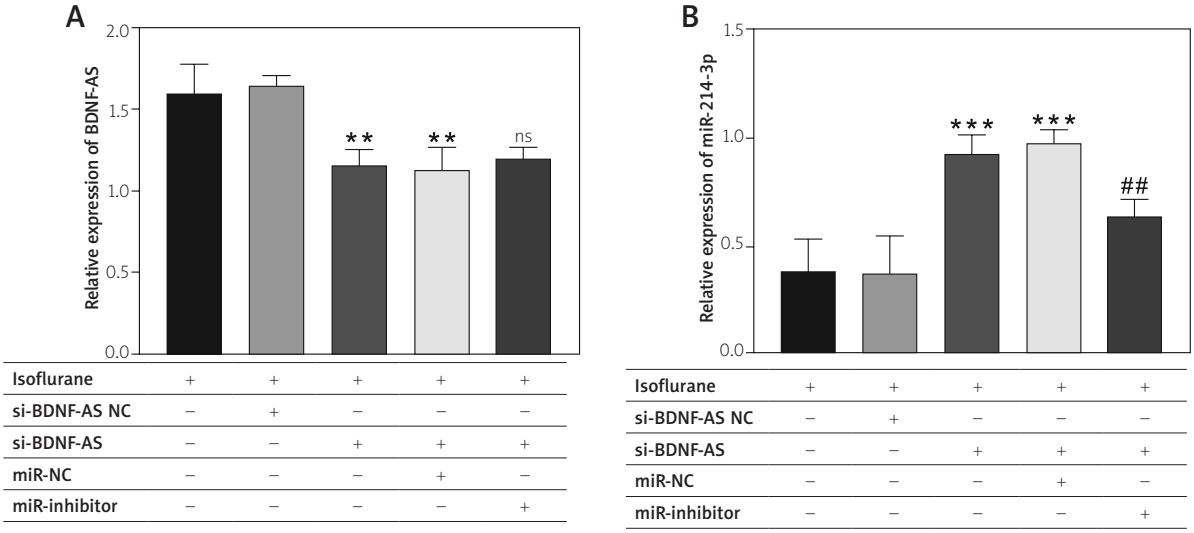

Fig. 2. Transfection of BDNF-AS siRNA dramatically suppressed elevated expression of BDNF-AS (**A**) and enhanced reduced miR-214-3p (**B**) in isoflurane-induced BV2 cells, which was reversed by transfection of miR-214-3p inhibitor. **$p < 0.01$, ***$p < 0.001$ compared with isoflurane group; ##$p < 0.01$ compared with BDNF-AS siRNA-transfected group.

Fig. 3A). Moreover, the isoflurane-treated cells showed relatively higher concentrations of MDA (Fig. 3B) and nitrite (Fig. 3C), and the decreasing activity of SOD (Fig. 3D) in comparison with untreated cells ($p < 0.01$). The reduced BDNF-AS could drop the MDA (Fig. 3B) and nitrite (Fig. 3C) concentrations and improve the activity of SOD (Fig. 3D) in the isoflurane-treated cells ($p < 0.01$), which was reversed by the knockdown of miR-214-3p ($p < 0.01$).

Effect of BDNF-AS and miR-214-3p on inflammation and cognitive function of isoflurane-induced rats

In isoflurane-induced rat models, the elevated expression of BDNF-AS (Fig. 4A) and reduced expression of miR-214-3p (Fig. 4B) were observed compared with the control group ($p < 0.001$), which was consistent with the abnormal expression levels in BV2 cells.

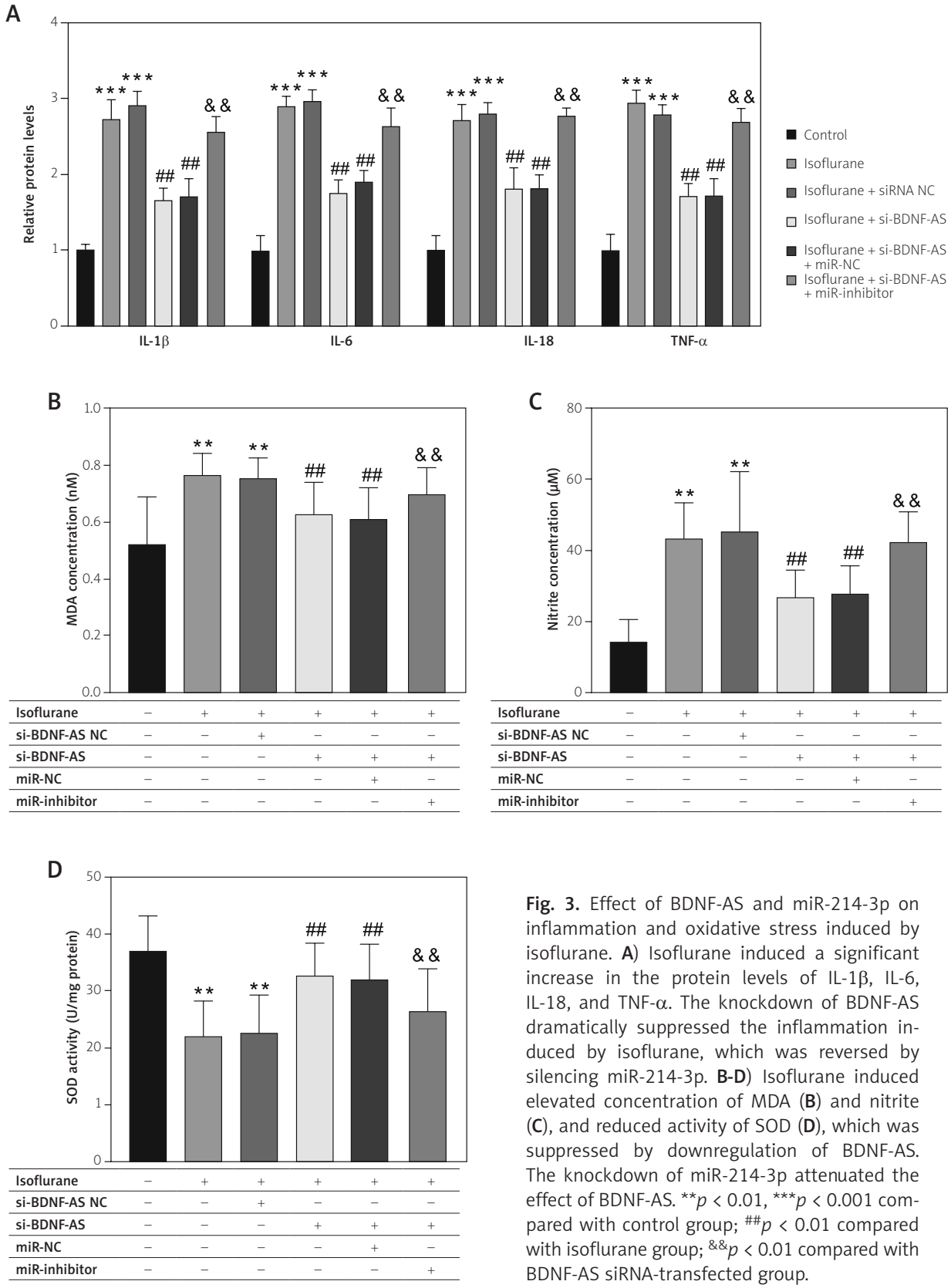

Fig. 3. Effect of BDNF-AS and miR-214-3p on inflammation and oxidative stress induced by isoflurane. **A**) Isoflurane induced a significant increase in the protein levels of IL-1β, IL-6, IL-18, and TNF-α. The knockdown of BDNF-AS dramatically suppressed the inflammation induced by isoflurane, which was reversed by silencing miR-214-3p. **B-D**) Isoflurane induced elevated concentration of MDA (**B**) and nitrite (**C**), and reduced activity of SOD (**D**), which was suppressed by downregulation of BDNF-AS. The knockdown of miR-214-3p attenuated the effect of BDNF-AS. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with control group; $^{\#\#}p < 0.01$ compared with isoflurane group; $^{\&\&}p < 0.01$ compared with BDNF-AS siRNA-transfected group.

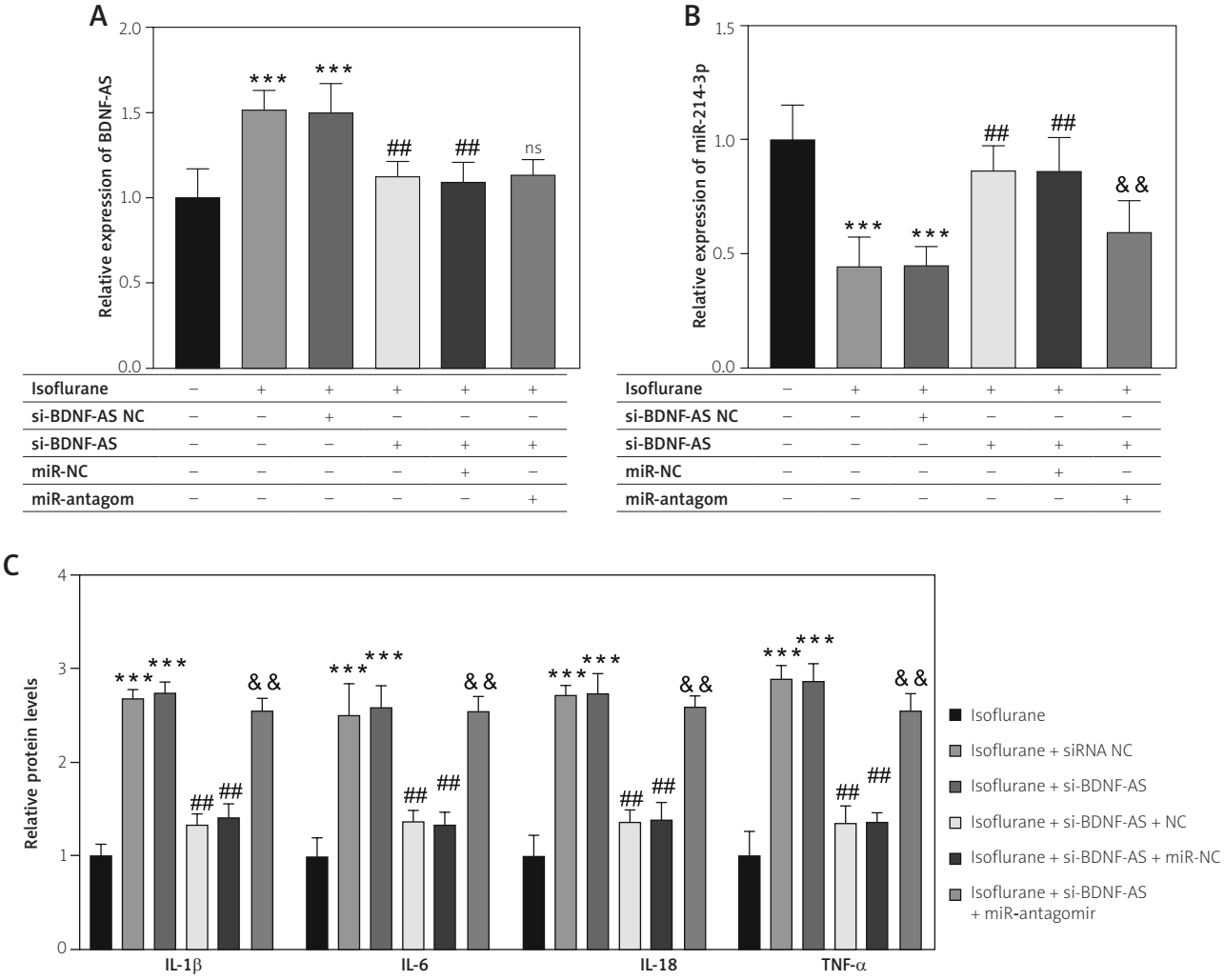

Fig. 4. Expression of BDNF-AS and miR-214-3p in isoflurane-induced rat models and their effect on the inflammatory response. **A**, **B**) Elevated BDNF-AS (**A**) and decreased miR-214-3p (**B**) were observed in isoflurane-induced rat models. The knockdown of BDNF-AS could enhance the expression of miR-214-3p. The silencing of miR-214 showed no significant effect on the expression of BDNF-AS. **C**) Improved levels of IL-1β, IL-6, IL-18, and TNF-α were observed in isoflurane-induced rats, which was suppressed by the knockdown of BDNF-AS. The downregulation of miR-214-wp reversed the protective effect of BDNF-AS silencing. ***$p < 0.001$ compared with the control group; ##$p < 0.01$ compared with isoflurane group; &&$p < 0.01$ compared with BDNF-AS siRNA-transfected group.

The elevated BDNF-AS level in the isoflurane-induced rats was knocked down by the transfection of BDNF-AS siRNA, but the silencing of miR-214-3p showed no significant influence on the expression of BDNF-AS ($p < 0.01$, Fig. 4A). The reduced miR-214-3p caused by isoflurane was elevated by BDNF-AS silencing, which was reversed by miR-214-3p antagomir ($p < 0.01$, Fig. 4B).

Significant inflammation was observed in the isoflurane-treated rats with increasing levels of pro-inflammation cytokines, including IL-1β, IL-6, IL-18, and TNF-α ($p < 0.001$, Fig. 4C). The knockdown of BDNF-AS showed a significant inhibitory effect on the inflammation response of isoflurane-induced rats, which was attenuated by the silencing of miR-214-3p ($p < 0.01$, Fig. 4C).

In the Morris water maze task, the escape latency of rats in all groups was decreased with time. The treatment of isoflurane significantly reduced the escape latency ($p < 0.01$), while the knockdown of BDNF-AS recovered the escape latency of isoflurane-induced

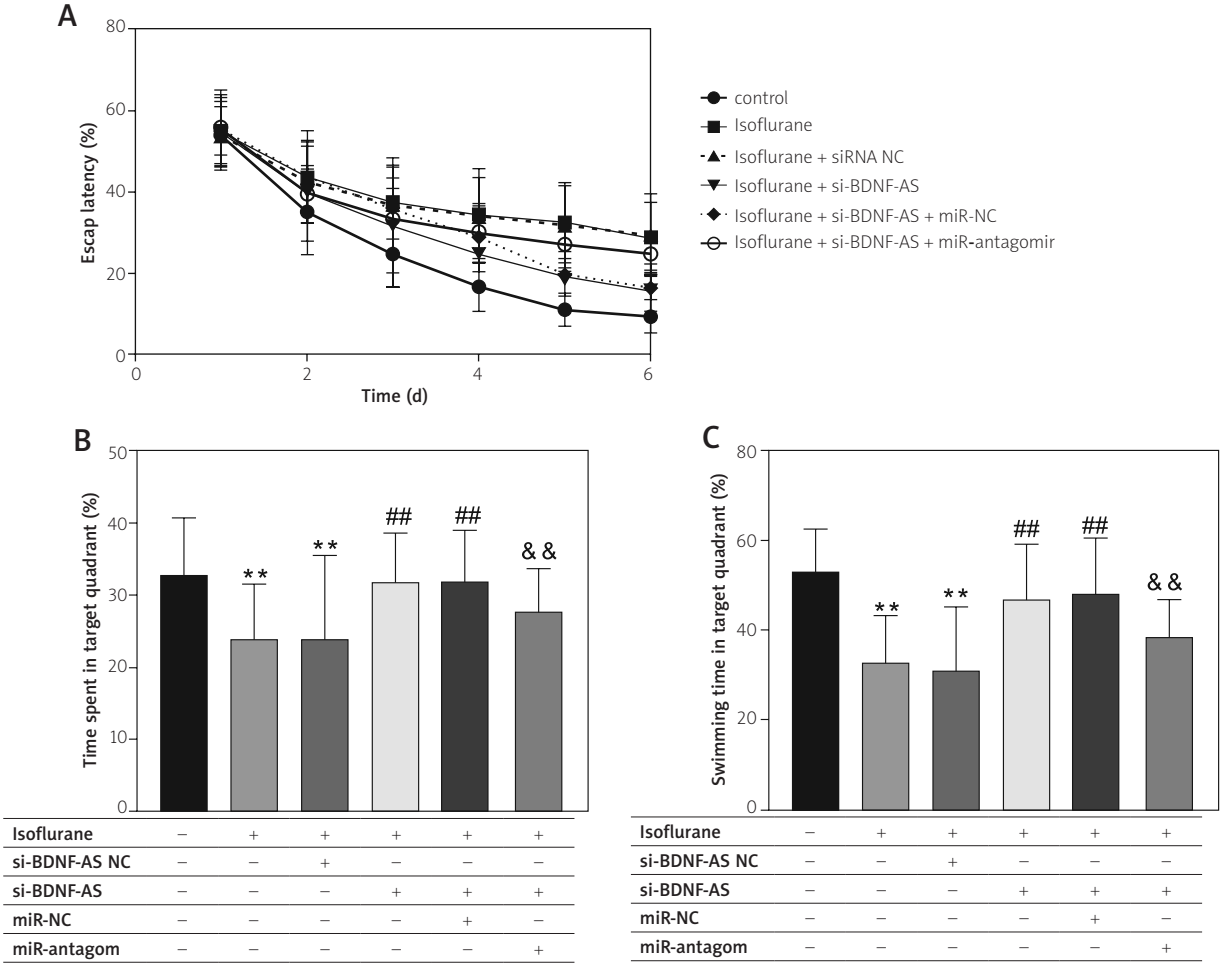

Fig. 5. Effect of BDNF-AS and miR-214-3p on cognitive and learning function of isoflurane-induced rat models. Isoflurane reduced the escape latency (**A**), time in the target quadrant (**B**), and the swimming time in the target quadrant (**C**). The knockdown of BDNF-AS significantly alleviated the cognitive impairment induced by isoflurane, which was reversed by miR-214-3p silencing. $^{**}p < 0.01$ compared with the control group; $^{\#\#}p < 0.01$ compared with isoflurane group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ compared with BDNF-AS siRNA-transfected group.

rats ($p < 0.01$), which was mediated by miR-214-3p ($p < 0.05$, Fig. 5A). Additionally, isoflurane also shortened the spent time (Fig. 5B) and swimming time (Fig. 5C) in the target quadrant of rats ($p < 0.01$). Downregulation of BDNF-AS could protect rats from the impairment, which was crippled by the knockdown of miR-214-3p ($p < 0.01$, Fig. 5B and C).

Discussion

Microglia cells play vital roles in the immune of the central nervous system, and mediate secretion of inflammation cytokines, eliminate injured neurons, and therefore protect neurons from damage induced by environmental factors [6]. However, in response to specific stimuli, microglia would release pro-inflammatory factors, and further lead to neuro-inflammation, damage, and even neuron's death [26]. It was reported that both inflammation and oxidative stress could promote the activation of microglia cells and even cause neurological impairment [12]. Previous studies demonstrated that the secretion of IL-1β and IL-18 by microglia cells could result in neuro-inflammation and cell death. With the wide application of isoflurane in clinics, different kind of harmful effects of isoflurane have been discovered, such as liver injury, ischemia-reperfusion damage, and neurological injury [15,18,32]. Herein, we found an enhanced levels of IL-1β, IL-6, TNF-α, and IL-18, and increasing oxidative stress in microglia cells induced by

isoflurane, revealing the potential of isoflurane in causing neuro-inflammation.

Neuro-inflammation could contribute to neuronal dysfunction and impaired neurogenesis, and chronic neuro-inflammation manifests as cognitive disorders and post-operative delirium [1]. Prior studies reported that patients with the usage of isoflurane presented a high-risk of post-operative cognitive dysfunction, which was confirmed in rat models [3,5]. The isoflurane-induced rat model was established in the present study, and isoflurane-induced rats showed a deteriorating memory and cognitive ability compared with untreated rats. Meanwhile, isoflurane was also found to induce an inflammatory response in rats, which is consistent with the results in the microglia cells.

Some dysregulated molecules were suggested to regulate the inflammatory response and oxidative stress, and therefore mediate the isoflurane-induced impairment [7,19,23]. BDNF-AS is an antisense lncRNA that repressed the expression of BDNF, which has been identified as a promising therapeutic target of various neurological disorders, such as Parkinson's disease, schizophrenia, and Alzheimer's disease. The significance of BDNF-AS in human disease has also been widely reported [10]. For instance, the downregulation of BDNF-AS in prostate and breast cancer was disclosed to correlate with patients' poor outcomes and malignant development [14,16]. In some neuro-degenerative diseases, such as Huntington's disease, the dysregulation of BDNF-AS was observed. BDNF-AS was also reported to mediate the mitigating effect of lithium on spinal cord injury through attenuating neuron apoptosis and inflammation [27]. Here, we observed a significant upregulation of BDNF-AS in the isoflurane-induced microglia cells and rat models. The knockdown of BDNF-AS could dramatically suppress neuro-inflammation and oxidative stress of microalga cells. Moreover, in the isoflurane-induced rat models, silencing BDNF-AS showed a significant protective effect that alleviated inflammatory response and recovered the cognitive and memory ability of rats. All these results indicated the involvement of BDNF-AS in the nerve destroying effect of isoflurane.

In mechanism, lncRNAs modulate downstream ceRNAs, such as miRNAs, to perform their function. The regulation of miR-214-3p by BDNF-AS was reported in esophageal cancer, where miR-214-3p mediated the tumor suppressor role of BDNF-AS in cell proliferation, metastasis, and EMT processes [31]. miR-214-3p was previously indicated to be abnormally expressed in neuro-pathological cognitive disorders, and to participate in the development of correlated diseases. For example, miR-214-3p reduced hippocampal neuron apoptosis and inhibited autophagy, and therefore alleviated cognitive defects in Alzheimer's disease [30]. As the ceRNA of lncRNA BACE1-AS, miR-214-3p reversed the protective effect of lncRNA BACE1-AS on Alzheimer's disease via regulating ATG5 [11]. The interaction between BDNF-AS and miR-214-3p was also confirmed in the microglia cells. miR-214-3p was found to negatively regulate the luciferase activity of BDNF-AS, and BDNF-AS negatively regulated the expression of miR-214-3p. Along with with its' role in esophageal cancer, miR-214-3p reversed the protective effect of BDNF-AS knockdown on isoflurane-induced neuro-inflammation and cognitive impairment.

According to the above results, it can be concluded that isoflurane could induce neuro-inflammation and cognitive and learning dysfunctions. The suppression of BDNF-AS alleviated neuro-inflammation and oxidative stress, and improved cognitive and learning impairment induced by isoflurane through sponging miR-214-3p.

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Disclosure

The authors report no conflict of interest.

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