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MicroRNA-204-5p mediates sevoflurane-induced cytotoxicity in HT22 cells by targeting brain-derived neurotrophic factor

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Summary. Background. Sevoflurane is widely used as an inhalational anesthetic in clinical practice. However, sevoflurane can cause cytotoxicity and induce learning capacity decline in patients. A previous publication indicated that miR-204-5p might have a close relationship with sevoflurane-induced neurotoxicity. When exposed to sevoflurane, the expression of miR-204-5p in neonatal hippocampus of rats was significantly increased. Hence, we aimed to investigate the role of miR-204-5p in sevoflurane-induced neurotoxicity using a mouse hippocampal neuronal cell line (HT22).

Methods. The levels of miR-204-5p in HT22 cells were detected by RT-qPCR. In addition, the effects of miR-204-5p on cell viability, apoptosis and proliferation were evaluated by CCK-8, flow cytometric, and immunofluorescence assay, respectively. Western blotting was used to detect expressions of Bax, Bcl-2, active caspase 3, BDNF, TrkB, p-TrkB, Akt and p-Akt in HT22 cells. ELISA assay was used to examine the levels of total superoxide dismutase (SOD), reduced glutathione (GSH), malondialdehyde (MDA) and reactive oxygen species (ROS) in cells. Meanwhile, the dual luciferase reporter system assay was employed to explore the interaction of miR-204-5p and BDNF in cells.

Results. The level of miR-204-5p was increased in sevoflurane-treated HT22 cells. Moreover, downregulation of miR-204-5p inhibited sevoflurane-induced apoptosis and promoted cell proliferation by upregulating the proteins of Bcl-2 and downregulating the expressions of Bax and active caspase-3 in HT22 cells. In addition, inhibition of miR-204-5p alleviated sevoflurane-induced oxidative injuries in HT22 cells via decline of ROS and MDA and upregulation of SOD and GSH. Furthermore, bioinformatics and dual luciferase assay demonstrated that miR-204-5p can inhibit the TrkB/Akt pathway by targeting BDNF.

Conclusion. Our findings indicated that downregulation of miR-204-5p can decrease oxidative status in HT22 cells and alleviate sevoflurane-induced cytotoxicity through stimulating the BDNF/TrkB/Akt pathway. Therefore, miR-204-5p might be a potential biomarker and therapeutic target for the treatment of sevoflurane-induced neurotoxicity.

Key words: MicroRNA-204-5p, Sevoflurane, HT22 cells, Brain-derived neurotrophic factor

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Introduction

Sevoflurane is a sort of inhalational anesthetic universally used in clinical practice (Li et al., 2018b). The applicable range of sevoflurane is fairly wide, from pediatric patients to the elderly (Qi et al., 2019). However, numerous reports have demonstrated that exposure to sevoflurane for an extended duration can cause neural toxicity and apoptosis of brain cells (Jevtovic-Todorovic et al., 2013). *In vivo* experiments indicated that 5% sevoflurane could disturb the normal

functions of the hippocampus of rodents (Takaenoki et al., 2014; Yu et al., 2015). These unexpected injuries might cause learning capacity decline and memory impairment (Takaenoki et al., 2014; Yu et al., 2015). However, effective strategies for alleviating sevoflurane-induced injuries have seldom been reported so far. Hence, investigation of the mechanism by which sevoflurane exhibits neurotoxicity is urgently needed to facilitate the development of safe treatment.

MiRNAs, as a class of small non-coding RNAs with length of approximate 20 nucleotides, exert a vital role in various biological processes, including cell proliferation and apoptosis (Pei et al., 2019; Sacconi et al., 2012). A previous report presented that when neonatal rats were exposed to sevoflurane, the expression of miR-204-5p in neonatal hippocampus was significantly increased (Ye et al., 2016). These data indicated that miR-204-5p might be associated with sevoflurane-induced neurotoxicity.

Brain-derived neurotrophic factor (BDNF), a neuroprotective factor, is important in impeding the apoptosis process of neuron cells (Yao et al., 2012). The function of BDNF is primarily controlled by a cell surface receptor, tyrosine kinase receptor B (TrkB) (Yin et al., 2019). Through TrkB receptor, BDNF is able to stimulate various signaling pathways including the PI3K/Akt (Cheng et al., 2018). The activation of Akt is proven to play an essential role in both development and function achievement of the nervous system (Ma et al., 2016). Thus, this study aimed to evaluate the relationship between miR-204-5p and sevoflurane-induced cytotoxicity using a mouse hippocampal neuronal cell line (HT22). Meanwhile, the potential mechanism by which miR-205-5p regulated sevoflurane-induced neurotoxicity was investigated.

Materials and methods

Cell culture

The hippocampal neuronal cell line HT22 was provided by American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in DMEM medium maintaining 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA), and 1% penicillin-streptomycin solution (100 U/ml, Sigma, Aldrich, St. Louis, MO, USA). All the cultures were placed in a humidified incubator with 5% CO_2 at 37°C. Sevoflurane was purchased from Marushi Pharmaceutical (Osaka, Japan). A culture plate was placed into a chamber, which was connected to an anesthesia machine (Cicero-EM 8060, Drager, Germany). The cells were treated with sevoflurane (3%) mixed with 95% air/5% CO_2 at 6 L/min for 12 h. After that, cells were cultured in normal conditions for another 36 h.

CCK-8 assay

Cell viability was determined using the CCK-8 assay kit (Beyotime, Haimen, China). HT22 cells were seeded into a 96-well plate at a density of 5×10^3 cells per well and incubated overnight. Upon reaching to 80% cell confluence, different concentrations of sevoflurane (0%, 1%, 2%, 3%, and 4%) were added. After co-incubation at 37°C for 48 h, 10 µL of CCK-8 solution was added into each well and cultured for another 3 h. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

MiR-204-5p mimics transfection

MiR-204-5p mimics, mimics control and miR-204-5p inhibitors were all purchased from GenePharma (Shanghai, China). HT22 cells were firstly seeded into a 6-well plate and cultured overnight. When the cells grew to 80% confluence, the original culture medium was replaced by fresh serum-free medium. MiR-204-5p mimics, mimics control or miR-204-5p inhibitors were transfected into HT22 cells for a final concentration of 10 nM using 5 µL Lipofectamine® 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfection period lasted for 6 h. Then, the medium of each well was replaced by fresh medium containing 10% FBS to terminate reaction. After being incubated with sevoflurane for 48 h at 37°C, HT22 cells were collected for subsequent analyses.

Quantitative real-time PCR

The total RNAs were extracted using the RNA extraction kit (TaKaRa Bio Inc. Shiga, Japan) according to the manufacturer's instructions. Next, the obtained RNAs were reverse-transcribed to cDNA using the RNA PCR Kit (TaKaRa, Ver.3.0) as per the instructions. Real-time PCR was performed using SYBR premix Ex Taq II kit (TaKaRa), based on the following description: primarily, 94°C for 2 min, followed by 40 cycles of 94°C for 30 s and 60°C for 45 s. All the reactions were carried out on an ABI 7500 Real-Time PCR system (ABI, NY, USA) and repeated in triplicate. The primer for miR-204-5p was purchased from GenePharma (Shanghai, China). U6 gene served as an endogenous reference. miR-204-5p: forward, 5'-TCCCTTTGTCAT CCTATGCC-3' and reverse, 5'-CTCAACTGGTGTCGT GGAGTC-3'. U6: forward, 5'- CTCGCTTCGGCAGCA CAT-3'; reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The relative quantification of target gene expression was calculated by the $2^{-\Delta\Delta CT}$ method and expressed as fold-changes.

Flow cytometric analysis of cell apoptosis

After transfection with miR-204-5p mimics control and miR-204-5p inhibitors, HT22 cells were seeded to 6-well plates at a density of 5×10^5 cells per well and co-incubated with 3% sevoflurane for 48 h. Subsequently, the collected cells were washed twice with cold PBS, and then resuspended in 500 µL binding buffer

containing Dual-staining Annexin V-FITC-propidium iodide (PI) (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C. Cellular fluorescence was measured by FCM flow cytometer (BD Bioscience, San Jose, CA, USA).

Western blot analysis

HT22 cells were pre-treated with miR-204-5p mimics control and miR-204-5p inhibitors for 6 h, respectively. The cells were incubated with 3% sevoflurane for 12 h, then cultured in normal conditions for another 36 h. Next, total cellular protein was extracted from collected cells and quantified using a BCA Protein Assay Kit (Beyotime, Haimen, China). Protein samples of equal amount were subjected to separation by electrophoresis using 10% polyacrylamide gel. After that, proteins were transferred to the polyvinylidene fluoride membranes (PVDF, Thermo Fisher Scientific) electrophoretically at 200 mA. The membranes were blocked with 5% nonfat milk (or BSA for phosphorylated protein) for 50 min, followed by incubation with primary antibodies overnight at 4°C. After that, the membranes were washed with PBS-T three times and then incubated with secondary antibody goat anti-rabbit IgG (1:5000) for 1 h at room temperature. The used primary antibodies were as follows: anti-Bax (1:1000), anti-Bcl-2 (1:1000), anti-active caspase 3 (1:1000), anti-BDNF (1:1000), anti-p-TrkB (1:1000), anti-TrkB (1:1000), anti-Akt (1:1000), anti-p-Akt (1:1000), anti-PI3K (p110β, 1:1000) and anti-β-actin (1:1000). All these antibodies were purchased from Abcam (Cambridge, MA, USA). Finally, the target protein band was visualized through the addition of ECL reagent (Santa Cruz Biotechnology). The density of blots was normalized by using β-actin as a reference.

Immunofluorescence assay

HT22 cells were pre-treated with the transfection of miR-204-5p mimics control and miR-204-5p inhibitors for 6 h, and then seeded into 6-well plates at a concentration of 5×10^5 cells per well. The cells were incubated with 3% sevoflurane for 12 h, then cultured in normal conditions for another 36 h. The group without sevoflurane addition was set as control. After that, cells were prefixed in 4% paraform at room temperature for 20 min and fixed in cold methanol for 10 min at -20°C. Next, the treated cells were incubated with primary antibodies for anti-Ki67 (Abcam, Cambridge, MA, USA) (1:1000) and DAPI (Abcam, Cambridge, MA, USA) (1:1000) at 4°C overnight. Later, cells were incubated with goat anti-rabbit IgG secondary antibodies (Abcam, Cambridge, MA, USA) (1:5000) at 37°C for 1 h. The images of cells were visualized using a fluorescence microscope (Olympus, Tokyo, Japan). 5 fields of each sample were randomly selected for quantification. Only double positive cells (pink color) were counted.

Intracellular ROS assay

According to a previous publication (Li et al., 2019), the level of intracellular ROS of HT22 cells was determined by the 2,7-dichlorofuorescin diacetate (DCFH-DA) kit (ROS kit, Beyotime, Haimen, China). The cellular fluorescence and cell quantity were measured by FCM flow cytometer (BD Bioscience, San Jose, CA, USA).

ELISA assay

ELISA kits (Beyotime, Haimen, China) were used to

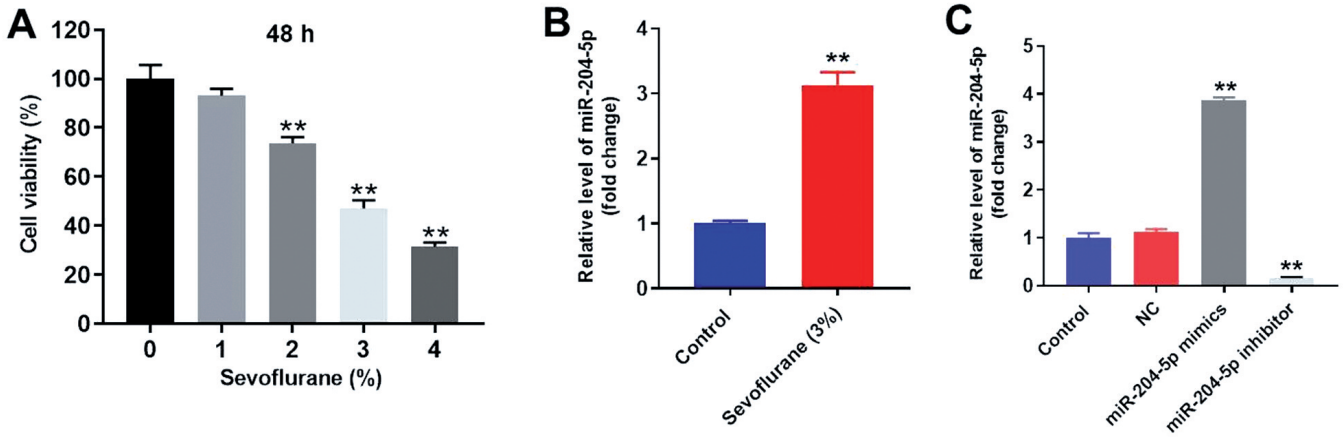

Fig. 1. Sevoflurane decreased the viability of HT22 cells and upregulated the expression of miR-204-5p. **A.** Cell viability of HT22 cells treated with different concentration (0%, 1%, 2%, 3% and 4%) of sevoflurane for 12 h then cultured for another 36 h in normal conditions. Cell viability was determined by CCK-8 assay. **B.** The relative level of miR-204-5p in HT22 cells incubated with 3% sevoflurane was detected using RT-qPCR. **C.** The relative level of miR-204-5p in HT22 cells transfected with 10 nM miR-204-5p mimics, mimics NC and miR-204-5p inhibitors was detected using RT-qPCR. **P<0.01 vs. control group, n=3.

evaluate three indicators related to oxidation, including intracellular total superoxide dismutase (SOD), reduced glutathione (GSH), and malondialdehyde (MDA). The absorbance was determined using a microplate reader (Thermo Fisher Scientific).

Luciferase reporter assay

The 3'-UTR sequences of BDNF were obtained by PCR and further cloned into psiCHECK-based luciferase plasmid (Promega, Madison, WI, USA). The mutated psiCHECK2-BDNF 3'-UTR was induced as the reference (Liu et al., 2014). The HT22 cells were treated with 100 nM miR-204-5p mimics, followed by co-transfection with 100 ng PsiCHECK2-BDNF-WT or PsiCHECK2-BDNF-MT using Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA). After incubation for 48 h, different groups of cells were digested by tritonX-100. The activities of firefly luciferase and renilla luciferase were detected via luminescent signals, using Tecan Infinite F200/M200 (Tecan, Männedorf, Switzerland) with the Gene5 software (BioTek).

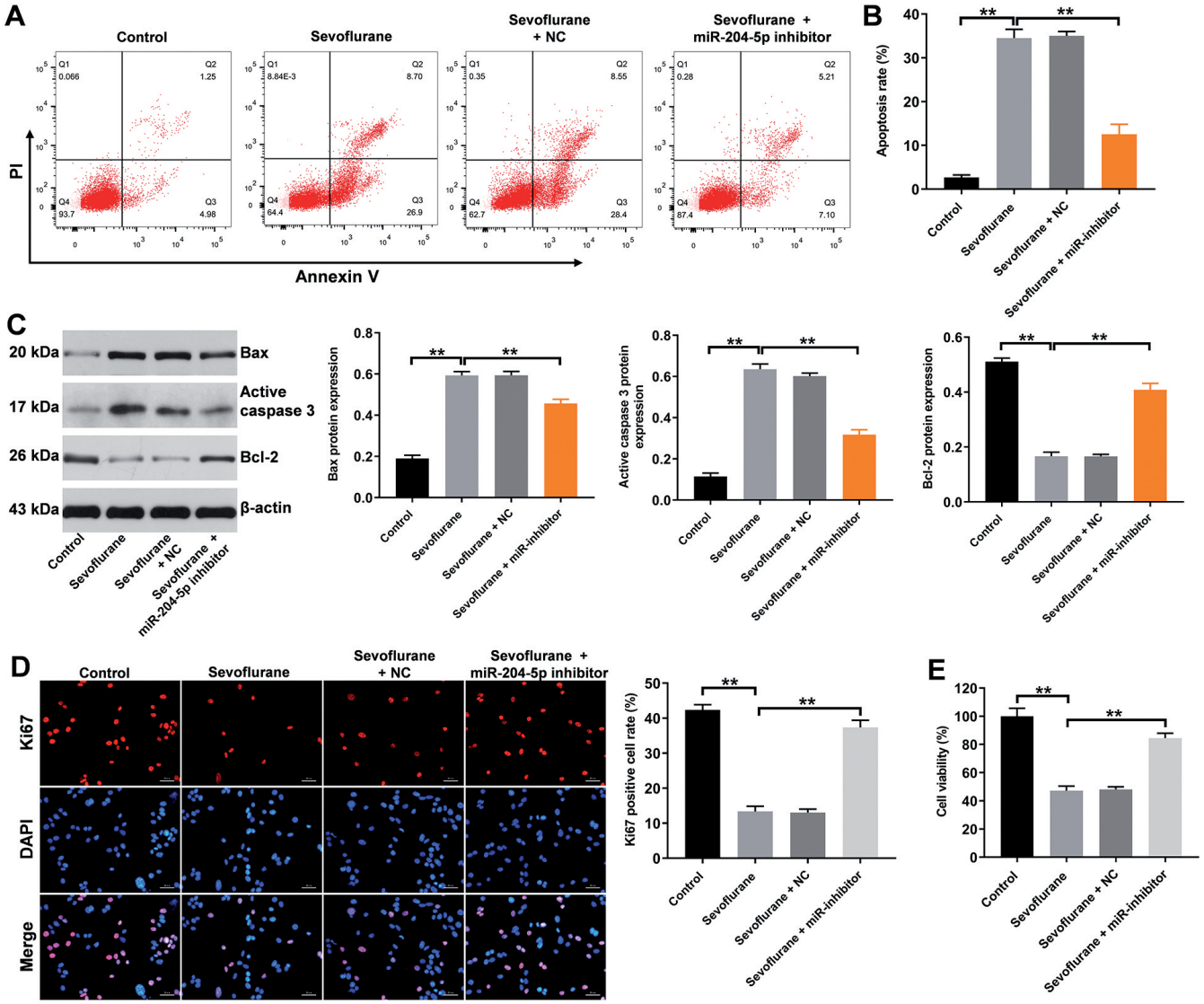

Fig. 2. MiR-204-5p inhibitors prevented sevoflurane-induced apoptosis of HT22 cells. HT22 cells were transfected with 10 nM miR-204-5p inhibitors and inhibitor NC for 6 h and incubated with 3% sevoflurane for 12 h, then cultured for another 36 h in normal conditions. **A.** Apoptosis of HT22 cells was detected with dual-staining Annexin V-PI. **B.** The calculated apoptosis rates. **C.** The expressions of Bax, active caspase 3, Bcl-2 and β-actin in HT22 cells were analyzed with western blotting. The relative expressions of proteins were quantified via normalization to β-actin. **D.** Representative fluorescent images and relative fluorescence expression levels of anti-Ki67 and DAPI using immunofluorescence assay. **E.** Cell viability was detected with CCK8 assay. **P<0.01. n=3.

Statistical analysis

All the tests were performed in triplicate and all the data are presented as the means ± standard deviation (SD). GraphPad Prism 7.0 (software, CA, USA) was used for data analysis. The comparisons among multiple groups were made with one-way analysis of variance (ANOVA) followed by Turkey's test. Student's t-test was used to analyze the comparison between two groups. The dose-dependent analysis was performed with Pearson analysis. The differences at a value of $P<0.05$ or $P<0.01$ were considered statistically significant (*$P<0.05$, ** $P<0.01$).

Results

The effects of sevoflurane on the viability of HT22 cells

In order to determine the impact of sevoflurane on the growth of HT22 cells, CCK-8 assay was employed. As shown in Fig. 1A, the addition of sevoflurane significantly decreased the viability of HT22 cells in a dose-dependent manner. Since 3% sevoflurane caused approximately 50% growth inhibition, this dose was chosen for use in the subsequent experiments.

To investigate the role of miR-204-5p in sevoflurane-induced cytotoxicity, the expressions of miR-204-5p in both normal and 3% sevoflurane treated cells were determined using RT-qPCR method. As indicated in Fig. 1B, the level of miR-204-5p in sevoflurane treated cells was significantly upregulated compared with that in the normal cells. In addition, normal HT22 cells were transfected with miR-204-5p mimics or NC or inhibitors. As shown in Fig. 1C, the expression of miR-204-5p was significantly upregulated in miR-204-5p mimics transfected cells and was dramatically downregulated in miR-204-5p inhibitor transfected cells, compared with NC.

MiR-204-5p inhibitors alleviated sevoflurane-induced cytotoxicity in HT22 cells via inhibition of apoptosis and increasing proliferation

Our findings (Fig. 2A,B) indicated that 48 h

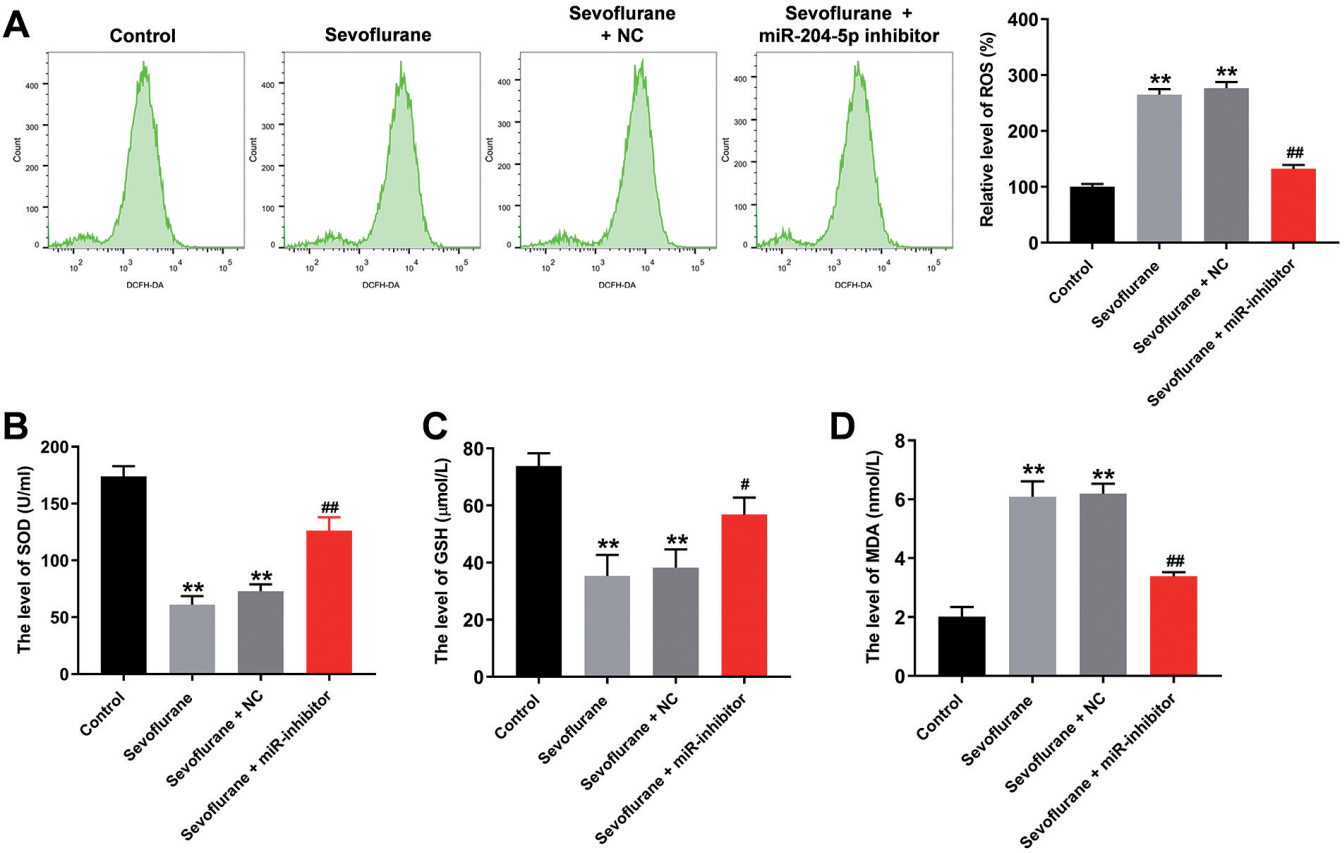

Fig. 3. MiR-204-5p inhibitors prevented sevoflurane-induced ROS generations in HT22 cells. HT22 cells were transfected with 10 nM miR-204-5p inhibitors and inhibitor NC for 6 h and incubated with 3% sevoflurane for 12 h, then cultured for another 36 h in normal conditions. **A.** The level of ROS in cells was detected using DCFH-DA kit. **B-D.** The levels of SOD, GSH and MDA in cell culture were determined by ELISA assay. **$P<0.01$ vs. control group, ##$P<0.01$ vs. sevoflurane group. n=3.

treatment of sevoflurane caused severer cell apoptosis compared with normal cells (P<0.01). Interestingly, this pro-apoptotic effect of sevoflurane was remarkably reduced by miR-204-5p inhibitors. So, western blotting was performed to detect apoptosis-related proteins. It has been reported that Bcl-2 is an anti-apoptotic protein, while Bax and caspase 3 are pro-apoptotic proteins (Sheikh et al., 2010). As indicated in Fig. 2C, sevoflurane treatment significantly increased the expressions of Bax and active caspase 3, which was attenuated by the transfection of miR-204-5p inhibitors. Meanwhile, miR-204-5p inhibitors promoted the levels of Bcl-2 protein in sevoflurane treated HT22 cells compared with sevoflurane group. Furthermore, sevoflurane-induced decrease of cell viability was significantly reversed by miR-204-5p inhibitors (Fig. 2D,E). All these results suggested that miR-204-5p downregulation could alleviate sevoflurane-induced cytotoxicity and increased the proliferation in HT22 cells via inhibition of apoptosis.

MiR-204-5p inhibitors prevented sevoflurane-induced oxidative status in HT22 cells

Oxidative status is an important cell characteristic included in response to cellular environment transformation (Wang et al., 2018). Generation of SOD and GSH can prevent apoptosis in neuroblastoma HT22 cells (Wei et al., 2016). ROS and MDA were served as oxidative indicators (Chen et al., 2017). As shown in Fig. 3A-D, sevoflurane was able to significantly increase the levels of ROS and MDA in HT22 cells, but

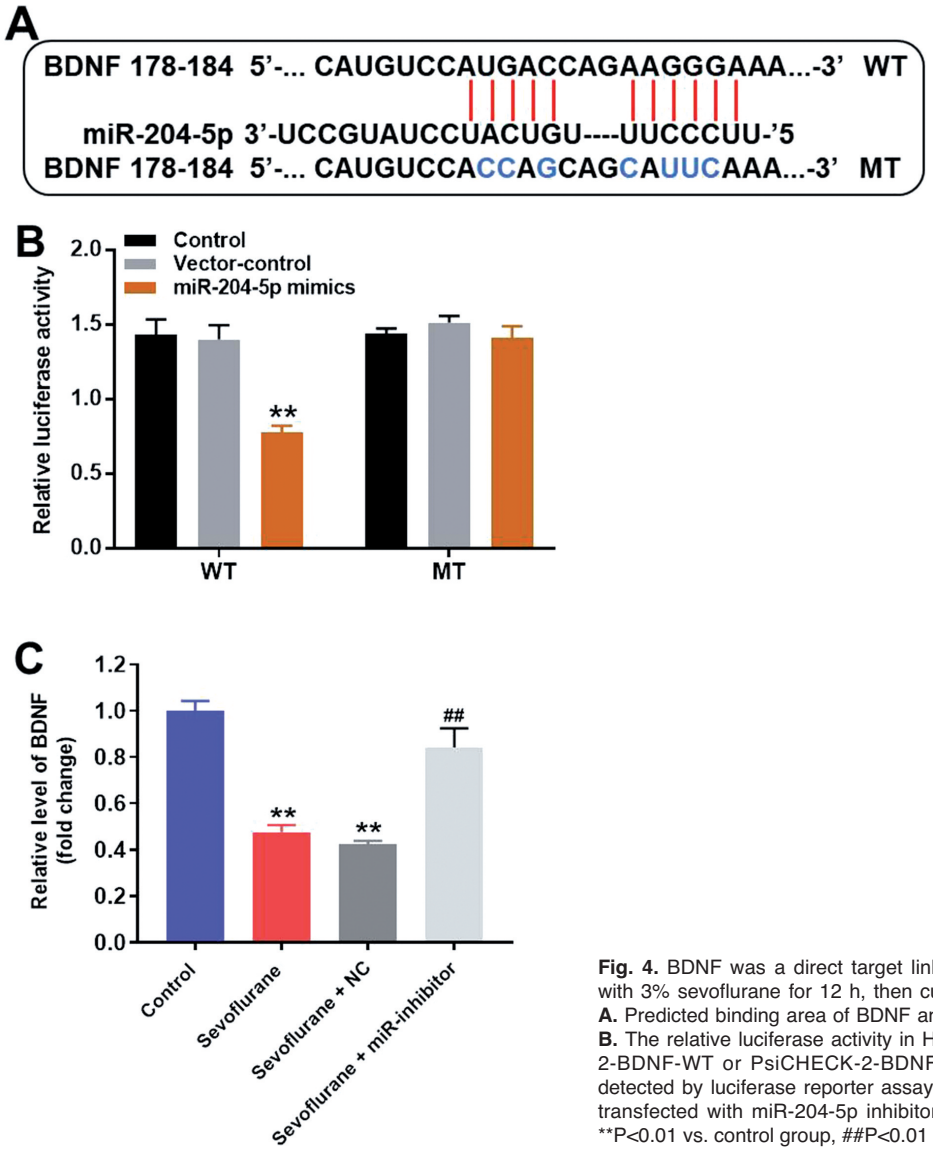

Fig. 4. BDNF was a direct target linking to miR-204-5p. HT22 cells were treated with 3% sevoflurane for 12 h, then cultured for another 36 h in normal conditions. **A.** Predicted binding area of BDNF and miR-204-5p using TargetScan and miRDB. **B.** The relative luciferase activity in HT22cells transfected with 100 ng PsiCHECK-2-BDNF-WT or PsiCHECK-2-BDNF-MT and 100 nM miR-204-5p mimics was detected by luciferase reporter assay. **C.** The relative level of BDNF in HT22 cells transfected with miR-204-5p inhibitors and NC was determined using RT-qPCR. **P<0.01 vs. control group, ##P<0.01 vs. sevoflurane group. n=3.

downregulated the expressions of SOD and GSH, compared with the control group. However, the levels of ROS and MDA in sevoflurane treated HT22 cells declined to almost normal status in the presence of miR-204-5p inhibitors (Fig. 3A,D). Meanwhile, the expressions of SOD and GSH were upregulated by miR-204-5p inhibitors. These results showed that downregulation of miR-204-5p might reduce sevoflurane-induced cytotoxicity by promoting antioxidative status in HT22 cells.

BDNF was a direct binding target of miR-204-5p

As found that miR-204-5p inhibitors were able to prevent sevoflurane-induced cytotoxicity in HT22 cells by inhibiting apoptosis and decreasing oxidative status, we next identified the potential downstream binding targets of miR-204-5p. As indicated in Fig. 4A, BDNF was predicted to be a binding target-mRNA of miR-204-5p, using bioinformatics tools including TargetScan and miRDB. Subsequently, the luciferase reporter assay was employed to verify this hypothesis. Seen from Fig. 4B, the luciferase intensity was significantly downregulated following co-transfection with miR-204-5p mimics and psiCHECK2-BDNF-WT, while almost no change was observed when the target-mRNA BDNF was mutated. In addition, the results of RT-qPCR showed that the expressions of BDNF were markedly promoted in sevoflurane-treated cells transfected with miR-204-5p inhibitors (Fig. 4C). Therefore, we concluded that BDNF was a direct target linking to miR-204-5p.

MiR-204-5p inhibitors decreased sevoflurane-induced cytotoxicity in HT22 cells via activating BDNF/TrkB/Akt pathway

BDNF is capable of stimulating the PI3K/Akt pathway by targeting downstream receptor, TrkB

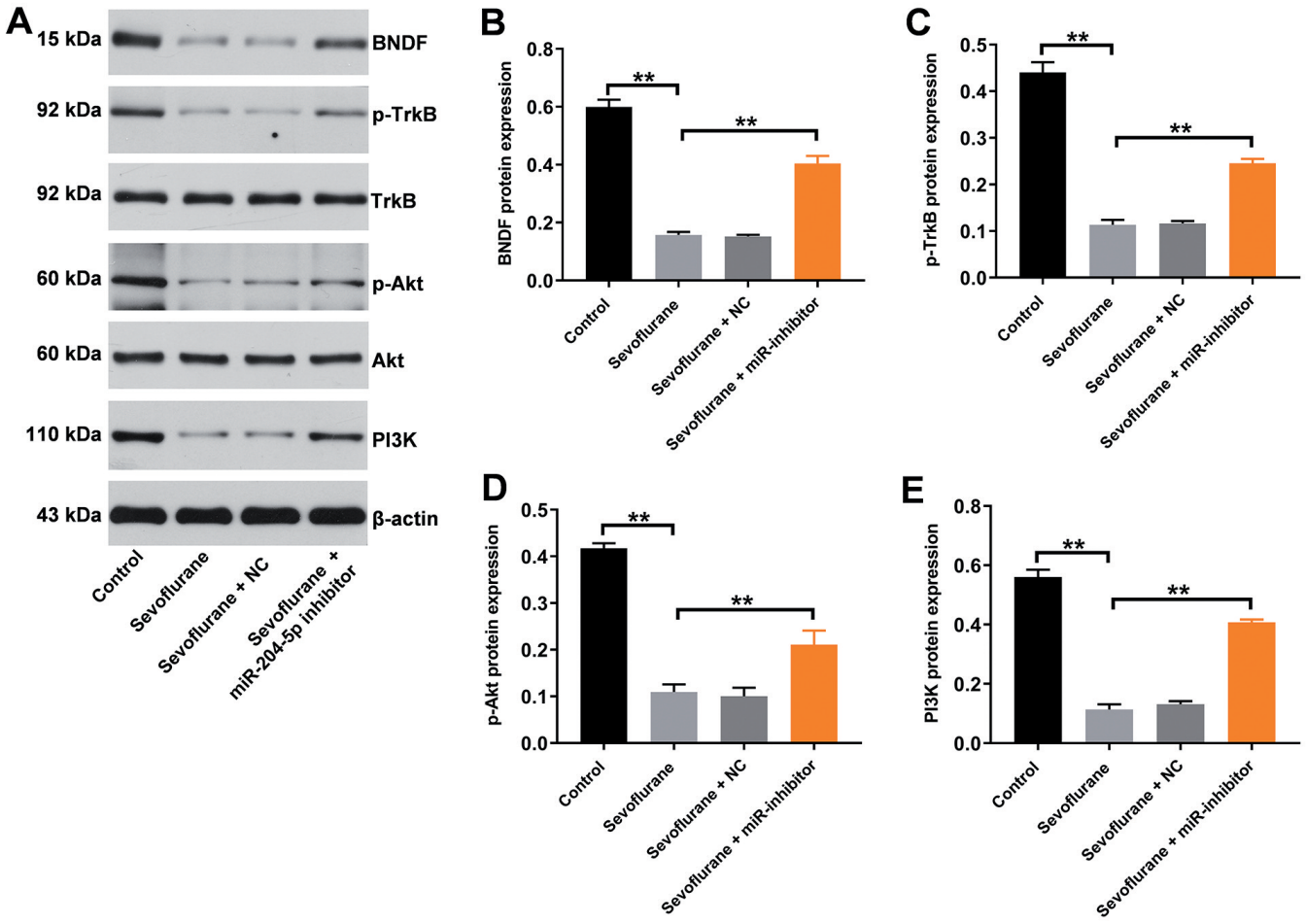

Fig. 5. MiR-204-5p inhibitors attenuated sevoflurane-induced cytotoxicity in HT22 cells via activating TrkB/Akt pathway. HT22 cells were transfected with 10 nM miR-204-5p inhibitors and NC for 6 h and incubated with 3% sevoflurane for 12 h, then cultured for another 36 h in normal conditions. **A.** The western blotting results of BDNF, p-TrkB, TrkB, p-Akt, Akt and β-actin. **B-D.** The BDNF, p-TrkB and p-Akt protein expression were quantified via normalization to β-actin. **P<0.01. n=3.

(Revest et al., 2014). Akt is proven to play an important role in neuron cell survival and death (Sheikh et al., 2010) As presented in Fig. 5A-D, sevoflurane treatment can significantly reduce the expression of BDNF, p-Akt, p-TrkB and PI3K. However, these effects can be dramatically reversed in the presence of miR-204-5p inhibitors (Fig. 5A-D). Hence, this evidence indicates that downregulation of miR-204-5p can attenuate sevoflurane-induced cytotoxicity in HT22 cells by activating the TrkB/Akt pathway.

Discussion

Previous publications reported that sevoflurane could induce apoptosis and damage of brain cells (Zhou et al., 2018). Consistent with these studies, CCK-8 assay analysis showed that the growth of HT22 cells was inhibited by sevoflurane. During this process, miR-204-5p, one of the miRNAs participating in complex cellular processes, was found to be upregulated in neonatal hippocampus of mice (Ye et al., 2016). In this study, we also found that the expression of miR-204-5p was increased in sevoflurane-treated HT22 cells using RT-qPCR. Du et al. found that miR-204-5p was able to induce apoptosis by regulating Bcl-2 in 3T3-L1 preadipocyte (Xu et al., 2018). Bcl-2 was considered as an anti-apoptotic protein. We examined the expression of Bcl-2 and two pro-apoptotic proteins that included Bax and active caspase 3 (Zhang et al., 2015). Results indicated that inhibiting the expression of miR-204-5p was able to prevent HT22 cells from sevoflurane-induced apoptosis through upregulating Bcl-2 and downregulating Bax and active caspase 3. Karkhah et al. have also demonstrated that the level of oxidative indicators such as ROS and superoxide anion were significantly increased under homocysteine-induced apoptosis in rat hippocampus cells (Li et al., 2018a). Similarly, the present findings revealed that sevoflurane can increase the ROS and MDA and reduce SOD and GSH levels, while these effects were reversed in the presence of miR-204-5p inhibitor.

Several targets of miR-204-5p that included RAB22A (Yin et al., 2014) and CREB1 (Bian et al., 2016) have been reported to be involved in considerable biological functions. To determine the target of miR-204-5p that was mainly associated with sevoflurane-induced apoptosis, bioinformatics approaches were implemented. It was found that BDNF might be the potential target of miR-204-5p, and a luciferase reporter assay was performed to confirm the predicted results. Previous studies showed that BDNF was controlled by the TrkB receptor and participated in the PI3K/Akt pathway to impede apoptosis of neuron cells (Yin et al., 2019). Consistent with these findings, we found the usage of miR-204-5p inhibitors upregulated the expressions of BDNF, p-Akt and p-TrkB. Therefore, the present study demonstrated that miR-204-5p downregulation can inhibit the apoptosis of HT22 cells by increasing the expression of BDNF, which further activated the TrkB/Akt pathway.

Conclusion

In the current study, we discovered that downregulation of miR-204-5p was able to decrease oxidative status and cell apoptosis in sevoflurane-treated HT22 cells, and alleviate sevoflurane-induced cytotoxicity by stimulating the BDNF/TrkB/Akt pathway. Thus, miR-204-5p might be a potential biomarker and therapeutic target for the treatment of sevoflurane-induced neurotoxicity.

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Conflicts of interest. The authors declare no competing financial interests.

Availability of data and materials. All data sets used in this study are available from the corresponding author upon reasonable request.

Author contribution. Hongchao Liu and Jun Wang conceived the study and supervised the experiments; Rongrong Yan, Shuangfen Jin, Zhenzhen Wan, Jing Cheng, Na Li and Lin Chen designed and conducted the experiments. Chengjin Le provided experimental materials and drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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