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Cerebellar Fastigial Nucleus Stimulation in a Chronic Unpredictable Mild Stress Rat Model Reduces Post-Stroke Depression by Suppressing Brain Inflammation via the microRNA-29c/TNFRSF1A Signaling Pathway

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: We previously reported that cerebellar fastigial nucleus stimulation reduced post-stroke depression in a rat model by reducing inflammation. This study aimed to investigate the molecular inflammatory signaling pathways associated with cerebellar fastigial nucleus stimulation in an established rat model of post-stroke depression.

Material/Methods: Twenty-four Sprague-Dawley rats included a sham group (N=6), an untreated stroke group (N=6), an untreated post-stroke depression model group (PSD) (N=6), and the model group treated with cerebellar fastigial nucleus stimulation (FNS) (N=6). The rat stroke model involved occlusion of the middle cerebral artery occlusion (MCAO). Post-stroke depression model was established using chronic unpredictable mild stress treatment and was verified using an open field test. Real-time polymerase chain reaction (PCR) and Western blot compared expression levels of microRNA-29c (miR-29c), miR-676, TNFRSF1A, tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β in cerebellar tissue. U251 human glioblastoma cells and SH-SY5Y human neuroblastoma cells were studied *in vitro*.

Results: Cerebellar fastigial nucleus stimulation reduced behaviors associated with depression in the rat model, upregulated the expression of miR-29c, and reduced the expression of TNFRSF1A and inflammatory cytokines, and mildly reduced neuronal apoptosis. Bioinformatics data analysis identified a regulatory relationship between miR-29c and TNFRSF1A. SH-SY5Y cells treated with a miR-29c mimic, or TNFRSF1A short interfering RNA (siRNA), identified a negative regulatory relationship between TNFRSF1A and miR-29c.

Conclusions: In a rat model, cerebellar fastigial nucleus stimulation reduced the expression of TNFRSF1A by upregulating miR-29c expression, which suppressed the expression of inflammatory cytokines, resulting in reduced severity of post-stroke depression.

MeSH Keywords: **Depression • Inflammation • MicroRNAs**

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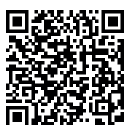

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Background

Post-stroke depression is considered to be a serious psychiatric complication in patients following stroke, with a prevalence of up to 40%, which affects approximately three million patients in the USA per year [1,2]. Stroke survivors often have difficulty in explaining their symptoms of depression, particularly among older patients. Also, patients suffering from both stroke and post-stroke depression are more susceptible to serious outcomes and tend to show less improvement during rehabilitation, and as a specific type of psychiatric disease, post-stroke depression can be difficult to treat [2]. Previously published studies have shown a correlation between mental disorders and cerebellar impairment, and have highlighted the important role of the cerebellum in the development of post-stroke depression [2,3]. Therefore, in order to improve cerebellar function in post-stroke depression, the type and dose of anti-depression drugs should be selected with care. Recently, physiological intervention for post-stroke depression has included electrical stimulation of the cerebellar fastigial nucleus, which may help to reduce the increasing burden of psychiatric illness in an aging population and an increasing incidence of stroke [3].

The treatment of cerebellar dysfunction using electrical stimulation has shown positive effects on the mood and overall wellbeing of patients with post-stroke depression [3]. Electrical stimulation of the fastigial nucleus has been shown to reduce the infarction volume in the forebrain, which is usually caused by occlusion of the middle cerebral artery [4,5]. Brain recovery induced by fastigial nucleus stimulation is unrelated to the changes in cerebral glucose metabolism and cerebral blood flow [6,7]. A neuroprotective role of fastigial nucleus stimulation may be related to a decreased level of neuronal excitability, which can decrease inflammation in the brain [7–9].

It has also been shown that fastigial nucleus stimulation reduced depressive behavior in a rat model of post-stroke depression, and that fastigial nucleus stimulation of the cerebellum could prevent Purkinje cell apoptosis and could decrease the level of inflammatory cytokines in brain tissues [7–9]. In a previous study, the authors confirmed that the abnormal function of the cerebellar fastigial nucleus is involved in the development of depression after stroke [10]. The development of an animal experimental model of post-stroke depression may aid the development of novel treatment for post-stroke depression, which may avoid the adverse effects of drug treatment for depression in patients who are recovering from stroke [10].

MicroRNAs (miRNAs) play essential roles in gene regulation and in the pathogenesis of a wide range of diseases and are highly evolutionarily conserved. The miRNAs are short RNA transcripts, less than 25 nucleotides in length, which are commonly expressed in eukaryotic cells and can regulate the transcription and translation of almost all known genes [11]. In general, miRNAs can exert inhibitory effects by interacting with the 3'-untranslated region (3'-UTR) of their target mRNAs and have also been shown to have a role in oncogenesis [11]. In a previous study, we showed that fastigial nucleus stimulation treatment could reduce the symptoms of post-stroke depression in rats by reducing inflammatory responses [10]. This finding was also supported by a study that showed that fastigial nucleus stimulation treatment upregulated the expression of miR-29c [12].

The TNFRSF1A gene encodes tumor necrosis factor receptor 1 (TNFR1), and the deregulation of TNFRSF1A expression may be responsible for the development of post-stroke depression [13]. Therefore, there is a possibility that fastigial nucleus stimulation could suppress the inflammation in the brain via the miR-29c and TNFRSF1A signaling pathway. To investigate this hypothesis, this study aimed to investigate the molecular inflammatory signaling pathways associated with cerebellar fastigial nucleus stimulation in an established rat model of post-stroke depression.

Material and Methods

Establishment of a rat model of post-stroke depression

This study was approved by the Ethics Committee of Shanxi Provincial Peoples' Hospital, Taiyuan, Shanxi 030012, China (Approval No: 2016012). Sprague-Dawley rats were purchased and housed in a clean animal facility at room temperature and under a relative humidity of 50–60%. The rats had free access to clean water and a standard diet during the course of the experiment. Twenty-four healthy rats were randomly selected and allocated into four groups, including a sham group (N=6), a stroke group (N=6), an untreated post-stroke depression (PSD) group (N=6), and a post-stroke depression fastigial nucleus stimulation (FNS)-treated group (N=6). In the sham group (N=6), the rats received the same procedure as those in other groups, but the middle cerebral artery was not sutured. The remaining rats (N=18) underwent a standard middle cerebral artery occlusion (MCAO) procedure to establish the stroke model.

The rats in the untreated post-stroke depression (PSD) group (N=6), and the post-stroke depression fastigial nucleus stimulation (FNS)-treated group (N=6) were exposed to chronic unpredictable mild stress treatment (N=12), which included electric shock to the foot, water deprivation, food deprivation, tail clamping, and behavioral restriction. The rats in the FNS-treated group were treated with cerebellar fastigial nucleus electrical stimulation. The body weight (gm), sucrose preference (%),

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locomotor activities (score) and rearing activities (score) were recorded as indicators to compare the differences among the rats in different model groups.

Cerebellar electrical fastigial nucleus stimulation

The rats in the post-stroke depression group treated with fastigial nucleus stimulation (FNS) (N=6) were anesthetized with an intraperitoneal injection of a 3.5% (w/v) chloral hydrate solution at a dose of 10 mL/kg. The rats were stabilized under a stereotactic endoscope (Mau Science Equipment, Tokyo, Japan) according to the manufacturer's instruction. The stereotaxic atlas of the rat brain was used as the reference to precisely locate the fastigial nucleus, with the posterior border of the anterior fontanelle set as the zero reference point. Then, an 11.1 mm incision was used to allow the insertion of electrodes of a programmed electrical stimulation device (Chengdu Instrument Factory, Chengdu, China). The fastigial nucleus stimulation was performed using a 70 μA direct current square wave pulse at 50 Hz. All experiments were performed in triplicate to record the values of regional cerebral blood flow, body weight, the amount of sucrose water consumption, and the level of locomotor and rearing activity of the experimental animals.

Locomotor activity

The locomotor activity of the rats was evaluated using an open field test. Briefly, the rats were housed in an acrylic box for 20 min. A computer system (San Diego Instruments, San Diego, CA, USA) was then used to measure the locomotor activity according to the manufacturer's instructions.

Regional cerebral blood flow measurement

A transcranial Doppler ultrasound system (Spencer Technologies, Seattle, WA, USA) was operated at a pulse frequency of 2 MHz to measure the values of regional cerebral blood flow in the posterior cerebral artery and middle cerebral artery, according to the manufacturer's protocol. Then, a standard procedure was used to locate the posterior cerebral artery and middle cerebral artery, as previously described [14].

Electron microscopy

After the rats were euthanized, brain tissue samples were collected from all the rats in the study, fixed, processed, and tissue sections were cut and stained with lead citrate and uranyl acetate, and evaluated using a Philips CM10 transmission electron microscope (TEM) (Philips Electron Optics, Eindhoven, The Netherlands).

RNA isolation and real-time polymerase chain reaction (PCR)

The total RNA in the samples was extracted using a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The absorption values at 260 nm and 280 nm were measured using an ultraviolet spectrophotometer and were used to determine the concentration and purity of isolated RNA. If the ratio of optical density (OD) 260/OD 280 was between 2.1 and 1.7, the purity was acceptable for the subsequent experiments.

Reverse transcription from RNA into cDNA was performed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) on a PCR amplifier (Applied Biosystems, Forster City, CA, USA). Then, an ABI Sequence Detection System 7900HT (Applied Biosystems, Forster City, CA, USA) was used to perform the real-time polymerase chain reaction (PCR). The reaction conditions of real-time PCR included pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, and 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 34 s. The real-time PCR reaction system consisted of 10 μl of SYBR Premix Ex Taq II (Tli RNase H Plus), a reagent designed for real-time PCR using SYBR Green I (Thermo Fisher Scientific, Waltham, MA, USA), 0.8 μl of PCR forward primer (10 μM), 0.8 μl of PCR reverse primer (10 μM), 0.4 μl of ROX Reference Dye to normalize the fluorescent reporter signal (Thermo Fisher Scientific, Waltham, MA, USA), 2.0 μl of cDNA template, and 6.0 μl of sterile distilled water. The U6 RNA gene was used as the internal control of real-time PCR, and the method of $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression of microRNA-29c (miR-29c), miR-676 and TNFRSF1A mRNA in samples from the four animal study groups.

Cell culture and transfection

SH-SY5Y human neuroblastoma cells and U251 human glioblastoma cells were cultured in RPMI 1640 culture medium (GIBCO, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA). The culture conditions were 37°C and 5% CO_2. When the cells reached the logarithmic growth phase, they were seeded into 24-well plates and transfected with miR-29c, TNFRSF1A, or TNFRSF1A short interfering RNA (siRNA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The cells were harvested at 48 hours post-transfection for subsequent measurements.

Luciferase assay

To determine the regulatory relationship between TNFRSF1A and miR-29c, a computational analysis was performed to locate the binding site of miR-29c in the 3' UTR of TNFRSF1A. The full length of TNFRSF1A 3' UTR was amplified and cloned

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into a pcDNA3.1 vector (Promega, Madison, WI, USA) downstream of the firefly luciferase reporter gene. A QuikChange II Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA) was used to perform site-directed mutagenesis in the miR-29c binding site of TNFRSF1A 3' UTR, which was also amplified and cloned into the pcDNA3.1 vector to create the mutant TNFRSF1A 3' UTR plasmid. The SH-SY5Y and U251 cells were co-transfected with the miR-29c mimic and mutant or wild-type TNFRSF1A 3' UTR. At 48 hours post-transfection, the cells were harvested and the luciferase activity of transfected cells was measured using a Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Madison, WI, USA), according to the manufacturer's instructions.

Western blot

The total proteins of samples were isolated, resolved using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (PVDF) membrane, and blocked in Tris-buffered saline containing 20% Tween (TBST) and 5% skimmed milk powder at room temperature for 1 h. The monoclonal primary antibodies to TNFRSF1A, interleukin (IL)-6, tumor necrosis factor-α (TNF-α), IL-1β, caspase-3, and the internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were all purchased from Abcam (Cambridge, MA, USA) and were added onto the membrane and incubated overnight at 4°C. After washing with phosphate-buffered saline (PBS), the membrane was incubated with secondary antibodies at room temperature for 1 h before the protein bands were developed using an enhanced chemiluminescence (ECL) kit (Thermo Fisher Scientific, Waltham, MA, USA). The relative expression of target proteins was analyzed using ImageJ software (National Institutes of Health, MD, USA).

Immunohistochemistry

Brain tissue samples were fixed in 10% formalin, processed and embedded in paraffin wax. Tissue sections were cut at a thickness of 4 µm onto glass slides. The sections were dried in a 60°C oven for 1 h, and dewaxed using a conventional xylene method, dehydrated in graded alcohols, incubated in 3% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min, boiled in 0.01 M citrate buffer at 95°C for 20 min, and blocked with normal sheep serum at 37°C for 10 min prior to immunostaining. The tissue sections were incubated with anti-TNFRSF1A primary antibodies (Abcam, Cambridge, MA, USA) at 4°C overnight, washed, and incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Abcam, Cambridge, MA, USA) at room temperature for 30 min. Following incubation and staining with 3,3'-diaminobenzidine (DAB) and hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), the tissue sections were mounted with a glass coverslip and observed by light microscopy at high magnification (×400) microscope to evaluate the expression of the TNFRSF1A protein.

Enzyme-linked immunosorbent assay (ELISA)

The protein expression of IL-6, TNF-α, and IL-1β was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The absorbance at a wavelength of 450 nm was measured by a microplate reader, and the measured OD values were used to quantify the expression of IL-6, TNF-α, and IL-1β.

TUNEL assay

The samples were stained using a TUNEL kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer's instructions. The apoptotic profiles of the cells were observed under a fluorescence microscope. A total of 10 visual fields were selected randomly for each slide, and the ratio between the number of apoptotic cells and the total number of cells was calculated as the apoptotic index.

Statistical analysis

Statistical analysis was performed using SPSS version 21.0 software (IBM, Chicago, IL, USA). Data were presented using the mean ± the standard deviation (SD). Comparisons between two groups were made using t-tests, while the comparisons among multiple groups were conducted using analysis of variance (ANOVA). The analysis of occurrence was performed using the chi-squared χ^2 test. A P-value of <0.05 considered to represent statistical significance.

Results

Post-stroke depression-like behaviors were reduced by cerebellar fastigial nucleus stimulation

An animal model of stroke was established by middle cerebral artery occlusion (MCAO) in previously healthy Sprague-Dawley rats, and a post-stroke depression model was established using chronic unpredictable mild stress treatment and was verified by an open field test. Twenty-four rats were studied in four groups that included a sham group (N=6), an untreated stroke group (N=6), an untreated post-stroke depression model group (PSD) (N=6), and the model group treated with cerebellar fastigial nucleus stimulation (FNS) (N=6). No deaths occurred in the rats studied, indicating the successful establishment of the rat model of post-stroke depression

In the rats who underwent stroke followed by chronic unpredictable mild stress treatment (N=18) showed symptoms of the post-stroke depression within one week, including slowed responses, drowsiness, and sluggishness. As shown in Figure 1,

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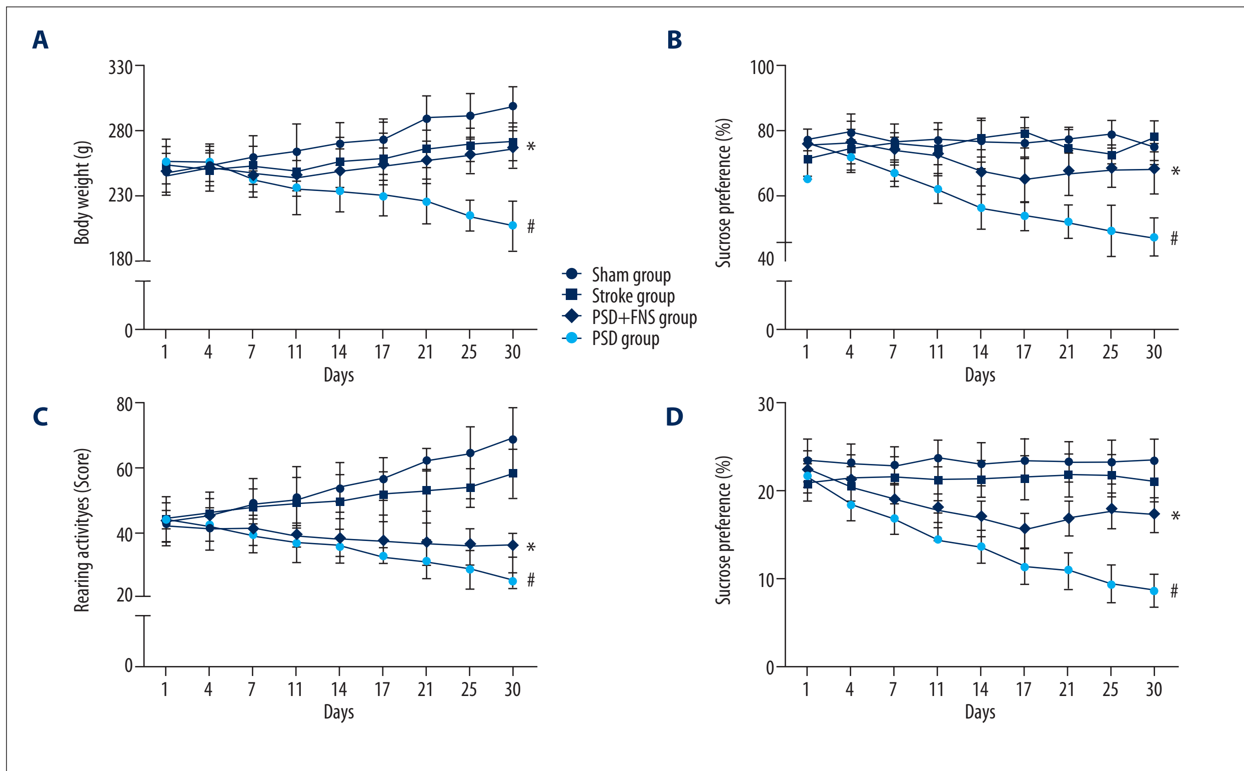

Figure 1. Dynamic changes of body weight, sucrose preference, locomotor activity, and rearing activity in the sham group, the stroke group, the post-stroke depression (PSD) group, and the fastigial nucleus stimulation (FNS) group showed that fastigial nucleus stimulation treatment could reduce behavior associated with post-stroke depression. (**A**) Dynamic changes in rat body weight. Compared with the sham group, the rat body weights in the stroke and fastigial nucleus stimulation (FNS) group began to increase after day 11. The rat body weights in the post-stroke depression (PSD) group continuously decreased and reached their lowest level on day 30. $^{\#}$ P<0.01 *vs.* the stroke group; * P<0.05 *vs.* the PSD group. The bars represent the standard deviation (SD). N=6. (**B**) Dynamic changes of sucrose preference. Compared with the sham and stroke groups, the consumption of sucrose water in the fastigial nucleus stimulation (FNS) group started to increase slightly after day 14 and remained constant thereafter. The consumption of sucrose water continued to decrease in the PSD group and reached the lowest level on day 25. $^{\#}$ P<0.01 *vs.* the stroke group; * P<0.01 *vs.* the PSD group. The bars represent the SD. N=6. (**C**). Dynamic changes of locomotor activity in the open field test. Compared with that in the sham group and stroke group, the locomotor activity in the PSD group continued to decrease and reached its lowest point on day 30. The locomotor activity in the fastigial nucleus stimulation group started to increase slightly after day 17. A significant difference was found between the PSD group and the FNS group after day 21. $^{\#}$ P<0.01 *vs.* the stroke group. * P<0.01 *vs.* the PSD group. The bars represent the SD. N=6. (**D**) Dynamic changes of rearing activity in the open field test. Compared with that in the sham group and stroke group, the rearing activity in the PSD group continued to decrease and reached its lowest point on day 30. The rearing activity in the FNS group started to increase slightly after day 17. A significant difference was found between the PSD group and the FNS group after day 21. $^{\#}$ P<0.01 *vs.* the stroke group. * P<0.01 *vs.* the PSD group. The bars represent the SD. N=6.

when compared with the untreated post-stroke depression rat model, the post-stroke depression rats treated with fastigial nucleus stimulation showed significantly increased body weight, from day 21 (Figure 1A), sucrose preference, from day 17 (Figure 1B), locomotor activities, from day 21 (Figure 1C), and rearing activities, from day 21 (Figure 1D). These indicators steadily decreased over time in the untreated rat model of post-stroke depression. Therefore, cerebellar fastigial nucleus stimulation improved the behaviors associated with post-stroke depression in the rat model.

Fastigial nucleus stimulation upregulated the expression of microRNA-29c (miR-29c)

To investigate the molecular mechanisms associated with the effects of fastigial nucleus stimulation in the alleviation of post-stroke depression, we measured the relative expression levels of miR-29c and miR-676 were measured in the four rat model groups. As shown in Figure 2A, when compared with the sham and stroke groups, the relative expression of miR-29c was reduced in the post-stroke depression group. However, the level of miR-29c was increased by the treatment with fastigial

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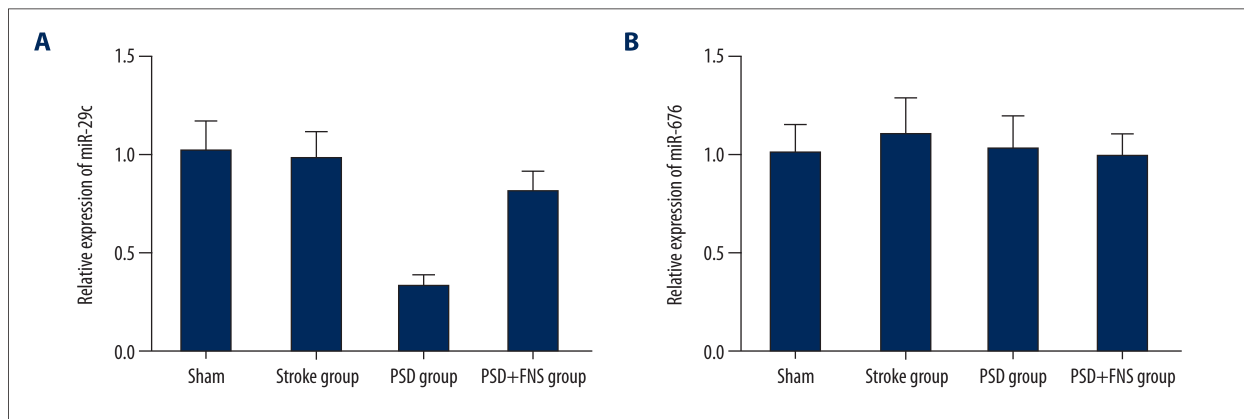

Figure 2. The relative expression of miR-29c and miR-676 in the sham group, the stroke group, the post-stroke depression (PSD) group, and the fastigial nucleus stimulation (FNS) group showed that cerebellar fastigial nucleus stimulation treatment increased the expression of miR-29c in the rat model of post-stroke depression (PSD). (**A**) The relative expression of miR-29c was reduced in the post-stroke depression (PSD) group compared with the sham and stroke groups, while the cerebellar fastigial nucleus stimulation (FNS) increased the expression of miR-29c. (**B**) The relative expression of microRNA-676 (miR-676) showed no significant difference among different model groups.

nucleus stimulation. However, the relative expression of miR-676 (Figure 2B) showed no significant change between the different rat groups. Therefore, cerebellar fastigial nucleus stimulation upregulated the expression of miR-29c.

Cerebellar fastigial nucleus stimulation reduced the expression of inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β

As shown in Figure 3A, the relative level of TNFRSF1A mRNA was significantly increased in rats with post-stroke depression compared with the sham-operated rats and rats with stroke but without post-stroke depression. Treatment with cerebellar fastigial nucleus stimulation treatment resulted in reduced expression of TNFRSF1A mRNA when compared with the non-treated sham and stroke groups. Also, the levels of inflammatory cytokines, including TNF-α (Figure 3B), IL-6 (Figure 3C) and IL-1β (Figure 3D) were all increased in the rat model of the post-stroke depression and were reduced following treatment with fastigial nucleus stimulation.

Cerebellar fastigial nucleus stimulation inhibited the progression of neuronal apoptosis

The expression of pro-apoptosis markers, including total caspase-3 and active caspase-3, were measured in the four rat groups. As shown in Figure 4A, Western blot analysis showed that the expression of TNFRSF1A protein and active caspase-3 were significantly increased in the untreated post-stroke depression group compared with the sham and stroke groups. The relative density of TNFRSF1A protein was slightly decreased in the fastigial nucleus stimulation-treated group compared with that in the untreated post-stroke depression group; the relative density of TNFRSF1A protein in the sham group and the stroke group were the lowest. Treatment with fastigial nucleus stimulation reduced the expression of TNFRSF1A and active caspase-3. However, the level of total caspase-3 showed no significant difference between the different groups. These results were presented as the relative density index of TNFRSF1A (Figure 4B), total caspase-3 (Figure 4C) and active caspase-3 (Figure 4D). The results showed that the cerebellar fastigial nucleus stimulation could downregulate the expression of both active caspase-3 and TNFRSF1A. As shown in Figure 5, the TUNEL assay of neuron apoptosis showed that the apoptosis index of sham-operated rats and untreated rats with stroke were both lower when compared with the rat model of post-stroke depression, while the fastigial nucleus stimulation treatment reduced the apoptosis index in the rat model of post-stroke depression. These results support that cerebellar fastigial nucleus stimulation could partly inhibit neuron cell apoptosis.

miR-29c targeted the 3'UTR of TNFRSF1A

The above results indicated a potential role for miR-29c in fastigial nucleus stimulation treatment of post-stroke depression by targeting the expression of TNFRSF1A. Therefore, the regulatory relationship between miR-29c and TNFRSF1A were investigated further. As shown in Figure 6A, the results of an online bioinformatics analysis located a putative binding site of miR-29c in the 3'UTR of TNFRSF1A. The luciferase activity of SH-SY5Y human neuroblastoma cells (Figure 6B) and U251 human glioblastoma cells (Figure 6.C) transfected by wild-type 3'UTR of TNFRSF1A was significantly decreased in the presence of miR-29c mimics, suggesting that TNFRSF1A acted as a molecular target for miR-29c.

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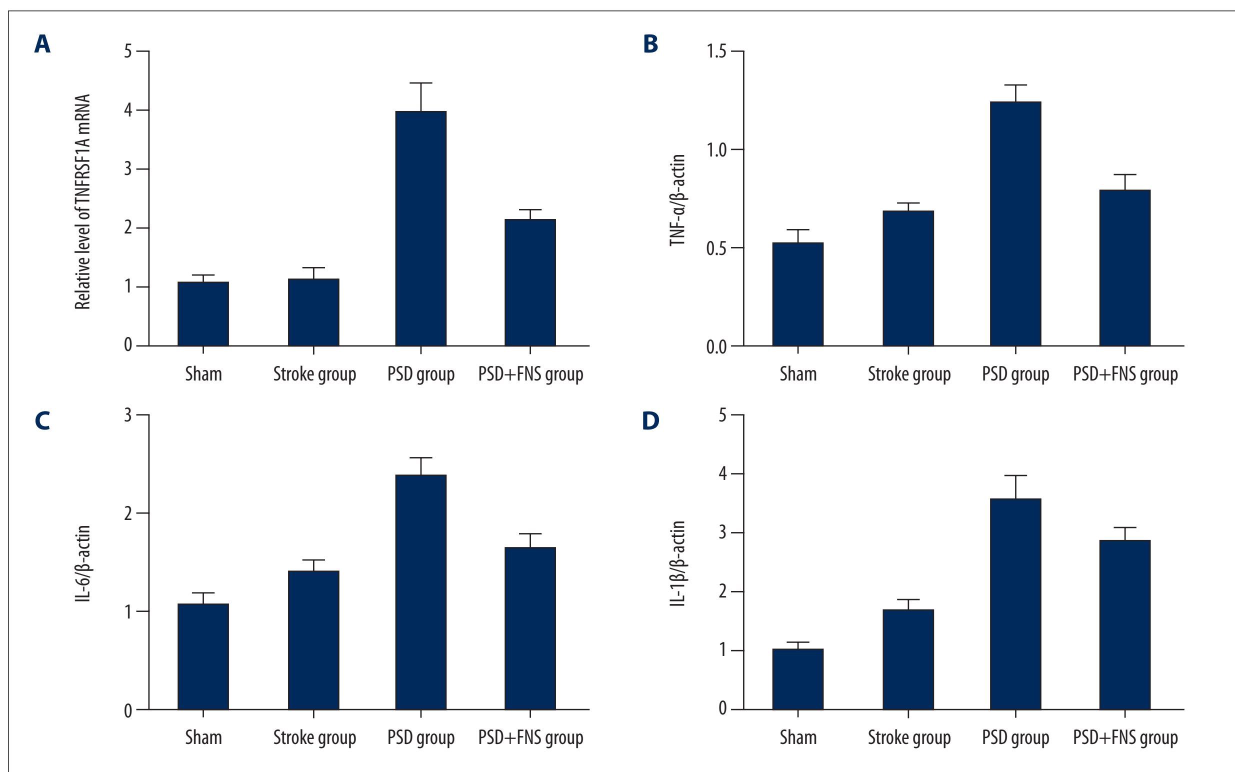

Figure 3. The relative level of TNFRSF1A mRNA and the inflammatory cytokines, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β, in the sham group, the stroke group, the post-stroke depression (PSD) group, and the fastigial nucleus stimulation (FNS) group showed that cerebellar fastigial nucleus stimulation reduced the expression of TNFRSF1A mRNA and inflammatory cytokines. (**A**) The relative expression of TNFRSF1A mRNA was increased in the rat model of post-stroke depression (PSD) compared with that in the sham-operated rats and stroke rats, whereas the fastigial nucleus stimulation treatment reduced the level of TNFRSF1A mRNA. (**B**) Compared with the sham group and the stroke group, the level of tumor necrosis factor-α (TNF-α) was upregulated in the PSD group, and the fastigial nucleus stimulation treatment reduced the level of TNF-α. (**C**) Compared with that in the sham group and stroke group, the level of IL-6 was evidently upregulated in the PSD group, and the fastigial nucleus stimulation treatment reduced the level of IL-6. (**D**) Compared with that in the sham group and stroke group, the level of IL-1β was upregulated in the PSD group, and the fastigial nucleus stimulation treatment reduced the level of IL-1β.

miR-29c negatively regulated the expression of TNFRSF1A

To further study the regulatory relationship between miR-29c and TNFRSF1A, the relative expression of miR-29c and TNFRSF1A mRNA was compared in SH-SY5Y human neuroblastoma cells and U251 human glioblastoma cells treated with a miR-29c mimic, or TNFRSF1A short interfering RNA (siRNA), and a negative control. As shown in Figure 7A, the relative expression of miR-29c was increased in the SH-SY5Y human neuroblastoma cells treated with the miR-29c mimic. Also, treatment with TNFRSF1A siRNA or the miR-29c mimic significantly reduced the relative expression of TNFRSF1A mRNA (Figure 7B) and protein (Figure 7C and 7D) in SH-SY5Y cells. The experiments were also repeated in U251 human glioblastoma cells (Figure 8) and similar results were obtained, indicating a negative regulatory relationship between miR-29c and TNFRSF1A. Therefore, treatment with cerebellar fastigial nucleus stimulation may aid the recovery from post-stroke depression by increasing the expression of miR-29c, which reduces the expression of TNFRSF1A and inflammatory cytokines, including TNF-α, IL-6, and IL-1β, so that the overall level of inflammation in post-stroke depression is reduced.

Discussion

Depression can present in a variety of ways and with different degrees of severity in patients following stroke, due to the presence of psychological and physiological stress. The symptoms of post-stroke depression include feelings of inadequacy, hopelessness, anxiety, loss of concentration, loss of appetite with weight loss, somnolence, sleepiness, social and behavioral inhibition, and reduced motor activity [15]. These behavioral disorders associated with depression have been shown to be regulated by pro-inflammatory cytokines, including interferon-α (IFN-α), tumor necrosis factor-α (TNF-α), interleukin

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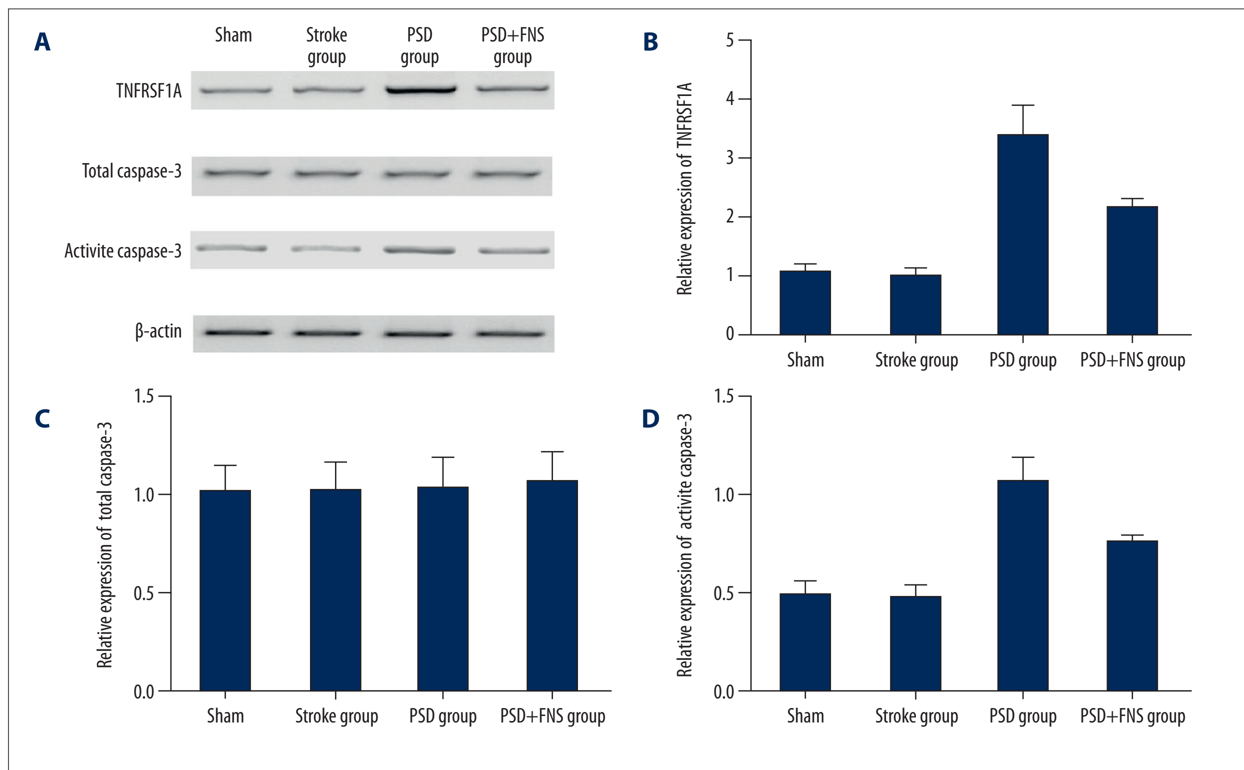

Figure 4. Expression of the TNFRSF1A protein, total caspase-3, and active caspase-3 in the sham group, the stroke group, the post-stroke depression (PSD) group, and the fastigial nucleus stimulation (FNS) group showed that the cerebellar fastigial nucleus stimulation reduced the protein expression of TNFRSF1A and caspase-3. (**A**) Western blot analysis showed that the expression of TNFRSF1A protein, total caspase-3, and active caspase-3 in the post-stroke depression (PSD) group were significantly increased compared with the sham group and stroke group, and fastigial nucleus stimulation treatment reduced the expression of these proteins. (**B**) The relative density of TNFRSF1A protein was slightly decreased in the fastigial nucleus stimulation (FNS) treated group compared with that in the untreated PSD group; the relative density of TNFRSF1A protein in the sham group and the stroke group were the lowest. (**C**) The relative density of total caspase-3 was slightly decreased in the FNS group compared with that in the PSD group; the relative density of total caspase-3 in the sham group and the stroke group were the lowest. (**D**) The relative density of active caspase-3 was slightly decreased in the FNS group compared with that in the PSD group; the relative density of active caspase-3 in the sham group and stroke group were the lowest.

(IL)-18, IL-8, IL-6, IL-1β, and IFN-γ, and stroke may increase inflammation, particularly in the limbic regions [16].

Previously published studies have produced results that support the hypothesis that symptoms of post-stroke depression might be alleviated by cerebellar stimulation. Sui et al. found that the emotional disorders in patients with post-stroke depression could be reduced by cerebellar fastigial nucleus stimulation [17]. Su et al. also demonstrated that the electrical stimulation of the cerebellar fastigial nucleus could significantly improve the symptoms of post-stroke depression [18]. Electrical stimulation of the fastigial nucleus could excite the nerve fibers passing through this region, resulting in increased regional cerebral blood flow, reflexive vasodilatation, and increased blood pressure [19]. Also, fastigial nucleus stimulation could significantly improve the tolerance of the brain for cerebral ischemia, and several published studies have now shown that electrical stimulation of the fastigial nucleus in the cerebellum could provide protection from ischemic brain damage [4,5,7].

In the present study, a rat model of post-stroke depression was established with the combination of middle cerebral artery occlusion (MCAO) and the use of chronic unpredictable mild stress treatment, verified using an open field test, with and without treatment with cerebellar fastigial nucleus stimulation. The results showed that the relative expression of miR-29c was reduced in the rat group with post-stroke depression compared with that in the sham and stroke groups, whereas treatment with fastigial nucleus stimulation increased the expression of miR-29c, and the relative expression of miR-676 was similar among the different groups.

In a previous study, the relationship between miR-29c expression and the neuroprotective role of fastigial nucleus stimulation was confirmed in a rat model of post-stroke depression [12].

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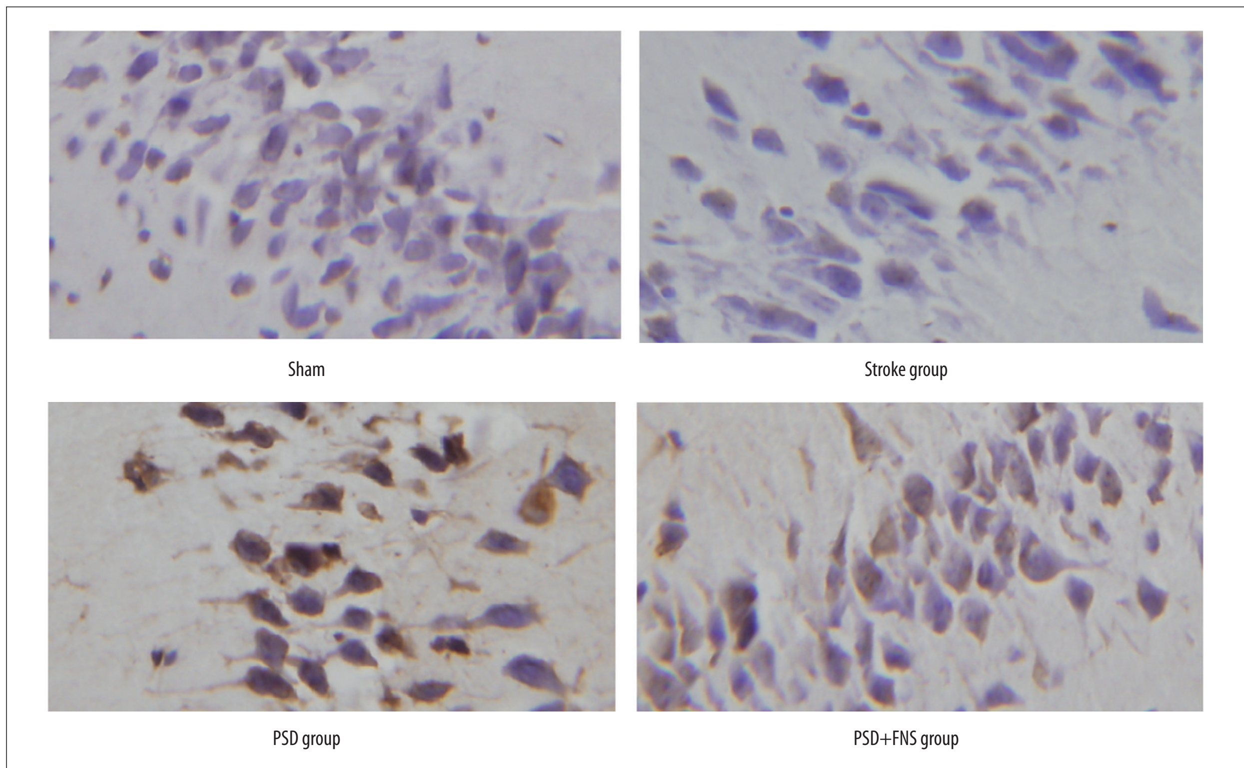

Figure 5. The TUNEL assay was used to measure neuron apoptosis in the sham group, the stroke group, the untreated post-stroke depression (PSD) group, and the fastigial nucleus stimulation (FNS) group. The post-stroke depression (PSD) group was associated with the highest apoptosis index. The sham group and the stroke group were associated with the lowest apoptosis index. The apoptosis index in the fastigial nucleus stimulation (FNS) group was slightly decreased, indicating that cerebellar fastigial nucleus stimulation had a mild inhibitory effect on neuronal apoptosis.

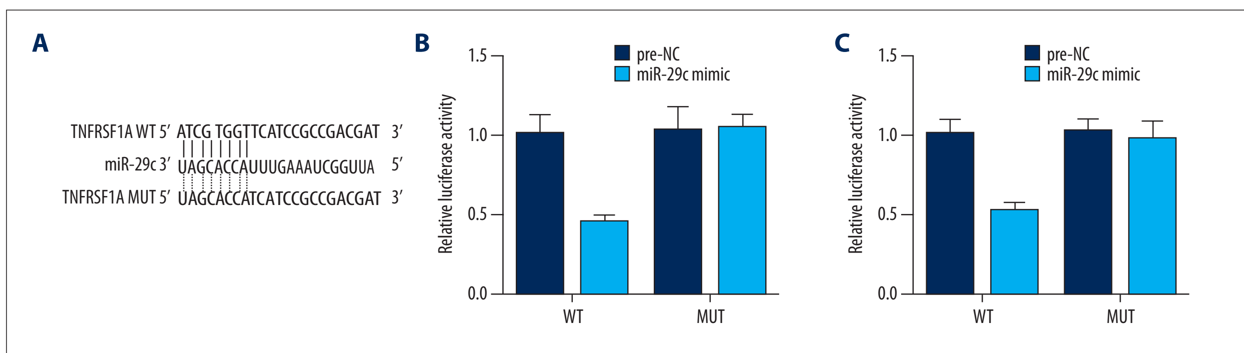

Figure 6. Computational analysis identified the TNFRSF1A gene as a target of miR-29c, with a binding site of miR-29c located in the 3'UTR of TNFRSF1A and the regulatory relationship between TNFRSF1A and miR-29c was confirmed using luciferase assays and cells co-transfected with miR-29c mimics and plasmids carrying wild-type or mutant 3'UTR of TNFRSF1A. (**A**) Online bioinformatics analysis tools were used to detect a putative binding site of miR-29c in the 3'UTR of TNFRSF1A. (**B**) Luciferase activity was decreased when SH-SY5Y human neuroblastoma cells, which were co-transfected with miR-29c mimics and plasmids carrying wild-type 3'UTR of TNFRF1A. (**C**) Luciferase activity was decreased when the U251 human glioblastoma cells, which were co-transfected with miR-29c mimics and plasmids carrying wild-type 3'UTR of TNFRF1A.

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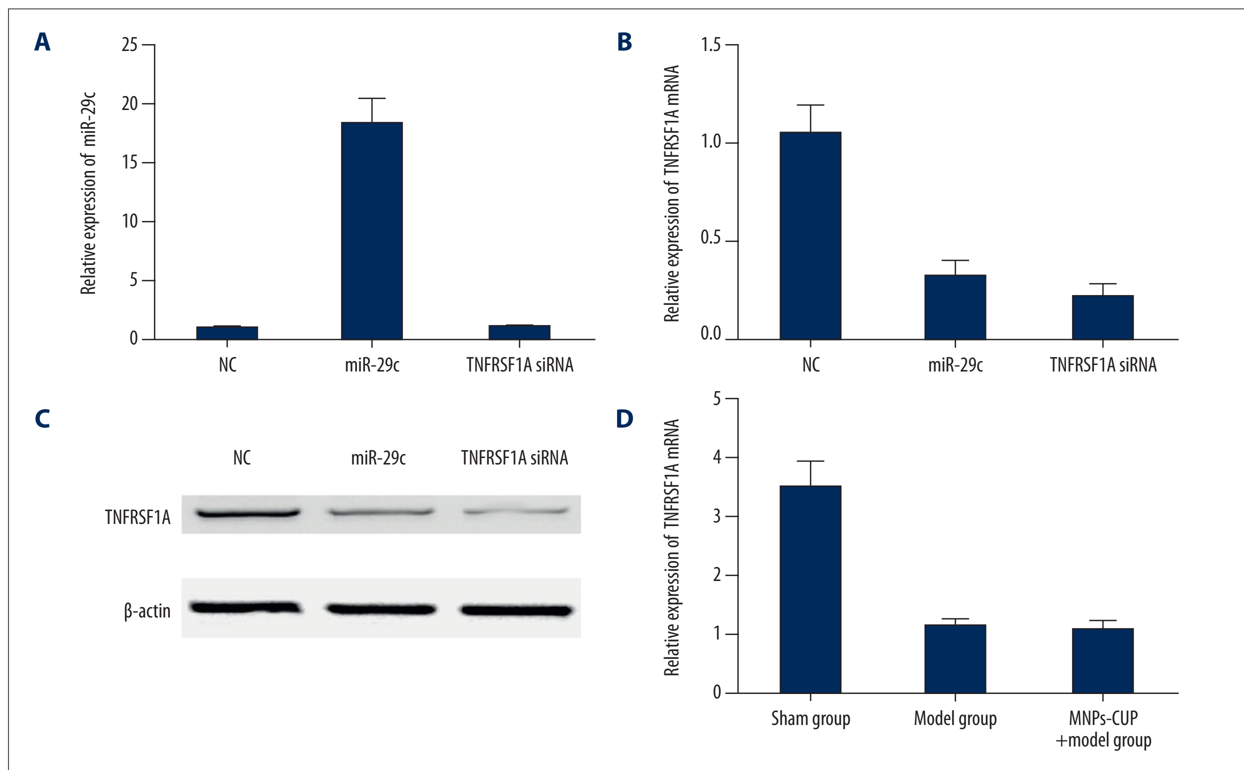

Figure 7. SH-SY5Y human neuroblastoma cells treated with miR-29c mimic, TNFRSF1A short interfering RNA (siRNA), or a negative control resulted in a negative regulatory relationship between the expression of TNFRSF1A and miR-29c. (**A**) The relative expression of miR-29c was increased in SH-SY5Y human neuroblastoma cells treated with miR-29c mimic compared with cells treated with TNFRSF1A short interfering RNA (siRNA) or a negative control. (**B**) The relative expression of TNFRSF1A mRNA was reduced in SH-SY5Y human neuroblastoma cells treated with miR-29c mimic or TNFRSF1A siRNA. (**C**) Western blot showed that the relative expression of TNFRSF1A protein was markedly reduced in SH-SY5Y human neuroblastoma cells treated with miR-29c mimic or TNFRSF1A siRNA. (**D**) Relative levels of TNFRSF1A in SH-SY5Y human neuroblastoma cells treated with miR-29c mimic or TNFRSF1A siRNA were reduced.

Also, miR-29c has been shown to reduce ischemia-induced neuronal cell apoptosis by inhibiting the activation of the pro-apoptotic genes BAK1 and BIRC2, and the effects of miR-29c on apoptosis be part of its neuroprotective role during the treatment of post-stroke depression by fastigial nucleus stimulation, as miR-29c has also been shown to affect the activation of multiple apoptotic pathways, including the PI3K-Akt pathway [12].

In this study, a binding site for miR-29c was located in the 3'UTR of TNFRSF1A mRNA using computational analysis, and the regulatory relationship between miR-29c and TNFRSF1A was further verified using the luciferase assay. The results showed that the luciferase activity in the U251 human glioblastoma cells and SH-SY5Y human neuroblastoma cells co-transfected with miR-29c mimics and the plasmids carrying the wild-type 3'UTR of TNFRSF1A was significantly reduced when compared with the luciferase activity in the cells co-transfected with miR-29c mimics and the plasmids carrying the mutant 3'UTR of TNFRSF1A. Furthermore, the relative expression of TNFRSF1A mRNA was reduced by treating the cells with either miR-29c mimic or TNFRSF1A short interfering RNA (siRNA). These results support that a negative regulatory relationship exists between miR-29c and TNFRSF1A.

Tumor necrosis factor alpha-induced protein 3 (TNFAIP3) is an essential regulator of inflammation, and its expression has been shown to be negatively correlated with the level of miR-29c both *in vivo* and *in vitro* [20,21]. Also, TNFAIP3 has been confirmed to be a target for miR-29c, and the upregulation of miR-29c expression in HepG2.2.15 cells has been shown to significantly suppress the expression of TNFAIP3, inhibiting cell proliferation and enhancing apoptosis of HepG2.2.15 cells [20,21]. The authors of these studies concluded that miR-29c was an important tumor suppressor during the development and progression of hepatitis B virus (HBV)-induced hepatocellular carcinoma (HCC) by targeting TNFAIP3 [20,21]. Therefore, TNFAIP3 and miR-29c might have potential as diagnostic biomarkers and therapeutic targets in the diagnosis and treatment of HBV-induced HCC [20,21]. TNFRSF1A encodes

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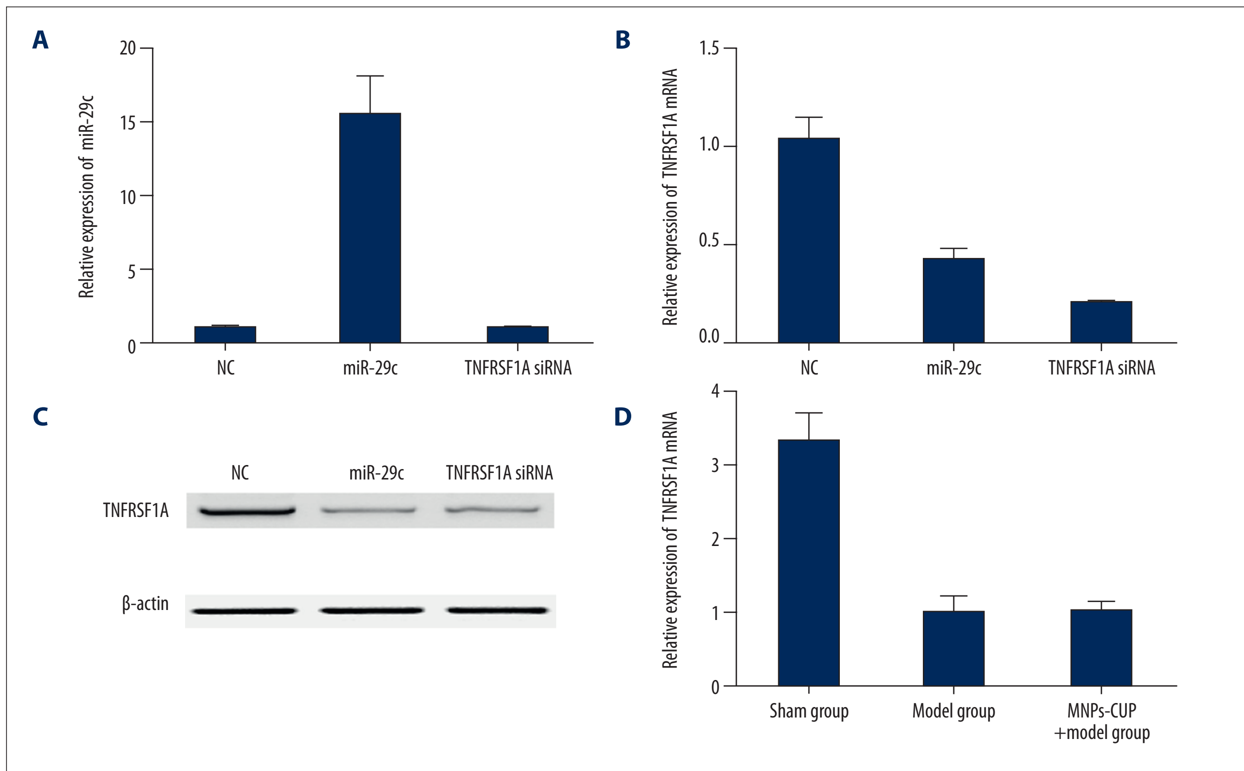

Figure 8. U251 human glioblastoma cells were treated with miR-29c mimic, TNFRSF1A short interfering RNA (siRNA), or a negative control resulted in a negative regulatory relationship between the expression of TNFRSF1A and miR-29c. (**A**) The relative expression of miR-29c was increased in U251 human glioblastoma cells treated with miR-29c mimic compared with that in the cells treated with TNFRSF1A short interfering RNA (siRNA) or negative control. (**B**) The relative expression of TNFRSF1A mRNA was markedly reduced in U251 cells treated with miR-29c mimic or TNFRSF1A siRNA. (**C**) Western blot showed that the relative expression of TNFRSF1A protein was reduced in U251 cells treated with miR-29c mimic or TNFRSF1A siRNA. (**D**) The relative density of TNFRSF1A in U251 cells treated with miR-29c mimic or TNFRSF1A siRNA was reduced.

a 55 kDa receptor for tumor necrosis factor (TNF), a pro-inflammatory cytokine implicated in a wide range of disorders [20,21]. A 75 kDa receptor for TNF is mainly expressed in endothelial cells and leukocytes [22]. Previous studies in TNF receptor knockout mice have shown that the pro-inflammatory role of TNF is primarily mediated via the 55 kDa TNF receptor [23–25].

In this study, the results showed that, when compared with the sham-operated rats and stroke rat model, the relative level of TNFRSF1A mRNA was significantly increased in the rat model of post-stroke depression, and fastigial nucleus stimulation treatment could reduce the expression of TNFRSF1A mRNA to a certain degree, with the level of TNFRSF1A mRNA in the fastigial nucleus stimulation group being greater than that in the sham and stroke groups. Also, the levels of inflammatory cytokines, including TNF-α, IL-6, and IL-1β, were all increased in the rat model of post-stroke depression and were reduced by treatment with fastigial nucleus stimulation. The findings of this study showed that fastigial nucleus stimulation could suppress the degree of inflammatory response, reflected by cytokine levels, in the rat model of post-stroke depression. Western blot analysis was conducted to measure the expression of TNFRSF1A and total and active caspase-3 protein in the four rat study groups. The results showed that the expression of TNFRSF1A and active caspase-3 protein was increased in the rat model of post-stroke depression compared with that in the sham and stroke groups, while treatment with fastigial nucleus stimulation reduced the expression of TNFRSF1A and active caspase-3 protein. However, the level of total caspase-3 showed no significant difference between the study groups. These results were also consistent with those obtained from the TUNEL assay of neuron apoptosis. Previously published studies have shown that different levels of TNF, TNFRSF1A, and TNFRSF1B gene expression were found between normal subjects and patients with drug treatment-resistant depression and that people suffering from recurrent depressive disorders have increased expression of the TNF, TNFRSF1A, and TNFRSF1B genes on both the protein and the mRNA levels [26–33].

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Conclusions

The findings of this present study showed that cerebellar fastigial nucleus stimulation reduced the expression of TNFRSF1A, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in cerebellar tissue by upregulating microRNA-29c (miR-29c) expression, which reduced the severity of post-stroke depression in a rat model. The use of cerebellar fastigial nucleus stimulation has the potential to reduce levels of inflammation to improve recovery from post-stroke depression.

Conflict of interest

None.

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