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MiR-7 alleviates secondary inflammatory response of microglia caused by cerebral hemorrhage through inhibiting TLR4 expression

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Abstract. – OBJECTIVE: This study was conducted to analyze the effect of miR-7 on the inflammatory response of microglia *in vitro* and *in vivo* by constructing an intracerebral hemorrhage model.

PATIENTS AND METHODS: In this study, we first established a model of cerebral hemorrhage in rat for *in vivo* experiments, and used lipoprotein (LPS) to induce an inflammatory response development in microglial cells, and constructed microglial inflammation models for *in vitro* experiments. Quantitative Real-time-polymerase chain reaction (qRT-PCR) was used to detect the expression of miR-7 in the rat model of cerebral hemorrhage and microglia with inflammation. The effect of miR-7 on the inflammation caused by intracerebral hemorrhage was evaluated through measuring the expression of IL-1β, IL-8 and TNF-α by enzyme-linked immunosorbent assay (ELISA). Dual luciferase reporter assay was used to detect the binding site of miR-7 to TLR4. Western blot was used to evaluate the level of TLR4 after overexpression and knockdown of miR-7 and to evaluate whether miR-7 alleviated the secondary inflammatory response of microglia after cerebral hemorrhage by inhibiting the expression of TLR4.

RESULTS: The expression of miR-7 in the rat cerebral hemorrhage model and microglial inflammation model tissue was significantly lower than that in the normal control group. Expression of inflammatory cytokines including IL-1β, IL-8 and TNF-α was significantly increased in rats with intracerebral hemorrhage and microglial inflammation in rats, and the expression of these inflammatory cytokines was partially reversed after overexpression of miR-7. Double luciferase reporter gene and ELISA results showed that miR-7 could inhibit the expression of TLR4 and relieve the secondary inflammatory response of microglia after cerebral hemorrhage.

CONCLUSIONS: We demonstrated that, in in vivo and *in vitro* experiments, miR-7 could reduce the LPS-induced inflammatory response produced by microglial cells, and alleviate the inflammation in the brain of rats with cerebral hemorrhage.

Key Words
Cerebral hemorrhage, Inflammation, Microglia, MiR-7.

Introduction

Intracerebral hemorrhage (ICH) refers to primary non-traumatic intracerebral hemorrhage, which is caused by a ruptured blood vessel in the brain. It is a subtype of stroke and accounts for approximately 20-30% of all strokes, whose 6-month mortality rate exceeds 50%, making ICH a catastrophic disease for humans[1]. Researches have demonstrated that the inflammation induced by cerebral hemorrhage plays a crucial role in the secondary injury of cerebral hemorrhage. The microglia is the most important inflammatory effector cell in the central nervous system and accounts for 10-15% of the total glial cells in the brain. It can be activated several minutes after cerebral hemorrhage[2-4]. Activated microglial cells release a large number of pro-inflammatory cytokines and chemokines, and promote the accumulation of inflammatory cells at the site of cerebral hemorrhage. However, inhibiting the activation of microglia after cerebral hemorrhage can greatly reduce the inflammatory response and the secondary damage. There is a lack of an effective way to inhibit its activation, since the mechanism of sustained activation of microglia is not yet clear.

MicroRNAs are a type of endogenous non-encoded single-stranded RNA molecules discovered in recent years that are highly conserved in evo-

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lution and are approximately 17-25 nucleotides in length. MicroRNAs are widely present in animal and plants and can bind to mRNA of their target gene in a complementary base pairing manner, thereby degrading the target gene or inhibiting the target gene translation process, and then playing a role in post-transcriptional regulation. Some studies[5-7] have showed that certain abnormal miRNAs are closely related to the occurrence and development of cerebral hemorrhage and secondary inflammation after cerebral hemorrhage. However, the research is rarely on whether the miRNAs that are abnormally expressed after cerebral hemorrhage directly act on inflammatory effector cells, microglia, to induce secondary inflammation.

In recent years, many studies[8-12] have suggested that miR-7 is a tumor suppressor that can inhibit the proliferation and promote the apoptosis of tumor cells in various tumors such as malignant neuroblastoma, lung cancer, and tongue squamous cell carcinoma. However, its role in the inflammatory response after cerebral hemorrhage and its mechanism remains to be studied.

Patients and Methods

Research Object and Sample Collection

We selected 53 patients with cerebral hemorrhage who were hospitalized in The First People's Hospital of Jiande from July 2012 to July 2017, among which 33 were male and 20 were female. All patients with mean age of 48.8 were diagnosed with intracranial hemorrhage when the bleeding volume was over 35 mL, and hematoma removal surgery was performed. All patients voluntarily participated in the study and signed written informed consent. This study has been approved by the Ethics Committee of The First People's Hospital of Jiande. The obtained specimens were pieces of tissue around the hematoma. The brain tissue around the hematoma was taken as the experimental group, and that away from the hematoma was used as a control. There was no statistical difference in age, gender, and weight between the two groups. Immediately after collection, all samples were placed at -80°C refrigerator and stored for use.

Establishment of Rat Cerebral Hemorrhage Model

In this experiment, 50 μL of fresh autogenous arterial blood were collected and injected into the right basal ganglia to establish a rat model with intracerebral hemorrhage. After weighing, the rats were anesthetized with 10% chloral hydrate (0.03 mL/kg). After deep anesthesia, the rats were routinely sterilized, and the right femoral artery was separated. 60 μL of blood were drawn using a microsyringe, then the blood vessels were ligated and the wound was sutured. The rats were fixed in a prone position on a stereotaxic apparatus. The skin was incised and the periosteum was peeled off to expose the anterior ridge. A cranial drill was used to drill a hole that was 0.2 mm in front of the forehead and 3.5 mm on the right side of the midline, then a microsyringe was used to inject fresh femoral artery blood from the borehole through a stereotaxic instrument, with a speed of 7-10 μL/min. The depth was 6 mm, making the microsyringe in the brain basal ganglia area. After the injection, the needle was kept for 15 min. The hole was closed by the sterile bone wax and the skin was sutured.

Preparation of LPS-Induced Microglial Inflammation Model

Rat microglia BV2 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell suspension (about 104 cells) was seeded in a 24-well plate and incubated in a 37°C, 5% CO_2 incubator. Phosphate-buffered saline (PBS) solution was used to dissolve LPS powder into mother liquor with concentration of 1 mg/mL. Next, LPS solution was added to the cell culture plate. After 12 hours and 24 hours of action, the cells were collected for subsequent experiments.

RNA extraction

The cells and tissues required for the experiment were collected and lysed by adding 1 mL TRIzol (Invitrogen, Carlsbad, CA, USA). Then, 250 μL of chloroform were added; after shaking for 30 s, the mixture was centrifuged at 4°C. The supernatant was aspirated and an equal volume of pre-chilled isopropanol was added. After centrifugation, the precipitate was gently washed with 75% ethanol and then dissolved in 20 μL of diethyl pyrocarbonate (DEPC) water. The RNA concentration was measured using a spectrophotometer and placed in a refrigerator at -80°C until use.

Quantitative Real-time-polymerase chain reaction (qRT-PCR)

A reverse transcription reaction system was prepared on ice using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan, Code No. RR037A), and cDNA was obtained after the re-

action. The miRNA quantitative PCR procedure was performed according to the miScript SYBR Green PCR Kit instructions. Total reaction system was 10 μL. PCR amplification conditions were: pre-denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and 30 s. The primer sequences were: miR-7 (F: 5'-TGGAAGACTAGTGATTTTGTTGT-3', R: 5'- TACTGGCACCACTGGAAACC-3'), TLR4 (F: 5'- GGACTCTGCCCTGCCACCATTTA-3', R: 5'-CTTGTGCCCTGTGAGGTCGTTGA-3'), GAPDH (F: 5'- AGCCACATCGCTCAGACAC-3', R: 5'- GCCCAATACGACCAAATCC-3'), U6 (F: 5'-CTCGCTTCGGCAGCAGCACATATA-3', R: 5'-AAATATGGAACGCTTCACGA-3').

Cell Transfection

The cells in log phase were transfected according to Lipofectamine 2000 instructions with miR-7 mimic, inhibitor, and pcDNA-TLR4, which were all from GenePharma (Shanghai, China). After 48 hours of transfection, cells were collected for other experiments.

The cells were then seeded in a 12-well plate (20*10^4 cells/well) and incubated in a 37°C incubator with 5% CO_2. An appropriate amount of the virus of interest and the negative control virus (MOI=50) were added to the cells according to the virus titer, respectively. After 24 hours of infection, the expression of the reporter gene GFP on the adenovirus was observed, and a photograph was taken to record the result.

Transfection of Viral Vector in Rats

At 12 h after intracerebral hemorrhage, the rats were anesthetized with 3.6% chloral hydrate, and 50 ul mixture of the rAAV9-ZsGreen-miR-7 virus transfection agent were injected into the hematoma region of the rat with a microsyringe under a stereotaxic instrument for 10 minutes. After the injection, the needle was retained for 10 min and then slowly withdrawn. The rats in control group were injected with 50 _l blank rAAV9-ZsGreen-control virus in the right hematoma area in the same way. The bone window was closed with sterile bone wax, the scalp was sutured and 20*10^4U of penicillin was injected subcutaneously. After the rats were resuscitated, they were isolated and fed into a feeding cage with food and water.

Elisa Detection

The antibody was diluted with coating buffer to 1 to 10 μg/mL and reacted in the wells overnight at 4°C. On the next day, the wells were washed three times with wash buffer for 3 minutes each. Subsequently, 0.1 mL of a certain dilution of the sample was added to the above-mentioned reaction wells and incubated at 37°C for 1 hour. After wash, 0.1 mL of freshly diluted enzyme-labeled antibody (titration after titration) was added in each reaction well, which was then incubated at 37°C for 0.5 to 1 hour. After wash, 0.1 mL of a tentatively prepared TMB substrate solution was added to each well and incubated at 37°C for 10-30 min. The reaction was terminated using 0.05 mL of 2 M sulfuric acid. The OD value of each well was measured on ELISA tester at a wavelength of 450 nm after zeroing with a blank control well.

Western Blot Assay

Cells were collected after centrifugation. They were disrupted by sonication and centrifuged, and the supernatant was removed. Bromophenol blue (9.5 mL to 0.5 mL) was added to the protein to boil for 10 min, and then stored at -20°C until use. After the samples run in electrophoresis, they were transferred to polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland), and corresponding size of the membrane was cut according to the molecular weight. The antigen was sealed in blocking solution of 5% skim milk powder, and the specific primary antibody was used to incubate overnight. After washing the membrane, the secondary antibody was used for incubation for 1 h on the next day. Finally, exposure was performed.

Dual Luciferase Reporter Assay

The 3' UTR sequence of TLR4 was downloaded from the NCBI website, which was used for constructing plasmid containing the TLR4 wild-type sequence (TLR4 WT 3' UTR) or the mutant sequence (TLR4 MUT 3' UTR). The cells were seeded in 96-well plates, and 50 pmol/L miR-7 mimics or negative controls were co-transfected with 80 ng TLR4 wild type or mutant plasmid. After 48 hours of transfection, a system of luciferase reporter gene assay was used to detect the fluorescence intensity.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) were used for analysis. x^2-test was used for analyzing classification data, and *t*-test was used for measurement data, which were expressed as mean±standard deviation. The difference was statistically significant when $p<0.05$.

Results

MiR-7 was Lowly Expressed in Patients and Rat Models with Cerebral Hemorrhage

We detected the levels of miR-7 in patients with intracerebral hemorrhage and controls by fluorescence Real-time quantitative PCR. The results showed that miR-7 was lowly expressed in patients with cerebral hemorrhage (Figure 1A). Subsequently, miR-7 was also found lowly expressed in the brain tissue of SD rats (Figure 1B). MiR-7-mimic and miR-7-inhibitor were transfected into rat microglial cells to detect the expression of miR-7. It was found that miR-7-mimic dramatically enhanced the miR-7 level, while miR-7-inhibitor conspicuously reduced that, confirming that both of them were effective (Figure 1C).

MiR-7 Alleviated the Inflammatory Response Induced by Cerebral Hemorrhage in Vitro

MiR-7 expression was detected in rat microglia after LPS treatment. The results showed that miR-7 level in microglia was significantly down-regulated after LPS treatment compared to normal control treatment (Figure 2A). After the microglial cells were treated with LPS, the expression of inflammatory cytokines including IL-1β, IL-8 and TNF-α, was detected. The results showed that after LPS treatment, the expression of IL-1, IL-6, IL-8 and TNF-α in cells was significantly up-regulated. After overexpression of miR-7, the expression of these inflammatory factors was significantly reduced (Figure 2B-2D). These results implied that miR-7 could suppress the expression of inflammatory factors in an *in vitro* model, thereby alleviating the inflammatory response induced by cerebral hemorrhage.

MiR-7 Relieved Inflammation Induced by Cerebral Hemorrhage in Rats

LPS was used to treat the Sprague Dawley (SD) rats with cerebral hemorrhage; next, the expression of IL-1β, IL-8 and TNF-α was detected. The results showed that after LPS treatment, the levels of IL-1, IL-6, IL-8 and TNF-α in the tissue were significantly increased. At the same time, overexpression of miR-7 significantly decreased the expression of above inflammatory factors (Figure 3A-3C). The above results demonstrated that miR-7 could inhibit the expression of inflammatory cytokines.

MiR-7 Could Target and Degrade TLR4

We predicted target genes of miR-7 through TargetScan and performed functional analysis; after that, TLR4 was selected (Figure 4A). Results of reporter gene assay showed that after transfecting miR-7 in cells, the luciferase activity in the TLR4-WT 3'UTR group was decreased, while no significant difference of luciferase activity was found in the TLR4-MUT 3'UTR group (Figure 4B), suggesting that TLR4 could interact with miR-7. The mRNA expression of TLR4 was detected after miR-NC, miR-7 mimics, and miR-7 inhibitor were transfected into rat microglia. Results indicated that compared with miR-NC, TLR4 level was decreased in miR-7 mimics group while increased in miR-7 inhibitor group (Figure 4C). Western Blot results showed that

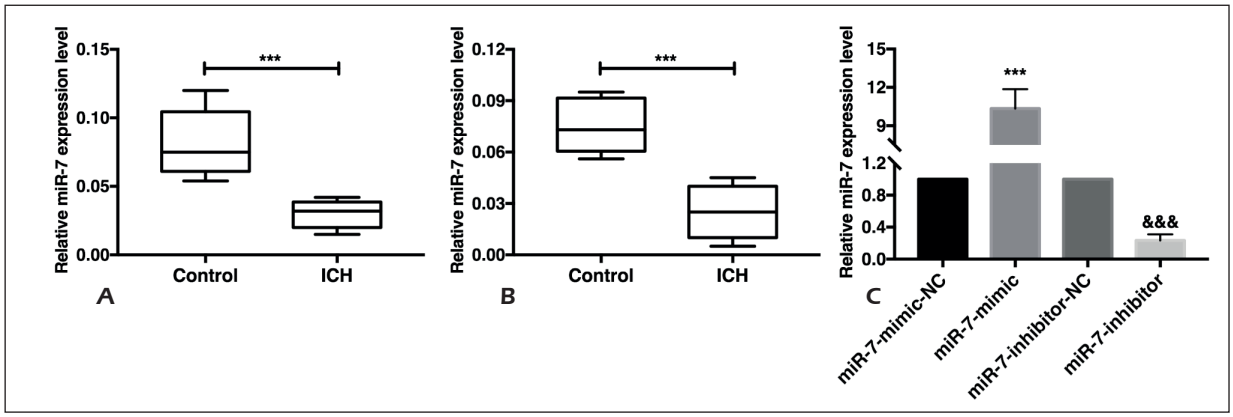

Figure 1. MiR-7 was lowly expressed in patients or rats model with intracerebral hemorrhage. **A**, MiR-7 expression was significantly reduced in patients with cerebral hemorrhage. **B**, MiR-7 expression was significantly reduced in Sprague Dawley (SD) rat cerebral hemorrhage model. **C**, After overexpression and interference of miR-7, miR-7 expression was significantly changed.

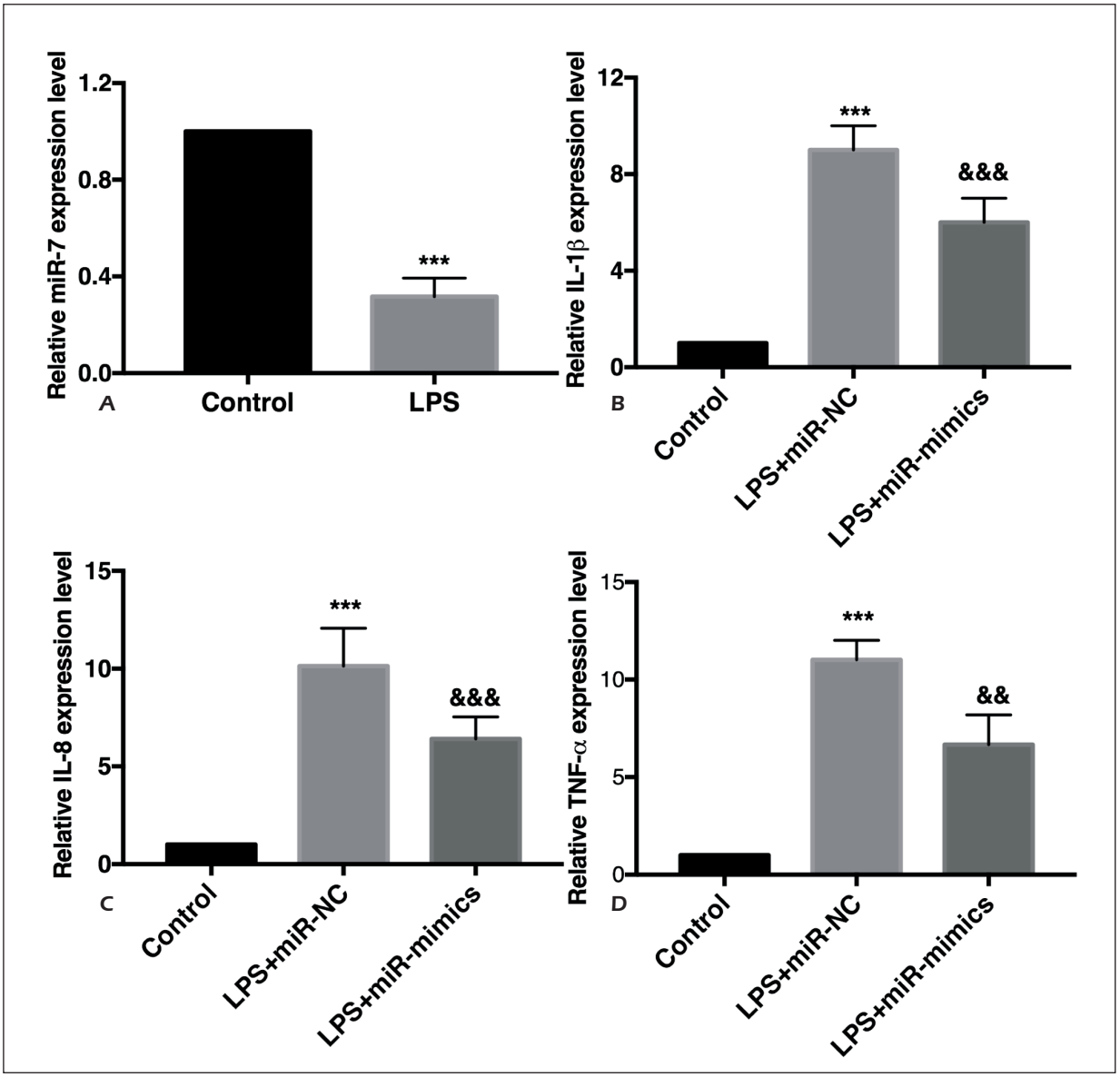

Figure 2. MiR-7 alleviated the inflammatory response induced by cerebral hemorrhage in an *in vitro* model. **A**, After LPS treatment in rat microglia, miR-7 expression was significantly decreased. **B**, **C**, and **D**, After treatment with LPS, the expression of IL-1β (**B**), IL-8 (**C**) and TNF-α (**D**) in rat glial cells was significantly up-regulated, but overexpression of miR-7 significantly reduced their expression. (***, $p<0.001$, compared with the control group; $^{\&\&\&}$, $p<0.001$, compared with the LPS group).

after overexpression of miR-7, the level of TLR4 protein was decreased, and after interference with miR-7, the level of TLR4 protein increased (Figure 4D). The above results indicated that miR-7 could target and bind to TLR4.

MiR-7 Reduced Inflammation By Degrading TLR4

TLR4 was over-expressed in the cells and the expression of IL-1β, IL-8 and TNF-α genes was detected. The results showed that after overexpression of TLR4, the expression levels of IL-1β, IL-8 and TNF-α in the cells were significantly increased. After overexpression of miR-7, the expression of these inflammatory factors was partially reversed but still higher than that in the control group (Figure 5A-5C). These results showed that miR-7 could inhibit the development of inflammatory response by inhibiting the expression of TLR4 in rat microglia.

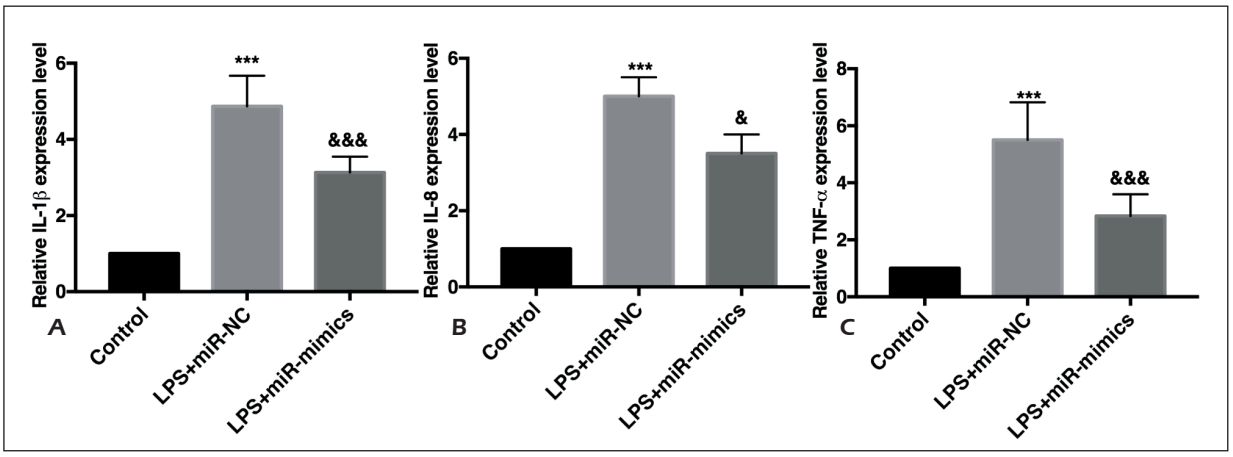

Figure 3. miR-7 was able to relieve inflammation in an intracerebral hemorrhage model. **A**, **B**, and **C**, MiR-7 significantly reduced the expression of IL-1β (**A**), IL-8 (**B**) and TNF-α (**C**) in rats after intracerebral hemorrhage.

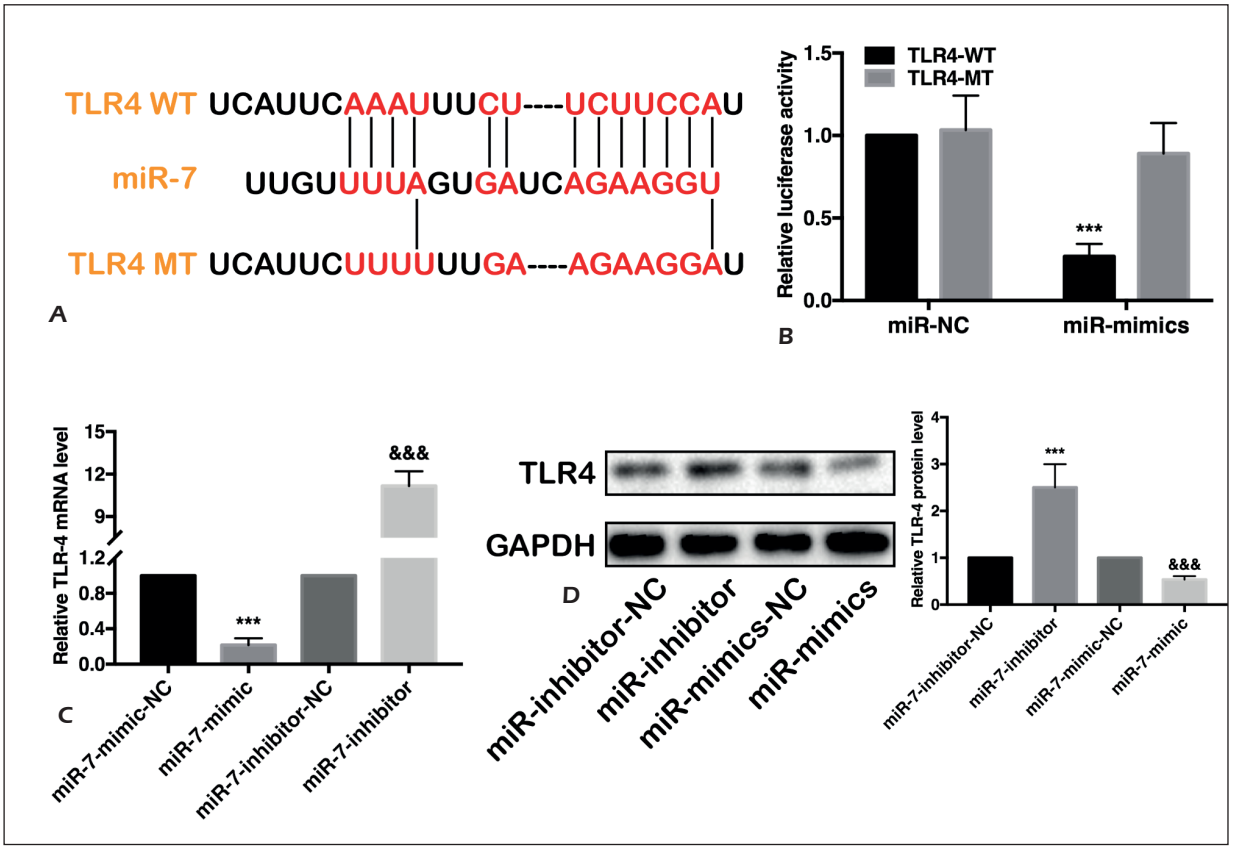

Figure 4. MiR-7 was able to target TLR4 to degrade it. **A**, TargetScan was used to predict that miR-7 could bind to TLR4 3'UTR. **B**, Dual luciferase reporter gene results showed that the group of miR-7 and TLR4 wild-type had the lowest luciferase activity. **C**, After overexpression of miR-7, the level of TLR4 mRNA decreased, and interference with miR-7 increased the level of TLR4 mRNA. **D**, After over-expression of miR-7, the level of TLR4 protein was decreased, and interference with miR-7 increased the level of TLR4 protein.

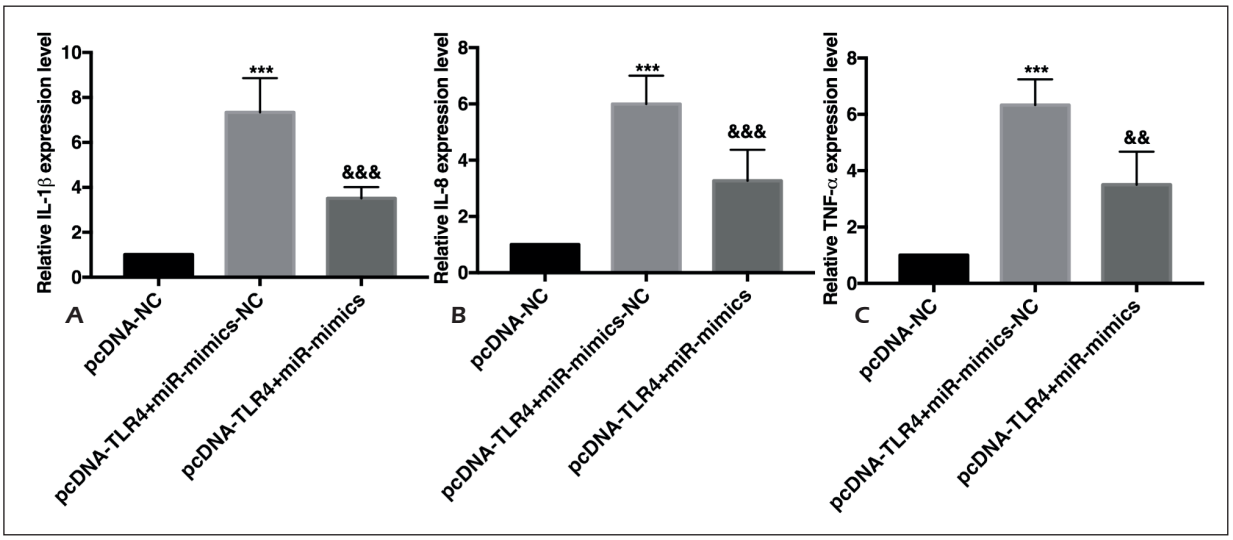

Figure 5. MiR-7 reduced inflammation by degrading TLR4. **A**, **B**, and **C**, After overexpression of TLR4, the expression levels of IL-1β (**A**), IL-8 (**B**), and TNF-α (**C**) were significantly up-regulated. After overexpression of miR-7, expression levels of IL-1β (**A**), IL-8 (**B**), and TNF-α (**C**) decreased significantly.

Discussion

In this study, we demonstrated that miR-7 has a role in alleviating the inflammatory response after cerebral hemorrhage both *in vitro* and *in vivo* experiments. Firstly, we established LPS-induced microglial inflammation models. Microglia are neuro-inflammatory effector cells that play an important role in the nervous system. When brain tissue is damaged, microglia are first activated to release a large number of pro-inflammatory cytokines and chemokines, driving blood-borne inflammatory cells to accumulate at the site of injury[13]. Therefore, it is of great significance to study the inflammatory signaling pathway of microglia in the treatment of secondary inflammatory injury after cerebral hemorrhage. LPS, as a strong pro-inflammatory substance, is widely studied in the treatment of inflammatory diseases. LPS can activate multiple signaling pathways. For example, LPS binds to CD14-like receptors on cell membranes and then binds to the extracellular ends of TLR4. After activation of TLR4, its intracellular ends are polymerized and then bind to MyD88 and interact with each other through a series of related factors. Subsequently, the signal transduces into the nucleus and eventually activates the NF-kB-mediated inflammatory signaling pathway[14].

We found that the expression levels of miR-7 in patients and the rats model with intracerebral hemorrhage were significantly reduced. *In vitro* experiments showed that miR-7 down-regulated the levels of IL-1β, IL-8, TNF-α, which confirmed its strong anti-inflammatory effect *in vitro*. Based on above results, we constructed a model of intracerebral hemorrhage in rats and injected adenovirus vector overexpressing miR-7 to regulate the expression of miR-7 in the brain tissue of rats. The changes in the expression of these inflammatory factors were observed. The results showed that the expression of IL-1β, IL-8 and TNF-α increased in the surrounding tissue of hematoma. Further, it was found that miR-7 can target and bind to TLR4 and promote its degradation, and overexpression of miR-7 can reverse the inflammatory response caused by TLR4.

Conclusions

We showed that miR-7 can inhibit LPS-induced inflammatory responses in microglia *in vitro* and inhibit secondary inflammation in the rats with intracerebral hemorrhage through inhibiting TLR4 expression and reducing the levels of inflammatory factors.

Conflict of Interests:

The authors declared no conflict of interest.

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