Journal of Musculoskeletal and Neuronal Interactions JMNI

Original Article

Study on the Mechanism of BMSCs in Regulating NF-κB Signal Pathway by Targeting miR-449a to Improve the Inflammatory Response to Peripheral Nerve Injury

Hongjiao Wang[1], Fangyuan Wang[2], Yuejing Wang[3], Xiaonan Li[1], Cihan Di[1], Chunming Liang[1], Yuyuan Mu[1], Jiexin Zhou[1]

[1]Department of Neurology, The Second Affiliated Hospital of Qiqihar Medical College, China;
[2]Department of General Surgery, Qiqihar First Hospital, China;
[3]Department of Histology and Embryology, Qiqihar Medical College, China

Abstract

Objective: To evaluate the mechanism of Bone Marrow Mesenchymal Stem Cells (BMSCs) in regulating NF-κB signal pathway by targeting miR-449a. **Methods**: Stem cells were transfected by over-expressing and inhibiting miR-449a to detect the levels and viability of miR-449a in stem cells after transfection. Stem cells and neurons were co-cultured *in vitro* to evaluate the *in vitro* mechanism of stem cells over-expressing miR-449a on neurons. **Results**: After the addition of neurons, the neuronal activity of miR-449a over-expression group increased significantly, the expression of NF-κB signal pathway proteins (IκBα, p50, and p65) decreased, and the inflammatory cytokines (TNF-α and IL-1β) decreased significantly (P<0.05). *In vivo* experiments in rats also showed that rats were unresponsive, did not chirp or elude after being stimulated. After stem cell therapy, the weight and response of rats gradually returned to normal levels. miR-449a expression significantly increased in the stem cell + miR-449a over-expression group, expression of NF-κB signal pathway proteins (IκBα, p50, and p65) decreased, inflammatory cytokines (TNF-α and IL-1β) significantly decreased, and cell activity significantly increased (P<0.05). **Conclusions**: BMSCs can modulate NF-κB signaling pathway by targeting miR-449a, so as to reduce the inflammatory response to peripheral nerve injury and repair nerve injury.

Keywords: Inflammatory Response, miR-449a, NF-κB Signal Pathway, Peripheral Nerve Injury

Introduction

Peripheral nerve injury is a common neurological complication, which can lead to regional motor, sensory and functional dysfunction of the nervous system. When an injury occurs, the injured nerve fails to function properly. Peripheral nerve injury can be divided into central injury and axonal injury. Central nerve injury is generally irreversible, and axonal injury can recover, but its independent recovery is generally long. Mild injury can be self-healing, but severe injury will cause patients to lose all functions, which can only be repaired through auxiliary means[1]. At present, better treatment measures include surgery, transplantation, and repair, but the surgery cannot be accurately positioned, which is easy to lead to dysfunction, difficult to recover, and cause secondary injury[2]. Neural transplantation can trigger immune rejection and increase the risk of tumor growth at the injured site. Artificial repair is relatively limited, which can only repair injuries less than 1 cm, with a long recovery and poor treatment effect[1]. From pathological studies, many factors were found involved in the repair of nerve injury, such as macrophages, Schwann cells, inflammatory responses, as well as vascular regenerative[3]. With the extensive study on stem cells, it has been found that stem cells play an important role in repairing nerve injury[4].

Bone marrow mesenchymal stem cells (BMSCs) can differentiate into a variety of cells, as well as release

The authors have no conflict of interest.

Corresponding author: Hongjiao Wang, Department of Neurology, The Second Affiliated Hospital of Qiqihar Medical College, No.37 Zhonghua West Road, Jianhua District, Qiqihar 161000, Heilongjiang Province, China
E-mail: whjjj012@163.com

Edited by: G. Lyritis
Accepted 24 August 2022

Table 1. The designed qPCR primers.

Primer	Sequence	
miR-449a	Upstream: 5′-TGGCGGTGGCAGTGTATTGTTA-3′	Downstream: 5′-GTGCAGGGTCCGAGGT-3′
Internal reference U6	Upstream: 5′-GCTTCGGCAGCACATATACTAAAAT-3′	Downstream: 5′-CGCTTCACGAATTTGCGTGTCAT-3′

regulators that promote neural axonal regeneration[5-9]. However, the molecular mechanism of action for the rapid repair of peripheral nerve injury by BMSCs has not been clearly defined. It has been shown that micro RNA (miRNA) is a conserved single-stranded RNA that causes its degradation by directly binding mRNA, thus regulating the expression of target genes[10]. And miR-449a can promote the differentiation of BMSCs into neurogenic cells and promote the repair of SCI. Liu et al. also showed that miR-449a can target protein deacetylase 1 to inhibit its expression, and thereby promote neuron-like differentiation, while peripheral nervous system injury can lead to a series of responses caused by NF-κB activation. NF-κB can inhibit further injury to the peripheral nerves and inhibit the death of neurons, with an important protective effect on the injured nervous system[11]. Therefore, the application of BMSCs treatment to the peripheral nervous system injury repair should first start from the source of the system injury, to inhibit this target, and thus repair nerve injury. Currently, this study targeted BMSCs and found that miR-449a can regulate activated NF-κB signal and promote the protein expression of p65 and P50. This study aimed to reveal the action mechanism of BMSCs to repair peripheral nervous system injury, and to provide a theoretical and experimental basis for the clinical treatment of BMSCs.

Experimental Methods

Reagent and materials

A total of 55 healthy Wistar female rats weighed from 200 to 250g and aged 7 to 8 weeks were selected. They were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd., the animal production license number is SYXK (Shandong) 2017-0001, and the certificate number is 370092000009757. BMSCs from Wistar rats were purchased from Cyagen Biosciences. Sodium pentobarbital, parenzyme, F12 complete media and chloroacetaldehyde hydrate were purchased from Aladdin. TRIzol reagent and reverse transcription kit were purchased from Solarbio. Primers for miR-449a were purchased from Applied Biosystems. BCA protein concentration detection kit and polyacrylamide gel were purchased from Applygen, Beijing. Polyclonal goat anti-rabbit antibodies, IκBα, p50 and p65 antibodies were purchased from Cell Signaling Technology. TNF-α and IL-1β antibodies were purchased from ThermoFisher, China.

Stem cell culture

The cell freeze-dried powder was added into a low-glucose incubator containing Dulbecco's modified eagle medium (DMEM) (20% FBS) with 5% CO_2 at 37°C for culture. The medium was changed in the incubator for continued culture after 5 days. After about 90% cell fusion was observed, adherent cells were washed with DMEM and digested with 2.5% parenzyme. Then, parenzyme was discarded and fetal bovine serum (FBS) was added to stop the digestion, DMEM was added to wash the cells, and then low-glucose DMEM (containing 20% FBS) was added to disperse the cells for subculture.

Cell transfection

miR-449a inhibitor, miR-449a mimic, and the negative control (NC) were synthesized by RiBoBio (Guangzhou, China). Stem cells were plated and cultured for 24h before adding miR-449a inhibitor, miR-449a mimic, and NC for transfection. After transfection for 48h, the cells were collected, relaid, and cultured, for *in vitro* experiments. Cell viability was first determined through lactate dehydrogenase (LDH) release and Thiazolyl Blue Tetrazolium Bromide (MTT) test.

RNA extraction and qPCR amplification

Total RNA was extracted from each group of cells with the TRIzol reagent. The RNA (1,000 ng) was reverse transcribed to cDNA with the High Capacity RNA Reverse Transcription Kit (Applied Biosystems) and was conducted for qPCR amplification with SYBR Green (Qiagen). The resulting values were normalized according to GAPDH of each parallel sample as the internal reference gene, and the designed qPCR primers are shown in Table 1. For cDNA synthesis, the mixture was reacted at 42°C for 15min and at 85°C for 5s, and then stored at 4°C. Amplification was performed with SYBR Green PCR Master Mix and stored at 95°C for 10min, for 40 cycles, at 94°C for 15s and at 60°C for 1 min, and then stored at 4°C. Primers for miR-449a were purchased from Applied Biosystems, and the relative expression levels of miR-449a were calculated by $2^{-\Delta\Delta CT}$. All experiments were performed in triplicate.

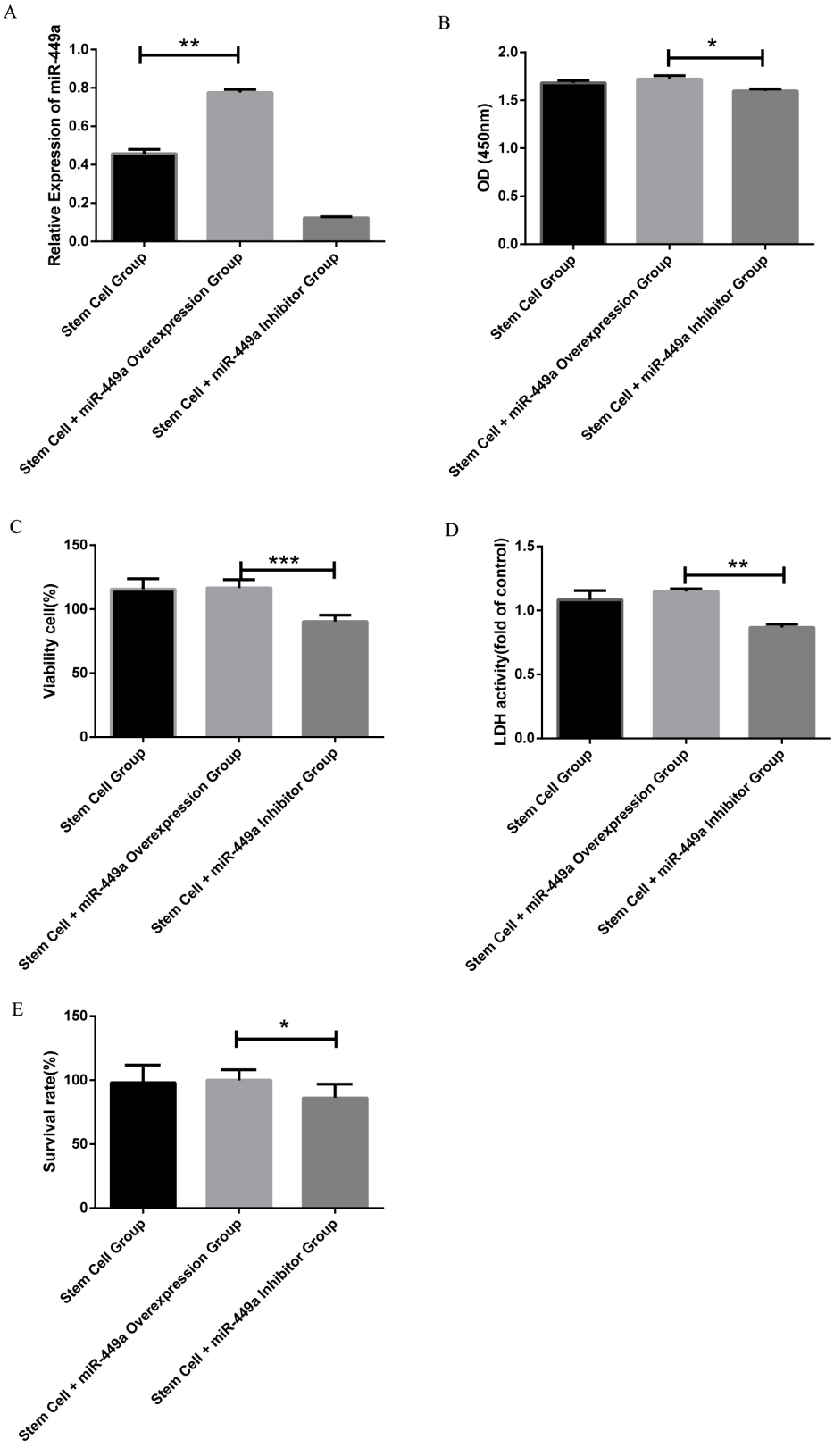

Figure 1. Validation of stem cell viability after transfection of miR-449a; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (BMSCs were transfected with miR-449a inhibitor, miR-449a mimic and NC; RT-qPCR analysis of mRNA expression in transfected stem cells; A: mRNA expression in transfected stem cells; B: Effect on stem cell proliferation after transfection; C: MTT of stem cell viability in each group; D: LDH of stem cell viability in each group; E: Live/dead cells counting).

Table 2. Limb stimulation responses.

Group	Chirping response	Eluding reaction	Number of animals n	Process mode
Blank group	100%	100%	3	1cm to outer ankle, with a depth of 2mm
Model group	0%	0%	3	
Stem cell group	30%	30%	3	
Stem cells + miR-449a over-expression group	100%	100%	3	
Stem cells + miR-449a inhibition group	0%	0%	3	

Detection of stem cell viability

miR-449a inhibitor, miR-449a mimic and negative control (NC) group stem cells were paved with 96-well plates as required and were diluted at 2×10^3 per well. Then, the cells were evenly spread into cell culture plates and labeled. A total of 5 plates were made. One of the plate was added with 10ul of CCK8 and another plate was added with 10ul of XTT, both of which were cultured at 37°C for 2h. The optical density (OD) value of each well was measured at 450nm with an ELISA reader, to calculate the survival rate. Another two plates were incubated with 10ul of MTT and 20ul of LDH at 37°C for 4h. The OD value of each well was measured at 490 nm with an ELISA reader, to calculate the survival rate. The last plate was added with placental blue staining, and dead cells were stained as blue, while living cells were colorless.

Co-culture of stem cells with neurons

The experiments were divided into four groups, including Group A with neurons, Group B with stem cells and neurons, Group C with stem cells and neurons over-expressing miR-449a, and Group D with stem cells + neurons inhibiting miR-449a expression. After neurons were cultured for 3 generations, wells with well-growing cells were selected and labeled by groups, 2 wells were included in each group, for a total of 8 wells. Different stem cells were inoculated in Transwell culture plates by groups, adding 1 ml of cell suspension per well and the cells of 1×10^5/well. The medium was replaced after 24h of inoculation, and the double plate was placed in an anaerobic incubator with 5% CO_2 at 37°C for 48h. neurons were collected from each group and neuronal cell activity was measured as per "1.5", and the expression levels of proteins (IκBα, p50 and p65) and inflammatory cytokines (TNF-α and IL-1β).

Establishment of peripheral nerve injury models of rats

Fifty healthy Wistar female rats were selected, 10 of which were grouped into the normal control group, and the remaining 40 were divided into four groups to establish peripheral nerve injury models, including the model group, stem cell group, stem cell + miR-449a over-expression group and stem cell + miR-449a inhibitor group. After rats were anesthetized with 2% pentobarbital at 40 mg/kg, they were fixed in the supine position. After disinfection, an incision of about 2 cm was made in the right buttock of rats, and the muscles were separated layer by layer to expose the sciatic nerve, and the sciatic trunk was completely cut off at a position of 0.5 cm below the rat sciatic nodule. The rats were randomly divided into four experimental groups, among which one group was given no treatment, and the remaining three groups were injected with 10 μl of DMEM containing BMSCs with different treatments, with about 1.0×10^6 cells, healthy and model groups were injected with only 10μl of DMEM. Then, non-invasive suture was used to anastomose the nerves at the two broken ends of all anatomical rats, and the wounds were sutured layer by layer[11,12]. The movement, sensation and reaction ability of rats in different groups were observed, and the body weight of rats was monitored. The animal experiments were strictly in accordance with relevant national laws, regulations and standards on laboratory animals, including but not limited to the Regulations on the Management of Laboratory Animals (revised version on March 1, 2017) and the Guidelines for Ethical Review of Laboratory Animal Welfare (GB/T 35892-2018).

Neuronal cell extraction and RT-qPCR detection

Neurons from normal control and 4 SCI rats were taken, and SCI neuron cells were transferred to 6-well plates, and cultured with 5% CO_2 at 37°C for 24h, 48h and 72h. 0.125% parenzyme was added for 15min of digestion, which was terminated after adding FBS, and then centrifuged at 2,000 r/min for 5 min. Neurons were collected and cultured with F12 complete medium. While healthy BMSCs were found in the incubator with 5% CO_2. The cells were grown with F12 complete medium to the third generation, digested with papain at 2 ug/u L for 15min and centrifuged to obtain the supernatant. miR-449a test was performed according to the method mentioned in "RNA extraction and qPCR amplification" section.

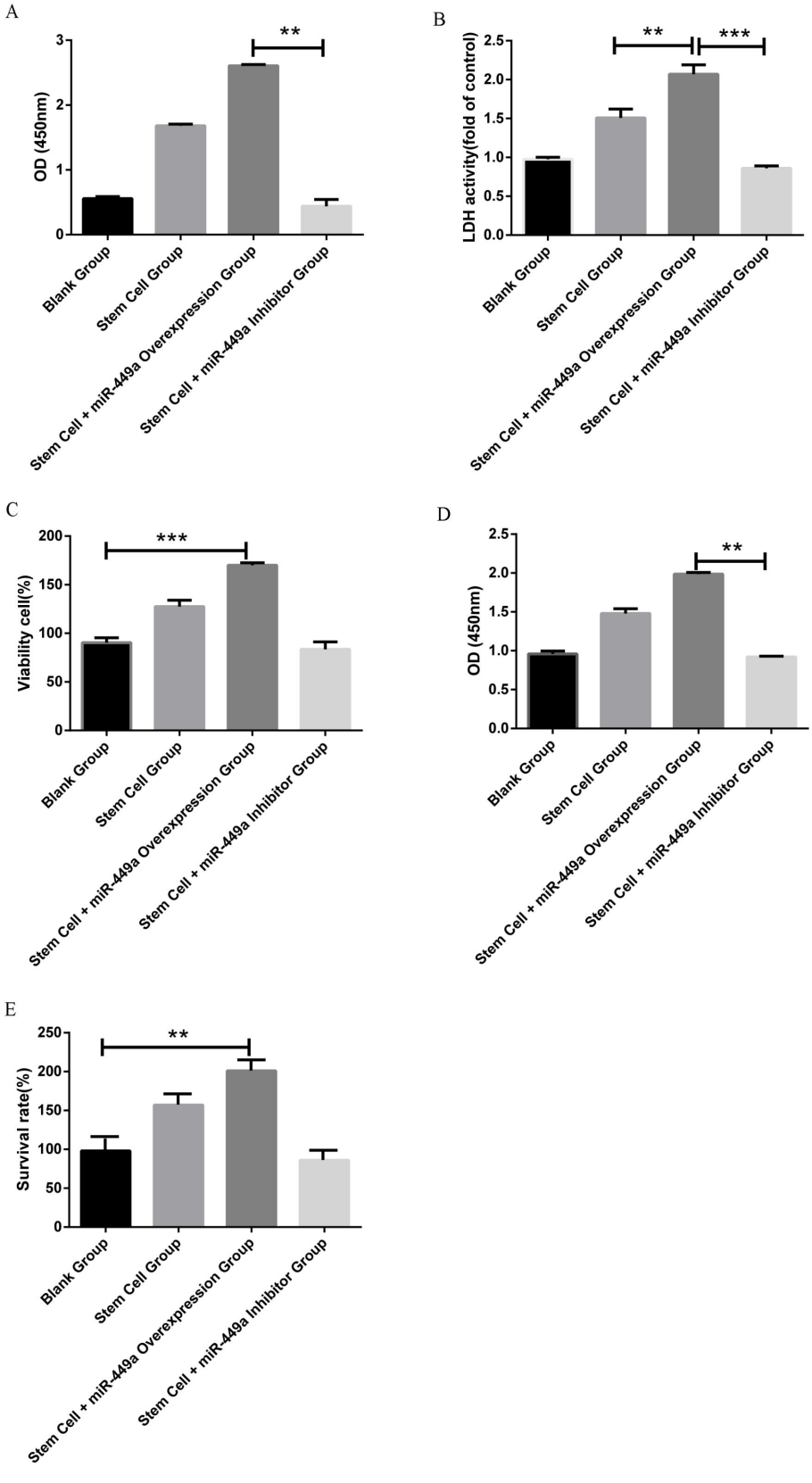

Figure 2. Validation of neuronal activity by stem cells over-expressing miR-449a; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (A: CCK8 test of neuronal cell proliferation; B: LDH of neuronal cell activity in each group; C: MTT of neuronal cell activity in each group; D: XTT (a cell viability detection method) of neuronal cell activity in each group; E: Live/dead cell counting).

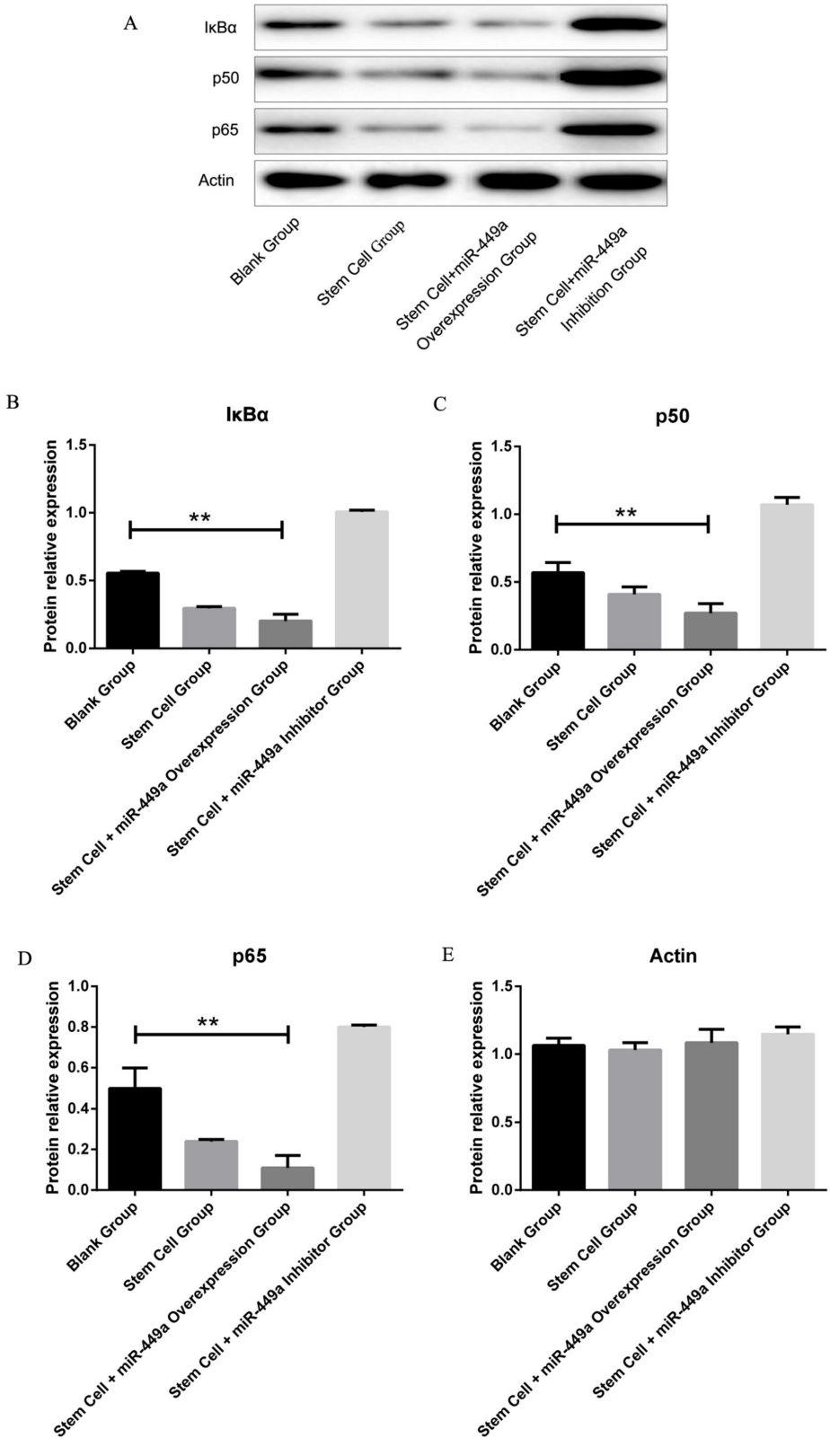

Figure 3. Verification of the regulatory effect of miR-449a on NF-κB signal pathway with *in vitro* co-cultured stem cells and neurons; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (From left to right, they are blank control group, stem cell group, stem cell + miR-449a over-expression group and stem cell + miR-449a inhibition group. In the transfected cells, the expression of proteins (IκBα, p50 and p65) was determined by Western blotting).

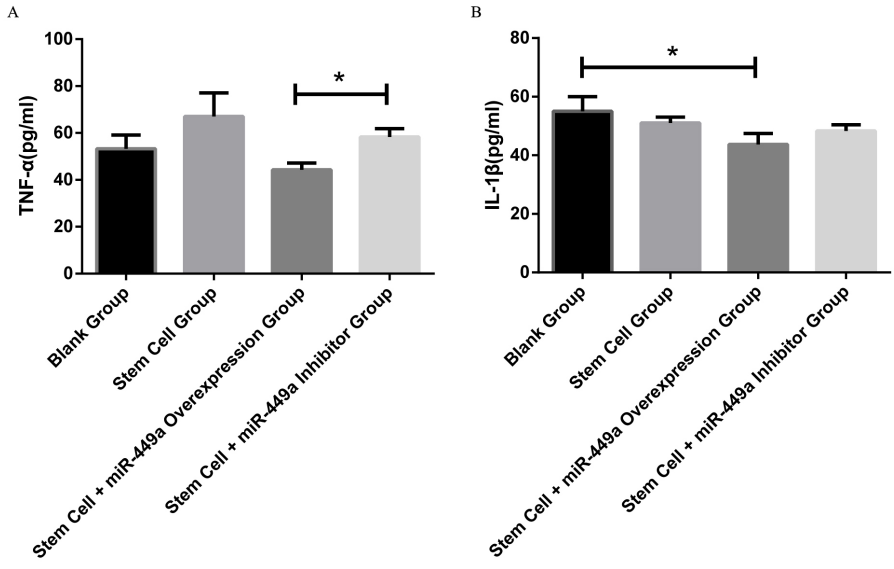

Figure 4. *In vitro* validation showed that the up-regulation of miR-449a expression can decrease the expression of inflammatory cytokines (both TNF-α and IL-1β). (Note: A: TNF-α content in cells among different groups after transfection; B: IL-1β content in cells among different groups after transfection).

Observation of pathological tissues

Three mice from the healthy, model and stem cell groups were selected to obtain histopathological slides after conducting surgery for 1 and 6 weeks, respectively. Rats were anesthetized with 2% sodium pentobarbital, sciatic nerves were taken about 3 mm, fixed in 10% paraformaldehyde, dehydrated with different concentrations of ethanol for 2h, embedded in paraffin, sectioned with hematoxylin-eosin, and then observed.

Western blot analysis

Neurons from healthy and each experimental groups were quantified according to the BCA protein concentration detection kit. Protein expression was determined by a Western blot Protein Assay kit (BJ-S963557, MLBio, Shanghai, China), with 50 g of proteins collected and separated by electrophoresis on a 10% polyacrylamide gel. The fiber membrane was transferred to the membrane with semi-dry electroimprinting, and sealed at room temperature for 1h. Rabbit anti-rat choline acetyltransferase (ChAT) antibody (diluted at 1:500) was incubated at 4°C overnight. The membrane was washed the next day, and goat anti-rabbit antibody (diluted at 1:5000) was added. One hour later, the membrane was incubated and washed as the treatment mentioned above again, and was exposed in the ECL chemiluminescence darkroom. The expression levels of NF-κB signaling pathway proteins (IκBα, P50 and P65) were analyzed with the Quantity One treatment system. Protein bands were observed with the ECL system (Millipore, USA).

ELISA method

TNF-α and IL-1β antibodies (diluted at 1:1000) were coated in microplates and cellular protein samples tested separately to act with the corresponding antibodies. Microbiized antibodies were added, then incomplete binding biotin antibodies were washed. Horseradish peroxidase (HRP) labeling and avidin were added, and finally, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added after complete washing. TMB turned blue after catalysis by peroxidase, and finally yellow with acid. Deeper color indicates higher sample concentration. The blank control well and standard curves with concentrations of 0.1, 1 and 10 μg/mL were set. All samples were parallel 3 times. Samples were detected for the OD_{450} with an ELISA reader, and the protein concentration of the measured samples was calculated.

Statistical analysis

All analyses were performed using SPSS 16.0 statistical software. All values were presented as mean ± SD. ANOVA was used among multiple groups, and Bonferonni method was used for pairwise comparisons between two groups. Statistical significance was set at $P<0.05$.

Results

Verification of stem cell viability after transfection of miR-449a

After transfection of miR-449a, miR-449a expression was significantly increased in stem cells (Figure 1A) and

decreased in the inhibitor group, indicating successful stem cell transfection. Successfully transfected stem cells were used for validation of cell viability. As shown in Figure 1B-1E, cell viability was almost unchanged in the miR-449a over-expression group compared to the blank stem cell group, but slightly decreased in the stem cell + miR-449a inhibitor group, indicating that stem cells increase miR-449a expression after successful transfection of miR-449a.

In vitro effects of stem cells over-expressing miR-449a on neurons

As shown in Figure 2A-2E, neuronal cell viability was gradually enhanced in the stem cells and miR-449a groups compared to the blank group (P<0.05), which was significantly decreased in the stem cell + miR-449a inhibitor group, indicating that over-expressing miR-449a can promote stem cells, and thus regulate neuronal cell viability and promote neuronal proliferation.

In vitro mechanism of stem cells over-expressing miR-449a on neurons

To verify the effect of stem cells on neurons *in vivo*, *in vitro* verification found that stem cells with different treatments decreased the expression of proteins (IκBα, p50 and p65) in neurons, with statistically significant difference (P<0.05). When inhibiting the over-expression of miR-449a, IκBα, p50 and p65 increased, which further verified that miR-449a can regulate NF-κB signal pathway and reduce the expression of inflammatory proteins (Figure 3). In Figure 4A and 4B, the content of inflammatory cytokines (TNF-α and IL-1β) also significantly decreased in the stem cell miR-449a over-expression group. *In vitro* results also showed that miR-449a promotes neuronal effects by targeting the NF-κB signal pathway in neurons, inhibiting the levels of inflammatory proteins, and reducing the production of inflammatory cytokines, which plays the main role in repairing peripheral nerve injury.

Establishment of the rat pathology model

The rat pathological model was established to observe the status of different rat groups. Rats in the model group and miR-449a inhibition group showed tardiness and decreased response (Figure 5A), and almost no response after stimulation (Table 2), with significantly reduced intake of food and water. However, there was no significant difference between miR-449a over-expression and blank groups. The speed of nerve signal propagation in rats gradually recovered, and the wet weight recovery rate of gastrocnemius muscle reached over 80% (Figure 5B). After treatment, the body weight of the rats also returned to normal (Figure 5C). The above results illustrate the success of rat modeling and the gradual return to normal movement after the therapeutic effect of stem cells.

Therapeutic effects of stem cells over-expressing miR-449a in tissue-injured rats

The rat injury model was established to detect miR-449a expression in different groups, showing that miR-449a expression significantly increased in stem cells and stem cell + miR-449a over-expression groups compared with the model group, with statistical significance (P<0.05), in which the stem cell + miR-449a over-expression group was similar to the blank control group, which was less in the stem cell group than that in the stem cell + miR-449a over-expression group (Figure 6B). The results also initially illustrate the role of miR-449a over-expression. The proliferation of neurons also increased in the stem cell + miR-449a over-expression group (Figure 6A). As shown in Figure 6C and 6D, the content of inflammatory cytokines in the sciatic nerve of the stem cell and stem cell+ miR-449a over-expression groups was similar to that in the blank control group, but the content in stem cell + miR-449a inhibition group became higher than that in the model group, indicating a role of over-expressing miR-449a pathway in improving the inflammatory response to peripheral nerve injury. Comparing with different *in vivo* model groups, the expression of proteins (IκBα, p50 and p65) significantly decreased in neurons of the stem cell + miR-449a over-expression group, with significant significance (P<0.05), while inhibiting miR-449a over-expression, IκBα, p50 and p65 increased, which further verified that miR-449a can regulate NF-κB signal pathway and reduce the expression of inflammatory proteins (Figure 7).

In vivo mechanism of stem cells over-expressing miR-449a on neurons

As shown in Figure 8, the comparison between different *in vivo* model groups showed that the expression of proteins (IκBα, p50 and p65) significantly decreased in neurons of stem cell + miR-449a over-expression group, with significant significance (P<0.05). Upon inhibition of miR-449a over-expression, IκBα, p50 and p65 increased by over-expression, which further verified that miR-449a can regulate NF-κB signal pathway and decrease the expression of inflammatory proteins. TNF-α and IL-1β are representative factors of inflammatory response, whose inflammation can lead to the increase of inflammatory cytokines, as the model group shown in Figure 9A-9B. The expression of inflammatory cytokines in neurons of stem cell and stem cell + miR-449a over-expression groups was similar to that in the blank control group, while the content in stem cell + miR-449a inhibition group exceeded that in the model group, suggesting that over-expressing miR-449a pathway can ameliorate the inflammatory response of peripheral nerve injury, which also validates that miR-449a can promote neurons for auto-repair by targeting the NF-κB pathway, as well as inhibiting the levels of proteins (IκBα, p50 and p65) and play a major role in repairing peripheral nerve injury by reducing the production of inflammatory cytokines (TNF-α and IL-1β).

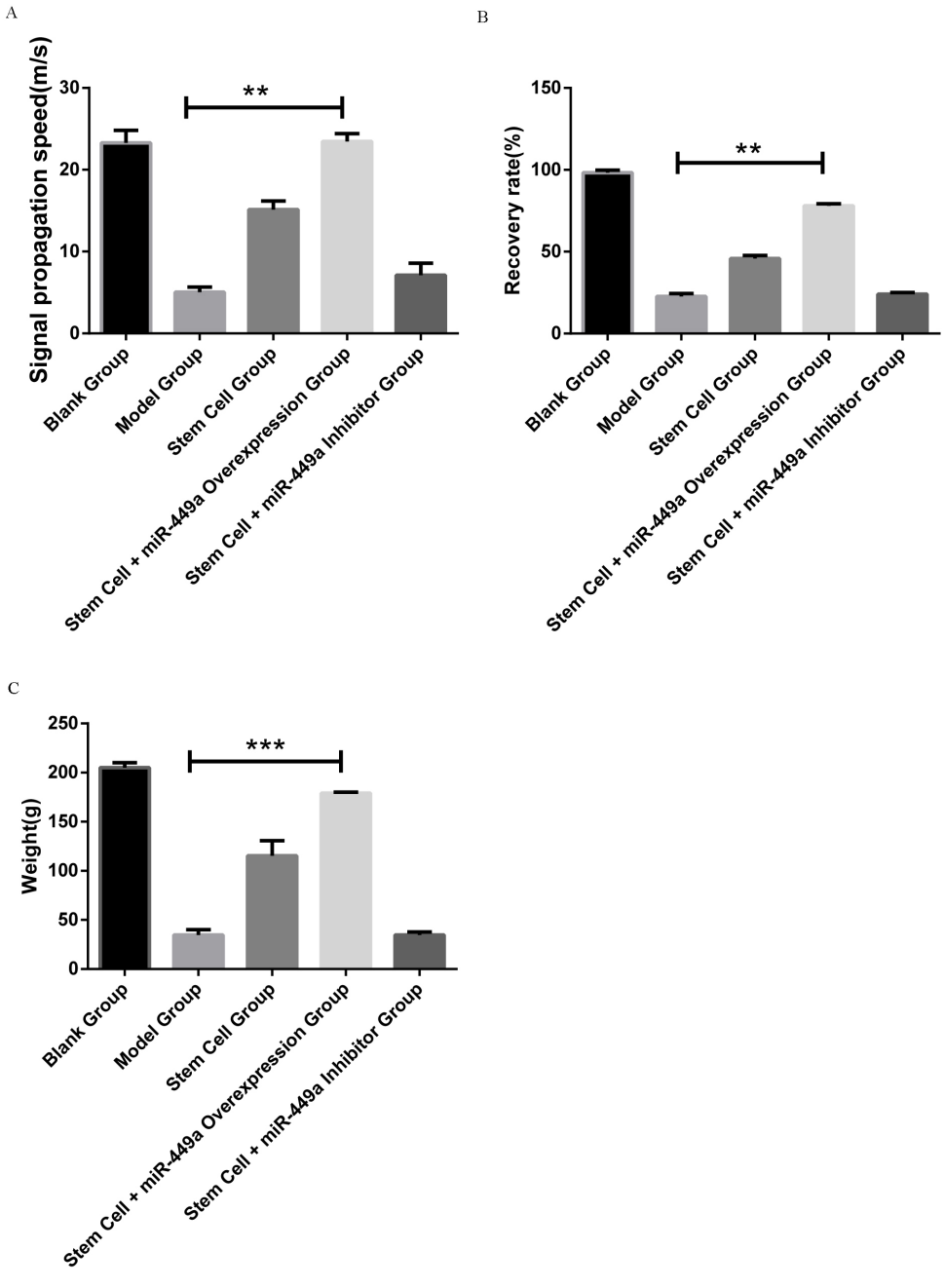

Figure 5. Establishment of the rat pathology model; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (A: Neuroelectrophysiological response, label with signal propagation speed; B: Recovery rate of rat gastrocnemius muscles; C: Change of rats' weight).

Discussion

Mesenchymal stem cells (MSCs) are heterogeneous stromal stem cells that can be isolated from adult tissues, such as umbilical cord, placenta and bone marrow. Stem cells have the ability to proliferate and differentiate into mesodermal cells, such as adipocytes, osteocytes and chondrocytes. BMSC can produce cell differentiation under specific stimulation, so BMSC has a very important role in tissue injuries[13-15]. Yan et al. verified that after loading hPDGF-A modified pig BMSCs and keratinocytes on the deepithelial human amniotic membrane, the healing of skin injury of radial skin wounds on small pigs can be promoted[15]. Ding et al. indicated that the nutritional value and vascular

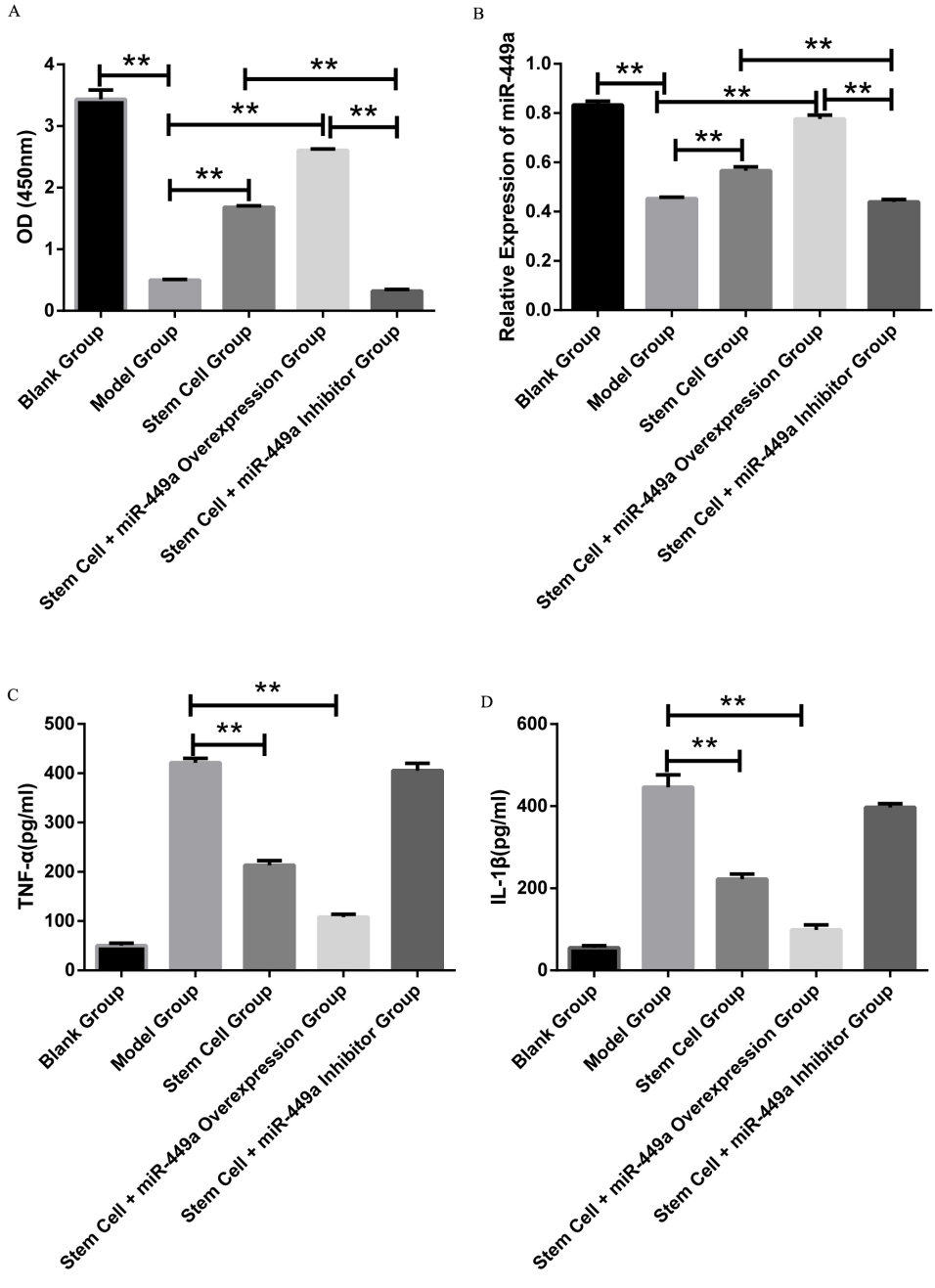

Figure 6. Therapeutic effects of stem cells over-expressing miR-449a on tissue-injured rats; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (A: Verification of cell viability in different groups; B: RT-qPCR analysis of mRNA expression of miR-449a in cells of injured spinal cord neurons; C: TNF-α content among different groups; D: IL-1β content among different groups).

regeneration function of BMSCs have restorative effects on perforated flap injury[16]. Ye et al. studied the transplantation of human CSF-induced rat BMSC-Ns into the brains of middle cerebral artery occlusion (MCAO) rats. The results showed that human CSF-induced BMSC-Ns significantly improved neural function and reduced infarct area in MCAO rats[17]. Ritfeld et al. demonstrated the neuroprotective effect of BMSCs through the rat spinal cord contusion model, the data of which broaden our understanding of the BMSC-mediated neuroprotective effects[18,19]. By stimulating internal or external sources, BMSCs can be directed to differentiate, which can promote the recovery of axons and the formation of blood vessels, thus avoiding nerve damage, and can also play a role by being directly transformed into neuron-like cells[20].

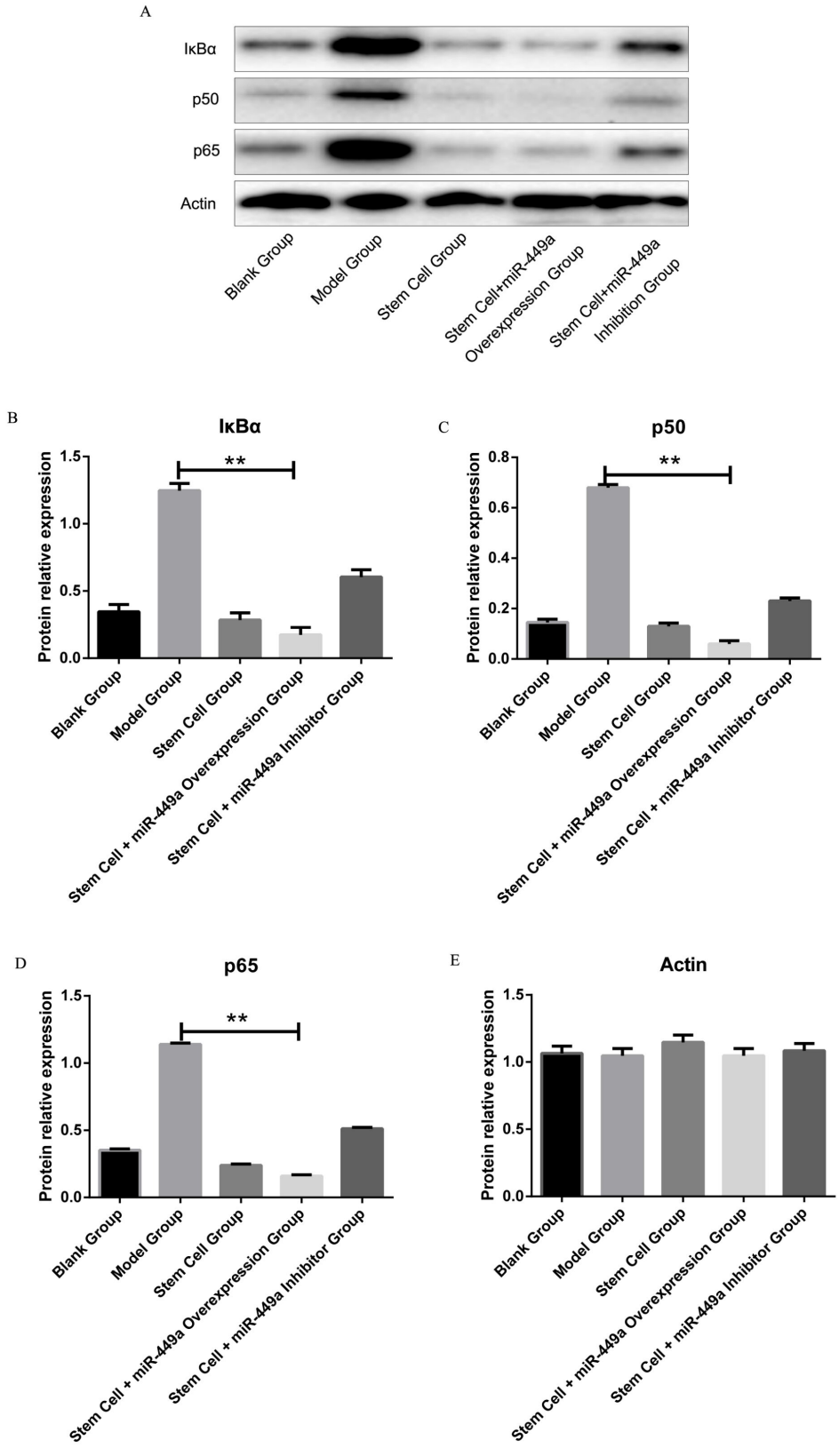

Figure 7. Regulation of over-expressing miR-449a on NF-κB signaling pathway in tissues; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (A: Western blotting of protein imprinting of IκBα, P50 and P65 of cells of rats in different groups; B: Protein expression of IκBα; C: Protein expression of p50; D: Protein expression of p65; E: Reference expression).

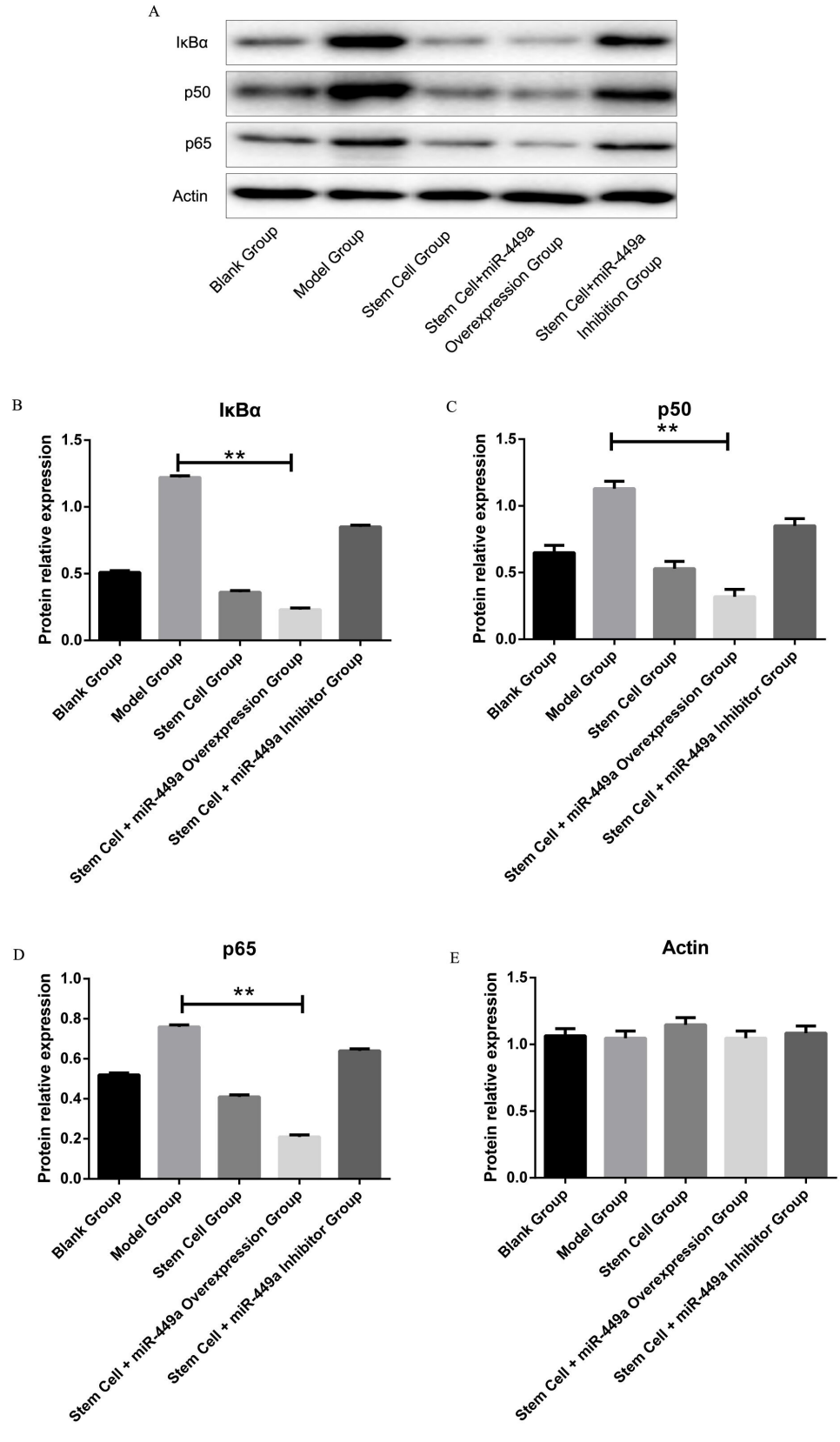

Figure 8. Stem cells could regulate NF-κB signal pathway via miR-449a and reduce the expression of inflammatory proteins; ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (From left to right, they are blank control group, stem cell group, stem cell + miR-449a over-expression group and stem cell + miR-449a inhibition group. Western blotting of the expression of proteins (IκBα, p50, and p65) in transfected cells).

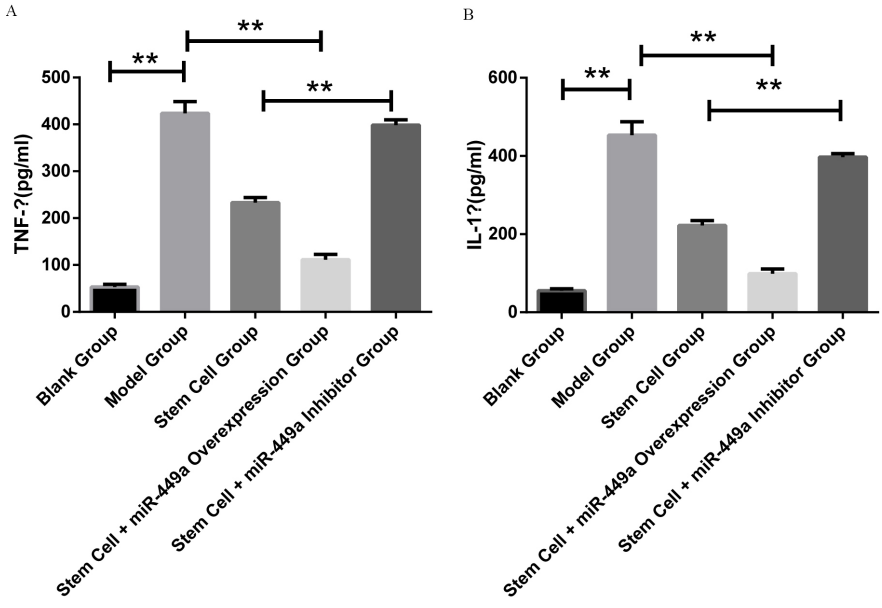

Figure 9. Up-regulation of miR-449a expression could decrease the expression of inflammatory cytokines (TNF-α and IL-1β); ANOVA was used among multiple groups, and pairwise comparisons were performed with the Bonferonni method (*P<0.05, **P<0.01, ***P<0.001). (A: TNF-α content in cells in different groups; B: IL-1β content in cells in different groups).

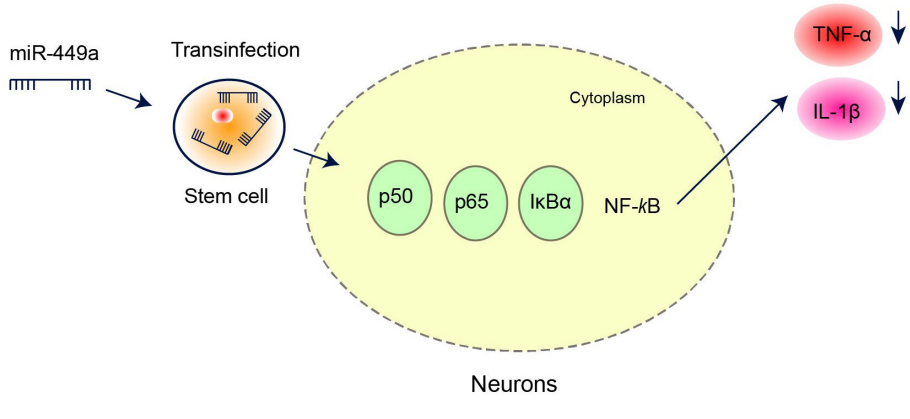

Figure 10. Study hypothesis graph. The mechanism of BMSCs in regulating NF-κB signal pathway by targeting miR-449a to improve the inflammatory response to peripheral nerve injury.

In addition, BMSCs can promote the repair of injured brain tissues and spinal cord, and differentiate into neurons to play an anti-inflammatory repair role[21]. However, little research has been conducted on neuroinflammation after neuronal injury, so it is of great significance to explore the mechanism of differentiation of BMSCs to reduce the inflammatory response of peripheral nerve injury to repair nerve injury.

MicroRNA (miRNA) is a small non-coding transcript that provides critical and universal post-transcriptional gene regulatory layers, and has become the focus of intensive research. To date, a large body of literature suggests that miRNAs play a critical role in delivering information to intracellular cells, which, therefore, target transcription factors and genes in multiple cellular pathways, including angiogenesis, cell transport, apoptosis, and proteolysis[22,23]. Although nearly 500 human miRNA sequences are known with only about 21 nucleotides each that bind to multiple mRNA targets, the accurate prediction of miRNA targets seem to be beyond our grasp. Bioinformatics analysis suggests that more than one-third of the human genes may

be regulated by miRNA, suggesting that they play a critical role in regulating gene expression[24]. In the nervous system, miRNA has also been shown to play an important role in regulating regeneration, and increasing evidence illustrate that miRNAs are essential for vascular regeneration and miRNA can promote differentiation of stem cells to neurons, and thus restore[25]. Studies show that miRNA-21, miRNA-31, miRNA-668 and miRNA-672 are all localized to neurons in ganglia when the nerve is injured, further indicating that miRNA may be a therapeutic target for multiple forms of neurological injury. Meanwhile, high miR-449a expression can inhibit the expression of proinflammatory cytokines such as TNF-α and IL-1β[26]. According to the above reports, a hypothesis can be presented (Figure 10), which summarizes the potential mechanism of BMSCs in the repair of nerve injury. To assess the mechanism of action of BMSCs in targeting NF-κB signal pathway via miR-449a to improve the inflammatory response to peripheral nerve injury, the miR-449a-transfected cell line was established in this study, and the recovery of peripheral nerve injured rats injected with different groups of stem cells was evaluated. The results indicate that after injecting stem cells only, the rats were unresponsive, did not chirp or elude in time after being stimulated. The recovery of tissues was better in the stem cell + miR-449a over-expression group, with reduced inflammatory cytokines. It showed that BMSCs with over-expressed miR-449a can reduce the harm to peripheral nerves, promote neural repair, which may be achieved by inhibiting neuroinflammation or by differentiation into neurons. Whether this process is indirect or direct, and the role of NF-κB, are discussed in the following sections.

NF-κB is a classical inflammatory signal pathway that is present almost exclusively in the cells of the nervous system, controls cell signal pathway, cellular stress response, cell growth, survival, and apoptosis, as a major transcriptional factor expressed by most inflammatory cytokines, and activating NF-κB can promote the over-expression of IL-1β and tumor necrosis factors[27-30]. It has been shown that when the peripheral nervous system were harmed, the proteins (IκBα, p50 and p65) in Schwann cells would be activated, which causes a pathological response in the nervous system. In resting cells, NF-κB dimers with transcriptional activation potential are sequestered in the cytoplasm and interact with the inhibitor family of IκBα. Activated NF-κB signal pathway system will bring more than 150 target genes expressing IκBα to inhibit NF-κB, including the most abundant p50/p65. In response to a large number of different stimulation, IκBα is phosphorylated, ubiquitinated and degraded, releasing NF-κB nuclear localization signal (NLS) targeting NF-κB to the nucleus. Liu et al. showed that exosome stem cells released by bone marrow mesenchymal can reduce lung damage by targeting the TLR4/NF-KB pathway for intestinal ischemia reperfusion[31]. The results of Yang et al. showed that the anti-inflammatory protein TSG-6 secreted by BMSCs can alleviate neuropathic pain by inhibiting the TLR2/MyD88/NFkB signal pathway in spinal microglia[32]. However, NF-κB expression may be regulated as targeted by miRNA, for example, Dexmedetomidine exerts cardioprotective effects by targeting miR-146a-3p of IRAK1 and TRAF6 by inhibiting the NF-kB pathway, and miR-449a inhibits the growth, migration and invasion of tumors in non-small cell lung cancer by targeting HMGB1-mediated NF-kB signaling pathway, thereby improving the initiation and development of cancer[33]. In peripheral nerve injury, NF-kB can promote the differentiation of stem cells into neurons, thus promoting recovery. To further validate the relationship between miR-449a and NF-κB, the effect of miR-449a over-expression on NF-κB signal pathway proteins was verified in this study, which showed that in the stem cell + miR-449a over-expression group, the expression of proteins (IκBα, p50 and p65) was significantly inhibited, and the amount in inflammatory cytokines (TNF-α and IL-1β) was reduced. NF-κB signal pathway lies in neurons and plays an important role in inflammation, while inhibiting miR-449a expression, the expression of inflammation-related proteins in neuronal cells decreased significantly, and pathological slides showed that the injury barely recovered in rats. In summary, first, miR-449a over-expressed BMSCs can reduce peripheral nerve injury, promote nerve repair, inhibit the levels of proteins (IκBα, p50 and p65), and mainly repair peripheral nerve injury by reducing the production of inflammatory cytokines (TNF-α and IL-1β).

Taken together, we demonstrate in this study that miR-449a can improve peripheral nerve injury by promoting BMSCs, which is a potential target for repairing peripheral nerve injury. We also verified that the up-regulation of miR-449a expression can reduce the amount of inflammatory cytokines in peripheral nerve injury, which can be repaired by alleviating the inflammatory response. NF-κB signal pathway plays an important role in the differentiation of miR-449a-induced BMSCs in improving the inflammatory response to peripheral nerve injury. Up-regulating miR-449a in BMSCs can target the regulation of NF-κB signal pathway to promote the differentiation of stem cells and reduce the inflammatory response of peripheral nerve injury to repair nerve injury, and inhibiting miR-449a expression can reverse this effect.

In conclusion, this study validated that up-regulation of miR-449a expression in BMSCs can specifically regulate NF-κB signal pathway, so as to promote the differentiation of stem cells and reduce the inflammatory reaction of peripheral nerve injury and repair nerve injury. It depends on the possible mechanism by inhibiting the levels of proteins (IκBα, p50 and p65), and reducing the amount of inflammatory cytokines (TNF-α and IL-1β). However, its deeper mechanism still needs to be further verified *in vivo*.

Authors' contributions

HW and FW conceived and designed the study, and drafted the manuscript. HW, YW, XL, CD, CL, YM and JZ collected, analyzed and interpreted the experimental data. FW and YW revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

References

1. Oñate M, Catenaccio A, Martínez G, et al. Activation of the unfolded protein response promotes axonal regeneration after peripheral nerve injury [published correction appears in Sci Rep 2021;11(1):24424]. Sci Rep 2016;6:21709.
2. Ko HG, Choi JH, Park DI, et al. Rapid Turnover of Cortical NCAM1 Regulates Synaptic Reorganization after Peripheral Nerve Injury. Cell Rep 2018;22(3):748-759.
3. Zamora R, Ravuri SK, Plock JA, Vodovotz Y, Gorantla VS. Differential inflammatory networks distinguish responses to bone marrow-derived versus adipose-derived mesenchymal stem cell therapies in vascularized composite allotransplantation. J Trauma Acute Care Surg 2017;83(1 Suppl 1):S50–s8.
4. Wang C, Lu CF, Peng J, Hu CD, Wang Y. Roles of neural stem cells in the repair of peripheral nerve injury. Neural Regen Res 2017;12(12):2106-2112.
5. Wakao S, Matsuse D, Dezawa M. Mesenchymal stem cells as a source of Schwann cells: their anticipated use in peripheral nerve regeneration. Cells Tissues Organs 2014;200(1):31-41.
6. Fernandes M, Valente SG, Sabongi RG, Gomes Dos Santos JB, Leite VM, Ulrich H, Nery AA, da Silva Fernandes MJ. Bone marrow-derived mesenchymal stem cells versus adipose-derived mesenchymal stem cells for peripheral nerve regeneration. Neural Regen Res 2018;13(1):100-104.
7. Chen Z, Han X, Ouyang X, Fang J, Huang X, Wei H. Transplantation of induced pluripotent stem cell-derived mesenchymal stem cells improved erectile dysfunction induced by cavernous nerve injury. Theranostics 2019; 9(22):6354-6368.
8. Yi S, Zhang Y, Gu X, Huang L, Zhang K, Qian T, Gu X. Application of stem cells in peripheral nerve regeneration. Burns Trauma 2020;8:tkaa002.
9. Schäfer S, Berger JV, Deumens R, Goursaud S, Hanisch UK, Hermans E. Influence of intrathecal delivery of bone marrow-derived mesenchymal stem cells on spinal inflammation and pain hypersensitivity in a rat model of peripheral nerve injury. J Neuroinflammation 2014;11:157.
10. Naiyu K, Liru C, Kuohu C. The Role of Micro RNA and Long-Non-Coding RNA in Osteoporosis. Int J Mol Sci 2020;21(14):4886.
11. Te L, Lengchen H, Yanhui Z, Yongyi H. Epigenetic silencing of HDAC1 by miR-449a upregulates Runx2 and promotes osteoblast differentiation. Int J Mol Med 2015;35(1):238-46.
12. Ke X, Li Q, Xu L, Zhang Y, Li D, Ma J, Mao X. Netrin-1 overexpression in bone marrow mesenchymal stem cells promotes functional recovery in a rat model of peripheral nerve injury. J Biomed Res 2015; 29(5):380-9.
13. Kizilay Z, Aktas S, Kahraman Cetin N, Bakay Ilhan D, Ersoy G, Erken HA. Effect of systemic application of bone marrow-derived mesenchymal stem cells on healing of peripheral nerve injury in an experimental sciatic nerve injury model. Turk Neurosurg 2017.
14. Chen J, Zhou J, Chen X, Yang B, Wang D, Yang P, He X, Li H. miRNA-449a is downregulated in osteosarcoma and promotes cell apoptosis by targeting BCL2. Tumour Biol 2015;36(10):8221-9.
15. Yan G, Sun H, Wang F, et al. Topical application of hPDGF-A-modified porcine BMSC and keratinocytes loaded on acellular HAM promotes the healing of combined radiation-wound skin injury in minipigs. Int J Radiat Biol 2011;87(6):591-600.
16. Ding JP, Chen B, Qian WJ, Bao SW, Zhao HY. Effect of bone marrow mesenchymal stem cells on perforator skin flap survival area in rats. Br J Oral Maxillofac Surg 2020;58(6):669-674.
17. Ye Y, Peng YR, Hu SQ, Yan XL, Chen J, Xu T. *In Vitro* Differentiation of Bone Marrow Mesenchymal Stem Cells into Neuron-Like Cells by Cerebrospinal Fluid Improves Motor Function of Middle Cerebral Artery Occlusion Rats. Front Neurol 2016;7:183.
18. Saeyoung P, Jiyeon K, Seoha M, Namhee J, Yeonzi C, Sungchul J. Differentiation of Motor Neuron-Like Cells from Tonsil-Derived Mesenchymal Stem Cells and Their Possible Application to Neuromuscular Junction Formation. Int J Mol Sci 2019;20(11):2702.
19. Ritfeld GJ, Patel A, Chou A, Novosat TL, Castillo DG, Roos RA, Oudega M. The role of brain-derived neurotrophic factor in bone marrow stromal cell-mediated spinal cord repair. Cell Transplant 2015;24(11):2209-20.
20. Pang CJ, Tong L, Ji LL, Wang ZY, Zhang X, Gao H, Jia H, Zhang LX, Tong XJ. Synergistic effects of ultrashort wave and bone marrow stromal cells on nerve regeneration with acellular nerve allografts. Synapse 2013;67(10):637-47.
21. Wang Y, Jia H, Li WY, Guan LX, Deng L, Liu YC, Liu GB. Molecular examination of bone marrow stromal cells and chondroitinase ABC-assisted acellular nerve allograft for peripheral nerve regeneration. Exp Ther Med 2016;12(4):1980-1992.
22. Ebert MS, Sharp PA. Roles for microRNAs in conferring robustness to biological processes. Cell 2012; 149(3):515–524.
23. Kosik KS. The neuronal microRNA system. Nat Rev Neurosci 2006;7(12):911–920.
24. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15–20.
25. Kang H, Hata A. The role of microRNAs in cell fate determination of mesenchymal stem cells:balancing adipogenesis and osteogenesis. BMB Rep 2015; 48(6):319–323.
26. Wang A, Ren M, Song Y, Wang X, Wang Q, Yang Q, Liu H, Du Z, Zhang G, Wang J. MicroRNA expression profiling of bone marrow mesenchymal stem cells in steroid-induced osteonecrosis of the femoral head associated

with osteogenesis. Med Sci Monit 2018;24:1813–1825.
27. Ahmed OB, Bamidele VO. Bromelain reduced pro-inflammatory mediators as a common pathway that mediate antinociceptive and anti-anxiety effects in sciatic nerve ligated Wistar rats. Sci Rep 2021;11(1):289.
28. Xiaoxu C, Xiaoke N, Jiamin M, Yan Z, Kaizhi Y, Pingping S, Jiashan L, Yiming L, Shengyang J, Lingli S. Perfluorooctane sulfonate mediates secretion of IL-1β through PI3K/AKT NF-κB pathway in astrocytes. Neurotoxicol Teratol 2018;67:65-75.
29. Yanfang W, Jie L, Xiaoli C, Huimin S, Sheng P, Yashu K, Jingjiang P, Tao Z, Lin Z, Zuoren Y, Brain Tomlinson, Paul C, Yihan C, Yuzhen Z, Ying L. Dysfunctional endothelial-derived microparticles promote inflammatory macrophage formation via NF-κB and IL-1β signal pathways. J Cell Mol Med 2019;23(1):476-486.
30. Zhuyin J, Yiwei H, Xiaojun J, Jiaju S, Guosheng F. Ticagrelor and clopidogrel suppress NF-κB signaling pathway to alleviate LPS-induced dysfunction in vein endothelial cells. BMC Cardiovasc Disord 2019; 19(1):318.
31. Jianpei L, Tufeng C, Purun L, Xiao T, Pinjie Huang. Exosomes Released by Bone Marrow Mesenchymal Stem Cells Attenuate Lung Injury Induced by Intestinal Ischemia Reperfusion via the TLR4/NF-κB Pathway. Int J Med Sci 2019;16(9):1238-1244.
32. Hao Y, Lingmin W, Huimin D, Yuanli C, Huanping Z, Meiyun L, Shaochen W, Li Z, Lina Z, Xin L. Anti-inflammatory protein TSG-6 secreted by bone marrow mesenchymal stem cells attenuates neuropathic pain by inhibiting the TLR2/MyD88/NF-κB signaling pathway in spinal microglia. J Neuroinflammation 2020;17(1):154.
33. Dandan W, Jun L, Jianliang C, Haiyan H, Hang M, Xuedong L. miR-449a Suppresses Tumor Growth, Migration, and Invasion in Non-Small Cell Lung Cancer by Targeting a HMGB1-Mediated NF-κB Signaling Pathway. Oncol Res 2019;27(2):227-235.