MiR-146a regulates osteogenic differentiation and proliferation of bone marrow stromal cells in traumatic femoral head necrosis

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Abstract. – OBJECTIVE: To investigate the regulatory mechanism of micro ribonucleic acid (miR)-146a in osteogenic differentiation and proliferation of bone marrow stromal cells (BMSCs) in traumatic femoral head necrosis.

PATIENTS AND METHODS: Femoral neck fracture patients undergoing surgery were divided into necrosis group and non-necrosis group. The expression level of miR-146a in BMSCs isolated from these patients was detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The clinical correlation of miR-146a with BMSCs in traumatic femoral head necrosis was explored. The regulatory effects of miR-146a on osteogenic differentiation and proliferation of BMSCs in traumatic femoral head necrosis were detected. Moreover, the cell proliferation was analyzed via cell counting kit-8 (CCK-8) assay. The deposition of calcium on the cell surface was detected via alizarin red staining to evaluate the osteogenic differentiation. The messenger RNA (mRNA) expressions of osteogenesis-specific genes, alkaline phosphatase (ALP), and osteocalcin (Ocn) in BMSCs undergoing osteogenic differentiation were detected via qRT-PCR.

RESULTS: Expression level of miR-146a in BMSCs of necrosis group was significantly lower than that in non-necrosis group, and the difference was statistically significant (p<0.01). CCK-8 assay revealed that the proliferation of BMSCs was significantly enhanced in miR-146a-mimic group compared with that in miR-NC group, while it significantly declined in miR-146a-inhibitor group compared with that in miR-NC group. The results of alizarin red staining showed that the deposition of calcium obviously increased in miR-146a-mimic group compared with that in miR-NC group, indicating that the osteogenic differentiation ability is significantly enhanced, while it markedly decreased in miR-146a-inhibitor group compared with that in miR-NC group. The detection of osteogenesis-specific genes via qRT-PCR manifested that the mRNA expressions of ALP and Ocn remarkably increased in miR-146a-mimic group compared with those in miR-NC group, and there were statistically significant differences (p<0.05). The mRNA expressions of ALP and Ocn remarkably decreased in miR-146a-inhibitor group compared with those in miR-NC group, and there were statistically significant differences (p<0.05), suggesting the inhibited osteogenic differentiation ability.

CONCLUSIONS: We showed that miR-146a regulates the osteogenic differentiation and proliferation of BMSCs in traumatic femoral head necrosis.

Key Words: MiR-146, Bone marrow stromal cells, Traumatic femoral head necrosis.

Introduction

Femoral head necrosis is a severe hip joint disease characterized by insufficient blood supply in the femoral head and intraosseous hypertension. Traumatic femoral head necrosis is an important type mostly caused by femoral neck fracture, dislocation of hip joint and other hip joint diseases. In recent years, the incidence rate of femoral head necrosis is increasing year by year, ranking first in tuberculosis of hip joint. Multiple therapeutic approaches have been applied for femoral head necrosis, such as nucleus pulposus decompression, bone grafting, joint formation, and joint replacement. Nowadays, the modified bone marrow stromal cells (BMSCs) via gene transfection technique have become a new therapeutic regimen for femoral head necrosis.

BMSCs are adult stem cells with high self-renewal ability and multi-directional differentiation potential, existing in almost all human tissues. They have been considered as a promising cell source for regenerative medicine due to their capacity to differentiate into various cell types including osteoblasts, chondrocytes, and adipocytes. The regulation of osteogenic differentiation of BMSCs is a complex process involving multiple factors and signaling pathways. One of these factors is miR-146a, a micro RNA that has been implicated in various biological processes, including cell proliferation, differentiation, and apoptosis. The objective of this study was to investigate the regulatory mechanism of miR-146a in osteogenic differentiation and proliferation of bone marrow stromal cells in traumatic femoral head necrosis.

Patients and Methods

Femoral neck fracture patients undergoing surgery were divided into necrosis group and non-necrosis group. BMSCs were isolated from these patients and their expression level of miR-146a was detected via quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The clinical correlation of miR-146a with BMSCs in traumatic femoral head necrosis was explored. Additionally, the regulatory effects of miR-146a on osteogenic differentiation and proliferation of BMSCs in traumatic femoral head necrosis were assessed. The cell proliferation was analyzed via cell counting kit-8 (CCK-8) assay. The deposition of calcium on the cell surface was detected via alizarin red staining to evaluate the osteogenic differentiation. The mRNA expressions of osteogenesis-specific genes, such as alkaline phosphatase (ALP) and osteocalcin (Ocn), were measured via qRT-PCR.

Results

The expression level of miR-146a in BMSCs of the necrosis group was significantly lower than that in the non-necrosis group (p<0.01). CCK-8 assay revealed that the proliferation of BMSCs was significantly enhanced in the miR-146a-mimic group compared to the miR-NC group, whereas it significantly declined in the miR-146a-inhibitor group compared to the miR-NC group. Alizarin red staining showed that the deposition of calcium was obviously increased in the miR-146a-mimic group compared to the miR-NC group, indicating a significant enhancement in osteogenic differentiation ability. In contrast, the mRNA expressions of ALP and Ocn were remarkably increased in the miR-146a-mimic group and decreased in the miR-146a-inhibitor group, suggesting a inhibited osteogenic differentiation ability.

Conclusions

We demonstrated that miR-146a regulates the osteogenic differentiation and proliferation of BMSCs in traumatic femoral head necrosis. This finding has implications for understanding the mechanisms underlying osteogenic differentiation and may pave the way for the development of novel therapeutic strategies for traumatic femoral head necrosis.

Key Words: MiR-146, Bone marrow stromal cells, Traumatic femoral head necrosis.
BMSCs can not only proliferate widely, but also differentiate into different types of cells, such as adipocytes, osteoblasts, and chondrocytes. BMSCs can also secrete various cytokines to regulate immune response and promote angiogenesis through paracrine and autocrine\(^6\). BMSCs, as one of the important seeds of bone tissue engineering, play an important role in proliferation, osteogenic differentiation, and bone regeneration in the bone defect region. Therefore, studying the mechanism of proliferation and osteogenic differentiation of BMSCs provides an important solution for nonunion, bone cyst, bone defect, and ischemic necrosis of femoral head in the future.

Micro-ribonucleic acids (miRNAs) are a kind of small-molecular non-coding RNA with 18-24 nucleotides in length. MiRNAs alter the messenger RNA (mRNA) stability or protein translation at the post-transcriptional level through binding to the 3’-untranslated region (UTR) of their target mRNA. The complete complementary pairing between miRNA and its target gene can directly degrade the target gene, while the incomplete complementary pairing between them leads to inhibited translation of the target gene\(^-10\). MiRNA, as an important regulatory molecule, is involved in a series of important pathophysiological processes, such as BMSCs proliferation, differentiation, and migration, as well as cytokine secretion\(^11\). A large number of researches\(^2\) have demonstrated the vital role of miRNAs in regulating the osteogenic differentiation and proliferation of MSCs. According to a large number of microarray studies, there are a large number of differentially expressed miRNAs in BMSCs, showing important roles in the functional maintenance and directed differentiation of stem cells\(^17\). In this work, the expression of miR-146a in BMSCs in necrosis group and non-necrosis group after operation of femoral neck fracture was detected via qRT-PCR. The clinical correlation between miR-146a and traumatic femoral head necrosis was explored. Moreover, overexpression or knockdown of miR-146a expression was achieved via lentiviral transfection in BMSCs in necrosis group. The influences of miR-146a on proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis was further analyzed.

**Patients and Methods**

**Clinical Samples**

All patients signed the informed consent, and this study was approved by the Ethics Committee of Affiliated Hospital of Jining Medical University. All patients with femoral neck fracture who underwent percutaneous hollow screw internal fixation after traction reduction from 2016 to 2017 in our hospital were selected. Among them, 19 patients meeting the diagnostic criteria for traumatic femoral head necrosis reported in the guidelines\(^8\) were enrolled into necrosis group, while 34 patients without traumatic femoral head necrosis were enrolled into non-necrosis group. None of the enrolled patients received drug therapy (especially steroid hormones) 6 months before and after the period, and they had no history of arthritis. In addition, patients with systemic inflammatory response, autoimmune diseases, malignant tumors, etc., were excluded.

BMSCs were obtained from the bone marrow in the upper femoral marrow cavity of patients with a traumatic femoral neck fracture and isolated via density gradient separation as previously described\(^17\). An equal volume of Percoll separation solution (Solarbio, Beijing, China) was added for centrifugation at 2000 rpm for 30 min. Cells were suspended in the normal growth medium [90% low-glucose Dulbecco’s Modified Eagle Medium (DMEM; HyClone Laboratories Inc., Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA), penicillin (100 U/mL) and streptomycin (100 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA)]. Then, the cells were inoculated in a 55 cm² flask and cultured in an incubator with 5% CO₂ at 37°C. The medium was replaced once every 2-3 d.

BMSCs were identified based on cell surface antigen determination (CD29 and CD34) via flow cytometry.

**qRT-PCR**

MiR-146a expression in BMSCs between necrosis group and non-necrosis group after operation of femoral neck fracture was detected via qRT-PCR. The clinical correlation between miR-146a and traumatic femoral head necrosis was explored. Moreover, overexpression or knockdown of miR-146a expression was achieved via lentiviral transfection in BMSCs in necrosis group. The influences of miR-146a on proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis was further analyzed.
The total RNA was extracted using TRIzol reagent (Sangon Biotech, Shanghai, China) and reversely transcribed using the NanoDrop 2000 device (Thermo Fisher Scientific, Rockford, IL, USA). The cDNA samples were obtained using TaKaRa RNA PCR kit (TaKaRa, Dalian, China) and Oligo dT primers (Invitrogen, Shanghai, China). The miRNA and mRNA expression levels were measured via qPCR using SYBR master mixture (TaKaRa, Dalian, China) on a Light Cycler 480 device (Roche, Basel, Switzerland). Each sample was measured for 3 times. The primer design and synthesis were shown in Table I. The expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the data were analyzed using the 2−ΔΔCT method. Primer sequences used in this study were as follows: ALP, F: 5'-GCAGAACAACTCCTTACTC-3', R: 5'-GCTATTGGGTGTCCGAAGGA-3'; MiR-146a, F: 5'-GACTTCTCCACAACCCTCTG-3', R: 5'-AGAGGGAAGAGTTCCCCAG-3'; Ocn, F: 5'-AGATGCACCTGTACGATCAA-3', R: 5'-CTTTCAACCTGCAATTTG-3'. U6: F: 5'-GCTTCGCAGCACATATACTAAAT-3', R: 5'-CGACTGTGCCGTTGAATTTG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTGACTCCGACCTTCAC-3'.

**Construction and transfection of lentiviral vector**

The miR-NC, miR-146a-mimic, and miR-146a-inhibitor lentiviral vectors were purchased from Genechem (Shanghai, China). The multiplicity of infection (MOI) of all lentiviruses reached 50. MiR-146a expression after lentiviral transfection was detected via qRT-PCR.

**Cell Proliferation Analysis**

Cell proliferation was analyzed using the cell counting kit-8 (CCK-8) kit (Dojindo, Kumamoto, Japan). After transfection of BMSCs with miR-NC, miR-146a-mimic or miR-146a-inhibitor lentiviruses, the CCK-8 solution was added at 1 d, 2 d, 3 d, 4 d, and 5 d, respectively. The absorbance was measured at 450 nm using a microplate reader (Infinite 200, Tecan, Hombrechtikon, Switzerland).

**In vitro Induction of Osteogenic Differentiation**

Osteogenic differentiation-inducing medium containing 10% FBS, 10 mM β-glycerophosphate, 1.9×10⁻⁸ M dexamethasone, 50 μg/mL L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA), 0.01 μM 1,2,5-dihydroxyvitamin D₃, 100 U/mL penicillin, and 100 μg/mL streptomycin was used to induce the osteogenic differentiation of BMSCs in vitro. The deposition of calcium on the cell surface was detected via alizarin red staining at 14 d, and expression levels of osteogenesis-specific genes (ALP and Ocn) in the three groups were detected via qRT-PCR.

**Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. The comparison between the two groups was done using the t-test. p-values < 0.05 were considered statistically significant.

**Results**

**Clinical Data of Patients**

A total of 19 patients meeting the diagnostic criteria for traumatic femoral head necrosis were enrolled into necrosis group, while 34 patients without traumatic femoral head necrosis were enrolled into non-necrosis group. The X-ray displayed severe ONFH in patients after operation of femoral neck fracture. The clinical image of the patient was shown in Figure 1.

**Morphology of BMSCs**

With the prolongation of cell culture, the spindle-shaped BMSCs began to adhere to the wall. At 14 d after continuous culture, the cell proliferation was rapid. The morphology of BMSCs was shown in Figure 2.

**Identification of BMSCs**

To identify the isolated BMSCs, two specific surface antigens of BMSCs were examined using a flow cytometer. The results revealed that the cells cultured showed strong positive expression
of CD29 and negative expression of CD34 (Figure 3). Based on the above results, we confirmed the purity of extracted BMSCs.

**Clinical Correlation Between MiR-146 and Traumatic Femoral Head Necrosis**

The expression level of miR-146a was detected via qRT-PCR in necrosis group and non-necrosis group. The results showed that the expression level of miR-146a in necrosis group was significantly lower than that in non-necrosis group, and the difference was statistically significant ($p<0.01$) (Figure 4). It is indicated that miR-146a may be involved in regulating traumatic femoral head necrosis, and miR-146a overexpression may inhibit traumatic femoral head necrosis.

**MiR-146a Expression in the Three Groups (MiR-NC, MiR-146a-mimic, and MiR-146a-inhibitor) Detected Via qRT-PCR**

The three lentiviruses (miR-NC, miR-146a-mimic, and miR-146a-inhibitor) were transfected into BMSCs in necrosis group to overexpress or inhibit miR-146a in BMSCs. After transfection, the miR-146a expression in the three groups (miR-NC, miR-146a-mimic, and

![Figure 1. Clinical image of patient: Femoral head necrosis occurs at 1 year after hollow screw internal fixation.](image1)

![Figure 2. Observation of BMSCs under a light microscope (40×) at 14 d after continuous culture.](image2)

![Figure 3. Identification results of flow cytometry. A, Positive expression of CD29, B, negative expression of CD34.](image3)
Role of miR-146a in traumatic femoral head necrosis

**CCK-8 Proliferation Assay Results**

The proliferation ability of BMSCs in the three groups was detected via CCK-8 assay for consecutive five days. The results manifested that the proliferation ability of BMSCs was significantly enhanced in miR-146a-mimic group compared with that in miR-NC group, while it significantly declined in miR-146a-inhibitor group compared with that in miR-NC group. It suggested that miR-146a may be involved in regulating the proliferation of BMSCs. Overexpression of miR-146a enhanced the proliferation of BMSCs, while miR-146a knockdown reduced the proliferation of BMSCs.

**Culture of BMSCs in Osteogenic Differentiation-Inducing Medium in the Three Groups**

BMSCs transfected with miR-NC, miR-146a-mimic, and miR-146a-inhibitor in the three groups were cultured in the osteogenic differentiation-inducing medium. The deposition of calcium on the cell surface was detected via alizarin red staining after 14 d. It was found that calcium deposition markedly increased in miR-146a-mimic group compared with that in miR-NC group, indicating the elevated osteogenic differentiation ability. On the contrary, it significantly decreased in miR-146a-inhibitor group compared with that in miR-NC group, indicating that the osteogenic differentiation ability was significantly weakened (Figure 7). These results indicated that miR-146a may be involved in regulating the osteogenic differ-
osteocyte death after traumatic fracture due to the special anatomical location of the femoral neck. Traumatic femoral head necrosis is the severest complication of a femoral neck fracture. Prevention and treatment of femoral head necrosis have become a hot spot recently. In this study, miR-146a expression in necrosis group and non-necrosis group was detected via qRT-PCR. It is found that the expression of miR-146a in BMSCs in necrosis group was significantly lower than that in non-necrosis group. Studies have demonstrated that miRNA is involved in regulating BMSCs in traumatic femoral head necrosis. Ying et al. found that miR-93-5p inhibits osteogenic differentiation of BMSCs in traumatic femoral head necrosis through targeting BMP-2. MiR-146a may be involved in regulating traumatic femoral head necrosis, and miR-146a overexpression may inhibit traumatic femoral head necrosis.

Proliferation ability of BMSCs in the three groups was detected via CCK-8 assay for consecutive five days. The results manifested that the proliferation ability of BMSCs was significantly enhanced in miR-146a-mimic group compared with that in miR-NC group, while it significantly declined in miR-146a-inhibitor group compared with that in miR-NC group. We may conclude that the overexpression of miR-146a enhanced the proliferation of BMSCs, while miR-146a knockdown inhibited the proliferation of BMSCs. Some studies have revealed that the number of local BMSCs in patients with femoral head necrosis declines, and the osteogenic differentiation ability of BMSCs is significantly reduced. 

MRNA Expressions of Osteogenesis-Specific Genes in BMSCs in Osteogenic Differentiation-Inducing Medium Detected Via qRT-PCR

BMSCs transfected with miR-NC, miR-146a-mimic, and miR-146a-inhibitor in the three groups were cultured in the osteogenic differentiation-inducing medium. The mRNA expressions of osteogenesis-specific genes (ALP and Ocn) in BMSCs in the three groups were detected via qRT-PCR. The results showed that the mRNA expressions of ALP and Ocn remarkably increased in miR-146a-mimic group compared with those in miR-NC group, and there were statistically significant differences ($p<0.05$). It is suggested that the osteogenic differentiation ability was significantly enhanced. The mRNA expressions of ALP and Ocn remarkably decreased in miR-146a-inhibitor group compared with those in miR-NC group, and there were statistically significant differences ($p<0.05$). It is believed that the osteogenic differentiation ability was inhibited by miR-146a knockdown (Figures 8 and 9).

Discussion

Traumatic femoral head necrosis is caused by the interruption of blood supply to the femoral head, component changes in bone marrow and osteocyte death after traumatic fracture due to the special anatomical location of the femoral neck. Traumatic femoral head necrosis is the severest complication of a femoral neck fracture. Prevention and treatment of femoral head necrosis have become a hot spot recently. In this study, miR-146a expression in necrosis group and non-necrosis group was detected via qRT-PCR. It is found that the expression of miR-146a in BMSCs in necrosis group was significantly lower than that in non-necrosis group. Studies have demonstrated that miRNA is involved in regulating BMSCs in traumatic femoral head necrosis. Ying et al. found that miR-93-5p inhibits osteogenic differentiation of BMSCs in traumatic femoral head necrosis through targeting BMP-2. MiR-146a may be involved in regulating traumatic femoral head necrosis, and miR-146a overexpression may inhibit traumatic femoral head necrosis.

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of BMSCs is weakened, which are consistent with our findings. In surgically-treated patients with traumatic femoral head necrosis developing from femoral neck fracture, the expression of miR-146a in BMSCs significantly reduced. In addition, Chen et al. found that the osteogenic differentiation of BMSCs declines in femoral head necrosis. It is reported that the osteogenic differentiation and proliferation of BMSCs play important roles in femoral head necrosis. In this study, miR-146a was also involved in regulating the osteogenic differentiation of BMSCs in patients with traumatic femoral head necrosis. The deposition of calcium on the cell surface in the osteogenic differentiation-inducing medium in the three groups was detected via alizarin red staining. The results demonstrated that the deposition of calcium markedly increased in miR-146a-mimic group compared with that in miR-NC group, indicating that the osteogenic differentiation ability is significantly enhanced. Calcium deposition decreased in miR-146a-inhibitor group compared with that in miR-NC group, indicating that the osteogenic differentiation ability was significantly weakened. Moreover, the mRNA expressions of osteogenesis-specific genes (ALP and Ocn) in BMSCs in the three groups were detected via qRT-PCR. The results demonstrated that the mRNA expressions of ALP and Ocn remarkably increased in miR-146a-mimic group compared with those in miR-NC group, suggesting the enhanced osteogenic differentiation ability. On the contrary, mRNA expressions of ALP and Ocn remarkably decreased in miR-146a-inhibitor group compared with those in miR-NC group, suggesting that the osteogenic differentiation ability was inhibited. Compared with that in surgically-treated patients with femoral neck fracture without traumatic femoral head necrosis, the expression of miR-146a significantly declined in surgically-treated patients with traumatic femoral head necrosis developing from a femoral neck fracture.

Conclusions
We showed that miR-146 regulated the proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis. Further experiments are needed in the future to investigate the downstream signaling pathway of miR-146 in regulating the proliferation and osteogenic differentiation of BMSCs in traumatic femoral head necrosis and its specific mechanism.

Conflict of Interest
The Authors declare that they have no conflict of interest.

References
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