MiR-145 silencing promotes steroid-induced avascular necrosis of the femoral head repair via upregulating VEGF


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Abstract. – OBJECTIVE: To investigate the role of miR-145 silencing in the expression of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), B-cell lymphoma-2 (Bcl-2), BCL2-Associated X(bax) and caspase-3 in avascular necrosis of femoral head (ANFH).

MATERIALS AND METHODS: A total of 12 healthy wild-types (the control group) and 12 miR-145 knock-out (miR-145-/−) (the experimental group) adult New Zealand white rabbits were selected to construct ANFH model with steroid. Four weeks later, immunohistochemistry, qRT-PCR and Western blot were performed to measure the VEGF, bFGF, Bcl-2, bax, caspase-3, β-catenin as well as c-Myc expression. Terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining analysis was used to detect the apoptosis of bone cells in each group.

RESULTS: Compared with the control group, the expression of VEGF, bFGF, Bcl-2, β-catenin and c-Myc in the miR-145-/− group raised (p<0.05). Moreover, the expression level of bax and caspase-3 significantly decreased in the miR-145-/− group (p<0.05). TUNEL staining showed decreased apoptosis in the miR-145-/− group.

CONCLUSIONS: MiR-145 silencing promotes bone repair of ANFH via upregulating VEGF, bFGF and inhibiting the bone cells apoptosis through Wnt/β-catenin pathway.

Key Words: miR-145, Avascular necrosis of femoral head, Angiogenesis, Apoptosis.

Introduction

Avascular necrosis of femoral head (ANFH) is a kind of refractory orthopedics disorder that is due to blood supply disruption. ANFH often affects young people aged between 30 and 50 years old. Patients diagnosed with ANFH manifests as the collapse of femoral head and destruction of the hip joint. ANFH can be classified as traumatic and non-traumatic avascular necrosis of femoral head. Compared with traumatic ANFH, non-traumatic ANFH is not only a kind of femoral head disease, but also as a kind of partial manifestation of systemic disorders. In recent years, the incidence of non-traumatic ANFH increases due to the wide use of glucocorticoid and excessive drinking. However, the common pathophysiologic feature of different kind ANFH is disorder of blood supply. Although some treatments including conservative treatments and operations have been attempted to manage ANFH, there is no accepted effective method until now due to the complex pathophysiologic process of ANFH. In the treatment of ANFH, smooth blood supply is necessary for the bone healing and bone formation. The capability of bone cells and bone marrow stromal cells differentiating into osteoblasts decreases when blood supply disorders. In this condition, the bone trabecular is ruptured by osteoclasts because of the loss of barrier from osteoblasts attached to bone trabecular. If the absorbed bone could not be replaced by new bone, bone fracture would happen and then bone collapsed eventually. Vascular endothelial growth factor (VEGF) plays critical roles in the process of physiological and pathological angiogenesis and many other cytokines, which could promote angiogenesis function by enhancing VEGF expression partially or fully. Basic fibroblast growth factor (bFGF) was reported take part in the angiogenesis and bone regeneration. In addition, it was reported that increased apoptotic bone cells were found in ANFH. MicroRNAs (miRNAs) are small, noncoding RNAs and play important roles in gene posttranslational expression. Recent studies reported that miRNAs were involved in a series of pathological and physiological processes such as the initiation of neoplasms, bone formation, neonatal development and so on. MiR-145 was reported to be related to angiogenesis in metabolic syndrome.
ANFH, the role of miR-145 in bone healing and bone regeneration has not been clarified. In our study, the silencing of miR-145 promoted the bone healing and bone formation in ANFH rats. The silencing of miR-145 upregulated the VEGF and bFGF expression and inhibited the apoptosis of bone cells. Therefore, the silencing of miR-145 promoted bone healing in ANFH.

Materials and Methods

Animals and Grouping

A total of 12 healthy wild type (the control group) and 12 miR-145 knocked-out (miR-145⁻/⁻) (the experimental group) New Zealand white rabbits were purchased from the SLAC Laboratory Animal Company (Shanghai, China). All experimental rabbits were at 6-7 weeks old and weighted between 2.5-3.0 Kg. 50% of animals were male and 50% were female. Rabbits were kept in a room with temperature at 25°C ± 3°C and humidity of 40-60% and on a cycle of 12 h light and 12 h dark. This study was approved by the Animal Ethics Committee of Hebei University of Engineering Animal Center (Hebei, China).

Construction of ANFH Model

All rabbits in the two groups were injected with lipopolysaccharide purchased from Sigma-Aldrich (St. Louis, MO, USA) intravenously at a dose of 10 µg/Kg once a day for 2 days. 48 h later, methylprednisolone (MPS) were injected intramuscularly at a dose of 20 mg/Kg body weight for three times at 24-h intervals. All rabbits received 100,000 U penicillin (Yangtze Pharma Company Taizhou, Jiangsu, China) intraperitoneally to prevent infection.

Confirmation of ANFH by Magnetic Resonance Imaging (MRI) Examination

After 4 weeks from MPS injection, MRI was used to confirm ANFH induced by steroid. 3% pentobarbital sodium (Yangtze Pharma Company, Taizhou, Jiangsu, China) was injected into rabbits (1 mg/kg) intravenously. Philips Gyroscan T5-NT MRI was used to scan bilateral femoral heads (T1WI: TR 550 ms, TE 14 ms; T2WI: TR 2100 ms, TE 87 ms; stir: TR 1456 ms, TE 70 ms) with slice thickness of 1.5 mm and interval of 0.12 mm (Eindhoven, The Netherlands). T1WI low and T2WI high signal was considered as ANFH.

Sample Collection

Rabbits were sacrificed at four weeks from MPS injection for the first time. We collected bilateral femoral heads of rabbits in two groups, which were washed with 0.9% saline. Femoral heads from the left side were stored at -80°C for qRT-PCR analysis and Western blot. The right femoral heads were fixed with 4% paraformaldehyde at 4°C.

Immunohistochemistry

The fixed femoral heads tissues were processed for immunohistochemistry analysis. Briefly, slices were immersed in graded alcohol and dimethyl benzene to deparaffinize and dehydrate sequentially. Next, citrate buffer was used to retrieve antigen in boiling water for 5 min. After the slices were cooled in room temperature, they were incubated with primary antibodies overnight. The slices were incubated with secondary antibodies for 30 min at 37°C after they were washed with phosphate buffered saline (PBS) for three times. Sections were treated with diaminobenzidine (DAB) for 4 min. We observed the staining under microscope and analyzed the images with Image-Pro-plus software. We randomly chose five visual fields in each slice to capture a photo and compared their average values.

TUNEL Staining Analysis

To detect the apoptosis rate, we performed terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining analysis with an in situ cell death detection kit purchased from Jiancheng Bioengineering Institute of Nanjing (Nanjing, China). Hematoxylin was used to counterstain the paraffin slices and TUNEL-positive cells were considered as apoptotic cells. By randomly selecting five regions and counting positive cells at 200 x, we evaluated the apoptosis rate in each group.

QRT-PCR Analysis

RNAiso plus was added into the tissues. Total RNA was collected and resolved using an appropriate volume of DEPC water. Thereafter, reverse transcription was carried out following the protocols of reverse transcription kit, and samples were added and amplified according to the instructions of the amplification kit. The expression of VEGF, bFGF, bcl-2, bax and caspase-3 were detected on the ABI7900HT PCR System (Foster City, CA, USA) with the SYBR Premix Ex TaqTM II kit purchased from TaKaRa (Otsu, Shiga, Japan). GAPDH was used as an internal control.
**Western Blot Analysis**

After washing with ice-cold PBS, the femoral heads were disrupted mechanically within liquid nitrogen. Then, the disrupted tissues were lysed with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Nanjing, China) to get total protein. We determined protein concentration with a protein assay kit. 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate protein. Then, it was shifted to polyvinylidene fluoride (PVDF) membranes purchased from Millipore (Billerica, MA, USA). 5% fat-free milk was used to block non-specific protein interactions in TBST buffer. The membranes loaded with proteins were incubated at 4°C with primary antibody and incubated at room temperature with secondary antibody conjugated with horseradish peroxide (two hours). After washing these membranes in tris buffered saline tween (TBST), we developed the membranes using chemiluminescence to detect antibodies concentration and took β-actin as our internal control. The antibodies were purchased from Abcam (Cambridge, MA, USA).

**Statistical Analysis**

SPSS11.0 (SPSS Inc., Chicago, IL, USA) was used to analyze our data. Quantitative data was expressed as mean ± SD. Non-paired $t$-test was used to analyze data between groups. One-way ANOVA test was used to analyze comparisons between groups followed by Post Hoc Test (Least Significant Difference). $p<0.05$ was determined as statistically significant.

**Results**

**ANFH Confirmations via MRI Scan**

MRI was performed to all rabbits four weeks after MPS administration to confirm ANFH induced by steroid. Results from MRI examination showed abnormal MRI signals in bilateral femoral heads in all rabbits with fat or mucoid changes. The intensity of T1WI (Figure 1A) decreased while T2WI signals intensity (Figure 1B) was heterogeneously high.

**MiR-145 Silencing Increased the VEGF and bFGF Expression**

We detected the expression levels of VEGF and bFGF via immunohistochemistry analysis, qRT-PCR, and Western blot analysis. After four weeks from the construction of steroid-induced ANFH, the expression levels of VEGF and bFGF in the miR-145 silencing group were higher than that in the control group (Figure 2).

**MiR-145 Silencing Inhibited the Apoptosis of Bone Cells in ANFH**

TUNEL staining was done to detect the apoptosis of bone cells in ANFH. Results

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**Figure 1.** ANFH confirmed by MRI scan. Bilateral femoral heads of all rabbits displayed abnormal MRI signals with lipid or myxoid changes. The intensity of T1WI signals (**A**) was reduced showing a line-like low-signal zone while T2WI signals (**B**) intensity was heterogeneously high.
**Figure 2.** The expression of VEGF and bFGF in the control group and experimental group. (A), Immunohistochemistry (magnification ×100) showed enhanced expression of VEGF and bFGF in the experimental group. Blue and red arrows indicated bFGF expression. (B), QRT-PCR showed increased VEGF and bFGF expression in the experimental group. GAPDH was used as an internal control. (C), Western blot analysis showed increased VEGF and bFGF expression in the experimental group. β-actin was used as an internal control. **, \( p < 0.01 \), ***, \( p < 0.001 \).

**Figure 3.** MiR-145 silencing inhibited the bone cells apoptosis. (A), TUNEL staining (magnification ×100) showed decreased apoptosis rate in the experimental group. Red arrows indicated TUNEL-positive cells. (B), QRT-PCR results showed increased bcl-2 expression and decreased bax and caspase-3 expression. GAPDH was used as an internal control. (C), Western blot analysis showed increased bcl-2 expression and decreased bax and caspase-3 expression in the experimental group. β-actin was used as an internal control. ***, \( p < 0.001 \).
showed that miR-145 silencing significantly inhibited the apoptosis of bone cells (Figure 3). Then, qRT-PCR and Western blot results indicated that the silence of miR-145 increased the bcl-2 expression and decreased the bax and caspase-3 expression (Figure 3).

**MiR-145 Silencing Inhibited the Apoptosis of Bone cells via Wnt/β-catenin Pathway**

Immunohistochemistry and Western blot were conducted to investigate the activated β-catenin and c-Myc expression in the control group and the experimental group. Results were showed in Figure 4. The levels of β-catenin and c-Myc increased greatly in the miR-145−/− group compared with the control group.

**Discussion**

ANFH is a kind of common disease, which could be caused by a series of reasons. To date, the mechanisms of ANFH are still unclear. Animal models are used widely to study the mechanisms of ANFH especially steroid-induced ANFH. Pathologically speaking, the features of ANFH are the empty lacunae and ghost nuclei in the lacunae and a lot of fat cells in the bone marrow. Strikingly, the defect of blood supply is the main reason of blocking the bone repair. VEGF is one of the most effective factors in vasculogenesis. Studies showed that VEGF induced the proliferation of vascular endothelial cells and, therefore, promoted the differentiation, migration and accumulation of bone cells. BFGF is involved in the regeneration of vessels and osteoblasts especially the arteriole. We observed that miR-145 silencing upregulated the expression of VEGF and bFGF. Glucocorticoid could induce cell apoptosis consisting of osteocytes. In our study, TUNEL staining indicted that miR-145 silencing rescued bone cells from apoptosis. Caspase-3 plays a vital role in apoptosis of various forms. Our work showed that miR-145 silencing decreased the expression of caspase-3 compared with the control group. In addition, the ratio of bcl-2 and bax regulates the apoptosis. We found that miR-145 increased the level of bcl-2 while decreased the bax confirmed by PCR as well as Western blot analysis. Therefore, the above results indicated that miR-145 could inhibit the apoptosis of bone cells in ANFH. To investigate the mechanisms involved in the inhibition effect, we detected the signaling proteins that are related to apoptosis. Both immunohistochemistry and Western blot results showed the β-catenin expression and c-Myc expression were blocked in the control group while the miR-145 silence increased the β-catenin and c-Myc expression. Several types of research reported that targeting downstream genes of the Wnt/β-catenin signaling pathway including c-Myc could regulate apoptosis.
Conclusions

miR-145 silencing could increase the expression levels of VEGF and bFGF and promote angiogenesis. Also, the silence of miR-145 inhibited the apoptosis of bone cells in ANFH through the Wnt/β-catenin pathway. Therefore, miR-15 silencing promoted the repairmen of ANFH, which may be a promising effective treatment in the management of ANFH.

Conflict of interest
The authors declare no conflicts of interest.

References
Role of MiR-145 in necrosis of the femoral head


