European Review for Medical and Pharmacological Sciences 2017; 21: 3754-3762

# MiR-146a inhibits proliferation and induces apoptosis in murine osteoblastic MC3T3-E1 by regulating Bcl2

B. ZHANG[1], J. YI[1], C.-L. ZHANG[1], Q.-H. ZHANG[1], J.-F. XU[1], H.-Q. SHEN[2], D.-W. GE[1,3]

[1]Department of Orthopedics, Shuyang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Chinese Medicine, Suqian, China
[2]Department of Orthopedics, Nanjing Medical University Affiliated Nanjing First Hospital, Nanjing, China
[3]Department of Orthopedics, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

*Bin Zhang and Jun Yi contributed equally*

**Abstract. – OBJECTIVE: The dysregulation of proliferation and apoptosis plays a significant role in the pathogenesis of hormone-induced osteonecrosis of femoral head (ONFH). The research aimed to explore the regulatory role of miR-146a in dexamethasone (DEX)-induced proliferation and apoptosis change in MC3T3-E1 cells from murine osteoblastic.**

**MATERIAL AND METHODS: In this study, MC3T3-E1 was co-cultured with 10-7 DEX for 6 h, then RT-PCR was employed to test the expression level of miR-146a and Bcl2. CCK8 assay and flow cytometry were adopted to verify miR-146a could regulate proliferation and apoptosis. After transfected MC3T3-E1 with mimics and inhibitor, RT-PCR and Western blot was used to detect Bcl2 expression level.**

**RESULTS: In DEX treated MC3T3-E1 cells showed higher MiR-146a expression level and lower Bcl2 expression level. MiR-146a could inhibit proliferation and promotes apoptosis in murine osteoblastic MC3T3-E1 cells. Additionally, Bcl2 gene is regulated by MiR-146a.**

**CONCLUSIONS: The MiR-146a expression level increased, while Bcl2 has low expression level in dexamethasone treated MC3T3-E1 cells. MiR-146a regulates proliferation and apoptosis of mouse bone cells. The low expression level of Bcl2 in DEX treated MC3T3-E1 cells is caused by increased MiR-146a level.**

Key Words:
Apoptosis, Bcl2, Femur head necrosis, MiR-146a.

## Introduction

Osteonecrosis of the femoral head (ONFH), also known as ischemic femoral head necrosis, is divided into traumatic ONFH and non-traumatic ONFH1. Non-traumatic hormone-induced necrosis of the femoral head is the most general one, mostly happened in young people[2]. The pathogenesis of steroid-induced femoral head necrosis is complex. The direct effect of hormones on ONFH is regulating the differentiation, proliferation and apoptosis of osteocytes, eventually leading to loss of bone mass, femoral head collapse[3-5]. The indirect effect of hormones on ONFH is leading to thrombosis generation by promoting the apoptosis of vascular endothelial cells, affecting the vasoconstriction and diastolic activity of the expression of substances and lipid metabolism by inhibiting the expression of vascular endothelial growth factor (VEGF) and making obstacle to the generation of neovascularization[6].

Clinical data shows that osteoblast apoptosis contributes the pathogenesis of hormone-induced femoral head necrosis, which is the cytological basis of necrosis of femoral head[7,8]. Since osteoblasts are the main functional cells of the femoral head, the abnormal proliferation and apoptosis of osteoblasts in the femoral head are the hotspots of the study of steroid-induced ONFH. Intensive bone cell apoptosis contributes to ONFH[9]. Bcl2 is an important anti-apoptotic member in Bcl2 family, which can enhance the resistance of cells to most DNA damage factors, inhibit cell apoptosis and necrosis[10]. Studies have shown that high doses hormone inhibit the expression of Bcl2 gene and promote osteocyte apoptosis[11]. Avascular necrosis and apoptosis were caused by variety factors in the destruction of bone cells, in which miRNA played an important regulatory role. In recent years, in-

*Corresponding Authors:* Haiqi Shen, MD; e-mail: zhangbinsy@126.com; Dawei Ge, MD; e-mail: gdw02115252@163.com

vestigations have confirmed that microRNAs are widely involved in the regulation of differentiation, proliferation, apoptosis, angiogenesis, and other physiological processes[12-14]. MiR-146a is primarily involved in the regulation of inflammation in innate immune system[15,16]. In early osteoarthritis, IL-1β stimulation in cultured normal human articular cartilage chondrocytes increased the expression level of miR-146a17. MiR-146a knockout spontaneous OA mice exhibited alleviated articular cartilage degeneration, presenting as a potential new therapeutic target for bone destruction in OA[18,19]. In the paper of altered microRNAs in ONFH, researchers found the upregulation of MiR-146a in ONFH patients compared with the control samples20. MiR-146a could inhibit proliferation and enhance chemo sensitivity in epithelial ovarian cancer[21]. However, the specific mechanism of MiR-146a in ONFH is not clear. In this study, we focused on the role of increased expression of miR-146a in ONFH progress and its mechanism. We found that the transcriptional level of miR-146a in osteoblasts was significantly increased while the transcription level and protein level of anti-apoptotic gene Bcl2 were significantly decreased in steroid-induced ONFH. Further researches have shown that MiR-146a can inhibit osteoblast proliferation and promote its apoptosis, and this function is achieved by regulating the expression of Bcl2.

## Materials and Methods

### Chemicals and Materials

Dexamethasone was purchased from Abcam (Cambridge, MA, USA). MC3T3-E1 cells were obtained from ATCC (Manassas, VA, USA). Lipofectamine® 3000 Transfection Reagent was obtained from Invitrogen (Carlsbad, CA, USA). FBS was purchased from Gibco (Grand Island, NY, USA). CCK8 Assay Kit was obtained from Keygen (Nanjing, China). β-actin and Bcl2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### MC3T3-E1 Cell Culture

Murine osteoblastic MC3T3-E1 cells were maintained in a modified minimum essential medium (MEM) with 10% fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), 2 mM L-glutamine, and 25 μg/mL gentamicin at 37°C in a humidified atmosphere of 5% CO2. For exponential growth, MC3T3-E1 cells were passaged every 2-3 days.

### RNA Isolation and Real-time PCR

After MC3T3-E1 cells were stimulated with dexamethasone as described above, cells were collected by centrifugation, the supernatant was discarded and RNA was extracted with TRIzol reagent (Thermo Scientific, Waltham, MA, USA). Extraction procedures were carried out according to the operating instructions, RNA was extracted and analyzed by UV spectrophotometer for RNA quantification and purity. The integrity of RNA was detected by 0.8% agarose gel electrophoresis. Then, 1 μg of total RNA was taken by ABI (Foster City, CA, USA) reverse transcription kit according manufacturer's instructions. The reverse transcription reaction program was as follows: 25°C, 10 min; 37°C, 120 min; 85°C, 5 min. Real-time PCR amplification conditions were 95°C for 10 min, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 1 min.

### Western Blot Assay

After MC3T3-E1 cells were stimulated with dexamethasone as described above, cells were collected by centrifugation, and the supernatant was discarded. Cell pellets were suspended in cell lysis buffer containing protease inhibitors. The supernatant of cell suspension was separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels after boiled for 5 min in SDS-loading control. Subsequent the protein was transferred in gel to polyvinylidene difluoride (PVDF) membranes. To block nonspecific protein-protein interactions, PVDF membranes were blocked in Tris Saline Tween (TST) buffer containing 5% non-fat milk at room temperature. After that the antibodies were diluted (Bcl2, 1: 4000; β-Actin, 1: 5000) in TST buffer, the PVDF membranes were gently shaken in the diluted antibodies overnight at 4°C. The next day, PVDF membranes were washed with TST buffer, then incubated with peroxidase-conjugated individual secondary antibodies and shaken for 1 h. Eventually, enhanced chemiluminescence (ECL) solution in the dark room was prepared; the exposure time was determined according to the fluorescence intensity.

### Cell Proliferation and Viability

The MC3T3-E1 cells were suspended in complete medium to adjust the cell concentration to 5*106 cells/mL, and cells were grown into 96-well cell culture plates for 100 μL each well. For cell proliferation detection, cells were harvest in medium 24 h after transfection. Cell suspension, after normal culture, was find 24 h after the de-

tection of cell proliferation activity. Cell proliferation was detected by CCK8 Assay Kit according to the manufacturer's steps. Absorbance was detected at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was determined by methylthiazolyl tetrazolium bromide (MTT) assay. After MC3T3-E1 cells were treated with dexamethasone and transfected with miR-146a mimics or inhibitor for 24 h, cell absorbance was measured by using microplate reader.

### Cell Apoptosis Assay

After MC3T3-E1 cells were treated with dexamethasone, cells were collected and suspended in binding buffer. Then, annexin V-fluorescein isothiocyanate (V-FITC) and propidium iodide (PI) were added in cell suspension, and incubated in the ice for 15 min in the dark. We observed the apoptosis rate by FACScan (BD Biosciences, Franklin Lakes, NJ, USA). The apoptosis rate was detected by using apoptotic detection kit (Keygen Tech., Nanjing, China). All the procedures were carried out according to the manufacturer's instruction.

### Statistical Analysis

Each experiment in this article was repeated at least in triplicate. Results were showed as the mean value ± standard deviation (SD). Statistical analysis was carried out using Student's t-test. Each p-value less than 0.05 is thought to be with significance.

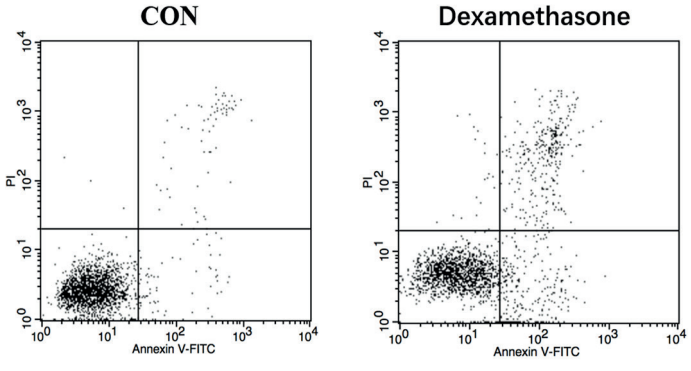
## Results

### Dexamethasone induced MC3T3-E1 Apoptosis

In order to find the appropriate concentration of dexamethasone to MC3T3-E1 cells, several doses of DEX were tested and 10-7 was the most suitable concentration. We treated MC3T3-E1 with 10-7 dexamethasone for 24 h; next, we applied flow cytometry to detect the apoptotic rate in DEX treated cells. Compared with the solvent control, the apoptotic rate is increased in DEX treated MC3T3-E1 cells (Figure 1).

### miR-146a was Increased and Bcl2 was Down Regulated in Dexamethasone Treated MC3T3-E1

We treated MC3T3-E1 with 10-7 dexamethasone for 6 h, then we compared the expression level of miR-146a in dexamethasone treated MC3T3-E1 cells. The result showed that miR-146a expression level was significantly increased in DEX treated MC3T3-E1 cells compared with controls (Figure 2). At the same time, we detected Bcl2 mRNA expression level. On the contrary, dexamethasone significantly decreased Bcl2 expression level. Western Blot demonstrated the protein level of Bcl2 also decreased (Figure 2).




| Quad | % Total |
|---|---|
| UL | 0.39±0.16 |
| UR | 3.71±0.45 |
| LL | 92.13±0.94 |
| LR | 3.77±0.57 |

| Quad | % Total |
|---|---|
| UL | 1.07±0.21 |
| UR | 16.3±0.81 |
| LL | 70.81±1.54 |
| LR | 11.79±0.57 |

**Figure 1.** The apoptotic rate in dexamethasone stimulated MC3T3-E1 cells. MC3T3-E1 cells were treated with 10-7 dexamethasone for 24 h, the cells were harvested for apoptosis detecting. The results showed dexamethasone increased the apoptotic percentage in MC3T3-E1 cells.

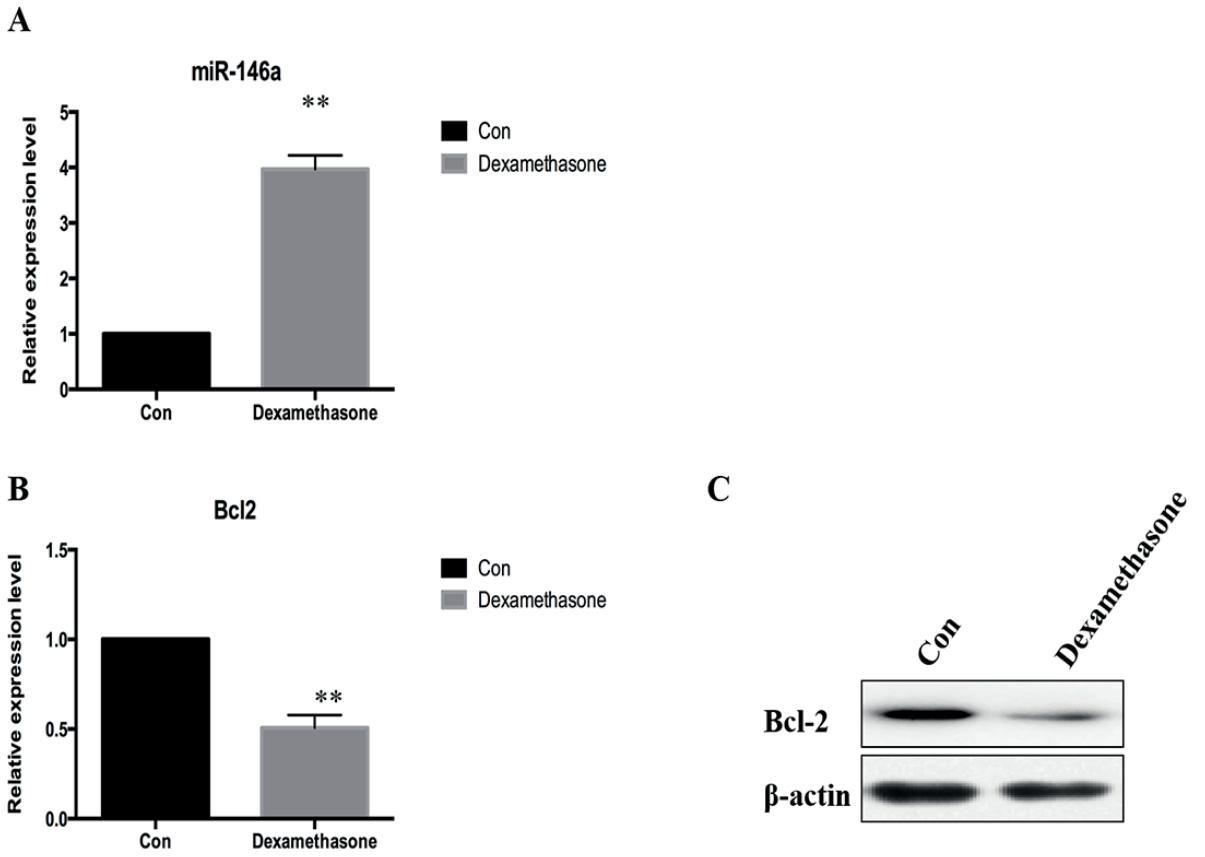



**Figure 2.** Expression levels of miR-146a and Bcl2 in dexamethasone stimulated MC3T3-E1 cells. RNA was extracted from MC3T3-E1 cells treated with 10-7 dexamethasone for 6 h. The miR-146a and Bcl2 expression level were detected by Real-time-PCR. The result showed miR-146a expression level significantly increased ***(A)***, and Bcl2 gene expression level significantly decreased ***(B)***.

### *Overexpression of MiR-146a Inhibited Proliferation and Induced Apoptosis*

In order to evaluate the function of miR-146a on bone cell proliferation ability, we transfected miR-146a mimics in MC3T3-E1 cells; 24 h after transfection the CCK-8 results showed that the cell proliferation of miR-146a mimics group was significantly decreased compared with controls (Figure 3). The apoptosis level of the transfected cells was detected by flow cytometry. The result showed that overexpression of miR-146a increased the percentage of apoptotic rate of MC3T3-E1 cells. Additionally, the function of miR-146a mimics on the sensitivity to dexamethasone in MC3T3-E1 cells was also determined. The cells were transfected with miR-146a mimics and at the same time they were treated MC3T3-E1 with 10-7 dexamethasone for 24 h; the results showed that over-expression of miR-146a enhanced dexamethasone induced apoptotic rate. The cell viability was also measured in the same condition. Over-expression of miR-146a enhanced dexamethasone induced decreasing of MC3T3-E1cells viability (Figure 3).

### *Decrease in MiR-146a increased Proliferation and inhibited apoptosis*

MC3T3-E1 cells were transfected with miR-146a inhibitor to detect the effect of miR-146a on bone cell proliferation and apoptosis. We transfected miR-146a inhibitor in MC3T3-E1 cells; the CCK-8 results showed that, compared with controls, the cell viability of miR-146a mimics group was significantly increased. The effect of miR-146a inhibitor on the sensitivity to dexamethasone in MC3T3-E1 cells was also determined. miR-146a inhibitor was transfected, and at the same time MC3T3-E1 was treated with 10-7 dexamethasone for 24 h; the results showed that miR-146a inhibitor decreased dexamethasone inducing apoptotic rate. Besides, the inhibitor of miR-146a inhibited

dexamethasone inducing the decrease of cell viability (Figure 4).

### MiR-146a Regulated the Expression Level of Bcl2

To investigate the mechanisms by which miR-146a influences cellular proliferation and apoptosis, we searched for putative protein coding gene targets of miR-146a. According to a previous study[22], miR-146a could regulate Bcl-2 expression level during autophagy. Bcl-2 expression was significantly reduced in DEX treated MC3T3-E1 cells, whereas miR-146a expression level increased. To evaluate whether Bcl-2 was regulated by miR-146a, we transfected MC3T3-E1 with miR-146a mimics and inhibitor; Bcl2 expression level was detected by Q-PCR. Bcl2 expression level changed contrary to miR-146a level (Figure 5). miR-146a could regulate bcl2 expression level.

## Discussion

The femoral head necrosis caused by high dose hormones is a common non-traumatic ONFH. The detrimental effect of glucocorticoids on osteoblast proliferation and apoptosis is clear, glucocorticoids could inhibit bone cell proliferation and induce apoptosis. In this work we show that dexamethasone (a synthetic hormone of glucocorticoids) can increase the transcription level of miR-146a and decrease the level of anti-apoptotic gene Bcl2 transcription. MiR-146a regulates the proliferation and apoptosis of murine osteoblastic MC3T3-E1 cells, which are a novel mechanism of femoral head necrosis. Previous studies have found miR-146a was increased in ONFH patient. In the present work, we found miR-146a expression level was upregulated in dexamethasone stimulated MC3T3-E1 cells, indicating a

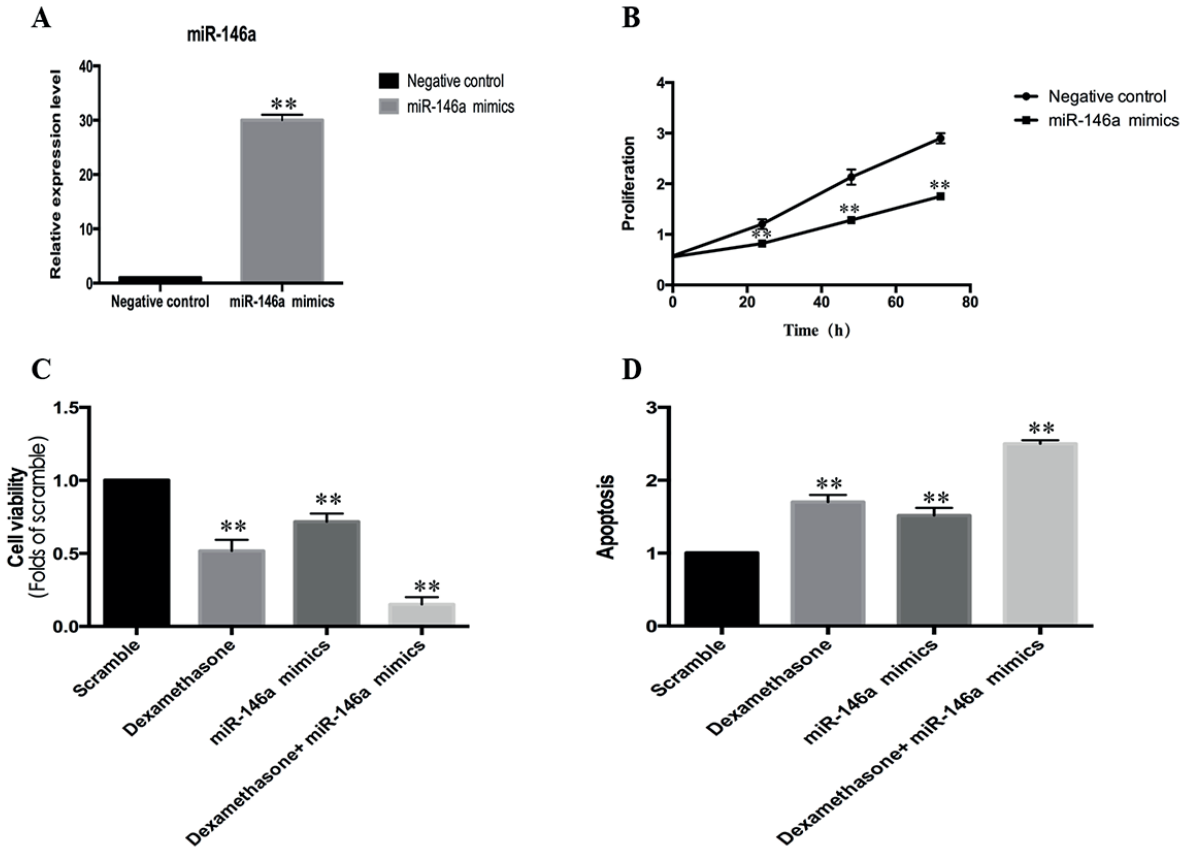



**Figure 3.** Overexpression of MiR-146a inhibited proliferation and induced apoptosis. MC3T3-E1 cells were transfected with miR-146a mimics, the mRNA level of miR-146a was increased compared with control ***(A)***, the cell proliferation ability was decreased in miR-146a mimics transfected cells compared with control ***(B)***. MC3T3-E1 cells were transfected with miR-146a mimics and treated dexamethasone for 24 h, the cell viability result ***(C)*** showed the viability was significantly decreased in miR-146a mimics group, dexamethasone treated group and the combined group. The apoptosis result showed the apoptosis rate was significantly increased in miR-146a mimics group, dexamethasone treated group and the combined group ***(D)***.

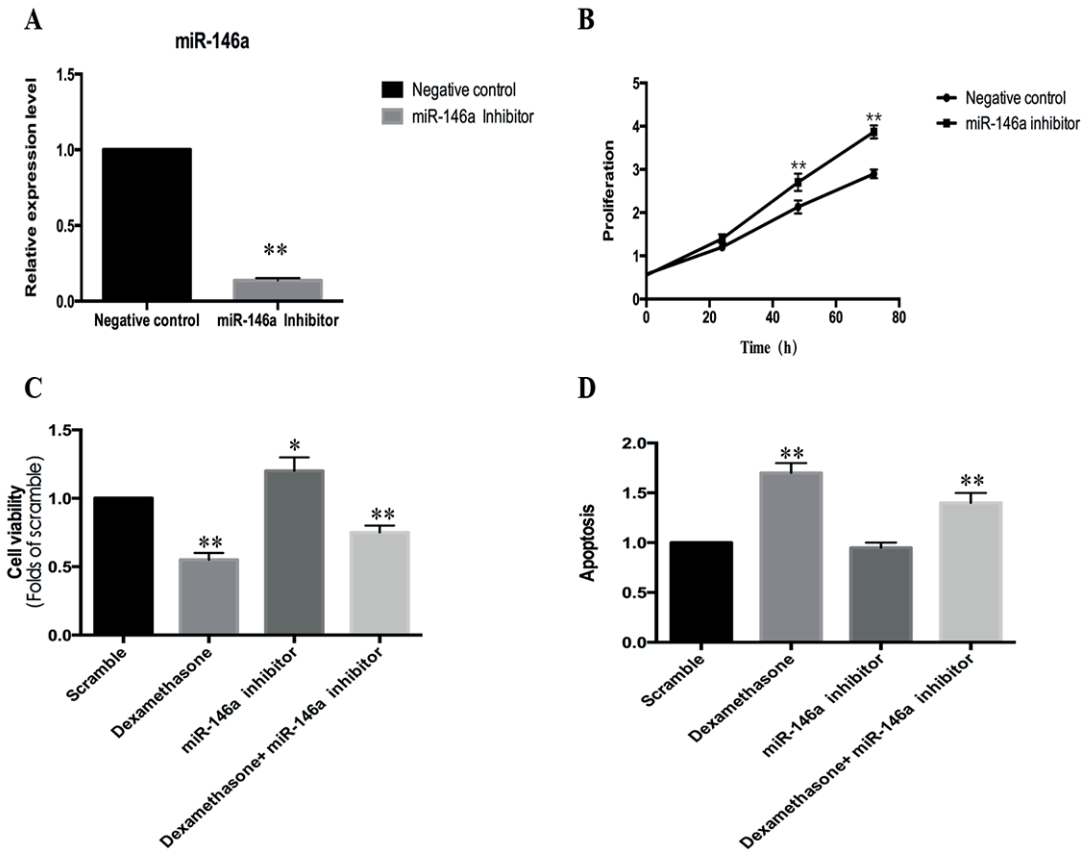



**Figure 4.** Decrease in MiR-146a increased proliferation and inhibited apoptosis. MC3T3-E1 cells were transfected with miR-146a inhibitor, the mRNA level of miR-146a was decreased compared with control ***(A)***, the cell proliferation ability was increased in miR-146a inhibitor transfected cells compared with control ***(B)***. MC3T3-E1 cells were transfected with miR-146a inhibitors and treated dexamethasone for 24h, the cell viability result showed the viability was significantly increased in miR-146a inhibitor group, the viability was significantly decreased in dexamethasone treated group and the combined group ***(C)***. The apoptosis result showed the apoptosis rate was significantly increased in dexamethasone treated group and the combined group ***(D)***.

potential target for ONFH treatment. To investigate whether upregulation of miR-146a was participated in bone cell proliferation and apoptosis, MC3T3-E1 cells were transfected with miR-146a mimics and inhibitor, then the proliferation and apoptotic rate were analyzed. Upregulation of miR-146a could inhibit cell proliferation and induce apoptosis. At the same time, the results also showed that overexpression miR-146a could enhance MC3T3-E1 cells' sensitivity to dexamethasone stimulation. The dysregulation of proliferation and apoptosis plays a significant role in the pathogenesis of hormone-induced osteonecrosis of femoral head (ONFH)[9,23]. In this study, the upregulation of miR-146a may be one of the ONFH pathogenesis mechanisms. The reduction of the increased miR-146a caused apoptotic rate in bone cells would protect the progression of ONFH. Studies[24,25] proved that MiR-146a could regulate cell survival. It has been reported that miR-146a knockout mice showed increased number of regulatory T cells and impaired capacity to suppress the Th1 response[26]. Our investigation also provides support to this theory; however, all the researches are obtained in murine MC3T3-E1 cells. In the next studies, we will focus on the function of miR-146a in steroid-induced femoral head necrosis rat model. The pathogenesis of ONFH is complex. The dysregulation of proliferation and the aberrant apoptosis regulated by miR-146a, contribute to the necrosis of bone cells. However, other functions of miR-146a in different diseases were discovered in other studies. MiR-146a could inhibit the expression of IRAK1 and TRAF6 and impair NF-κB signaling in the immune system[27]. Besides, in the study of the progression of cartilage degradation, miR-146a promoted autophagy by decreasing Bcl2 expression level[17,28]. miR-146a

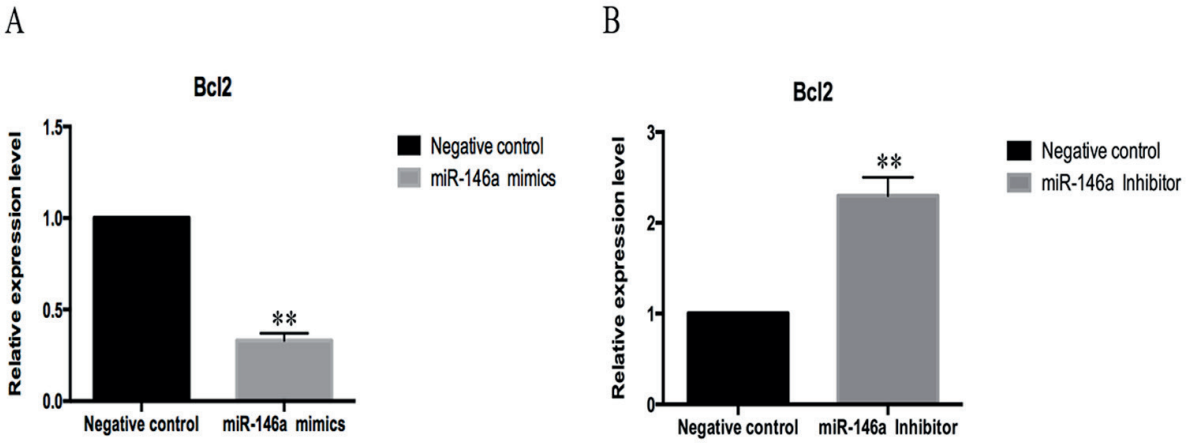



**Figure 5.** MiR-146a regulated the transcription level of Bcl2. MC3T3-E1 cells were transfected with miR-146a mimics and inhibitors, the Bcl2 mRNA level was detected by Real-time-PCR. ***(A)*** The Bcl2 expression level was significantly decreased when MC3T3-E1 cells were transfected with miR-146a mimics. ***(B)*** The Bcl2 expression level was significantly increased when MC3T3-E1 cells were transfected with miR-146a inhibitors.

could regulate the other physiological process in ONFH. It has been reported that miR-146a could regulate differentiation. Dysregulation of microRNA is associated with the pathogenesis of ONFH. Previous investigations have found elevated levels of miR-146a in OFNH specimens11. However, the mechanism of miR-146a in ONFH is not clear. MiR-146a was found to mediate the transcription of the anti-apoptotic gene Bcl2 in MC3T3-E1 for the first time in our study. MiR-146a, which regulates apoptosis by down regulating Bcl2 gene transcription, is one of the possible mechanisms. In previous reports, miR-146a plays a regulatory role in ischemic stroke by targeting gene Fbxl10[29]. Additionally, the effect of MiR-146a on the proliferation and apoptosis of human osteoarthritis chondrocytes is targeting gene TRAF630. MiR-146a also has a large number of unreported target genes. We have predicted Bcl11A may be one of miR-146a target genes and plays a regulatory role in the process of apoptosis. Bcl11A gene encodes a C2H2 type zinc-finger protein by its similarity to the mouse Bcl11a/Evi9 protein[31]. During hematopoietic cell differentiation, the expression level of Bcl11A is decreased. Its deregulation has an important role in lymphoma pathogenesis[32]. Bcl11A also own the anti-apoptotic ability similar to Bcl2. In the study of breast cancer, knockdown of Bcl11A expression in MDA-MB-231 could promote breast cancer cell apoptosis[33]. In the following researches, we will focus on the potential target genes of miR-146a.

## Conclusions

We found miR-146a as a potential reason for the apoptosis of bone cells in non-traumatic ONFH. The upregulation of miR-146a in dexamethasone stimulated MC3T3-E1 cells, and decreased the expression level of anti-apoptotic gene Bcl2. Then, leading to an increased apoptosis and decreased proliferation of MC3T3-E1 cells, overexpression of miR-146a increased the apoptotic sensitivity of MC3T3-E1 cells to dexamethasone stimulation. We demonstrated that miR-146a/Bcl2 pathways may serve as a potential therapeutic target in steroid-induced osteonecrosis of femoral head.

### Conflict of interest

The authors declare no conflicts of interest.

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