

Overactivated autophagy contributes to steroid-induced avascular necrosis of the femoral head

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Abstract. Steroid-induced avascular necrosis of the femoral head (SANFH) is a mainly bilateral complication of steroid therapy that involves extensive necrosis, and frequently occurs in young and middle-aged individuals, with a high disability rate. Autophagy is an intracellular lysosomal degradation process occurring in numerous diseases. However, the effect of dexamethasone (DXM)-induced autophagy on osteoblasts is unclear. The aim of the present study was to investigate the effects of autophagy on SANFH. In the present study, femoral head of SANFH patients was collected, and the autophagy in the samples was evaluated. In addition, cell proliferation, membrane integrity and differentiation of osteoblasts were also detected to confirm the effect of DXM on a mouse osteoblasts cell MC3T3-E1 *in vitro*. Beclin 1 and microtubule-associated protein 1 light chain 3 were used as the markers of autophagy, while the autophagy inhibitor 3-methyladenine (3-MA) was used to investigate the role of autophagy in DXM-challenged osteoblasts. Immunohistochemistry results demonstrated that Beclin1 was markedly increased in the femoral head of SANFH patients. Furthermore, the treatment of osteoblasts with DXM decreased cell viability, increased lactate dehydrogenase activity in the cell culture supernatant, and reduced the alkaline phosphatase activity and bone morphogenetic protein-2 expression in osteoblasts *in vitro*. By contrast, 3-MA treatment attenuated the cell injury induced by DXM. The present study indicates that overactivated autophagy may be an important factor contributing to SANFH, and autophagy may be a potential target for the prevention of SANFH.

Introduction

Steroids are useful drugs that can be applied in numerous serious diseases due to their ability to profoundly affect the disease course, including improvement of symptoms and reduction of disease duration (1). However, high dosages and prolonged use of steroids induces high incidence of complications and side effects, leading to severe consequences (2). Steroid-induced avascular necrosis of the femoral head (SANFH) is the most commonly reported steroid-associated osteonecrosis complication (3). SANFH is mainly bilateral and involves extensive necrosis, frequently occurring in young and middle-aged individuals, and resulting in a high disability rate (4). Numerous theories have been proposed concerning the pathogenesis of SANFH, including osteoporosis, cumulative osteocyte dysfunction, enlarged fat cells and fat embolism (5). However, the exact mechanism remains incompletely understood.

Autophagy is a complex and evolutionarily conserved process, in which abnormal cellular proteins and organelles are engulfed in autophagosomes and fused with lysosomes, forming autolysosomes (6). The fundamental function of autophagy is to maintain the metabolic balance in the cell, since autophagy is able to produce essential nutrients such as amino acids under various stress conditions, including nutrient starvation, oxidative stress and endoplasmic reticulum stress (7). Autophagy is beneficial for repair after injury; however, it is also known to also have unfavorable results. For instance, the physiological levels of autophagy are favorable to neuronal survival, but excessive or inadequate levels can be harmful and cause injury. In addition, the degree of autophagy is critical in ischemic stroke (8). In the field of cancer research, autophagy has positive and negative effects in tumorigenesis and serve an important role in the resistance of cancer cells to chemotherapy (9). Recently, studies have focused on the role of autophagy in the bone. For examples, Liu *et al* observed that the natural flavonoid isoliquiritigenin decreased microtubule-associated protein 1 light chain 3 (LC3) and Beclin 1 accumulation, suppressed autophagy and exerted anti-osteoclastogenic effects (10). Yang *et al* also identified that autophagy protected osteoblasts from apoptosis induced by advanced glycation end products through the intracellular reactive oxygen species pathway (11). However, the degree of autophagy activity in SANFH remains unclear.

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Glucocorticoids, such as dexamethasone (DXM), may regulate the autophagy activity. A previous study indicated that autophagy may be induced by DXM in chondrocyte cells, associated with a reduction in cell viability (12). Furthermore, DXM stimulates an early activation of autophagy in L6 myotubes, regulating the muscle atrophy program (13). To the best of our knowledge, it is uncertain whether DXM induces autophagy in osteoblast, or what the effect of autophagy is in SANFH.

The aim of the present study was to investigate the autophagy activity in the femoral head of SANFH patients. *In vitro*, a mouse osteoblast was used to investigate the effect of DXM-induced autophagy on proliferation, integrity and differentiation. It was demonstrated that the degree of autophagy activity in the femoral head was overactivated, while inhibition of autophagy attenuated DXM-induced cell injury in osteoblasts *in vitro*.

Materials and methods

Hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) analysis of femoral head samples. The protocol described herein were approved by the Ethics Reviewing Council of Xiangya Hospital (Changsha, China). A total of 6 femoral heads were collected from SANFH patients (4 males and 2 females; 64.3±6.4 years), and 8 femoral heads from individuals undergoing total hip replacements (5 males and 3 females, 61.5±8.7 years) were collected as the control group (between May 2013 and December 2013; Xiangya Hospital). All patients signed an informed consent approved by the Institutional Review Board of Xiangya Hospital. All harvested femoral head samples were decalcified for 14 days in 10% EDTA (pH 7.4), dehydrated and subsequently embedded in paraffin. The samples were cut into 5-μm sections and stained with H&E at room temperature to investigate the morphologic changes of femoral head from SANFH patients. Subsequently, autophagy in the sections was evaluated by IHC staining with a Beclin 1 primary antibody (sc-11427; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilution, 1:100) applied overnight at 4°C.

Cell culture. Mouse osteoblast MC3T3-E1 cell line was purchased from the Institute of Cell Bank for Biological Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal-calf-serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 mg/l streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Cell proliferation assay. The MC3T3-E1 cells were seeded into 96-well culture plates (5×10³ cells/well) and treated with DXM (0.01, 0.1, 1 and 10 μmol/l; cat. no. D1756; Sigma-Aldrich; Merck KGaA) with or without an autophagy inhibition (3-methyladenine; 3-MA; 2.5 mM; Sigma-Aldrich; Merck KGaA), for 24 or 48 h. Next, 10 μl cell counting kit-8 reagent (CK04; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well and incubated for 40 min. The viability of the cells was measured at 450 nm

using a microplate reader (SpectraMax 250; GE Healthcare Life Sciences, Chalfont, UK). Cell viability (%) was calculated based on the optical density (OD) values, as follows: (OD of treated sample - blank)/(OD of control sample-blank) × 100.

Collection of supernatant of cell treatment. The MC3T3-E1 cells were seeded into 6-well culture plates (1×10⁶ cells/well) and treated with DXM (0.01, 0.1, 1 and 10 μmol/l) with or without 3-MA for 24 or 48 h at 37°C. The cell culture medium was subsequently centrifuged at 2,500 × g for 10 min at 4°C. The supernatant was collected for analysis.

Lactate dehydrogenase (LDH) activity assay. Cell injury was evaluated using the LDH Cytotoxicity Assay kit (C0016; Beyotime Institute of Biotechnology, Haimen, China). Cells were seeded in 96-well culture plates (1×10⁴ cells/well) and the LDH activity was determined following the protocol provided by the kit's manufacturer. The absorbance of the samples at 490 nm was measured using a microplate reader (SpectraMax 250; GE Healthcare Life Sciences).

Alkaline phosphatase (ALP) activity. Cells were seeded into 12-well plates at a density of 5×10⁴ cells per well. Subsequent to culturing for 24 h or 48 h at 37°C, osteogenic differentiation of the cells was measured on the basis of ALP activity using an ALP assay kit (Sigma-Aldrich; Merck KGaA).

Protein extraction and western blot analysis. Total proteins were isolated from cells using radioimmunoprecipitation assay lysis buffer (P0013; Beyotime Institute of Biotechnology). A BCA protein assay (Thermo Fisher Scientific, Inc.) was adapted to measure the total protein concentration in the samples. Equal amounts of proteins (40 μg) were separated by 10% SDS-PAGE (Amresco, LLC, Solon, OH, USA) and transferred onto polyvinylidene difluoride membranes (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The membranes were then blocked with 5% w/v non-fat dried milk dissolved in Tris-buffered saline and 0.1% Tween-20 (pH 8.3; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) for 1 h. Subsequently, the membranes were incubated with primary antibodies at 4°C overnight, following standard procedures. The primary antibodies used were: Bone morphogenetic protein-2 (BMP-2; sc-9003; 1:1,000), Beclin 1 (sc-11427; 1:1,000) and LC3 (sc-292354; 1:1,000; all from Santa Cruz Biotechnology, Inc.). β-actin was used as an internal control (SAB5500001, Sigma-Aldrich; Merck KGaA; 1:7,500). Samples were then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (A0545, Sigma-Aldrich; Merck KGaA; 1:1,000) at room temperature. Protein band intensities were quantified using Quantity One® software (version 4.62; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard deviation and were analyzed using one-way analysis of variance. Statistical analyses were performed using SPSS version 19.0 for Windows (IBM SPSS, Armonk, NY, USA). For all tests, differences with P<0.05 were considered as statistically significant. Each experiment was repeated at least three times.

Results

Autophagy is overactivated in SANFH. In the control group, there were rich hematopoietic cells and relatively fewer lipocytes. The bone trabeculas were regularly arrayed, with complete structure and clearly visible osteocytes. However, in the patient group, H&E staining demonstrated bone marrow structure disturbance, marrow cell necrosis and debris assembly (Fig. 1, upper images). Subsequently, Beclin 1 expression in the femoral head sections was detected by IHC in order to evaluate the autophagy activity. It was observed that Beclin 1 expression was upregulated in the patient group when compared with the normal group (Fig. 1, lower images). These results indicate that overactivated autophagy may be associated with the pathology of SANFH.

DXM impairs the proliferation, integrity and differentiation of mouse osteoblasts. DXM is one of the most commonly used steroids in clinical practice and experimental research. Various concentrations of DXM (0.01, 0.1, 1 and 10 $\mu\text{mol/l}$) were added to MC3T3-E1 osteoblast cultures for 24 or 48 h. The results indicated that DXM inhibited the proliferation of osteoblasts in a dose-depended manner (Fig. 2A and B). In addition, the inhibition ratio of DXM was significantly higher at 48 h in comparison with that after 24-h incubation ($P<0.05$; Fig. 2C).

LDH is released from the cells when the cellular integrity is damaged, thus the LDH release from osteoblasts was also investigated in the present study. LDH activation in the supernatant of osteoblasts was significantly increased after DXM treatment (0.1, 1 and 10 $\mu\text{mol/l}$) for 24 h, and this increase was more apparent following DXM treatment for 48 h (Fig. 2D and E).

ALP activity and BMP-2 expression are typically used as the markers of osteoblast differentiation. In the current study, DXM was not found to affect the ALP activity after treatment for 24 h; however, ALP activity was significantly reduced after treatment with DXM (0.01, 0.1, 1 and 10 $\mu\text{mol/l}$) for 48 h (Fig. 2F and G). Furthermore, the BMP-2 expression was also significantly decreased after treatment with DXM (1 and 10 $\mu\text{mol/l}$) for 48 h (Fig. 3). These results indicate that DXM treatment impairs the proliferation, integrity and differentiation of mouse osteoblasts.

DXM triggers autophagy in osteoblasts. Subsequently, the present study investigated the potential mechanism underlying the osteoblast injury induced by DXM. As the autophagy is overactivated in SANFH, it was presumed that DXM triggers autophagy contributing to osteoblast injury. Beclin 1 and LC3 were selected as the markers for autophagy. The results revealed that DXM (1 and 10 $\mu\text{mol/l}$) significantly increased Beclin 1 and LC3 expression levels after treatment for 24 h ($P<0.05$), whereas lower concentrations of DXM (0.01 and 0.1 $\mu\text{mol/l}$) had no marked effect ($P>0.05$). However, after treatment for 48 h, 0.1 $\mu\text{mol/l}$ DXM treatment also significantly increased Beclin 1 and LC3 expression levels ($P<0.05$; Fig. 4). These results indicate that DXM triggers autophagy in osteoblasts in dose- and time-depended manners.

Inhibition of autophagy rescues osteoblast cell injury induced by DXM. Since DXM was found to induce osteoblast cell

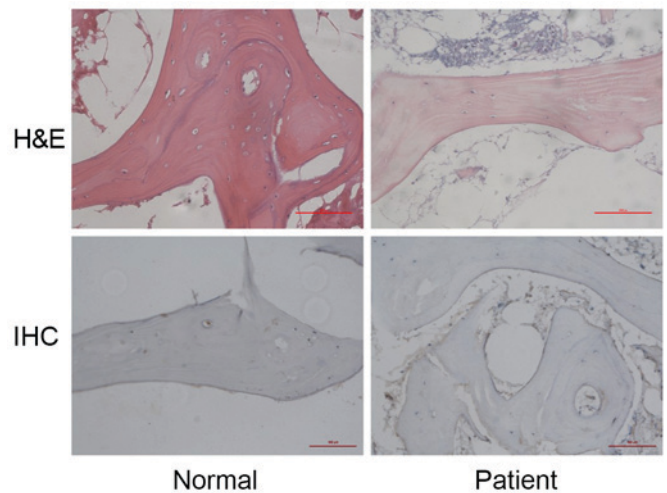


Figure 1. H&E staining (upper panel) and IHC staining for Beclin 1 antibody (lower panel) in femoral head samples from controls and SANFH patients. Bar=100 μm . H&E, hematoxylin and eosin; IHC, immunohistochemical; SANFH, steroid-induced avascular necrosis of the femoral head.

injury and trigger autophagy, the current study further investigated whether inhibiting autophagy rescues from cell injury induced by DXM. Thus, 2.5 mM 3-methyladenine (3-MA) was added to inhibit autophagy. The 3-MA treatment significantly decreased the DXM (1 $\mu\text{mol/l}$)-induced Beclin 1 expression after 24 and 48 h incubation ($P<0.05$; Fig. 5A). In addition, the number of osteoblasts was evidently increased in the 3-MA + DXM group compared with the DXM alone group at the two time points ($P<0.05$; Fig. 5B). 3-MA also reduced the LDH activity in the supernatant of osteoblasts treated with DXM (1 $\mu\text{mol/l}$) for 24 and 48 h ($P<0.05$; Fig. 5C), while it significantly increased the ALP activity of osteoblasts treated with DXM (1 $\mu\text{mol/l}$) for 48 h ($P<0.05$; Fig. 5D). These results indicate that inhibition of autophagy with 3-MA is able to rescue cell proliferation, integrity and differentiation of osteoblasts induced by DXM.

Discussion

The present study revealed that autophagy was overactivated in SANFH samples. In addition, DXM was demonstrated to trigger autophagy, as well as to decrease cell proliferation, cell integrity and differentiation of osteoblasts in dose- and time-depended manners. Inhibition of autophagy with 3-MA was shown to rescue from osteoblast cell injury induced by DXM. To the best of our knowledge, this is the first study reporting that overactivated autophagy is a mechanism underlying osteoblast loss in SANFH.

SANFH is a progressive disease with bone marrow cell and osteocyte death, resulting in collapse of the femoral head. Osteonecrosis can result in debilitation and adversely affect the quality of life, frequently requiring surgical intervention. Thus far, there are no effective preventive measures for SANFH (14). Although multiple theories underlying this complication have been proposed, no pathophysiologic mechanism has been identified as the etiology for the development of osteonecrosis of the femoral head (15). In the present study, the IHC results revealed that Beclin 1 protein expression was increased in the

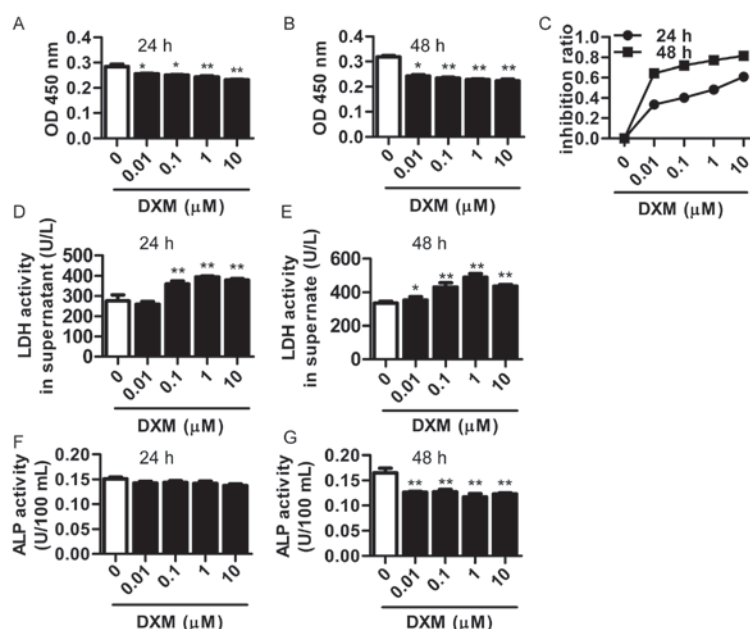


Figure 2. DXM impaired the proliferation and integrity of mouse osteoblasts *in vitro*. Proliferation of osteoblasts after DXM treatment for (A) 24 h and (B) 48 h, and (C) inhibition ratio of osteoblasts. DXM increased the LDH release at (D) 24 h and (E) 28 h after treatment, in a dose-dependent manner. ALP activity was decreased after DXM treatment for (F) 24 h and (G) 48 h. * $P < 0.05$ vs. 0 μM group; ** $P < 0.01$ vs. 0 μM group. DXM, dexamethasone; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

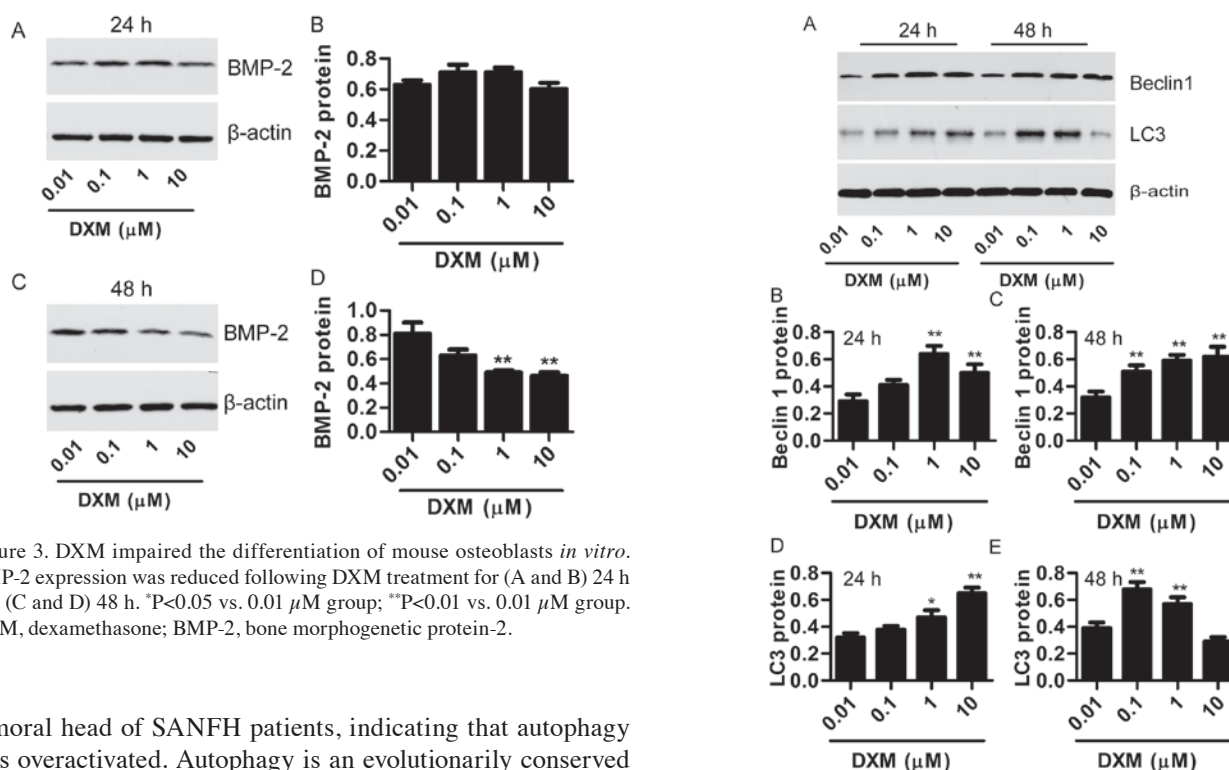


Figure 3. DXM impaired the differentiation of mouse osteoblasts *in vitro*. BMP-2 expression was reduced following DXM treatment for (A and B) 24 h and (C and D) 48 h. * $P < 0.05$ vs. 0.01 μM group; ** $P < 0.01$ vs. 0.01 μM group. DXM, dexamethasone; BMP-2, bone morphogenetic protein-2.

femoral head of SANFH patients, indicating that autophagy was overactivated. Autophagy is an evolutionarily conserved mechanism that links to several cellular pathways (16). An increasing number of studies support that autophagy can have both positive and negative effects in various diseases, such as in anti-angiogenesis therapy (17), the development of cancer (18) and cerebral ischemia (8). However, it remains unclear whether or not overactivated autophagy serves a beneficial role in SANFH.

Glucocorticoid administration is often overlooked as the most common cause of nontraumatic osteonecrosis. Glucocorticoids are a class of corticosteroids that are prescribed

Figure 4. DXM triggered autophagy in osteoblasts in dose- and time- dependent manners. (A) Western blot analysis of osteoblast lysates to detect the expression of Beclin 1, LC3 and β -actin proteins. The relative expression of Beclin 1 in osteoblasts after DXM treatment for (B) 24 h and (C) 48 h. The relative expression of LC3 in osteoblasts after DXM treatment for (D) 24 h and (E) 48 h. * $P < 0.05$ vs. 0.01 μM group; ** $P < 0.01$ vs. 0.01 μM group. DXM, dexamethasone.

for numerous diseases, including rheumatoid arthritis (19), septic shock (20) and IgA nephropathy (21). However, high-dose or

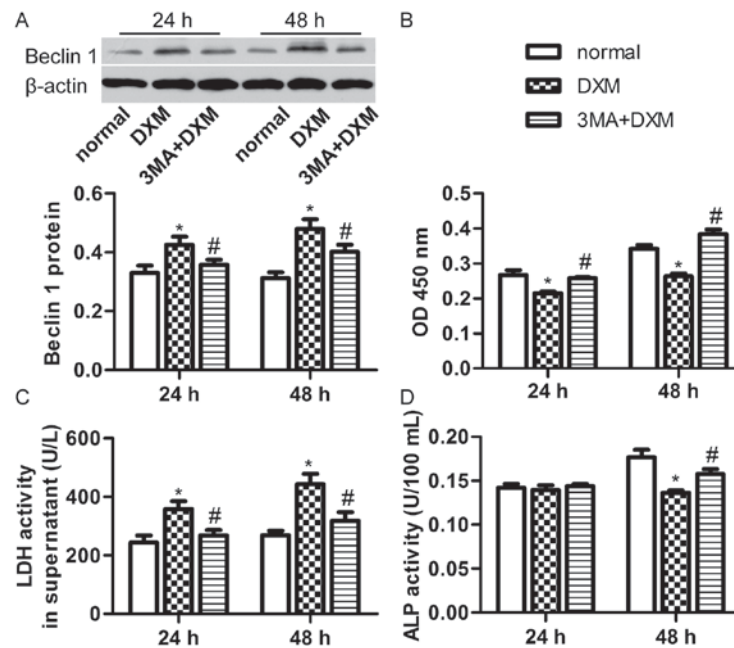


Figure 5. 3-MA, an inhibitor of autophagy, rescued the proliferation, integrity and differentiation of osteoblasts induced by DXM. (A) Western blot analysis of osteoblast lysates detecting Beclin 1 and β -actin. (B) Cell proliferation was detected by cell counting kit-8, showing that 3-MA increased the cell number of osteoblasts. (C) LDH activity in supernatant of DXM-challenged osteoblasts was reduced by 3-MA. (D) ALP activity of DXM-challenged osteoblasts at 48 h was increased by 3-MA. * $P < 0.05$ vs. normal group; # $P < 0.05$ vs. DXM group. DXM, dexamethasone; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

abnormal use of glucocorticoids results in bone disease. DXM is one of the most commonly administered glucocorticoids in clinical practice. The current study observed that DXM inhibited the cell proliferation of osteoblasts, increased the LDH activity in the supernatant, and decreased the ALP activity and BMP-2 expression in osteoblasts. These results indicate that DXM is an injury factor in osteoblasts. Similarly, Ding *et al* demonstrated that DXM induced apoptosis of osteocytic and osteoblastic cells via activating TAK1 (22), which supports the findings of the present study. The current results also revealed that DXM triggered autophagy in osteoblasts; therefore, in consideration of the overactivated autophagy in the femoral head, it is presumed that autophagy serves an important role in DXM-induced osteoblast cell injury. Finally, incubation of osteoblasts with 3-MA, an inhibitor of autophagy (23), resulted in decreased expression of Beclin 1, while it also rescued the cell proliferation, integrity and differentiation of osteoblasts induced by DXM.

However, the effect of autophagy in DXM-induced osteonecrosis remain controversial. Shen *et al* observed that DXM induced apoptosis in chondrocytes, and autophagy protected chondrocytes from glucocorticoids-induced apoptosis via ROS/Akt/FOXO3 signaling (24). Zhao *et al* also demonstrated that high doses of DXM reduce the ATDC5 chondrocyte cell viability by inducing autophagy (25). Therefore, in the current study, it is hypothesized that the degree of autophagy determines the protective or adverse role in DXM-induced cell injury.

In conclusion, the present study revealed that autophagy was overactivated in the femoral head of SANFH patients, while DXM treatment impaired the cell proliferation, integrity and differentiation of osteoblasts *in vitro*. Finally, inhibiting autophagy with 3-MA attenuated the cell injury induced by DXM treatment. Thus, autophagy may be a potential target for the prevention of SANFH.

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