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Original Article

Alendronate prevents glucocorticoid-induced osteonecrosis of the femoral head in rats

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Abstract: Objective: Osteonecrosis of the femoral head (ONFH) frequently results in collapse and subsequent degenerative joint disease of the hip. Bisphosphonates (BPs) therapy has been reported to retard osteoporosis; however, the effect of BPs on the prevention of glucocorticoid-induced ONFH remains controversial. *Methods*: In the current study, we investigated the state of MC3T3-E1 in vitro, in the presence of dexamethasone (DEX) with or without alendronate (ALN) treatment and detected osteogenesis-associated proteins, namely osteopontin (OPN) and osteocalcin (OCN). In in vivo studies, we investigated the preventive effect of ALN on methylprednisolone (MP)-induced ONFH in rats. We employed micro-CT scanning, angiography, and histologic and immunohistochemical analysis to demonstrate ALN's effect on the subchondral trabeculae of the femoral head. *Results*: In in vitro study, we observed that high-dose DEX clearly inhibited osteogenic differentiation of MC3T3-E1. OPN and OCN expression levels in MC3T3-E1 were downregulated by DEX; however, the suppressive effect could be reserved when ALN was supplemented. The mineralization of MC3T3-E1 was improved significantly in the DEX+ALN group compared to that of DEX solely. In in vivo study, the parameters of micro-structure of trabecular bone were greatly ameliorated by ALN. Moreover, angiography of the femoral head revealed a protective role of ALN on osseous circulation. Combined effects of ALN contributed to decreased incidence of MP-induced ONFH in rats. *Conclusion*: ALN counteracts the suppressive effect of DEX on MC3T3-E1 cells, which benefits the trabecular bone and microcirculation of the femoral head in rats and can prevent glucocorticoid-induced ONFH.

Keywords: Osteonecrosis of femoral head, dexamethasone, alendronate, MC3T3-E1, rat

Introduction

Osteonecrosis of the femoral head (ONFH) is a progressive and degenerative disease that commonly occurs in young adults. It is mainly due to the loss or compromise of blood flow to the femoral head and bone progenitor deficiency. It may progress to femoral head collapse and require a total hip replacement if the necrotic bone lesion is not treated early and efficiently. Core decompression associated with autologous bone marrow grafting is usually used in the early precollapse stage of ONFH to avoid arthroplasty.

Glucocorticoids are effective for many therapeutic purposes such as autoimmune and inflammatory disorders, but long-term and high-dose glucocorticoid administration usually causes side effects such as hyperglycemia, obesity, hypertension, osteoporosis, and osteonecrosis [1]. No effective preventive measures or therapies for glucocorticoid-induced ONFH are currently available. Zhang *et al.* found that Vitamin K_2 could prevent glucocorticoid-induced osteonecrosis of the femoral head in rats-a finding that, however, lacks clinic evidence [2]. Activation of the glucocorticoid receptor can induce both autophagy [3-5] and apoptosis [3, 4, 6-9]. Further, glucocorticoid-induced cell death is mediated through initiation of autophagy [10]. Low-dose dexamethasone (DEX) induced osteogenesis, and the maximal stimulatory effect was seen at 1×10^{-8} M, while high-dose DEX inhibited the growth of osteoblast precursor cells and the maximum inhibition was observed at 10^{-5} M [11].

Bisphosphonates (BPs) are potent inhibitors of bone resorption and are widely used for pre-

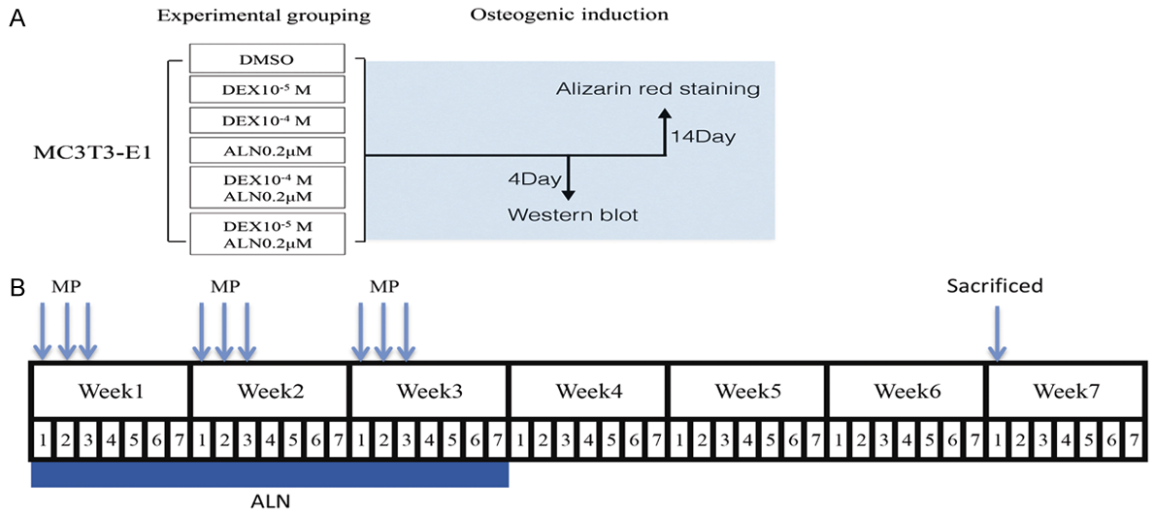

Figure 1. In vitro experiment and animal treatment protocol. (A) In in vitro experiment, cells were divided into six groups as shown in (A). (B) Animals were divided into four groups. The control group received no treatment. Animals in the MP group were intramuscularly injected with MP. Animals in the MP+ALN group were fed ALN 0.2 mg/kg/d daily and intramuscularly injected with MP as in the MP group. All samples were obtained 6 weeks after the first injection of MP.

venting and treating osteoporosis [12, 13] and other diseases that cause bone mass loss, such as Paget's disease, bone metastases, and multiple myeloma, to prevent pathologic fractures [14]. Some physicians also use BPs to treat ONFH in clinical practice. As the representative of BPs, alendronate (ALN) is developed to reduce vertebral and non-vertebral osteoporosis by inhibiting osteoclast function [15] Another role of ALN is prevention of osteocyte and osteoblast apoptosis by activating ERK [16]. ALN can also induce autophagy [17]. It is an effective agent against resorption of bone matrix by osteoclasts and has been widely used in patients with established osteoporosis and in patients with risk factors for developing osteoporosis. Clinicians and researchers believe that ALN could prevent or delay femoral head collapse and reduce the need for total hip arthroplasty in ONFH. Evidence from human studies suggests that prolonged treatment with ALN suppresses bone formation *in vivo*. However, the controversial mechanism is still unknown.

Recently, great interest has emerged regarding the possible mechanisms that BPs may affect other bone cells besides osteoclasts, such as osteoblasts and osteocytes [16, 18]. Indeed, some BPs can regulate osteoblast functions that depend on the concentration and type of BPs as well as the experimental models used, although with varying or contradictory effects, for example, osteonecrosis of the jaw [19-21]. In this context, we hypothesized that ALN can prevent the incidence of glucocorticoid-induced ONFH. To support our analysis, we evaluated the effects of ALN on cell differentiation and osteogenesis-associated protein expression *in vitro* as well as changes in subchondral trabeculae and blood supply of the femoral head *in vivo*.

Materials and methods

Cell culture

As previously described in another study [41], the murine calvaria-derived osteoblastic-like MC3T3-E1 cells were maintained in α minimum essential medium (α-MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. They were used in all experiments when adherent MC3T3-E1 propagated to a density of 80%~90%. The cells were plated at the same cell density and divided into six groups: (1) control group; (2) DEX group 1 fed with 10^{-5} M DEX; (3) DEX group 2 fed with 10^{-4} M DEX; (4) ALN group treated with 0.2 μM ALN; (5) DEX+ALN

group 1 treated with 10^{-5} M DEX and additional 0.2 μM ALN; (6) DEX+ALN group 2 treated with 10^{-4} M DEX and 0.2 μM ALN (**Figure 1A**).

Osteogenic induction

MC3T3-E1 cells were plated at a density of 5,000 cells/cm^2 in 48-well plates and cultured in α-MEM with 10% FBS. Each group's basic medium was supplemented with 10^{-2} M β-sodium glycerophosphate, 50 μg/m ascorbic acid, and 10^{-7} M DEX after 48 h to induce osteogenic differentiation. The medium was replaced every two or three days. For Alizarin red staining, MC3T3-E1 cells were taken from all the above-mentioned six groups.

Alizarin red staining

After osteogenic induction for 14 days, cells grown in 48-well plates were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 45 min at 4°C, then rinsed twice with distilled water and stained with 40 mM alizarin red S (2% aqueous, Sigma) for 5 min. These cells were rinsed again with PBS before visualization by microscopy. Positive staining was represented as a red/purple color.

Western blot

Total proteins isolated from cells were extracted with a cell lysis buffer supplemented with proteinase inhibitor on ice, and protein concentration was detected with a bicinchoninic acid assay. Then, the protein extract was boiled before use. A 20 μg sample of proteins was separated by electrophoresis on SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels and transferred onto a PVDF (polyvinylidene difluoride) membrane. After blocking with 5% defatted milk at room temperature for 1.5 h, the membranes were labeled with primary antibodies of osteopontin (OPN) (Boster, Wuhan, China), osteocalcin (OCN) (Abcam, Cambridge, England), and β-actin (Cell Signaling Technology, Beverly, MA) at 4°C overnight and then immersed in the secondary antibody working reagent of anti-rabbit IgG (1:1000) at 37°C for 1 h. Immunodetection was performed using ECL reagent (Santa Cruz Biotechnology, Santa Cruz, CA). The target bands were semi-quantitatively quantified by analyzing blot intensity with a gel image-processing system using β-actin as the loading control.

Animal model and grouping

The current study was approved by the ethics committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Forty Sprague-Dawley rats were divided into four groups. The control group received no treatment. Animals in the methylprednisolone (MP) group were intramuscularly injected with MP 20 mg/kg/d for 3 continuous days per week, with an injection period of three weeks (total MP: 200 mg/kg). Animals in the MP+ALN group were fed ALN 0.2 mg/kg/d daily and intramuscularly injected with MP as in the MP group, accompanied by consecutive ALN feed until the fourth week. All samples were obtained 6 weeks after the first injection of MP (**Figure 1B**).

Micro-CT scanning

The femoral head of half the rats from four groups were scanned with a micro-CT scanner at a voxel of 9 microns to evaluate bone morphologic changes. We use CTAn software to transfer 2-D images and quantify the trabecular bone parameters of the upper-outer subchondral bone of the femoral head, including bone mineral density, bone volume, bone volume per tissue volume, trabecular thickness, trabecular pattern factor, and trabecular number.

Angiography

The remaining rats of the four groups were perfused with Microfil (MV-112, Flow Tech, Inc., Carver, MA), which was injected through the abdominal aorta until a persistent outflow of the compound exited the caudal vein after cardiac perfusion with heparinized saline and 5% paraformaldehyde solution. Subsequently, the rats were placed at 4°C for 2 h to ensure polymerization of the contrast agent. Femoral heads were fixed with 10% formalin for 24 h. The samples were scanned through micro-CT as described above and then decalcified with a 10% EDTA (ethylenediaminetetraacetic acid) solution. After decalcification, the samples were scanned again. Finally, the vessels of the femoral head were reconstructed using CTVol software.

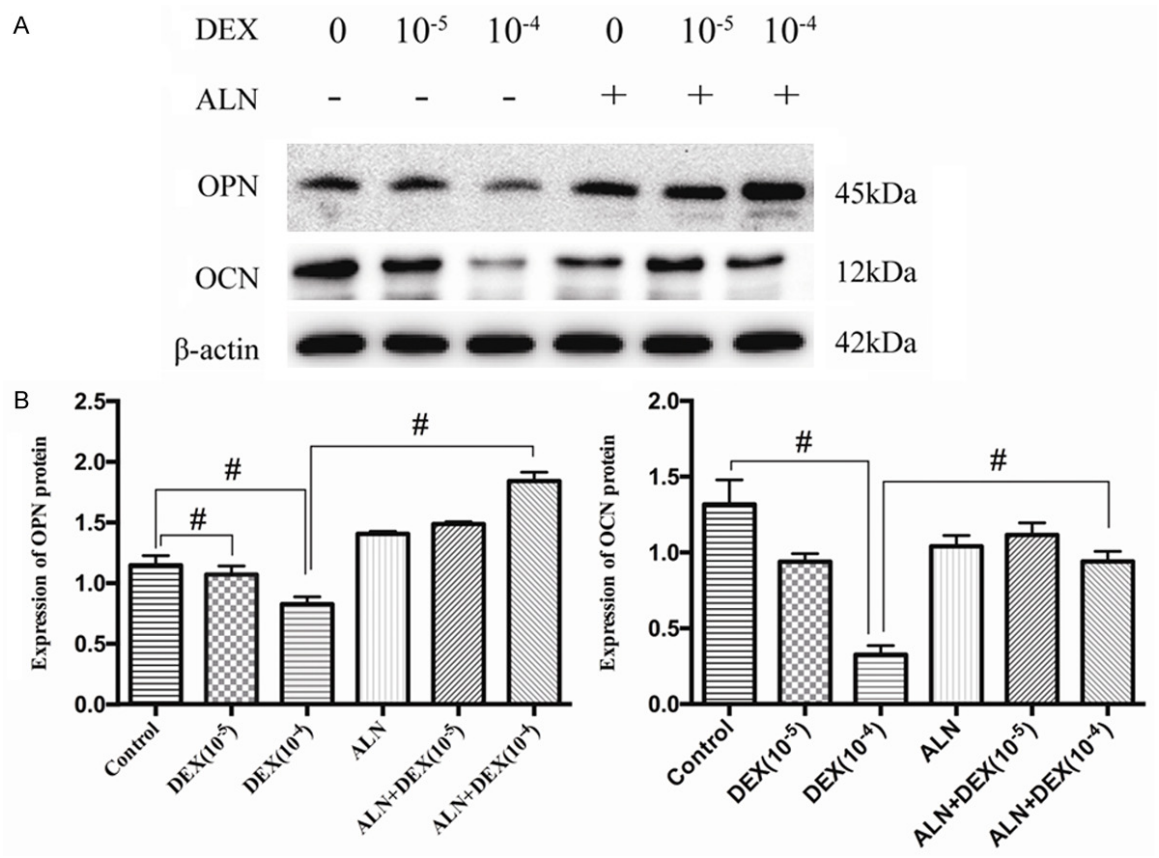

Figure 2. OPN and OCN expression in MC3T3-E1 treated with different concentrations of DEX and DEX plus ALN. A. Western blotting showed DEX and combined use of ALN significantly affected the expression of OPN and OCN. B. Analysis showed the expression of OPN and OCN was decreased by MP, and combined use of 0.2 μM ALN significantly enhanced the protein expression. All values were expressed as mean ± standard deviation. All results were repeated three times. (#, significant difference versus the MP group, P<0.05).

Histologic and immunohistochemical analyses

After decalcification and paraffin embedding, femoral heads were cut into 5-mm-thick sections in the coronal plane and then incubated at 60°C for 1.5 h. To evaluate the trabecular structure, the sections were stained with hematoxylin and eosin. The representative sections were incubated in 3% hydrogen peroxide for 5 min and then deparaffinized, antigen retrieved, stained with primary anti-OCN (Abcam, Cambridge, England) for 30 min, and then incubated with appropriate biotinylated secondary HRP-conjugated anti-rabbit antibodies. The sections were stained with DAB (diaminobenzidine), counterstained with hematoxylin, and then mounted with Permount™ solution (eBioscience, San Diego, CA). Photomicrographs were acquired using a LEICA DM 4000. Finally, the integrated option density of target protein and total area of trabecular bones were measured with the software Image-Pro Plus to analyze the images.

Statistical analysis

Experiments were repeated at least three times with consistent results obtained. SPSS 20.0 (SPSS, Inc., Chicago, IL) was used to analyze the values in each group. Data comparisons among the groups were performed using one-way analysis of variance with Dunnett's *post hoc* analysis. Quantitative data are expressed as means ± standard deviation. Significance was set at $P<0.05$ (two-tailed).

Results

ALN promotes osteogenic differentiation of MC3T3-E1 cells in vitro

Following treatment with different levels of DEX supplemented with/without ALN for preosteo-

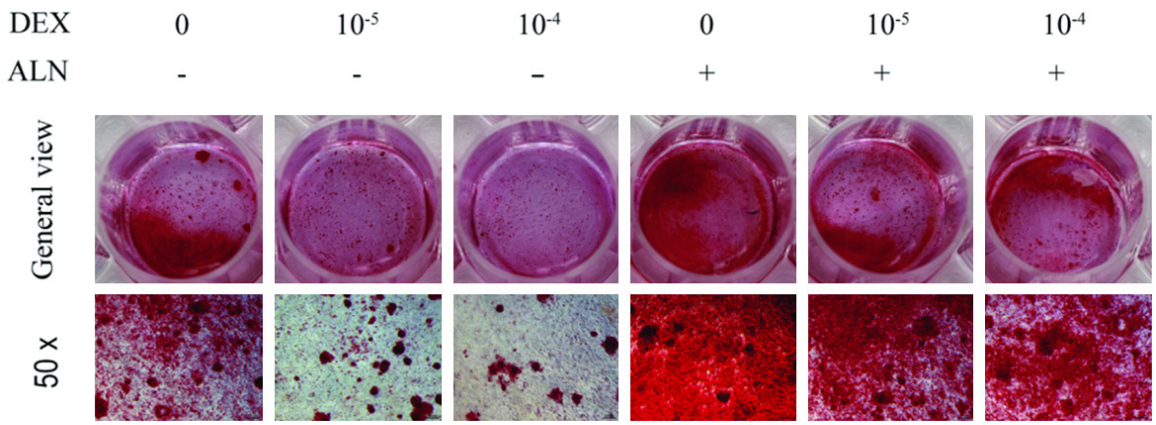

Figure 3. Effects of DEX and DEX plus ALN on osteogenic differentiation of MC3T3-E1. Alizarin red staining of MC3T3-E1 exposed to 10^{-5} M or and 10^{-4} M DEX and DEX plus 0.2 μM ALN as the control.

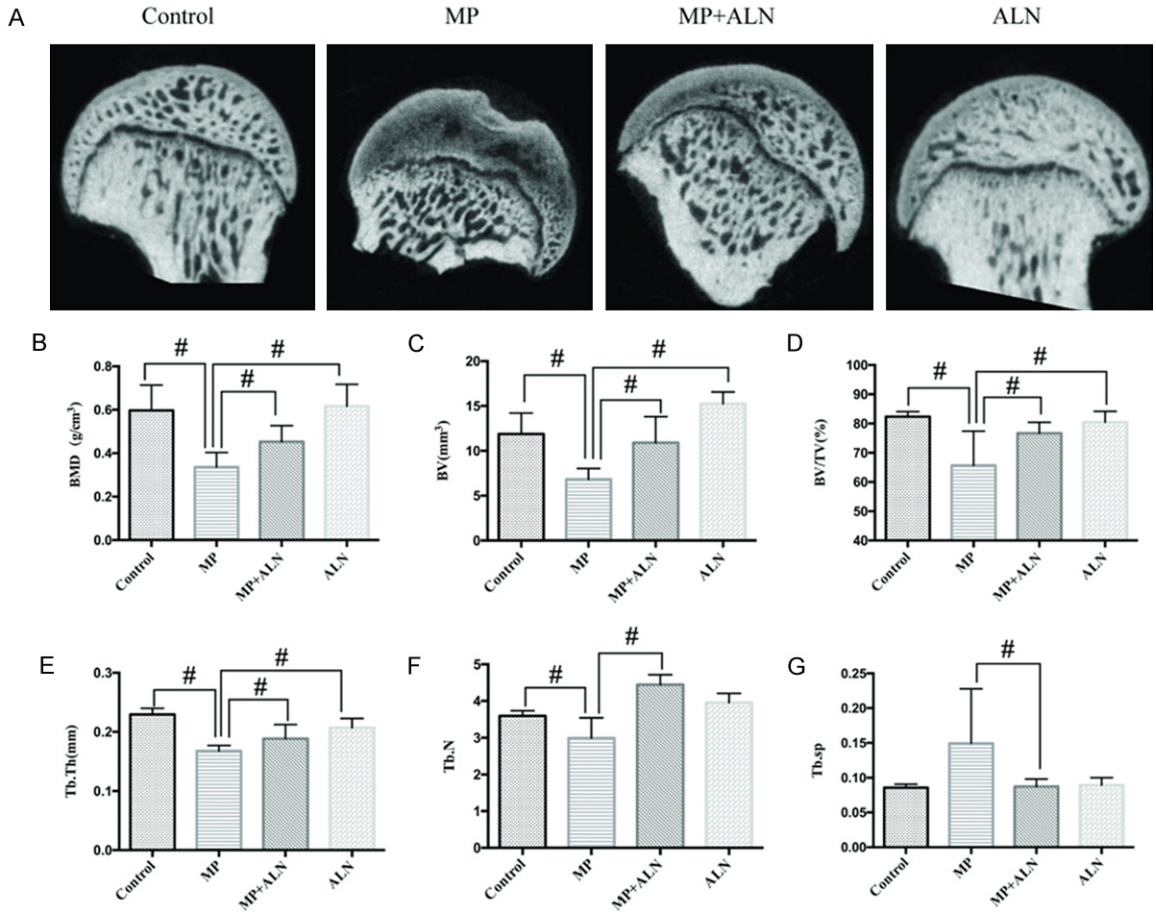

Figure 4. Micro-CT evaluation of the subchondral bone of the femoral head. A. 2-D images of the coronal section of the femoral heads. B-G. Morphometric analysis showed callus parameters of the upper-outer subchondral bone of the femoral heads. (#, significant difference between two groups, P<0.05).

blastic MC3T3-E1 cells, the expression levels of OPN and OCN were detected by western blotting. DEX treatment decreased the expression level of OPN and OCN, while supplementation of 0.2 μM ALN clearly upregulated OPN and OCN expression level. OPN and OCN activity in

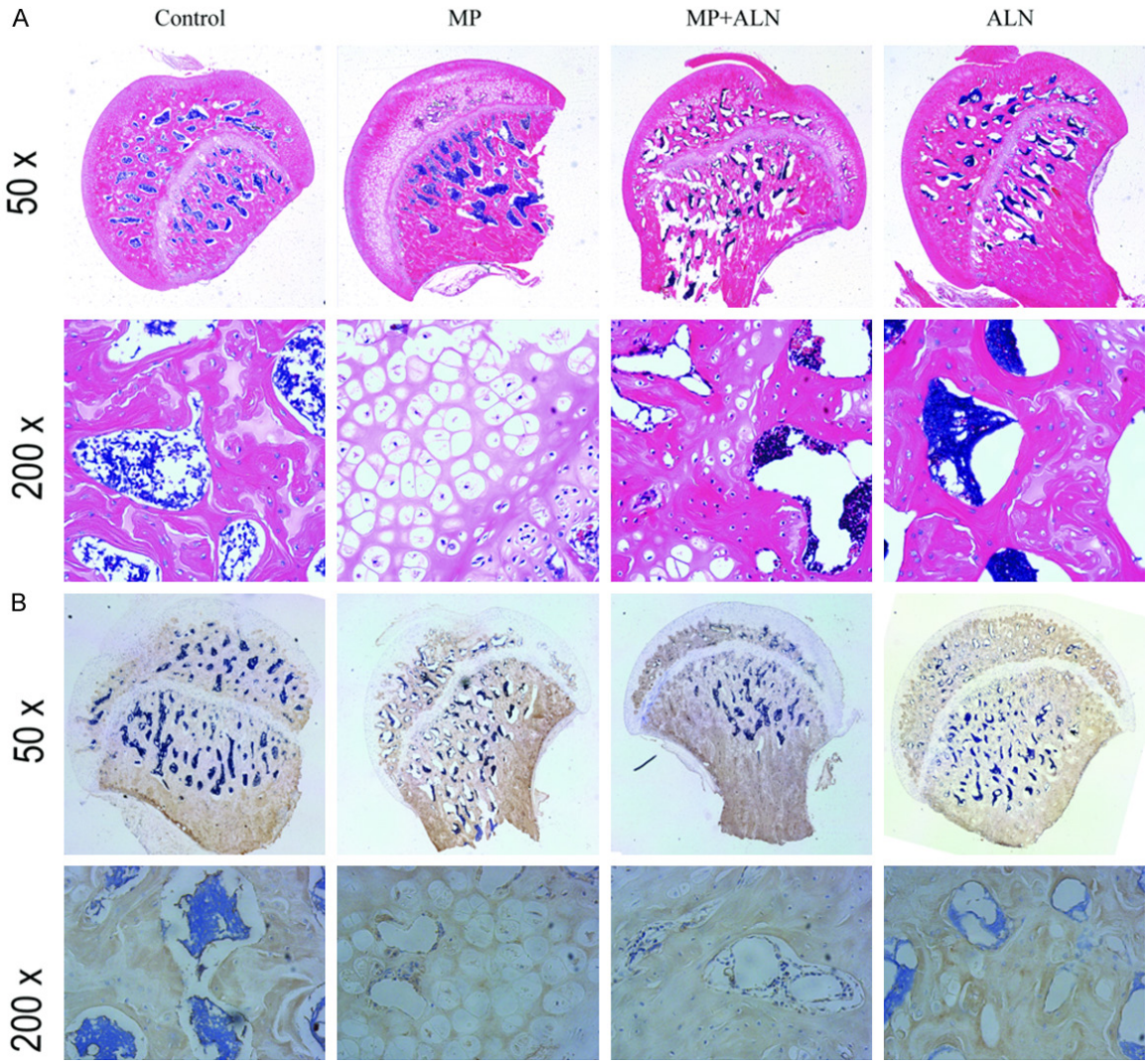

Figure 5. Histologic analyses of paraffin sections of the femoral head. A. Hematoxylin and eosin staining of coronal sections of representative femoral heads in each group. The MP group showed fewer trabeculae left than the other groups. B. Immunohistochemical staining of OCN of the coronal sections of representative femoral heads in each group. More positive staining was observed in the MP+ALN group than in the MP group.

MC3T3-E1 induced after 4 days were downregulated by DEX in different concentrations and upregulated when combined with ALN. (**Figure 2**).

Alizarin red staining showed less calcium nodules in the DEX group than in the control group after 2 weeks. The mineralization of MC3T3-E1 was improved more significantly when ALN was added to DEX-treated cells. (**Figure 3**).

ALN relieves incidence of ONFH and improves bone quality of the trabeculae

Trabecular changes of the subchondral area of the femoral head were detected through micro-CT scanning conducted six weeks after the first injection of MP. ONFH appeared in eight rats in the MP group based on micro-CT images, while no obvious osteonecrosis was observed in the ALN group. Bone mineral density of the femoral head in the MP group was 267.0 ± 15.47 mg/cm^3, which was significantly lower than that in the control group. The supplementation of ALN could significantly increase the mineral density. In addition, the bone parameters had similar performance between the MP+ALN and control groups. (**Figure 4**).

Hematoxylin and eosin staining showed typical subchondral necrosis with little trabeculae left

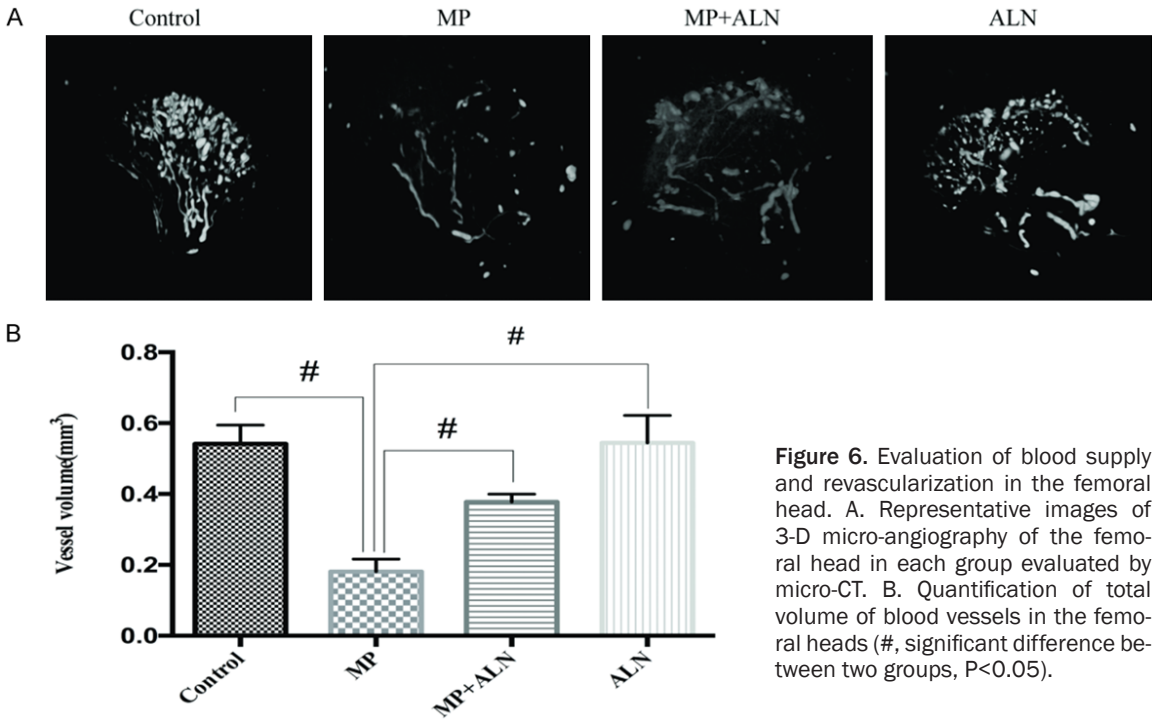

Figure 6. Evaluation of blood supply and revascularization in the femoral head. A. Representative images of 3-D micro-angiography of the femoral head in each group evaluated by micro-CT. B. Quantification of total volume of blood vessels in the femoral heads (#, significant difference between two groups, $P<0.05$).

in the subchondral area in animals of the MP group. No obvious osteonecrosis was observed in the majority of the femoral head in the ALN group. More trabeculae appeared in the femoral head in rats of the MP+ALN group than in rats of the MP group (**Figure 5A**). Immunohistochemical staining was used to detect OCN expression in each group. The results showed more positive staining in the ALN group than in the MP group, and there was no significant difference detected between the MP+ALN group and the control group (**Figure 5B**).

ALN ameliorates blood supply of the femoral head

It is convenient to use Microfil perfusion and micro-CT to detect the blood supply of the femoral head. Quantitative parameter analysis was conducted on the 3-D-reconstructed micro-CT images. The MP group had only trunk vessels left in the femoral head. Vessels of the femoral head in rats treated with MP+ALN were significantly denser, although there were fewer than in the control group. (**Figure 6A**).

Quantitative parameter analysis was conducted on the 3-D-reconstructed micro-CT images by morphometric analysis. The total volume of blood vessels of the femoral head in the MP group was significantly lower than those in the group without any treatment, while the whole volume of blood vessels treated with MP+ALN were greater than those in the MP group but lower than those in the control group. (**Figure 6B**).

Discussion

Clinically, patients often suffer osteoporosis with long-term glucocorticoid usage [22]. The reduced number of osteoblasts is believed to occur mainly due to decreased osteoblast differentiation and apoptosis of osteoblasts and osteocytes [22, 23]. These patients with osteoporosis have been shown to have fewer osteoblasts in their bone. The histologic studies showed that animals injected with glucocorticoid have decreased bone volume, osteogenesis-associated proteins, and blood vessel volume [24, 25]. Glucocorticoids could directly induce osteoblast injuries [26]. Thus, osteoblasts cultured with glucocorticoid are used to create the cellular model of glucocorticoid-associated osteoporosis.

Jureus *et al.* reported that patients with osteonecrosis treated with BPs have a better outcome than the placebo group [27]. However, the evidence for the clinical outcomes is limited

[28, 29]. BPs have been proven to play an important role in bone metabolism in *in vitro* and animal studies. The *in vitro* study by Basso *et al.* found that highly potent BPs caused cytotoxic effects on osteoblasts, which reduced the production and expression of proteins that play an important role in bone matrix synthesis and mineralization [12] In animal models, BPs were developed to decrease bone resorption and increase new bone formation during non-weight-bearing treatment of ischemic osteonecrosis in a pig model [30].

In our *in vitro* study, we observed that high-dose DEX clearly inhibited osteogenic differentiation of MC3T3-E1 and induced apoptosis, which is consistent with previous studies. Yamaguchi's study had revealed that BPs promoted osteoblast differentiation [31]. In the current study, we discovered ALN significantly increased MC3T3-E1 differentiation treated with glucocorticoid. In addition, ALN may stimulate proliferation of osteoblasts and inhibit apoptosis of osteocytes and osteoblasts [32]. These may be related to the discovery that BPs could prevent the femoral head osteonecrosis.

OPN and OCN are initially identified in osteoblasts as mineralization-modulatory matrix proteins [33], which are markers of differentiation to control differentiation in bone marrow stromal cells [34, 35]. In *in vitro* studies, we observed similar results for the DEX group and some degree of upregulation in the ALN group compared to that in the DEX group, especially in the group containing 0.2 μM ALN with the most obvious promotion. These results have shown that ALN could activate transcription of some osteogenic-related proteins to promote mineralization.

In our *in vivo* study, we found that ALN could protect the blood supply of the femoral head. According to previous research, GC could decrease vessel volume and blood supply of femoral heads [36, 37]. This may evidence that ALN protects the femoral head from osteonecrosis, but the mechanism remains unclear.

Moreover, ALN administration induced clearly improved bone volume in the femoral head, while obvious decrease was found in rats with MP supplementation. No evident decrease was observed in rats in the ALN and MP group, and the trabecular parameters in the ALN group were also significantly improved. Therefore, we believe that ALN could protect the femoral head in rats with MP administration.

Many studies have proven that BPs could cause osteonecrosis of the jaw as a contradictory effect, but the pathologic mechanism is not clear. Some researchers found that BPs can upregulate the parathyroid hormone (PTH), which stimulates bone remodeling by activating PTH receptor (PTH1R). They considered it one of the causes of osteonecrosis of the jaw. In addition, the dose and duration time were close to the disease [38, 39].

This study has some limitations. We found ALN could prevent DEX-induced ONFH in the rat; however, many meta-analyses of randomized control trials have indicated BPs have no efficacy on ONFH [28, 40]. The current study cannot completely explain the different results between the humans and the rodents. Many studies confirmed beneficial results of BPs on the prevention of ONFH. Comprehensive effects on osteogenic homeostasis of BPs should be investigated further in the future. Randomized prospective controlled clinical trials and *in vitro* studies on the regulation mechanisms and signaling pathways are mandatory.

In conclusion, ALN is a beneficial antagonist for the suppressive effect of DEX on osteogenic progenitors. The underlying mechanisms include acceleration of osteoblast differentiation and promotion of osteogenesis-associated protein expression, which combine and contribute to the prevention of glucocorticoid-induced ONFH in rats. ALN is a good choice of treatment to prevent glucocorticoid-induced osteonecrosis.

Acknowledgements

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Disclosure of conflict of interest

None.

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