CLINICAL ARTICLE

Denosumab Can Prevent Collapse in Patients with Early-Stage Steroid-Induced Osteonecrosis of the Femoral Head by Inhibiting Osteoclasts and Autophagy

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Objective: The osteoclastic bone resorption inhibitors might have positive effect in preventing femoral head collapse in patients with osteonecrosis of the femoral head (ONFH). However, as a novel osteoclastic inhibitor, whether denosumab can prevent collapse in steroid-induced ONFH remains unknown. This study aims to evaluate the treatment effect of denosumab and the potential protective mechanism.

Methods: This was a retrospective study. A total of 161 patients with steroid-induced ONFH who underwent denosumab treatment were reviewed, and 209 untreated patients were selected as controls. Their clinical characteristics and radiological exam results were obtained. Patients were treated with 60 mg denosumab every 6 months for 2 years. The primary outcome was the incidence of femoral head collapse at 2 years after the initial diagnosis of ONFH. Secondary outcomes included the Harris hip score, progression of osteosclerosis, increase in necrotic area, bone marrow oedema relief, and bone mineral density increase in the femoral head. The Mann–Whitney U test and chi-square tests were performed to identify the differences between the continuous and categorical variables, respectively. A multivariate logistic regression model was built to identify the factors associated with the treatment effect of denosumab.

Results: The incidence of femoral head collapse was 42.24% (68/161) in the denosumab group and 54.07% (113/209) in the control group ($\chi^2 = 5.094$, $p = 0.024$; relative risk = 0.787, 95% CI = 0.627–0.973). The excellent-good rates of the Harris hip score were 63.98% (103/161) in the denosumab group and 44.98% (94/209) in the control group ($\chi^2 = 13.186$, $p < 0.001$). The incidence of osteosclerosis progression in the denosumab group was 55.28% (89/161), which was significantly higher than that in the control group (43.54%, 91/209, $\chi^2 = 5.016$, $p = 0.025$). Meanwhile, a significant increase in bone mineral density was identified in 29.19% (47/161) and 7.18% (15/209) of patients in the denosumab and control groups, respectively ($\chi^2 = 31.600$, $p < 0.001$). The osteoclastic cytoplasm expression of LC3-II was more positive in the control group than in the denosumab group (immunohistochemistry scoring: 3.58 ± 2.27 *vs* 6.33 ± 2.64, $Z = -2.684$, $p = 0.007$). A total of three independent factors were considered to be associated with the positive treatment effect of denosumab, the time of first denosumab administration (OR = 2.010, 95% CI = 1.272–3.177), osteosclerosis (OR = 1.583, 95% CI = 1.024–2.445), and the necrotic area before denosumab administration (medium necrotic area: OR = 2.084, 95% CI = 1.245–3.487; large necrotic area: OR = 2.211, 95% CI = 1.255–3.893).

Conclusions: The current study demonstrated that denosumab had a positive effect on preventing femoral head collapse in patients with steroid ONFH. This effect might be closely associated with the inhibition of osteoclasts and their autophagy.

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Whether osteoclastic bone resorption inhibitors (such as bisphosphonates) can provide a positive effect in preventing femoral head collapse in patients with osteonecrosis of the femoral head (ONFH) is still debated. For instance, the S3 Guideline[1] for nontraumatic adult femoral head necrosis suggested that bisphosphonates delayed structural femoral head damage and collapse and reduced the amount of pain, with a grade 2+ level of evidence and medium recommendation grade. Jamil *et al.*[2] carried out a multicenter, randomized control trial and concluded that bisphosphonates should be recommended as an effective treatment for childhood femoral head avascular necrosis due to Perthes disease. Agarwala *et al.*[3] stated that both oral alendronate-only therapy and bisphosphonate combination therapy retard the progression of the disease, reduce the rate of collapse, and, hence, reduce the need for joint replacement surgery. However, some other studies have drawn opposite conclusions. In a systematic review from Villa *et al.*,[4] 12 randomized control trials were reviewed and analyzed. The bisphosphonate treatment showed no improvement in radiologic or functional outcomes. Lee *et al.*[5] carried out a multicenter, randomized controlled trial with 110 patients. They did not find any evidence that zoledronate is effective in preventing the collapse of the femoral head or the necessity of total hip arthroplasty in patients with ONFH.

These studies demonstrated that bone resorption inhibitors could prevent collapse in some patients diagnosed with ONFH, but not all. Some researchers have tried to explain the reason for the inconsistency of these results. Hong *et al.*[6] pointed out that the detailed indications of ONFH for alendronate treatment should be further clarified, as should a number of patient-specific factors. Therefore, the most important question is which patients might benefit from bone resorption inhibitors. Among them, the etiology of ONFH is considered to be the most important. For example, Li *et al.*[7] reviewed several studies and concluded that bisphosphonates could improve bone architecture in animal studies, but those results did not translate into either symptomatology or end-stage complications and management in human studies. This might be because most animal models of femoral head necrosis are steroid induced. However, an epidemiological study showed that the majority (45.3%) of femoral head necrosis in humans is idiopathic.[3] Only 12% of femoral head necrosis cases are steroid induced.[3] Sheng *et al.*[8] found that in an animal model of steroid osteonecrosis, a significant effect of alendronate on inhibiting osteoclastic-regulated bone resorption and maintaining the bone structure in the femoral head could be identified. Therefore, considering the osteoclastic activation effect of steroids, it is reasonable to believe that osteoclastic bone resorption inhibitors might have a positive treatment effect for steroid-induced ONFH, and it might reduce the occurrence of femoral head collapse. However, in ONFH caused by other etiology, anti-osteoclast treatment might not have a significant effect, since osteoclastic activation might not play such an important role in the pathogenesis of non-steroid-ONFH.

Compared with bisphosphonates, denosumab is an alternative choice that could also inhibit osteoclastic and bone resorption. Cummings *et al.*[9] reported that denosumab is a fully human monoclonal antibody to the receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL) that blocks its binding to RANK, inhibiting the development and activity of osteoclasts, decreasing bone resorption, and increasing bone density. It is being used for the treatment of osteoporosis and giant cell tumors. For instance, Bone *et al.*[10] reported that twice-yearly treatment with 60 mg denosumab could provide sustained reductions of bone turnover in both early and later postmenopausal women with low bone mass. Deeks *et al.*[11] suggested that denosumab reduced the risk of vertebral, nonvertebral, and hip fractures and increased BMD across skeletal sites *versus* placebo in a clinical trial, with these benefits maintained over up to 10 years' therapy in the extension. The drug was also more effective in improving BMD than bisphosphonates, including in women switched from a bisphosphonate regimen, in 1-year trials. Some other studies also suggested that denosumab also had treatment effect on bone metastases and giant cell tumor.[12,13] However, there is no report regarding whether denosumab could prevent femoral head collapse in patients with ONFH. As the osteoclastic inhibitor, denosumab might have pharmacological effect similar to bisphosphonates. Due to the suspicion above that an osteoclastic inhibitor could only prevent collapse in steroid ONFH, all of the patients included in this study had steroid-induced ONFH. The hypothesis is that denosumab could reduce the incidence of femoral head collapse. The aims of this study were (1) to determine whether denosumab can prevent femoral head collapse in steroid ONFH; (2) to determine the possible mechanism of this preventive effect; and (3) to identify patients more likely to have a satisfactory treatment effect from denosumab.

Patients and Methods

Participants

Patients diagnosed with steroid-induced ONFH treated at our institute from January 2018 to July 2020 were retrospectively reviewed. The inclusion criteria were as follows: (1) a history of steroid use, with a total equivalent

methylprednisolone dose >20 mg/kg; (2) ONFH diagnosed within 2 years after the initial use of steroids; (3) precollapse of the femoral head (Association Research Circulation Osseous, ARCO stage I–II); and (4) patients who received denosumab. The exclusion criteria were as follows: (1) combined use of other anti-osteoclastic drugs or bisphosphonates; (2) collapse of the femoral head already identified at initial diagnosis; (3) other hip disease or injury, such as femoral neck fracture, hip dysplasia, or osteoarthritis; (4) other potential causes of ONFH, such as sickle cell anemia or abuse of alcohol; and (5) follow-up less than 2 years, lack of medical records or radiological images. If bilateral ONFH was identified, the patient was regarded as two independent individuals.

This study was approved by the Institutional Review Board of the Third Hospital of Hebei Medical University (No. K2021-027-1) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients before participation in the study.

Initial sample size calculations assumed that the incidence of femoral head collapse in patients who were treated with denosumab would be 30%. In the control group, the incidence of collapse would be 50%. When the power of the test and significance level were set to 0.80 and 0.05, respectively, the number of required patients and controls were 93 each. Therefore, the sample size in this study was considered to be sufficient.

Clinical and Radiological Evaluation

Data Collection

Baseline information and radiological exams were obtained from the medical records and the Picture Archiving and Communication Systems of our institute. These included the demographic characteristics, lifestyle factors, steroid dose, radiological evaluation, and treatment strategy. The radiological exams in this study included anterior–posterior view and lateral view X-ray exams of the hip joint, computed tomography (CT) and magnetic resonance imaging (MRI). The Association Research Circulation Osseous (ARCO) system was used for the classification of the patients. All of these data were obtained and analyzed at the initial diagnosis of ONFH (baseline) and at the 2-year follow-up (last follow-up).

Harris Hip Score

The Harris hip score, which comprehensively evaluates the status of pain, function, and range of motion, was used for clinical evaluation. The result was considered to be excellent or good if the Harris hip score was ≥80 points; otherwise, the result was considered to be fair or poor.

Collapse of the Femoral Head

Collapse was determined based on the regular anterior–posterior view and lateral view X-ray exams of the hip joint. An electronic ruler (Fujifilm Medical Systems, Stamford) was used to measure the femoral head height. Collapse was defined as a femoral height decrease of over 2 mm on either view of the X-rays.

Necrotic Area

The necrotic area was determined on the MRI images. The necrotic area was considered to be small if <15% of the femoral head was involved, 15%–30% for medium and >30% for large. The MRI images at baseline and the last follow-up were compared to identify whether the necrotic area had expanded.

Bone Marrow Oedema

Bone marrow oedema was also determined on the MRI images. Beyond the necrotic area and sclerotic band, if homogeneous high signal intensity could be found in the screening of T2-weighted MRI, it was considered to be bone marrow oedema. Similarly, the MRI images at baseline and the last follow-up were compared to identify whether the bone marrow oedema was relieved.

Osteosclerosis

Osteosclerosis, which included both local osteosclerosis lesions and extended osteosclerosis bands, was identified on CT scans. The progression of osteosclerosis was identified by comparing the baseline CT scans and the last follow-up CT scans.

Bone Mineral Density

The mean bone mineral density of the necrotic area was evaluated by quantitative computed tomography. If the bone mineral density at the last follow-up increased more than 20% compared to the baseline bone mineral density, the increase was considered significant.

Quality Control

All radiological measurements were performed by the same surgeon. All of the measurements were repeated three times and averaged. The intraclass coefficient was used to assess intraobserver reliability. The results showed good reliability (intraclass correlation coefficient >0.9 in all measurements). The clinical evaluation (namely, the Harris hip scores) of patients was performed by two surgeons independently. If unanimous agreement on a patient's evaluation was not achieved, the interpretation by another surgeon was used as the final evaluation result.

Histological and Biochemical Exams

For patients who had undergone core decompression surgery *via* thick passage, bone biopsies were performed. The bone sample was acquired by using a trephine when preparing the decompression channel. The specimen was obtained from the necrotic area of the femoral head. This bone biopsy was routinely performed since they were in our institute and since a pathological exam could help establish the definitive diagnosis of ONFH and identify the severity osteonecrosis. If isolated thin channel core decompression surgery was

performed, bone biopsy would not be performed since no bone tissue could be acquired during this surgical procedure. The bone biopsy, namely histological exam, including hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining, were performed to explore the protective mechanism of denosumab (In fact, these histological exams also had other clinical effect, such as predicting the prognosis of ONFH. But in this study, we mainly focused on the protective mechanism of denosumab). Two standard procedures for H&E staining and IHC were performed. The pathological changes were observed under a light microscope. The results of IHC were semiquantitatively analyzed by the IHC score, which combined an intensity score and a percentage score. The intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong. The percentage of positive cells was scored as follows: 0, <5%; 1, 5%–25%; 2, 26%–50%; 3, 51%–75%; and 4, >75%. The final score was the product of the intensity score and the percentage score. The anti-LC3-II polyclonal antibody (dilution, 1:1000–1:1500; cat. no. 14,600-1-AP) and the anti-Beclin-1 polyclonal antibody (dilution, 1:1000–1:10,000; cat. 11,306-1-AP) were purchased from Wuhan Sanying Biotechnology Co. Ltd.

Intervention and Comparison

Patients were divided into two groups according to the pharmacological treatments. In this study, patients were administered 60 mg denosumab (Prolia® 1.0 ml: 60 mg) every 6 months. The first administration was begun within 2 weeks of the initial diagnosis of ONFH. The course of denosumab treatment was 2 years, which means that the total dose of denosumab was 240 mg. In the control group, no anti-osteoclastic drug was administered. In both groups, patients were advised to consume foods that were rich in vitamin D and calcium. However, routine administration of pharmacological vitamin D and calcium was not performed.

Other treatment methods for ONFH were also performed in both groups, such as reducing weight bearing and core decompression surgery. In this study, a patient was considered to be partial weight bearing if walking aids were continuously used for at least 6 months. The patient was considered to be non-weight bearing if they stayed in bed or used a wheelchair for at least 6 months. Additionally, thick passage decompression, thin passage decompression, and decompression with bone grafting were not distinguished.

Outcome

The primary outcome of interest was the incidence of femoral head collapse 2 years after the initial diagnosis of ONFH. Secondary outcomes included the Harris hip score, progression of osteosclerosis, increase in necrotic area, bone marrow oedema relief and bone mineral density increase (also at 2 years after the initial diagnosis of ONFH). The adverse effect of denosumab was also investigated.

Meanwhile, for some patients who underwent core decompression, bone biopsy was performed to evaluate the potential protective mechanism of denosumab.

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 statistical software for Windows (IBM, Armonk, NY) and Excel 2016 for Windows (Microsoft Corporation). Continuous variables are expressed as the mean ± standard deviation, and categorical variables are expressed as frequencies. Student's *t*-test was performed if the data followed a normal distribution. Otherwise, the Mann–Whitney U test was performed for comparisons between continuous variables. Chi-square tests were used to identify the differences between the categorical variables. A multivariate logistic regression model was built to identify the factors associated with the treatment effect of denosumab (only patients in the denosumab group were included in this regression analysis). For continuous variables, receiver operating characteristic curves were drawn, and the cut-off points were selected by Youden's index. A stepwise regression method was used. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated for each independent factor. A p value <0.05 was considered to be statistically significant.

Results

Baseline Characteristics

A total of 370 individuals were included in this study. Among them, 161 individuals had undergone denosumab treatment, and 209 individuals did not and were selected as controls. The details of their baseline characteristics are shown in Table 1. No difference between the groups was found regarding these characteristics, such as demographic characteristics, ARCO stage, or radiological findings.

Clinical Outcomes of Interest

The incidence of femoral head collapse was 42.24% (68/161) in the denosumab group and 54.07% (113/209) in the control group ($\chi^2 = 5.094$, $p = 0.024$). Compared to individuals who did not undergo denosumab treatment, the relative risk of femoral head collapse was 0.787 (95% confidence interval = 0.627–0.973) among individuals who were treated with denosumab. Namely, the administration of denosumab could reduce total femoral head collapse by 21.3% among individuals with steroid ONFH.

The excellent-good rates of the Harris hip scores were 63.98% (103/161) in the denosumab group and 44.98% (94/209) in the control group ($\chi^2 = 13.186$, $p < 0.001$). In the denosumab group, 88/161 (54.66%) patients showed enlargement of the necrotic area on radiological exams. In addition, 25/161 (15.53%) patients showed evidence of bone marrow oedema relief. Correspondingly, in the control group, there were 118/209 (15.53%) patients with necrotic area enlargement ($\chi^2 = 0.120$, $p = 0.730$) and 36/209 (17.22%) patients with bone marrow oedema relief ($\chi^2 = 0.190$, $p = 0.663$). No significant difference was found. In the denosumab group, osteosclerosis and bone mineral density increases were more commonly identified. The incidence of osteosclerosis progression in the denosumab group was 55.28%

TABLE 1 Baseline demographic and clinical information of patients with osteonecrosis of the femoral head receiving denosumab treatment

Demographic	Denosumab ($n = 161$)	Control ($n = 209$)	Test statistics	p
Age (years)	42.89 ± 15.11	40.52 ± 12.48	−0.193	0.847
Sex				
Male	121 (75.16%)	144 (68.9%)	1.751	0.186
Female	40 (24.84%)	65 (31.1%)		
Body mass index (kg/m^2)	25.34 ± 3.20	25.24 ± 4.03	−0.257	0.797
Smoking				
No	113 (70.19%)	139 (66.51%)	0.567	0.452
Yes	48 (29.81%)	70 (33.49%)		
Alcohol consumption				
No	127 (78.88%)	150 (71.77%)	2.444	0.118
Yes	34 (21.12%)	59 (28.23%)		
Weight bearing				
Full weight bearing	75 (46.58%)	85 (40.67%)	4.001	0.135
Partial weight bearing	20 (12.42%)	42 (20.1%)		
Non weight bearing	66 (40.99%)	82 (39.23%)		
Steroid dose[a] (mg/kg)	60.14 ± 15.98	58.17 ± 15.49	−0.390	0.697
ARCO stage				
Stage I	46 (28.57%)	53 (25.36%)	0.479	0.489
Stage II	115 (71.43%)	156 (74.64%)		
Necrotic area				
Small	116 (72.05%)	142 (67.94%)	0.770	0.680
Medium	28 (17.39%)	43 (20.57%)		
Large	17 (10.56%)	24 (11.48%)		
Bone marrow oedema				
No	116 (72.05%)	145 (69.38%)	0.312	0.576
Yes	45 (27.95%)	64 (30.62%)		
Osteosclerosis				
No	54 (33.54%)	72 (34.45%)	0.033	0.855
Yes	107 (66.46%)	137 (65.55%)		
Bone mineral density (g/cm^3)	1.06 ± 0.18	1.05 ± 0.19	−0.266	0.790
Decompression surgery				
No	67 (41.61%)	85 (40.67%)	0.034	0.855
Yes	94 (58.39%)	124 (59.33%)		

Abbreviations: ARCO, Association Research Circulation Osseous; ONFH, osteonecrosis of femoral head.; [a]Converted to the corresponding dose of equivalent methylprednisolone.

(89/161), which was significantly higher than that in the control group (43.54%, 91/209, $\chi^2 = 5.016$, $p = 0.025$). Meanwhile, a significant increase in bone mineral density was identified in 29.19% (47/161) and 7.18% (15/209) of patients in the denosumab and the control group, respectively ($\chi^2 = 31.600$, $p < 0.001$). Finally, no significant adverse effect was identified in all patients in this study.

Detailed information on the primary and secondary outcomes is shown in Table 2.

Histological and Biochemical Exams

A total of 12 patients in the denosumab group and 15 patients in the control group underwent bone biopsy when core decompression was performed. The results of H&E staining showed that in both groups, bone structure disorders could be identified, such as necrotic bone marrow cells and aggregated fragments, thinned trabeculae, and numerous empty lacunae, especially in the control group. The osteoclastic content was significantly decreased in the denosumab group (Fig. 1A). The numbers of osteoclasts were 1.84 ± 0.99 in every medium-power field (× 200) in the denosumab group and 3.22 ± 1.03 in the control group ($Z = -3.064$, $p = 0.002$; Fig. 1B).

Using IHC staining, the expression levels of LC3-II and Beclin-1 were examined. Interestingly, IHC results of LC3-II demonstrated that most positive results were located in the osteoclastic cytoplasm. In particular, in the control group, a large number of LC3-II-positive osteoclasts with large cell sizes and massive cytoplasm could be identified around the trabecula. However, in the denosumab group, in addition to lower numbers of osteoclasts, the expression of LC3-II was also decreased in osteoclasts (Fig. 2A). Meanwhile, in the denosumab group, the IHC score of LC3-II in osteoclasts was lower than that in the control group (3.58 ± 2.27 *vs* 6.33 ± 2.64, $Z = -2.684$, $p = 0.007$; Fig. 2B). In both groups, the expression of Beclin-1 was detected (Fig. 3A). However, no difference was found for the Beclin-1 IHC score (8.33 ± 2.15 *vs* 8.27 ± 2.52, $Z = -0.051$, $p = 0.981$; Fig. 3B).

TABLE 2 Primary and secondary outcomes of patients with osteonecrosis of the femoral head receiving denosumab treatment

	Denosumab (n = 161)	Control (n = 209)	Test statistics	p
Collapse of femoral head				
No	93 (57.76%)	96 (45.93%)	5.094	0.024
Yes	68 (42.24%)	113 (54.07%)		
Harris hip score				
Excellent-good	103 (63.98%)	94 (44.98%)	13.186	<0.001
Fair-poor	58 (36.02%)	115 (55.02%)		
Progress of necrotic area				
No	73 (45.34%)	91 (43.54%)	0.120	0.730
Yes	88 (54.66%)	118 (56.46%)		
Bone marrow oedema relief				
No	136 (84.47%)	173 (82.78%)	0.190	0.663
Yes	25 (15.53%)	36 (17.22%)		
Progress of osteosclerosis				
No	72 (44.72%)	118 (56.46%)	5.016	0.025
Yes	89 (55.28%)	91 (43.54%)		
Bone mineral density increasing				
No	114 (70.81%)	194 (92.82%)	31.600	<0.001
Yes	47 (29.19%)	15 (7.18%)		

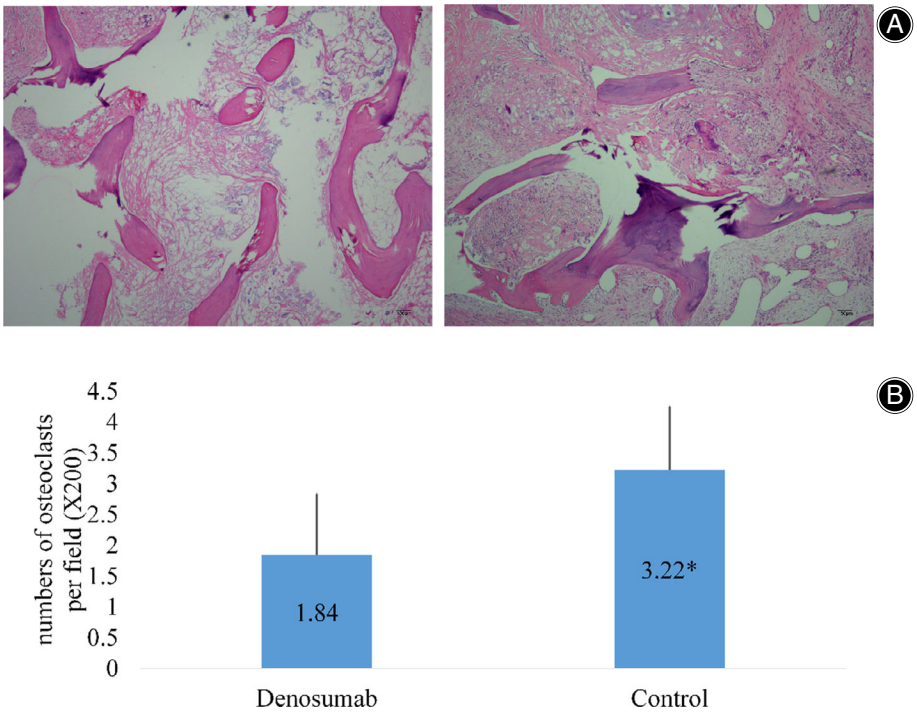

Fig. 1 Observation of femoral head structures by hematoxylin and eosin staining. (A) In both groups, bone structure disorders such as aggregated fragments, thinned trabeculae, and fibrous tissue proliferation could be identified, especially in the control group. (B) The numbers of osteoclasts in every medium-power field. *p < 0.05, compared with the control group

Factors Associated with the Treatment Effect of Denosumab

Three independent factors were found to be associated with the positive treatment effect of denosumab in this study (Table 3), the time of denosumab administration, osteosclerosis, and the necrotic area before denosumab administration. These findings indicate that denosumab should be used as soon as possible after an ONFH diagnosis. Compared with patients who underwent denosumab administration within 3.1 months after the initial diagnosis of ONFH, patients were at an approximately two-fold higher risk (OR = 2.010, 95% CI = 1.272–3.177) of femoral head collapse if denosumab was first given after 3.1 months. Second, collapse was more commonly identified in patients with osteosclerosis before denosumab treatment than in those patients without osteosclerosis (OR = 1.583, 95% CI = 1.024–2.445). Finally, the necrotic area was associated with the treatment effect of

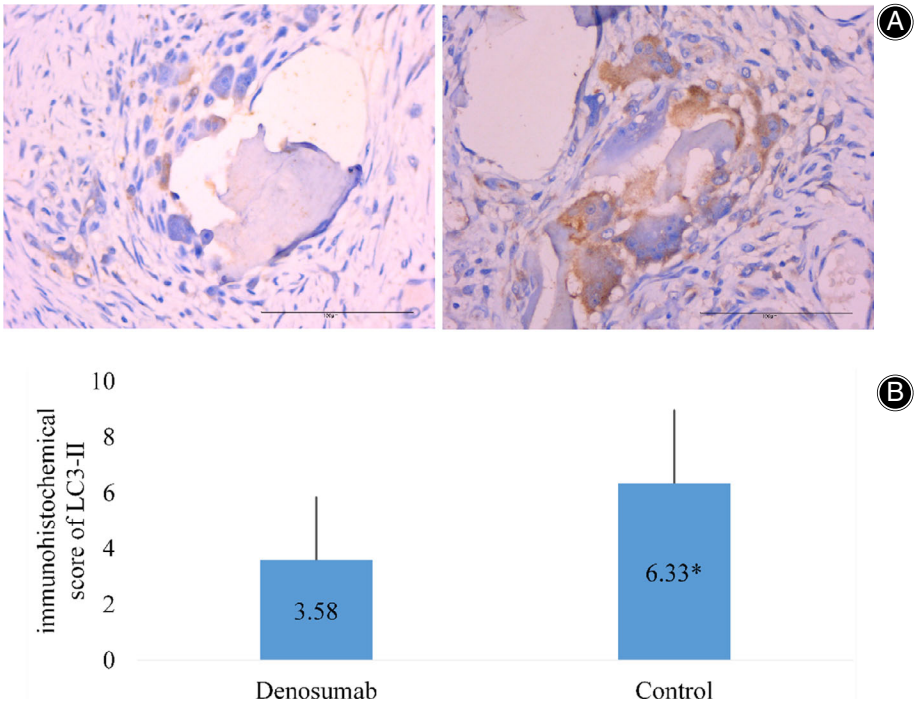

Fig. 2 Observation of osteoclasts in the femoral head by immunohistochemical staining of LC3-II. (A) In the control group, a strong positive reaction could be identified in the cytoplasm of osteoclasts, in significant contrast to the background. A trabecula was surrounded by several LC3-II-positive osteoclasts, which were in close contact with the trabecula. Part of this trabecula had already been reabsorbed by these osteoclasts. In the denosumab group, fewer osteoclasts with significantly smaller sizes were identified than in the control group. These osteoclasts were not in contact with the trabecula and were LC3-II negative. The trabecula was also intact. (B) The immunohistochemical scores of the osteoclasts. *$p < 0.05$, compared with the control group

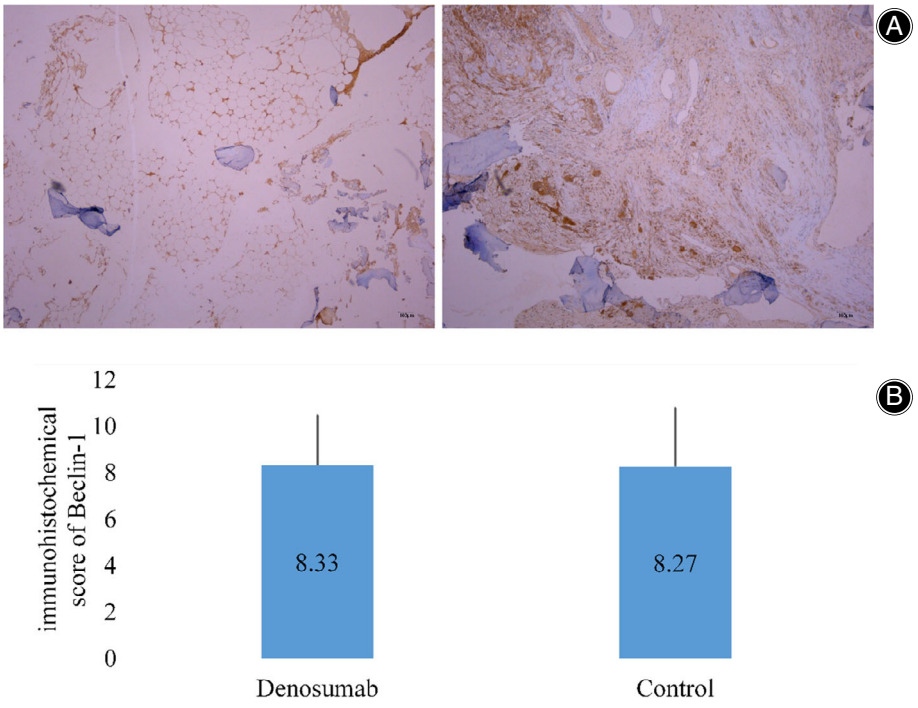

Fig. 3 Observation of femoral head structures by immunohistochemical staining for Beclin-1. (A) Positive reactions were observed in both groups. However, the expression of Beclin-1 in the background was significantly higher than that of LC3-II. (B) The immunohistochemical scores of the osteoclasts. No significant difference was found

denosumab. Compared with patients with small necrotic areas, patients with medium (OR = 2.084, 95% CI = 1.245–3.487) and large (OR = 2.211, 95% CI = 1.255–3.893) necrotic areas were more likely to suffer from collapse of the femoral head.

Discussion

This study mainly demonstrated that, as an osteoclastic inhibitor, denosumab could prevent the collapse of femoral head in steroid-induced ONFH. This protective effect might associate with the direct reducing the number of osteoclasts

TABLE 3 Factors associated with femoral head collapse in patients treated with denosumab

Factors	Odds ratio	95% Confidence interval	*p*
Time from ONFH diagnosis to denosumab use[a]			
≤3.1 months	Reference		
>3.1 months	2.010	1.272–3.177	0.003
Osteosclerosis			
No	Reference		
Yes	1.583	1.024–2.445	0.039
Necrotic area			
Small	Reference		
Medium	2.084	1.245–3.487	0.005
Large	2.211	1.255–3.893	0.006

Abbreviations: ONFH, osteonecrosis of femoral head.; [a] Cut-off point made by using the receiver operating characteristic curve and Youden's index.

and inhibition osteoclastic function by downregulating the level of autophagy in osteoclasts. Meanwhile, this study also suggested that the time of drug administration, existed osteosclerosis in femoral head, and the necrotic area were associated with the positive treatment effect of denosumab.

Treatment Effect of Denosumab

The predominated treatment effect of denosumab in patients with ONFH included three aspects: reducing the occurrence of collapse, increase the occurrence of osteosclerosis of the femoral head, and increase the bone mineral density of the femoral head. First, as hypothesized, this study demonstrated that denosumab did have a protective effect on steroid-induced ONFH. This is in contrast to several other studies that suggested that ONFH patients would not benefit from bisphosphonates.[5–7,14] We believe the reason for this difference was the patient selection. Namely, in this study, all included cases of ONFH were steroid induced. It has already been well established that glucocorticoids directly affect bone metabolism, greatly enhancing osteoclastic bone resorption and inhibiting osteogenesis.[15,16] As a monoclonal antibody against receptor-activator of nuclear factor κ-B ligand (RANKL), denosumab could promote the apoptosis of osteoclasts and inhibit the bone resorption induced by osteoclasts,[17,18] which might be the predominant reason for femoral head collapse in patients with steroid ONFH.[18] However, in ONFH induced by other aetiologies, the overactivation of osteoclasts might not play an important role in the pathogenesis of ONFH or the occurrence of femoral head collapse.[19] Because the majority of ONFH is idiopathic,[3] many studies have concluded that bisphosphonates could not prevent the collapse of the femoral head. In addition to reducing the occurrence of collapse, in this study, denosumab could also increase the occurrence of osteosclerosis of the femoral head and increase the bone mineral density of the femoral head. The former is considered to be a manifestation of bone union and necrotic area regeneration. The latter is closely associated with the mechanical strength of the bone.[20] Increased bone mineral density might also protect the affected femoral head from collapse.

Protective Mechanism of Denosumab

Some studies revealed the potential protective mechanism of bisphosphonates in patients with ONFH. In this study, the histological exams suggested that denosumab could directly reduce the number of osteoclasts in the femoral head. This was confirmed by several other studies, which showed that denosumab could either induce osteoclast apoptosis or reduce osteoclast precursor differentiation into osteoclasts.[13,17,21,22] However, in this study, the protective mechanism of denosumab went beyond that. By IHC staining, it was found that the expression of LC3-II, which is considered to be a crucial protein associated with autophagy,[23] could also be inhibited in osteoclasts by denosumab. Autophagy is a key cell physiological process that can help produce new building blocks and energy for cellular renovation and homeostasis, as well as decompose metabolic substrates.[23] A well-established example that autophagy contributes to degrading metabolic substrates[24] was that enhanced autophagy could help eliminate abnormal huntingtin protein in neurons and thus treat Huntington's disease. Furthermore, it is clear that autophagy increases the secretion of RANKL, which results in the activation of osteoclasts and bone resorption. Meanwhile, it increases the expression level of autophagic-related genes such as Atg5 and Atg12, recruits LC3 to autophagosome, and enhances the expression of RANKL, cathepsin K, NFATc1, and MMPs, which leads to increased osteoclastogenesis.[25] In the control group, multiple osteoclasts around trabeculae could be found. These osteoclasts were in contact with the trabecula. In the cytoplasm, strong positive LC3-II expression could be identified (Fig. 2A). This suggested that the trabeculae were absorbed by these osteoclasts, and the expression of LC3-II indicated that absorbed substrate (bone matrix) was decomposed by autophagy in osteoclast. However, in the denosumab group, the osteoclasts did not contact the trabeculae. Meanwhile, LC3-II was not expressed, which suggested that autophagy was not activated. This finding demonstrated that in addition to reducing the number of osteoclasts, denosumab might further inhibit osteoclastic function by downregulating the level of autophagy in osteoclasts. Previously, Fu *et al.*[26] reported that glucocorticoids could enhance osteoclast autophagy, which might also exist in steroid ONFH. This study demonstrated that denosumab have the negative effect against the autophagy activation induced by glucocorticoid, which might be a protective mechanism of denosumab. Note that this is a key point that distinguishes the protective mechanism of denosumab from that of bisphosphonates. Wasko *et al.*[27] believed that bisphosphonates could induce autophagy by depleting geranylgeranyl diphosphate. Yu *et al.*[28] suggested that autophagy could be promoted by enhancing RANKL-RANK-TRAF6 signaling. In this study, as a RANKL inhibitor, by downregulating its signaling pathway, denosumab had an autophagy inhibition effect, which was the opposite to the autophagy activation effect caused by

bisphosphonates. However, the expression of Beclin-1, an upstream protein of LC3-II, showed no difference between the two groups.[29] This finding indicated that denosumab might mainly affect the formation of autophagosomes. The induction of autophagy might be independent of Beclin-1. Of course, the sample size of the patients who underwent bone biopsy was limited. This might also be a reason why there was no difference detected regarding the expression of Beclin-1 between the two groups. Of particular note was that in several other studies focusing on the relationship between autophagy and ONFH, enhanced autophagy was considered to be a protective mechanism in ONFH. For instance, Han *et al.*[30] found that autophagy could relieve the functional inhibition and apoptosis-promoting effects on osteoblasts induced by glucocorticoids. Zhou *et al.*[31] suggested that autophagy played a crucial protective role in the pathogenesis of steroid ONFH. The enhancement of autophagy levels could be an important target for the prevention or treatment of ONFH. Zhu *et al.*[32] found that parathyroid hormone could induce autophagy to protect osteocyte cell survival from dexamethasone damage. However, in this study, the opposite conclusion was drawn: autophagy might not be a protective mechanism for the progression of ONFH but a risk factor. The different findings might be because in previous studies, osteoblasts were mainly investigated.[30–32] However, in our study, the function of osteoclasts was the focus. Therefore, this study demonstrated that the role of osteoclasts and their bone resorption function must be emphasized in the treatment of ONFH, especially in the aspect of collapse prevention.

Factors Associated with the Treatment Effect of Denosumab

Although the incidence of femoral head collapse did decrease after denosumab treatment, the current study demonstrated that over 40% of patients still suffered from femoral head collapse. The next problem is determining which patients could benefit from denosumab treatment. In the regression model in this study, three factors associated with femoral head collapse were identified. The first one was the treatment time of denosumab. The natural history of ONFH includes an ischaemia period in which the bone tissue and the trabecula become necrotic and are absorbed, followed by a regeneration period in which the blood supply is reconstructed and the bone is healed or substituted by fibro tissues.[33] Because the main pharmacological effect of denosumab is to protect the trabecula from osteoclastic bone resorption, rather than recovering after a collapse,[21] it should be administered in the early stage of ONFH. Note that even if a cut-off point is identified, we strongly recommend that denosumab should be used as soon as possible after the establishment of an ONFH diagnosis. The second factor is osteosclerosis, which is a common pathological change in the femoral head in patients with ONFH.[34] In fact, extended osteosclerosis or the formation of an osteosclerotic rim would further block the blood supply inside the necrotic area.[35–37] Therefore, denosumab might not be able to enter the necrotic area. Obviously, the local osteoclast inhibition effect would be limited by this insufficient drug concentration. A large necrotic area was associated with ONFH treatment failure.

Strengths and Limitations

The main strengths of this study included that it clinically investigated the treatment of denosumab, a novel osteoclastic inhibition, in patients with steroid ONFH. Meanwhile, the protective mechanism of denosumab had also been established *via* biopsy and biochemistry and molecular biological methods. Furthermore, factors associated with the positive reaction of the drug were also detailly investigated. However, several limitations of this study must be considered. First, collapse might have occurred during the 2-year treatment period. Therefore, the actual accumulated dose of denosumab before collapse might vary between patients. In other words, the total dose for preventing collapse was <240 mg if the collapse occurred before the last administration of denosumab. This would cause the real treatment effect of denosumab to be underestimated. Second, although no differences were found between the two groups, the treatment of ONFH was different for each individual. Therefore, the prognosis of the patient was affected by both denosumab administration and other treatment methods, such as core decompression. This might affect the accuracy of the results. Finally, only a small portion of patients underwent biopsies. Therefore, the study of the protective mechanism of denosumab might not be as accurate as the clinical evaluation. Furthermore, in this study, inhibition of autophagy was considered to be an important mechanism of action of denosumab, which could prevent the collapse of the femoral head. However, the relationship between RANKL inhibition and autophagy inhibition was not investigated further.

Conclusion

This short-term clinical follow-up demonstrated that denosumab, an osteoclastic inhibitor, had a positive effect on preventing femoral head collapse in patients with steroid ONFH. After a treatment course of 2 years, denosumab reduced femoral head collapse by 21.3%. However, the mid- to long-term clinical outcome of denosumab treatment still needs further investigation. The protective effect of denosumab might be closely associated with the inhibition of osteoclasts and their autophagy. Three independent factors associated with femoral head collapse during denosumab treatment were identified. Based on these factors, it is recommended that denosumab be administered to patients with small necrotic areas before the formation of osteosclerosis rims. Furthermore, denosumab is suitable for early-stage osteonecrosis, and which should also be administered as soon as possible after an ONFH diagnosis, especially in the first 3 months.

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Author's Contribution

All authors have read and approved the manuscript. Conceptualization: Bo Liu, Yongtai Han. Data curation: Bo Liu, Feng Gao, Xiaofei Xiu, Tao Wu. Methodology: Bo Liu, Zeming Liu, Bingshi Zhang. Writing: Bo Liu, Sikai Liu, Yongtai Han.

Authorship Declaration

All authors listed meet the authorship criteria according to the latest guidelines of the International Committee of Medical Journal Editors, and all authors approved the final version of the manuscript.

Conflict of Interest

All authors have declared that they have no conflicts of interest.

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