Abnormal hyperplasia of chondrocytes in a rat model of glucocorticoid-induced osteonecrosis of the femoral head

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Abstract. – OBJECTIVE: The aim of our study was to determine whether abnormal hyperplasia of chondrocytes occurs in glucocorticoid-induced osteonecrosis of the femoral head (GC-ONFH) using a well-established rat model.

MATERIALS AND METHODS: Rats were injected with lipopolysaccharide and methylprednisolone to induce GC-ONFH, while control animals were injected with saline (12 animals per group). Establishment of the disease model was confirmed using micro-computed tomography and hematoxylin-eosin (HE) staining of femoral head tissue sections. Chondrocyte hyperplasia was detected using HE staining and semi-quantitated using toluidine blue and saffron O staining. Expression of the autophagy marker LC3B was assessed in cartilage tissues of femoral head using immunohistochemistry.

RESULTS: GC-ONFH animals showed significantly greater area of abnormal chondrocyte hyperplasia in femoral head tissue sections than control animals. They also showed significantly higher expression of LC3B in articular cartilage of the femoral head.

CONCLUSIONS: GC-ONFH may be associated with abnormal chondrocyte hyperplasia in articular surface cartilage, which may be related to glucocorticoid-induced overactivation of autophagy.

Key Words:
   Osteonecrosis of the femoral head, Glucocorticoid, Chondrocyte, Hyperplasia, Autophagy.

Introduction

Osteonecrosis of the femoral head is a common refractory orthopedic disease, which tends to occur in young and middle-aged people[1]. One of the most common causes appears to be long duration or high doses of glucocorticoid therapy[2]: the disease occurs in 9-40% of people on such therapy[1]. The mechanisms behind glucocorticoid-induced osteonecrosis of the femoral head (GC-ONFH) are unclear. In previous research work, we discovered abnormal chondrocyte hyperplasia in the articular surface cartilage in a widely used rat model of GC-ONFH. Given that glucocorticoids can induce autophagy of chondrocytes[3-5], we wondered whether this might be related to the observed abnormal hyperplasia. Under normal conditions, autophagy provides nutrients to cells because lysosomes degrade damaged organelles, misfolded proteins, and intracellular pathogens[6]. Several studies[7-11] have suggested that autophagy in GC-ONFH serves as a stress response. It might be that glucocorticoid-induced overactivation of autophagy leads to chondrocyte hyperplasia, which in turn contributes to the pathological changes in GC-ONFH.

In our study we used the well-established rat model of GC-ONFH to verify the occurrence of chondrocyte hyperplasia and to explore a potential link with autophagy in chondrocytes.

Materials and Methods

Animals

The animal protocols in this study were approved by the Animal Care and Use Committee of West China Medical School, Sichuan University, and they complied with the "Guidelines on the humane use and care of laboratory animals" of the US National Institutes of Health. The experiments involved a total of 24 adult

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male Sprague-Dawley rats weighing 450-500 g (Dashuo, Chengdu, China). Animals were housed for 6 weeks in the Animal Center of Sichuan University before experiments.

Establishment of GC-ONFH Model

The 24 rats were randomly divided evenly into two groups. The model group was injected intraperitoneally twice with lipopolysaccharide (10 μg/kg, Sigma, St. Louis, MO, USA); the interval between injections was 24 h. At 24 h after the last injection, they were injected once a day intramuscularly for three consecutive days with methylprednisolone (40 mg/kg, Pfizer, New York, NY, USA). The control group was injected with the same volume of saline and otherwise treated in the same way as the model group.

Sample Collection

At 8 weeks after the first methylprednisolone injection, all animals were sacrificed, and the bilateral femoral heads were dissected. All the collected samples were washed twice with ice-cold phosphate-buffered saline (PBS), then one femoral head from each animal was randomly selected and fixed for 24 h in 10% formalin for further analysis as described below.

Micro-Computed Tomography

The femoral head was scanned using small-animal micro-computed tomography (PerkinElmer, Waltham, MA, USA) and 3D reconstruction was performed using the manufacturer's software. Scanning parameters were voltage, 90 kV; current, 88 A; resolution, 50 μm; and scanning time, 14 min. Trabecular bone parameters were assessed over the entire femoral head area. Bone volume/total volume (%) and trabecular number (mm^{-1}) were calculated automatically using the manufacturer's software.

Hematoxylin-Eosin Staining

Femoral head tissue was sectioned and stained with hematoxylin-eosin (HE, ServiceBio, Wuhan, China) in order to assess histomorphology, detect empty osteocyte lacunae, and assess the extent of trabecular bone destruction as described[12,13]. Formalin-fixed femoral heads were decalcified for four weeks in a 10% solution of ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, cut into sections 3 μm thick, deparaffinized in xylene, and dehydrated in ethyl alcohol. Finally, the sections were stained with HE.

Animals were considered to have GC-ONFH if their femoral head tissue showed at least one lesion with empty osteocyte lacunae or bone nucleus pyknosis, accompanied by necrosis of surrounding bone marrow cells[14]. Abnormal chondrocyte hyperplasia was detected in HE-stained sections.

Toluidine Blue Staining

Femoral head tissue sections were stained with toluidine blue (Sigma) for 30 min, which colored cartilage and osteoblasts dark blue against a light blue background. The percentage of subchondral bone area that was cartilage tissue was calculated using Image J (version 1.8.0; US National Institutes of Health, Bethesda, MD, USA) and the following formula: Cartilage % = (cartilage area above the epiphyseal line / total area of femoral head above the epiphyseal line) × 100%.

Safranin O Staining

Femoral head tissue sections were stained with Weigert hematoxylin staining solution, solid green staining solution and safranin O staining solution (Sigma). After staining, cartilage matrix appeared dark red; cartilage nuclei, blue; chondrocyte cytoplasm, red; and chondrocyte nuclei, gray-black. The percentage of subchondral bone area that was cartilage tissue was calculated using Image J and the same formula as above.

Immunohistochemistry

Femoral head tissue sections were subjected to immunohistochemistry using primary antibody (Abcam, Cambridge, UK) against the autophagy marker LC3B, in order to examine autophagy levels in the articular cartilage. After incubation with primary antibody, sections were incubated with streptavidin-biotin complex (Jackson ImmunoResearch, Philadelphia, PA, USA). Primary antibody binding was detected using a DAB kit (Abcam), and sections were counterstained with hematoxylin. Areas positive for LC3B were quantitated under an optical microscope (Zeiss, Oberkochen, Germany) using Image J.

Comparison of Different Species

Femoral head tissue sections from five patients diagnosed with GC-ONFH and from five rabbits with GC-ONFH, which were generated in the same manner as the rats, were stained with HE and compared. This part of the study was approved by the Clinical Trials and Biomedical Ethics Committee of Sichuan University West

China Hospital. Written informed consent was obtained from all patients for their tissue samples to be used in medical research.

Statistical Analysis

Statistical analysis was performed using SPSS 26.0 (IBM Corp., Armonk, NY, USA). Continuous data were presented as mean and standard deviation. Categorical data were presented as numbers or percentages. Inter-group differences in continuous data were assessed for significance using Student's t test, and differences in categorical data were found using Pearson's Chi-squared test or Fisher's exact probabilities test. Differences were considered significant if $p < 0.05$.

Results

During the experiment, one rat in the control group died of unknown causes. The model and control groups did not differ significantly from each other in body weight at baseline or at sacrifice (Table I). Micro-computed tomography showed significantly lower percentage of bone volume and bone trabecular number in the model group (Figure 1, Table I).

HE staining of femoral head sections from the control group showed even staining, normal appearance of bone cells and their nuclei, regular bone trabecular arrangement with small spacing, and smooth articular surface cartilage (Figure 2). In contrast, tissue sections from the model group showed typical signs of osteonecrosis, including many empty lacunae filling the bone trabeculae, disordered arrangement of bone trabeculae with widened spacing, some destroyed trabeculae,

and necrosis of bone marrow cells. In addition, the articular surface cartilage showed abnormal hyperplasia that had replaced the subchondral trabecular area. Nuclei in areas of abnormal hyperplasia appeared pyknotic and denatured. The incidence of GC-ONFH was significantly higher in the model group than in the control group (83.3% *vs.* 0%, $p < 0.001$), as was the incidence of abnormal chondrocyte hyperplasia (66.7% *vs.* 0%, $p = 0.004$; Table I).

Toluidine blue staining revealed no obvious pathology in the artificial surface cartilage of control rats, whereas it revealed abnormal hyperplasia in the model group, which had replaced the subchondral trabecular area (Figure 3). The artificial surface cartilage contained chondrocyte clusters of various sizes, distributed as spots, sheets and bands. The percentage of cartilage in the femoral head was significantly higher in the model group than in the control group (50.67% *vs.* 30.03%, $p < 0.001$; Table I). Similar results were observed with safranin O staining (51.55% *vs.* 31.03%, $p < 0.001$).

The histopathology in the model group correlated with upregulation of autophagy: expression of the autophagy marker LC3B in articular cartilage of the femoral head was significantly higher in the model group (Figure 4, Table I). LC3B remained stable on the autophagosome membrane until it fused with lysosomes. Therefore, the level of LC3B was proportional to the number of autophagosomes[15,16], with higher levels indicating greater autophagy.

Abnormal hyperplasia of articular surface cartilage was not observed in patients diagnosed with GC-ONFH or rabbit models of GC-ONFH (Figure 5).

Table I. Comparison between control and GC-ONFH rats.

Parameter	Control group (n = 11)	Model group (n = 12)	p
Body weight before injections (g)	496.2 ± 37.2	499.7 ± 45.9	0.844[a]
Body weight before sacrifice (g)	557.9 ± 31.4	562.7 ± 32.1	0.721[a]
Bone volume/total volume (%)	63.08 ± 3.92	45.85 ± 8.29	< 0.001[a]
Bone trabecular number per mm	6.09 ± 0.57	4.86 ± 0.62	< 0.001[a]
Incidence of GC-ONFH (n, %)	0	10 (83.3)	< 0.001[b]
Incidence of abnormal chondrocyte hyperplasia (n, %)	0	8 (66.7)	0.004[b]
Percentage of cartilage tissue area, based on			
Toluidine blue staining	30.03 ± 3.62	50.67 ± 16.94	< 0.001[a]
Safranin O staining	31.03 ± 3.87	51.55 ± 16.40	< 0.001[a]
Percentage of LC3B-positive area, based on immunohistochemistry	3.12 ± 0.75	9.52 ± 2.31	< 0.001[a]

Values are mean ± SD or n (%), unless otherwise noted. GC-ONFH: glucocorticoid-induced osteonecrosis of the femoral head.
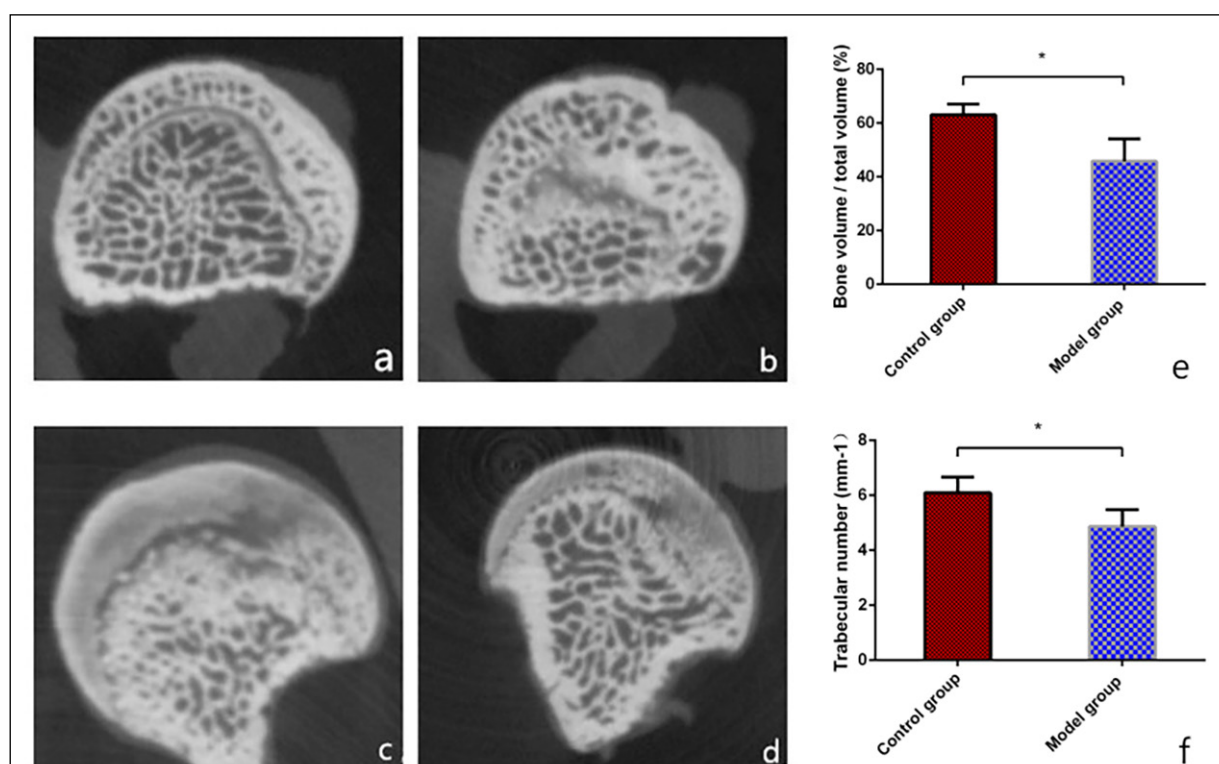[a]Student's *t*-test. [b]Pearson's Chi-squared test.

Figure 1. Micro-computed tomography. **a-b**, Two representative femoral heads in the control group. **c-d**, Two representative femoral heads in the model group. **e**, Bone volume as a percentage of total volume. **f**, Trabecular number per mm. *$p < 0.001$ between the two groups. The error bars indicate the standard deviation of the mean.

Discussion

The present study documented, for the first time, abnormal hyperplasia of articular surface cartilage in rats with GC-ONFH. We also extended this finding by linking it to upregulation of autophagy in chondrocytes, which may be helpful for future research to further clarify its mechanism.

Glucocorticoids can affect the normal physiological function of chondrocytes in some diseases of the skeletal system[17-22], impairing growth and skeletal development[23,24]. Decreased proliferation of chondrocytes has been linked to thinning of the growth plate, leading to inadequate longitudinal bone growth[17-19]. At the same time, glucocorticoids can promote chondrocyte apoptosis, as well as inhibit their growth and activity[20,21]. Apoptosis may reduce the number of chondrocytes and reduce the extracellular matrix, including proteoglycan and collagen type II, contributing to osteoarthritis[22]. The present work strongly suggests that in addition to these effects in other skeletal disorders, glucocorticoids induce abnormal chondrocyte hyperplasia in GC-ONFH.

Our work justifies further study into how glucocorticoids compromise chondrocyte function, and it points to at least one culprit: dysregulated autophagy. GC-ONFH has already been linked to autophagy[7-11] and glucocorticoids have been shown to induce autophagy in chondrocytes[3-5], but exactly how autophagy may contribute to the disease has remained unclear. Autophagy appears to be a 'double-edged sword': it can protect cells from apoptosis by removing oxidatively damaged organelles, but excess autophagy can destroy cellular components and lead to cell death[25]. We found that expression of the autophagy marker LCB in articular cartilage of the femoral head was significantly higher in the model group than in the control group. Our results here are consistent with the idea that abnormal hyperplasia of articular surface cartilage may be related to excess autophagy induced by high-dose glucocorticoids.

We further speculate that metabolic abnormality of chondrocytes contributes to the abnormal hyperplasia. Cartilage, subchondral cortical bone and subchondral trabecular bone lie near one another, and their metabolisms are coordinated.
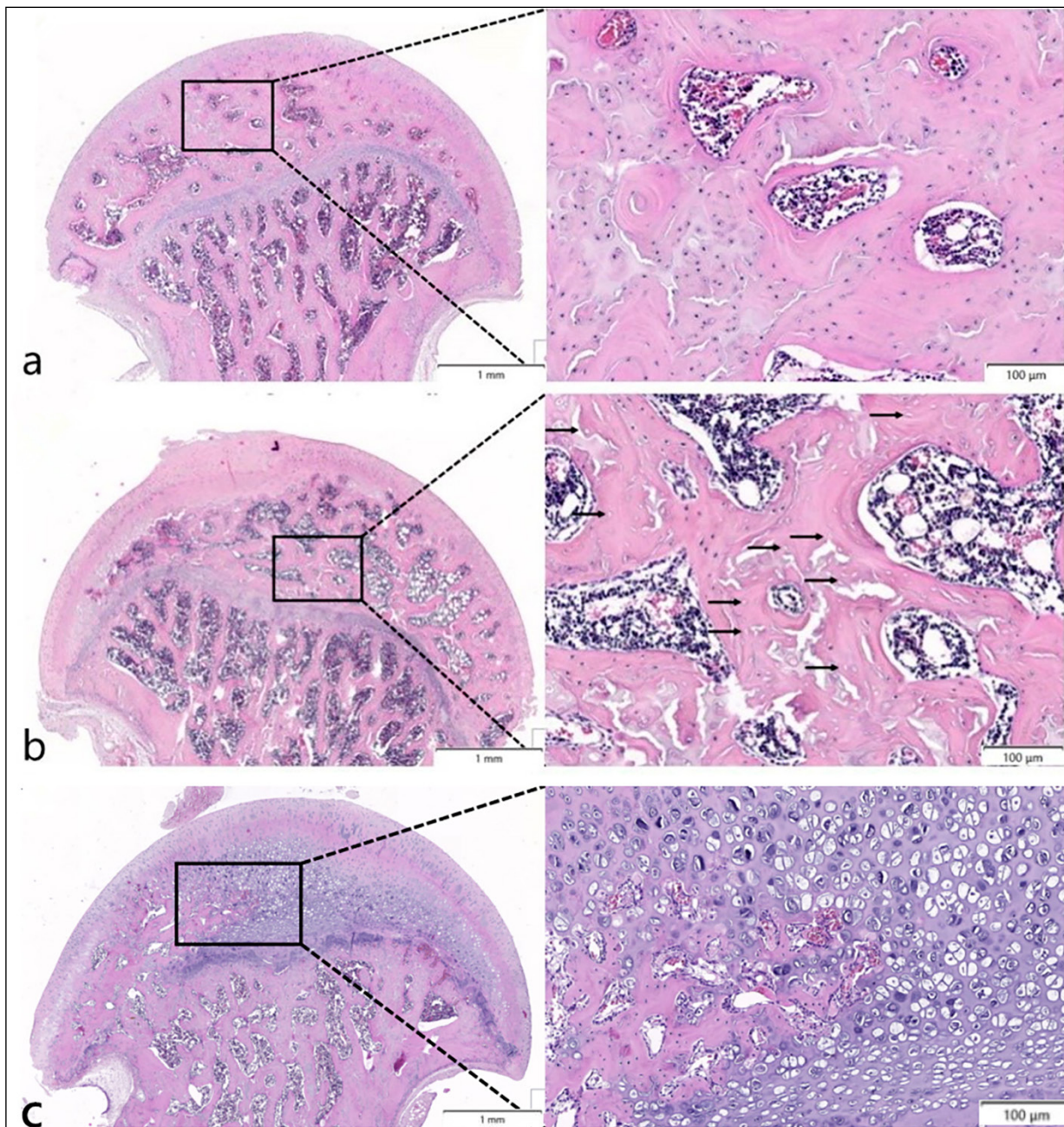
Figure 2. Representative results of HE staining of the femoral head in the control group (**a**, one animal) and the model group (**b-c**, two animals). **b**, Arrows indicate empty lacunae or pyknotic nucleus. **c**, Abnormal hyperplasia of articular surface cartilage. Boxes in the images at *left* (magnification, 20×) show the areas enlarged on the *right* (magnification, 100×).

Subchondral bone necrosis may create pressure, which normally propagates *via* the porous cortical bone endplate into the calcified cartilage layer. In GC-ONFH, these pores are blocked, preventing the cartilage from receiving adequate nutrients or eliminating metabolic waste products. As a result, adjacent subchondral cortical bone and cartilage suffer metabolic damage.

An important next step in elucidating the mechanism of abnormal hyperplasia of articular surface cartilage is to clarify why this pathological manifestation is observed in rat models of the disease, but not in patients or rabbit models of the disease. Studies should examine whether our observation of abnormal hyperplasia is species-specific.
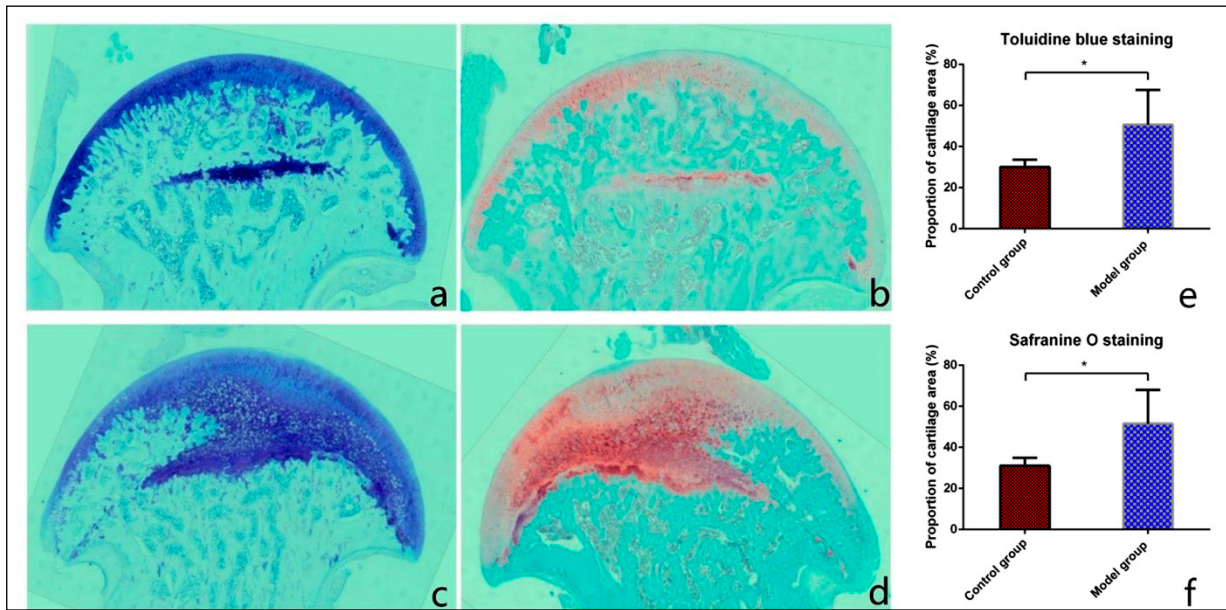
Figure 3. Representative images of the staining of femoral head with (**a, c**) toluidine blue or (**b, d**) safranine O. Magnification, 20×. **a-b**, Samples from two animals from the control group. **c-d**, Samples from two animals from the model group. **e**, Semi-quantitative analysis of cartilage area based on toluidine blue staining. **f**, Semi-quantitative analysis of cartilage area based on safranine O staining. *$p < 0.001$ between the two groups. The error bars indicate the standard deviation of the mean.

Limitations

This study presents two limitations. One is that we did not explore the potential molecular mechanisms linking abnormal hyperplasia of articular surface cartilage with GC-ONFH. Another is that we did not verify or extend the results from animal studies using cell culture or biochemical experiments. Further studies are needed to address these limitations.

Conclusions

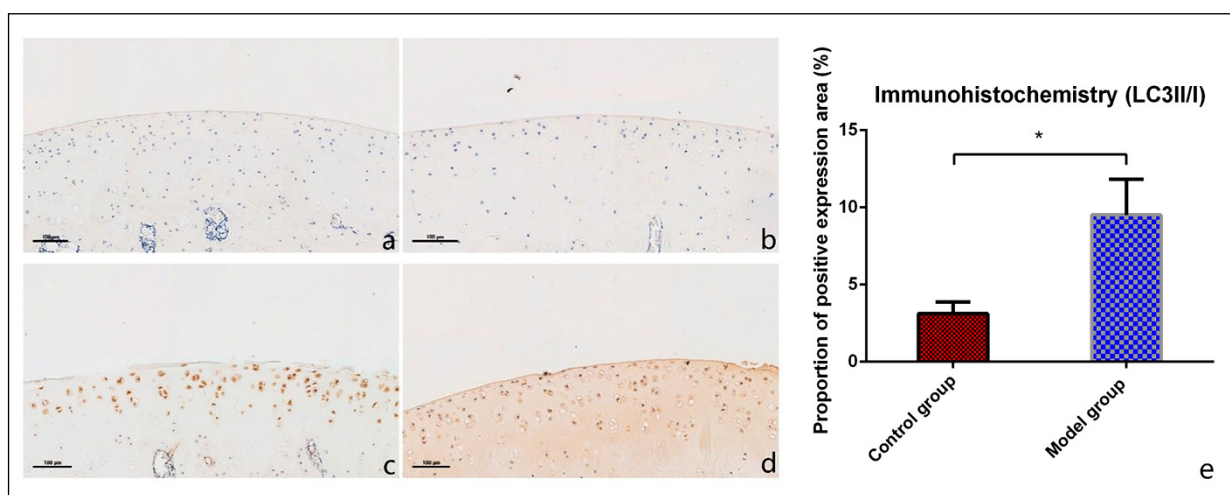Abnormal hyperplasia of articular surface cartilage can be observed in a rat model of GC-ONFH,

Figure 4. Immunohistochemistry against LC3B in articular surface cartilage. Magnification, 100×. **a-b**, Two representative samples (two animals) from the control group. **c-d**, Two representative samples (two animals) from the model group. **e**, Semi-quantitative analysis of positive expression area. *$p < 0.001$ between the two groups. The error bars indicate the standard deviation of the mean.
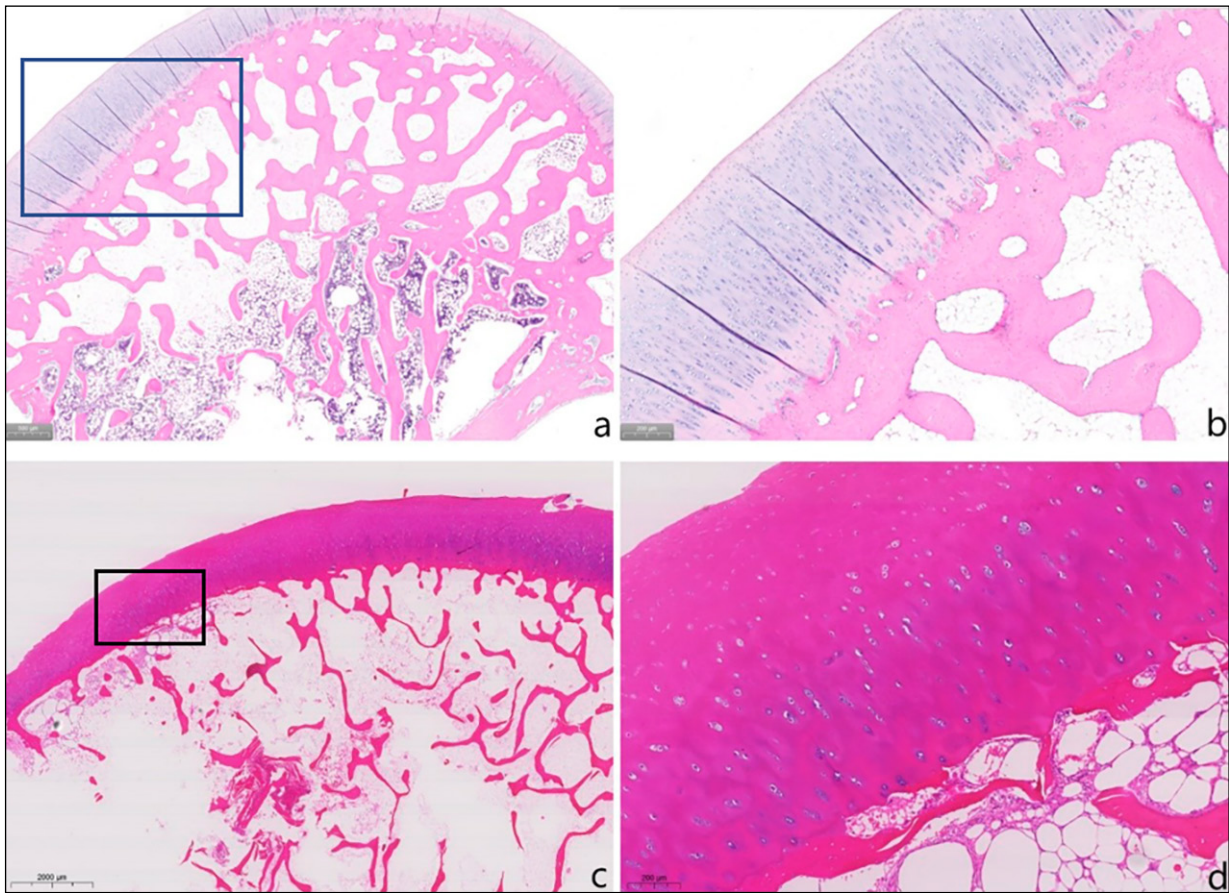
Figure 5. Representative images after HE staining of femoral heads from (**a-b**) one rabbit with GC-ONFH and (**c-d**) one patient with GC-ONFH. Abnormal hyperplasia of articular surface cartilage was not observed in either species. Boxes on the left show the areas enlarged on the right. Magnification: **a**: 20×, **b**: 50×, **c**: 5×, and **d**: 50×.

and this phenomenon may be related to glucocorticoid-induced overactivation of autophagy in chondrocytes. Further studies should clarify whether these observations are species-specific and what mechanisms cause them. Such insights may help to clarify the human disease.

Conflict of Interest
The Authors declare that they have no conflict of interests.

Ethical Approval
The guidelines of the National Institution of Health on the humane use and care of laboratory animals were applied on all the animal experiments in this study and the protocols were agreed upon by the Institutes Animal Care and Use Committee of West China Medical School of Sichuan University. The second part of the study was approved by the Clinical Trials and Biomedical Ethics Committee of Sichuan University West China Hospital.

Informed Consent
Written informed consent was obtained from all patients for their tissue samples to be used in medical research.

Data Availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' Contribution
Q.W. and Z.Y. conducted experiments and wrote the manuscript. W.Z. and Q.L. analyzed data and helped write the manuscript. P.K. oversaw the study and made important intellectual contributions to the manuscript. All authors read and approved the final manuscript.

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