Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Therapeutic Effect of Huzhangoside D in Rats with Knee Osteoarthritis Induced by Anterior Cruciate Ligament Transection

Ruo Jing Zhang, Cheng Chen Cai[1], Jian Pang[2], Xiao Li Xu[1], Hai Xin Gou[1], Guo Wen Li

Department of Pharmacy, Shanghai TCM-Integrated Hospital, Shanghai University of Traditional Chinese Medicine, [1]Department of Traditional Chinese Medicine, HuaDong Hospital, FuDan University, [2]Shi's Center of Orthopaedics and Traumatology, ShuGuang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China

Submitted: 20-Jul-2020 Revised: 09-Sep-2020 Accepted: 05-Jan-2021 Published: 15-Apr-2021

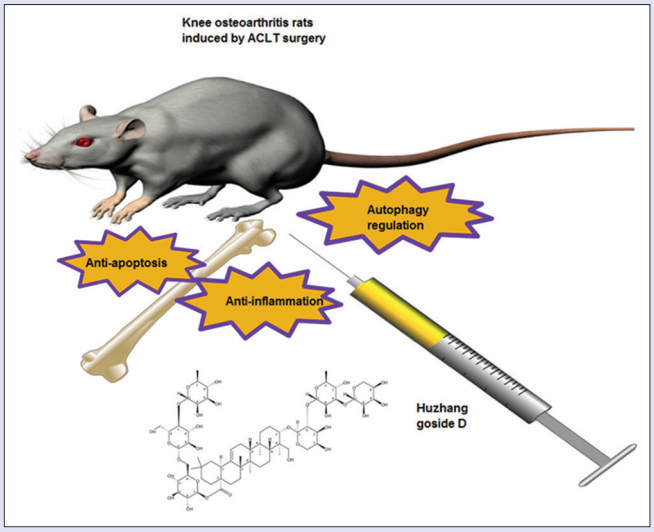
ABSTRACT

Background: Knee osteoarthritis (KOA) is an age-related disease. Huzhangoside D is a saponin isolated from genus *Clematis* L. (*Ranunculaceae)*. The aim of this study is to explore the anti-inflammatory, apoptotic, and autophagy regulation effects of huzhangoside D on KOA in a rat model. **Materials and Methods:** The KOA model was established by an anterior cruciate ligament transection surgery. Huzhangoside D was administered for 4 weeks. The weight-bearing assay, morphology observation, and intrinsic mechanism exploration were performed. **Results:** After administration, the weight-bearing assay showed that huzhangoside D promoted joint function recovery. Hematoxylin-eosin and safranin O-Fast green staining indicated that huzhangoside D ameliorated the structural damage. The Mankin scores were decreased in the huzhangoside D groups. Huzhangoside D enhanced cartilage thickness. Enzyme-linked immunosorbent assay study revealed that huzhangoside D downregulated the proinflammatory cytokine (tumor necrosis factor alpha, interleukin-6, and interleukin-1β) levels, while it upregulated the anti-inflammatory cytokine (interleukin-10) level in rat serum. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay showed that huzhangoside D downregulated the apoptosis ratio of cartilage cells. Immunohistochemical staining showed that huzhangoside D upregulated the autophagy-related protein beclin-1, ATG5, ATG7, and light chain 3 levels and downregulated the p62 level. Moreover, the AKT and mTOR signaling pathway activities were downregulated. The 3-MA combination with huzhangoside D downregulated the weight-bearing function and morphology of the knee and upregulated the proinflammatory cytokines, which showed the role of autophagy as a protective mechanism in the effect of huzhangoside D. **Conclusion:** This study revealed that huzhangoside D is a promising agent in KOA treatment.

Key words: Agent, apoptosis, autophagy, huzhangoside D, inflammation, knee osteoarthritis

SUMMARY

- In this study, we explored the pharmacological effects of a natural saponin, huzhangoside D, in the anterior cruciate ligament transection-induced knee osteoarthritis (KOA) rat model and the underlying molecular mechanisms. Huzhangoside D promoted the joint function recovery and ameliorated the histological change in cartilage loss in KOA rats. This effect was mediated by the anti-inflammatory, anti-apoptotic, and autophagy regulation abilities of huzhangoside D. This study showed that huzhangoside D is a promising agent for KOA therapy.



Abbreviations used: ACLT: Anterior cruciate ligament transection; KOA: Knee osteoarthritis; IOD: Integrated optical density; IHC: Immunohistochemical; LC3: Light chain 3; ELISA: Enzyme-linked immunosorbent assay; TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin-6; IL-10: Interleukin-10; IL-1β: Interleukin-1β; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.

Correspondence:

Prof. Hai Xin Gou,
No. 221, Yan'An West Road, Shanghai, 200040, China.
E-mail: gouhaixin@fudan.edu.cn

DOI: 10.4103/pm.pm_298_20

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Osteoarthritis (OA) is an age-related disease, which affects about a quarter of the middle-aged people and most of the elderly people.[1] The most commonly affected joints in OA are the knee and hip. The main symptoms of knee osteoarthritis (KOA) are pain, knee swelling, and physical disability, which cause a gradual loss of walking ability.[2] In many countries, KOA causes an unaffordable economic burden and healthcare costs to the families and society.[3] Inflammation plays a crucial role in KOA. Proinflammatory cytokines are generated from inflammatory

Cite this article as: Zhang RJ, Cai CC, Pang J, Xu XL, Gou HX, Li GW. Therapeutic effect of Huzhangoside D in rats with knee osteoarthritis induced by anterior cruciate ligament transection. Phcog Mag 2021;17:112-9.

cells and synovial cells under the circumstance, and they consequently stimulate the production of proteolytic enzymes.[4] The characteristic pathological features of KOA are articular cartilage degeneration and chondrocyte death under the stimulation of biochemical, biomechanical, and even genetic changes in knee joints, especially in cartilage.[1] The only type of cell that exists in the cartilage is the chondrocyte. Under stimulation, the chondrocytes are subjected to gradual degradation and death. Cell death can be divided into several types, and the most common type is apoptosis.[5] Current pharmacological therapies, such as nonsteroidal anti-inflammatory drugs and glucosamine, are limited and palliative because treatment only focuses on anti-inflammation and apoptosis in the chondrocytes.[6]

Autophagy is a conservative mechanism of cell self-protection, which plays an important role in maintaining metabolism and internal environment stability in cells.[7] Autophagy is mainly characterized by the compromise of autophagosomes and lysosomes to create autolysosomes.[8] Intracellular contents, including damaged and dysfunctional macromolecules and organelles in autolysosomes, are then degraded by lysosomal enzymes. Numerous autophagy-related genes, including microtubule-associated protein 1 light chain 3 (LC3), ATG, beclin-1, and sequence 1 (sqstm1/p62), are involved in autophagy under a stimulated environment.[9] Accumulating reports have revealed that autophagy is tightly associated with inflammation, metabolism, and apoptosis in KOA.[10] Hence, a pharmacological intervention should be developed to focus not only on anti-inflammation and chondrocyte apoptosis but also on autophagy regulation in chondrocytes.

Bioactive compounds from the plants are favored because plants provide a variety of resources, especially plants that have been commonly applied in the past in folk medicine.[11] They can provide an effective drug by screening and diminishing the chemical establishment procedures. Numerous plant compounds have been validated for their efficacy in OA.[12] Huzhangoside D [Figure 1a] is a saponin isolated from genus *Clematis* L. (*Ranunculaceae*),[13] as an example *Clematis graveolens*.[14] The genus *Clematis* L. (*Ranunculaceae*) plants consist of 295 species and plants worldwide. They have been employed to treat inflammation in folk medicine. Although huzhangoside D has been found about 20 years ago,[15] its pharmacological effect is not fully known to date. A study exhibited the anticancer effect of huzhangoside A, a similar structure compound, by the inhibition of the pyruvate dehydrogenase kinase activity.[16] The aim of this study is to explore the anti-inflammatory, apoptotic, and autophagy regulation effects of huzhangoside D on KOA in a rat model.

MATERIALS AND METHODS

Chemicals

Quantitation enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-1β (IL-1β) were obtained from Beyotime Institute of Biotechnology (Nantong, JiangSu, China). Huzhangoside D standard was obtained from Nature-Standard Corporation (>98%, Nature-Standard, Shanghai, China). Mild decalcification solution was purchased from Sigma-Aldrich (St. Louis, MO, USA). The *TransDetect® In Situ* Fluorescein Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Cell Apoptosis Detection Kit was provided by Roche (Roche, Basel, Switzerland). Primary antibodies and secondary antibodies were obtained from Abcam (Abcam plc, Cambridge, UK).

Animal, surgery, and treatment

All animal experiments in this study were performed following the Declaration of Helsinki, and procedures were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM190906003). Sprague–Dawley (SD) rats (150–160 g, 6 weeks, specific pathogen-free [SPF], healthy, and males) were obtained from Shanghai SIPPR-Bk Laboratory Animals Ltd and housed in the Shanghai University of Traditional Chinese Medicine with a temperature of 20°C–25°C, humidity of 40% ± 5%, and 12-h light/12-h dark cycle environment at SPF grade. Rats were maintained in a cage with bedding of sawdust and free to diet and water. After 7 days of accommodation, 42 rats were randomized and divided into seven groups (n = 6): sham group as normal control (sham group), one model group that underwent anterior cruciate ligament transection surgery (ACLT model group), three groups that underwent ACLT surgery and were treated with huzhangoside D at three increasing doses, one positive group that received glucosamine sulfate (positive group, 200 mg/kg/day),[17] and one huzhangoside D group (68 mg/kg/day) combined with the 3-MA (an autophagy inhibitor, 15 mg/kg/day).[18] ACLT surgery was performed using pentobarbital sodium 40 mg/kg i.p. at first following previous literature.[19] After that, anesthesia was maintained under a mixture gas of O_2 and isoflurane 2.5% from a small animal anesthesia machine (SAR-1000, Bioseb, Vitrolles, France). The anterior cruciate ligament was carefully transected in the right knee to avoid injury

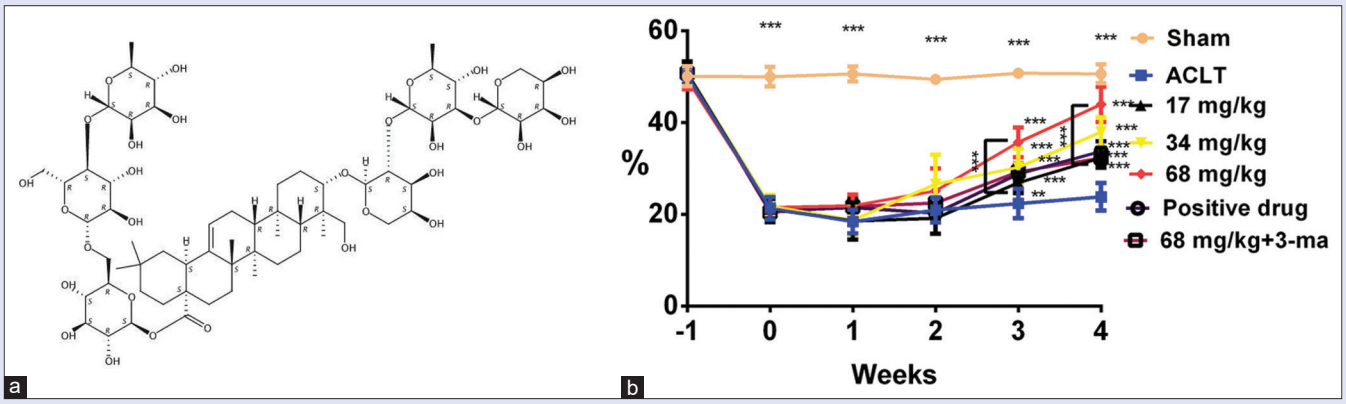


Figure 1: The chemical structure and behavior assay of huzhangoside D in knee osteoarthritis rats. (a) The chemical structure of huzhangoside D. (b) The weight-bearing assay. The statistical difference among the anterior cruciate ligament transection model group and other groups was considered significant at the level of $^{**}P < 0.01$ or $^{***}P < 0.001$. The statistical difference among 68 mg/kg group and 68 mg/kg + 3-MA group was considered significant at the level of $^{***}P < 0.001$. ACTL: Anterior cruciate ligament transection

to the other muscles and ligaments. In the sham group, the wounds were sutured after exposing the knee joint cartilage surface at the same position. After surgery, rats were subjected to the drawer test to ensure the success of the operation. Meanwhile, one million units of penicillin (Yansheng, Shanghai, China) was intramuscularly injected in each rat for 3 days to avoid infection. Huzhangoside D was dissolved in PBS and injected i.p. into the rats at a dose of 17, 34, and 68 mg/kg daily in the huzhangoside D groups for 4 weeks. An equivalent volume vehicle was injected into the rats in the ACLT model group.

Weight-bearing distribution

The weight-bearing distribution test was employed to investigate the joint weight-bearing function using a dynamic weight-bearing instrument (Taimeng, Chengdou, China) following previously described methods.[20,21] Concisely, a dual-channel weight average was employed to investigate the weight borne by the two hind paws per week. The index of joint discomfort and function was calculated as the ratio of the experimental hind paw (right) to total hind paws (right + left). Weight-bearing tests were performed two times at an interval of 10 min and recorded as the mean of two trials. The weight-bearing test was initiated at −1 week from ACLT surgery to the last administration of huzhangoside D at 4 weeks.

Enzyme-linked immunosorbent assay

About 24 h after the behavior assay, rats were executed using cervical dislocation under anesthesia. Then, the serum of rats was collected immediately, and the proinflammatory (TNF-α, IL-1β, and IL-6) cytokine and the anti-inflammatory IL-10 cytokine levels in the serum were detected following the manufacturer's method.

Morphology observation

The right knee joints of rats were fixed in 4% paraformaldehyde after dissection for 24 h, washed three times using PBS, and decalcified in mild decalcification solution for approximately 5 weeks with continuous solution exchange per week. Decalcification was complete when the needle could pass the tissues easily without any obvious resistance. After that, joint tissues were embedded in paraffin wax and cut into 5 μm sections. Hematoxylin-eosin (HE) staining and safranin O-Fast green staining (1% safranin O counterstained with 0.75% hematoxylin and then 1% Fast Green, Service bio, Wuhan, China) were conducted to visualize the structural changes of cartilage and proteoglycan content in the cartilage using a light microscope (MF31, Mshot, Guangzhou, China). Semi-quantitative histopathological grading of the structure was performed by a third-party pathologist following a modified Mankin scoring system concerning the structure.[22] Masson staining (Servicebio, Wuhan, China) was employed to identify the type II collagen fibers. Toluidine blue staining was employed to investigate the cartilage thickness.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

TUNEL assays were employed to detect apoptosis *in situ* according to a previous report[23] and manufacturer's instructions. Briefly, 5 μm sections of the joint tissue were soaked with dimethylbenzene and subjected to gradient dehydration (100%, 95%, 90%, 80%, and 70% alcohol). Then, the sections were digested with proteinase K (Dako, Glostrup, Denmark) action solution for 15 min and incubated with terminal deoxynucleotidyl transferase action solution at 37°C for 1 h. Furthermore, sections were counterstained with 4',6-Diamidino-2-Phenylindole (DAPI). The images of the approximate region of the joint were visualized by a slide-driver fluorescence microscope (3D Histech Ltd., Budapest, Hungary) at an excitation wavelength of 468 nm. The percentage of apoptotic chondrocytes was evaluated as the green fluorescence of cartilage versus blue nuclei (DAPI).

Immunohistochemical analysis

Immunohistochemical (IHC) analysis was performed on the articular cartilage to investigate the expressions of related signaling pathway and autophagy-related proteins. As mentioned above, the joint sections were cut into 5 μm sections. The joint sections were incubated overnight at 4°C with rabbit polyclonal antibodies developed against the desired proteins (beclin-1:Ab62557, 1:100; ATG5:Ab103827, 1:100; ATG7:Ab133528, 1:100; p62:Ab56416, 1:100; LC3B:Ab48394, 1:100; p-mTOR:Ab84400, 1:100; p-AKT: Ab38449, 1:100). Immune complexes were then incubated with the anti-rabbit HRP-conjugated secondary antibody for 20 min at 37°C. The images of the approximate region of the joint were captured using a microscope (MF31, Mshot, Guangzhou, China). The integrated optical density and area of immunostaining were assessed using Image-Pro Plus 6.0 image analysis software (Media Cybernetics Co., USA).

Statistical analysis

Results of the animal study were expressed as means ± standard deviation and determined using one-way ANOVA to assess statistical significance. $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using commercially available statistical software (SPSS 22.0, Chicago, IL, USA) and presented by GraphPad Prism 6.0 (GraphPad Software Inc., Diego, CA, USA).

RESULTS

Huzhangoside D promoted the recovery of joint function and ameliorated the pain in KOA rats. First, during the total animal study, there were no toxic signs, such as diminished activity, vomiting, bleeding, or death, when doses of 17, 34, and 68 mg/kg huzhangoside D were administered. All rats maintained good-eating and normal activity. Weight-bearing test was employed to investigate the endurance of knee function. Figure 1b shows that weight bearing of the right limb was nearly 50% at −1 week, suggesting that left and right knee function was balanced among all groups. After ACLT surgery, weight bearing of the right limb was decreased in KOA model rats. Huzhangoside D at doses of 17, 34, and 68 mg/kg/day ameliorated the decrease gradually [Figure 1b], demonstrating that huzhangoside D promoted the recovery of right knee joint function.

Huzhangoside D ameliorated the histological assay of the cartilage

HE staining [Figure 2a] and safranin O-Fast green staining [Figure 2b] indicated the histological changes among the seven groups. The ACLT model group showed changes, which were classified as inflammatory infiltrates, angiogenesis, edema, pannus formation and granuloma, focal loss of cartilage, bone erosion, and presence of extra-articular inflammation. However, huzhangoside D treatment alleviated the damage. Mankin scores were increased in the ACLT model group compared with the sham group. After huzhangoside D administration, Mankin scores were decreased in huzhangoside D groups compared with the ACLT model group [Figure 2e]. Moreover, huzhangoside D also enhanced cartilage thickness [Figure 2c], which suggested that huzhangoside D ameliorates the symptoms of KOA. Furthermore, Masson's staining also showed disorganized collagen fibers in the ACLT model group, while the collagen fibers were well arranged in the huzhangoside D groups [Figure 2d].

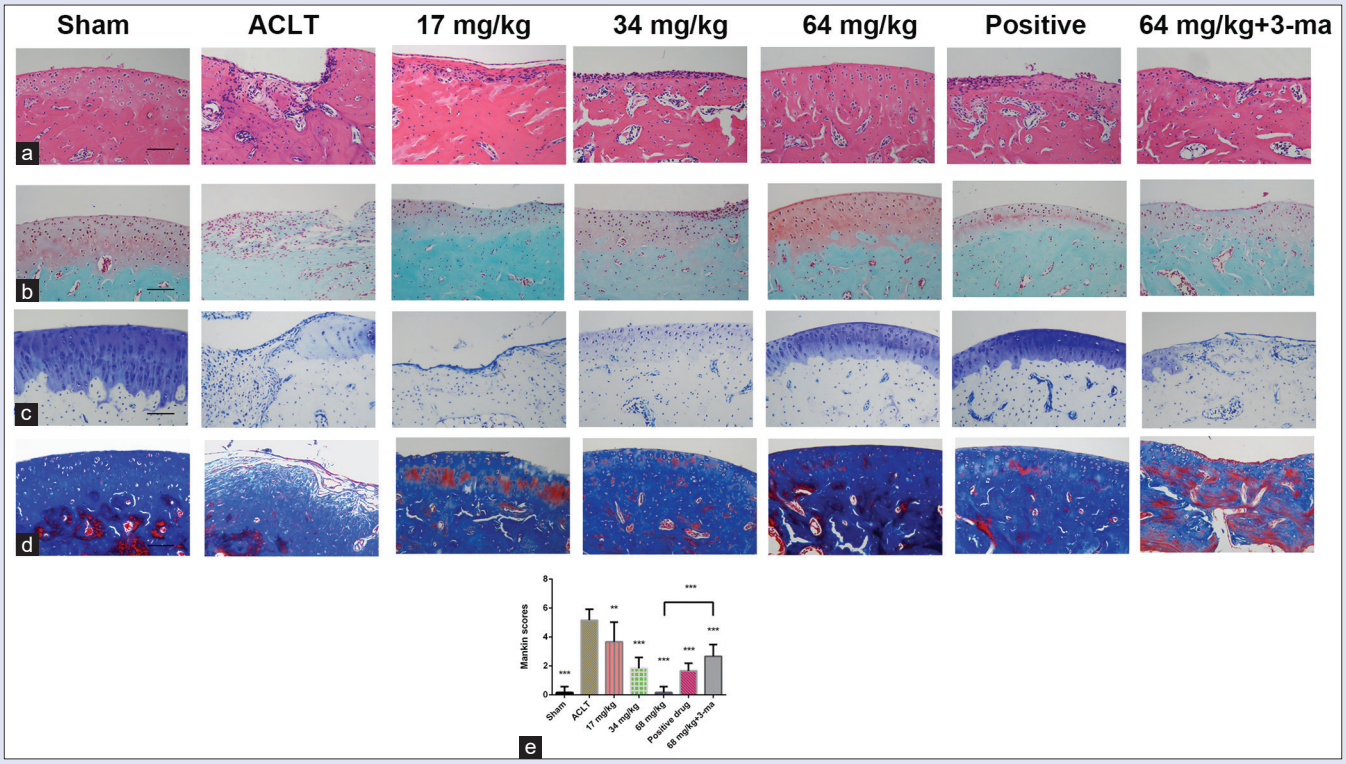


Figure 2: The morphology assay of huzhangoside D in knee osteoarthritis rats. (a) Hematoxylin-eosin staining of knee joint cartilage. (b) Safranin O-fast green staining. (c) Masson's staining. (d) Toluidine blue staining. (e) Mankin scores among groups. Scale bar = 200 μm. The statistical difference among the anterior cruciate ligament transection model group and other groups was considered significant at the level of $^{**}P < 0.01$ or $^{***}P < 0.001$. The statistical difference among the 64 mg/kg model group and 64 mg/kg + 3-MA group was considered significant at the level of $^{***}P < 0.001$. ACTL: Anterior cruciate ligament transection

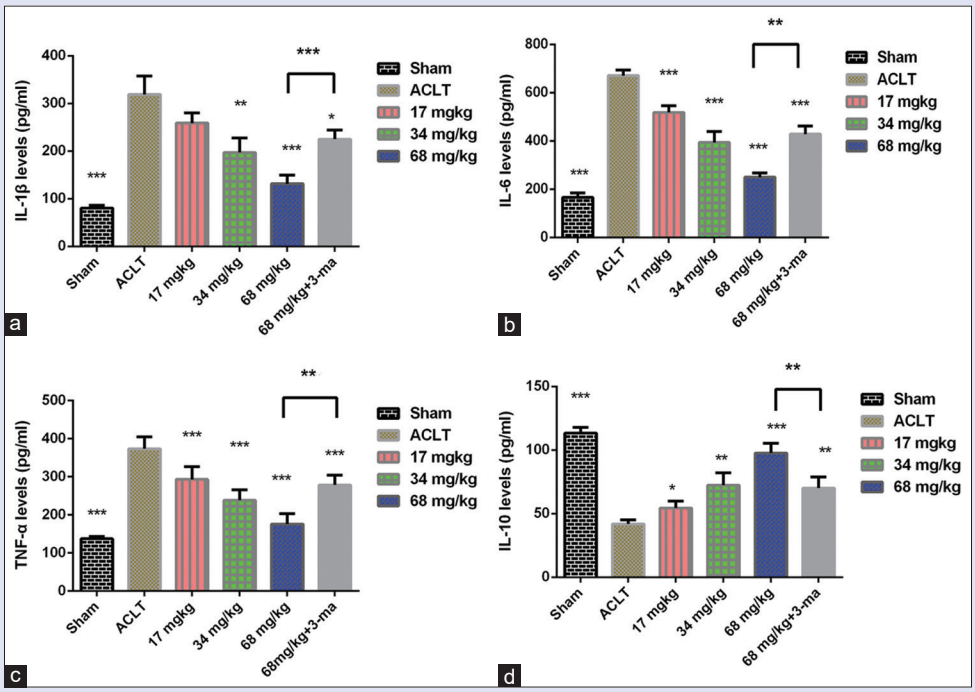


Figure 3: Inflammatory cytokine levels in rat serum. (a) The expression level of interleukin-1β. (b) The expression level of interleukin-6. (c) The expression level of tumor necrosis factor alpha. (d) The expression level of interleukin-10. The statistical difference among the anterior cruciate ligament transection model group and other groups was considered significant at the level of $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. The statistical difference among the 64 mg/kg model group and 64 mg/kg + 3-MA groups was considered significant at the level of $^{**}P < 0.01$ or $^{***}P < 0.001$. ACTL: Anterior cruciate ligament transection; TNF-α: Tumor necrosis factor alpha, IL-6: Interleukin-6; IL-10: Interleukin-10; IL-1β: Interleukin-1β

Huzhangoside D downregulated the proinflammatory cytokine levels in the serum

ELISA assay showed the proinflammatory cytokine level changes in the serum of SD rats. Proinflammatory cytokine levels (TNF-α, IL-1β, and IL-6) were increased in the ACLT model group compared with the sham group. Huzhangoside D administration attenuated the increase in proinflammatory cytokines, as shown in Figure 3a-c. These results showed that huzhangoside D has an inhibitory effect of inflammation. Meanwhile, huzhangoside D upregulated the IL-10 anti-inflammatory cytokine level [Figure 3d].

Huzhangoside D downregulated the apoptosis of cartilage cells

As shown in Figure 4, apoptotic chondrocytes with green fluorescence were apparently increased in the ACLT-induced KOA model. However, after 4 weeks of huzhangoside D administration, the apoptosis ratios in administration groups were decreased, especially in the 68 mg/kg group. TUNEL assay showed that huzhangoside D downregulated the apoptosis ratio of cartilage cells [Figure 4].

Huzhangoside D upregulated the autophagy-related proteins and role of the AKT-mTOR signaling pathway

Moreover, IHC studies showed that huzhangoside D upregulated the autophagy-related protein beclin-1, ATG5, ATG7, and microtubule-associated protein 1 LC3B levels in the cartilage compared with the ACLT model group, as shown in Figure 5a-d, indicating that huzhangoside D promoted the autophagy levels in the cartilage. Meanwhile, the level of another autophagy-related protein p62 was downregulated in Figure 5e. Moreover, p-mTOR and p-AKT levels were inhibited in the huzhangoside D groups compared with the ACLT model group, as shown in Figure 5f and g.

Role of autophagy in the pharmacological effect of huzhangoside D

In addition, 3-MA, a well-known autophagy inhibitor, was injected to validate the role of autophagy in the pharmacological effect of huzhangoside D. Compared with the sole administration of

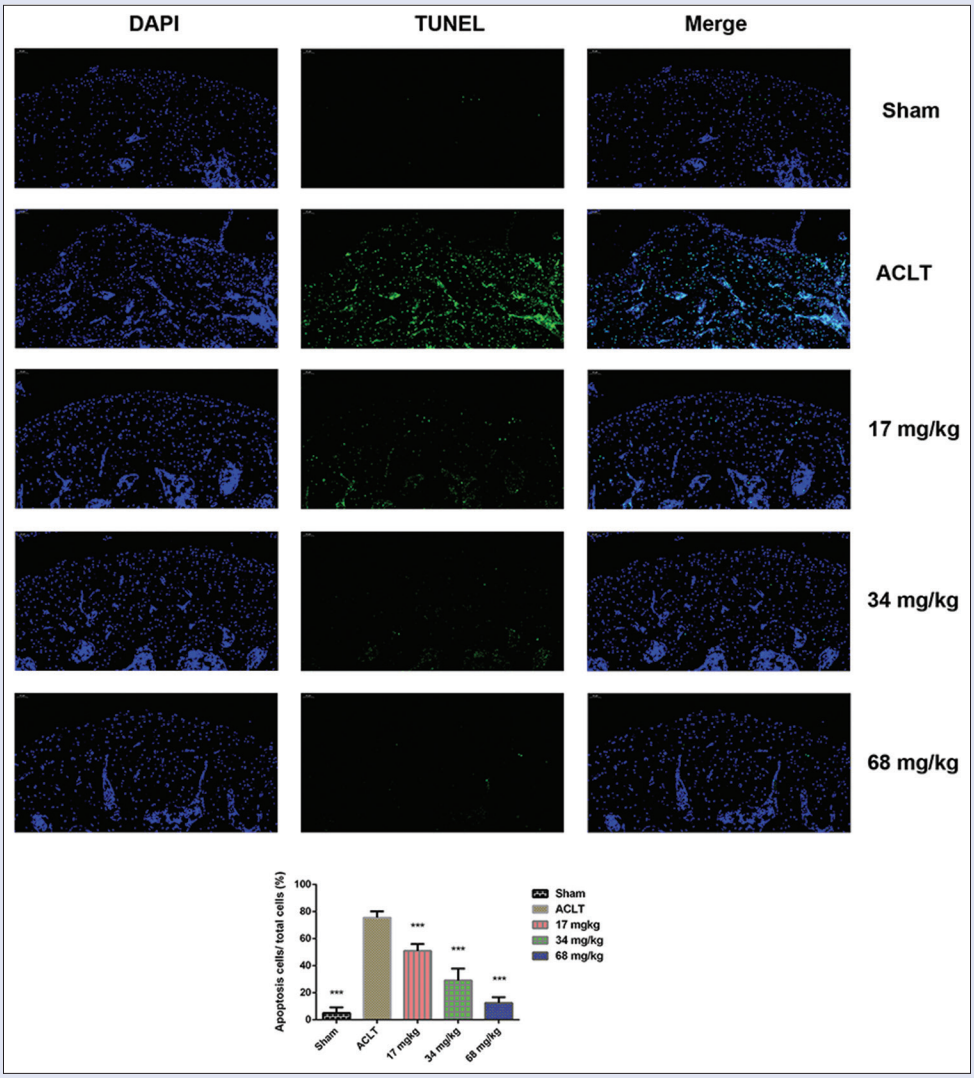


Figure 4: Terminal deoxynucleotidyl transferase dUTP nick end labeling staining of chondrocyte apoptosis. Scale bar = 50 μm. The statistical difference among the anterior cruciate ligament transection model group and other groups was considered significant at the level of ***$P < 0.001$. ACTL: Anterior cruciate ligament transection; TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

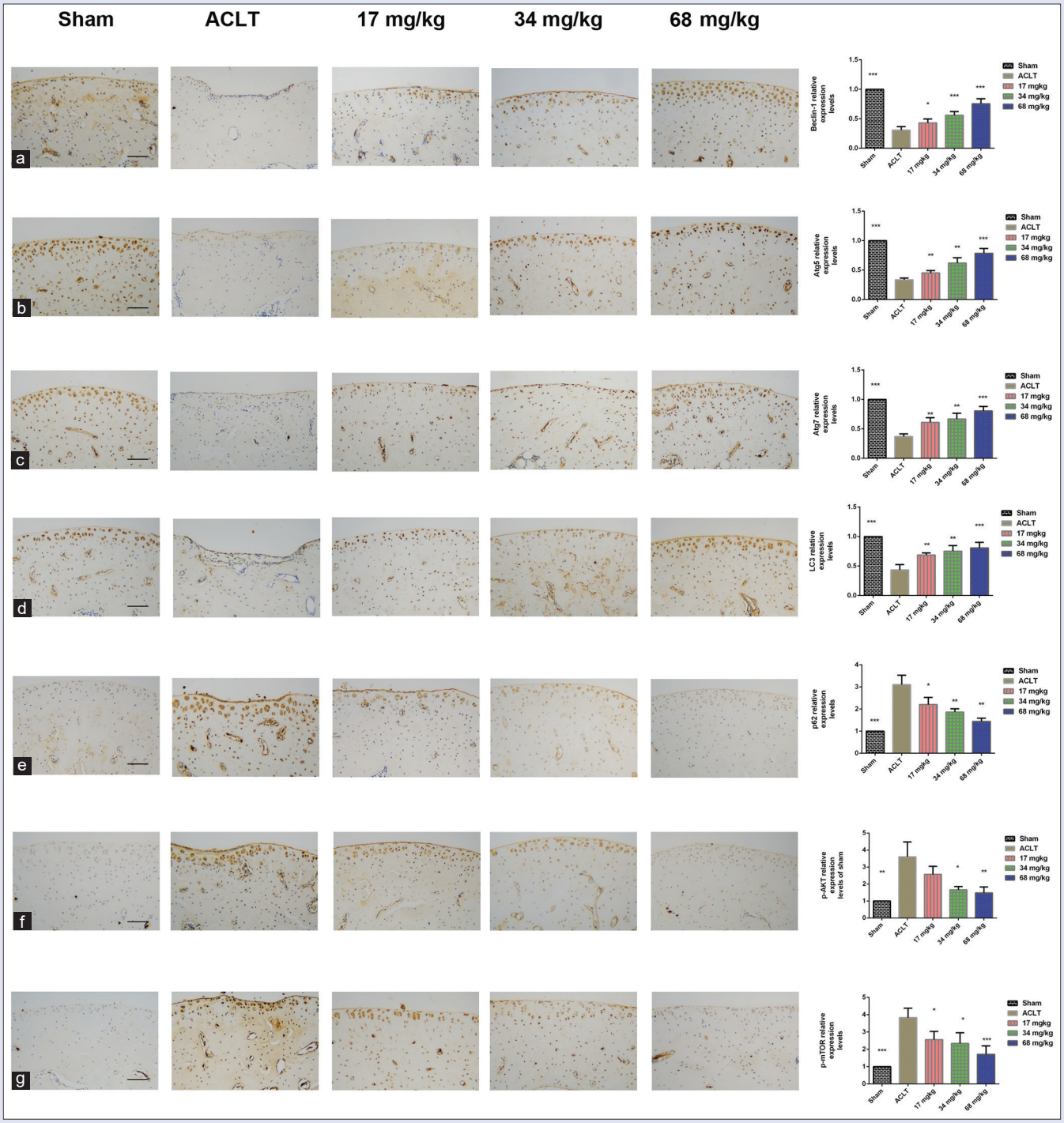


Figure 5: The autophagy-related protein expression in rat knee osteoarthritis bone cartilage using immunohistochemical assay. (a) Beclin-1 expression. (b) ATG5 expression. (c) ATG7 expression. (d) LC3 expression. (e) P62 expression. (f) p-AKT expression. (g) p-mTOR expression. Scale bar = 200 μm. The statistical difference among the model group and other groups was considered significant at the level of $^{*}P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$. ACTL: Anterior cruciate ligament transection

huzhangoside D (68 mg/kg), combined administration of huzhangoside D and 3-MA weakened the improved effect on the weight-bearing ability [Figure 1b]. Furthermore, combined administration with 3-MA worsened the knee joint morphology [Figure 2a-d] and upregulated the inflammatory cytokines [Figure 3a-d]. This result confirmed that huzhangoside D induced autophagy as an important protective mechanism.

DISCUSSION

To date, many drugs derived from plants have been screened, and their efficacy has been confirmed in the preclinical study. In this study, huzhangoside D, a saponin compound, rarely studied in the past was found to show anti-inflammatory, apoptotic, and autophagy regulation effects in an ACLT-induced KOA rat model.

Some study has revealed the association between worse joint function and high levels of joint degeneration biomarkers, for instance, high levels of TNF-α, IL-6, and IL-1β.[24] The articular cartilage is composed of chondrocytes and extracellular matrix. Chondrocytes are responsible for tissue maintenance, which has an impact on joint function and performance. IL-1β and TNF-α are inflammatory mediators involved in joint degeneration caused by KOA. Inhibition of these proinflammatory cytokines in the knee joint can elicit the synthesis of proteoglycans and collagen, and then, it can inhibit joint swelling. IL-10 expression plays a key role in anti-osteoclastogenesis, and it is downregulated in OA patients.[25] In this study, huzhangoside D inhibited the increase in proinflammatory cytokines in the ACLT-induced KOA rat model, demonstrating that huzhangoside D is a promising anti-inflammatory compound. Moreover, autophagy is tightly linked with inflammation in tissues, especially in OA.[10] Generally, autophagy can regulate inflammation. Absence of B-cell autophagy inhibited the production of inflammatory cytokines. Meanwhile, autophagy of the immune system affects T-cell proliferation and survival.[26] The inhibition of mTOR, an autophagy-related signaling pathway, resulted in a decrease in cartilage injury[27] and reduced secretion of the synovial inflammatory cytokines, such as IL-1β and TNF-α.[28]

Meanwhile, autophagy is a protective mechanism that can help chondrocytes to adapt to various stresses in the joint.[29] As KOA is a bone degradation disease, autophagy can digest the necrotic organelles in chondrocytes, thus suppressing damage and maintaining the cellular metabolism.[30] Accumulating reports have revealed that autophagy is indispensable for chondrocytes, and autophagy deficits play crucial roles in the pathogenesis of KOA.[30] Atg5 and LC3 were markedly downregulated in the cartilage of aging mice.[31] In addition, lower levels of autophagy-related proteins aggravated the cartilage loss and apoptosis. Hence, regulation of autophagy is an important cellular target of new drug development. Many new drugs, treatment, and physical therapy have been developed to promote the autophagy levels against KOA development. For instance, the treadmill exercise enhanced the autophagy-related protein levels; thus, ameliorating the KOA scores in a KOA rat model,[32] adipose-derived mesenchymal stem cells elicited the autophagy and helped the recovery from KOA.[33,34] Curcumin and BST106 alleviated the cartilage impair in KOA *in vivo*.[35] On the contrary, microRNA-128a downregulated the chondrocyte autophagy levels, thus exacerbating KOA.[36] There are many biomarkers of autophagy in chondrocytes, such as Unc-51-like kinase 1, beclin-1, microtubule-associated protein 1 LC3, ATG7, and ATG5.[37] Beclin-1, which is also named BECN1, is a key regulator of autophagy in the cartilage. The activation of p62 is tightly linked with the inhibition of autophagy.[38] Huzhangoside D inhibited the activation of p62 in this study. Most importantly, LC3 is an essential autophagy-related protein, which indicated the formation of an autophagosome directly. In this study, huzhangoside D promoted the upregulation of autophagy-related proteins, LC3B, ATG5, ATG7, and beclin-1. Numerous signaling pathways, such as MAPK signaling pathway,[39] NF-kappa B signaling pathway,[40] and PI3K/AKT signaling pathway,[41] were involved in the autophagy regulation *in vitro* and *in vivo*. Among them, the AKT/mTOR signaling pathway was frequently involved and commonly exerted a protective effect.[42-44] The phosphorylation of AKT and downstream mTOR signaling pathway could elicit the upregulation of autophagy. In this study, huzhangoside D downregulated the p-AKT/p-mTOR expression levels and elicited the upregulation of autophagy-related protein levels.

CONCLUSION

To sum up, the *in vivo* pharmacological effects of huzhangoside D in the ACLT-induced KOA rat model and the underlying molecular mechanisms were investigated. In this study, huzhangoside D promoted the joint function recovery and ameliorated the histological change in cartilage loss in KOA rats. This effect was mediated by the anti-inflammatory, anti-apoptotic, and autophagy regulation abilities of huzhangoside D. Therefore, huzhangoside D is a promising agent for KOA treatment.

Financial support and sponsorship

This study was supported by Shanghai Science and Technology Commission Project (No. 18401901700).

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Bijlsma JW, Berenbaum F, Lafeber FP. Osteoarthritis: An update with relevance for clinical practice. Lancet 2011;377:2115-26.
2. Crawford C, Lee C, Bingham J; Active Self-Care Therapies for Pain (PACT) Working Group. Sensory art therapies for the self-management of chronic pain symptoms. Pain Med 2014;15 Suppl 1:S66-75.
3. Dulay GS, Cooper C, Dennison EM. Knee pain, knee injury, knee osteoarthritis and work. Best Pract Res Clin Rheumatol 2015;29:454-61.
4. Clemett D, Goa KL. Celecoxib: A review of its use in osteoarthritis, rheumatoid arthritis and acute pain. Drugs 2000;59:957-80.
5. Huang Q, Li F, Liu X, Li W, Shi W, Liu FF, *et al.* Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy. Nat Med 2011;17:860-6.
6. Hussain SM, Neilly DW, Baliga S, Patil S, Meek R. Knee osteoarthritis: A review of management options. Scott Med J 2016;61:7-16.
7. Mariño G, Niso-Santano M, Baehrecke EH, Kroemer G. Self-consumption: The interplay of autophagy and apoptosis. Nat Rev Mol Cell Biol 2014;15:81-94.
8. Zhao F, Huang W, Zhang Z, Mao L, Han Y, Yan J, *et al.* Triptolide induces protective autophagy through activation of the CaMKKβ-AMPK signaling pathway in prostate cancer cells. Oncotarget 2016;7:5366-82.
9. Zhou C, Zhong W, Zhou J, Sheng F, Fang Z, Wei Y, *et al.* Monitoring autophagic flux by an improved tandem fluorescent-tagged LC3 (mTagRFP-mWasabi-LC3) reveals that high-dose rapamycin impairs autophagic flux in cancer cells. Autophagy 2012;8:1215-26.
10. Luo P, Gao F, Niu D, Sun X, Song Q, Guo C, *et al.* The role of autophagy in chondrocyte metabolism and osteoarthritis: A comprehensive research review. Biomed Res Int 2019;2019:5171602.
11. Ansari MY, Khan NM, Haqqi TM. A standardized extract of Butea monosperma (Lam.) flowers suppresses the IL-1β-induced expression of IL-6 and matrix-metalloproteases by activating autophagy in human osteoarthritis chondrocytes. Biomed Pharmacother 2017;96:198-207.
12. Cameron M, Chrubasik S. Oral herbal therapies for treating osteoarthritis. Cochrane Database Syst Rev 2014;(5):CD002947.
13. Ishtiaq CM, He Q, Feng S, Wang Y, Xiao PG, Cheng YY. Chemodiversity of saponins and their taxonomic importance in Clematis genus (*Ranunculaceae*). Pak J Biol Sci 2007;10:2066-72.
14. Rattan R, Reddy SG, Dolma SK, Fozdar BI, Gautam V, Sharma R, *et al.* Triterpenoid saponins from clematis graveolens and evaluation of their insecticidal activities. Nat Prod Commun 2015;10:1525-8.
15. Kawata Y, Kizu H, Tomimori T. Studies on the constituents of Clematis species. VII. Triterpenoid saponins from the roots of *Clematis terniflora* DC. var. robusta Tamura. Chem Pharm Bull (Tokyo) 1998;46:1891-900.
16. Kwak CH, Lee JH, Kim EY, Han CW, Kim KJ, Lee H, *et al.* Huzhangoside a suppresses tumor growth through inhibition of pyruvate dehydrogenase kinase activity. Cancers (Basel) 2019;11:712.
17. Sudirman S, Chang HW, Chen CK, Kong ZL. A dietary polysaccharide from Eucheuma cottonii downregulates proinflammatory cytokines and ameliorates osteoarthritis-associated cartilage degradation in obese rats. Food Funct 2019;10:5697-706.
18. Zhou R, Zhu F, Wu X, Song S, Chen Y, Zhu C, *et al.* Effects of autophagy on apoptosis of articular chondrocytes in adjuvant arthritis rats. J Cell Mol Med 2019;23:7879-84.
19. Chen CH, Ho ML, Chang LH, Kang L, Lin YS, Lin SY, *et al.* Parathyroid hormone-(1-34) ameliorated knee osteoarthritis in rats via autophagy. J Appl Physiol (1985) 2018;124:1177-85.

20. Yu X, Zhao L, Yu Z, Yu C, Bi J, Sun B, *et al.* Sivelestat sodium hydrate improves post-traumatic knee osteoarthritis through nuclear factor-κB in a rat model. Exp Ther Med 2017;14:1531-7.
21. Ma Y, Guo H, Bai F, Zhang M, Yang L, Deng J, *et al.* A rat model of knee osteoarthritis suitable for electroacupuncture study. Exp Anim 2018;67:271-80.
22. Mankin HJ, Dorfman H, Lippiello L, Zarins A. Biochemical and metabolic abnormalities in articular cartilage from osteo-arthritic human hips. II. Correlation of morphology with biochemical and metabolic data. J Bone Joint Surg Am 1971;53:523-37.
23. Wang X, Shen P, Tang D, Xu H, Qiu H, Wu T, *et al.* Effects of Qi-Fang-Xi-Bi-Granules on Cartilage Morphology and C/ebpα Promoter Methylation in rats with knee osteoarthritis. Evid Based Complement Alternat Med 2018;2018:2074976.
24. Martins JB, Mendonça VA, Aguiar GC, da Fonseca SF, Dos Santos JM, Tossige-Gomes R, *et al.* Effect of a moderate-intensity aerobic training on joint biomarkers and functional adaptations in rats subjected to induced knee osteoarthritis. Front Physiol 2019;10:1168.
25. Kwon JY, Lee SH, Na HS, Jung K, Choi J, Cho KH, *et al.* Kartogenin inhibits pain behavior, chondrocyte inflammation, and attenuates osteoarthritis progression in mice through induction of IL-10. Sci Rep 2018;8:13832.
26. Weindel CG, Richey LJ, Bolland S, Mehta AJ, Kearney JF, Huber BT. B cell autophagy mediates TLR7-dependent autoimmunity and inflammation. Autophagy 2015;11:1010-24.
27. Cejka D, Hayer S, Niederreiter B, Sieghart W, Fuereder T, Zwerina J, *et al.* Mammalian target of rapamycin signaling is crucial for joint destruction in experimental arthritis and is activated in osteoclasts from patients with rheumatoid arthritis. Arthritis Rheum 2010;62:2294-302.
28. Caramés B, Hasegawa A, Taniguchi N, Miyaki S, Blanco FJ, Lotz M. Autophagy activation by rapamycin reduces severity of experimental osteoarthritis. Ann Rheum Dis 2012;71:575-81.
29. Jeon H, Im GI. Autophagy in osteoarthritis. Connect Tissue Res 2017;58:497-508.
30. Caramés B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M. Autophagy is a protective mechanism in normal cartilage, and its aging-related loss is linked with cell death and osteoarthritis. Arthritis Rheum 2010;62:791-801.
31. Caramés B, Olmer M, Kiosses WB, Lotz MK. The relationship of autophagy defects to cartilage damage during joint aging in a mouse model. Arthritis Rheumatol 2015;67:1568-76.
32. Zhang X, Yang Y, Li X, Zhang H, Gang Y, Bai L. Alterations of autophagy in knee cartilage by treatment with treadmill exercise in a rat osteoarthritis model. Int J Mol Med 2019;43:336-44.
33. Zhou J, Wang Y, Liu Y, Zeng H, Xu H, Lian F. Adipose derived mesenchymal stem cells alleviated osteoarthritis and chondrocyte apoptosis through autophagy inducing. J Cell Biochem 2018:1-15.
34. Zhang G, Cao J, Yang E, Liang B, Ding J, Liang J, *et al.* Curcumin improves age-related and surgically induced osteoarthritis by promoting autophagy in mice. Biosci Rep 2018;38:BSR20171691.
35. Hong JM, Shin JK, Kim JY, Jang MJ, Park SK, Lee JH, *et al.* BST106 protects against cartilage damage by inhibition of apoptosis and enhancement of autophagy in osteoarthritic rats. Biol Pharm Bull 2018;41:1257-68.
36. Lian WS, Ko JY, Wu RW, Sun YC, Chen YS, Wu SL, *et al.* MicroRNA-128a represses chondrocyte autophagy and exacerbates knee osteoarthritis by disrupting Atg12. Cell Death Dis 2018;9:919.
37. Musumeci G, Castrogiovanni P, Trovato FM, Weinberg AM, Al-Wasiyah MK, Alqahtani MH, *et al.* Biomarkers of chondrocyte apoptosis and autophagy in osteoarthritis. Int J Mol Sci 2015;16:20560-75.
38. Komatsu M, Kageyama S, Ichimura Y. P62/SQSTM1/A170: Physiology and pathology. Pharmacol Res 2012;66:457-62.
39. Shi J, Zhang C, Yi Z, Lan C. Explore the variation of MMP3, JNK, p38 MAPKs, and autophagy at the early stage of osteoarthritis. IUBMB Life 2016;68:293-302.
40. Yan H, Duan X, Pan H, Holguin N, Rai MF, Akk A, *et al.* Suppression of NF-κB activity via nanoparticle-based siRNA delivery alters early cartilage responses to injury. Proc Natl Acad Sci U S A 2016;113:E6199-E6208.
41. Sun W, Li Y, Wei S. MiR-4262 regulates chondrocyte viability, apoptosis, autophagy by targeting SIRT1 and activating PI3K/AKT/mTOR signaling pathway in rats with osteoarthritis. Exp Ther Med 2018;15:1119-28.
42. Zhang Q, Lai S, Hou X, Cao W, Zhang Y, Zhang Z. Protective effects of PI3K/Akt signal pathway induced cell autophagy in rat knee joint cartilage injury. Am J Transl Res 2018;10:762-70.
43. He W, Cheng Y. Inhibition of miR-20 promotes proliferation and autophagy in articular chondrocytes by PI3K/AKT/mTOR signaling pathway. Biomed Pharmacother 2018;97:607-15.
44. Khan NM, Ansari MY, Haqqi TM. Sucrose, but not glucose, blocks IL1-β-Induced inflammatory response in human chondrocytes by inducing autophagy via AKT/mTOR pathway. J Cell Biochem 2017;118:629-39.