Tetrahydrohyperforin prevents articular cartilage degeneration and affects autophagy in rats with osteoarthritis

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Abstract. Osteoarthritis (OA) is a highly prevalent disease, which is associated with extracellular matrix degradation and cell death in articular cartilage. The aim of the present study was to identify whether tetrahydrohyperforin (IDN5706) ameliorates the degeneration of articular cartilage and affects autophagy in OA. The rat model of experimental OA was induced by intra-articular injection of collagenase solution. IDN5706 was administered intragastrically to rats for 6 weeks. Histopathological changes in articular cartilage were examined using hematoxylin and eosin (H&E) and safranin O staining, and Mankin scoring systems. The effect of IDN5706 on autophagy was examined using western blotting. ELISA was performed to detect cartilage inflammation. H&E and safranin O staining, Mankin scores, and electron microscopy indicated that IDN5706 could lessen the degeneration of articular cartilage in OA rats. In addition, western blotting revealed that IDN5706 treatment may activate the suppressed autophagy in OA rats. In conclusion, the present study demonstrated that IDN5706 was able to reduce the severity of experimental OA, alleviate the degeneration of articular cartilage, and affect autophagy in OA model rats.

Introduction

Osteoarthritis (OA) is the most prevalent degenerative joint disease worldwide, which typically causes pain, joint dysfunction, stiffness, swelling, limited motion and long-term disability (1). The onset of OA is associated with a variety of factors, such as age (2), obesity (3) and inflammation (4). OA is characterized by thickening of subchondral bone, degradation of articular cartilage and formation of osteophytes (5). Increasing morbidity rates of OA make this condition a major healthcare problem and, therefore, a serious health burden to society (6,7). However, there remains no effective prevention and treatment for OA. Traditional therapies only temporarily alleviate the clinical symptoms, but do not effectively inhibit the pathological process (8,9). Therefore, the probe of key molecules in the pathogenesis is important for improving the prevention, mitigation and treatment of OA.

Autophagy is a self-protection mechanism for cells, which serves a vital role in the removal of dysfunctional and damaged organelles and macromolecules, and also in cellular homeostasis and metabolism (10,11). At the cellular level, the dysfunction of autophagy can lead to increased expression of abnormal genes, production of reactive oxygen species, and cell death (12). Consequences of autophagy failure at the organismal and tissue level include abnormal skeletal development, cardiomyopathy, neurodegeneration and premature mortality (13-15). Previous studies have demonstrated that the downregulation of autophagy is closely associated with the pathology of OA (16-19). Mammalian target of rapamycin (mTOR) is a crucial suppressor of autophagy, involving a number of autophagy-related proteins (Atg) (20-22). Rapamycin, a specific mTOR inhibitor, has been used as an immunosuppressive drug in solid organ transplantation and has been demonstrated to induce autophagy in a variety of cell types, including malignant glioma U87-MG cells and chondrocytes (23,24). It has previously been reported that rapamycin can inhibit neurodegenerative diseases, inflammation, infection, spinal cord injury, kidney damage and other diseases via activation of autophagy (25-28). In addition, it has been demonstrated that rapamycin is able to reduce the severity of experimental OA, at least in part, via autophagy activation (29).

It was recently observed that tetrahydrohyperforin (IDN5706) is a tetrahydro derivative of hyperforin, which is one of the main active components mediating the antidepressant activity of Hypericum perforatum L. extracts with many pharmacological uses, including anti-depression, anti-inflammation and anti-tumor properties (31-33). Together, these findings suggest that the pharmacological activation of autophagy may be an effective treatment for OA (29). Therefore, in the present study,
it was investigated whether IDN5706 ameliorated the degeneration of articular cartilage and affected autophagy in OA.

**Materials and methods**

**Establishment of experimental OA model.** A total of 30 male Sprague-Dawley rats (weight, 200±10 g; age, 8-10 weeks) were obtained from The Laboratory Animal Unit, Zhengzhou University (Zhengzhou, China). The rats were housed at the Animal Center of Anhui Medical University (Hefei, China). All experimental procedures were performed in strict accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) (34). All animals were housed in a well-ventilated holding room at a controlled temperature (24°C) and humidity (55%) with a 12 h light/dark cycle and ad libitum access to water and food. All animal experiments were approved by the Institutional Animal Care and Use Committee at The First Affiliated Hospital of Anhui Medical University (Hefei, China). Following 1 week of acclimation, rats were weight-matched and randomly assigned into three groups (n=10 each). Rats were anesthetized and received a 0.5 ml intra-articular injection of collagenase solution (Clostridium histolyticum, type II; 456 U/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in the right knee joint of the rat hindleg. Rats in the normal control group were administered with an equal volume of saline as a control. The administration was performed twice, at days 1 and 4, in accordance with the previously detailed method of papain injection (35). Following two weeks, 6 g/kg IDN5706 (Indena S.p.A., Milan, Italy; IDN5706 group) or an equal volume of saline (OA group) was intraarticularly administered once weekly for 6 weeks. The normal control group also received intraarticular administration of an equal volume of saline once for 6 weeks.

**Histological evaluation.** Following 6 weeks of drug treatment, rats were decapitated under intraperitoneal anesthesia with 1,000 mg/kg urethane, and articular cartilage was separated from the knee joint in rats (N=5/group). Knee cartilage tissue was fixed with 4% formaldehyde at 20°C for 30 min, embedded in paraffin and cut to obtain serial 5-µm thick sections. Subsequently, these sections were stained with hematoxylin and eosin (H&E) at room temperature for 10 min, and safranin O solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at room temperature for 5 min. Safranin O solution (prepared in water) was added at 0.1-0.5 mg/ml as a counterstain at room temperature for 5 min. Samples were observed under a light microscope (Nikon Corporation, Tokyo, Japan; magnification, x200) and the histological changes of articular cartilage were evaluated using the Mankin scoring system (36).

**Autophagy observed via transmission electron microscopy.** Knee cartilage tissue from the other rats (n=5/group) was fixed in 2.5% glutaraldehyde at room temperature for 4 h, washed with 0.1 mol/l PBS (pH 7.2) for 2 h and post-fixed in 1% osmium tetroxide at room temperature for 1 h. Following dehydration in ethyl alcohol, the tissue was embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, USA). Tissue blocks were cut serially into ultrathin (0.07 mm) sections, which were stained with uranyl acetate at 4°C for 2 h and lead citrate at 4°C for 20 min. Sections were subsequently observed under transmission electron microscopy (magnification, x12,000).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** The mRNA levels of matrix metalloproteinase-13 (MMP-13) and vascular endothelial growth factor (VEGF) were detected via RT-qPCR according to previously detailed methods (37). The total RNA was extracted from rat knee cartilage (n=10/group) via TRIzol reagent (Thermo Fisher Scientific, Inc.). RNA samples were subjected to reverse transcription using a GoScript Reverse Transcription system (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. RT-qPCR was performed using the SYBR® Premix Ex Taq™ kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's instructions.

The sequences of the PCR primers used were as follows: VEGF, forward 5'-CACCCCACCCCCCATATACA-3' and reverse 5'-CTCAAATGCTACAGGCTACA-3'; MMP-13, forward 5'-TGAATGGCCGATGCTGAA-3' and reverse 5'-AACGTGAATTCTGCCTTTGA-3'; and GAPDH, forward 5'-CCGAGTCAACCGATTTGGTCTAT-3' and reverse 5'-GCTCTTGGAAGATGGTATGGGGATTTC-3'. qPCR analysis was performed using ABI 7900 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Quantitative analysis of the relative expression of RNA was calculated using the 2-ΔΔCq method (38). GAPDH was used as an internal reference.

**Western blotting.** The protein expression of mTOR, Atg5, Beclin-1, microtubule-associated protein 1 light chain 3 (LC3)-I, LC3-II and phosphorylated (p-)mTOR was detected via western blotting. Rat knee cartilage (n=10/group) were lysed in 200 µl radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 1% Triton X-100 and protease inhibitors. The protein concentrations were quantified using a Bio-Rad Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (20 µg/lane) were then separated by 10% SDS-PAGE prior to being transferred onto nitrocellulose membranes (GE Healthcare, Chicago, IL, USA). Following blocking with 5% skimmed milk (Merck KGaA) overnight at 4°C, membranes were incubated with the following primary antibodies; anti-mTOR (cat. no. SAB2701842; 1:500), Atg5 (cat. no. A0731; 1:500), Beclin-1 (cat. no. SABI306484; 1:1,000), LC3-I and LC3-II (both from Anti-LC3B antibodies; cat. no. SAB2700738; 1:1,000), p-mTOR (cat. no. SAB4504476; 1:500), and anti-GAPDH (cat. no. SAB2100894; 1:500) (Sigma-Aldrich; Merck KGaA) at 4°C overnight. Subsequently, protein bands were detected by incubation with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (cat. no. A50-106P; 1:1,000; Origene Technologies, Inc, Beijing, China) at room temperature for 1 h. The chemiluminescent signal was visualized using an ECL Detection Reagents (GE Healthcare). GAPDH was used as a loading control. Each protein sample was examined in triplicate.

**Cell culture.** Chondrocytes were isolated from the knee joint of OA model rats (n=5/group). In brief, the articular cartilage
tissue from the right knee joint of OA rats was cut into small pieces (<1 mm\(^3\)) and incubated with 0.2% trypsin at 37°C for 30 min. Following removal of the trypsin solution, the tissue was treated with 0.2% Type II collagenase at 37°C for 2 h. Released cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.), L-glutamine, and antibiotics including penicillin (100 u/ml) and streptomycin (100 g/ml). The medium was replaced every 3 days. Following the growth of cells to 80-90% confluence, third passage chondrocytes were used for all further experiments. Then, the chondrocytes were cultured with IDN5706 (50, 100, 150 and 200 µmol/l) in DMEM, supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), and penicillin/streptomycin (Thermo Fisher Scientific, Inc.), in a 5% CO\(_2\) atmosphere at 37°C for 16 h. Untreated chondrocytes were used as a control.

**Cell counting kit-8 (CCK-8) assay.** Cell viability was assessed using a CCK-8 assay kit according to the manufacturer's protocol (MedChem Express, Monmouth Junction, NJ, USA). In brief, cells were seeded in a 96-well plate at a density of 1x10\(^5\) cells/well in 100 µl culture medium in a CO\(_2\) incubator at 37°C for 24 h. Afterwards, cells were cultured with cisplatin (10 µg/ml; Hospira Australia Pty, Ltd., Melbourne, Australia) for 72 h. Subsequently, 10 µl CCK-8 solution was added to each well followed by incubation at 37°C for 4 h. Absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). All experiments were performed in triplicate.

**ELISA.** Chondrocytes were isolated from the knee joint of OA model rats (n=5/group). ELISA was used for the quantitative detection of MMP-13 and interleukin (IL)-6. The levels of MMP-13 and IL-6 were determined using a Matrix Metalloproteinase 13 ELISA kit (cat. no. DL-MMP13-Ra; DLdevelop; Wuxi Donglin Sci & Tech Development Co., Ltd., Wuxi, China) and an IL-6 Mouse ELISA kit (cat. no. BMS603HS; Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. All samples were measured in duplicate.

**Statistical analysis.** Data were analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) and expressed as the mean ± standard deviation. Student's t-test was used to analyze differences between two groups. One-way analysis of variance followed by a Dunnett's post-hoc test was used to perform multiple group analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**IDN5706 reduces the pathological injury of OA.** Initially, the effect of intragastric administration of IDN5706 on OA was evaluated by determining pathological changes using H&E and safranin O staining, and Mankin scoring system. H&E staining revealed normal morphology of joints in the normal group, whereas the surface of the joint cartilage exhibited defects, damage and structural breakage with a decreased number of cartilage cells in the articular cartilage tissue of the OA group. Treatment with IDN5706 alleviated the pathological changes in edema, degeneration, clustering and necrosis in articular cartilage of the knee joints, compared with the OA group (Fig. 1A). Safranin O staining demonstrated that nucleoli were stained brilliant red, and cytoplasm were stained light red in the normal group. However, markedly reduced staining was observed in articular cartilage of the knee joints from OA rats, indicating that degeneration and denaturing of the cartilage occurred. In addition, the nucleoli were stained brilliant red in the IDN5706 group (Fig. 1A), suggesting a therapeutic effect of IDN5706 on OA. Furthermore, Mankin scores were calculated in each group. As presented in Fig. 1B, the score was significantly elevated in the OA group compared with the normal group, and IDN5706 treatment significantly decreased this score compared with the OA group, indicating that IDN5706 may have a therapeutic effect on OA (P<0.01).
IDN5706 may protect articular cartilage and affect autophagy. To elucidate the influence of IDN5706 in OA, the degeneration of chondrocytes and autophagosome were observed using electron microscopy. In the normal group, a intact cell structure was observed, the rough endoplasmic reticulum was abundant and concentrated in the perinuclear regions, and surrounded various types of organelles and autophagosomes (Fig. 2A). Decreased numbers of organelles, swelling mitochondria and vacuolar degeneration were observed in the cytoplasm of the OA group, and autophagosomes with double membranes were also decreased (Fig. 2A). Following treated with IDN5706, autophagosomes with double membranes were increased compared with the OA group (Fig. 2A). These results indicated that IDN5706 alleviated the degeneration of chondrocytes caused by OA.

To further investigate the detailed role of IDN5706 in autophagy and OA, OA- and autophagy-related genes and proteins were detected by RT-qPCR and western blotting. The results demonstrated that the expression of MMP-13 and VEGF in the OA group was significantly increased compared with the normal group (P<0.01), and the relative levels of MMP-13 and VEGF were significantly reduced in the IDN5706 group compared with the OA group (P<0.01) (Fig. 2B). The results mentioned above indicated that IDN5706 may have a protective role in articular cartilage. As presented in Fig. 2C, the phosphorylation of mTOR was markedly increased in the OA group compared with the normal group, however, IDN5706 treatment resulted in a marked amelioration in the levels of p-mTOR. Furthermore, the protein levels of autophagy-related proteins, including LC3, Beclin-1 and Atg5, were reduced in the OA group compared with the normal group, and displayed a marked increase in the IDN5706 group compared with the OA group. These findings suggest that IDN5706 had a protective role in OA.
IDN5706 reduces the injury of chondrocyte. To further confirm the therapeutic effect of IDN5706 in OA, chondrocytes were isolated from the knee joints of OA rats and cultured with IDN5706 (50, 100, 150 and 200 µmol/l). As presented in Fig. 3A, cell viability in the five groups of chondrocytes exhibited no difference compared with the chondrocytes without treatment of IDN5706. The finding indicated that there was no injury of IDN5706 for the chondrocytes. Thus, 50 µmol/l IDN5706 was chosen for the subsequent assays. The results from ELISA indicated that the levels of MMP-13 and IL-6 were significantly decreased in the IDN5706 group compared with the control group (P<0.01; Fig. 3B). As presented in Fig. 3C, IDN5706 treatment resulted in a marked decrease in the protein levels of p-mTOR, and a significant increase in the protein levels of LC3, Beclin-1 and Atg5, in the IDN5706 group compared with the control group. These findings suggest that IDN5706 had a protective effect on chondrocytes and affected the level of autophagy in vitro.

Discussion

OA is a highly prevalent disease and principally affects the biomechanical and structural properties of the focal regions of articular cartilage tissue (39). It is characterized by limited intra-articular inflammation with synovitis, degeneration of articular cartilage, and changes in subchondral bone and periarticular bone (5). Substantial progress has been achieved in understanding pathogenesis pathways in established OA, and a large number of drug candidates have been effective in OA animal models (40-43). In the present study, justification was provided for the therapeutic use of IDN5706, a synthetic derivative of the chemical compound hyperforin of the St John's Wort plant, which may lessen symptoms of OA and alleviate the degeneration of articular cartilage.

Intra-articular injection of collagenase has been widely used to induce the murine model of OA (18,44-47). Intra-articular injection with collagenase induces damage to collagen type I-containing joint structures, such as tendons, ligaments and menisci, leading to the osteoarthritic joint lesions observed in this model (48). It is a rapid and economic method to induce OA-like lesions (47,48). In addition, collagenase-induced OA was characterized by severe degenerative cartilage lesions on the medial side of the femorotibial joint associated with patellar dislocation to the medial side of the joint, sclerosis of subchondral bone below the cartilage erosions, osteophyte formation and consequent deformity of the knee joints. The collagenase-induced OA model offers the possibility of studying experimental OA in large animal groups (47). In the present study, OA was induced in rats with intra-articular injection with collagenase. Largely consistent with previous studies that successfully established OA rat models, the collagenase-induced OA rats displayed degeneration and denature of the articular cartilage.

In the experimental OA model, it was observed that treatment with IDN5706 caused a marked reduction in OA severity.
The Mankin scoring system of OA indicated the degree of the cartilage lesions, which reflect extracellular matrix degradation and cell loss. It was observed that the Mankin score was significantly elevated in the OA group compared with the normal group, whereas IDN5706 treatment significantly decreased the score, indicating that IDN5706 exerted a definite effect on OA. Furthermore, transmission electron microscopy revealed that IDN5706 alleviated the degeneration of chondrocytes caused by OA. The expression of VEGF and MMP-13 was significantly decreased in cartilage from IDN5706-treated OA rats, compared with the OA group. The association of increased production of proteinases, such as MMP-13, with cartilage damage has been previously established (49,50). Furthermore, expression of VEGF and MMP-13 may disrupt normal homeostasis, resulting in abnormal cartilage and bone metabolism (51). As autophagy level was closely associated with OA, autophagosomes were examined using transmission electron microscopy and protein levels of autophagy-related proteins Beclin-1 and LC3 were detected using western blotting. Results from electron microscopy demonstrated decreased autophagosomes in the OA rats. Furthermore, autophagosomes with double membranes were increased in IDN5706-treated rats compared with the experimental OA rats. Furthermore, it has been reported recently that autophagy activation by IDN5706 is dependent on mTOR inactivation and Atg5 levels (30). Consistent with this, the present results demonstrated that IDN5706 treatment increased the number of autophagosomal structures, and levels of LC3-II, Beclin-1 and Atg5, and decreased the levels of p-mTOR, which further indicated that autophagy loss partially resulted in the degeneration of articular cartilage following induction of OA with collagenase injection.

To confirm this hypothesis, the impact of IDN5706 on chondrocytes was detected in vitro. At the onset of OA, articular cartilage differentiates into hypertrophic cartilage, and the hypertrophic chondrocytes express MMP-13 and further degrade the cartilage matrix (52-54). MMP-13 was previously confirmed to be associated with the regulation of chondrocyte proliferation and chondrocyte hypertrophy (55,56). In addition, OA in experimental models and humans is associated with inflammatory changes (5). Previous research has demonstrated that IL-6 can increase inflammatory cells and stimulate the proliferation of chondrocytes (57). In the present study, it was demonstrated that IDN5706 also reduced the levels of MMP-13 and IL-6 in chondrocytes. Meanwhile, the levels of LC3-II, Beclin-1 and Atg5 were increased and the level of p-mTOR was decreased. The main findings of the present study are presented in Fig. 4. These findings proved once again that IDN5706 prevented articular cartilage degeneration in rats with OA and affected autophagy.

However, an increasing number of studies have demonstrated that, monitoring autophagic flux in vivo or in organs is limited at present, and ideal methods associated with cell culture techniques may not exist (58,59). One potential method for in vivo studies is the analysis of green fluorescent protein-light chain 3/Atg8 using fluorescence microscopy, and another is immunohistochemical staining (59). Therefore, the use of transmission electron microscopy in the present study is not sufficient to monitor autophagy in vivo. Furthermore, other autophagy-related proteins, such as Atg8/LC3, which is an excellent marker for autophagic structures, are recommended to be measured. In addition, another factor that should be considered is that the effect of intra-articular injection of collagenase solution itself on the change of autophagy level. All of these factors affect the interpretation of the present results. The detected level of autophagy may be the result of comprehensive effects mediated by both known and unknown factors. Collectively, these are limitations of the present study. Detection of in vivo autophagy using other methods should be performed in future studies.

In conclusion, the present study is, to our knowledge, the first to establish the efficacy of IDN5706 in an animal model of OA. The present data demonstrated that IDN5706 reduced the severity of experimental OA, alleviated the degeneration of articular cartilage, and affects autophagy. These results suggest that IDN5706 is a potentially effective therapeutic approach for OA.

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Availability of data and materials
The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors’ contributions
CS and JZ conceived and designed the study. JZ, SW, GR, BG, FC performed the experiments. JZ and SW wrote the paper. CS reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate
All experiments related to the use of animals were approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Anhui Medical University (Hefei, China).

Consent for publication
Not applicable.
Compeing interests
The authors declare that they have no competing interests.

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