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DHA attenuates cartilage degeneration by mediating apoptosis and autophagy in human chondrocytes and rat models of osteoarthritis

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Abstract

Osteoarthritis (OA) is a degenerative joint disease that usually occurs in the elderly, and docosahexaenoic acid (DHA) plays a therapeutic role in cardiovascular disease, diabetes, and rheumatoid arthritis (RA) with its anti-inflammatory and antioxidant effects. The objective of this study is to investigate the effect and mechanism of DHA on hypertrophic differentiation and senescence of OA chondrocytes to provide a theoretical basis for the effect of OA clinical treatment. A human OA chondrocyte model was established by IL-1β, and a rat model of OA was established by anterior cruciate ligament (ACL) transection and medial meniscectomy. The result showed DHA promoted chondrocyte proliferation and reduced apoptosis. Transmission electron microscopy (TEM) analysis showed that there were more autophagosomes in the cytoplasm under the treatment of DHA. Compared to the OA group, samples from the OA + DHA group showed thickened cartilage, reduced degeneration, and an increased rate of collagen II–positive cells, while the Mankin score was significantly lower. In addition, DHA decreased the expression of phosphorylated mammalian target of rapamycin (p-mTOR) and the ratio of light chain 3-I/II (LC3-I/II) and increased the expression of Beclin-1 and Bcl-2 measured by western blot analysis. Therefore, DHA promotes chondrocyte proliferation, reduces apoptosis, and increases autophagy in OA chondrocytes, a process that is accomplished by inhibiting the expression of mTOR, c-Jun N-terminal kinase (JNK), and p38 signaling pathways, providing new perspectives and bootstrap points for the prevention and treatment of OA.

Keywords Osteoarthritis · Docosahexaenoic acid · Apoptosis · Autophagy · Chondrocyte

Introduction

Osteoarthritis (OA) is a degenerative joint disease, which usually affects the elderly. Studies have shown that more than half of the people over 65 yr of age have symptomatic or radiological OA (Bijlsma *et al.* 2011). With an increase in the aging population around the world, there has been a gradual increase in the prevalence of OA (Murphy and Helmick 2012), causing a huge social and economic burden. Pathologic processes in OA involve multiple molecular pathways and biomarkers, such as matrix metalloproteinases (MMPs), the tissue inhibitors of metalloproteinases (TIMPs), nitric oxide, c-myc, p53, mitogen-activated protein kinase/c-JunN terminal kinase(MAPK/JNK), Toll-like receptors 1/2 (TLR1/2), poly(ADP-ribose) polymerase (PARP-1), and tumor necrosis factor-α(TNF-α) (Vincenti 2001; Musumeci *et al.* 2015).

Apoptosis, also known as programmed cell death, plays a crucial role in maintaining the homeostasis of various tissues in the body. Dysregulation of apoptosis leads to pathological conditions such as cancer, developmental disorders, and degenerative diseases (Hwang and Kim 2015). Autophagy is a metabolic process in which the lysosomes degrade damaged proteins and organelles. It is an important survival mechanism when cells are under stress, and it plays an important role in hypoxia, starvation, and other stress conditions (Caramés *et al.* 2015; Riffelmacher *et al.* 2018).

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Docosahexaenoic acid (DHA) is an n-3 polyunsaturated fatty acid, which is commonly found in deep-sea fish oil and vegetable oil, making it an important nutrient in the diet (Shahidi and Ambigaipalan 2018). According to recent studies, DHA has extensive and definite anti-inflammatory effects on multiple systems, which has a wider range of physiological and anti-inflammatory functions than other n-3 PUFAs (such as eicosapentaenoic acid and alpha-linolenic acid) in the human body (Richard and Calder 2016; Kuda 2017; David *et al.* 2022). In lipopolysaccharide-induced (LPS-induced) THP-1 (The human monocytic cell line) cells, 100 μmol/L DHA could reduce the level of TNF-α, IL-1β, IL-6, and other inflammatory factors (Gorjão *et al.* 2009). When rats were fed with fish oil containing DHA and eicosapentaenoic acid (EPA), intercellular adhesion molecule 1 (ICAM-1) expression on the surface of macrophages was decreased, and the synthesis of TNF-α, IL-1β, and IL-6 was significantly reduced (Renier *et al.* 1993). But the activity of DHA on the inflammatory response of OA chondrocytes and cartilage is still unclear. We speculated that DHA played an anti-inflammatory role in OA by regulating apoptosis and autophagy of chondrocytes. Thus, we determined the effect of DHA on OA chondrocytes in cell culture and in an OA rat model.

Material and methods

Isolation, culture, and identification of chondrocytes The surface of cartilage was resected from the normal area of the femoral head in patients with femoral neck fractures undergoing artificial femoral head replacement. The cartilage was from seven patients (2 males and 5 females with no prior relevant surgical history, aged 63–82 yr). Ethics approval was granted by the Bioethics Committee of Beijing Friendship Hospital, Capital Medical University, China (approval code 2020-P2-205-01). Informed consent was obtained from all patients before surgery. The cartilage tissue was rinsed in PBS (Solarbio, Wuhan, China) and cut into small pieces. Next, 0.2% type II collagenase (Sigma, St.Louis, MO) was added to isolate the chondrocytes, and the vessel was vibrated in a 37°C water bath for 3–4 h. The supernatant was centrifuged at 1200 rpm for 5 min, and the precipitated cells were rinsed with PBS, incubated with DMEM medium (Solarbio) supplemented with 10% fetal bovine serum (Sigma), 100 IU/mL penicillin and 100 μg/mL streptomycin, at 37°C in humidified air containing 5% CO_2.

We used safranin O and toluidine blue staining to identify chondrocytes. Cells were seeded in cell climbing sheets, dehydrated with 95% ethanol, stained with safranine O and toluidine blue, respectively, and then cleaned and sealed with neutral resin. Cells from the same patient were used in a single replicate of the same in vitro experiment.

In the first passage, chondrocytes were cultured up to 80% confluency, counted, and divided into four groups. The control group was treated with normal saline in the same amount as the other groups. The IL-1 group was stimulated with 100 pg/mL IL-1 (Sigma) to establish the OA chondrocyte model. The IL-1 + DHA group was pre-treated with 50 μg/mL DHA (Sigma) 1 h before 100 pg/mL IL-1. The IL-1 + DHA + 3-MA group was pre-treated with 50 μg/mL DHA and 5 mmol/L 3-Methyladenine (3-MA) (Sigma), an autophagy inhibitor 1 h before treatment with 100 pg/mL IL-1 as previously described (Wang *et al.* 2016). Protein expression was measured 24 h after the stimulation by IL-1.

Animal models A total of 24 Sprague Dawley male rats (8 wk old) were divided into the control group, OA group, sham + DHA group, and OA+ DHA group ($n = 6$ each). The control group and OA group were fed a normal AIN-93G (American Institute of Nutrition-93G) diet (3.96 kcal/g) (Beijing Keao Biological Pharmaceutical Company, Beijing, China), and the sham + DHA group and OA + DHA group were fed an AIN-93G diet rich in DHA (add 5 g DHA per kg diet) (3.98 kcal/g). It is thought that DHA has an absorption rate of 62.3±30% from the intake (Moya *et al.* 2001) and small amounts of DHA (200mg) result in a large increase in the proportion of DHA in blood lipids (Sanders 2009). In the OA group and OA + DHA group, OA rat models were prepared by anterior cruciate ligament transection and medial meniscectomy. The control group and sham + DHA group were compared by sham operation (only the articular capsule was cut open). All rats were euthanized by cervical dislocation after 6 wk, while the cartilage and subchondral bone of the knee on the femur and tibia were removed and stored in formaldehyde solution for further histology. The flow chart of the *in vivo* experiment is showed in Fig. 1. All procedures complied with the ARRIVE guidelines, were carried out in accordance with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Bioethics Committee of Beijing Friendship Hospital, Capital Medical University, China (approval code 2020-P2-205-01).

Cell viability analysis The cell viability analysis was done using the CCK8 (cell counting kit-8) assay. Chondrocytes were seeded in 96-well plates (5000 cells/well) and incubated at 37°C for 24 h. Then, the cells were divided into four groups and pre-treated as previously mentioned. After 24 h, 10 μL of CCK-8 (Solarbio) reagent was added to each well, and the cells were incubated for another 4 h. The absorbance was measured using a multi-functional microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm. The relative cell viability (RCV) to the controls was calculated using the following equation:

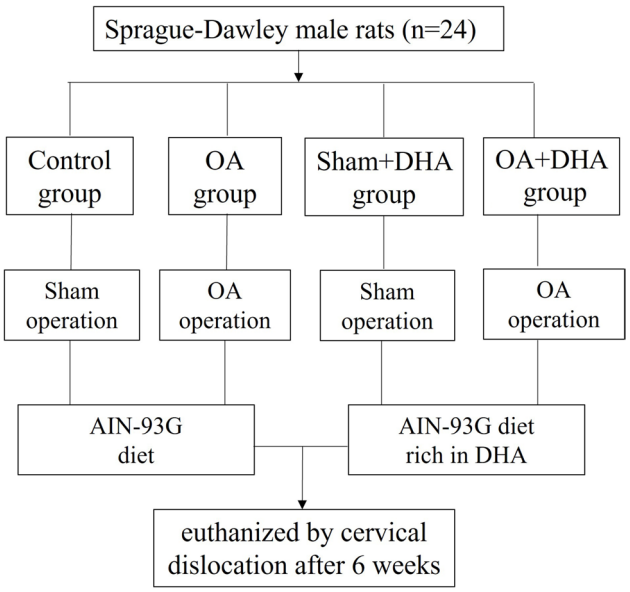

Figure 1. The flow chart of in vivo experiment about operations and drug delivery. Twenty-four Sprague Dawley male rats (8 wk old) were divided into the control group, OA group, sham + DHA group, and OA+ DHA group ($n = 6$ each). Sham operation: only the articular capsule was cut open. OA operation: anterior cruciate ligament transection and medial meniscectomy.

$$\mathrm{RCV} = \left(\mathrm{OD}_{\mathrm{test}} - \mathrm{OD}_{\mathrm{blank}}\right) / \left(\mathrm{OD}_{\mathrm{control}} - \mathrm{OD}_{\mathrm{blank}}\right) \times 100\%.$$

Flow cytometry assay Chondrocytes were cultured with various treatments for 24 h in 6-well plates, and the apoptosis rates were determined by Annexin V-FITC/PI apoptosis kit according to the instructions using flow cytometry (FCM) analysis. The cells were resuspended in 1X binding buffer and stained with 5 μL of fluorescently labeled Annexin V (Annexin V-FITC) and 5 μL of propidium iodide (PI), and then cultured in the dark at 25°C for 15 min. After culture, the samples were detected by flow cytometry. The cells considered to be in early apoptosis were Annexin V-FITC positive and PI negative; Annexin V-FITC and PI were both positive in late apoptotic or dead cells. The experiment was performed thrice.

Transmission electron microscopy Chondrocyte degeneration and autophagosomes were observed by transmission electron microscopy. Cells were collected and fixed with 1% OsO_4 in PBS (pH 7.0) for 2 h and washed 3 times in PBS. The chondrocytes were then continuously washed and dehydrated using ethanol. After that, samples were incubated in a mixture of alcohol and isoamyl acetate and embedded in gold palladium. Ultrathin slices were produced and observed under transmission electron microscopy (TEM, Hitachi, Tokyo, Japan). Five cells in each group were randomly selected and the numbers of autophagosomes were recorded for statistical analyses.

Western blot analysis The cells were washed in ice-cold PBS and lysed in total protein lysis buffer (Ukzybiotech, Beijing, China) supplemented with 1 mg/mL protease inhibitor cocktail (Roche, Indianapolis, IN) at 4°C for 30 min. After protein extraction, the bicinchoninic acid (BCA) (Solarbio) method was used for protein quantification. Next, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed at 95°C for 5 min, with the final sample concentration of 0.8 μg/μL adjusted by radioimmunoprecipitation assay (RIPA) buffer. After electrophoresis, the protein bands were transferred to a 0.45 μm polyvinylidene fluoride (PVDF) membrane (Millipore, St.Louis) for 90 min. The membrane was completely immersed in 5% bovine albumin-TBS with Tween 20 (BSA-TBST) and incubated in a horizontal shaker for 1 h. Then, the membrane was incubated with 5% BSA-TBST and diluted with primary antibody to p-mTOR (1:1000) (Cell signaling, MA, Danvers), Beclin-1 (1:2000) (Abcam, Cambridge, UK), Bcl-2 (1:1000) (Abcam), light chain 3 (1:2000) (Abcam), extracellular signal-regulated kinase (ERK) (1:1000) (Cell signaling), p-ERK (1:1000) (Cell signaling), p-JNK (1:1000) (Cell signaling), JNK (1:1000) (Cell signaling), p-p38 (1:1000) (Cell signaling), p38 (1:1000) (Cell signaling), and β-actin (1:1000) (ZS, Beijing, China) overnight at 4°C. The blots were then developed using a horseradish peroxidase (HRP)–conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody (1:10,000) (Jackson, Westgrove PA), incubated at room temperature for 40 min, and subjected to signal development using enhanced chemiluminescence (ECL) substrate (Thermo, Waltham, Ma). β-actin was used as an internal parameter; the expression of each protein was equal to the gray value of target protein/β-actin. Each sample was analyzed thrice, and the mean values of the immunoblot band were calculated.

Cartilage and subchondral bone histology The distal femur and proximal tibia fixed with formalin were decalcified in 0.5 M EDTA for 6 wk and then embedded in paraffin. A series of 4 μm sections were made on the sagittal plane along the middle of the medial femoral condyle of the knee joint. This area was the weight-bearing surface of the medial femoral condyle, showing the earliest and most serious histological abnormalities in the joint (Calvo *et al.* 2004). Next, sections were stained with H&E, toluidine blue, and safranin O Fast Green to evaluate histological changes. A modified version of Mankin's grading score system was used on safranin O Fast Green staining to assess cartilage damage (Table 1) (Tiraloche *et al.* 2005). Images with an original magnification of ×400 were obtained by optical microscope. Each

Table 1. The modified version of Mankin's grading score system for histologic evaluation

Safranin O Fast Green staining
0 = uniform staining throughout articular cartilage
1 = loss of staining in the superficial zone for less than one-half of the length of the condyle or plateau
2 = loss of staining in the superficial zone for one-half or more of the length of the condyle or plateau
3 = loss of staining in the superficial and middle zones for less than one-half of the length of the condyle or plateau
4 = loss of staining in the superficial and middle zones for one-half or more of the length of the condyle or plateau
5 = loss of staining in all 3 zones for less than one-half of the length of the condyle or plateau
6 = loss of staining in all 3 zones for one-half or more of the length of the condyle or plateau
Chondrocyte loss
0 = no decrease in cells
1 = minimal decrease in cells
2 = moderate decrease in cells
3 = marked decrease in cells
4 = very extensive decrease in cells
Structure
0 = normal
1 = surface irregularities
2 = 1–3 superficial clefts
3 => 3 superficial clefts
4 = 1–3 clefts extending into the middle zone
5 => 3 clefts extending into the middle zone
6 = 1–3 clefts extending into the deep zone
7 => 3 clefts extending into the deep zone
8 = clefts extending to calcified cartilage
Clone formation
0 = normal
1 = minimal (≤4)
2 = moderate (>4 but ≤8)
3 = marked (>8)
Tidemark integrity
0 = intact
1 = crossed by blood vessels

image was evaluated thrice by three experienced observers, and the average score was used in statistical analyses.

Immunohistochemical analysis Four micrometers of formalin-fixed paraffin sections was prepared for each sample, then isolated in xylene, hydrated with graded ethanol, rinsed in PBS for 5 min, and incubated in 0.125% trypsin (Sigma) for 15 min at 37°C. The slices were then cultured in 0.3% H_2O_2/methanol for 30 min to inhibit endogenous peroxidase activity and rinsed three times with PBS for 3 min. Next, the slides were blocked with 100 μL normal goat serum (Solarbio) at room temperature for 15 min. After the blockade, the slides were incubated overnight with rabbit anti-collagen II antibody (1:200, Bioss, Beijing, China) at 4°C. The next day, a secondary biotinylated goat anti-rabbit antibody (Bioss) was added for 30 min and then incubated with an avidin-biotin complex reagent containing horseradish peroxidase (Bioss) for 30 min. A negative control was also performed which is without collagen II first antibody, only incubated with an isotype-matched secondary antibody. Then, the sections were stained with 3,3'-diaminobenzidine (DAB) (Sigma). The positivity rate was calculated as positive cells/total cells.

Statistical analysis The data are expressed as the mean and standard deviation (sd). Statistical analysis was performed using SPSS 19.0. The student's *t*-test was used to compare two groups. One-way analysis of variance was used for multiple comparisons between independent groups. A statistically significant difference was considered as $P < 0.05$.

Results

DHA increased human chondrocyte viability There was no significant difference between the RCV of the IL-1 group and the control group ($P = 0.3$). However, after the addition of DHA, the cell proliferation rate increased by 6.53% compared to the control group ($P = 0.017$). After the addition of 3-MA into the medium, the change in relative cell viability (RCV) was not significant compared to the IL-1+ DHA group ($P = 0.651$). These results indicated that DHA acted as a positive regulator for the cell proliferation of osteoarthritic chondrocytes (Fig. 2).

DHA suppressed IL-1 induced chondrocyte apoptosis Flow cytometry was used to evaluate cell apoptosis to further explore the effects of DHA on the apoptosis of human chondrocytes (Fig. 3*A–D*). The results showed that when IL-1β acted on chondrocytes, there was a significant increase in the percentage of apoptotic chondrocytes ($P < 0.001$). After the treatment with DHA, the percentage of apoptotic chondrocytes decreased ($P < 0.001$). However, when IL-1-induced chondrocytes were incubated with DHA and 3-MA, the percentage of cell apoptosis increased significantly compared to the IL-1 + DHA group ($P < 0.001$) (Fig. 3*E*).

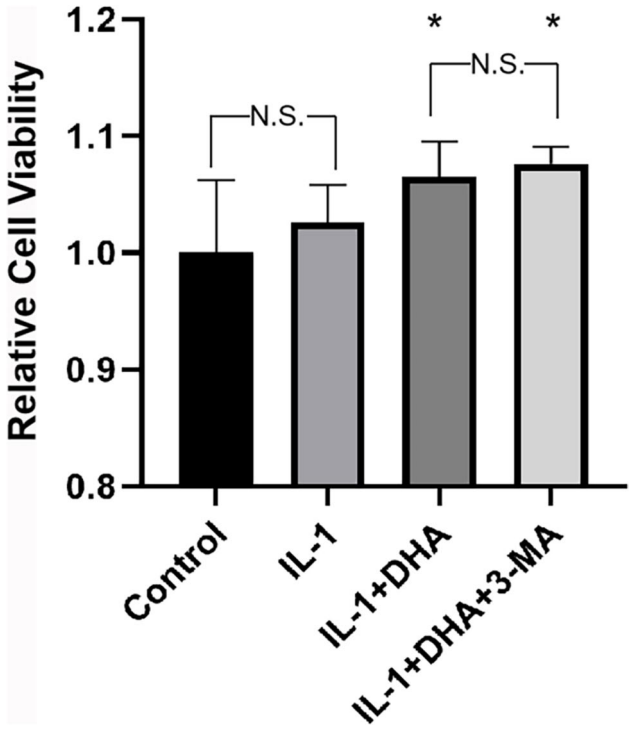

Figure 2. Comparison of the relative cell viability (RCV) in chondrocytes between four groups 24 h after stimulation (n=5). *$P < 0.05$ compared to the control group. N.S. not significant.

DHA elevated autophagic activity of OA chondrocytes Chondrocytes were observed via TEM to detect autophagic bodies and other organelles. In the control group, chondrocytes appeared with a normal structure, with several autophagic bodies with bilayer membrane structures in the cytoplasm. In the IL-1 group, fewer autophagic bodies were observed in chondrocytes. In the IL-1+DHA group, a greater number of autophagic bodies and rough endoplasmic reticulum were found in the cytoplasm. In the IL-1+DHA+3-MA group, fewer autophagic bodies and more vacuoles were seen in the cells (Fig. 4).

DHA reduced protein expression of p-JNK and p-p38 in OA chondrocytes Next, we examined the expression of p-ERK/t-ERK, p-JNK/t-JNK, and p-p38/t-p38 by western blotting (Fig. 5). The results showed that IL-1 induced an increase in the expression of p-JNK/t-JNK ($P = 0.002$) and p-p38/t-p38 ($P < 0.001$), while treatment with DHA significantly decreased the expression of p-JNK/t-JNK ($P = 0.001$) and p-p38/t-p38 ($P < 0.001$) compared to the IL-1 group.

DHA decreased p-mTOR expression and LC3-I/II ratio while increased Beclin-1 and Bcl-2 expression The expression of p-mTOR, Beclin-1, Bcl-2, and LC3-I/II was detected. The results showed that p-mTOR expression ($P = 0.003$) and LC3-I/II ratio ($P < 0.025$) were elevated in OA chondrocytes, while the expression of Beclin-1 ($P < 0.001$) and Bcl-2 ($P < 0.001$) was significantly decreased. Compared to the IL-1 group, DHA decreased p-mTOR expression ($P = 0.001$) and LC3-I/II ($P < 0.037$) and increased the expression of Beclin-1 ($P < 0.001$) and Bcl-2 ($P < 0.001$). However, when the cells were incubated with DHA and autophagy inhibitor 3-MA, p-mTOR expression increased significantly ($P = 0.001$), and the expression of Beclin-1 ($P < 0.001$) and Bcl-2 ($P < 0.001$) decreased, indicating that the effect of DHA was blocked by 3-MA (Fig. 6).

DHA alleviated cartilage damage and reduced the Mankin score in the OA rat model Cartilage damage in the knees was shown by H&E, toluidine blue, and safranin O Fast Green staining (Fig. 7*A*) and was evaluated using a modified histopathological Mankin grading score (Fig. 7*B*). Sections from the OA group showed more erosion, less thickness of the cartilage, and more irregular cartilage surface than the control group and the sham + DHA group. Total Mankin scores of the OA group were significantly higher than the control group and the sham + DHA group (both $P< 0.001$). In the OA + DHA group, the Mankin scores were also higher than that of the control group and the sham + DHA group (both $P< 0.001$). However, when compared with the OA group, cartilage samples from the OA + DHA group showed thicker cartilage and decreased degradation. The Mankin scores were significantly lower in the OA + DHA group compared

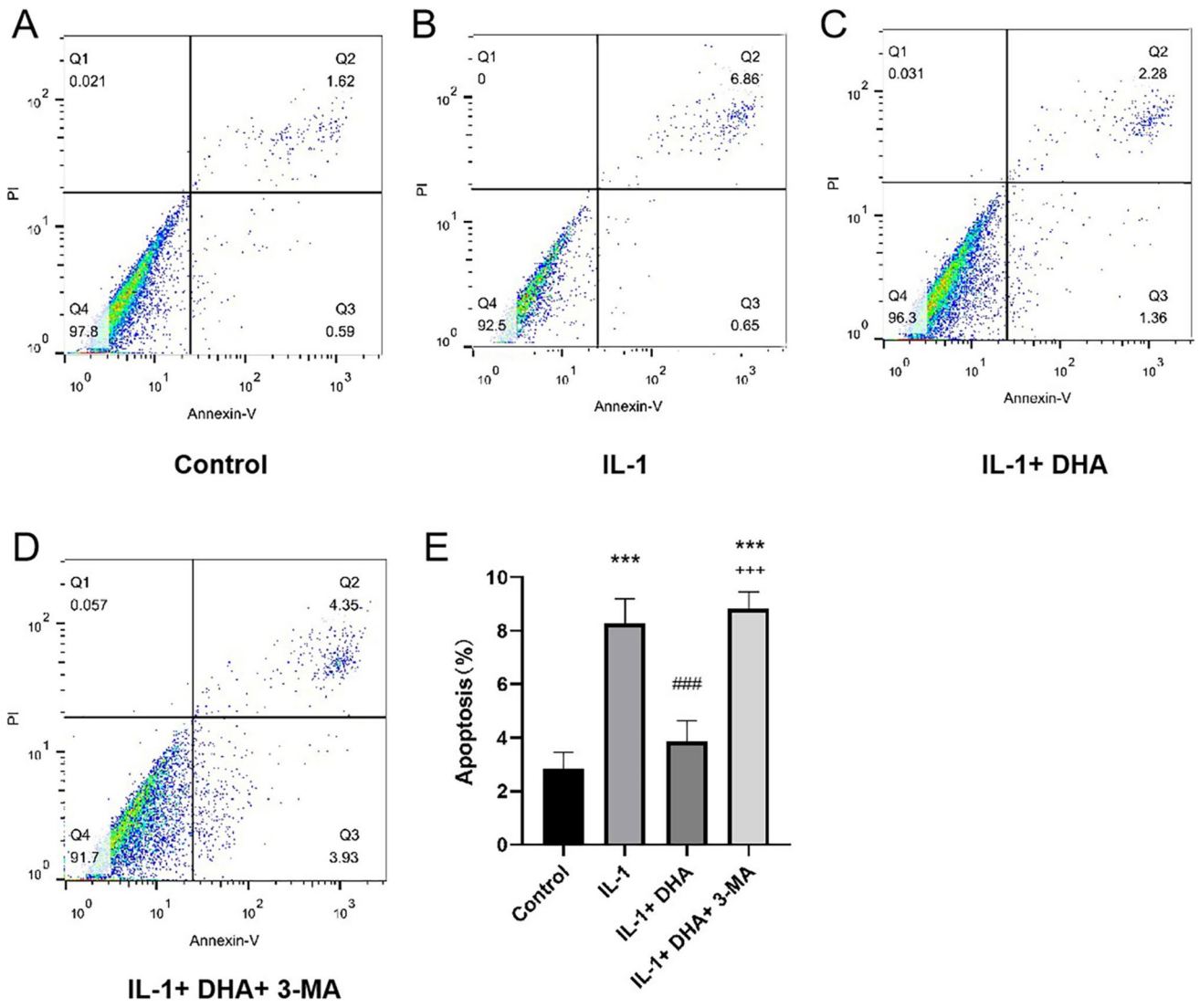

Figure 3. ***(A–D)*** Flow cytometric detection of chondrocyte apoptosis between four groups 24 h after stimulation ($n = 3$). Apoptotic cells labeled with Annexin V and propidium iodide (PI) fluorescence were estimated. Signals of Annexin V showed the proportion of cells with early apoptosis and PI showed the proportion of cells with late apoptosis and death. Q1: cell fragments. Q2: late apoptosis and cell death. Q3: early apoptosis. Q4: live cells. (***E***) Apoptotic rates are shown by histogram. $^{***}P < 0.001$ compared to the control group, $^{\#\#\#}P < 0.001$ compared to the IL-1 group, $^{+++}P < 0.001$ compared to the IL-1 + DHA group.

to the OA group (6.55 ± 1.28 vs. 8.06 ± 1.04, respectively; $P = 0.018$).

DHA increased collagen II–positive cell rate in the OA rat model Collagen II expression of the cartilage was measured by immunohistochemical staining (Fig. 8). The sections showed significantly fewer positive cells in the OA group compared with the control and the sham + DHA group (both $P < 0.001$). However, when the OA model rats were fed DHA, there was a significant increase in the collagen II–positive cell rate (46.16 ± 11.79% vs. 57.50 ± 6.60%, respectively; $P = 0.046$), indicating more collagen II expressions in the cartilage.

Discussion

Osteoarthritis, also known as degenerative arthropathy, is characterized by articular cartilage degeneration, subchondral bone remodeling, synovitis, and osteophyte formation (Loeser *et al.* 2012; Jiang 2022). OA leads to joint pain, stiffness, deformity, and dysfunction. It occurs commonly in the elderly and is associated with limb disability and a severe decline in the quality of life of the patients (Glyn-Jones *et al.* 2015; Abramoff and Caldera 2020). According to statistics, the incidence of osteoarthritis accounts for 15–18% of the total population and includes approximately 80% of

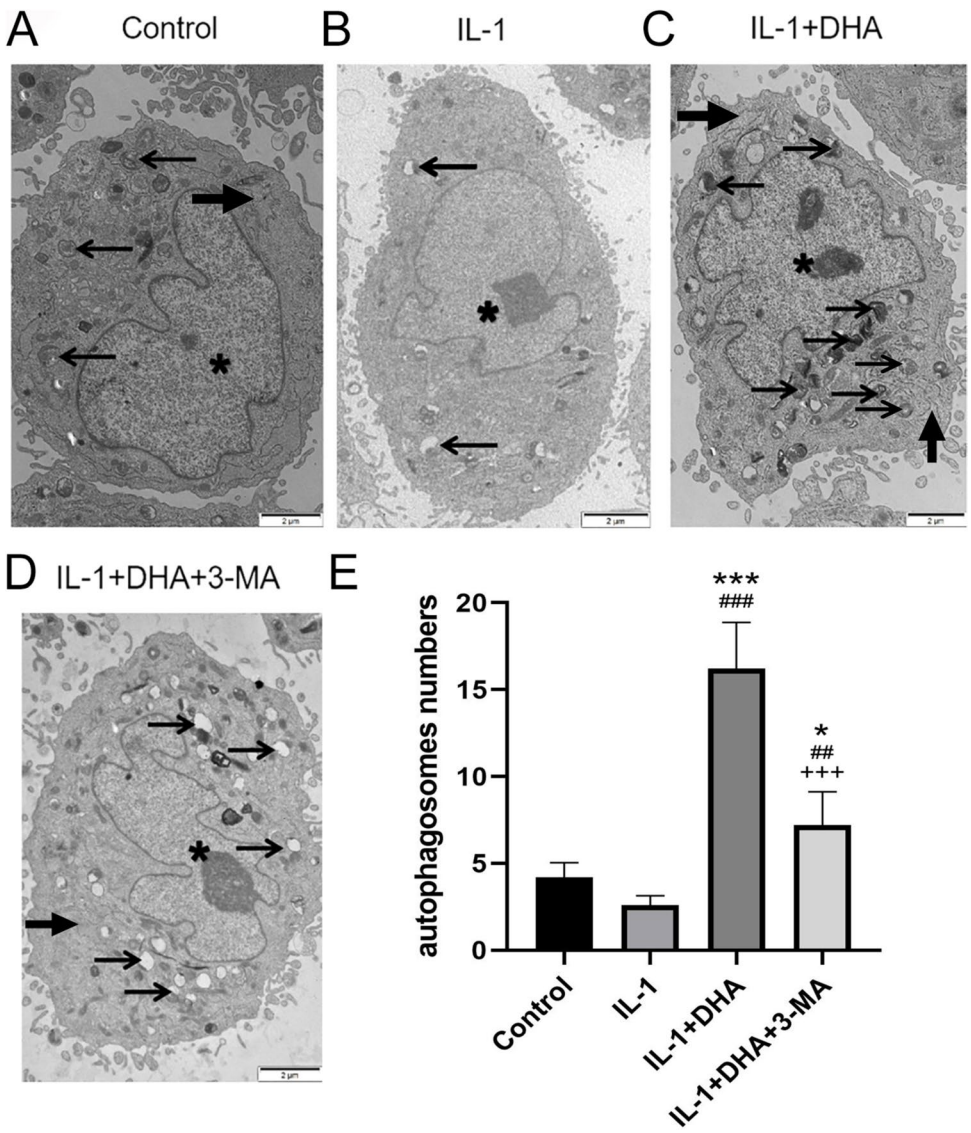

Figure 4. ***(A–D)***Representative images of transmission electron microscopy. Autophagic bodies were observed and marked with thin *black arrows* and the rough endoplasmic reticulum was marked by thick black arrows. The nuclei are marked with *asterisks*. *Scale bar*, 2 μm. (***E***) The *histogram* displayed the quantitative results of autophagosomes (n=5). $^{*}P < 0.05$, $^{***}P < 0.001$ (compared to the control group), $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared to the IL-1 group), $^{+++}P < 0.001$ (compared to the IL-1+DHA group).

elderly people over 75 yr old (Garver *et al.* 2015). The major treatment for OA includes reducing joint bearing, medicine, physical therapy, and surgery, which are mainly symptomatic treatments rather than etiological treatments (Houard *et al.* 2013).

DHA (docosahexaenoic acid) is a type of n-3 polyunsaturated fatty acid (n-3 PUFA) (Sun *et al.* 2018). Since it is difficult to be synthesized by the human body and needs to be ingested from food, DHA along with EPA (eicosapentaenoic acid) are called "essential fatty acids." Recent studies have suggested that DHA and EPA play a strong anti-inflammatory role in the inflammation process of the human body (Pu *et al.* 2013; Gabbs *et al.* 2015; Calder 2017). Wang *et al.* (2016) found that DHA inhibited the overexpression of IL-1 and MMP-13 through the p38-mediated mitogen-activated protein kinases (MAPK) pathway, thereby alleviating articular cartilage degeneration. In the study by Mehler *et al.* (2016), daily supplementation of dogs' diet with EPA and DHA could shift the blood fatty acid concentrations correlated to the relief of clinical signs associated with OA in dogs. Xu *et al.* (2019) study showed that DHA had anti-apoptotic effects in IL-1β-induced human chondrosarcoma cell death through the MAPK signaling pathway. Gruenwald *et al.* 2009) demonstrated that patients with 26 wk of oral administration of glucosamine sulfate and omega-3 polyunsaturated fatty acids (EPA and DHA) had a lower WOMAC pain score than patients with glucosamine sulfate only. These studies suggest that DHA has an anti-inflammatory effect on the body. However, the effect of DHA on the apoptosis and autophagy of articular chondrocytes has not been studied, and the mechanism is still unclear.

Both apoptosis and autophagy are important pathophysiologic processes during cartilage degeneration. Apoptosis is a programmed form of cell death in which a predetermined

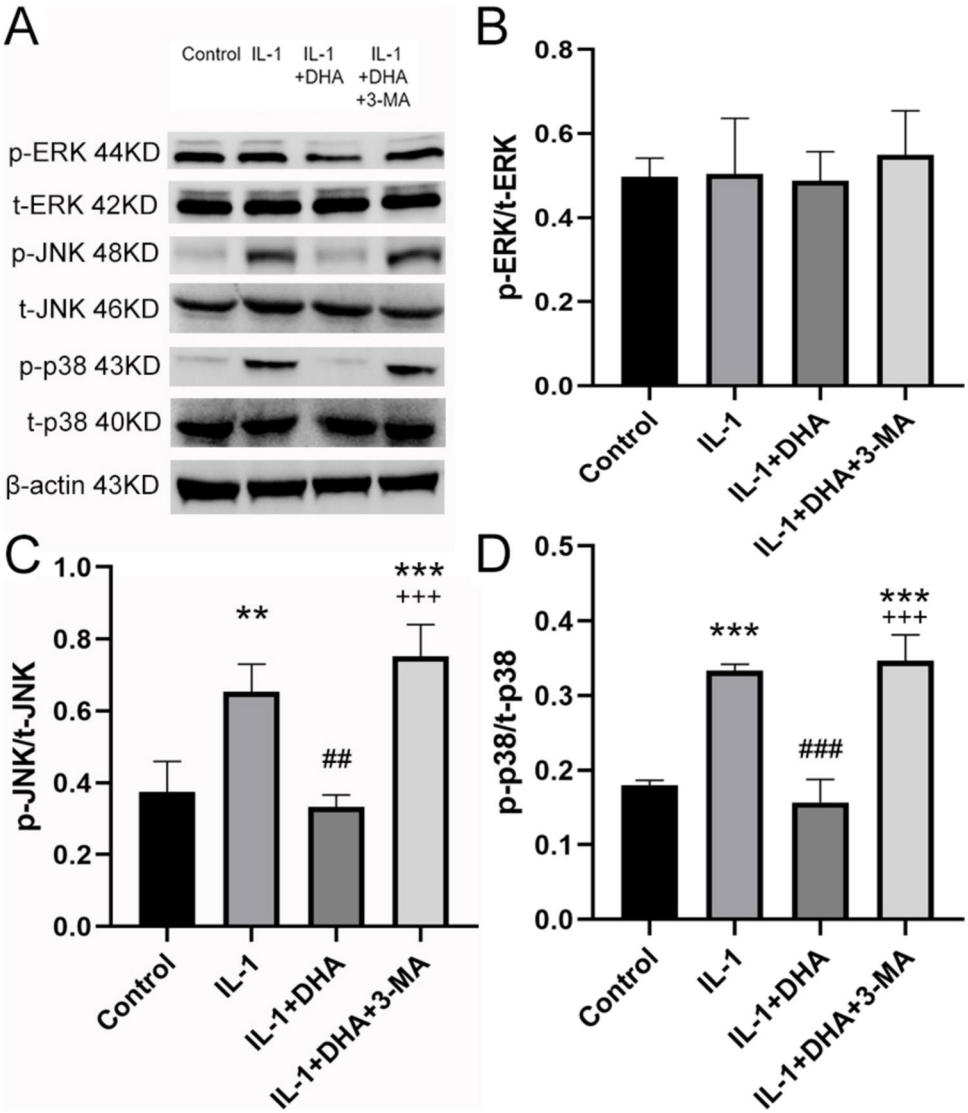

Figure 5. (***A***) Expression of p-ERK/t-ERK, p-JNK/t-JNK, and p-p38/t-p38 in human chondrocytes. Control group: normal cells. IL-1 group: 100 pg/mL IL-1β-induced cells. IL-1 + DHA group: 100 pg/mL IL-1β-induced cells incubated with 50 μg/mL DHA. IL-1 + DHA + 3MA group: 100 pg/mL IL-1β-induced cells incubated with 50 μg/mL DHA and 5 mmol/L 3-MA. Protein expression was evaluated by western blotting ($n = 3$). (***B–D***) Quantitative analysis of the p-ERK/t-ERK, p-JNK/t-JNK, and p-p38/t-p38 expression in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to the control group), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared to the IL-1 group), $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ (compared to the IL-1+DHA group).

set of events results in the removal of old, unnecessary, and unhealthy cells without releasing harmful substances into the surrounding area (Bertheloot *et al.* 2021). Apoptotic cell death of OA cartilage is common. This is associated with matrix degradation and calcification, suggesting that cell death/survival plays a role in OA pathogenesis (Galanti *et al.* 2013; Musumeci *et al.* 2015). IL-1β is a common pro-inflammatory cytokine that promotes the progression of OA by inducing apoptosis of chondrocytes in vivo and in vitro, leading to cartilage matrix degradation and joint inflammation (Wang *et al.* 2015). Our results showed that IL-1β significantly increased cell apoptosis, which was consistent with a previous study showing that IL-1 possessed the potential to induce apoptosis in chondrocytes (Zhou *et al.* 2008). However, treatment with DHA reduced IL-1β-induced chondrocyte apoptosis and increased chondrocyte viability. These results suggested that DHA protects OA chondrocytes by inhibiting apoptosis.

Autophagy is a cellular self-protection mechanism, which keeps energy regeneration and nutrition recycling (Feng and Qiu 2018; Ceccariglia *et al.* 2020). In the case of inflammation, exogenous chemicals, oxides, infection, and hypoxia, autophagy maintains cellular homeostasis by removing damaged organelles and intracellular unfolded proteins, playing an important role in cell survival under stress conditions (Deretic and Levine 2018). mTOR, Beclin-1, and LC3 are known to play important roles in all autophagy-related proteins; mTOR has significant negative control over cellular autophagy associated with LC3-I and LC3-II involvement (Yeh *et al.* 2016). Beclin-1 participates in the initial stage of the formation of autophagolysosomes (Jeon and Im 2017). Autophagy requires LC3 participation, which has two forms: LC3-I and LC3-II. During autophagy, LC3-I is transformed into LC3-II, which then binds to autophagic vesicles (Kabeya *et al.* 2000). Cheng *et al.* (2017) found that autophagy affected chondrocyte

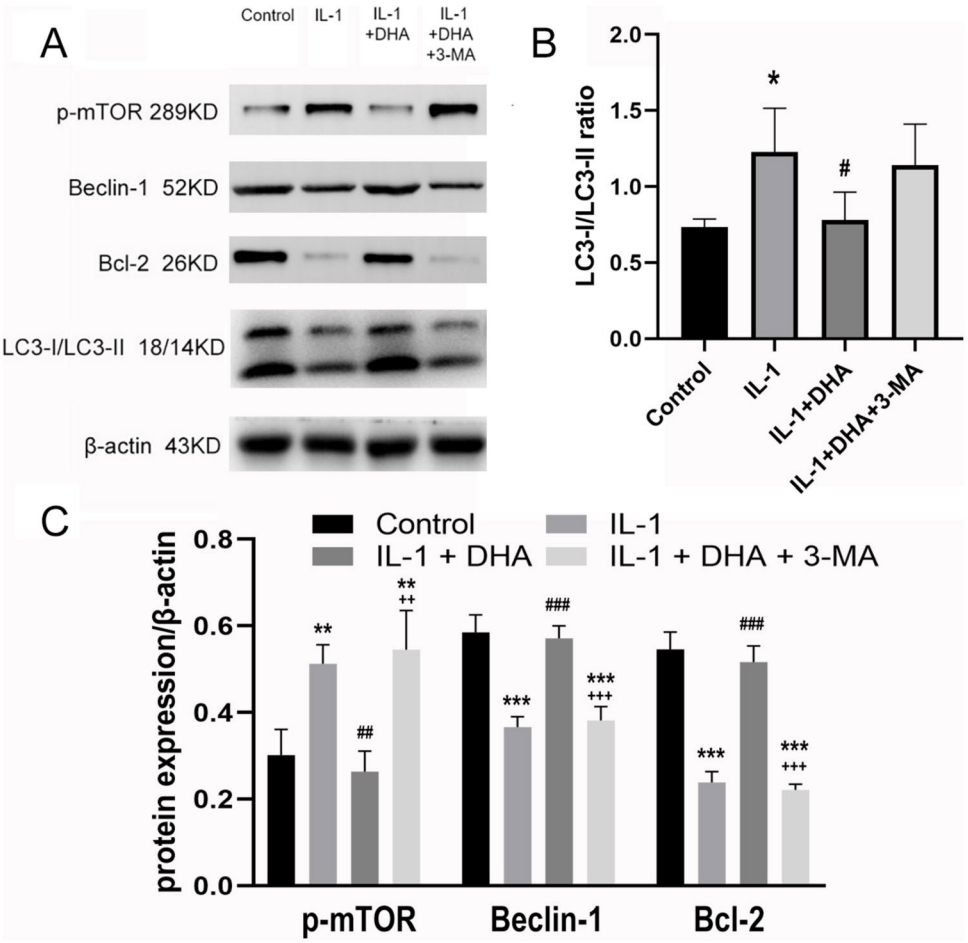

Figure 6. (***A***)Expression of p-mTOR, Beclin-1, bcl2, and LC3-1/LC3-II in human chondrocytes. Control group: normal cells. IL-1 group: 100 pg/mL IL-1β-induced cells. IL-1 + DHA group: 100 pg/mL IL-1β-induced cells incubated with 50 μg/mL DHA. IL-1 + DHA + 3MA group: 100 pg/mL IL-1β-induced cells incubated with 50 μg/mL DHA and 5 mmol/L 3-MA. Protein expression was evaluated by western blotting ($n = 3$). (***B***) Quantitative analysis of the LC3-I/II ratio. (***C***) Quantitative analysis of the p-mTOR, Beclin-1, and Bcl-2 expression. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to the control group), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ (compared to the IL-1 group), $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ (compared to the IL-1+DHA group).

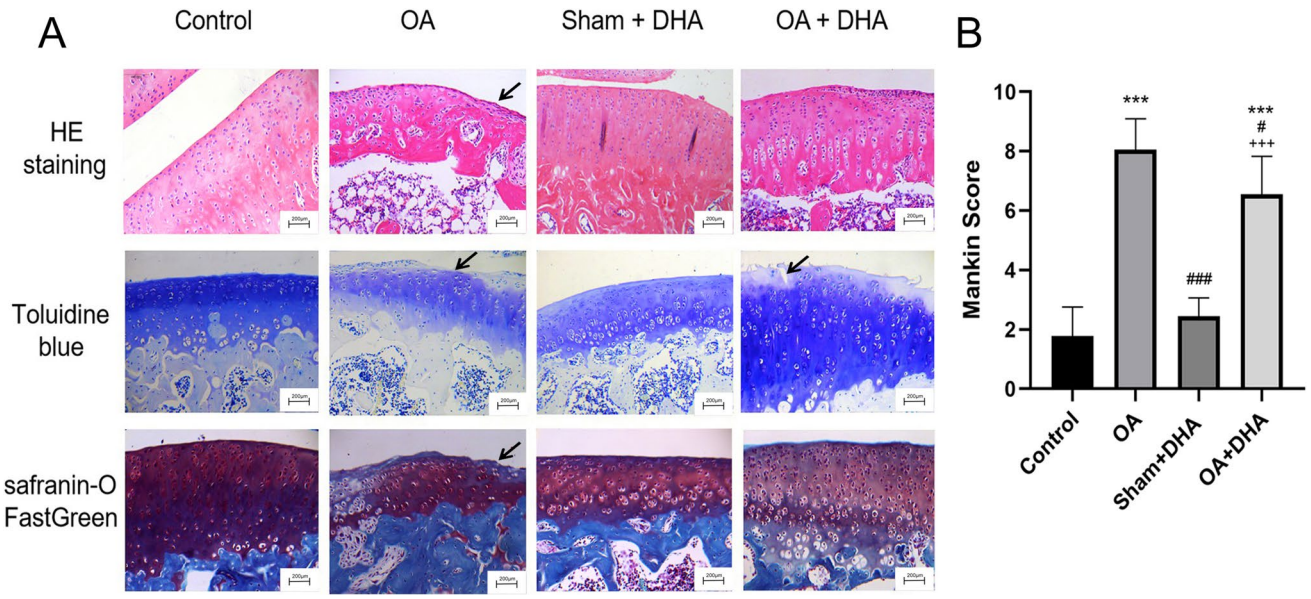

Figure 7. (***A***) Representative sections of H&E, toluidine blue, and safranin O Fast Green staining of each group (original magnification ×400). (***B***) *Bar graph* showing the results of modified Mankin score evaluation. The results are expressed as means ± standard deviation (SD). $^{***}P < 0.001$ compared to the control group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to the IL-1 group, $^{+++}P < 0.001$ compared to the IL-1 + DHA group.

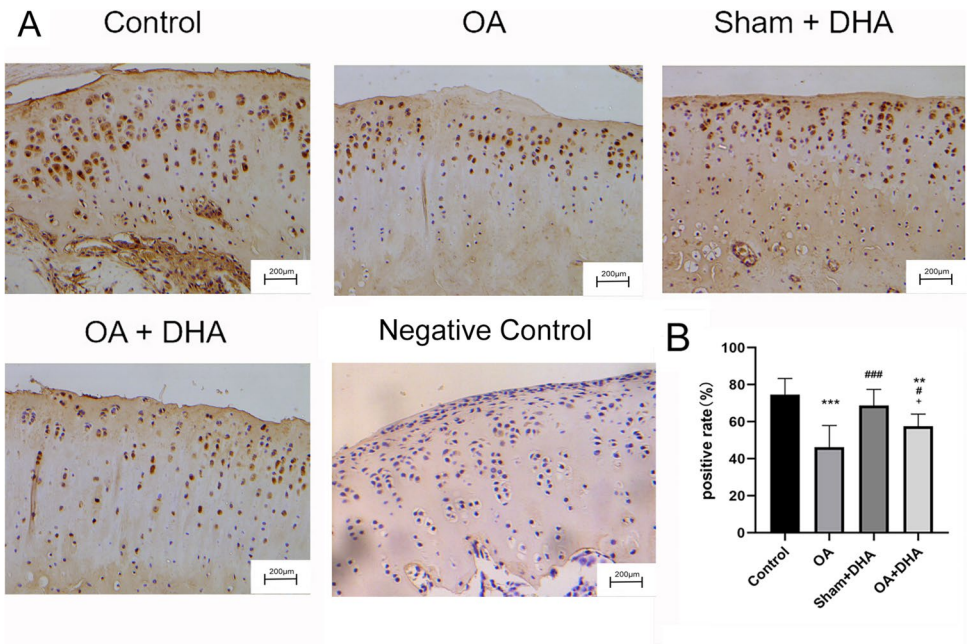

Figure 8. (***A***) Representative sections of immunohistochemical analysis of collagen II in each group (original magnification ×400). (***B***) *Bar graph* showing the results of positive rate. The results are expressed as mean ± standard deviation (SD). $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to the IL-1 group, $^{+}P < 0.05$ compared to the IL-1 + DHA group.

degeneration. In OA models in vitro and in vivo, autophagy inhibitor 3-MA inhibited autophagy, aggravating chondrocyte damage and knee cartilage degeneration. In a rat OA model, intra-articular injection of Torin (a specific inhibitor of the mTOR pathway) upregulated autophagy, decreased MMP13 and VEGF expressions (Cheng *et al.* 2016a), enhanced TIMP-1, and Beclin-1 expression, and LC3-II/I ratio, and alleviated the degeneration of articular cartilage (Cheng *et al.* 2016b).

In our study, an in vitro OA model was established to investigate the function of DHA on cell autophagy. The result showed that p-mTOR expression and LC3-I/II ratio were elevated in OA chondrocytes, while the expression of Beclin-1 and Bcl-2 was significantly decreased, suggesting that IL-1 inhibited autophagy. Representative TEM images also found fewer autophagic bodies in the cytoplasm. After the addition of DHA, p-mTOR expression and LC3-I/II ratio were significantly lower than that of the IL-1 group, while the protein synthesis of Beclin-1 increased significantly. This process demonstrated that DHA could activate autophagy by inhibiting mTOR expression in OA chondrocytes. Similarly, Wann *et al.* (2010) found both EPA and DHA at 0.1 and 1 μM concentrations, respectively, could significantly reduce IL-1β-mediated cartilage degradation through competitive inhibition of the arachidonic acid oxidation pathway. However, after the autophagic pathway was inhibited by 3-MA, p-mTOR expression and LC3-I/II ratio increased again while Beclin-1 decreased, indicating that the activation of autophagy by DHA was blocked by 3-MA.

The chondroprotective effect of DHA was also demonstrated in OA rat models. The models were established by anterior cruciate ligament transection and medial meniscectomy. The results of histological staining showed more erosion, less thickness of the cartilage, higher Mankin scores, and fewer collagen II expressions. However, when OA model rats were fed DHA, histology sections showed less erosion, Mankin scores decreased, and collagen II expressions were enhanced. These results suggested that DHA could alleviate the damage and degeneration of OA articular cartilage.

The potential pathway of DHA action on chondrocytes was detected by western blotting. The results showed that IL-1 increased p-JNK and p-p38 expression, but not p-ERK. After treatment with DHA, the expression of p-JNK and p-p38 plummeted significantly. MAPK (mitogen-activated protein kinase) signaling pathways regulate a variety of biological processes mainly through three pathways: ERK, JNK, and p38. Among them, the ERK pathway is mainly involved in cell growth and differentiation, while JNK and p38 pathways are related to inflammation and apoptosis (Guo *et al.* 2020; Yue and López 2020). Therefore, inhibition of JNK and p38 pathways is the possible reason why DHA reduces the level of apoptosis and protects chondrocytes. However, when 3-MA was added into the medium, p-JNK and p-p38 expressions increased significantly, suggesting that the inhibitory effect of DHA on the JNK and p38 signaling pathway was blocked to some extent by 3-MA. Because the main action of 3-MA is an autophagy inhibitor, this result suggests a correlation between cell apoptosis and autophagy. In other studies, Phitak *et al.* (2018) found that leptin (10μg/ml) alone or in combination with IL-1β could induce cartilage destruction by activating NF-κB,

ERK, JNK, and p38 in human articular chondrocytes, whose effect was inhibited by EPA and DHA. Feng and Qiu (2018) found that artesunate inhibited chondrocyte proliferation and accelerated cell apoptosis and autophagy via the suppression of the PI3K/AKT/mTOR signaling pathway.

Conclusion

DHA could promote chondrocyte proliferation, decrease cell apoptosis, and increase autophagic activity in inflammatory articular chondrocytes. These effects of DHA reduced the damage and degeneration of OA cartilage. This process was accomplished by inhibiting the expression of mTOR, JNK, and p38 signaling pathways. This study revealed that DHA could be used as a potential drug for the treatment of OA, which could alleviate the degradation of articular cartilage and delay the progression of OA.

Author contribution All authors contributed to the study conception and design, especially Lifeng Ma and Ai Guo. Material preparation and data collection were performed by Zishun Gong, Haomiao Yu, Guodong Wang, and Ruiqi Cao. Data analysis was performed by Zishun Gong, Haomiao Yu, and Heyong Yin. The first draft of the manuscript was written by Zishun Gong and Haomiao Yu, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript. Zishun Gong and Haomiao Yu are the co-first authors of this paper.

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Data availability The dataset supporting the conclusions of this article is available upon request to the corresponding author.

Declarations

Ethics approval and consent to participate Ethics approval was granted by the Bioethics Committee of Beijing Friendship Hospital, Capital Medical University, China (approval code 2020-P2-205-01). All procedures were performed in accordance with the Declaration of Helsinki and complied with the ARRIVE guidelines, and were carried out in accordance with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Informed consent was obtained from all patients included in the study before surgery.

Consent for publication No individual person's data were included in this research.

Competing interests The authors declare no competing interests.

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