Asiatic Acid Attenuates Osteoarthritis by Regulating NF-κB Signaling Pathway in Chondrocytes

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Abstract

Background: Natural small molecules have become more attractive as alternatives to non-steroidal anti-inflammatory drugs in osteoarthritis (OA) treatments. This study aims to investigate the effects of Asiatic acid (AA) on OA in chondrocytes and the surgery-induced OA animal model.

Methods: Cytotoxicity of AA in primary rat articular chondrocytes was determined. Chondrocytes were pretreated with AA at the safe concentrations and subsequently treated with IL-1β. The production of inflammatory mediators including nitric oxide (NO), nitric oxide synthase (iNOS), as well as cyclooxygenase (COX)-2, and the expression of chondrogenic and hypertrophic markers including Sox 9, Aggrecan, Col 2a1, and matrix metalloproteinase-13 (MMP13) in the cells were measured. The effect of AA on nuclear factor-kappa B (NF-κB) signaling pathway was further determined by dual luciferase assay and western blot. The surgery-induced OA animals were treated with AA or saline for 6 weeks. The pathological changes in the affected joints were measured by micro-CT and histological analysis.

Results: We found a broad safety spectrum of AA from 0 to 25 μM. A dose-dependent inhibitory effect of AA on NO production, as well as iNOS and COX-2 expression were found. Meanwhile, AA promoted chondrogenesis and inhibited hypertrophy in chondrocyte treated with IL-1β. In addition, AA inhibited NF-κB signaling pathway with a dose-dependent manner. Furthermore, results from animal study revealed that AA prevented articular cartilage damage as well as subchondral bone remodeling in the surgery-induced OA animal.

Introduction

With the aging population increase worldwide, age-related degenerative bone disorders have occupied enormous clinical resources. Osteoarthritis (OA), the most popular age-related chronic disease of synovial joints, can damage articular cartilage, cause severe pain in joint, even finally result in disability [1]. Unfortunately, there are very limited managements or treatments to halt osteoarthritis progression.

Although OA is classified as a noninflammatory arthropathy, many inflammatory components have been implicated in the disease process and have been observed in the synovial fluid. These include inflammatory cytokines, immunoglobulins, and other mediators in the joints [2]. As a main component of articular cartilage, chondrocytes maintain the matrix components under normal and low turnover conditions. However, chondrocytes would shift their phenotype to a catabolic one and result in OA initiation and progression when responding to abnormal environmental insults [3]. Aberrant expression of inflammation-related genes in the chondrocytes would do more harm to the synovial joint, which becoming trapped in a vicious negative-feedback cycle. These genes include nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, and several matrix metalloproteinases (MMPs), including MMP-13, and a disintegrin and metalloproteinase (ADAM) with thrombospondin-1 domains (ADAMTS)-4 and 5 [3]. From previous experiments, researchers have found that proinflammatory cytokines, such as interleukin (IL)-1β, can induce articular cartilage destruction by directly triggering the production of other inflammatory

mediators like matrix MMPs, COX-2 and iNOS in either synovial membrane as well as chondrocytes [4][5][6]. Those inflammatory mediators can induce nuclear factor-kappa B (NF-κB)/ mitogen-activated protein kinase (MAPK) signal activation and then over-produce nitric oxide (NO) and PGE2 [7]. The additional NO subsequently up-regulates MMPs expression which accounting for the degradation of cartilage matrix network [8], while the increased PGE2 causes bone resorption and joint pain [9][10]. Hence, inflammation has become one of the most important therapeutic targets of OA.

In terms of pharmacological intervention, pain killers including paracetamol or non-steroidal anti-inflammatory drugs (NSAIDs) are the most often recommended frontline medication [1]. For instance, celecoxib, a representative NSAID and COX-2 specific inhibitor, has shown to be effective in terms of clinically relevant improvement of both pain and function, with limited side effects [2] [11]. Paracetamol used to be the first frontline pain medication for OA, however, it is now of little use as a single agent given the very small effect size comparing to placebo [1] [12]. However, the effects of these drugs on the progression of OA remain unclear. For instance, several types of NSAIDs like sodium salicylate, indomethacin may restrain the synthesis of cartilage matrix component [13]. The preliminary clinical trials also revealed that some NSAIDs like indomethacin, had negative influence on joint structure [14]. The other side effect of long-term NSAIDs including gastrointestinal toxicity, liver functions disorder and hepatocellular injury [15]. Therefore, there are still many hurdles in the current treatments of OA to overcome and achieve pain relief and joint function improvement. It is a great demand to find out a specific treatment to revive the damaged articular cartilage.

Recent years, there are more and more interests in natural products, such as avocado soybean unsaponifiables [16], cordycepin [8], piperine [9], astragalin [10], etc., extracted from herbs as alternatives because of their potential anti-arthritic effects. Evidences have shown the potential role of natural products in the treatment of OA by regulating IL-1β-induced inflammatory mediators [17][18][19]. Asiatic acid (AA), a pentacyclic triterpene isolated from various plants including *Centella asiatica*, has shown anti-inflammation effect in many inflammatory conditions. Researchers found out that AA has beneficial effects in preventing inflammation occurred within lung or liver through down-regulating TGF-beta/Smad or NF-κB signaling pathway, respectively [20][21]. People also found out that AA could ameliorate dextran sodium-induced murine experimental colitis [22], attenuated kainic-induced seizure by inhibiting hippocampal inflammatory and oxidative stress [23], and lipopolysaccharide-induced inflammation in human corneal epithelial cells [24]. Our recent research finding also showed AA attenuated bone loss by regulating NF-κB signaling pathway during osteoclastic cells differentiation [25]. Given a close relationship in the biological process between osteoclastic differentiation and macrophage activation during inflammation, togethering with others finding above, we hypothesized that AA may be a potential anti-inflammation choice for OA treatment. Nevertheless, AA was found to be effective in stimulate collagen synthesis, which has been recommended for wound healing or skin care [26][27], indicating the potential therapeutic effects of asiatic acid by not only regulating inflammation but also promoting collagen synthesis in OA. In this study, we have investigated the anti-inflammatory effect of AA in IL-1β-stimulated chondrocytes and the therapeutic effect of AA in surgery-induced OA animal model.

Materials And Methods

Chondrocyte culture and treatment

Male Sprague-Dawley (SD) rats (1-month-old, Laboratory Animal Services Centre, the Chinese University of Hong Kong, HK) were sacrificed and used for isolation of chondrocytes under institutional ethical approval. Articular cartilage was isolated from right femoral head cap, and chondrocytes were obtained after digestion of cartilage fragments by 0.25% trypsin (w/v) for 30 minutes followed by about 12 hours of digestion within 0.2% collagenase II (w/v) in serum-free Dulbecco's Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA). After that, the chondrocytes were collected and cultured in DMEM containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 100 units/ml of penicillin and 100 mg/ml of streptomycin and cultured at 37 °C, 5% CO_2. The staining for collagen type II and Toluidine blue O staining were used for chondrocyte identification. To avoid dedifferentiation, only the passage 1 to 3 was used for our experiment.

Cell viability assay

MTT (Sigma-Aldrich, St Louis, MA) assay was used to measure the effect of AA on the viability of chondrocytes. In brief, the chondrocytes at a density of 6×10^3/well were cultured in a 96-well plate overnight and treated by various concentrations (0, 5, 10, 25 μM) of AA (Chengdu Biopurify Phytochemical, Chengdu, China) for additional 24 h. Then, 20 μl MTT (5 mg/ml) was loaded into each well for 4 h. Next, the supernatant was discarded and 150 μl DMSO was applied for solubilization of formazan. The absorbance at 570 nm was measured with the micro-plate reader (Bio-red, Hercules, USA).

Transfection and dual-luciferase reporter assays

NF-κB / p65 Renilla/firefly dual luciferase reporter system was constructed in this research as the method described previously [28]. 1 μg of NF-κB/p65 promoter/Luciferase Plasmid DNA along with 1μg of internal control pRL-TK plasmid was transiently transfected into 1×10^5 293T cell/well in a 6-well plate using lipofectamine 2000 (Thermo Fisher Scientific, USA) reagents for 24 h according to manufactures' instruction. After that, the cells were treated with AA (0, 5, 10, 25 μM) for 24 h. Each well was then washed twice by cold PBS and harvested by Dual-Luciferase® Reporter Assay System (Promega, USA). After sample preparation, luminescence was measured by PerkinElmer VictorTM X2 2030 multilabel reader (Waltham, USA). The luciferase activity was normalized with expression of control pRL-TK.

NO assay

Chondrocytes were pretreated by AA 24 h and then stimulated with interleukin (IL)-1β (Cell Signal Technology, USA) for 24 h. The concentration of NO within the culture medium was measured by the Griess reagent (Beyotime Biotechnology, Guangzhou, China) according to the manufacturer's instructions.

Chondrocyte real-time quantitative PCR

Total RNAs of chondrocytes were extracted by using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA concentrations were measured using Nanodrop method (Thermo Scientific, Wilmington, NC, USA). Then, single-stranded cDNA was synthesized from 500 ng of total RNA by reverse transcriptase (TaKaRa Biotechnology, Otsu, Japan). Real-time PCR was applied using SYBR Green Master mix (Thermo Fisher, Waltham, USA) and results were detected by ABI 7500 Sequencing Detection System (Applied Biosystems, Foster City, CA, USA). The primers were designed corresponding to the coding region of genes as listed: Aggrecan (forward: GAAGTGGC-GTCCAAACCAAC, reverse: AGCTGGTA-ATTGCAGGGGAC), SOX9(forward: ATCTGAAGAAGGAGAGCG-AG, reverse: CAAGCTCTGGAGACTGCTGA), Col2 a1(forward: ATCGCCACG-CTCCTACAATG, reverse: GGCCCTAATTTTCGGGCATC), MMP-13 (forward: TGACTATGCGTGGCTGGAA, revise: AAGCTGAAATCTTGCCTTGGA), PPAR-γ (forward: forward: GAGTCCGTCTAGCAGTGT, reverse: CGAGGACATCCA AGACAAC), GAPDH (forward: CCT-CGTCTCATAGACAAGATGGT, reverse: GGGTAGAGTCATACTGGAACAT-G). The final data were analyzed using the $2-\Delta\Delta CT$ method. All values were normalized to the level of the housekeeping gene GAPDH.

Western Blot Analysis

Chondrocyte were washed three times with cold PBS and whole cell protein were extracted by using Radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris–HCl, pH 7.5, 150 μmol/L sodium chloride, 0.5% cholic acid, 0.1% SDS, 2 mmol/L EDTA, 1% Triton, and 10% glycerol) including protease and phosphatase inhibitors. The protein within cytoplasma and nuclear was extracted by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, USA) according to the manufacturer's protocol. Then, all the protein samples were normalized by BCA protein assay kit. Aliquots of the samples (40 μg) were separated within 10% SDS-polyacrylamide gel and transferred to PVDF membranes. After blocked with 10% FBS, the membrane was incubated overnight under 4°C with primary antibodies to COX2 (sc-376861), iNOS (ab-3523), MMP-13 (ab219620), PPAR-γ (ab209350), phospho-IκB-alpha (CST #2859), IκB-alpha (CST #4814), NF-κB p65 (ab16502), GAPDH (CST #5174). After that, the membrane was incubated with horseradish-peroxidase (HRP)-conjugated secondary antibody for additional 1.5 h (Cell Signaling, Danvers), flowed with visualization by the chemiluminescence.

OA animal model

This animal surgical procedures were approved by the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (CUHK, ref. 18-089-GRF) and performed according to previous reports [29]. Male ICR outbred mice (Laboratory Animal Services Centre, CUHK, HK), 12-week old and body weighing 20-25 g, were used in this study. Animals were kept in local vivarium conditions at a temperature of 24–26 °C and humidity of 70% with free access to water and a pelleted commercial diet in the mouse house under specific pathogen-free (SPF) conditions. To induce mice OA, the right knee joints were received anterior cruciate ligament and medial collateral ligament transection (ACLT) surgery as previously mentioned [30]. After surgery, OA mice were randomly treated with AA (5 μg/g in saline, twice per week, *s.c.*, n = 8) or vehicle (saline, 100 μl, *s.c.*, n= 8) for 6 weeks. In the sham-operated animals (n =

8), only a small piece of skin covering each right knee joint was resected. Animals were euthanasia by carbon dioxide after 6 weeks. The knee joints were then harvested and fixed in 10% buffered formalin.

Microcomputed tomography (μCT) analysis

The right knee joints were analyzed using high-resolution μCT (μCT40, Scanco Medical, Basserdorf, Switzerland) as previous described [29]. Image acquisition was performed at 70 kV and 118 μA, with a resolution of 12 μm per voxel. Three dimensional (3D) reconstructions of the mineralized tissues were performed by using a global threshold (158 mg hydroxyapatite/cm^3) and a Gaussian filter (sigma = 0.8, support = 2) was applied for noise suppression. One hundred sagittal images of the subchondral bone of medial tibial plateau was selected to perform the 3D Histomorphometry analysis. The bone volume/total tissue volume (BV/TV) was analyzed as the 3D structural parameters.

Histological and immunochemical examinations

The right knee joints of mice were decalcified within 10 % EDTA for about 21 days and embedded in paraffin. Five micrometer-thick sagittal-oriented sections of the sample were used for Safranin-O/fast green and immunohistochemistry (IHC) staining.

IHC staining was performed as our previous mentioned [29]. We incubated sections with primary antibodies to rabbit collagen type II (Abcam, 1:20, ab34712), MMP-13 (Abcam, 1:200, ab39012) and Osterix (Abcam,1:200, ab22552) overnight at 4 °C. For

immunohistochemical staining, a horse radish peroxidase-strep-tavidin detection system (Dako, Carpinteria, CA, USA) was used according to manufacturer's instruction. Photographs of the selected areas were taken under a light microscope (Leica, Wetzlar, Germany). We counted the number of positively stained cells and repeated in triplicate in three randomly selected sections at the area of interest in each specimen, and the numbers of cells were statistically analyzed.

Result

Effect of AA on chondrocyte viability

The primary chondrocytes specifically expressed collagen type II. The cells were also stained positively in proteoglycans and glycosaminoglycans with Toluidine blue O (Figure1 A). The MTT assay was used to evaluate the potential cytotoxicity of AA on chondrocytes (Figure1 B). The result indicated AA at the concentrations from 0 to 25 μM were not contributed any cytotoxicity (Figure 1B). However, when the concentrations equal or higher than 50 μM, the cell viability was remarkably decreased in day 3 and day 7 (-28.9% in 50 μM, $p < 0.01$; -75.3% in 100 μM, $p < 0.01$) in comparison to that in the concentration of 0 μM (Figure1 B). Thus, we chose the safe concentrations of AA (5, 10, and 25 μM) in the subsequent experiments.

Effect of AA on IL-1β-induced NO production and iNOS, COX-2 expression

The effect of asiatic acid on IL-1β-induced NO production in chondrocytes was evaluated by Griess reagent. Results showed that IL-1β significantly up-regulated NO production after 24 h (+293.2%, $p < 0.05$), comparing to the control group (Figure 2A). However, the production of NO was significantly inhibited in the group pretreated with AA (-39.3% in 5 μM, $p < 0.01$; -47.6% in 10 μM, $p < 0.01$; -67.4% in 25 μM, $p < 0.01$) in a dose dependent way (Figure 2A). The protein expression levels of iNOS, one of the key enzymes generating NO, and COX-2, an inflammation-related molecule was detected by Western blot (Figure 2B). Results showed that IL-1β distinctly induced the expression levels of iNOS and COX-2 (+612.7%, $p < 0.05$; +493.2%, $p < 0.05$) , while AA dose-dependently suppressed their expression levels (COX-2: -57.3% in 5 μM, $p < 0.01$; -71.6% in 10 μM, $p < 0.01$; -73.4% in 25 μM, $p < 0.01$ and iNOS: -27.5% in 5 μM, $p < 0.01$; -57.6% in 10 μM, $p < 0.01$; -77.2% in 25 μM, $p < 0.01$) (Figure 2B & 2C).

Effect of AA on the expression of chondrogenic differentiation and hypertrophic markers within chondrocytes.

MMP-13 is a hypertrophic marker which plays an important role in cartilage degradation. Here the results of real-time PCR and Western blot indicated that AA could significantly inhibit the MMP-13 expression (-67.8%, $p < 0.05$; -69.5%, $p < 0.05$) induced by IL-1β (Figure 3A, 3C & 3D). In addition, AA activated PPAR-γ expression which was suppressed by IL-1β (+37.7%, $p < 0.05$; +87.8%, $p < 0.05$) (Figure 3 A, 3C & 3D). Results of real-time PCR showed that IL-1β significantly inhibited the expression of chondrogenic markers, including Aggrecan, SOX-9 and colleague type 2a1(-78.9%, $p < 0.05$; -74.8%, $p < 0.05$; -85.4%, $p < 0.05$) when comparing to those in the control group (Figure 3B). AA upregulated the gene expression of SOX-9 (+15.9% in 5 μM, $p > 0.05$; +112% in 10 μM , $p < 0.01$; +120.5% in 25 μM, $p < 0.01$) and Col 2a1 (+86.1% in 5 μM, $p > 0.05$; +111.5% in 10 μM , $p < 0.01$; +198.3% in 25 μM, $p < 0.01$) in a dose-dependent manner (Figure 3B). At a dose of 10 μM, AA also significantly upregulated the gene expression of Aggrecan (+259.6 %, $p < 0.01$) (Figure 3B). However, there is no significant change of Aggrecan expression when the dose of AA was higher to 25 μM.

Effect of AA on NF-κB activation

To determine if AA has a direct inhibition on NF-κB activation, NF-κB / p65 Renilla/firefly gene reporter was transfected into 293T cell line. The result of dual-luciferase assay showed that AA could reduce the luciferase activity driven by NF-κB promoter in a dose dependent manner (-25.3% in 5 μM, $p > 0.05$; -68.3% in 10 μM, $p < 0.01$; -61.4% in 25 μM, $p < 0.01$) (Figure 4A). To further identify the underlying mechanism of AA on NF-κB signaling pathway, the expression level of some key molecules of NF-κB signaling pathway were measured. Data showed that AA inhibited the phosphorylation of IκB induced by IL-1β (-53.6% in 5 μM, $p<0.05$; -67.1% in 10 μM, $p < 0.01$; -105.4% in 25 μM, $p < 0.01$) (Figure 4B & 4C). Moreover, the result also indicated that the level of NF-κB/p65 was decreased within nucleus in AA treatment group (-32.5% in 5 μM, $p < 0.05$; -58.3% in 10 μM, $p < 0.01$; -60.9% in 25 μM, $p < 0.01$) comparing to the IL-1β treatment group (Figure 4B & 4C). These findings suggested that AA may inhibit NF-κB signaling through down-regulating IκB phosphorylation as well as blocking the entry of NF-κB/p65 to the nucleus.

Effects of AA on cartilage phenotype in OA mouse model

The result of Safranin-O/fast green staining indicated that there was obviously cartilage degradation characterized by a significant higher OA scores (+635% in OARSI, $p < 0.001$) and higher expression level of MMP13 (+616.7%, $p < 0.001$) as well as Col X (+455.9%, $p < 0.001$) in the articular cartilage of OA group compare to the sham-operated group after 6 weeks post operation (Figure 5). However, the systemic application of AA significantly retarded the degeneration of articular cartilage of OA animals (-67.4% in OARSI, $p < 0.001$) (Figure 5A & 5B). Moreover, results of the IHC staining showed that AA significantly inhibited the expression of MMP-13 (-89.89%, $p < 0.001$) and Col-X (-80.9, $p < 0.001$) (Figure 5B & 5C).

Effects of AA on microstructure of subchondral bone in OA mouse model

The 3D images obtained from μCT displayed the microarchitecture of mice subchondral bone (Figure 6). Result showed that, after 6 weeks of operation, significant bone lost happened in the OA animals comparing with the sham group (-38.1% in BV/TV, $p < 0.001$) (Figure 6A & 6B). Interestingly, AA treatment efficiently inhibited abnormal bone remodeling within subchondral bone induced by OA, exhibiting a higher BV/TV (-15.3 % in BV/TV, $p < 0.001$) (Figure 6A & 6B). Moreover, results of the IHC staining showed that Ostrix expression was suppressed in the OA subchondral bone (87.7%, $p < 0.001$). However, AA significantly promoted the expression of Ostrix (+67.6%, $p < 0.001$) (Figure 6 A & 6C).

Discussion

In this study we demonstrated the evidence that AA exhibited a convincing rescue effect on IL-1β-induced inflammation in chondrocytes and surgery-induced osteoarthritis in animals.

IL-1β, a proinflammatory cytokine, has been regarded as a potent mediator of catabolic processes, inducing other inflammatory mediators including iNOS, COX-2, MMPs, and IL-6 [31]. Abnormally elevated levels of IL-1β was also observed in the synovial fluid and articular cartilage of OA patients [32]. The highly expressed iNOS produces additional NO, then subsequently up-regulates MMPs which are responsible for degradation of extracellular matrix, like collagen type II [33] [34] [8]. A higher level of COX-2 may induce over production of PGE2, which then causes pain and bone resorption [6,7]. The current cellular results demonstrated that chondrocytes exhibited an inflammatory status characterizing as high expression levels of COX-2, NO, and MMP-13 induced by IL-1β, which are consistent with the previous findings [8,9,28].

Furthermore, the inflammatory processes are frequently accompanied by extracellular matrix degradation in chondrocytes. Our results also found that remarkably reductions in the gene expression level of chondrogenic markers including Aggrecan, Sox 9 and Col 2a1 in chondrocytes impaired by IL-1β, suggesting a catabolic change in chondrocytes. It is reported that IL-1β usually involved in the destruction of cartilage matrix in chondro-destructive diseases by inhibiting expression of extracellular matrix (ECM) components, while increasing expression of ECM-degrading proteases [35] [36].

Considering the unmet demands in OA therapy with NSAIDs, natural products have shown the potential capability for OA treatment with some inspiring outcomes [8,9, [37][38]. *Centella asiatica* has been regarded as a traditional Chinese medicine for cleaning heat and disinhibiting dampness, resolving toxin and dispersing swelling [39]. Pharmacological research revealed that *Centella asiatica* exhibits the capabilities of anti-inflammation, promoting wound healing, preventing liver fibrosis, and reducing anxiety, et al [39]. As one of the main components within *Centella asiatica*, asiatic acid (AA) is potent in preventing inflammation in many systems [18,19,29].

In this study, AA effectively inhibited IL-1β-induced high levels of inflammatory mediators including NO, COX-2, MMP-13 and PGE-2 in chondrocytes, in a dose dependent manner. Furthermore, AA significantly revived the expression of chondrogenic markers which inhibited by IL-1β. These results indicated that AA had an anti-inflammation effect as well as an anti-catabolic effect in chondrocytes. Results of animal study also verified that AA effectively prevented articular cartilage from further degeneration and destruction, and significantly decrease the expression levels of hypertrophic markers including MMP-13 and Col X. These results revealed that systemic delivery of AA could inhibit hypertrophy and degradation of articular cartilage, then protect the cartilage from the harsh environment in the joint caused by mechanical instability [40]. Taking together, results from these functional studies show a beneficial effect of AA in the treatment of OA in both cellular and animal levels, indicating a translational potential of AA as an alternative for OA therapy.

Regarding the underlying mechanisms, we found that NF-κB signaling pathway might involve in the processes. NF-κB signaling pathway is a family of ubiquitously expressed transcription factors (TFs) that play an essential role in most immune and inflammatory responses [41] [42]. The abundant NF-κB/p65 and p50 was observed within rheumatoid (RA) and OA patients [43]. NF-κB is retained in the cytoplasm with the inactivation IκB-α. The present of IL-1β activates p65 NF-κB through triggering IκB-α degradation in chondrocytes [44]. After activated, NF-κB unit p65 protein was dissociated from its inhibitory protein IκB-α and translocate from the cytoplasm to the nucleus, then it may trigger the transcription of specific target genes such as iNOS and COX-2 [45] [46]. In the current research, we investigated the NF-κB activation level to study molecular mechanisms behind AA effects. We found that AA significantly inhibited NF-κB activation within 293T cell line *in vitro*. In addition, results of western blot indicate that AA effectively inhibited NF-κB activation within chondrocytes by restraining phosphorylation of IκB-α and limiting NF-κB/p65 translocated from cytoplasma into nucleus as well. On the other hand, as a nuclear hormone receptor, PPAR-γ has been found not only expressing in adipocytes, but also in other cells including chondrocytes. It is reported that PPAR-γ activator could inhibit IL-1-induced NO and MMP13 production in chondrocytes [47][48]. The inhibition may be through a PPAR-γ-dependent pathway, by interfering with the activation of AP-1 and NF-κB [49]. In current study, we also found a similar result that AA significantly activated PPAR-γ expression in OA chondrocyte, which may subsequently inhibit NF-κB signaling pathway.

Our previous research have shown that AA exhibits anti-resorption in osteoclast progenitors by regulating TGF-β and NF-κB pathways [25]. Moreover, AA has inhibitory effect on osteoclastic differentiation of the

precursor cells including bone marrow-derived mononuclear cells and RAW264.7 cell line with a dose dependently manner. As the RAW264.7 cells are also regarded as macrophage progenitors, which have been identified involving in OA and RA process by driving the production of several proinflammatory cytokines (e.g., IL-6, IL-8) and several MMPs as well [32]. Here, we believed that the effects of AA on RAW264.7 mentioned within our previous work may also partially reveal the mechanisms behind the anti-inflammatory effect of AA in OA treatment.

Besides articular cartilage destruction, abnormal subchondral bone sclerosis is another phenotype frequently observed within OA [50] [51]. It has been well elucidated that there is a closely relationship between thickening of the subchondral bone with an abnormally low mineralization pattern and osteoarthritis [52] [53]. However, phenotype in subchondral bone remodeling changed dramatically and dynamically in OA animal models, changing dominantly from bone resorption at the early phase to bone formation at the later phase [51]. In this study, we found that significant bone loss in the subchondral bone of the OA animal model, indicating the active bone resorption at the early phase of OA. AA effectively prevented subchondral bone from absorption and maintained the structure, which was also consistent with our previous finding that AA prevented osteoclastic differentiation [25].

However, there are still some limitations existing in this study. Firstly, we used different species between animal and cellular studies. ICR mice were used in the animal study. However, rat chondrocytes were used in the cellular study as relatively easier to isolate chondrocytes from articular cartilage rather than that in mouse. Secondly, the surgery-induced OA model in our study could not fully mimic the real pathological phenotype in patients. OA models could be established in several weeks. However, OA progression in patients could be months to years. However, there is still no ideal animal models of OA from the research aspect. Thirdly, regarding the mechanism, we focused on NF-κB pathway in this study. Actually, OA is a quite complicated disorders which involves enormous cell types and signaling pathways. Further studies are needed to verify the protective effect of AA in large animal and to explore more details on the underlying mechanism.

Conclusion

Our data demonstrated that Asiatic acid successfully inhibited production or expression of inflammatory mediators induced by IL-1β in the chondrocytes, rescued their chondrogenic properties, and effectively prevented articular cartilage from destruction and subchondral bone from resorption. The promising anti-inflammatory mechanisms of AA may be through the inhibition in NF-κB activation. These studies suggested that AA may be a novel anti-arthritic agent for OA therapy providing its protective effect on both cartilage and subchondral bone.

Abbreviations

Osteoarthritis, OA; Asiatic acid, AA; nitric oxide, NO; nitric oxide synthase, iNOS; cyclooxygenase, COX; matrix metalloproteinase-13, MMP13; nuclear factor-kappa B, NF-κB; computed tomography, CT;

metalloproteinase, ADAM; thrombospondin-1 domains, ADAMTS; interleukin, IL; mitogen-activated protein kinase, MAPK; prostaglandin, PEG; non-steroidal anti-inflammatory drugs, NSAIDs; Sprague-Dawley, SD; fetal bovine serum, FBS; 4,5-dimethylthiazolyl-2, MTT; dimethyl sulfoxide, DMSO; specific pathogen-free, SPF; anterior cruciate ligament and medial collateral ligament transection, ACLT; immunohistochemistry, IHC; extracellular matrix, ECM; transcription factors, TFs; rheumatoid, RA; Peroxisome proliferator-activated receptor, PPAR; Transforming Growth Factor Beta, TGF-β;

Declaration

Availability of data and materials

All data generated and analyzed during this study are included in this article. Materials used in this study are available from the corresponding author on reasonable request.

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Not applicable.

Authors' contribution

GL and SL designed the study. LF, ZY, XTZ, WPL, and BW performed the experiments and acquired the data. LF and ZY analyzed and interpreted the data and draft the article and JH revised it. GL, CL and HL finalized the manuscript for submission. All authors read and approved the final manuscript.

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Declarations

Ethic approval

This study protocol received prior approval from the Animal Experimental Ethics Committee of the Chinese University of Hong Kong (CUHK, ref. 18-089-GRF).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

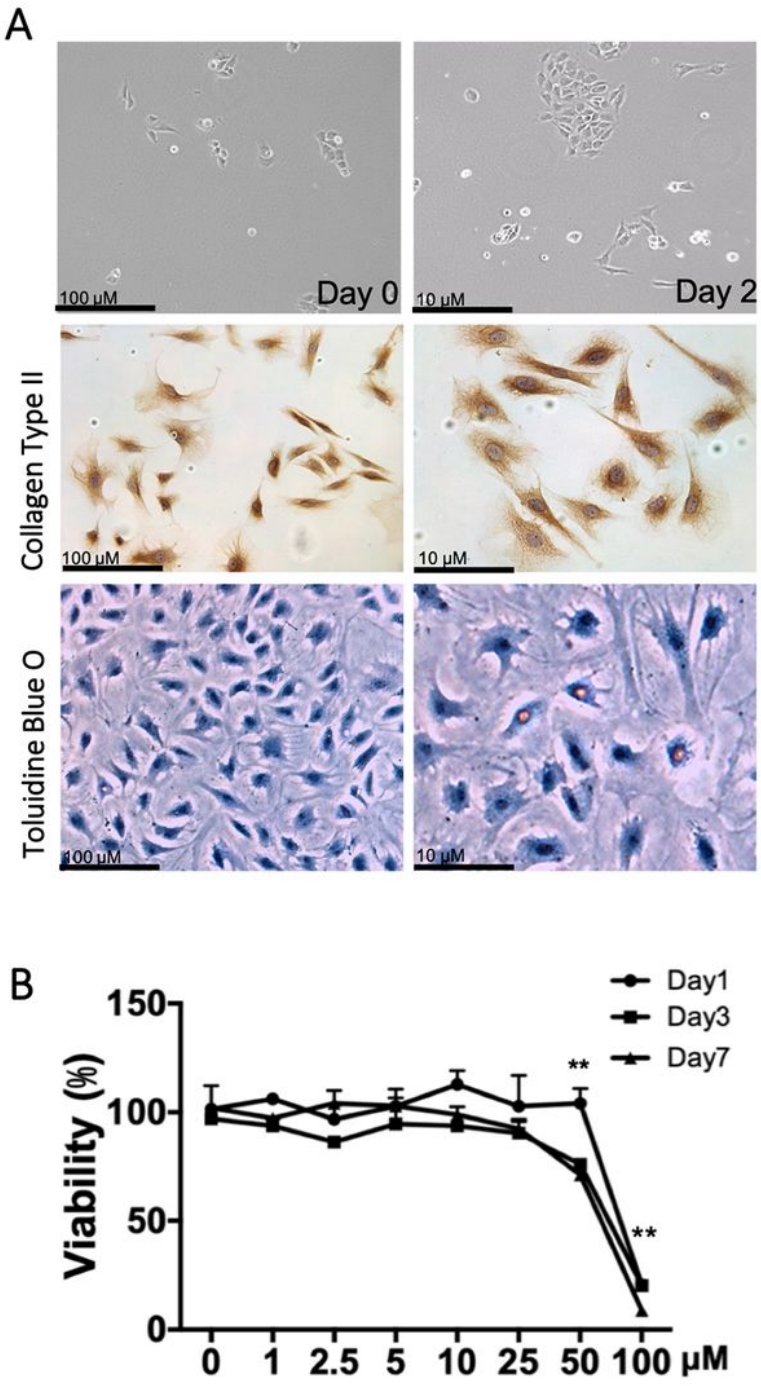

Figure 1

Identification of chondrocytes and their viability after treated with various concentrations of AA. (A) Chondrocytes were cultured within 6-well plates and identified by collagen type II staining by immuhistochemistry as well as Toluidine blue O. (B) Cells were cultured with different concentrations of

AA (from 0 to 100 μM) for 1, 3, 7 days selectively. The cell viability was evaluated by MTT assay. The values presented are the means ± SEM of three independent experiments. **p <0.01, vs. 0 μM.

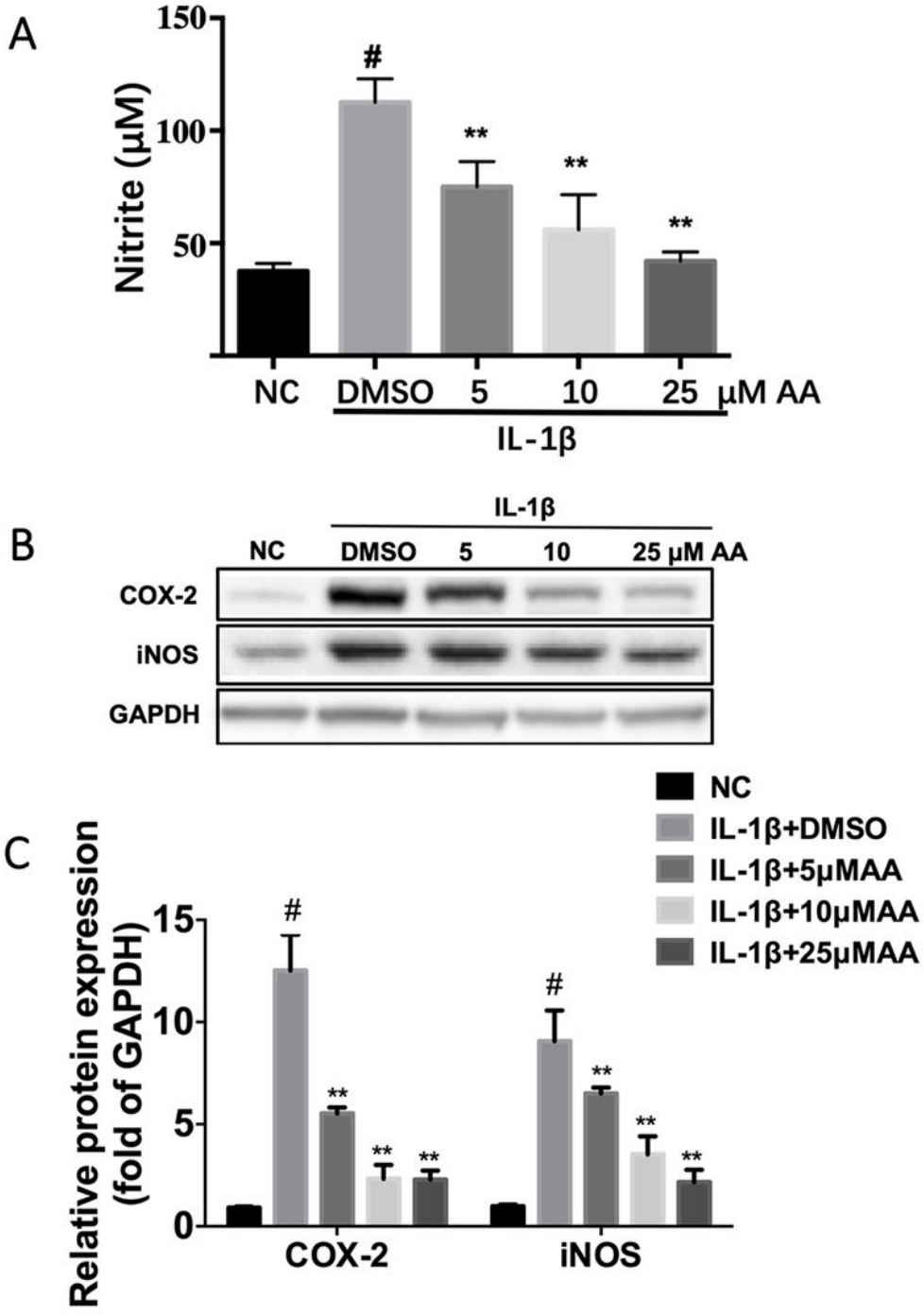

Figure 2

Effect of AA on the production or expression of inflammatory mediators induced by IL-1β. AA dose-dependently inhibited production of nitric oxide (NO) or expression of COX-2 and iNOS which increased by IL-1β. (A) The effects of AA on IL-1β induced NO production were detected by Griess reagent. (B&C)

The effects of AA on IL-1β induced COX-2 and iNOS up-regulate were detected by Western blotting. The data presented are means± SEM of three independent experiments. #p< 0.05 vs. control group (NC); *p <0.05, **p < 0.01 vs. (DMSO + IL-1β) group.

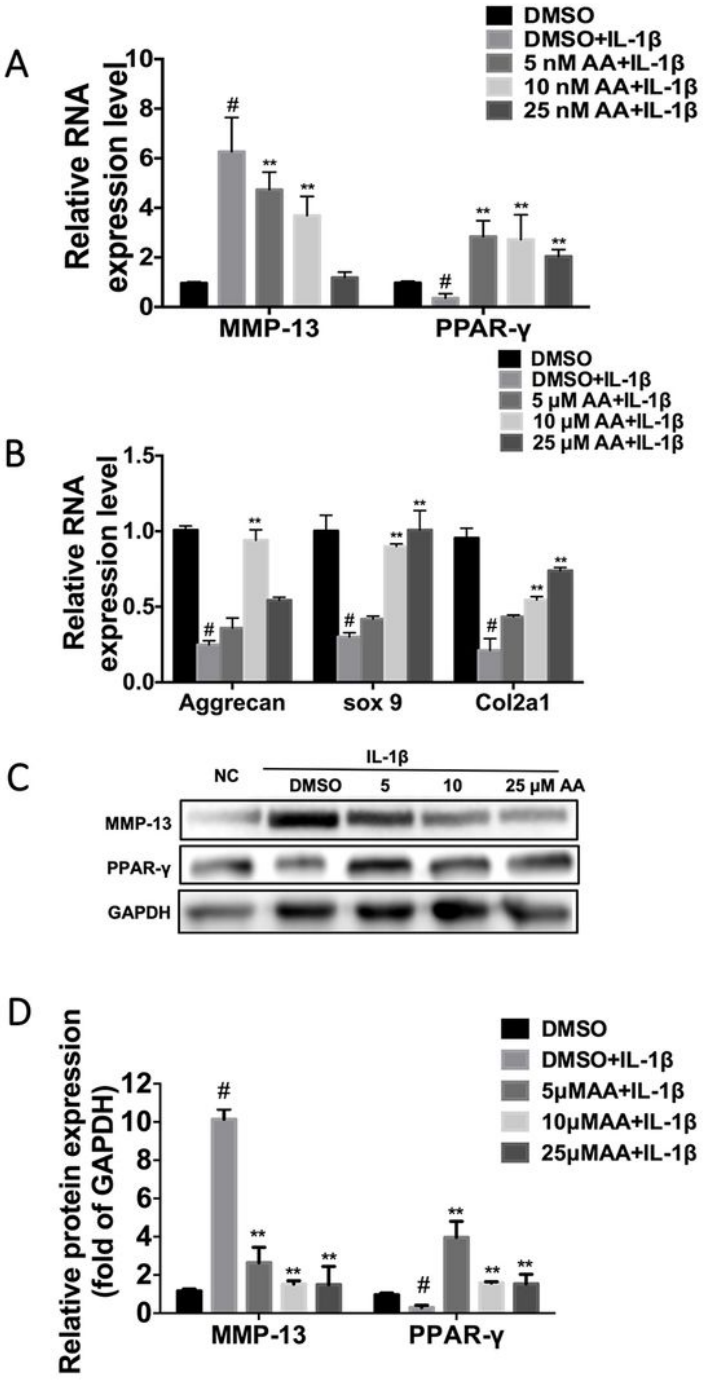

Figure 3

Effect of AA on the expression of hypertrophic markers and chondrogenic differentiation markers in rat chondrocytes. (A) Effects of AA on MMP-13 and PPAR-γ expression after IL-1β treatment. (B) Gene

expression of chondrogenic differentiation markers after AA and IL-1β treatment. (C&D) The protein expression of MMP-13 and PPAR-γ. The data presented are means ± S.D of three independent experiments. #p< 0.05 vs. control group (NC); *p <0.05, **p < 0.01 vs. (DMSO + IL-1β) group.

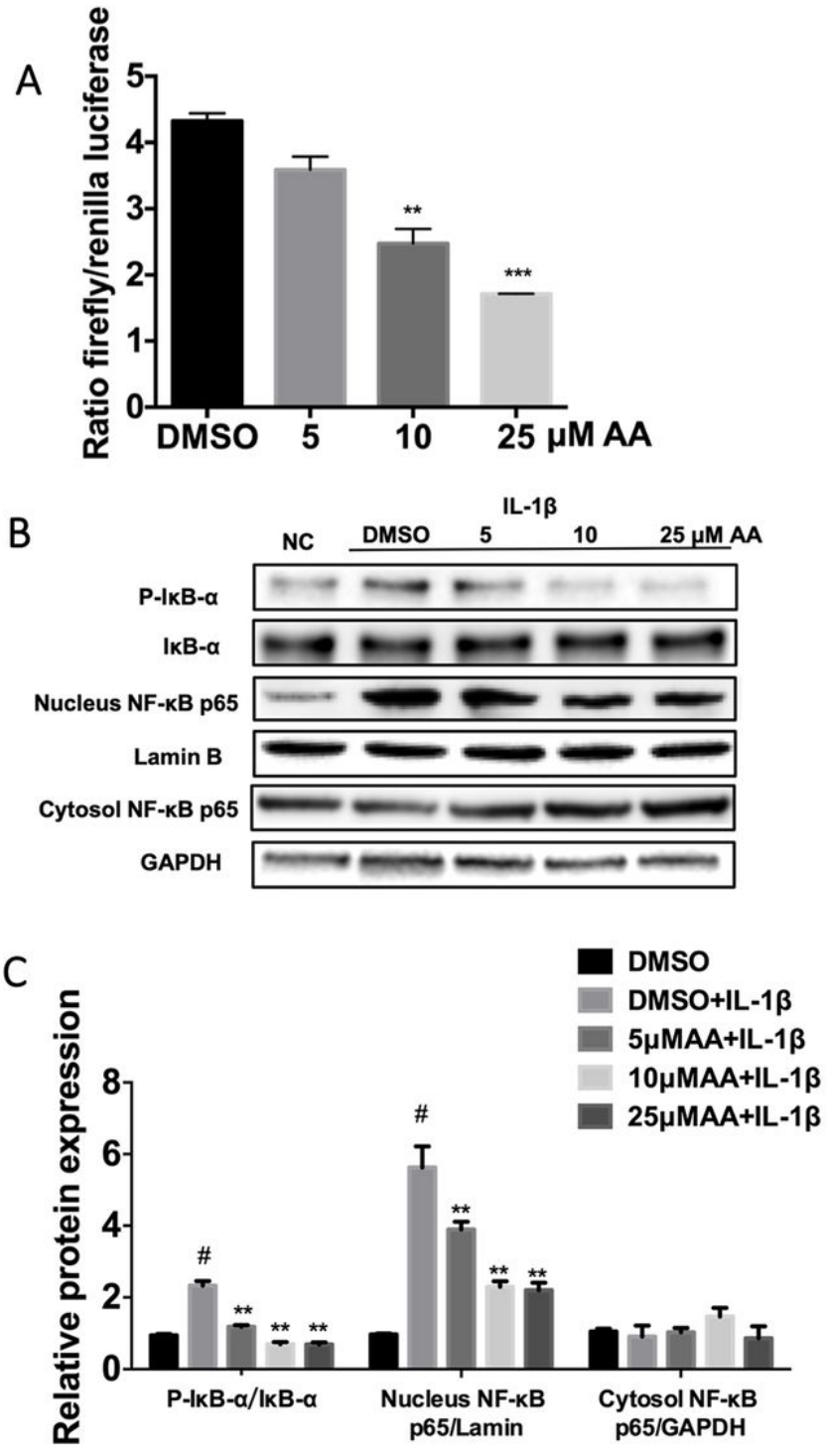

Figure 4

Effects of AA on NF-κB / p65 signal pathway. (A) Result of AA on NF-κB / p65 Renilla/firefly dual luciferase reporter system. (B&C) Result of western blotting showing the phosphorylation level of IκB-α

and distribution of NF-κB P65 within nucleus or cytoplasma after AA treatment. The data presented are means± SEM of three independent experiments. #p < 0.05 vs. control group (NC); *p < 0.05, ** p < 0.01 vs. (DMSO + IL-1β) group.

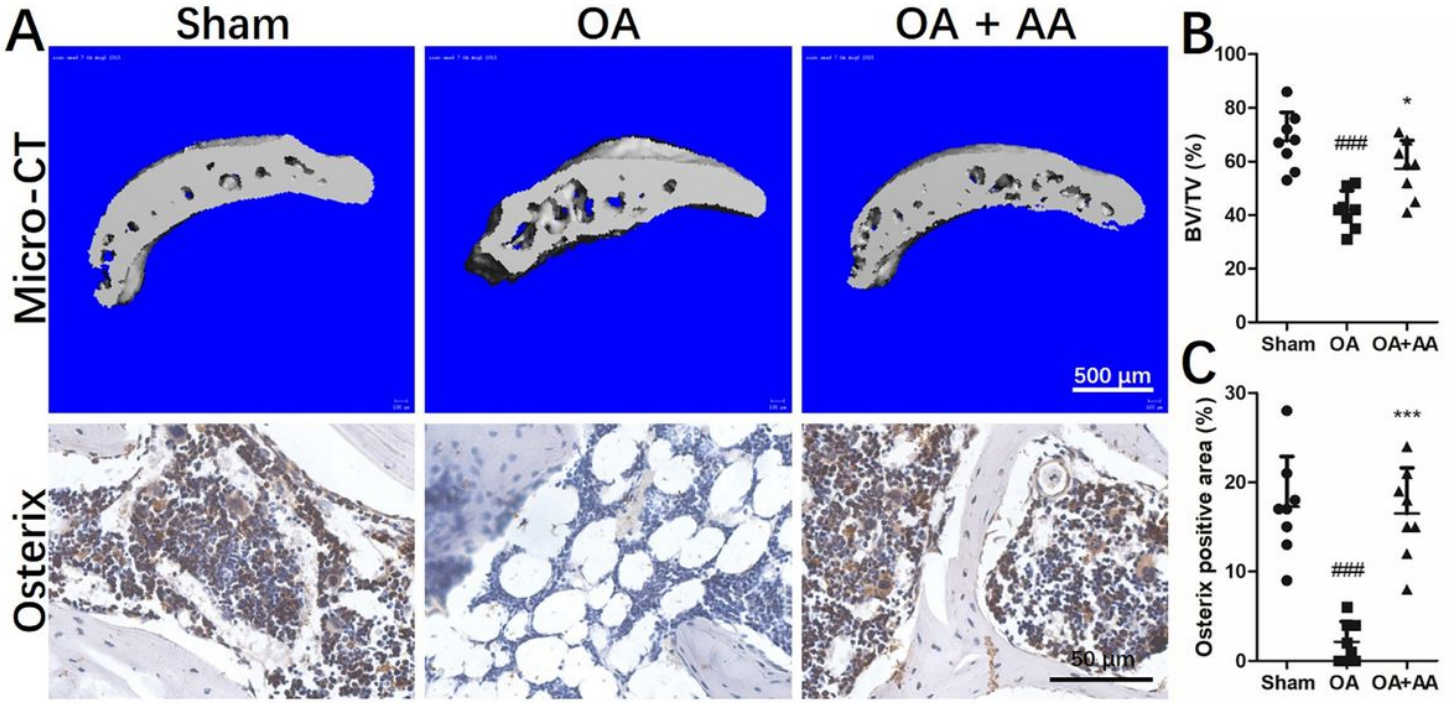

Figure 5

Effects of AA on cartilage phenotype in OA mouse model. (A) Results of Safranin O & Fast Green (SO & FG) staining, and MMP13 or Col X expression in cartilage examined by immunohistochemistry. (B) Cartilage pathological scoring results (OARSI). (C & D) Semi-quantitative results of MMP13 (C) or Col X (D) expression in the cartilage. # p< 0.05, # # p< 0.01, # # #p< 0.001 vs. Sham group; *p <0.05, **p < 0.01, ***p < 0.001 vs. OA group; n = 8 per group.

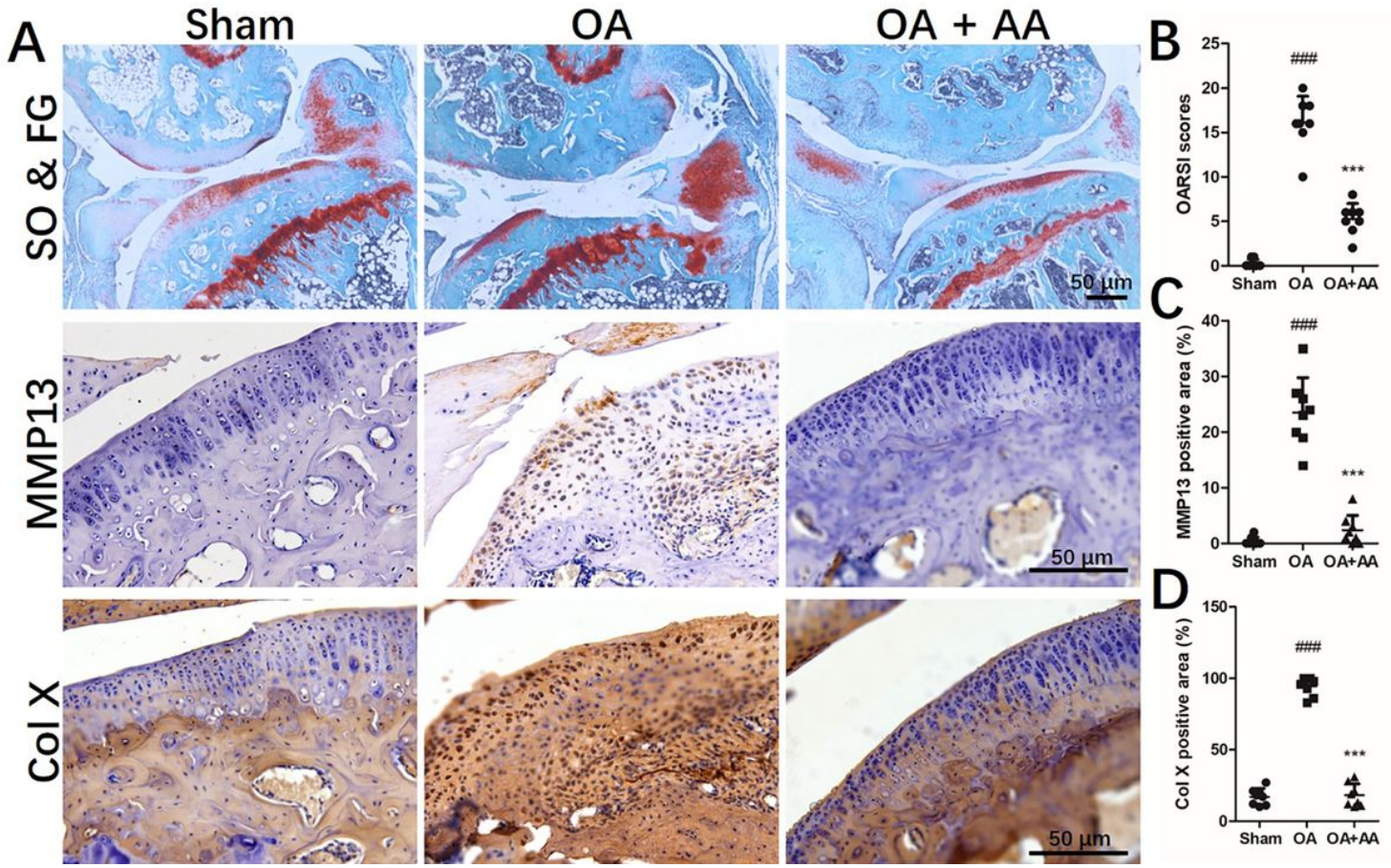

Figure 6

Effects of AA on microstructure and osteogenesis property of subchondral bone in OA mouse model. (A) 3D images of subchondral bone reconstructed by micro-CT, and the expression of osterix, an osteogenic marker, in subchondral bone examined by immunohistochemistry. (B) Quantitative result (bone volume/ tissue volume, BV/TV) of the subchondral bone. (C) Semi-quantitative results of osterix expression in the subchondral bone. # p< 0.05, # # p< 0.01, # # #p< 0.001 vs. Sham group; *p <0.05, **p < 0.01, ***p < 0.001 vs. OA group; n = 8 per group.