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ORIGINAL ARTICLE

Geniposide Suppresses Interleukin-1β-Induced Inflammation and Apoptosis in Rat Chondrocytes *via* the PI3K/Akt/NF-κB Signaling Pathway

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Abstract— Osteoarthritis (OA) is a chronic degenerative joint disease that is principally characterized by progressive joint dysfunction and cartilage degradation. Inflammation and apoptosis play critical roles in the progression of OA. Geniposide (GPO), one of the principal components of the fruit of *Gardenia jasminoides* Ellis, has been reported to have anti-inflammatory and other pharmacological effects. In this study, we performed *in vitro* experiments on rat chondrocytes to examine the therapeutic effects of GPO on OA and investigated its effects *in vivo* in a rat model of OA induced by medial meniscal tear (MMT). The results suggest that GPO can inhibit the expression of INOS, COX-2, and MMP-13 *in vitro*, and promote the expression of collagen II in rat chondrocytes stimulated with interleukin-1β (IL-1β). In addition, we also found that GPO can inhibit the expression of pro-apoptotic proteins such as Bax, Cyto-c, and C-caspase3 and increase the expression of the anti-apoptotic protein Bcl-2. These changes may be related to GPO-induced inhibition of the IL-1β-induced activation of the PI3K/Akt/NF-κB signaling pathway. *In vivo*, we also found that GPO can limit the development of OA in a rat model. Taken together, the above results indicate that GPO has potential therapeutic value for treating OA.

KEY WORDS: geniposide; osteoarthritis; chondrocyte; inflammation; apoptosis; PI3K/Akt/NF-κB.

INTRODUCTION

Osteoarthritis (OA) is a chronic degenerative joint disease that is characterized by progressive joint dysfunction and cartilage degradation [1, 2]. A variety of risk factors including age, obesity, joint trauma, metabolic diseases, and genetic factors are linked to its pathogenesis [3, 4]. Chondrocytes are the sole cell type in articular cartilage and are responsible for the maintenance of normal synthesis and renewal of the extracellular matrix (ECM) [2, 3]. Interleukin-1β (IL-1β), operating as an inflammatory mediator, can induce chondrocytes to express inflammation-associated proteins, including INOS, COX-2, and MMP-13, while reducing the secretion of collagen II, one of the main components of cartilage ECM [4–7].

These changes lead to an imbalance in the normal physiological state of the ECM, resulting in chondrocyte dysfunction and even apoptosis [8], which is thought to play a key role in human and animal OA [9]. The Bcl-2

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family of proteins, which comprises both pro- and anti-apoptotic molecules, serves as a key regulator of apoptosis [10]. In the Bcl-2 family, anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are members of a critical group of regulatory proteins that modulate apoptosis. Inhibition of the expression of Bax and promotion of the expression of Bcl-2 can play a role in the protection of chondrocytes [11]. It is thought that the PI3K/Akt/NF-κB signaling pathway is prominent in the regulation and progression of OA as its over-activation has been reported to lead to symptoms of inflammation and apoptosis of chondrocytes [12, 13]. Many experimental studies have shown that inflammation and apoptosis play key roles in the progression of OA [8, 12–14]. Hence, inhibition of inflammation and apoptosis may be valid strategies for the treatment of OA.

Geniposide (GPO), an iridoid glycoside compound purified from the fruit of *Gardenia jasminoides* Ellis, is a traditional medicine in many Asian countries because of its anti-inflammatory and other pharmacological effects [15]. Although the literature has many reports of studies using GPO [16–18], its study in the treatment of OA has not yet been reported. Therefore, in this study, the potential beneficial effects and the underlying mechanism of GPO on the pathogenesis of OA were investigated both in *in vitro* and *in vivo* experimental models.

MATERIALS AND METHODS

Chemicals and Reagents

For this study, GPO (purity ≥ 98%, HPLC, SML0153), collagenase type II and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium, bovine serum albumin (BSA), fetal bovine serum (FBS), and 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Gibco (Life Technologies Corp. Carlsbad, CA, USA). Antibiotic solution (penicillin/streptomycin) and phosphate-buffered saline (PBS) were purchased from Solarbio (Beijing, China). RIPA lysis buffer, bicinchoninic acid (BCA), and Griess reagent were purchased from the Beyotime Institute of Biotechnology (Shanghai, China). The following primary antibodies used in this study: rabbit anti-collagen II, rabbit anti-INOS, rabbit anti-COX-2, rabbit anti-MMP 13, rabbit anti-p65, rabbit anti-p-p65, rabbit anti-IκB, rabbit anti-p-IκB, rabbit anti-Cyto-c, rabbit anti-GAPDH, rabbit anti-PI3K, rabbit anti-p-PI3K, rabbit anti-Akt, and rabbit anti-p-Akt were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-Bcl-2, rabbit anti-Bax, and rabbit anti-cleaved caspase 3 were obtained from Cell Signaling Technology (Beverly, MA, USA). Recombinant Rat IL-1β was purchased from PeproTech (Rocky Hill, NJ, USA). Goat anti-rabbit horseradish peroxidase conjugates were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for prostaglandin E_2 (PGE_2) were purchased from R&D systems (Minneapolis, MN, USA).

Primary Chondrocyte Isolation and Culture

Normal articular cartilage was removed from male Sprague-Dawley rats that were less than 2 weeks old (Wenzhou Medical University) that had been sacrificed using 10% chloral hydrate. After joint surgery, the pieces of articular cartilage were aseptically dissected and separated from the underlying bone and connective tissues. The cartilage was then cut into $1 \times 1 \times 1$ mm^3 pieces and washed three times with PBS after which they were digested with 0.2% collagenase type II for 4–6 h at 37 °C. This suspension was then centrifuged at 180×*g* for 3 min to collect the chondrocytes. The extracted chondrocytes were cultured in DMEM/F-12 (with 10% FBS plus a 1% antibiotic mixture of penicillin and streptomycin) at a density of 1×10^5 cells/ml and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. Culture medium was changed every 2–3 days, and cells were passaged using a 0.25% trypsin-EDTA solution when 80–90% confluence had been attained. In order to avoid loss of phenotype, only the second or third generation of cells was used in subsequent experiments.

Cell Viability Assay

Cell viability was determined using a CCK-8 kit according to the manufacturer's instructions. Briefly, rat chondrocytes were incubated in 96-well plates at a density of 1×10^4 cells per well for 24 h. Chondrocytes were subsequently treated with different concentrations of GPO (0, 2.5, 5, 10, or 25 μM) for 24 h and then for a further 24 h either co-incubated with IL-1β (10 ng/ml) or retained in just the GPO alone. Next, the cells were washed with PBS, then 10 μl of the CCK-8 solution added to each well and cultured at 37 °C for 4–6 h. Absorbance at a wavelength of 450 nm was measured with a microplate reader (Leica Microsystems, Germany). All experiments were performed in triplicate.

Nitric Oxide (NO) and PGE_2 Measurement

Chondrocytes were cultured in DMEM/F-12 at a density of 3×10^5 cells/ml in 6-well plates and incubated with GPO (0, 2.5, 5, 10, or 25 μM) for 24 h prior to IL-1β (10 ng/ml) stimulation for 24 h. Quantification of NO concentration within the supernatant was achieved using the Griess reaction. Briefly, 100 μl of the cell suspension was mixed with the same volume of Griess reagent. Each sample was incubated at room temperature for 10 min, and the absorbance at 540 nm measured using a microplate reader (Leica Microsystems, Germany), using fresh culture medium as a blank. The culture medium supernatant from each sample was collected, and PGE_2 concentration measured using an ELISA kit (R&D Systems, Minneapolis, MN USA) according to the manufacturer's instructions. All assays were performed in duplicate.

Protein Extraction and Western Blot Analysis

Total protein from chondrocytes was extracted using RIPA lysis buffer (with 1% phenylmethylsulfonyl fluoride [PMSF]). Protein concentration was measured using a BCA protein assay kit. Equal quantities of 40 μg of total protein were loaded onto 10% SDS-PAGE gels and then afterwards transferred to PVDF membranes. The membranes were incubated in blocking buffer (5% skim milk in TBS-T) at room temperature for 2 h and washed three times with TBS-T for 5 min. The blocked membranes were then incubated with primary antibodies against collagen II (1:1000), INOS (1:1000), COX-2 (1:1000), MMP-13 (1:1000), Bcl-2 (1:1000), Bax (1:1000), C-caspase-3 (1:1000), Cyto-c (1:1000), p65 (1:1000), p-p65 (1:1000), IκB (1:1000), p-IκB (1:1000), PI3K (1:1000), p-PI3K (1:1000), Akt (1:1000), p-Akt (1:1000), or GAPDH (1:5000) overnight at 4 °C and washed three times with TBS-T for 5 min, followed by incubation with the appropriate secondary antibody (1:3000) at room temperature for 2 h. After washing in TBS-T, luminescence on the membranes was detected using an enhanced chemiluminescence (ECL) kit and quantified by Quantity ONE (Bio-Rad Laboratories Inc., Hercules, CA, USA) software. In addition, GAPDH was used as an internal control for the quantification of the target proteins. All experiments were performed in triplicate.

Immunofluorescence Microscopy

Chondrocytes were seeded on glass coverslips in a 6-well plate at a density of 3×10^5 cells/ml and incubated for 24 h. Samples were fixed with 4% paraformaldehyde for 15 min and permeabilized in PBS containing 0.5% Triton X-100 for 15 min at room temperature. Cells were blocked with 5% goat serum for 1 h and then incubated with a primary antibody, either collagen II antibody (diluted 1:200), Bcl-2 antibody (diluted 1:200), Bax antibody (diluted 1:200), or p65 antibody (diluted 1:200) overnight at 4 °C. The following day, cells were washed with PBS and then incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (diluted 1:400) prior to labeling with DAPI (Invitrogen) for 1 min. Finally, images of the cells were captured using a fluorescence microscope (Olympus Inc., Tokyo, Japan).

Establishment of a Rat OA Model and Experimental Animal Design

Six-week old male Sprague-Dawley rats (200 ± 20 g) were used in the following experiments. They were purchased from Wenzhou Medical University, the protocol for their care and use conforming to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, which was approved by the Animal Care and Use Committee of Wenzhou Medical University. The animals were acclimatized to a laboratory environment for a week prior to the start of experiments. Rats were randomly divided into three groups ($n = 6$), namely a sham-operated group, an osteoarthritis group, and an osteoarthritis treated with GPO group. The right knee joint of each rat was used as the experimental joint. The OA and GPO group rats were subjected to surgically induced OA by medial meniscal tear (MMT) model. Briefly, after anesthesia with intraperitoneal injection of 4% chloral hydrate, the medial collateral ligament and medial meniscus of the right knee were identified and excised in operation, while the other structures were preserved. The control group rats received sham surgery, consisting of arthrotomy without transection of the medial meniscus ligament and medial meniscus. After surgery, the joint surface was washed with sterile saline, and both capsule and skin sutured. From the fourth week after surgery, rats in the GPO group received an intraperitoneal injection of GPO (10 mg/kg, dissolved in 100% DMSO and then diluted it with saline) every 2 days for 8 weeks after surgery while rats in the OA group received an intraperitoneal injection of a vehicle (DMSO). The volume of GPO that injected into the rats was 1.0–2.0 ml/100 g. All animals were sacrificed

8 weeks after surgery and joint tissue collected for further evaluation.

Histological Analysis

Knee joints were dissected and fixed in 4% paraformaldehyde for 24 h, then subsequently decalcified in 10% EDTA at 37 °C for 4 weeks. The specimens were then embedded in paraffin and cut into 5 μm-thick sections prior to staining with hematoxylin and eosin (H&E) and Safranin-O-Fast green (S-O) to assess the condition of the cartilage. Then, we used a summed OARSI score (0–12) from medial femoral condyle and medial tibial plateau to evaluate the degree of articular cartilage destruction in a blinded manner [19]. Finally, AxioVision software was used to measure the thickness of the medial subchondral bone plate according to S-O stained sections.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) for at least three separate determinations for each group. The statistical significance of differences between groups was assessed using a Student's *t* test and one-way analysis of variance (ANOVA) using SPSS version 16.0 software. A P value < 0.05 was considered statistically significant.

RESULTS

Potential Cytotoxicity of GPO on Rat Chondrocytes

Potential cytotoxicity of GPO on rat chondrocytes was determined using a CCK-8 assay. Cells were cultured with increasing concentrations of GPO (0, 2.5, 5.0, 10, and 25 μM) for 24 h. According to Fig. 1a, cell viability was not influenced by GPO at concentrations up to and including 10 μM. Consequently, those concentrations of GPO (2.5, 5.0, and 10 μM) were used in subsequent experiments.

Effect of GPO on IL-1β-Induced Production of NO and PGE_2 in Rat Chondrocytes

Experiments were conducted to investigate the effect of GPO on IL-1β-induced NO and PGE_2 production in rat chondrocytes. Cells were pretreated with different concentrations of GPO (2.5, 5.0, or 10 μM, 24 h) before stimulation with IL-1β (10 ng/ml, 24 h). The concentration of NO in the cell suspensions was quantified using the Greiss reaction, while PGE_2 secretion was assessed using an ELISA kit. As shown in Fig. 1b, c, NO production in cell suspensions increased after IL-1β treatment ($P < 0.05$) compared with the control group, while IL-1β-induced NO production was inhibited in a dose-dependent manner by GPO. A concentration of 10 μM was the most effective ($P < 0.01$). IL-1β stimulation also markedly increased the production of PGE_2 ($P < 0.05$) compared with the control group, while GPO decreased IL-1β-induced PGE_2 release in a dose-dependent manner. This was consistent with the results of NO production.

Effect of GPO on IL-1β-Induced Protein Expression of Collagen II, INOS, COX-2, and MMP-13 in Chondrocytes

The protein levels of collagen II, INOS, COX-2, and MMP-13 were quantified by Western blot analysis. The results showed that collagen II expression was substantially down-regulated whereas INOS, COX-2, and MMP-13 were up-regulated after stimulation with IL-1β (10 ng/ml for 24 h). In contrast, pretreatment with GPO (2.5, 5.0, or 10 μM, 24 h) significantly reversed the IL-1β-induced decrease in collagen II and release of INOS, COX-2, and MMP-13 (Fig. 1f–j). In addition to the results of Western blot analysis, immunofluorescence experiments with collagen II corroborated the above results (Fig. 1d, e).

Effect of GPO on IL-1β-Induced Apoptosis in Rat Chondrocytes

In order to study whether IL-1β-induced apoptosis in rat chondrocytes was inhibited by GPO, we pretreated the cells with different concentrations of GPO (2.5, 5.0, or 10 μM, 24 h) before stimulation with IL-1β (10 ng/ml for 24 h).

The protein expression levels of Bcl-2, Bax, C-caspase3, and Cyto-c were quantified by Western blot analysis. As shown in Fig. 2a–e, chondrocytes stimulated with IL-1β (10 ng/ml for 24 h) significantly increased their expression of Bax, Cyto-c, and C-caspase3, and reduced protein production of Bcl-2. Pretreatment with GPO caused an inversion of this phenomenon in a dose-dependent manner. Immunofluorescence experiments with Bcl-2 and Bax corroborated the above results (Fig. 2f–i) in addition to the results of Western blot analysis. Meanwhile, a CCK-8 assay was used to test whether the cell viability of rat chondrocytes was affected by IL-1β. As shown in Fig. 2j, cell viability of the IL-1β group was reduced

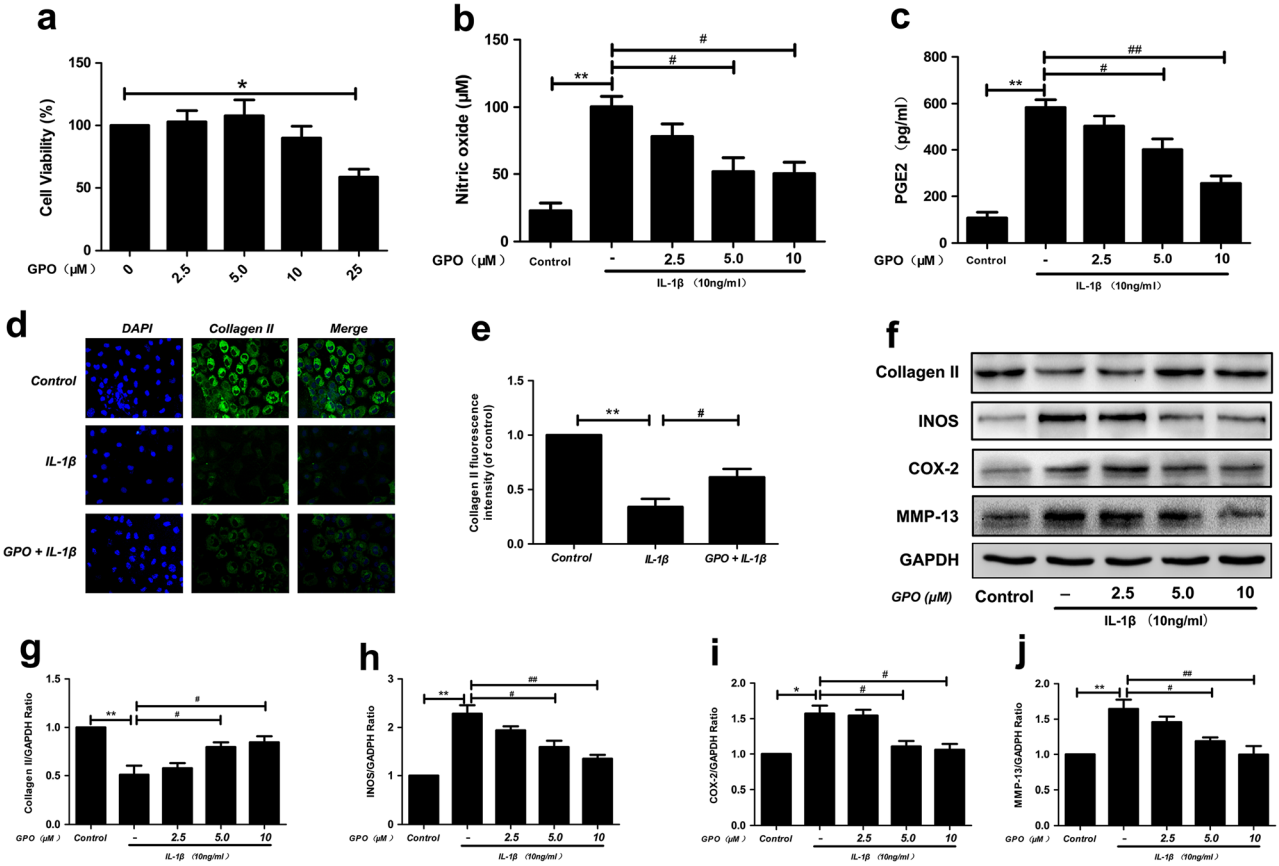

Fig. 1. Effect of GPO on *in vitro* cell cytotoxicity. Cells were cultured with various GPO concentrations (0, 2.5, 5.0, 10, or 25 μM) for 24 h. Cell viability was determined using CCK-8 assay (**a**). Cells were treated with different concentrations of GPO (0, 2.5, 5.0, and 10 μM, 24 h) before stimulated with IL-1β (10 ng/ml, 24 h). GPO inhibited IL-1β-induced NO and PGE_2 production in chondrocytes. NO concentration in the culture medium was determined by the Griess reaction (**b**). PGE_2 concentration was determined by an enzyme-linked immunosorbent assay (ELISA) kit (**c**). Effect of GPO on IL-1β-induced protein expression of collagen II, INOS, COX-2, and MMP-13 in chondrocytes. Immunofluorescence staining of collagen II protein in chondrocytes, and the intensity of green fluorescence represents the amount of collagen II expressed (**d**), and the specific fluorescence intensity is shown on the bar chart (**e**). The expression of collagen II, INOS, COX-2, and MMP-13 was tested by Western blot (**f**) and quantification analysis (**g**–**j**). Values represent mean ± SD of the three independent experiments. Significant differences from the control group are indicated by * $P < 0.05$ and ** $P < 0.01$. Significant differences from the IL-1β group are indicated as by $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$.

compared with that of the control group. As with previous results, pretreatment with GPO also reversed this effect in a dose-dependent manner.

Effect of GPO on IL-1β-Induced Activation of the NF-κB Signaling Pathway in Rat Chondrocytes

The NF-κB signaling pathway is important in the regulation of inflammation and apoptosis. To investigate the specific molecular mechanisms of action of GPO, we investigated its impact on the NF-κB signaling pathway. As shown in Fig. 3a–e, the expression levels of p-p65 and p-IκB were markedly increased in the IL-1β group compared with that in the untreated group. However, IL-1β-induced expression of p-p65 and p-IκB was reversed by pretreatment with GPO in a dose-dependent manner. We subsequently attempted to detect potential nuclear translocation of NF-κB p65 activated by IL-1β through immunofluorescence microscopy (Fig. 3f). The control group showed that the majority of p65 was located in the cytoplasm, whereas the IL-1β group revealed a distinct and intense nuclear staining for p65, indicating nuclear translocation of the NF-κB subunit. Co-treatment of chondrocytes with IL-1β and GPO resulted in a reduced translocation of the p65 subunits into the nuclei and demonstrated

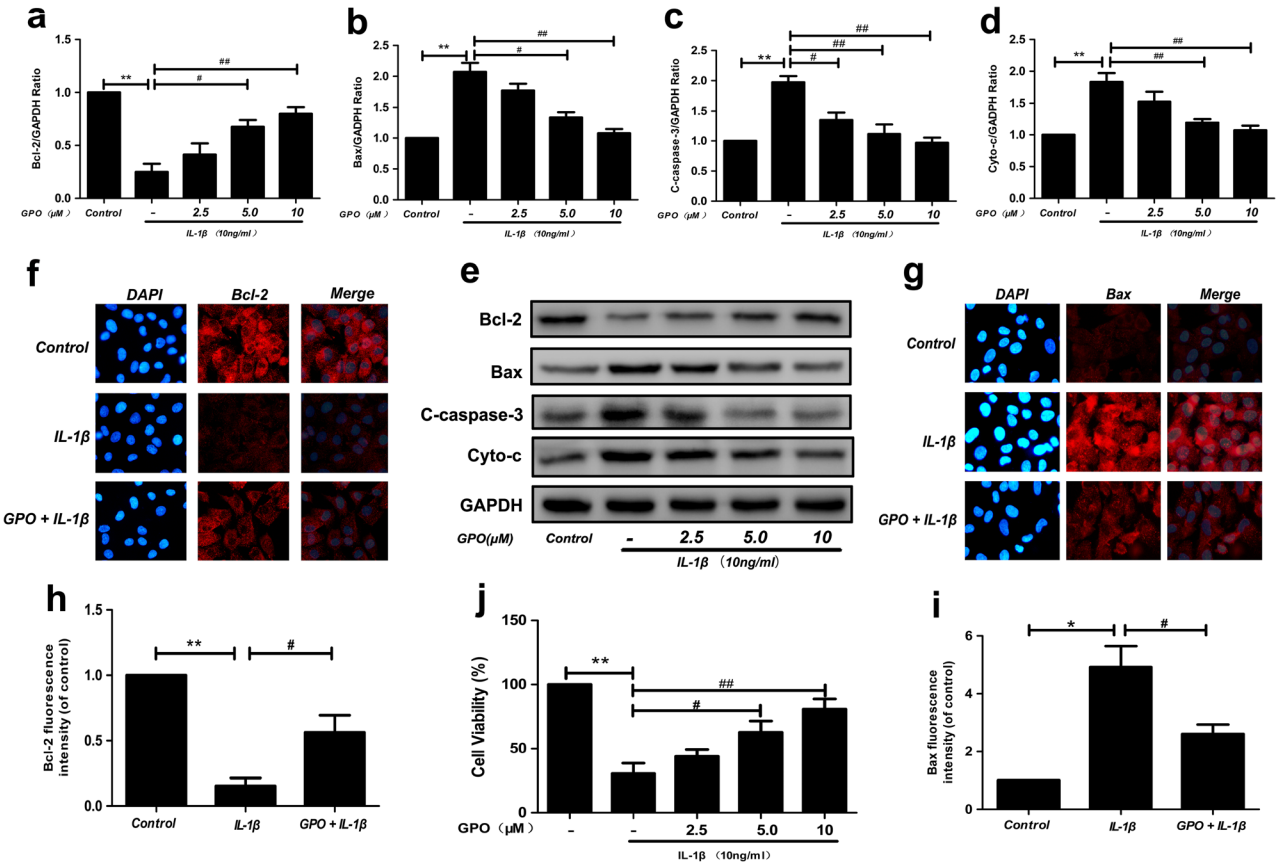

Fig. 2. Effect of GPO on IL-1β-induced apoptosis in rat chondrocytes. Cells were treated with different concentrations of GPO (0, 2.5, 5.0, and, 10 μM, 24 h) before stimulated with IL-1β (10 ng/ml, 24 h). The expression of Bcl-2, Bax, Cyto-c, and C-caspase-3 was tested by Western blot (**e**) and quantification analysis (**a**–**d**). Immunofluorescence staining of Bcl-2 and Bax protein in chondrocytes, and the intensity of red fluorescence represents the amount of Bcl-2 and Bax expressed (**f**, **g**), and the specific fluorescence intensity is shown on the bar chart (**h**, **i**). Cell counting Kit-8 (CCK-8) results of chondrocytes cells treated with different GPO concentrations for 24 h before IL-1β (10 ng/ml) stimulation for 24 h (**j**). Values represent mean ± SD of the three independent experiments. Significant differences from the control group are indicated by * $P < 0.05$ and ** $P < 0.01$. Significant differences from the IL-1β group are indicated as by $^{\#}$ $P < 0.05$ and $^{\#\#}$ $P < 0.01$.

decreased activation of NF-κB. The results indicate that GPO is able to reverse NF-κB p65 nuclear translocation in IL-1β-stimulated chondrocytes.

Effect of GPO on IL-1β-Induced PI3K and Akt Phosphorylation in Rat Chondrocytes

To further investigate the anti-inflammatory and anti-apoptotic molecular mechanisms of GPO, we investigated the regulation of GPO on IL-1β-induced PI3K/Akt phosphorylation using Western blot analysis. The results showed that GPO clearly inhibited IL-1β-induced PI3K and AKT phosphorylation (Fig. 3g–k).

Effect of GPO on Cartilage Degradation in a Rat Model of OA

A rat model of OA was established so as to determine whether GPO has a protective effect against the occurrence and progression of osteoarthritis *in vivo*. Rats received an intraperitoneal injection of GPO (10 mg/kg) or a vehicle (DMSO) every other day during the 8 weeks following surgery. Histological analysis of OA was evaluated using H&E and S-O staining and OARSI scores. As shown in Fig. 4a, S-O staining for proteoglycans was clearly reduced in the cartilage matrix of the OA group compared with that of the control group, as revealed by the ratio of red staining to total area (red/total), indicating OA-related matrix degradation. However, matrix degradation was less apparent in

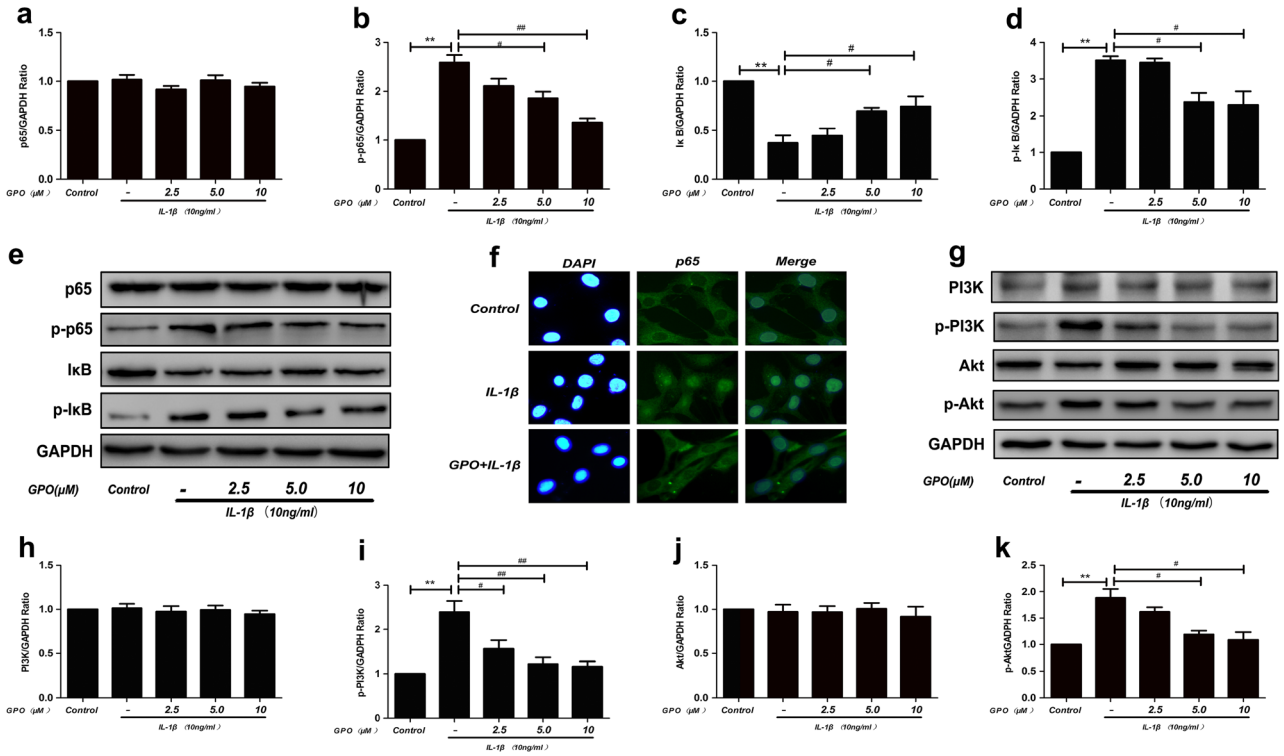

Fig. 3. Effect of GPO on IL-1β-induced PI3K/Akt/NF-κB phosphorylation in rat chondrocytes. Cells were treated with different concentrations of GPO (0, 2.5, 5.0, and 10 μM, 24 h) before stimulated with IL-1β (10 ng/ml, 24 h). The expression of p65, p-p65, IκB, p-IκB, PI3K, p-PI3K, Akt, and p-Akt was tested by Western blot (**e**, **g**) and quantification analysis (**a**–**d**, **h**–**k**). The effect of GPO on nuclear localization of the NF-κB p65 protein in chondrocytes stimulated by IL-1β was examined by immunofluorescence assay (**f**). Values represent mean ± SD of the three independent experiments. Significant differences from the control group are indicated by * $P < 0.05$ and ** $P < 0.01$. Significant differences from the IL-1β group are indicated as by $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

the GPO group than that in the OA group. Concomitantly, H&E staining showed that the articular cartilage in the control group had normal morphology whereas the OA group exhibited an evident reduction in chondrocyte number and articular cartilage thickness in addition to an irregular morphological structure. However, the results revealed a significantly thicker articular cartilage and reduced cartilage damage in the GPO group.

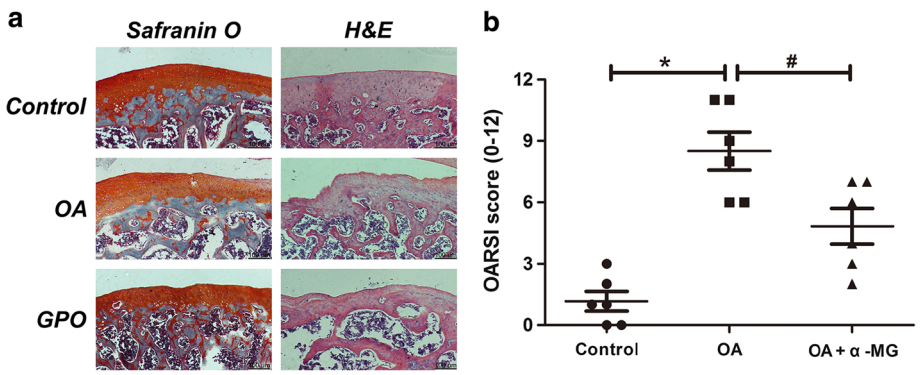

Fig. 4. The addition of GPO reduced the cartilage destruction in the OA rat model, assayed by Safranin-O staining, as well as H&E staining (**a**). Rat received intraperitoneal injections of GPO or vehicle (DMSO) during the fourth week after surgery. The OARSI score was calculated for each group (**b**). Values are mean ± SD. Significant differences from the control group are indicated by * $P < 0.05$. Significant differences from the OA group are indicated by $^{\#}P < 0.05$.

The OARSI scores (Fig. 4b) of the OA group were significantly higher than that of the sham control group ($P < 0.05$), demonstrating consistency with the results of the S-O and H&E staining described above. In contrast, the GPO group exhibited significantly lower OARSI scores than the OA group ($P < 0.05$). Thus, these results indicate that GPO may exhibit protective effects that prevent the occurrence and development of OA.

DISCUSSION

Osteoarthritis (OA) is a chronic degenerative joint disease that is characterized by progressive joint dysfunction, cartilage degradation, and pain [1, 2]. Inappropriate biomechanics, namely wear-and-tear, have been long believed to be the principal cause of OA [20]. Furthermore, surgical amputation, denervation, and load deprivation appear to be important factors driving articular tissue degeneration [21, 22]. In the cartilage damage caused by the factors described above, the dysfunction and even destruction of chondrocytes caused by inflammation and apoptosis may be common mechanisms. As the sole cell type in articular cartilage, chondrocytes play a crucial role in cartilage damage and repair [5, 6]. However, chondrocytes themselves have poor regeneration and repair function, and once seriously damaged, they do not commonly recover [23]. Therefore, the protection of chondrocytes is critical in the treatment of OA.

Treatment of OA patients generally aims to control pain and joint swelling, delay disease progression, and improve their quality of life. Until now, many drugs have been used in the treatment of OA, such as steroids and nonsteroidal anti-inflammatory drugs (NSAIDs), glucosamine, hyaluronic acid, or chondroitin [24]. However, many of these drugs alleviate only the clinical symptoms and are unable to prevent the progression of OA. In recent years, studies of the extracts of compounds from plants as treatments for human diseases have increased in number [12, 13, 16–18]. These plant extracts are widely studied because of reports of therapeutic efficacy with reduced side effects, which could be an ideal drug for the treatment of OA. Geniposide (GPO), an iridoid glycoside compound purified from *Gardenia jasminoides* Ellis, is a traditional medicine in many Asian countries because of its anti-inflammatory and other pharmacological effects [15]. In this study, we have demonstrated that GPO can inhibit the inflammation and apoptotic response induced by IL-1β in rat chondrocytes and that it can slow the progression of OA in an animal model.

Our study revealed the following novel observations: (1) GPO suppressed IL-1β-induced pro-inflammatory and matrix degrading enzyme production; (2) IL-1β-induced expression of apoptosis-related protein products was inhibited by GPO in rat chondrocytes; (3) GPO inhibited PI3K/Akt/NF-κB phosphorylation in a dose-dependent manner; and (4) GPO had the potential to slow the progression of OA in a rat OA model.

It has been demonstrated that inflammatory cytokines are involved in the pathological progress of OA. As a key inflammatory factor, IL-1β is widely used to simulate the microenvironment of OA in *in vitro* studies [11–13]. In the present study, the expression of NO, PEG_2, INOS, COX-2, and MMP-13 increased when cells were treated with IL-1β. These results demonstrate that IL-1β can simulate the OA microenvironment *in vitro*. As a member of the nitric oxide synthase (NOS) family of enzymes, INOS is able to synthesize the inflammatory mediator NO [25–27]. Likewise, COX-2, a critical mediator of pain and inflammation in OA, can produce another inflammatory mediator, PGE_2 [25, 27]. NO and PGE_2 play key roles in the pathogenesis of OA, such as promoting the synthesis and release of matrix metalloproteinase-13 (MMP-13), inhibiting the expression of collagen II, and ultimately the apoptosis of chondrocytes [28]. Studies have found that in most patients with OA, the levels of NO and PGE_2 are markedly elevated, and the pathological process of OA can be significantly delayed by inhibiting their expression [29]. In other experiments, it was found that selective inhibition of COX-2 and INOS expression could effectively protect cartilage from degeneration [7, 30]. In this study, we found that GPO significantly inhibited the expression of IL-1β-induced expression of NO, PGE_2, INOS, and COX-2 in a dose-dependent manner in chondrocytes. This indicates that GPO could act in a therapeutic manner against OA by alleviating inflammation.

Apoptosis of chondrocytes is thought to play an important role in the pathological process of OA [31]. As described above, inflammatory factors such as IL-1β, which is released by an overly aggressive inflammatory response, can trigger apoptosis in chondrocytes, which undoubtedly exacerbates cartilage degeneration [28]. As the only type of cell in cartilage, chondrocytes rarely recover once apoptosis is triggered [23]. Many experiments have demonstrated that Bcl-2, Bax, Cyto-c, and C-caspase-3 can regulate the apoptosis of cells [11, 31, 32]. In our experiments, we found that the expression of Bcl-2 in the IL-1β group was at a lower level than that of the control, while the expression of Bax, Cyto-c, and cleaved-caspase3 was higher. However, GPO reversed this

phenomenon in a dose-dependent manner. In order to verify this conclusion and increase the robustness of the results, both Western blot analysis and immunofluorescence experiments were performed. To some extent, these results demonstrate that GPO can protect cartilage by regulating the expression of apoptosis-related proteins.

The NF-κB signaling pathway is involved in regulating important physiological activities in many cells [33]. It can influence the expression of apoptosis-related proteins (Bcl-2, Bax, Cyto-c, and C-caspase3) and the synthesis of catabolic factors (INOS, COX-2, and MMP-13), which are vital in OA, as described previously. PI3K/Akt signaling is a major upstream element of the NF-κB signaling pathway. By inhibiting its activity, phosphorylation of NF-κB can be markedly reduced [13, 34, 35]. In order to reveal the anti-inflammatory and anti-apoptotic mechanisms of GPO, PI3K/Akt phosphorylation and NF-κB activation were quantified in our study. The results show that GPO can inhibit the inflammation and apoptosis induced by IL-1β, which may be closely related to modulation of the PI3K/Akt/NF-κB signaling pathway. But this is only one part of the mechanism, and it is likely that additional mechanisms will be elucidated in further studies.

To further investigate the effects of GPO *in vivo*, we established a rat OA model in this study. The model is based on MMT, whose effectiveness has been demonstrated in many studies due to the model's mechanical instability [36]. As a result, it is a very slowly progressive model compared to other models, but to some extent, this model also simulates the pathological changes of OA's slow progression [36, 37]. In the present study, OARSI score was used to evaluate the severity of cartilage degeneration. In the GPO group, the OARSI score was lower than that in the OA group, suggesting that GPO could slow the progression of OA in rats. This result along with the *in vitro* experiments and further suggests that GPO is likely to provide potential value in the therapy of OA.

In conclusion, we have demonstrated the anti-inflammatory and anti-apoptotic effects of GPO in rat chondrocytes stimulated by IL-1β. Its protective effect on cartilage may be accomplished by inhibiting the expression of inflammation and apoptosis-related proteins. Inhibition of the activation of the PI3K/Akt/NF-κB signaling pathway may play a key role in the treatment of OA using GPO. However, there may be other molecular mechanisms that may not yet have been explored and which require further study. In the rat model, we also observed the presence of synovitis, but we did not study the effect of GPO in synovitis in this experiment, perhaps for this experiment is an important supplement. In addition, the efficacy of GPO compared with traditional drugs remains to be verified and whether the same effect on human OA chondrocytes would be observed requires further validation. Therefore, many studies still need to be performed before GPO can be used in the clinical treatment of OA patients.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was in accordance with the Declaration of Helsinki and Tokyo.

Conflict of Interest. The authors declare that they have no conflict of interest.

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