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The protective effect of xanthan gum on interleukin-1β induced rabbit chondrocytes

Guanying Han[a,b,c,*,1], Huarong Shao[b,c,1], Xiqiang Zhu[b], Guilan Wang[b,c], Fei Liu[b], Fengshan Wang[c,**], Peixue Ling[b,c,***], Tianmin Zhang[b,c]

[a] School of Pharmaceutical Sciences, Liaoning Medical University, Jinzhou 121001, China
[b] Post-doctoral Scientific Research Workstation, Institute of Biopharmaceuticals of Shandong Province, Jinan 250101, China
[c] School of Pharmaceutical Sciences, Shandong University, Jinan 250101, China

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ABSTRACT

We have previously shown that intra-articular injection of xanthan gum (XG) could protect the joint cartilage and reduce osteoarthritis progression. In this study, we investigated the preliminary cytotoxicity of XG on chondrocytes, evaluated the effects of XG on the proliferation and the protein expression of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase-1 (TIMP-1) in interleukin-1β (IL-1β)-induced rabbit chondrocytes. Primary rabbit chondrocytes were cultured. After treatment with various concentrations of XG with or without 10 ng/mL IL-1β, the proliferation of chondrocytes was evaluated using the MTT assay and the expression levels of MMPs and TIMP-1 were evaluated using ELISA. The results showed that XG alone displayed no adverse effects on cell viability and reversed significantly IL-1β-reduced cell proliferation in a dose-dependent manner. Furthermore, XG showed a dose-dependent inhibition in the IL-1β-induced release of MMPs while increasing TIMP-1 expression. These results strongly suggest that XG affords protection on IL-1β induced rabbit chondrocytes.

1. Introduction

Osteoarthritis (OA) is one of the most common age-related degenerative joint disorders. Cartilage degradation is one of the pathological changes in OA, and is characterized by extracellular matrix (ECM) damage and tissue cellularity loss (Martel-Pelletier, 1998). Chondrocytes are the only cells of articular cartilage, and they play a central role in the degradation of ECM. Chondrocytes apoptosis is important in pathogenesis of OA (Johnson, Charchandi, Babis, & Soucacos, 2008). The models which mimic the circumstances leading to *in vivo* cartilage degradation are valuable in the evaluation of pathogenesis and therapeutic methods of OA. Interleukin-1β (IL-1β) has been shown to induce chondrocytes degradation *in vitro* and treatment of chondrocytes with IL-1β serve as a model for experimental inflammation seen in OA (Yasuhara et al., 2005).

IL-1β, a cytokine released by synovial cells and macrophages, plays a decisive role in amplifying inflammation in OA. IL-1β is responsible for accelerating the degradation of cartilage matrix and inducing chondrocytes apoptosis (Chowdhury, Bader, & Lee, 2006). IL-1β also alters chondrocytes anabolism by repressing the synthesis of proteoglycan and enhancing proteoglycan degradation. IL-1β also up-regulates matrix degrading enzymes such as matrix metalloproteinases (MMPs) and down-regulates the tissue inhibitor of metalloproteinases (TIMPs) in chondrocytes (Aida et al., 2005). Accumulating evidence suggests that an excess of MMPs plays an important role in cartilage breakdown. The contributions of these proteolytic enzymes to OA are associated with their ability to degrade the ECM proteins (Burrage, Mix, & Brinckerhoff, 2006). The activity of MMPs is controlled by TIMPs which inhibit all MMPs at a stoichiometry of 1:1 by forming high-affinity complexes (Brew, Dinakarpandian, & Nagase, 2000). Imbalance between MMPs and TIMPs is well known to be of importance in the progression of OA. Therefore, the down-regulation of MMPs and up-regulation of TIMPs will be reasonable therapeutic targets for the treatment of OA.

Apart from surgical measures, treatment of OA has generally been aimed at maintaining the mobility in the joint and retarding the degeneration of cartilage (Alcaraz, Megías, García-Arnandis, Clérigues, & Guillén, 2010). It is believed that many glycosaminoglycans have chondroprotective effects (Chen, Liu, Du, Peng, & Sun,

* Corresponding author. Tel.: +86 531 81213077; fax: +86 531 81213083.
** Corresponding author. Tel: +86 531 88382589; fax: +86 531 88382548.
*** Corresponding author at: Institute of Biopharmaceuticals of Shandong Province, Jinan 250101, China. Tel.: +86 531 81213002; fax: +86 531 88524738.
E-mail addresses: hgy19800223@yahoo.com.cn (G. Han), fswang@sdu.edu.cn (F. Wang), lingpeixue23@yahoo.com.cn, lpx@sdfmg.com (P. Ling).
[1] These authors contributed equally to this work.

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2006; Legendre, Baugé, Roche, Saurel, & Pujol, 2008). Intra-articular (IA) injection of hyaluronic acid (HA) is indicated as an effective treatment for OA due to it decreases inflammation and coats the pain receptors (Abate, Pulcini, Di Iorio, & Schiavone, 2010; Gomis, Miralles, Schmidt, & Belmonte, 2007). Furthermore, HA can protect chondrocytes against proteoglycan depletion and apoptosis induced by IL-1β (Akmal et al., 2005; Hashizume & Mihara, 2010). However, HA is instable and will be quickly degraded by hydrolytic or enzymatic reactions *in vivo* (Zhong et al., 1994). Therefore, a compound which is similar to HA in the structure and function, but with a longer effect in the joint, will be needed.

Xanthan gum (XG) is a natural microbial extracellular heteropolysaccharide which contains repeating units of five monosaccharides formed by two D-glucose, two D-mannose and one D-glucuronic acid (García-Ochoa, Santos, Casas, & Gómez, 2000). XG is similar to HA in rheology and viscosity. Furthermore, XG is a non-toxic, biodegradable and biocompatible glycosaminoglycan. Our recent study had indicated that IA injection of XG could protect the joint cartilage and reduce the papain-induced OA progression as assessed by morphological and histological analyses in rabbits. Furthermore, the protective effects on OA of IA injection of XG once every two weeks for 5 weeks and IA injection of HA once every week for 5 weeks did not have statistical significance (Han, Ling, Wang, Wang, & Shao, 2012; Han, Wang, et al., 2012). In the present study, we reported the cytotoxicity of XG on chondrocytes, evaluated the protective effect of XG on chondrocytes through investigating the proliferation and the protein expression of MMP1, -3, -13, TIMP-1 in IL-1β-induced rabbit chondrocytes.

2. Materials and methods

2.1. Materials

Recombinant human IL-1β, 3-(4,5-dimethylthiazolyl-2)-2,5-diphe-nyltetrazoliumbromide (MTT), dimethylsulfoxide (DMSO), trypsin and type II collagenase were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, penicillin, streptomycin, fetal bovine serum (FBS) were obtained from Gibco BRL (Grand Island, NY, USA). The monoclonal type II collagen antibody was purchased from the Boster (Wuhan, China). Alexa 555-conjugated goat anti-rabbit IgG, diaminido phenyl indol (DAPI) were obtained from Invitrogen (CA, USA). MMPs and TIMP-1 enzyme-linked immuno sorbent assay (ELISA) kits were purchased from EIAab (Wuhan, China). All common chemicals and reagents were purchased from Tairuida (Jinan, China).

2% (w/v) XG injection was prepared as described previously by Han, Ling, et al. (2012) and Han, Wang, et al. (2012), and supplied by Post-doctoral Scientific Research Workstation, Institute of Biopharmaceuticals of Shandong Province, Jinan, China. The pH value was 7.2 and the molecular weight was about 5200 kDa.

2.2. Isolation and culture of chondrocytes

The animal experiment was carried out according to the internationally accredited guidelines with the approval of the Institutional Animal Care and Use Committee of Drug Safety Evaluation Center of Shandong Institute of Pharmaceutical Industry (Jinan, China). As prescribed in the articles (Shakibaei, Csaki, Nebrich, & Mobasheri, 2008), cartilage slices were collected from the bilateral knee joints of 4-week-old New Zealand white rabbits. The slices were washed with D-Hanks buffer solution containing antibiotics twice and cut into small pieces (<1 mm^3). These pieces were digested primarily with 0.25% (w/v) trypsin for 30 min at 37 °C and the supernatants were discarded. They were further digested with 0.2% (w/v) type II collagenase for 4 h at 37 °C. The extracted chondrocytes were passed through a 70-μm pore size cell sieve and cultured at 1×10^6 cells/mL in 25 cm^2 plastic culture flask at 5% CO_2 and 37 °C in a tissue culture incubator. The complete DMEM/F12 medium was supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. Confluent chondrocytes were passaged using trypsin at a ratio of 1:3 and chondrocytes passage 2 were used throughout the study.

2.3. The identification of chondrocytes

The morphology of chondrocytes was observed by inverted phase microscopy, and chondrocytes were identified by histological examination of toluidine blue (Terry, Chopra, Ovenden, & Anastassiades, 2000) and type II collagen immunofluorescence staining. To verify the expression of type II collagen, chondrocytes were fixed with 4% paraformaldehyde for 30 min and blocked with 5% normal goat serum for 30 min. Then chondrocytes were incubated with rabbit polyclonal antibodies against types II collagen overnight at 4 °C and incubated with Alexa 555-conjugated goat anti-rabbit IgG (1:2000) as a secondary antibody for 1 h at 37 °C. Negative control was treated with PBS rather than primary antibody. Nuclei were counterstained with DAPI for 10 min. Finally, chondrocytes were washed three times in PBS and then observed directly on laser scanning confocal microscopy (LSM710; Carl Zeiss, Oberkochen, Germany).

2.4. Cell proliferation assays

The chondrocytes passages 2 were placed in 96-well microplates at different densities of 1.5×10^4 and 6×10^4 cells/cm^2 in 100 μL of growth medium for 24 h. After that time different concentrations of XG (0, 10, 100, 500, 1000 2000 μg/mL) with or without IL-1β (10 ng/mL) in 5% or 0.1% FBS were added and the cells were cultured for a further 24 h or 48 h. The medium containing 0.1% FBS was "serum-starved medium". The proliferation of cells was determined using the MTT assay (Csaki, Keshishzadeh, Fischer, & Shakibaei, 2008; Tinti et al., 2011). 20 μL of MTT solution (5 mg/mL in PBS, sterile) was added to each well and the culture continued to incubate for another 4 h in a 5% CO_2 atmosphere at 37 °C. The supernatants were discarded, and DMSO (150 μL) was added to each well to solubilize the formed formazane crystals. The absorbance at 570 nm was measured for each well using a Sunrise-Basic Tecan microplate reader (Tecan, Austria). The wells without cells were used as the zero point of absorbance.

2.5. ELISA

The chondrocytes passages 2 were placed in 24-well culture plates at a density of 3×10^4 cells/cm^2 in 1 mL of growth medium for 24 h. After that time different concentrations of XG (0, 10, 100, 500, 1000 2000 μg/mL) with or without IL-1β (10 ng/mL) in 0.1% FBS were added and the cells were cultured for a further 24 h or 48 h. At the end of incubation, the media were collected and the levels of MMP-1, -3, -13, and TIMP-1 proteins in media were determined using commercially available ELISA kits according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software package. All data were expressed as means ± standard deviation (SD). The data were initially evaluated for normal distribution. Statistical significances among groups were then tested using a one-way analysis of variance (ANOVA). Differences between

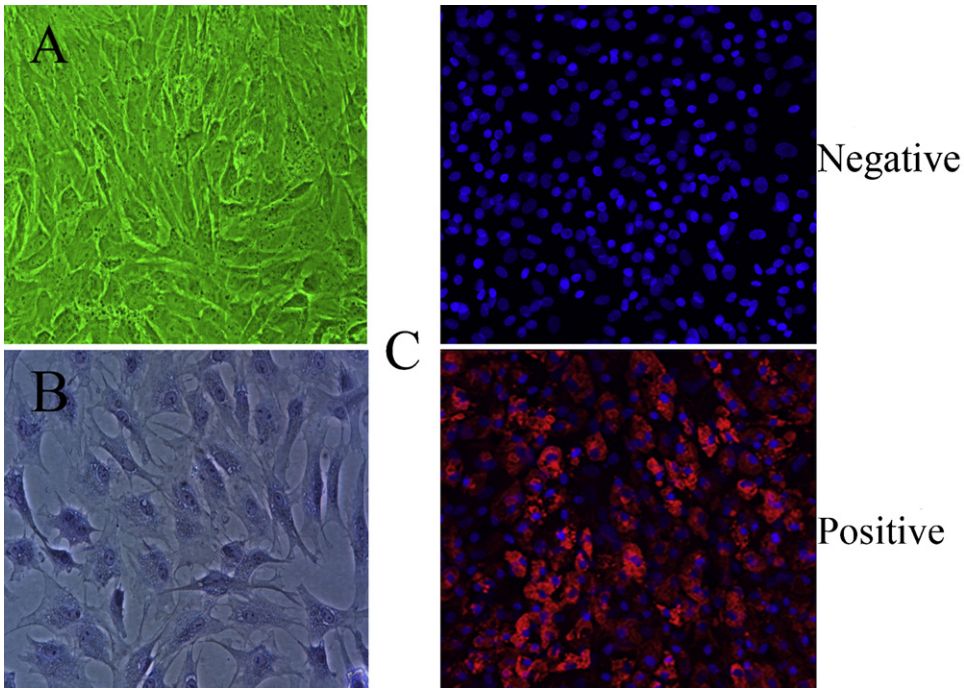

Fig. 1. Morphology and identification of rabbit chondrocytes (200× magnification). (A) Morphology of chondrocytes; (B) toluidine blue staining; (C) type II collagen immunofluorescence staining. The chondrocytes formed a monolayer in slabstone shape (A). The color of chondrocytes stained by toluidine blue was blue and some small blue particles were localized in cytoplasm (B). DAPI blue staining of cell nuclei was shown in (C). Type II collagen immunofluorescence positive signals (red) were localized in cytoplasm and cell membrane, but there was not red fluorescence in negative control group (C).

groups were further confirmed by Student's *t*-test and considered to be statistically significant if $P < 0.05$.

3. Results

3.1. The morphology and identification of chondrocytes

At first, the seeded chondrocytes were sphere-shaped and floated. At 24–48 h, cells showed cell attachment, deformation, polygon or shuttle shape, and the cells formed a monolayer in slabstone shape on plastic surface about 8 days (Fig. 1A). Passaged chondrocytes attached within 24 h, with a similar appearance as primary cells. When stained with toluidine blue and anti-type II collagen antibody, the results were positive. The color of the chondrocytes stained by toluidine blue was blue and some small blue particles were localized in cytoplasm (Fig. 1B). Type II collagen immunofluorescence positive signals which presented with red fluorescence were localized in cytoplasm and cell membrane, but there was not red fluorescence in negative control group (Fig. 1C).

3.2. Cell proliferation

In chondrocytes which cultured in medium containing 0.1% FBS or 5% FBS for 24 h or 48 h at densities of 1.5×10^4 or 6×10^4 cells/cm^2, the additions of XG alone at these concentrations studied did not modify significantly cell viability when compared to untreated control cells ($P > 0.05$) (Fig. 2A–D). These results indicated that chondrocytes could proliferate actively in the presence of XG and XG displayed no adverse effects on cell viability.

The MTT analysis demonstrated that IL-1β (10 ng/mL) only stimulated chondrocytes showed a significant decrease in cell viability. Treatment with IL-1β alone for 24 h or 48 h, the decline ratios of viability of chondrocytes which cultured in medium containing 0.1% FBS or 5% FBS at densities of 1.5×10^4 or 6×10^4 cells/cm^2 were about 33.78% and 21.98% (Fig. 2A), 24.49% and 27.61% (Fig. 2B), 35.13% and 45.42% (Fig. 2C), 30.38% and 37.98% (Fig. 2D) of untreated control, respectively. However, the viability of the chondrocytes cultivated only in the presence of IL-1β was significantly lower compared to those of chondrocytes treated with XG in doses ranging from 100 to 2000 μg/mL (Fig. 2A–D). Furthermore, the effects of XG were dose-dependent in doses ranging from 100 to 1000 μg/mL. The percentages ranged from about 19.62–41.14% and 5.13–20.5% (Fig. 2A), 6.70–23.25% and 21.91–29.56% (Fig. 2B), 9.08–41.64% and 21.03–65.95% (Fig. 2C), 21.57–37.88% and 17.82–51.92% (Fig. 2D), compared to the values obtained for cells stimulated with IL-1β alone. The data presented in this report suggested that treatment with 100, 500 and 1000 μg/mL XG in IL-1β-induced chondrocytes resulted in a dose-dependent restoration of chondrocytes proliferation.

3.3. MMPs and TIMP-1 protein expression

The proteins expressions of MMP-1, -3, -13 and TIMP-1 in the culture medium of rabbit articular chondrocytes were determined by ELISA. These results were shown in Fig. 3. Chondrocytes stimulated with IL-1β (10 ng/mL) showed enhanced release of MMP-1, -3 and -13 at 24 h and 48 h compared to untreated controls ($P < 0.05$). The percentages were about 85.43% and 198.60% (Fig. 3A), 61.08% and 72.47% (Fig. 3B), 108.55% and 141.96% (Fig. 3C) of untreated control, respectively. Furthermore, following 24 h and 48 h in the presence of IL-1β, a decrease in TIMP-1 protein expression was observed and the percentages were about 53.98% and 50.67% (Fig. 3D) of untreated control, respectively. However, treatment of chondrocytes in the presence of 100, 500, 1000 and 2000 μg/mL XG significantly inhibited the IL-1β-induced release of MMP-1, -3 and -13, increased TIMP-1 production in a dose-dependent manner in doses ranging from 100 to 1000 μg/mL. The percentages ranged from about 21.13–41.77% and 20.12–56.44% (Fig. 3A), 15.12–30.70% and 10.59–32.93% (Fig. 3B), 21.12–40.36% and 16.56–47.37% (Fig. 3C), 15.22–95.13% and 24.48–86.02% (Fig. 3D), compared to the

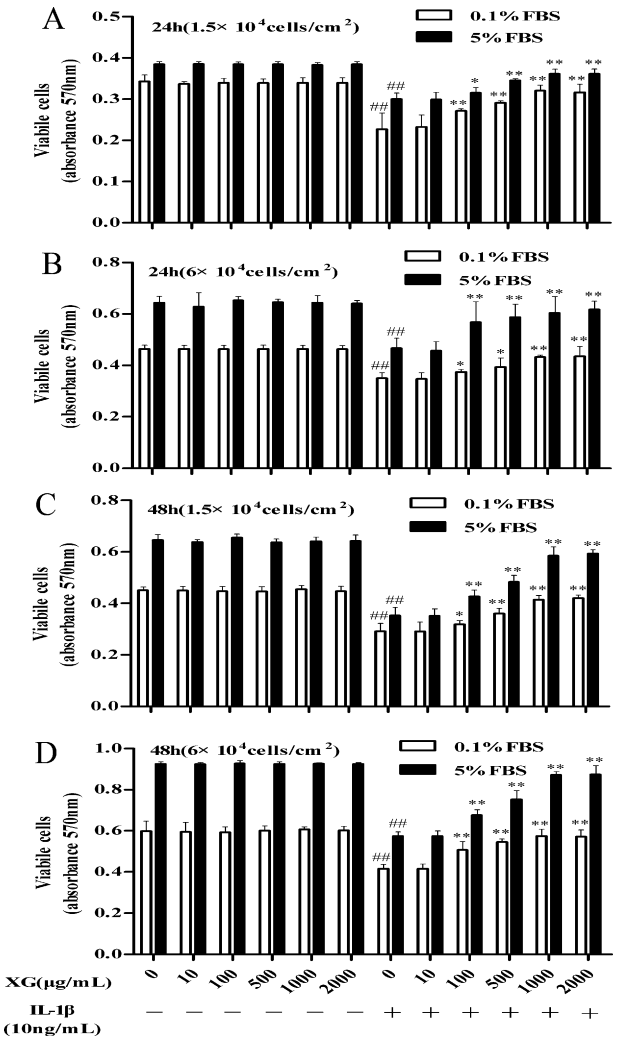

Fig. 2. Effect of XG (0, 10, 100, 500, 1000, 2000 μg/mL) with or without IL-1β (10 ng/mL) on the proliferation of chondrocytes. (A) The proliferation of chondrocytes which cultured at the density of 1.5×10^4 cells/cm^2 for 24 h. (B) The proliferation of chondrocytes which cultured at the density of 6×10^4 cells/cm^2 for 24 h. (C) The proliferation of chondrocytes which cultured the density of 1.5×10^4 cells/cm^2 for 48 h. (D) The proliferation of chondrocytes which cultured at the density of 6×10^4 cells/cm^2 for 48 h. $^{\#\#}P < 0.01$ as compared to negative control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to IL-1β group.

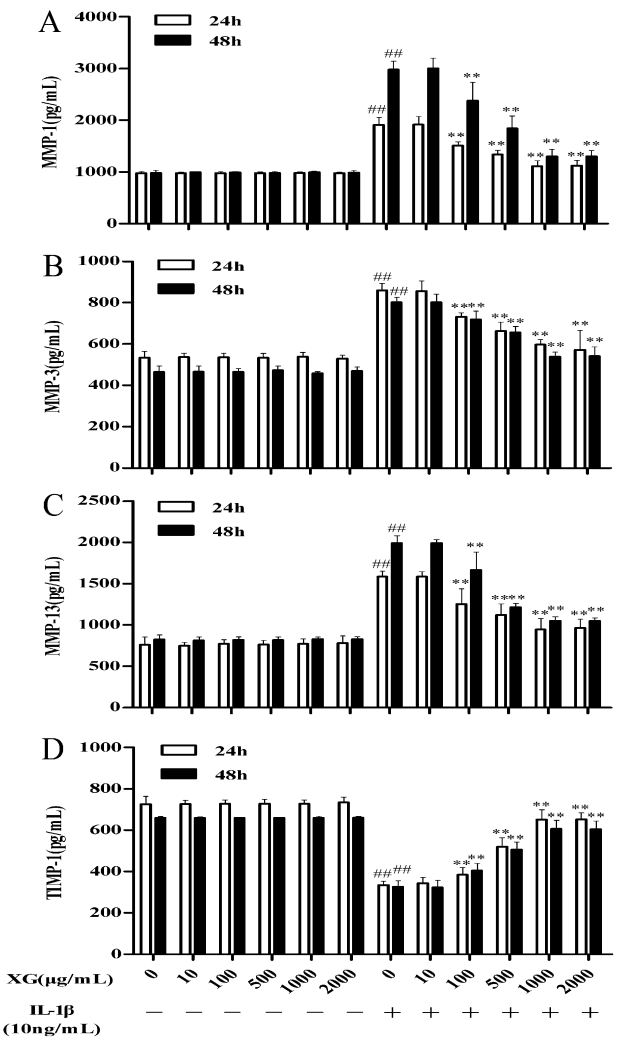

Fig. 3. Effect of XG (0, 10, 100, 500, 1000, 2000 μg/mL) with or without IL-1β (10 ng/mL) on MMPs and TIMP-1 protein expression. Treatment with XG alone showed no significantly marked modulation in the basal levels of MMP-1 (A), -3 (B), -13 (C) and TIMP-1 (D) when compared to untreated controls ($P > 0.05$). IL-1β (10 ng/mL) alone enhanced the release of MMP-1 (A), -3 (B), -13 (C) and decreased the protein expression of TIMP-1 (D) at 24 h and 48 h compared to untreated controls. However, treatment of rabbit chondrocytes in the presence of 100, 500, 1000 and 2000 μg/mL XG significantly inhibited the IL-1β-induced release of MMP-1 (A), -3 (B), -13 (C) and increased TIMP-1 (D) production in a dose-dependent manner in doses ranging from 100 to 1000 μg/mL. $^{\#\#}P < 0.01$ as compared to negative control group and $^{**}P < 0.01$ as compared to IL-1β group.

values obtained for cells stimulated with IL-1β alone. In addition, treatment with XG alone showed no significantly marked modulation in the basal levels of MMP-1, -3, -13 and TIMP-1 when compared to untreated controls ($P > 0.05$).

4. Discussion

The therapeutic use of polysaccharides in OA has been markedly developed in the recent years, due to a clearer understanding of their biological and pharmacological properties. XG is made from fermentation of *Xanthomonas campestris* in well-agitated fermenter. The molecular weight of XG distribution ranges from 2×10^6 to 20×10^6 Da (García-Ochoa et al., 2000). XG is of many properties such as high viscosity at low concentrations, temperature stability, pseudoplastic rheology and safety. XG is used in various medical and pharmaceutical applications such as controlled-release carriers and antineoplastic (Shalviri, Liu, Abdekhodaie, & Wu, 2010; Takeuchi et al., 2009). Our results had showed that IA injection of XG could protect the joint cartilage and reduce the papain-induced OA progression. The effect of XG on chondrocytes proliferation has not been previously reported and is described for the first time in the present study. In the current study, we reported the *in vitro* cytotoxicity of XG on chondrocytes, evaluated the protective effect of XG on chondrocytes through investigating the cell proliferation and the production of MMPs, TIMP-1 in rabbit chondrocytes in the presence or absence of IL-1β (10 ng/mL).

The present study leaded to the following findings. XG (1) did not significantly modify chondrocytes viability and the production of MMP-1, -3, -13, TIMP-1 in rabbit chondrocytes in doses ranging from 10 to 2000 μg/mL, (2) restored chondrocytes proliferation, suppressed protein expression of MMP-1, -3, and -13, increased TIMP-1 production in a dose-dependent manner in doses ranging from 100 to 1000 μg/mL in IL-1β-induced rabbit chondrocytes.

The *in vitro* culture and amplification of chondrocytes are suitable for the tissue engineered repair and clinical use (Lin, Willers, Xu, & Zheng, 2006). Monolayer culture of chondrocytes is the most

economic way of *in vitro* amplification. Chondrocytes have no specific marker, however, glycosaminoglycans and type II collagen secreted by chondrocytes can be stained by toluidine blue and anti-type II collagen antibody, respectively. These two staining in combination with sampling site can be used to identify chondrocytes. Chondrocytes may dedifferentiate when they are cultured *in vitro*. As a result of this process of dedifferentiation, chondrocytes are generally considered to loss the capacity of synthesizing type II collagen following passage 3 (Kamil et al., 2007) in monolayer culture. Thus, chondrocytes following passage 3 are no longer suitable for tissue engineered study of the cartilage. In this study, chondrocytes in monolayer culture were harvested from the bilateral knee joints cartilage of rabbits and identified by histological examination of toluidine blue and type II collagen immunofluorescence staining. Chondrocytes passages 2 were used throughout the study. The results showed that chondrocytes passages 2 grew well, when stained with toluidine blue and anti-type II collagen antibody, the results were positive.

In vitro toxicity tests are crucial to provide appropriate data for drug safety assessment. Cells from various tissues have been used for toxicological assessment of drug. Significant correlations between cytotoxicity *in vitro* and animal lethality have been demonstrated on many papers (Zhang, Mu, Fu, & Zhou, 2007). We had evaluated the *in vivo* safety of intra-articular injection of XG into knee joint of rabbit and the results showed that the dosage (0.5–2 mg/kg) of XG intra-articularly injection into knee joint of rabbit were no significant toxicity (Han, Ling, et al., 2012; Han, Wang, et al., 2012). In the present study, the *in vitro* cytotoxicity assay with chondrocytes was performed to evaluate the safety of XG. Our results indicated that chondrocytes could proliferate actively in the presence of XG. XG did not significantly modify chondrocytes viability and the production of MMP-1, -3, -13, TIMP-1 in chondrocytes in doses ranging from 10 to 2000 μg/mL. XG displayed no cytotoxicity to rabbit chondrocytes.

The inflammatory cytokine IL-1β is a potent immunoregulatory agent involved in a variety of pathological processes such as the response to infection, microbial toxins and other stimuli (Taub & Oppenheim, 1994). Studies have shown that IL-1β is a pivotal factor in the catabolic metabolism in cartilage, it can promote the imbalance between excessive cartilage destruction and cartilage repair process. IL-1β induces inflammatory mediators and MMPs in OA. IL-1β has been widely used to mimic the microenvironment of OA for *in vitro* studies (Largo et al., 2003). MMPs are a large group of enzymes that play a crucial role in tissue remodeling as well as in cartilage degradation due to their ability of degrading ECM (Burrage et al., 2006). MMP-1 may break down the types I, II, III collagens and is more important for remodeling of newly synthesized collagen. MMP-3 which is known to be essential for the full activation of proMMP-1 can degrades several of matrix macromoleculars, including proteoglycans, several collagen and aggrecan link protein. MMP-13 degrades type II collagen which is the main component of the ECM and is responsible for degradation of native collagen fibers (Cawston & Young, 2010; Mitchell et al., 1996). The activities of MMPs are regulated by TIMPs which considered specific inhibitors of MMPs. TIMPs can inhibit the activities of MMPs at a stoichiometry of 1:1. In this research, we chose IL-1β-induced rabbit chondrocytes as a model to study the protective effect of XG on chondrocytes. The cultured chondrocytes were treated with IL-1β (10 ng/mL). Our results showed that IL-1β stimulated chondrocytes showed a significant decrease in cell viability, enhanced release of MMP-1, -3, and -13, significant decrease of TIMP-1 production. However, when XG was added to IL-1β-treated chondrocytes, there were significant restoration in chondrocytes proliferation, inhibition in the protein expression of MMP-1, -3, and -13, increase in TIMP-1 production in a dose-dependent manner in doses ranging from 100 to 1000 μg/mL. Thus, we speculated that the protective effect of XG on chondrocytes might be associated with the prevention of destruction of IL-1β on chondrocytes and the regulation of MMP-1, -3, -13, TIMP-1.

In conclusion, we demonstrated that XG did not significantly modify rabbit chondrocytes viability and the production of MMP-1, -3, -13, TIMP-1 in cells. Furthermore, XG restored chondrocytes proliferation, suppressed protein expression of MMP-1, -3, -13, and antagonized the inhibition of TIMP-1 in a dose-dependent manner in IL-1β-induced rabbit chondrocytes. XG displayed no cytotoxicity to rabbit chondrocytes and exhibited protective effect on rabbit chondrocytes in the presence of IL-1β. However, the mechanism of XG action in chondrocytes is not clear, further study is needed to understand the detailed action mechanism.

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