Xanthan gum protects temporomandibular chondrocytes from IL-1β through Pin1/NF-κB signaling pathway

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Abstract. Temporomandibular disorder (TMD) is a complicated and multi-factorial disease related to inflammation and cartilage destruction. Intra-articular injection of xanthan gum (XG) has been demonstrated to protect the joint cartilage and reduce osteoarthritis progression. However, the role and mechanism of XG in TMD is still unclear. In the present study, chondrocytes were isolated from rats and identified by immunofluorescence. Cells were stimulated by XG or interleukin (IL)-1β. Cell viability was analyzed by MTT assay. Tumor necrosis factor α (TNF-α) and IL-6 levels were determined by ELISA. The expression of monocyte chemoattractive protein-1 (MCP-1), inducible nitric oxide synthase (iNOS), collagens, matrix metalloproteinases (MMPs), peptidyl-prolyl isomerase 1 (Pin1) and phosphorylated nuclear factor κB (NF-κB) p65 (p-p65) was analyzed by quantitative PCR or western blotting. MMP activity was assessed by gelatin zymography. Compared with the control, XG treatment partially reversed the IL-1β-reduced cell viability. In addition, IL-1β stimulation increased inflammatory cytokine expression, including TNF-α, IL-6 secretion, MCP-1 and iNOS expression, whereas XG treatment reduced the expression of these inflammatory cytokines compared with that of the IL-1β-stimulated cells. Additionally, XG increased the expression of collagen, but reduced MMP expression and activity as compared with that in the IL-1β group. In addition, XG treatment prevented the IL-1β-increased Pin1 and p-p65 expression. These data suggested that XG reduced the expression of inflammatory cytokines and may maintain the balance between collagens and MMPs partially through the Pin1/NF-κB signaling pathway in IL-1β-stimulated temporomandibular chondrocytes. Therefore, XG may be useful in the treatment of TMD.

Introduction

Temporomandibular disorder (TMD) is a joint disease that causes pain and dysfunction of the temporomandibular joints (TMJ) (1). It is a relatively common disease and is currently estimated to afflict 5-12% of the United States population (2). Symptoms of TMD include decreased mandibular range of motion, pain in the muscles of mastication, joint pain, associated joint noise during function and a functional limitation or deviation of jaw opening (3).

Inflammation, which can occur in the synovial membrane or the capsule of the joint, is one of the reasons for the pain in patients with TMD (4,5). A variety of inflammatory cytokines have been observed in patients with TMD, such as interleukin (IL)-1, IL-8 and monocyte chemotactic protein 1 (MCP-1) (6). A number of studies support the involvement of matrix metalloproteinases (MMPs) in inflammatory processes by decomposition of the extracellular matrix (ECM) and chemokines (7,8). A close relationship has been observed between MMP-2 in the synovial fluid and the degeneration of disc and articular cartilage in patients with TMD (9). In addition, neuropathic pain after nerve injury requires MMP-2 or MMP-9 (10).

Peptidyl-prolyl isomerase 1 (Pin1) is a unique peptidyl-prolyl *cis/trans* isomerase binding to and isomerizing phosphorylated Ser/Thr-Pro motifs (11). Pin1 controls the function of certain key regulators and contributes to a number of diseases (12). It has been demonstrated that Pin1 is an effective therapeutic target for Alzheimer's disease, myocardial fibrosis, obesity and vascular dysfunction (13-16). Furthermore, Pin1 leads to vascular inflammation and atherosclerosis via the NF-κB signaling pathway (17). Inhibition of Pin1 alleviates diabetes-induced endothelial dysfunction via NF-κB signaling (16). However, the role of PIN1 in IL-1β-stimulated temporomandibular chondrocytes is still unclear.

Xanthan gum (XG) is a heteropolysaccharide consisting of repeated pentasaccharide units, which are formed by one glucuronic acid unit, two mannose units and two glucose units (18).

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It has been reported that XG exerts no cytotoxic effect on rabbit chondrocytes, but has protective effects on these cells and relieves pain in a model of osteoarthritis by modulating the production of MMPs and tissue inhibitor of metalloproteinases 1 (TIMP-1) proteins (19-21). Additionally, XG also suppresses matrix degradation by inhibiting the expression of MMPs and promoting aggrecan and collagen II content in the ECM by regulating the Wnt3α/β-catenin signaling pathway (22). However, the role and underlying mechanism of XG in TMD are still unclear.

The present aimed to determine the potential function and mechanism of XG in IL-1β-stimulated temporomandibular chondrocytes. The results indicated that XG may play a protective role via the inhibition of the Pin1/NF-κB signaling pathway in TMD. This study could provide a novel target for the treatment of TMD.

Materials and methods

Isolation and culture of chondrocytes. The animal care and procedures were approved by the Ethics Committee of the Shandong University (Jinan, China). Sprague-Dawley (SD) rats (n=10, male, 6-week-old, 140-160 g) were purchased from Shandong University Animal Center. Rats were housed in a pathogen-free animal care facility on a 12-h light/dark cycle at 24°C and allowed full access to standard chow and water. The health and behavior of rats were monitored once a day. The rats were allowed to adapt for one week, and an intraperitoneal injection of sodium pentobarbital (100 mg/kg) was administered to euthanize the rats. Death was confirmed if there was no movement and breathing. All experiments were performed in one day. The temporomandibular cartilage was harvested and minced into small pieces (<1 mm^3). The pieces were primarily digested with 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 30 min, and the supernatants were discarded. The sediment was further digested with 0.2% collagenase II (Sigma-Aldrich; Merck KGaA) for 4 h at 37°C. The extracted chondrocytes were passed through a 70-μm pore size cell sieve and cultured overnight in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) at 5% CO_2 and 37°C.

Cell grouping and stimulation. The primary temporomandibular chondrocytes were randomly divided into four groups: Control, xanthan gum (XG), IL-1β and XG+IL-1β. Cells in the IL-1β group were stimulated with 10 ng/ml IL-1β (Sigma-Aldrich; Merck KGaA) for 24 h at 24°C. Cells in the XG+IL-1β group were cultured with 500 μg/ml XG for 24 h at 24°C prior to IL-1β stimulation (Fig. 1).

ELISA. The secretion levels of inflammatory cytokines were measured using ELISA kits for TNF-α (cat. no. RTA00) and IL-6 (cat. no. R6000B; R&D Systems) as previously described (23). The plates were examined using an absorption spectrometer (Thermo Fisher Scientific, Inc.) by subtracting readings at 540 nm from the readings at 450 nm.

MTT assay. The primary chondrocytes were seeded into 96-well plates. After stimulation, the MTT solution was added to each well and then incubated for 2 h at 24°C. Cell viability was measured at 570 nm using a spectrophotometer (Thermo Fisher Scientific, Inc.). The relative cell viability was calculated and compared with the absorbance of the control group.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the primary chondrocytes using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.) for 5 min at 25°C, 30 min at 42°C and 5 min at 95°C. Then, SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) was used to assess the transcript levels. The primer sequences were as follows: MCP-1 forward, 5'-GAAGGAATGGGTCCAGA CAT-3' and reverse, 5'-ACGGGTCAACTTCACATTCA-3'; inducible nitric oxide synthase (iNOS) forward, 5'-GTTCTC AGCCCAACAATACAAGA-3' and reverse, 5'-GTGGACGG GTCGATGTCAC-3'; Pin1 forward, 5'-TCGGGAGAGAGGA GGACTTTG-3' and reverse, 5'-GGAGGATGATGTGGAT GCC-3'; β-actin forward, 5'-TGTTGCCCTAGACTTCGA GCA-3' and reverse, 5'-GGACCCAGGAAGGAAGGCT-3'. The thermocycling conditions included an initial denaturation period of 1.5 min at 95°C, 40 cycles at 95°C for 15 sec, and 60°C for 30 sec. β-actin was used as the reference gene. The reaction was performed using the iCycler real-time PCR system (Bio-Rad Laboratories, Inc.), and gene expression was determined by the $2^{-\Delta\Delta Cq}$ method (24).

Western blotting. Cells were harvested and lysed in RIPA buffer (Beyotime Institute of Biotechnology). The bicinchoninic acid method was used to determine the protein concentration. Proteins (50 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham; GE Healthcare). The membranes were blocked with 5% non-fat dry milk in TBS for 2 h at room temperature and incubated overnight with anti-MCP-1 (1:500; cat. no. ab25124; Abcam), anti-iNOS (1:500; cat. no. ab3523; Abcam), anti-collagen I (1:500; cat. no. ab90395; Abcam), anti-collagen III (1:500; cat. no. ab7778; Abcam), anti-MMP-2 (1:500; cat. no. ab92536; Abcam), anti-MMP-9 (1:500; cat.no. ab38898; Abcam), anti-Pin1 (1:500; cat. no. ab192036; Abcam), anti-NF-κB p65 (1:1,000; cat. no. 4764; Cell Signaling Technology, Inc.), anti-phosphorylated NF-κB p65 (p-p65; 1:1,000; cat. no. 3039; Cell Signaling Technology, Inc.) and anti-β-actin (1:1,000; cat. no. 4967; Cell Signaling Technology, Inc.) antibodies at 4°C. After washing with TBS-0.1% Tween-20, the membranes were incubated with the anti-rabbit horseradish peroxidase (HRP) conjugated-secondary antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) and anti-mouse HRP conjugated-secondary antibody (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.) at room temperature for 2 h. The protein bands were visualized by ECL reagent (Pierce; Thermo Fisher Scientific, Inc.) and analyzed by ImageJ software (v1.8.0, National Institutes of Health).

Gelatin zymography. Zymography was performed using a MMP gelatin zymography kit (GenMed Scientific Inc.), as previously described (25). Briefly, cellular proteins were extracted by Native lysis buffer (cat. no. R0030; Beijing Solarbio Science & Technology Co., Ltd.). Proteins
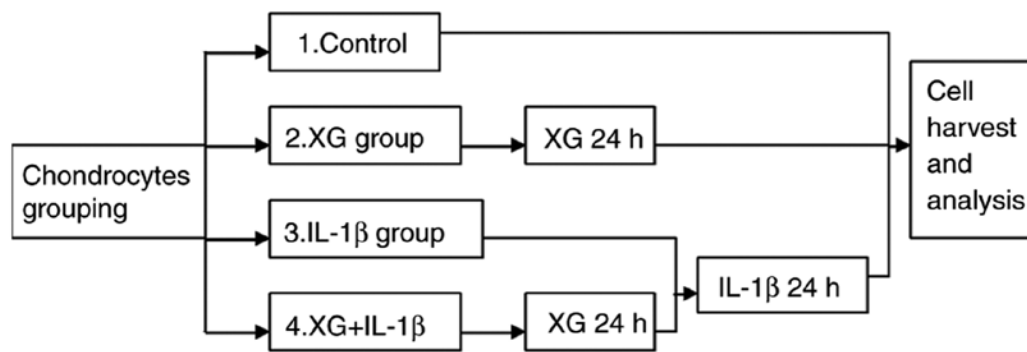
Figure 1. Experiment flowchart. Primary temporomandibular chondrocytes were randomly divided into four groups: Control, XG, IL-1β and XG+IL-1β. Cells in the XG group were cultured with 500 µg/ml XG for 24 h; cells in the IL-1β group were stimulated with 10 ng/ml IL-1β at for 24 h. The XG+IL-1β group was cultured with XG for 24 h before IL-1β stimulation. XG, xanthan gum; IL, interleukin.

(30 µg/lane) were separated by 10% SDS-PAGE polymerized in the presence of 0.1% gelatin. The gels were washed with the renaturing buffer for 2 h at room temperature, incubated with the developing buffer for 20 h at 37°C, stained with Coomassie brilliant blue for 30 min at room temperature and destained with the destaining buffer. Gel images were captured using a Kodak Imager (Kodak) and MMP activity was analyzed with the bright bands against the black background. The bands in the gel were quantified using ImageJ software (v1.8.0, National Institutes of Health).

Immunofluorescence. Chondrocytes were identified by staining collagen II. Cells were fixed in 4% paraformaldehyde for 30 min at room temperature (Beyotime Institute of Biotechnology) and permeabilized by 0.1% Triton X-100 for 30 min at room temperature. Samples were blocked with BSA for 30 min at room temperature, and then incubated with PBS (control) or anti-collagen II primary antibodies (1:1,000; cat. no. ab34712; Abcam) overnight at 4°C. Alexa 555-conjugated IgG (1:500; cat. no. A-21428; Invitrogen; Thermo Fisher Scientific, Inc.) was used as a secondary antibody. DAPI was used to stain nuclei for 10 min at room temperature. Images were captured by laser scanning confocal microscopy (LSM 710; Zeiss GmbH), magnification, x20. Data were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc.).

Statistical analysis. Statistical analysis was performed using SPSS 15.0 (SPSS, Inc.). Data are expressed as the mean ± SD. Intergroup comparisons were performed using Tukey's post hoc test following one-way ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of primary chondrocytes. Chondrocytes were observed under a microscope following extraction and an anti-collagen II antibody was used for identification. As demonstrated in Fig. 2A by immunofluorescence, the cytoplasm and the cell membrane were stained with red fluorescence by the anti-collagen II antibody; no staining was observed in the control group. Thus, chondrocytes were successfully extracted and cultured.
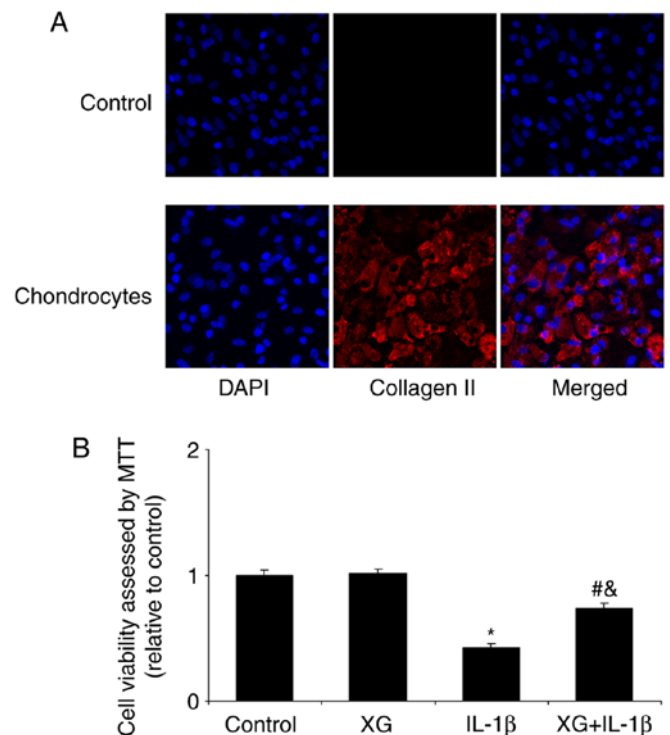

Figure 2. XG increases the IL-1β-reduced cell proliferation. Following pretreatment with XG and IL-1β stimulation, temporomandibular chondrocyte cell viability was analyzed by MTT assay. (A) Chondrocytes were identified by immunofluorescence. (B) IL-1β stimulation reduced cell viability compared with the control, whereas XG partially reversed this effect. Blue, DAPI; red, collagen II. *P<0.05 vs. control; #P<0.05 vs. XG; &P<0.05 vs. IL-1β. XG, xanthan gum; IL, interleukin.

XG increases the IL-1β-reduced cell viability. Cell viability was analyzed by MTT assay. Compared with the control, XG exhibited no significant effect on cell viability, whereas IL-1β stimulation significantly reduced chondrocyte viability (Fig. 2B). However, pretreatment with XG partially reversed the IL-1β-reduced cell viability, which suggested that XG exerted a protective effect on chondrocytes in the presence of IL-1β.

XG reduces the IL-1β-induced inflammatory response. Following chondrocyte stimulation with IL-1β, the secretion of TNF-α and IL-6 and the expression of MCP-1 and iNOS
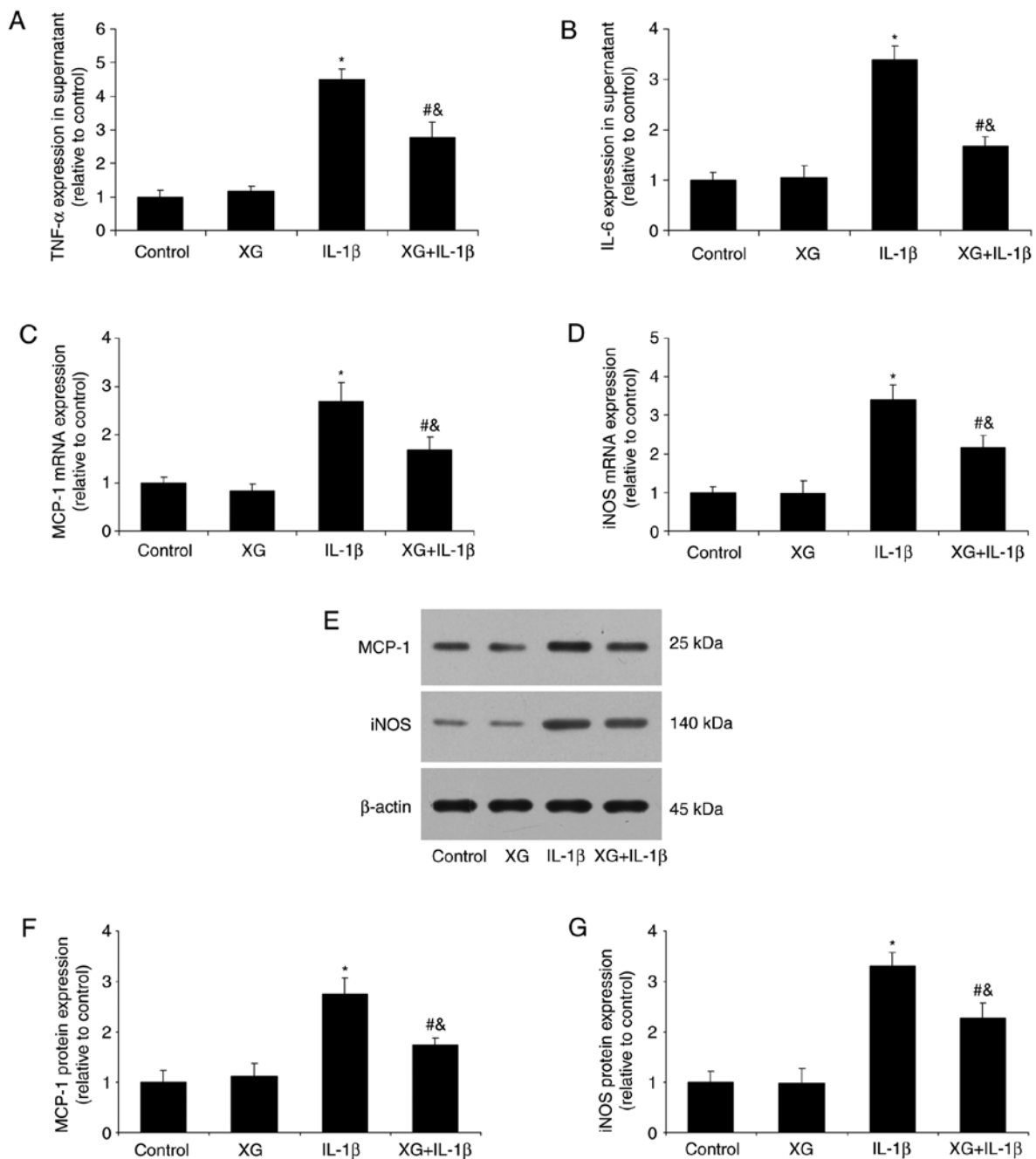
Figure 3. XG treatment reduces the IL-1β-induced inflammatory cytokine expression. After primary chondrocytes were stimulated by XG and IL-1β, TNF-α and IL-6 levels were determined by ELISA, and MCP-1 and iNOS expression was assessed by reverse transcription-quantitative-PCR and western blotting. (A and B) XG reduced the (A) TNF-α and (B) IL-6 expression compared with IL-1β. (C and D) XG reduced the (C) MCP-1 and (D) iNOS mRNA expression compared with the IL-1â group. (E-G) XG reduced the (F) MCP-1 and (G) iNOS protein expression compared with IL-1β. *P<0.05 vs. control; #P<0.05 vs. XG; &P<0.05 vs. IL-1β. XG, xanthan gum; IL, interleukin; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractive protein-1; iNOS, inducible nitric oxide synthase.

were determined. No differences were observed between the control and XG groups in inflammatory cytokine expression. However, compared with the control, IL-1β stimulation increased the TNF-α and IL-6 levels, whereas pretreatment with XG reduced them (Fig. 3A and B). In addition, XG significantly reduced the IL-1β-induced MCP-1 and iNOS mRNA expression (Fig. 3C and D), as well as MCP-1 and iNOS protein expression (Fig. 3E-G). These results suggested that XG may suppress the IL-1β-induced inflammatory response in chondrocytes.

XG increases collagen expression, but reduces MMP expression and activity. The effects of XG on the expression of collagens and MMPs, as well as MMP activity, were next investigated. Compared with the control, IL-1β stimulation reduced the expression of collagen, but increased MMP-2 and MMP-9 expression. However, XG treatment had a protective effect, with increased collagen and reduced MMPs expression (Fig. 4A-F). In addition, gelatin zymography revealed that IL-1β increased the activity of MMP-2 and MMP-9, and XG pretreatment reversed this effect (Fig. 4G-I). These results
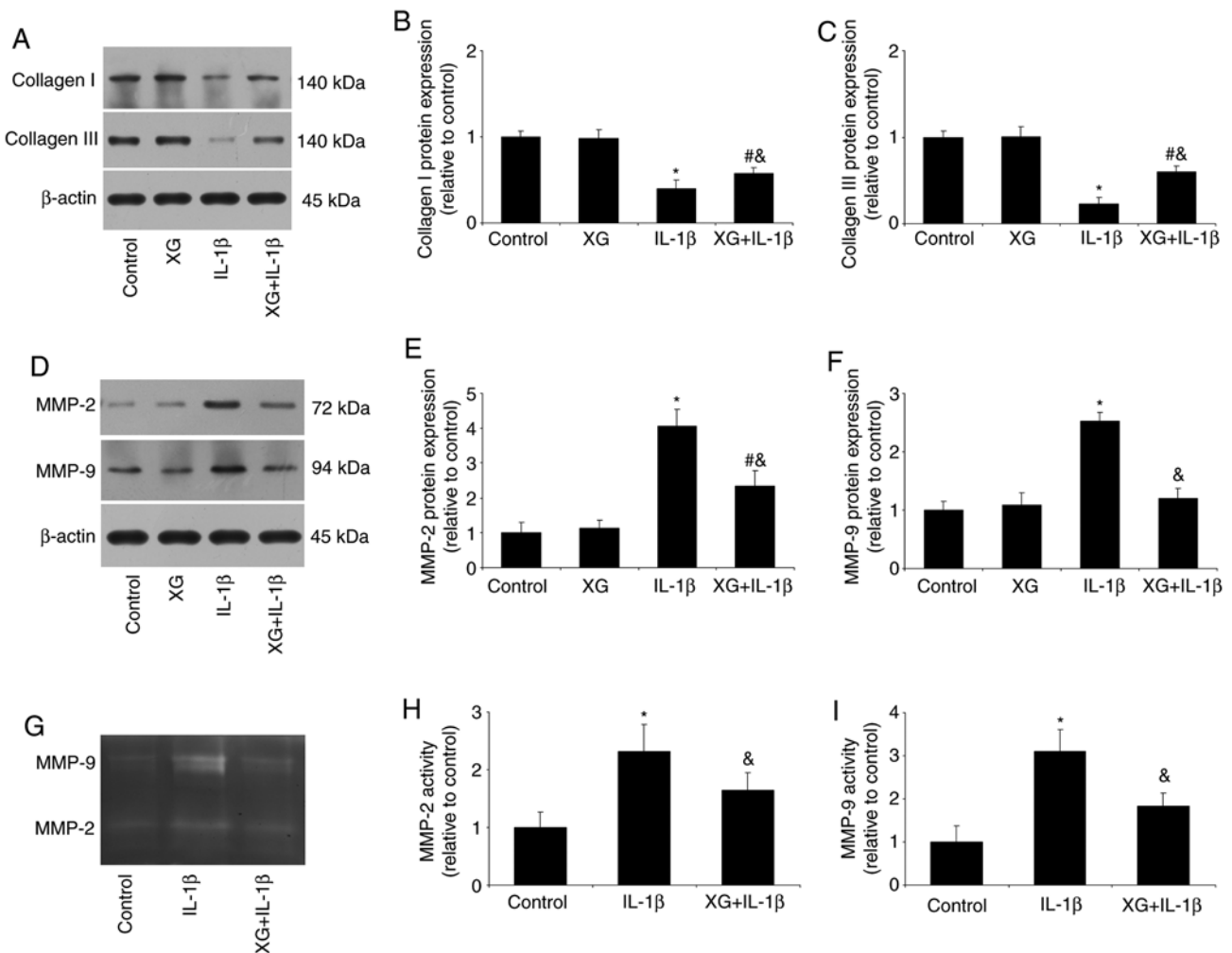
Figure 4. XG increases the expression of collagen, but reduces MMP expression and activity. Following pretreatment of temporomandibular chondrocytes with XG and IL-1β stimulation, collagen and MMP expression, as well as MMP activity were analyzed. (A-C) IL-1β reduced collagen I and III protein expression compared with the control group, whereas XG increased collagen expression compared with IL-1β. (D-F) IL-1β increased MMP-2 and MMP-9 protein expression compared with the control group, whereas XG reduced MMP expression compared with IL-1β. (G-I) Compared with their respective controls, IL-1β increased MMP-2 and MMP-9 activity, whereas XG reduced MMP activity. *$P<0.05$ vs. control; #$P<0.05$ vs. XG; &$P<0.05$ vs. IL-1β. XG, xanthan gum; MMP, matrix metalloproteinase; IL, interleukin.

suggested that XG may be responsible for maintaining the balance between collagens and MMPs.

XG reduces the IL-1β-induced Pin1 and p-p65 expression. After temporomandibular chondrocytes were stimulated with IL-1β, the expression levels of Pin1 and p-p65 were measured by RT-qPCR or western blotting. As presented in Fig. 5, no difference was observed between the control and the XG groups. However, compared with the control group, IL-1β significantly increased the mRNA and protein expression of Pin1 and p-p65, whereas the XG+IL-1β group exhibited reduced Pin1 and p-p65 expression compared with the IL-1β group. These results suggested that XG may exert protective effects on chondrocytes in presence of IL-1β through the Pin1/NF-κB signaling pathway.

Discussion

TMD is a complicated disease characterized by pain and dysfunction of TMJ (26). Owing to a clearer understanding

of its pathophysiological properties, the therapy of TMD has been markedly developed in recent years (27). XG is a natural polysaccharide and an important industrial biopolymer. XG is non-toxic, does not inhibit growth or cause skin or eye irritation (28). XG has a number of favorable properties, such as temperature stability, pseudoplastic rheology and safety (28). A previous study demonstrated that an intra-articular injection of XG alleviated pain and cartilage damage in a rat model of osteoarthritis (20). In the present study, compared with the control group, XG alone had no effect on cell viability, whereas IL-1β stimulation reduced cell viability. However, XG increased the IL-1β-reduced cell viability, suggesting a protective role of XG in TMD.

Based on the latest medical knowledge, inflammatory cytokines serve a crucial role in the pathogenesis of TMD (29). IL-1β is the most representative of these inflammatory cytokines (30). IL-1β is a pro-inflammatory cytokine that induces host defense processes, which can lead to joint destruction (31). It has been demonstrated that the level of IL-1β is significantly higher in patients with TMD compared with
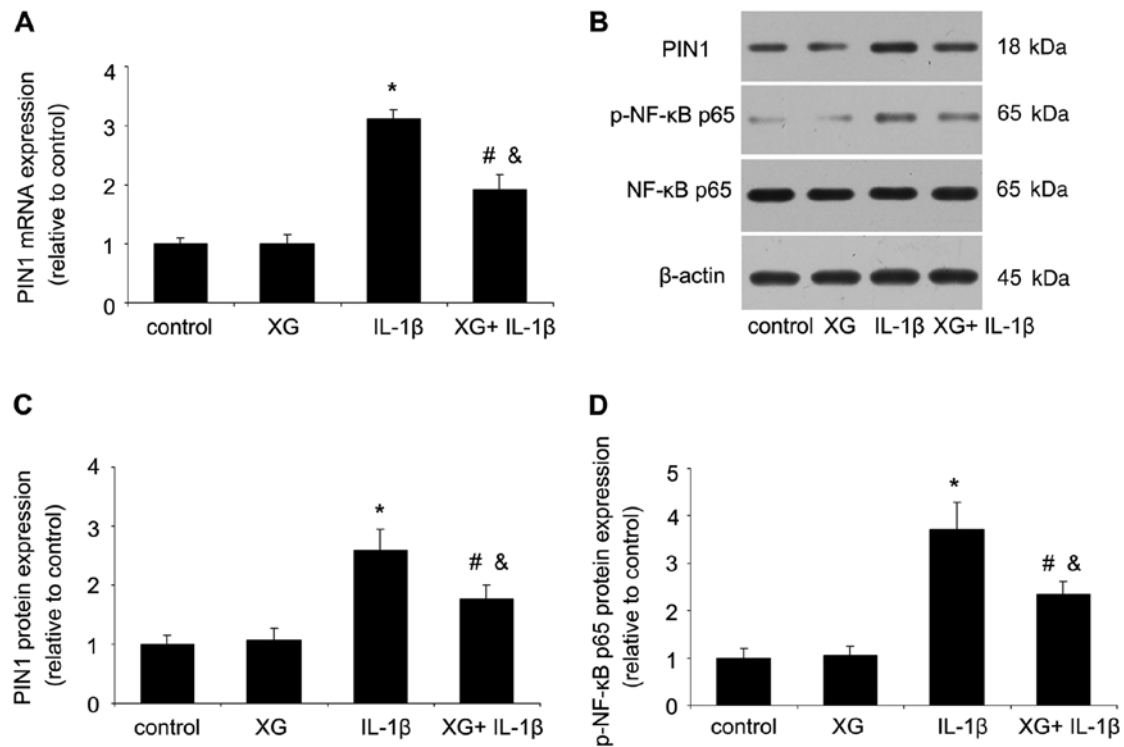
Figure 5. XG reduces Pin1 mRNA and protein expression as well as phosphorylation of NF-κB p65. Following pretreatment of temporomandibular chondrocytes with XG and IL-1β stimulation, IL-1β, Pin1 and p-p65 were analyzed by reverse transcription-quantitative-PCR or western blotting. (A) XG reduced Pin1 mRNA expression compared with the IL-1β group. (B) Western blot image showing the reduction of Pin1 and p-p65 expression following XG treatment. (C) XG reduced Pin1 protein expression compared with the IL-1β group. (D) XG reduced the p-p65 expression compared with the IL-1β group. $^*P<0.05$ vs. control; $^\#P<0.05$ vs. XG; $^\&P<0.05$ vs. IL-1β. XG, xanthan gum; Pin1, peptidyl-prolyl isomerase 1; IL, interleukin; NF-κB, nuclear factor κB; p-p65, phosphorylated NF-κB p65.
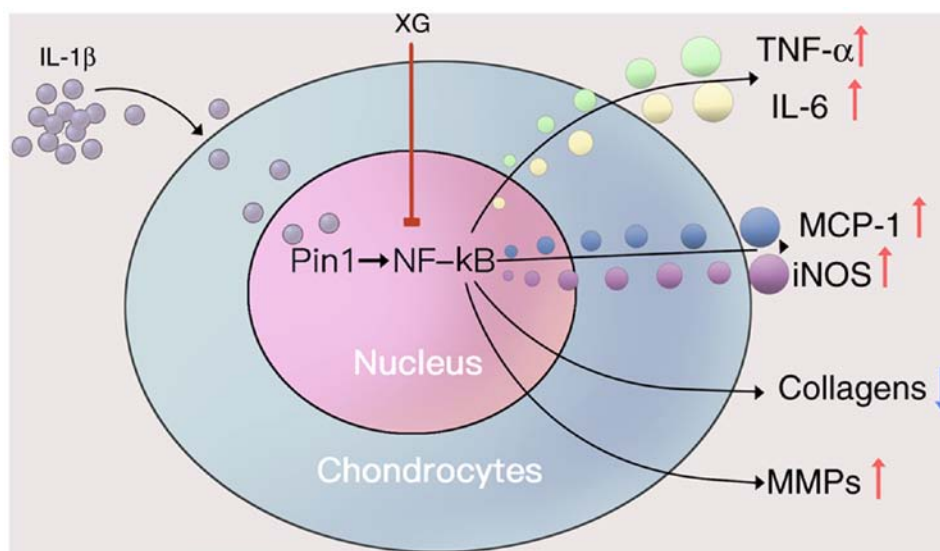

Figure 6. Proposed protective mechanism of XG on IL-1β-stimulated temporomandibular chondrocytes. IL-1β increased the secretion of TNF-α and IL-6, as well as the expression of MCP-1, iNOS and MMPs, but reduced the expression of collagens. XG may reduce the IL-1β-induced effects by inhibiting the Pin1/NF-κB signaling pathway. XG, xanthan gum; IL, interleukin; TNF-α, tumor necrosis factor-α; MCP-1, monocyte chemoattractive protein-1; iNOS, inducible nitric oxide synthase; MMPs, matrix metalloproteinases; Pin1, peptidyl-prolyl isomerase 1; NF-κB, nuclear factor κB.

that in asymptomatic volunteers (32,33). In the present study, IL-1β was used to stimulate primary temporomandibular chondrocytes. *In vitro*, compared with the control group, IL-1β induced an inflammatory response, including TNF-α and IL-6 expression, and increased the mRNA and protein expression

of MCP-1 and iNOS, whereas XG treatment reduced the IL-1β-induced expression of these inflammatory cytokines.

MMPs are a family of zinc-containing endopeptidases that serve important roles in tissue remodeling and cartilage degradation by degrading the ECM (7). Inflammation can induce

the production of MMPs and accelerate the degradation of the cartilage matrix (34). The development of inflammation increases the expression of MMP-2 and MMP-9 in the TMJ of the rat (35). The present study demonstrated that compared with the control group, IL-1β stimulation reduced the expression of collagen, but increased the expression of MMP-2 and MMP-9 compared with the control group, whereas XG alleviated these effects, with increased collagen and reduced MMP expression. In addition, XG reduced the IL-1β-induced MMP activity. Therefore, XG may exert protection through maintaining the balance between collagens and MMPs, which may result in a balance between cartilage construction and destruction.

Peptidylprolyl isomerases (PPIases) were discovered in 1984 as enzymes that catalyze the *cis/trans* isomerization of peptide bonds that precede the amino acid proline (36). As an important member of PPIases, Pin1 regulates gene transcription, cell proliferation, differentiation and apoptosis (37). Inhibition of Pin1 reduces airway inflammation and pulmonary collagen deposition in asthma, lung transplantation and liver fibrosis (38). Pin1 gene knockout attenuates angiotensin II-induced abdominal aortic aneurysm by downregulation of inflammation and MMPs in ApoE-knockout mice (39). In addition, Pin1 regulates IL-8 expression and the NF-κB signaling pathway in glioblastoma (40). NF-κB is a key mediator in the regulation of inflammatory genes (41). Various stimuli induce the NF-κB p65 nuclear translocation and subsequently upregulate the transcription of various proinflammatory cytokines, including TNF-α, IL-1, IL-6 and IL-8 (42). In addition, the phosphorylation of the p65 subunit regulates the activity of NF-κB by binding to its target sites on DNA (43). It has been reported that H_2O_2-induced oxidative stress increases Pin1 expression in PC12 cells (44). Furthermore, IL-1β upregulates Mucin 5ac expression via NF-κB-induced hypoxia-inducible factor-1α in asthma (45). IL-1β stimulation induces the activation of NF-κB and mitogen-activated protein kinases (MAPKs), as well as cytokines and chemokines (46). In the present study, IL-1β increased the mRNA and protein expression of Pin1 and NF-κB p65 phosphorylation compared with the control, whereas XG treatment reduced Pin1 and p-p65 expression. These results suggested that XG may serve a protective role partly through the Pin1/NF-κB signaling pathway. In addition, considering the role of MAPKs in inflammation, IL-1β may induce MAPK phosphorylation by binding to the IL-1β receptor, which needs further investigation.

There were some limitations to the present study. Firstly, only *in vitro* experiments were performed. Secondly, the role of apoptosis was not investigated. Thirdly, the nuclear translocation of NF-κB p65 and PI3K/Akt cell signaling pathways were not assessed. Thus, in our future experiments, *in vivo* effects and apoptosis, as well as PI3K/Akt cell signaling will be investigated.

In summary, the present study provided new insights into the protective role and regulatory mechanisms of XG in TMD. XG maintained cell viability and reduced IL-1β-induced expression of inflammatory cytokines, as well as the imbalance between collagens and MMPs. To the best of our knowledge, this study provided the first evidence that XG may effectively treat TMD through Pin1/NF-κB signaling pathway (Fig. 6).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WGY designed the experiments and wrote the manuscript. FY and JLX performed the experiments. KYL, JLS and YGS analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal care and procedures were approved by the Ethics Committee of the Shandong University (Jinan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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