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Xanthan gum protects rabbit articular chondrocytes against sodium nitroprusside-induced apoptosis *in vitro*

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ABSTRACT

We have previously reported that intra-articular injection of xanthan gum (XG) could significantly ameliorate the degree of joint cartilage degradation and pain in experimental osteoarthritis (OA) model *in vivo*. In this present study, we evaluated the protective effect of XG against Sodium nitroprusside (SNP)-induced rabbit articular chondrocytes apoptosis *in vitro*. Rabbit articular chondrocytes were incubated with various concentrations of XG for 24 h prior to 0.5 mmol/L SNP co-treatment for 24 h. The proliferation of chondrocytes was analyzed using MTT assay. The chondrocytes early apoptosis rates were evaluated using Annexin V-FITC/PI flow cytometry. The morphology of apoptosis chondrocytes were observed by scanning electron microscopy (SEM). The loss/disruption of mitochondrial membrane potential was detected using rhodamin 123 by confocal microscope. The concentration of prostaglandin E_2 (PGE_2) in cell culture supernatants was evaluated using ELISA assay. The results showed that XG could significantly reverse SNP-reduced cell proliferation and inhibited cell early apoptosis rate in a dose-dependent manner. XG alleviated loss/disruption of mitochondrial membrane potential and decreased the PGE_2 level of chondrocytes cell culture supernatants in SNP-induced chondrocytes. These results of the present research strongly suggest that XG can protect rabbit articular chondrocytes against SNP-induced apoptosis *in vitro*.

1. Introduction

Osteoarthritis (OA) is one of the most common chronic diseases, and is characterized by degradation of articular cartilage (Pelletier, Martel-Pelletier, & Abramson, 2001). Chondrocytes are the sole cell type found in mature articular cartilage and play a pivotal role in synthesis and maintenance of extracellular matrix (Pacifici, Koyama, & Iwamoto, 2005). Chondrocytes apoptosis is implicated in the occurrence and development of OA and inhibiting chondrocyte apoptosis has been shown to be an effective therapeutic approach for treating OA (Loeser, 2013; Goggs, Carter, Schulze-Tanzil, Shakibaei, & Mobasheri, 2003). Previous reports have indicated that proinflammatory mediators such as nitric oxide (NO), tumor necrosis factor-α (TNF-α), interleukin 1 (IL-1), and prostaglandin E_2 (PGE_2) are overexpressed in synovial fluid and serum of OA patients (Kapoor, Martel-Pelletier, Lajeunesse, Pelletier, & Fahmi, 2011). These mediators are responsible for chondrocyte apoptosis and have been widely employed to mimic the *in vivo* conditions of OA in established *in vitro* models of chondrocyte apoptosis (Alvarez-Soria et al., 2007; Han et al., 2012a; Liang, Wang, & Chen, 2014). An *in vitro* model of NO-induced chondrocyte apoptosis is a commonly used and informative one in evaluating therapeutic interventions for OA (Liang et al., 2014; Wimalawansa, 2008).

NO is a catabolic factor that contributes to the development of OA, and is synthesized in chrondrocytes from L-arginine through the activity of inducible nitric oxide synthase (iNOS) (Wimalawansa, 2008). NO has pleiotropic effects in regulating

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chondrocyte and cartilage function including: (1) accelerating the progression of chondrocyte apoptosis by increasing PGE_2 production, decreasing mitochondrial membrane potential and activating complex cell apoptosis pathways; (2) disrupting cartilage-specific extracellular matrix (ECM) homeostasis by inhibiting the synthesis of type II collagen and proteoglycans; and (3) upregulating matrix metalloproteinases, with subsequent degradation of the cartilage matrix (Kapoor et al., 2011; Kim et al., 2002). Sodium nitroprusside (SNP), a donor of exogenous NO, is a commonly used apoptotic stimulus for investigating NO-dependent chondrocyte cell death (Kim, Lee, & Bae, 2005). We have previously reported the establishment of an *in vitro* model of chondrocyte apoptosis using a 24-h exposure to 0.5 mM SNP (Chen, Han, Guo, & Guo, 2015).

Xanthan gum (XG) is a complex non-linear anionic microbial exo-polysaccharide which contains repeating units of five monosaccharides formed by two D-glucose, two D-mannose and one D-glucuronic acid (Shukla & Tiwari, 2012). Our previous studies have indicated that intra-articular injection of XG can inhibit articular cartilage degradation in a rabbit experimental OA model (Han et al., 2012a,b; Han et al., 2014; Shao et al., 2012, 2013). XG with molecular weights (M_r) ranging from 5.2×10^6 to 5.4×10^6 Da can decrease the rate of chondrocyte apoptosis *in vivo*, concomitant with down-regulated expression of matrix metalloproteinases-1 and -3, up-regulated expression of tissue inhibitor of metalloproteinase-1, and decreased levels of PGE_2 and NO in synovial fluid (Han et al., 2014). Our previous findings indicated that the treatment effect of intra-articular injection of XG (once every 2 weeks for 5 weeks) and sodium hyaluronate (SH) (once weekly for 5 weeks) had no statistically significant in a rabbit experimental OA model (Han et al., 2014; Shao et al., 2013); additionally, XG was not cytotoxic to rabbit chondrocytes *in vitro* (Han et al., 2012a). Intra-articular injection of SH has been used clinically for the treatment of OA (Bannuru, Natov, Dasi, Schmid, & McAlindon, 2011), and SH has been shown to suppress SNP-induced chondrocyte apoptosis *in vitro* (Peng et al., 2010). Based on the similarity of XG to SH with respect to rheology and viscosity (Shao et al., 2014), the purpose of present study was to investigate the protective effect of XG on SNP-induced rabbit chondrocyte apoptosis *in vitro*.

2. Materials and methods

2.1. Materials

Sodium nitroprusside dehydrate (SNP), Dimethylsulfoxide (DMSO), 3,5-diphenyl-1-(4,5-dimethyl-2-thiazolyl) formazan (MTT) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), trypsase and other cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA). fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (AV-PI) was obtained from BD Biosciences (Franklin Lakes, NJ, USA), Rhodamin 123 was obtained from Invitrogen (Eugene, Oregon, USA), rabbit prostaglandin E_2 (PGE_2) ELISA assay kits was obtained by Njjcbio (Nanjing, China).

The 1% SH (w/v) injection was obtained from by Bausch & Lomb-Freda Pharmacutical Co. (Jinan, China, Lot. 130510022). The 2% XG (w/v) injection was prepared as our previously described (Han et al., 2012b), and supplied by Post-doctoral Scientific Research Workstation, Institute of Biopharmaceuticals of Shandong Province, Jinan, China. The purity of XG was 99.6% (w/w). The viscosity was 1597 ± 32 mPa s (the flow rate was set to 0.6 mL/min). The pH value was 7.2 ± 0.2. The M_r was determined to be 5200 kDa by size exclusion chromatography-multiangle laser light scattering (SEC-MALLS) system (Wyatt Technology Corp., USA).

2.2. Culture of chondrocytes and treatment

The animal experiment was carried out in strict accordance with the institutional regulations regarding animal research of Liaoning Medical University. The 4-week-old female New Zealand white rabbits were killed by an injection of air in the marginal vein. The primary rabbit articular chondrocytes were cultured and identified as we previously described (Han et al., 2012a). The chondrocytes passages 2 were seeded and incubated with complete medium for 24 h in an incubator at 37 °C under 5% CO_2 atmosphere. Then complete medium was discarded and washed with 0.01 mmol/L phosphate buffered saline (PBS). Each well was replaced with serum free medium containing different concentrations of XG (0, 10, 100, 500, 1000 and 2000 μg/mL) and SH (1000 μg/mL). Further 24 h incubation at 37 °C in 5% CO_2 atmosphere was given. After that time, all wells were added with or without serum free medium containing SNP (0.5 mmol/L) and incubated for further 24 h. SNP were freshly dissolved in 0.01 mmol/L PBS and finally pass through filter membrane (aperture 0.22 μm) avoiding light.

2.3. Chondrocytes proliferation assays

The proliferation of cells was determined using the MTT assay as Han described previously (Han et al., 2012a). The chondrocytes passages 2 were seeded on 96-well plate at density of 1×10^5/mL in 100 μL of complete medium. Further 24 h incubation at 37 °C in 5% CO_2 atmosphere was given. After that time following various treatments as mentioned above, all wells were added MTT solution 20 μL (5 mg/mL) for 4 h in an incubator at 37 °C under 5% CO_2 atmosphere. The supernatants were discarded, and DMSO 150 μL was added to each well. The 96-well culture plate was shook in oscillators at room temperature for 10 min. Then the wells without cells were used as the zero point of absorbance. The absorbance was measured using microplate reader at 570 nm.

2.4. Flow cytometry analysis

Chondrocytes early apoptosis rates was evaluated using Annexin V-PI double staining (Liu et al., 2010). Briefly, the chondrocytes passages 2 were seeded on 6-well plate at density of 1×10^5 mL^{-1} in 1 mL of complete medium for 24 h. After that time following various treatments as mentioned above, chondrocytes were collected by trypsinization and washed 2 times with cool PBS. Chondrocytes were resuspended by 300 μL binding buffer and then adding 5 μL Annexin V and 5 μL PI in binding buffer while avoiding light. Further 15 min incubation at room temperature was given. Then samples were added 200 μL binding buffer prior to detection by flow cytometry.

2.5. Sample preparation and scanning electron microscopy (SEM)

The morphology of apoptosis chondrocytes was evaluated using SEM as Faria described previously (Faria, Cardoso, Larson, Silva, & Rossi, 2009). The chondrocytes passages 2 were seeded onto glass coverslips (diameter was 10 mm) in 24-well plate at density of 1×10^5 mL^{-1} in 0.5 mL of complete medium for 24 h. After that time following various treatments as mentioned above, chondrocytes were fixed in 2.5% glutaraldehyde for 1 h at 4 °C, washed with PBS (3 times, 5 min each time), stained in 1% osmium tetroxide for 1 h at 4 °C, washed with PBS (3 times, 5 min each time), dehydrated using 30%, 50%, 70%, 90% and 100% gradient of alcohol (2 times, 5 min each time), displaced in isoamyl acetate, dried at the critical point, gold evaporated, and finally observed using SEM (S-4800, Hitachi, Tokyo, Japan).