Saponin-rich fraction from *Clematis chinensis* Osbeck roots protects rabbit chondrocytes against nitric oxide-induced apoptosis via preventing mitochondria impairment and caspase-3 activation

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Abstract Our previous study reported that the saponin-rich fraction from *Clematis chinensis* Osbeck roots (SFC) could effectively alleviate experimental osteoarthritis induced by monosodium iodoacetate in rats through protecting articular cartilage and inhibiting local inflammation. The present study was performed to investigate the preventive effects of SFC on articular chondrocyte, and explore the underlying mechanisms. Primary rabbit chondrocytes were cultured and exposed to sodium nitroprusside (SNP), a NO donor. After treatment with different concentrations of SFC (30, 100, 300, 1,000 µg/ml) for 24 h, nucleic morphology, apoptotic rate, mitochondrial function and caspase-3 activity of chondrocytes were examined. The results showed that SNP induced remarkable apoptosis of rabbit chondrocytes evidenced by Hoechst 33258 staining and flow cytometry

analysis, and SFC prevented the apoptosis in a concentration-dependent manner. Further studies indicated that SFC could prevent the depolarization of mitochondrial membrane potential ($\Delta\psi/m$) in SNP-treated chondrocytes and suppress the activation of caspase-3. It can be concluded that the protection of SFC on articular chondrocytes is associated with the anti-apoptosis effects via inhibiting the mitochondrion impairment and caspase-3 activation.

Keywords *Clematis chinensis* · Osteoarthritis · Chondrocyte · Apoptosis · Nitric oxide

Introduction

OA is a chronic and degenerative joint disease with high morbidity that is primarily characterized by the erosion of articular cartilage, meniscus damage, low grade of synovitis, bone remodeling, sclerosis of subchondral bone and osteophyte formation in some cases (Lohmander 2000; Felson 2006; Krasnokutsky et al. 2007). Though the exact pathogenesis and etiology of OA remain unclear, it is no doubt that chondrocyte injury due to some inflammatory mediators (such as interleukin-1, tumor necrosis factor, nitric oxide, reactive oxygen species, etc.) is one of the key pathogenic events (Abramson and Krasnokutsky 2006; Goldring and Goldring 2007).

Conservative treatment for OA is based on some steroidal and nonsteroidal anti-inflammatory drugs,

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including glucocorticoids, diclofenac and ibuprofen. These drugs only alleviate early symptoms, relief pain and improve the joint function, but can not prevent progressive cartilage degradation. Recently, a great variety of herbal remedies have been proposed for OA therapy, such as *Capparis spinosa* extracts (Panico et al. 2005), green tea polyphenols (Ahmed et al. 2004), genistein (Hooshmand et al. 2007), resveratrol (Csaki et al. 2008), curcumin (Shakibaei et al. 2007), etc. Many plants from *Clematis* species have been demonstrated to possess anti-inflammatory and chondro-protective effects (Choi et al. 2002; Kim et al. 2005a; Kim et al. 2005b; Lee et al. 2005; Li et al. 2006; Park et al. 2006). Among them, *Clematis chinensis* Osbeck (Ranunculaceae), which is mainly distributed in southern China and contains several triterpenoid saponins (Mimaki et al. 2004), has long been used in traditional Chinese medicine to treat joint diseases including OA.

The present study was conducted to investigate the effects of Saponin-rich fraction from *Clematis chinensis* Osbeck roots (SFC) on the apoptosis of rabbit articular chondrocytes induced by nitric oxide (NO) in an attempt to support evidence for SFC in the treatment of OA. Furthermore, the effects of SFC on mitochondrion function and caspase-3 activity in NO-treated chondrocytes were examined to exploit the anti-apoptosis mechanisms of SFC.

Materials and methods

Preparation of SFC

Clematis chinensis Osbeck was supplied by the Department of Pharmacy, Anhui Province Hospital of Traditional Chinese Medicine. Its identity was confirmed as the roots of *Clematis chinensis* Osbeck by anatomical analysis. A voucher specimen was deposited in the Department of Pharmacology of Chinese Materia Medica, China Pharmaceutical University. The preparation process of saponin fraction was described by Xu and Xia (2006). Briefly, the crude power of the dried roots was extracted with 8 times volume of 70 % ethanol under reflux (2 h × 3), the extracted solution was then filtered and evaporated *in vacuo*, and the residue was diluted with distilled water and purified with D101 macroporous absorptive resin. After gradient eluting with distilled water and 10, 20, 30

and 50 % ethanol, the 50 % ethanol eluted fraction was collected, evaporated *in vacuo* and lyophilized to yield yellowish-brown powder (SFC, yield ratio: 2.1 %). The saponin content in SFC power is 58 %, determined by a colorimetry using a combination of vanillin, acetic acid as well as perchloric acid, and using oleanolic acid as a standard substance. The powder might contain more than fifty kinds of triterpenoid saponins, mainly with oleanolic acid or hederagenin as sapogenins (Liu et al. 2009; Shi et al. 2006; Shao et al. 1995).

SFC was first dissolved in dimethylsulfoxide (DMSO) with a concentration of 3,000 µg/µl and then diluted to the final concentration with DMEM/F-12 medium before use. The final concentration of DMSO did not exceed 3 ‰, which had no harm to cells and no effect per se is known.

Animals

12 New Zealand rabbits (6 weeks old) were obtained from the experimental animal center of the China Pharmaceutical University, and were housed under standard condition with food and water ad libitum. Animal experiments were conducted in accordance with current ethical regulations for animal care and use at China Pharmaceutical University.

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1) was purchased from Gibco Chemical Co., (Grand Island, NY, USA). Newborn calf serum was purchased from PAA Laboratories GmbH (Linz, Austria). Sodium nitroprusside (SNP) and Hoechst 33258 were purchased from Beyotime Institute of Biotechnology, (Haimen City, China). Annexin V-FITC apoptosis detection kit, mitochondrial membrane potential assay kit and caspase-3 activity assay kit were purchased from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, China). Trypsin and type II collagenase were obtained from Sunshine Biotechnology Co., Ltd. (Nanjing, China).

Chondrocyte preparation and treatment

Considering that the size of rat articular cartilage is very small, rabbits were used to get sufficient chondrocytes for the present study. Chondrocytes were isolated from the knee joints of 6-week-old New Zealand rabbits by enzymatic digestion (Chen et al.

2006). In brief, rabbits were killed by intravenous injection of pentobarbital 100 mg/kg, and then cartilage tissues were scraped from the knee joints and cut aseptically into small pieces ($<1 \text{ mm}^3$). Cartilage pieces were then digested with 0.25 % trypsin and 0.2 % type II collagenase for 30 min and 3 h, respectively. After digestion, chondrocytes were filtered through a mesh screen (mesh diameter $75 \text{ }\mu\text{m}$) to prepare single cell suspension. The cells were cultured in DMEM/F-12 medium (containing 20 % newborn calf serum, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin) in humidified air with 5 % CO_2 at 37°C . The primary cells reached confluence at about seven days after seeding, and were subcultured to generation 3.

Chondrocytes of generation 3 were changed to DMEM/F-12 medium with 10 % newborn calf serum for 24 h. Then, the medium was changed to DMEM/F-12 medium containing 5 % newborn calf serum. SFC (30, 100, 300 and 1,000 $\mu\text{g/ml}$) and SNP (2 mM) were added to the medium for further incubation for 24 h. Chondrocytes of the control group were cultured in medium without SFC or SNP. After treatments, cells and supernatants were used for Hoechst 33258 staining, flow cytometry analysis, determination of mitochondrial membrane potential and determination of caspase-3 activity.

Hoechst 33258 staining

Changes in nucleic morphology of chondrocytes were detected by staining with Hoechst 33258, a DNA-binding fluorochrome. The chondrocytes, isolated from the knee joints of rabbits, were seeded to the 96-well plates at 2×10^4 cells per well and stabilized for 24 h. After incubation with SFC (30, 100, 300 and 1,000 $\mu\text{g/ml}$) plus SNP (2 mM), the cells were fixed with methanol acetic acid for 10 min followed by staining with Hoechst 33258 1 mg/ml for 5 min at room temperature. The cells were then washed twice with PBS, observed and immediately photographed under a fluorescence microscope with excitation at 355 nm and emission at 465 nm (Terauchi et al. 2003).

Determination of apoptosis by flow cytometry analysis

Apoptotic rate was evaluated using a commercial Annexin V-FITC apoptosis detection kit. At early

stages of apoptosis, phosphatidylserine (PS) translocates from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can therefore be used as a sensitive probe for the exposure of PS on the cell membrane and hence as a marker of apoptosis (Chen et al. 2006). Briefly, after treatment, the cells were collected and suspended in 500 μl of binding buffer at the density of 4×10^5 cells/ml. Annexin V-FITC 5 μl and Propidium Iodide (PI) 5 μl were then added to the solution and incubated for 15 min at room temperature. After incubation, the cell suspension was analyzed by flow cytometry within 1 h.

Determination of mitochondrial membrane potential ($\Delta\psi_m$)

Chondrocyte $\Delta\psi_m$ was detected by staining with a lipophilic fluorescent cation JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazol carbocyanine iodide) which exhibit potential-dependent accumulation in mitochondria. JC-1 exists as a monomer at low values of $\Delta\psi_m$, while it forms aggregates at a high $\Delta\psi_m$. Thus, mitochondria with a normal polarized $\Delta\psi_m$ concentrate JC-1 into aggregates which emit red fluorescence, but with a depolarised $\Delta\psi_m$, JC-1 forms monomers which emit green fluorescence (López-Armada et al. 2006; Maneiro et al. 2005). Briefly, after treatment, the cells were collected and suspended in 500 μl JC-1 staining solution (10 $\mu\text{g/ml}$ JC-1) at the density of 4×10^5 cells/ml. After incubation in dark for 15 min at room temperature, the cells were collected and resuspended with 500 μl incubation buffer, which were then analyzed by flow cytometry. A 488 nm filter was used for excitation of JC-1. Emission filters of 535 nm and 595 nm were used to quantify the population of chondrocytes with green (JC-1 monomer) and red (JC-1 aggregates) fluorescence, respectively.

Determination of caspase-3 activity

The activity of caspase-3 was determined by using the caspase-3 colorimetric assay kit according to the manufacturer's protocol. Briefly, after treatment with SNP and SFC, cells were harvested and resuspended in 50 μl of ice-cold lysis buffer. After incubation on ice for 20 min, cell lysates were clarified by centrifugation (10,000g) for 1 min at 4°C . After centrifugation, 2 μl

supernatant of each sample were collected for a Bradford protein assay. 50 μ l of the supernatant containing about 100–200 μ g of total protein was mixed with $2 \times$ reaction buffer and 5 μ l of caspase-3 substrate, and immediately incubated at 37 °C for 4 h in the dark. The values of optical density (OD) at 405 nm were read by microplate reader. The activation of caspase-3 was calculated as OD per mg protein (OD/mg) (Beak et al. 2006).

Statistical analysis

Data are presented as mean $\pm$ SD. Statistical significance between groups was calculated by one way ANOVA and Dunnett's test. Differences were considered significant when *p*-values lower than 0.05.

Results

Effects of SFC on the nucleic morphology in SNP-treated chondrocytes

As is shown in Fig. 1, the nuclei of normal chondrocytes had a homogeneous pattern of staining, while in

SNP-treated group, numerous chondrocytes exhibited typical characteristics of apoptosis with highly fragmented, condensed or divided nuclei, while SFC (300 and 1,000 μ g/ml) significantly reversed SNP-induced apoptosis.

Effects of SFC on chondrocytes apoptosis induced by SNP

In Fig. 2, Q1 region represented the detection error. Q2 region (Annexin V⁺ and PI⁺ cells) represented the late apoptotic and necrotic cells. Q3 region represented normal cells that were neither stained by Annexin V nor PI. Q4 region (Annexin V⁺ and PI⁻ cells) represented the early apoptotic cells.

In control group, only a few cells underwent apoptosis. After incubating with SNP 2 mM for 24 h, the number of apoptotic chondrocytes significantly increased in comparison with control group. The apoptotic rate increased from 4.0 to 36.8 %. Treatment with SFC (30, 100, 300 and 1,000 μ g/ml) resulted in an obvious decline of apoptosis with apoptotic rates of 29.7, 24.5, 20.8 and 13.2 %, respectively. The apoptotic rates of chondrocytes in all groups are summarized in the histogram of Fig. 2.

Fig. 1 Representative fluorescence photomicrographs of chondrocytes stained with Hoechst 33258 (Magnification $\times 100$). Chondrocytes were incubated with different concentrations of

SFC plus SNP 2 mM for 24 h, then harvested and stained with Hoechst 33258, and finally examined under fluorescence microscope

Fig. 2 Effect of SFC on apoptotic rate in SNP-treated rabbit chondrocytes. After treated with SNP (2 mM) and SFC for 24 h, chondrocytes were collected and stained with Annexin V-FITC

and PI, and analyzed immediately by flow cytometry. Data are expressed as mean $\pm$ SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus SNP group

Effects of SFC on mitochondrial membrane potential ($\Delta\psi_m$) in SNP-treated chondrocytes

As represented in Q2 region of Fig. 3, normal chondrocytes with polarized $\Delta\psi_m$ exhibited red fluorescing aggregates. In Q4 region, cells that undergo apoptosis exhibited green fluorescing monomers.

In the control group, most chondrocytes exhibited red fluorescing aggregates, which indicated that they were live cells with polarized $\Delta\psi_m$. After incubation

with SNP 2 mM for 24 h, the number of chondrocytes with depolarized $\Delta\psi_m$ increased significantly in comparison with that in the control group, the percentage of chondrocytes with depolarized $\Delta\psi_m$ increased from 3.1 to 34.5 %. Treatment with SFC 30, 100, 300 and 1,000 µg/ml resulted in a significant decline of cells with depolarized $\Delta\psi_m$, the percentages of which was 29.7, 13.4, 6.2 and 4.1 %, respectively. The percentages of chondrocytes with depolarized $\Delta\psi_m$ in all groups are summarized in the histogram of Fig. 3.

Fig. 3 Effect of SFC on mitochondrial membrane potential in SNP-treated rabbit chondrocytes. After treated with SNP (2 mM) and SFC for 24 h, chondrocytes were collected and stained with

JC-1, and were analyzed immediately by flow cytometry. Data are expressed as mean $\pm$ SD of three independent experiments. * p < 0.05, ** p < 0.01 versus SNP group

Effect of SFC on caspase-3 activation in SNP-treated chondrocytes

The caspase-3 activity assay was based on the absorbance after cleavage of caspase-3 substrate Ac-DEVD-pNA. As shown in Fig. 4, after treatment with SNP (2 mM) for 24 h, the caspase-3 activity in chondrocytes increased up to approximately twofolds of the control group (1.06 ± 0.04 OD/mg vs. 0.48 ± 0.02 OD/mg).

SFC 100, 300 and 1,000 µg/ml inhibited the activation of caspase-3 caused by SNP.

Discussion

Chondrocytes undergo death through apoptosis and necrosis, two biochemically and morphologically distinct ways. Apoptosis is a form of programmed

Fig. 4 Effect of SFC on caspase-3 activity in SNP-treated chondrocytes. Chondrocytes were treated with SFC plus SNP (2 mM) for 24 h. Caspase-3 activity was then examined. Data are expressed as mean $\pm$ SD of triplicates. * $p < 0.05$, ** $p < 0.01$ versus SNP group

cell death and is either physiological or pathological, which includes manifest morphological and physiological features such as shrinking of cytoplasm and chromatin condensation, nuclear fragmentation, formation of apoptotic bodies, DNA laddering (Kühn et al. 2004), etc. while necrosis is caused only by pathological incidents and has some distinct characters, including swelling of cytoplasm and mitochondria, non-specific karyolysis, random digestion of nuclear DNA, etc. (Aigner 2002a). Accumulative evidence, including clinical studies and pre-clinical experiments (Blanco et al. 1998; Aigner and Kim 2002b; Kühn et al. 2004), has demonstrated that a large amount of chondrocytes extinct in cartilage of osteoarthritis mainly due to apoptosis. It should be noted that articular cartilage has no blood vessels, lymphatics or nerve fibers, and chondrocytes are the only cell type responsible for the elaboration and maintenance of the extra-cellular matrix (Blanco et al. 2004). In consideration with the weak repopulated capability of chondrocytes, the occurrence of apoptosis should break the homeostasis between anabolism and catabolism in cartilage (Blanco et al. 2004). Furthermore, the apoptotic body remains in cartilage for a long period due to inexistence of phagocytic cells in articular cartilage, which further impedes extra-cellular matrix metabolism and eventually results in impairment of whole cartilage (Lotz et al. 1999). Therefore, prevention of excessive apoptosis of chondrocytes is believed to be beneficial for OA management. Drugs that can ameliorate OA have been

reported to show anti-apoptotic effects on chondrocytes, such as sinomenine (Ju et al. 2010), ADAM15 (Böhm et al. 2010) and amiloride (Rong et al. 2011). Many triterpenoid saponin ingredients in traditional Chinese medicine have anti-apoptotic effects, such as astragaloside (Shang et al. 2011), Panax notoginseng saponins (Chen et al. 2011), Ginsenoside-Rg2 (Zhang et al. 2008). The present study was performed to address whether SFC possess protective effects on chondrocytes by an anti-apoptotic pathway.

Chondrocyte apoptosis may be caused by various factors, such as IL-1 β , TNF- α , LPS and NO, of which NO is an inter- and intracellular messenger molecule in a variety of cells and participates in many physiological and pathological processes (Blanco et al. 1995). Clinical (Blanco et al. 1995; Greisberg et al. 2002) and preclinical studies (Hashimoto et al. 1998; Beltrán et al. 2000) have indicated that NO level of osteoarthritis cartilages increased significantly in proportion with the number of apoptotic chondrocytes, demonstrating that NO is involved in the pathogenesis of osteoarthritis. Our previous study (Wu et al. 2010) has demonstrated that SNP concentration-dependently led to a reduction of the viability of chondrocytes in parallel with an elevation of NO level, indicating that the chondrocyte impairment was NO-dependent. And in this study, it is observed that NO-induced death of chondrocytes was mainly by apoptosis, which was in accordance with previous reports (Hashimoto et al. 1998; Beltrán et al. 2000). SFC substantially reversed SNP-induced chondrocyte apoptosis without significant impact on NO level, suggesting that SFC functions through antagonizing the action of NO or protecting chondrocytes, but not through scavenging NO.

Series of in vitro studies have identified that NO induces chondrocyte apoptosis via mitochondria-dependent pathway. NO, from either exogenous or endogenous sources, can activate p38 kinase (one subtype of MAP kinases) (Kim et al. 2002a; Kim et al. 2002b). The latter promotes phosphorylation and accumulation of p53 tumor suppressor protein (Kim et al. 2002a; Kim et al. 2002b). The p53 protein transcriptionally regulates expression and translocation of Bax, a proapoptotic member of Bcl-2 family. Bax is then redistributed from the cytosol to the mitochondria, where it causes a decline in mitochondrial membrane potential, followed by cytochrome c release and caspase activation (Kim et al. 2002b; Wu et al. 2007),

ultimately leading to apoptosis (Csaki et al. 2008). Among the signal pathways, mitochondria impairment is recognized as the classic sign of cell apoptosis. The mitochondrial membrane potential ($\Delta\psi_m$) is polarized in normal chondrocytes. Once mitochondria are impaired, $\Delta\psi_m$ should depolarize or even collapse, leading to an inevitably apoptosis of chondrocytes (Blanco et al. 2004; Maneiro et al. 2005). In the present study, SNP (2 mM) led to $\Delta\psi_m$ depolarization in a relatively large number of chondrocytes (ratio 34.5 %), which corresponds to the amount of chondrocytes undergoing apoptosis. The detrimental effect of SNP on chondrocyte mitochondria was significantly reversed by SFC. On the other hand, caspase-3, as the most important apoptotic executor in the caspase family, plays a critical role in cell apoptosis (Porter et al. 1999). SNP treatment for 24 h caused more than two folds of increase in the caspase-3 activity of rabbit chondrocytes. SFC showed an obvious reduction in the caspase-3 activity. These findings suggested that SFC may exert its anti-apoptotic effect mainly by hindering $\Delta\psi_m$ collapse and caspase-3 activation in chondrocytes.

Conclusion

SFC significantly ameliorates rabbit chondrocyte apoptosis induced by NO, which may be mediated by inhibiting mitochondria impairment and caspase-3 activation. To enable SFC going into clinical trials as an anti-OA agent, further studies are needed, such as safety evaluation in animals and human beings, definition of efficient dosage in patients suffering from OA, and elucidation of the active constituents and precise mechanisms.

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Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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