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Original Research

Myricitrin regulates proliferation, apoptosis and inflammation of chondrocytes treated with IL-1β

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Abstract: Osteoarthritis (OA) is a clinical disease which seriously affects the quality of life of sufferers. Although the pathogenesis of OA has not been fully unraveled, it is may be due to increased levels of pro-inflammatory cytokines, activation of inflammation-related signaling pathways, and degradation of extracellular matrix. Osteoarthritis is characterized by chronic joint pain, swelling, stiffness, limited movement or joint deformity, all of which seriously affect the quality of life and health of the affected individuals. Myroside (Myr) is a polyphenolic hydroxyflavone glycoside extracted from the fruits, bark and leaves of myroside and other natural plants. It has many pharmacological properties, especially anti-inflammatory effects. In the present study, primary chondrocytes of IL-1β rats were used to simulate pathological environment of chondrocytes in OA, and to explore the effect of Myr on chondrocytes. It was found that Myr improved the viability and proliferation of chondrocytes, and also inhibited apoptosis in these cells. Moreover, Myr reduced the expressions of inflammatory factors, and inhibited inflammatory reactions in chondrocytes. These findings provide good experimental basis for the clinical application of Myr in the prevention and treatment of progressive degeneration of cartilage in OA.

Key words: Myricitrin; Proliferation; Apoptosis; Inflammation; Chondrocytes.

Introduction

Osteoarthritis (OA) is a chronic degenerative joint disease which is clinically known as degenerative arthritis (1) . Due to changes in joint cartilage, subchondral bone, synovial capsule, synovial membrane, ligament and other changes in structures of the joints, the main clinical manifestations are gradually-developing joint pain, tenderness, swelling, deformity and joint stiffness (2). The pathological features of OA include cartilage degeneration, synovitis and subchondral bone changes (3). Globally, the incidence of OA occurs affects about 4 – 13 % of the world population, and it is one of the main causes of disability which seriously affects the quality of life of patients (4, 5). Currently, OA is managed with oral painkillers, non-steroidal anti-inflammatory drugs, and intra-articular injections of corticosteroids. However, these treatments only result in temporary relief from the symptoms of OA. Moreover, they are associated with adverse such as gastrointestinal stimulation and increased risk of cardiovascular disease (6, 7). Thus, the search for better treatment strategies for OA has been a major research interest in the field of orthopedics.

Myricetin (pentahydroflavanone-3-rhamnose, Myr, Figure 1) is a natural polyphenolic flavanone glycoside which is abundant in the fruit, bark and leaves of bayberry, vine tea, snake vines and longan leaves (8). Myricetin has antioxidant, free radical-scavenging, blood vessel-dilating, anti-inflammatory, analgesic, and antibacterial effects (9-11). It has been reported that pretreatment with myricetin inhibited the production of tumor necrosis factor-α(TNF-α) in LPS-stimulated RAW264.7 macrophages (12). Studies have shown that Myr reduced liver lipid peroxidation, glutathione, cyclooxygenase-2 and TNF-α overexpression and liver inflammation, and downregulated liver expression of transforming growth factor -β1 (TGF-β1) while decreasing liver fibrosis (9). These studies demonstrate the antioxidant and anti-inflammatory properties of Myr in various disease conditions. However, not much is known about the effect of Myr on IL-1β-induced inflammation in chondrocytes. Therefore, based on the anti-inflammatory properties of Myr, the present study investigated the effect of Myr on a model of interleukin (IL)-1β-induced chondrocyte apoptosis with a view to providing a basis for its use in the treatment of OA.

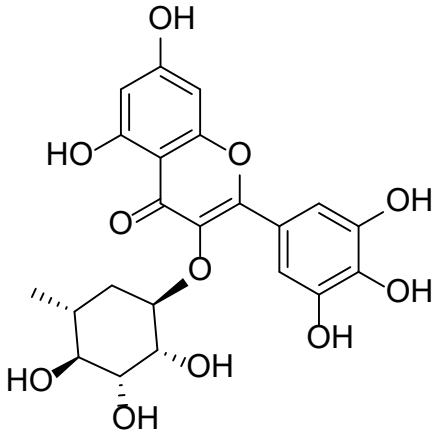

Figure 1. Chemical structure of myricitrin.

Materials and Methods

Drugs and reagents

Myricetin was purchased from National Institutes for Food and Drug Control, China (HPLC purity > 98%). Medium (DMEM/F12) was product of Hyclone, USA. Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd. Trypsin was obtained from Amresco, while IL-1β and thiazolium blue (MTT) were purchased from Sigma, USA. B cell lymphoma (Bcl) -2 and β-actin antibodies were purchased from Abcam. Bax, Caspase-3 and Caspase-9 antibodies were products of CST, USA. Diquinoline, formic acid, BCA protein concentration determination kit and ELISA kit were purchased from Beyotime Biotechnology Co. Ltd.

Culture of chondrocytes

Ten-day-old SPF rats (Shanghai SLAC Laboratory Animal Co. Ltd) were sacrificed via cervical dislocation. The cartilage tissues at the hip and knee joints were removed under aseptic conditions and placed in a PBS dish containing 10% double antibody on a clean bench. The tissues were washed once in the PBS dish, and then washed 3 times in PBS containing no double antibody. The cartilage tissue block was then transferred to an Eppendorf tube, and 2-3 drops of PBS were added. Then, the tissue block was cut to a size of about 1 mm^3 with ophthalmic scissors. The PBS in the EP tube was aspirated, and the tissue block was suspended in 0.2% type II collagenase and digested overnight at 37 °C. Thereafter, the EP tube was shaken on a shaker for 2 min to separate the digested cells from the tissue. The supernatant was discarded after centrifugation, and the lower layer of cells was taken out and added to a DMEM/F12 flask containing 10% FBS, and cultured in a 37 °C, 5% CO_2 incubator. After 72 h, the culture medium was changed, and the growth of the cells was observed. The culture medium was replaced once every 2 days, and the cells were sub-cultured when they were over 80 to 90% of the monolayer.

Grouping and treatment of chondrocytes

Second-generation articular chondrocytes with good growth were randomly divided into blank group, model group and Myr group. The blank group was cultured in DMEM medium containing 10% FBS, while the model group was cultured in DMEM with 10 ng/mL IL-1β and 10% FBS. The Myr group was cultured in DMEM with different concentrations of Myr solution, 10 ng/mL IL-1β and 10% FBS.

Determination of the effect of Myr on proliferation of chondrocytes

The second-generation chondrocytes were inoculated into 96-well plates (100 μL per well) at a density of 1×10^5 cells/mL. After 48 h, the culture medium was washed off.

An IL-1β-induced chondrocyte apoptosis model was established with addition of 10 ng/mL IL-1β in 6 replicate wells, followed by treatment with 5, 10, 20, 40 and 80 μM Myr. The cells were cultured for 24, 48 and 72 h, after which the culture medium was replaced with 100 μL of 0.1% MTT. Then, the cells were placed in an incubator for 4 h, after which the MTT solution was discarded from each well, and the resultant formazan crystals were solubilized in 150 μL of dimethyl sulfoxide (DMSO). The absorbance of the solution in each well was read at 490 nm in an enzyme label spectrophotometer, and the average value was obtained.

Flow cytometric determination of chondrocyte apoptosis

Chondrocytes at a concentration of 1×10^5 cells/mL were seeded in a 6-well culture plate, and after 24 h of culture, the cells were adhered. The culture medium was discarded, followed by addition of different concentrations of Myr (10, 40 and 80 μM), and incubation for another 48 h. There were three duplicate wells in each group. Wells without Myr served as control. Then, the culture solution was aspirated, and the cells were rinsed twice with PBS, and digested with 0.25% trypsin. The digest was centrifuged at 1000 g, and the cells were collected and washed twice with PBS. They were then re-suspended in 200 μL of binding buffer, and 10 μL of membrane-connected protein V-FITC and 5 μL of PI were added. The mixture was shaken gently at room temperature for 15 min. Then, 300 μL of binding buffer was added, and the percentage apoptosis was determined using flow cytometry.

Western blotting assay for protein expressions of Bax, Bcl-2, Caspase-3 and Caspase-9 in chondrocytes of each group

After 48 h of culture, the cells in each group were harvested, and 80 μL of protein lysate was added to each well, and lysed on ice for 30 min. The lysates were collected and transferred to a 1.5-mL Eppendorf tube and centrifuged at 14 000 rpm for 30 min at 4 °C. The supernatant was aspirated to obtain total protein which was quantified using the BCA assay method. Then, 25 μg of the protein was subjected to 12% sodium dodecyl sulfate - polyacrylamide gel electrophoresis. The separated protein bands were transferred to PVDF membrane and washed thrice with Tris-HCl buffer (TBST), followed by blocking of non-specific binding at room temperature for 1 h, and washing thrice with TBST. The membrane was then incubated with the respective primary antibodies at 4 °C, washed thrice with TBST, and incubated with the secondary antibody at room temperature for 1 h. The target bands were analyzed with Image Lab image processing software, and the expression levels of the target proteins was expressed as the ratio of gray value of target band to that of beta actin which was used as the internal reference. The experiment was repeated three times.

Determination of expression levels of TNF-a, MMP-13 and PGE2 in the supernatant using ELISA

Cells at a concentration of 1×10^5 cells/mL were seeded in a 24-well cell culture plate. The experimental grouping and treatment were the same as indicated previously. Three replicate wells were set up in each group, and after 48 hours of culture, the cells were removed, and the levels of TNF-*α*, MMP-13 and PGE2 in the supernatants were determined using ELISA.

Statistical analysis

Measurement data are expressed as mean ± SD, and *t*-test was used to compare between two groups, while one-way analysis of variance (ANOVA) was used for comparison amongst groups. All statistical analyses were done using Graphpad Prism 7.0 statistical software. Values of $p<0.05$ were considered statistically significant.

Results

Effect of Myr on the viability of normal chondrocytes

The MTT results showed that Myr significantly enhanced viability of normal chondrocytes in a dose- and time-dependent manner. These results are presented in Figure 2.

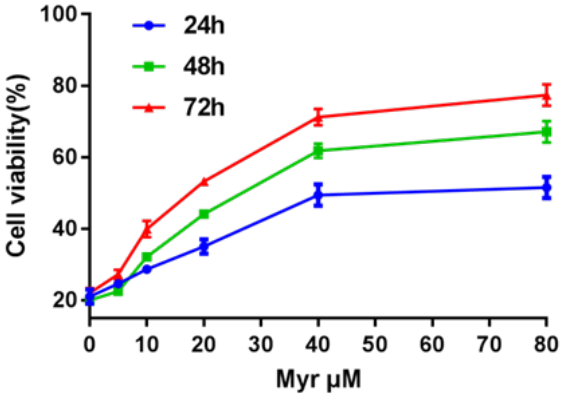

Figure 2. Effect of Myr on the viability of chondrocytes in each group. Model chondrocytes were treated with different concentrations of Myr (5, 10, 20, 40 and 80 μM) for 24, 48 and 72 hours. Data are shown as mean ± SD from three independent experiments.

Effect of Myr on apoptosis of OA chondrocytes

The effect of Myr on the apoptosis of OA chondrocytes was determined with flow cytometry. As shown in Figure 3, the % apoptosis of the model group was significantly higher than that of the control group ($p<0.001$). Compared with the model group, the degree of apoptosis of the Myr group was significantly lower ($p<0.05$). These results indicate that Myr inhibited IL-1β-induced apoptosis of OA chondrocytes.

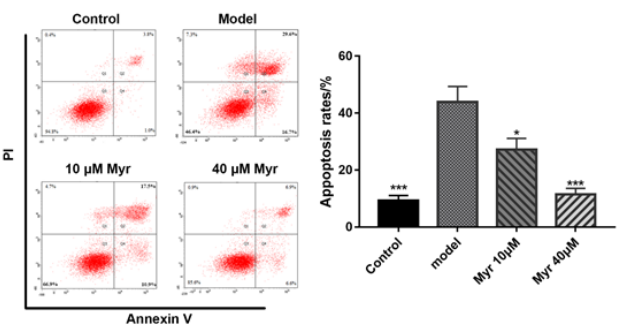

Figure 3. Effect of Myr on apoptosis of OA chondrocytes. *$p<0.05$, **$p<0.01$,***$p<0.001$), compared with model group.

Effect of Myr on expressions of Bax, Bcl-2, Caspase-3 and Caspase-9 in chondrocytes of each group.

Compared with the blank group, the expression of Bcl-2 protein in the chondrocytes of the model group was significantly decreased, while the protein expressions of Bax, Caspase-3 and Caspase-9 were significantly increased ($p<0.05$). Moreover, the expression of Bcl-2 protein in chondrocytes was increased significantly, while the protein expressions of Bax, Caspase-3 and Caspase-9 were decreased significantly, when compared with the myr group ($p<0.05$).

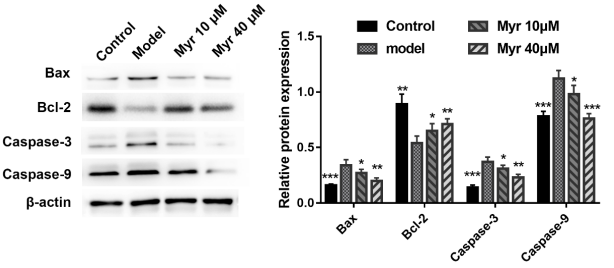

Figure 4. Effect of Myr on the expressions of Bax, Bcl-2, Caspase-3 and Caspase-9 in chondrocytes of each group. *$p<0.05$, **$p<0.01$, ***$p<0.001$), compared with model group.

Effect of Myr on expressions of TNF-α, MMP-13 and PGE2 in chondrocyte supernatants

The results showed that compared with the blank group, the expressions of inflammatory factors in the model group were significantly higher than those in the blank group. At the same time, compared with the model group, the expressions of inflammatory factors at different concentrations of Myr group decreased to different extents, indicating that Myr significantly inhibited the secretion of inflammatory factors of chondrocytes ($p<0.05$; Figure 5).

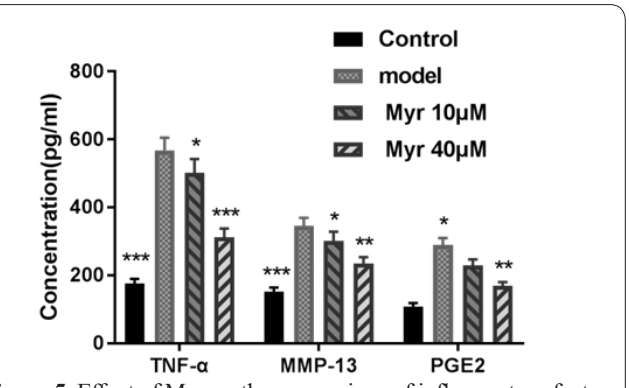

Figure 5. Effect of Myr on the expressions of inflammatory factors in chondrocytes of OA model. *$p<0.05$, **$p<0.01$, ***$p<0.001$), compared with model group.

Discussion

Osteoarthritis (OA) is characterized by joint pain, swelling, stiffness and deformity due to articular cartilage degeneration. Its occurrence is related to biomechanics, age, estrogen deficiency, heredity, trauma, osteoporosis and other factors. China is rapidly emerging as an aging society, resulting in increasing incidence of OA in the elderly, which is the main cause of joint pain and disability in the aged population (13). Myrroside (Myr) is a natural polyphenolic hydroxyflavone glycoside with numerous beneficial effects such as antioxidant, free radical-scavenging, blood vessel-dilating, anti-inflammatory, analgesic, and antibacterial effects. It has produced good antioxidant and anti-inflammatory effects in various disease conditions. However, very little

research has been done on its effect on IL-1β-induced inflammation of chondrocytes. In this study, primary chondrocytes of rats were treated with IL-1β to simulate the pathological environment of chondrocytes in OA, in order to investigate the effect of Myr on proliferation, apoptosis and inflammatory response of chondrocytes.

Abnormal apoptosis of chondrocytes is an important cause of OA (14). Decreases in the number of OA chondrocytes, thinning of cartilage thickness, and changes in phenotype are closely related to chondrocyte apoptosis. Increased apoptosis of chondrocytes promotes degradation of cartilage matrix and decrease in matrix synthesis. The expression level of IL-1β is positively correlated with the degree of degeneration of articular cartilage. It is the most direct factor in the destruction of articular cartilage; it inhibits the synthesis of chondrocytes proteoglycan and collagen type II, and promotes the degradation of cartilage matrix. Moreover, IL-1β induces apoptosis of cartilage cells. It is known that Bcl-2, Bax, Caspase-3 and Caspase-9 are important regulators of apoptosis. Caspase is an important executor of chondrocyte apoptosis. For example, when Caspase-9 is activated, it activates caspase-3 which degrades various protein substrates and triggers Caspase cascade reaction, leading to chondrocyte apoptosis (15).

It has been reported that Bcl-2 inhibited apoptosis of chondrocytes induced by various factors (16). However, Bax and Bcl-2 are antagonistic to each other. When Bcl-2 is highly expressed, Bcl-2 homologous dimer is formed to inhibit the apoptosis of chondrocytes. On the other hand, when Bax is highly expressed, it promotes the formation of heterodimer, inhibits the anti-apoptotic function of Bcl-2, and promotes the formation of Bax homologous dimer, triggering caspase-mediated chondrocyte apoptosis (17). The results of MTT study showed that Myr enhanced the viability of chondrocytes. Flow cytometry and Western blot showed that Myr increased Bcl-2 protein, decreased the protein expressions of Bax, Caspase-3 and Caspase-9 protein, and inhibited apoptosis of chondrocytes.

Inflammation-mediated degradation of cartilage matrix is one of the important causes of OA cartilage degeneration (18). Matrix metalloproteinases (MMPs) are the enzymes that destroy articular cartilage. The most destructive of the MMPs is MMP-13. It degrades collagen and proteoglycan in extracellular matrix, which is one of the important causes of OA. A large increase in IL-1β inflammatory cytokines disrupts chondrocyte function and the integrity of the cartilage extracellular matrix. In addition, IL-1β promotes the expressions of MMPs, inhibits the formation of cartilage matrix components, and causes imbalance in cartilage matrix homeostasis (19). The inflammatory cytokine IL-1β activates the production of MMP-1 and MMP-13 in synovial cells (20). In addition, IL-1β induces the production of cyclooxygenase in synoviocytes, thereby promoting the secretion of nitric oxide and PGE2. The latter is a pleiotropic inflammatory mediator which increases the production of MMPs and other catabolic substances (21). It affects the structure and function of joints. It has been reported that TNF-α inhibits cartilage synthesis and accelerates the degradation of articular cartilage, and that TNF-α, IL-1 and MMP-13 are positively correlated with the degree of lesion in OA (22). Results from ELISA revealed that the expressions of TNF-α, MMP-13 and PGE2 in the normal group and the Myr group were significantly decreased, when compared with the model group, indicating that Myr downregulated the expressions of inflammatory factors, inhibited inflammatory reaction in chondrocytes, and delayed the degeneration of the cartilage.

In summary, this study investigated the effect of Myr on IL-1β-induced chondrocyte apoptosis. The results revealed that Myr improved cell vitality, promoted chondrocyte proliferation, and inhibited chondrocyte apoptosis. Moreover, Myr reduced the expressions of inflammatory factors and inhibited inflammatory reaction in chondrocytes, thereby protecting them. These results provide experimental basis for the clinical application of Myr in the prevention and treatment of progressive degeneration of cartilage in OA.

Acknowledgements
None.

Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Xiao-Feng Wang; Tao Sun, Jian-Bo Xue, Ying-Ling Zhou, Xiao-Feng Wang collected and analysed the data; Tao Sun wrote the text and all authors have read and approved the text prior to publication.

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