Original Article
Effect of quercetin on interleukin-1β-induced apoptosis of chondrocyte

Hua-Jie Mao, Qi-Xin Chen, Bin Han

Department of Orthopedic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, People's Republic of China

Received July 9, 2017; Accepted May 3, 2018; Epub August 15, 2018; Published August 30, 2018

Abstract: Objective: To evaluate the effects of quercetin and study its mechanisms on chondrocyte apoptosis induced by interleukin-1β (IL-1β). Methods: Isolated chondrocytes were randomly divided into five groups, including a control group (without any intervention), an IL-1β group (50 μg/mL of IL-1β for 24 h), and three quercetin groups (100 μM, 200 μM and 400 μM quercetin in addition to IL-1β). All of the cells were incubated in pore plates, and the experiments were repeated three times. The apoptosis rates of the five groups were calculated, and the relative expression levels of Bcl-2, Bax and caspase-9 were detected using PCR and western blot analyses. The relative expression levels of cleaved caspase-3 were detected using western blot analyses. Results: Compared to the control group, the apoptosis rate of chondrocytes, iNOS expression levels, gene expressions of Bax and caspase-9 in other four groups significantly increased, but the gene expression of the anti-apoptotic Bcl-2 decreased significantly, and the differences were statistically significant (P<0.05). Compared to the IL-1β group, the apoptosis rates of chondrocytes, expressions of Bax and caspase-9 of chondrocytes in the quercetin group (400 μM) decreased significantly, but the expression of Bcl-2, an anti-apoptotic gene, increased significantly, and the differences were statistically significant (P<0.05). Compared to the IL-1β group, the protein expressions of cleaved caspase-3 was reduced, and the difference was statistically significant (P<0.05). Conclusion: Quercetin can reduce the expression of Bax and increase the expression of Bcl-2, resulting in a decrease in the expression of caspase-9, and thus can prevent chondrocyte apoptosis induced by IL-1β.

Keywords: Quercitrin, chondrocyte, apoptosis

Introduction

From a pathological perspective, osteoarthritis (OA) can be described as damage of the subchondral bone and articular cartilage along with a subsequent apoptosis of chondrocytes [1-3]. The worldwide incidence of OA accounts for more than 10% of people aged 60 and above. For a long time, OA was considered to be a result of pressure increase and cartilage matrix degeneration in the joint cavity, which caused the simultaneous occurrence and development of OA. With the development of molecular biology, researchers have found that OA involves cartilage, bone and synovium, and the release of inflammatory mediators and other processes, resulting in increased levels of matrix metalloproteinases and eventually leading to chondrocyte apoptosis.

Recent studies have reported that the development of OA is largely due to the imbalance between the synthesis and degradation of the extracellular matrix caused by inflammation, which damages of the soft tissues of the joints [4]. The shrinkage of chondrocytes and the formation of apoptotic bodies were observed in the intraoperative sections of clinical OA patients. Therefore, the apoptosis of chondrocytes is an important step in the pathologic mechanisms of OA [5]. Studies have identified interleukin-1β (IL-1β) as playing an important role in the development and progression of OA as a pro-inflammatory cytokine. IL-1β promotes the apoptosis of chondrocytes and results in further loss of cartilage through the caspase-9 pathway after increasing the levels of matrix-degrading enzymes and inducible nitric oxide synthase (iNOS) [6, 7]. Non-steroidal anti-inflammatory drugs are often used in clinical treat-
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In recent years, attention has gradually shifted toward traditional Chinese medicine. Quercetin, belongs to the genre of flavonols, is a drug with a variety of biological activities and widely available from various natural sources [8, 9]. Studies have shown that quercetin possesses multiple pharmacological effects, including the removal of oxygen free radicals, resisting inflammation and inhibiting osteoclastic bone resorption. However, the effects of quercetin on IL-1β-induced chondrocyte apoptosis and the potential mechanisms involved are unknown. In this study, different doses of quercetin were used to study the effects of quercetin on chondrocyte apoptosis.

Materials and methods

Laboratory reagents

Five healthy, clean, male SD rats aged 8 weeks [qualification: SCXK (Chongqing 2002008)], weighing 180 to 200 g were provided by the Animal Experiment Center of the Medical School. Quercetin (Dalian Meilunbio, China), IL-1β (Peprotech, U.S.), DMEM/F12 growth medium, PBS concentrate, fetal calf serum, pancreatin (Gibco, U.S.), Annexin V-FITC Apoptosis Assay Kit (Boster, China), DMSO (Ameresco, U.S.), TRIzol (Invitrogen, U.S.), mouse anti-rat primary antibodies against Bax, Bel-2, cleaved caspase-3 and caspase-9 (Abcam, U.S.), RIPA, PMSF, ECL substrate and goat anti-mouse secondary antibodies (Boster, China), ReverTra Ace qPCR reverse transcription kit (Takara, Japan), and SYBR® Green Realtime PCR Master Mix Assay Kit (Takara) were purchased from the indicated suppliers.

This study was approved by the animal ethics association of Second Affiliated Hospital of School of Medicine, Zhejiang University.

Extraction of chondrocyte

SD rats were disinfected with iodophor alcohol after they were euthanized through cervical dislocation. The exposed knee joints were approached from the anterior, and using a knife, the cartilage surfaces were carefully scraped, and the cartilage tissues were cut using scissors. Then, 0.25% trypsin was added to enzymatically digest and detach the chondrocytes. After the primary cells were detached, they were cultured in DMEM/F12 medium containing 10% fetal calf serum. The cells were incubated at 37°C in an incubator with 5% CO_2_ and saturated humidity. The media was changed for the first time after one week and then changed every 2 to 3 days. The cells were sub-cultured until 80 to 90% of cells were fused. The chondrocytes were identified from the second generation using Alcian blue staining and type II collagen staining (Supplementary Figure 1). After overnight starvation and digestion, the third generation chondrocytes were selected, and the cell density was adjusted to 3 × 10^5/ml. The cells were then randomly separated into 5 groups, including a control group (no intervention), an IL-1β group (50 μg/mL IL-1β for 24 hours), and three quercetin groups, which included groups that were treated with different concentrations of quercetin (100 μM, 200 μM and 400 μM) along with 50 μg/mL IL-1β for 24 hours. Each group included 5 replicates, and the experiment was repeated 3 times.

Flow cytometry and TUNEL for apoptosis

An Annexin V-FITC cell apoptosis detection kit was used to detect the rate of apoptosis for each group of cells after 24 hours of treatments. The digestive enzymes were used to digest the cells, and the NB serum was added to stop the digestion before centrifugation. After centrifugation for 5 minutes at 1000 g, the cells were resuspended in PBS with pH 7.4–7.6 and counted. Then, 5 × 10^4 cells were selected and added to 500 μL of Annexin V-FITC, and 5 μL propidium iodide staining solution was added and mixed thoroughly. The mixture was incubated for 30 min in the dark, flow cytometry (BD, the U.S.) was conducted, and the apoptotic rate was calculated as cells number in Q2 zone/total cell number × 100%.

Real-time RT-PCR

TRizol was added to the Petri dishes to extract cellular RNA samples from the cells. Using the ReverTra Ace qPCR RT kit, the mRNA samples were reversely transcribed into cDNA according to the description provided by the kit. The primers used were Bcl-2, upstream 5' CAA ACT GCT AAA TGA CGA GG 3', downstream 5' GGG AAA GGT TGT GTA GGG TC 3'; Bax, upstream 5'-AGATCCTGACCGAGCTGGC-3', downstream 5'-CCA GGG AGG AGG AGG ATG CG-3; cas-
pase-9, upstream 5'-AGATCCTGACCGAGCGTG- GC-3', downstream 5'-CCA GGG AGG AAG AGG ATG CG-3'; β-actin, upstream 5'-AGATCCTGACCGAGCGTG- GC-3', and downstream 5'-CCA GGG AGG AAG AGG ATG CG-3. The SYBR® Green Realtime PCR Master Mix assays kit was used to conduct QRT-PCR. The semi-quantitative evaluation was conducted by using the 2-ΔΔCt method and using β-actin as the internal reference.

Western blot

After digestion with 3 mL/g protein lysate, the protein was collected through centrifugation. The total protein concentration was measured using the BCA method. The same amount of protein from each sample was added to an equal volume of 2 × SDS buffer and boiled for 10 minutes. SDS-PAGE electrophoresis was performed, and the proteins were transferred to membranes and incubated primary antibodies overnight (1:150). The membranes were incubated in second antibodies (diluted to 1:5000) at 37°C for 2.5 h. ECL reagent was added to produce the gel image and imaged using the imaging system (Bole, China) and ImageJ (National Institutes of Health, the U.S.) was used to calculate the relative gray values using the following formula: measured protein gray value/gray value of the internal reference GAPDH) × 100%.

Statistical analysis

SPSS version 20.0 was used for statistical analyses. The data are expressed as (±S).
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The overall comparisons between the groups were conducted using the one-way ANOVA and the pairwise comparisons were conducted using the Dunnett test, using a significant level \( \alpha = 0.05 \).

Results

Quercetin decreased the apoptosis rate

Compared to the IL-1\( \beta \) group, the apoptotic rates of chondrocytes decreased after treatments with 400 \( \mu M \) quercetin, and the difference was statistically significant \( (P<0.05) \). The apoptosis rates of chondrocytes treated with 100 \( \mu M \) and 200 \( \mu M \) quercetin were lower than those of the IL-1\( \beta \) group; however, these differences were not statistically significant \( (P>0.05) \). The apoptosis rates for the 5 groups are shown in Figures 1 and 2.

Quercetin decreased the mRNA expressions of Bax, Caspase 9 and increased the mRNA expression of Bcl-2

PCR results showed that the expression levels of Bax and caspase-9 in chondrocytes significantly increased in the IL-1\( \beta \) group compared to the control group, whereas the levels of the anti-apoptotic gene Bcl-2 were significantly decreased, and both these differences were statistically significant \( (P<0.05, \text{Figure 3}) \).

Quercetin decreased the protein expression of Bax, Caspase 9 and increased the protein expression of Bcl-2

The western blot results indicated that, when compared to the control group, the expression of apoptosis proteins Bax and caspase-9 in the IL-1\( \beta \) group significantly increased, whereas the expression of anti-apoptotic protein Bcl-2 decreased significantly, and these differences were statistically significant \( (P<0.05, \text{Figure 4}) \).

Quercetin decreased the protein expression of cleaved Caspase 3

Compared to the control group, the expression of cleaved caspase-3 increased significantly after treatments with IL-1\( \beta \), and the differences

Figure 3. Gene expression of Bcl-2, Bax and Caspase 9 in chondrocytes in the 5 groups. Note: compared to the control group, *\( P<0.05 \); compared to the IL-1\( \beta \) group, †\( P<0.05 \).

Figure 4. Comparison of protein expression of Bcl-2, Bax and Caspase 9 in the chondrocytes in the 5 groups. Note: compared to the control group, *\( P<0.05 \); compared to the IL-1\( \beta \) group, †\( P<0.05 \).
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Compared to the IL-1β group, the expression of cleaved caspase-3 in chondrocytes treated with 100 μM, 200 μM and 400 μM quercetin significantly decreased, and the differences were statistically significant (P<0.05, Figure 5).

**Discussion**

We found that quercetin could decrease the apoptosis that inducted by IL-1β and the potential mechanism may be affected by influence the expression of Bcl-2/Bax and Caspase 9. The chondrocyte apoptosis model that uses 50 μg/mL IL-1β treatments was used. The apoptosis rates of chondrocytes decreased after quercetin treatments were applied. Our results that describe the expression of genes and proteins related to chondrocyte apoptosis show that quercetin may exert anti-apoptotic effects through influence the Bax/Bcl-2 expression.

In this study, the apoptotic chondrocytes were identified by the Annexin V-FITC/PI double staining method. The apoptosis rates of chondrocytes were increased by 5 times when 50 μg/mL IL-1β was added to chondrocytes, which agrees with results from existing literature [10-12]. The apoptotic rates of chondrocytes decreased due to the application of different concentrations of quercetin, and the effects of 400 μM quercetin were the most obvious. The potential reasons for these effects are as follows: (1) Quercetin is a flavonol and has a wide range of biological activities [14]. Recent literature shows that flavonoids have strong anti-inflammatory effects [15]. (2) Quercetin can inhibit the NF-kappaB signaling pathway, thus inhibiting the expression of p65 and modulating the expression of MMP-13, further delaying the degeneration of articular cartilage in a dose-dependent manner [16]. (3) Quercetin reduces oxidative stress by reducing the reactive oxygen species, thus lowering chondrocyte apoptosis. In vivo experiments conducted by Yuan Xiaoliang et al. [14] provided evidence for the role of quercetin in OA. The researchers identified quercetin’s effect in delaying OA through the use of quercetin treatments in a rat OA model. Bcl-2 and Bax belong to the Bcl-2 family, which includes pro-apoptotic molecules (Bax) and anti-apoptotic molecules (Bcl-2) [17]. However, these two molecules are associated with the mitochondrial apoptosis pathway and can cause apoptosis through the mitochondrial membrane. Bcl-2 increases with cell resistance. When Bcl-2 expression increases, homodimers are formed to inhibit apoptosis [19]. If the homodimers expression is too high, Bac/Bax homodimers are formed to accelerate the apoptosis. Results indicate that the expression of Bax and down stream gene caspase-9 in chondrocytes significantly increased due to IL-1β treatments. Quercetin can inhibit the expression of Bax and caspase-9, increase the expression of Bcl-2, and thus could delay the progress of OA. The reasons for these effects may be that quercetin has anti-oxidative and anti-inflammatory effects to prevent apoptosis of chondrocytes.

The matrix of chondrocytes and type II collagen is the microenvironment in which chondrocytes survive. Reducing the matrix and type II collagen synthesis in chondrocytes is not conducive to the survival of chondrocytes and therefore increases the apoptosis of the cartilage cells [12]. Overexpression of caspase-3 plays could disintegrate typeII collagen and promoting the development of knee OA. Quercetin can reduce the expression of cleaved caspase-3 and delay the development of OA, but the specific mechanisms that are involved need further exploration.

In short, quercetin can reduce the expressions of Bax and increase Bcl-2 to inhibit the IL-1β-induced apoptosis of chondrocytes. However, in vivo experiments are needed to verify the most appropriate dosage of quercetin.

**Acknowledgements**

This work was supported by grants from the Health Foundation of Zhejiang Province (No.
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2016142263, No. 2018241121) and Natural Science Foundation of Zhejiang Province (No. LY18H060004).

Disclosure of conflict of interest

None.

Address correspondence to: Qi-Xin Chen, Department of Orthopedic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University, No. 1511, Jianghong Road, Binjiang District, Hangzhou, Zhejiang Province, People’s Republic of China. Tel: +86 0571-89713667; E-mail: zrcqx@zju.edu.cn

References


Supplementary Figure 1. Alcian blue staining and type II collagen staining for chondrocytes (A. Alcian blue staining, B. type II collagen staining).