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Original Article

Resveratrol relive osteoarthritis through reducing inflammatory reactions

Jun Liu[1], Xiaoyan Lv[2]

[1]*Department of Orthopedics, Jingzhou Central Hospital, Jingzhou, Hubei, China;* [2]*Department of Rehabilitation, Jingzhou Central Hospital, Jingzhou, Hubei, China*

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Abstract: Objective: Resveratrol (RES) assisted in inhibiting proliferation of osteoarthritis (OA) chondrocytes *in-vitro*; however, the underlying mechanism is currently unclear. This study aimed to investigate the effects of RES on the apoptosis genes regulating OA chondrocytes. Methods: Rats OA chondrocytes were obtained and cultured while induced with IL-1β. Bax, Bcl-2, and Caspase-3 protein expression levels were tested by Western blotting. The viability of chondrocytes was detected by the MTT assay, and the expression levels of TNF-α, IL-6, p-PI3K, and p-AKT were measured by ELISA. Results: Compared with the DMSO group, IL-1β treatment significantly reduced the viability of cells ($P<0.01$). RES at 3.125-25 μmol/L significantly enhanced the viability of chondrocytes ($P<0.01$), which is opposite to RES at 50-100 μmol/L. At all tested concentrations, RES significantly reduced the expression of TNF-α and IL-6 in chondrocytes induced by IL-1β and increased the expression of p-PI3K and p-AKT in a dose-dependent manner ($P<0.05$). At different concentrations, RES significantly down-regulated Bax expression, up-regulated Bcl-2 expression and down-regulated Caspase-3 expression dose-dependently. Conclusion: RES significantly reduced the expression of inflammatory cytokines in chondrocytes, increased activation of the PI3K/AKT signaling pathway, and regulated expression levels of Bcl-2 and Bax, leading to alleviation of the inflammatory response of chondrocytes and a reduction in the apoptosis of chondrocytes.

Keywords: Inflammatory reactions, resveratrol, osteoarthritis chondrocyte, signaling pathway

Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting millions of people worldwide. It occurs when the protective cartilage on the ends of your bones wears down over time. The incidence of OA increases annually and seriously affects daily life of patients. Osteoarthritis could result in the formation and progression of cartilage lesion [1, 2]. Chondrocytes, the only cells found in healthy cartilage, are relatively inert cells with insufficient regenerative capacity, which, in turn, may further destroy the balance between synthesis and degradation of the extracellular matrix causing further OA aggravation [3, 4].

PI3K-Akt Pathway is an intracellular signal transduction pathway that promotes metabolism, proliferation, cell survival, growth and angiogenesis in response to extracellular signals. Studies indicate that sustained activation of the Phosphoinositide 3-kinase/Protein Kinase B (PI3K/AKT) signaling pathway may induce the expression of target genes such as Bcl-2 associated X protein (*Bax*), B-cell lymphoma *2* (*Bcl-2),* and *Caspase-3* in chondrocytes, resulting in apoptosis of chondrocytes [5]. Other studies reported that long lasting inflammation in the joint cavity was closely associated with the occurrence and development of OA [6, 7]. When an inflammatory reaction occurs in the synovial tissue of the joint, pro-inflammatory cytokines such as interleukin-1β (IL-1β) are secreted in order to slow matrix synthesis and inhibit chondrocyte proliferation [8]. Therefore, it is interesting to explore whether the progression of OA could be relieved by regulating the PI3K/AKT signaling pathway.

Resveratrol is an organic molecule with substantial effects on health. Modern pharmacological studies have demonstrated that RES is effective in anti-inflammation, anti-tumor, anti-

oxidation, and immune-regulation processes [9, 10]. RES is reported to significantly inhibit the proliferation of *in-vitro* OA chondrocytes, but the specific mechanism of action has not been clarified. Therefore, the current study observed the therapeutic effects of RES on OA, with a particular focus to its effect on cartilage inflammation and apoptosis.

Materials and methods

Animal specimens and reagents

Three SPF grade male rats aged 4 weeks and weighing 220-260 g were provided by the Laboratory Animal Center of Southern Medical University, Guangzhou, China. RES was obtained from Zhengzhou Zhongguan Jianye Biotechnology Co., Ltd. (Zhengzhou, China). Rabbit anti-Bax, anti-Bcl-2, anti-PI3K, anti-AKT, and anti-p-AKT antibodies were obtained from Epitmics (California, USA). Annexin V-FITC/PI flow double stained cells and the Caspase-3 detection kit were acquired from Nanjing Kylie Biotechnology Co., Ltd. (Nanjing, China). Fetal bovine serum, DMEM/F-12 medium, type II collagenase, and MTT were obtained from Gibco (New York, USA). A mouse IL-lβ enzyme-linked immunosorbent assay (ELISA) kit was acquired from Cell Signaling Technology (Boston, USA). BCA protein quantification kit and RIPA lysate were obtained from China Beyotime Biological Co., Ltd. (Shanghai, China). Every procedure was approved by the Animal Care and Use Committee of the Jingzhou Central Hospital and was in conformity with the guidelines of National Institute of Health (No. 81004).

Preparation of rat chondrocytes

Rat chondrocytes were prepared per previously specified methods [6], where the hyaline cartilage of the bilateral tibial plateau of the knee joint and the medial patellar were scraped under sterile conditions, and cut with a sterile blade or a pair of small tissue scissors to prepare a tissue mass with a volume of 1 mm^3. The tissue mass was sequentially digested with 0.25% trypsin and 0.2% collagenase until a single cell suspension was formed. DMEM/F-12 medium, containing 20% fetal bovine serum and 100 μmol/mL penicillin as well as 100 μmol/ml streptomycin, was used to re-suspend cells. Cell fusion was observed when cells were cultured at 37°C and 5% CO_2 for approximately 5-6 days. Cells were subcultured for 2 to 3 generations before being subjected to subsequent experiments.

Detection of chondrocyte viability by MTT

Following 2 to 3 passages, cultured chondrocytes were digested using trypsin, adjusted to 1×10^5 cells/mL and subsequently incubated at 37°C and 5% CO_2 for 24 h. The cells were divided into a blank control group, a solvent control group (2% DMSO), a RES (3.125, 6.25, 12.5, 25, 50, 100 μmol/L) group, an IL-1β (10 ng/ml) group, and a RES (3.125, 6.25, 12.5, 25, 50, 100 μmol/L) + IL-1β (10 ng/ml) group. These cell groups were cultured for additional 24 h and 48 h after treatment, respectively. Cells in each well were treated with 20 μL of MTT at a concentration of 5 mg/mL, and after incubation at room temperature for 4 h, centrifugation was performed and the supernatant discarded. Simultaneously, 150 μL of DMSO was added to each well before continuing incubation for a further 10 min and the OD value at 570 nm was measured with a microplate reader to detect cell viability.

Chondrocyte apoptosis detection

Following the logarithmic growth phase, the chondrocytes were subcultured for 2 or 3 passages, digested with trypsin, adjusted to 2×10^5 cells/mL and inoculated at 37°C and 5% CO_2 for 24 h. After treatment with different drugs, cells were subjected to cultivation at 37°C and 5% CO_2 for 48 hours, digested with 0.25% trypsin (without EDTA), washed with PBS, centrifuged for 5 minutes at 2000 r/min, and collected. Approximately 500 μL of binding buffer was added to suspend the cells and 5 μL of Annexin V-FIT and 5 μL of PI were added sequentially. After thorough mixing, the resulting solution was kept away from light at room temperature and allowed to react for 10 min. Flow cytometry was applied to detect apoptosis.

Detection of p-PI3K and p-AKT levels by ELISA

Cells treated according to the procedures stated previously were centrifuged for 5 min at 2000 r/min and cell culture supernatant was collected to detect the expression levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), phosphorylated phosphoinositide 3-kina-

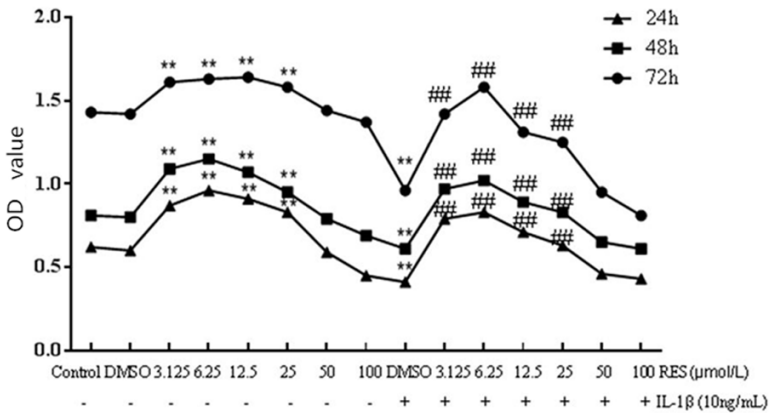

Figure 1. Effect of RES and IL-1β on the chondrocyte vitality. Note: compared with DMSO group **P<0.01; compared with IL-1β group, ##P<0.01.

se (p-PI3K) and phosphorylated protein kinase B (p-AKT) by ELISA.

Detection of protein expression by western blotting

Cells treated according to the procedures stated previously were collected and added to cell lysate to extract the total protein content. The sampling volume of each well was set to 20 μL and SDS - gel electrophoresis was conducted to transfer the protein to the PVDF membrane for sealing. The primary antibody (1:500) was added and incubated at 4°C overnight and on the next day, the secondary antibody (1:5000) was added and the incubation kept for 2 h. The ECL method was used for exposure and visualization, and the Quantity One software was applied to analyze the grayscale values of Bax, Bcl-2, and Caspase-3 proteins.

Statistical analysis

SPSS 19.0 statistical software was utilized for data analysis. Measurement data is expressed as mean ± standard deviation ($\bar{x} \pm s$), the independent samples t-test was used to compare means between two groups and the paired t-test was used to compare before and after intervention means of the same group. One-way analysis of variance was applied to compare means between groups and the least squared difference (LSD) method was used in the case of equal variance; otherwise, the Dennett T3 method was used. The rates were compared by using the Chi-square (χ^2) test at a significance level of P<0.05.

Results

Effect of RES and IL-1β on chondrocyte viability

Compared to the control group, DMSO did not significantly affect cell proliferation viability (P>0.05). Compared to the DMSO group, the viability of cells significantly increased 24 h, 48 h and 72 h after the RES at 3.125-25 μmol/L treatment (P=0.004), did not significantly changed 24, 48 and 72 h after the RES at 50-100 μmol/L treatment, and was inhibited in varying degrees 24, 48 and 72 h after the RES at 100 μmol/L. Compared with the DMSO group, the cell viability decreased significantly in the single IL-1β treatment group (P<0.01). Compared with the IL-1β group, the cell viability significantly increased 24, 48 and 72 h after the IL-1β + RES at 3.125-25 μmol/L treatment (P=0.002) and changed insignificantly in the RES at 50-100 μmol/L treatment group (**Figure 1**).

Effect of RES on IL-1β induced chondrocyte cytokines TNF-α, IL-6, p-PI3K and p-AKT

After induction with IL-1β, the levels of TNF-α and IL-6 significantly increased (P<0.01), but the levels of p-PI3K and p-AKT significantly decreased in the chondrocytes of the supernatant (P<0.01). Different concentrations of RES all significantly reduced the expression of TNF-α and IL-6 in chondrocytes induced by IL-1β, and increased the expression of p-PI3K and p-AKT in a dose-dependent manner, which was statistically significant compared to that of the IL-1β group (P<0.05) (**Table 1**).

Effect of RES on Bax and Bcl-2 expression in IL-1β induced chondrocytes

Western blotting results indicate that IL-1β significantly increased the expression of Bax and inhibited the expression of Bcl-2 when compared to that of the DMSO group (P<0.01). Different concentrations of RES all significantly down-regulated the expression of Bax and up-regulated the expression of Bcl-2 in a dose dependent manner, compared to that of the IL-1β group (P<0.05) (**Figures 2** and **3**).

Table 1. The effect of RES on the IL-1β-induced chondrocyte factors TNF-α, IL-6, p-PI3K and p-AKT ($\bar{x}$ ± s, ng/L)

Group	Drug dose	TNF-α	IL-6	p-PI3K	p-AKT
DMSO group	0	5.32 ± 1.18	23.51 ± 2.07	5.93 ± 0.71	375.61 ± 13.14
IL-1β group	0	17.56 ± 1.37	58.69 ± 3.28	2.06 ± 0.32	165.23 ± 11.16
RES (μmol/L)	3.125	15.31 ± 2.05#	52.35 ± 3.11#	2.75 ± 0.28#	182.37 ± 10.56#
	6.25	13.25 ± 2.14##	41.67 ± 3.36##	3.57 ± 0.31##	216.39 ± 12.35##
	12.5	10.18 ± 2.19##	36.13 ± 2.12##	4.34 ± 0.35##	241.87 ± 13.39##
	25	8.61 ± 2.12##	33.47 ± 3.92##	4.72 ± 0.29##	285.69 ± 13.54##
	50	8.15 ± 2.23##	31.51 ± 3.71##	5.04 ± 0.31##	327.52 ± 12.78##
	100	7.68 ± 2.17##	28.16 ± 3.59##	5.21 ± 0.34##	345.28 ± 13.34##

Remark: Compared with DMSO group, P<0.05; compared with IL-1β group, #P<0.05, ##P<0.01.

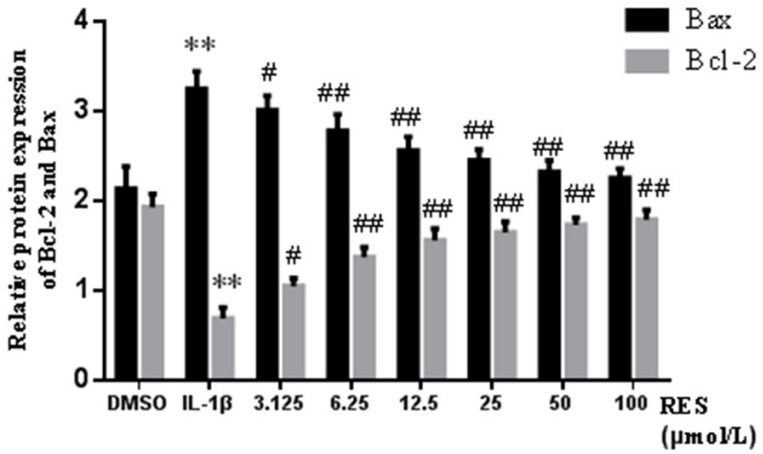

Figure 2. Differences in histogram expression of Bax and Bcl-2 proteins in chondrocytes. IL-1β may significantly increase the expression of Bax and inhibit the expression of Bcl-2, compared with the DMSO group, **P<0.01; compared with the IL-1β group, #P<0.05, ##P<0.01.

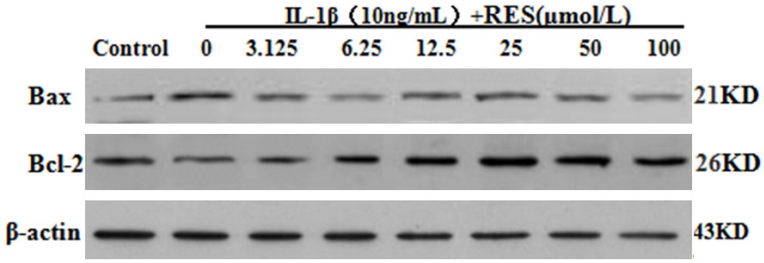

Figure 3. The difference of relative expression of Bax and Bcl-2 protein in chondrocytes of each group. Different concentrations of RES all significantly down-regulated the expression of Bax and up-regulated the expression of Bcl-2 in a dose dependent manner, compared to that of the IL-1β group (P<0.05).

Effect of RES on Caspase-3 activity in IL-1β induced chondrocytes

IL-1β significantly increased the activity of Caspase-3 in chondrocytes compared to the DMSO group (P<0.01). Different concentrations of RES significantly down-regulated the expression of Caspase-3 in a dose-dependent manner compared to the IL-1β group (P<0.05) (**Figures 4** and **5**).

Discussion

Studies have reported that apoptosis of chondrocytes and degradation of extracellular matrix are factors that play an important role in the pathogenesis of OA [10-12]. The apoptosis rate of chondrocytes is associated with the degree of cartilage matrix degradation and as apoptosis impacts the repair of the cartilage extracellular matrix, the severity of OA is further affected [13, 14]. Moreover, the apoptotic bodies that remain generate pyrophosphatic acid, leading to a pathological calcification of cartilage which further aggravates the progression of OA.

The PI3K/AKT pathway, an important signaling pathway, regulates the expression of a variety of anti-apoptotic genes, resulting in the inhibition of apoptosis and autophagy [15, 16]. The PI3K/AKT pathway controls the expression of *Bcl-2,* which encodes a protein that inhibits the mitochondrial membrane permeability of cytochrome C. The resulting reduction of cyto-

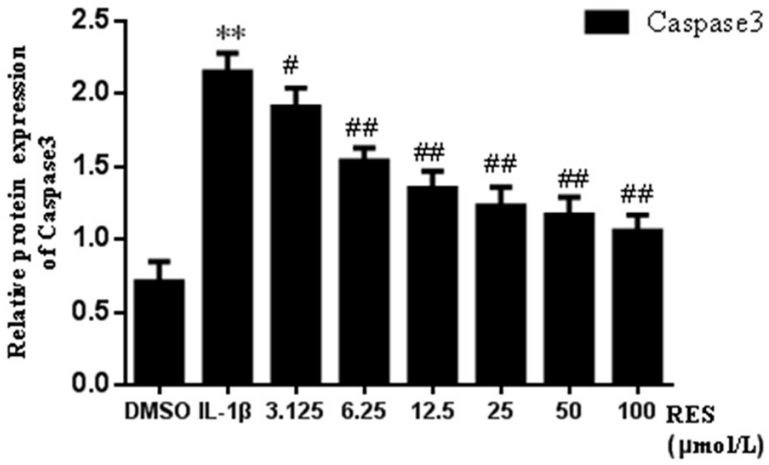

Figure 4. Differences in histogram expression of Caspase-3 in chondrocytes. IL-1β significantly increased the activity of Caspase-3 in chondrocytes compared to the DMSO group, *P<0.01; compared with IL-1β group, #P<0.05, ##P<0.01.

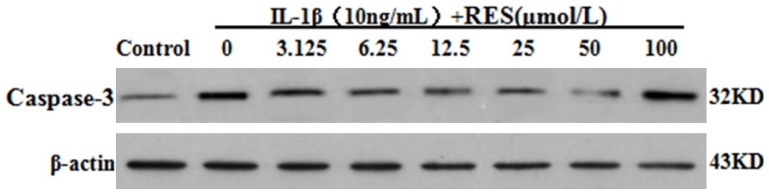

Figure 5. The difference of relative expression of Caspase-3 protein in chondrocytes of each group. Different concentrations of RES significantly down-regulated the expression of Caspase-3 in a dose-dependent manner compared to the IL-1β group (P<0.05).

chrome C impedes Caspase-3 activation and generates the anti-apoptosis function [17]. Bax forms a heterodimer with Bcl-2, and therefore Bax may play an antagonistic role in inhibiting apoptosis.

RES is a phytoalexin that is found in a variety of plants, where its biological functions include anti-inflammation, anti-oxidation and immune regulation. Our experiment demonstrated that RES may inhibit the degradation of cartilage matrix that is induced by IL-1β, and play an anti-OA role by decreasing the levels of matrix metalloproteinase and IL-6 in in-vitro cultured chondrocytes. RES also significantly reduced the loss of cartilage matrix proteoglycans and destruction of cartilage. RES resists IL-1β induced human osteoarthritis by inhibiting the Toll-like receptor 4 (TLR4) signaling pathway. However, it may be noted that RES at excessive concentrations is toxic to cells [18].

IL-1β was found in some studies at significantly elevated levels in the synovium, joint, synovial fluid, cartilage, and in the subchondral bones of OA patients [19]. The inflammatory cascade induced by IL-1β accelerates the destruction and apoptosis of OA chondrocytes in conjunction with inflammatory factors such as matrix metalloproteinase and TNF-α [20, 21]. IL-1β is activated in chondrocytes of OA patients where other inflammatory factors (such as IL-6) are also significantly increased at the same time [22]. By reducing the IL-1β content and inhibiting Caspase-3 activity, the apoptosis of chondrocytes may be restrained and cartilage destruction be further mitigated [23].

The study demonstrated that the viability of IL-1β induced chondrocytes significantly decreased (P<0.01) and that the levels of TNF-α and IL-6 were significantly higher than those of the control group (P<0.01), indicating that IL-1β may significantly increase the inflammatory reaction of chondrocytes. RES intervention at different concentrations significantly reduced TNF-α and IL-6 expression in IL-1β-induced chondrocytes, indicating that RES may act to inhibit the inflammatory response of chondrocytes.

Furthermore, our study determined the expression level of apoptosis markers and also demonstrated that the levels of p-PI3K and p-AKT were significantly decreased after induction of IL-1β (P<0.01), leading to a decrease in the anti-apoptotic effect with a resultant increase in apoptosis. Different concentrations of RES increased the expression of p-PI3K and p-AKT in a dose-dependent manner, suggesting that RES may significantly increase the activation of PI3K/AKT and exert an anti-apoptotic effect.

Western blotting showed that IL-1β significantly increased the expression of Bax and inhibited the expression of Bcl-2. In the meantime, RES down-regulated the expression of Bax and up-regulated the expression of Bcl-2, causing an increase in Bcl-2 protein levels which decrease

the level of Caspase-3. Therefore, RES may cause an anti-apoptotic effect by regulating gene expression in the PI3K/AKT pathway.

In summary, RES enables a significant reduction in the expression of inflammatory cytokines in cartilage cells, increases the activation of the PI3K/AKT signaling pathway and regulates the expression of *Bcl-2* and *Bax*, which results in alleviation of the inflammatory response in chondrocytes and leads to a reduction in chondrocyte apoptosis. Thus, the study indicates that RES may have a protect effect on the chondrocytes of OA patients via the mode of action suggested above.

Disclosure of conflict of interest

None.

Address correspondence to: Xiaoyan Lv, Department of Rehabilitation, Jingzhou Central Hospital, No. 1, Renmin Road, Jingzhou District, Jingzhou 434020, Hubei, China. Tel: +86 0716 8496417; E-mail: xiaoyanlv5434664@163.com

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