Resveratrol improves the progression of osteoarthritis by regulating cholesterol metabolism

ChuanCai Liang  
Renmin Hospital of Wuhan University

Hengte Xing  
Renmin Hospital of Wuhan University

ChenYu Wang  
Renmin Hospital of Wuhan University

XiongFeng Xu  
Renmin Hospital of Wuhan University

Yarong Hao  
Renmin Hospital of Wuhan University

Bo Qiu  
qbtg163@163.com  
Renmin Hospital of Wuhan University

Article

Keywords: chondrocytes, interleukin-1β, resveratrol, osteoarthritis, cholesterol metabolism

Posted Date: November 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-2236387/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Osteoarthritis (OA) is considered a metabolic disorder. This study investigated the effect of resveratrol (RES) on cholesterol accumulation in osteoarthritic articular cartilage via the SIRT1/FoxO1 pathway. Interleukin (IL)-1β-treated chondrocytes that mimic OA chondrocytes were used in in vitro experiments. The optimal RES concentration was selected based on the results of chondrocyte proliferation in the Cell Counting Kit-8 assay. Western blotting, immunofluorescence, and reverse transcription-quantitative polymerase chain reaction were performed. For the animal experiments, mice were randomly divided into the RES group (n = 15), medial meniscus destabilization group (n = 15), and Sham group (n = 15), and each group received the same dose of RES or saline. Articular cartilage tissue was obtained eight weeks after surgery for relevant histological analysis. Clinical tissue test results suggest that downregulation of the SIRT1/FoxO1 pathway is associated with cholesterol buildup in OA chondrocytes. For the in vitro studies, RES increased SIRT1 expression and FoxO1 phosphorylation in IL-1β-treated chondrocytes, promoted the expression of cholesterol efflux factors (LXRα, ABCA1, and ApoA1), and inhibited the expression of cholesterol synthesis-related factors (SREBP-2 and HMGCR). This reduced IL-1β-induced chondrocytes cholesterol accumulation. SIRT1 inhibition prevented the RES-mediated reduction in cholesterol buildup. Inhibiting FoxO1 but not SIRT1 reduced FoxO1 phosphorylation and increased cholesterol buildup in cultured chondrocytes. Additionally, in vivo experiments have shown that RES can alleviate cholesterol buildup and pathological changes in OA cartilage. Our findings suggest that RES regulates cholesterol buildup in osteoarthritic articular cartilage via the SIRT1/FoxO1 pathway, thereby improving the progression of OA.

Introduction

Osteoarthritis (OA) is an age-related condition of the articular cartilage that can cause chronic pain and limit joint function. Current treatments do not reverse the changes in OA but only reduce joint pain. Historically, OA has been considered a degenerative joint disease largely associated with sex, prior injuries, and age. It is related to metabolic disorders that speed up the progression of OA pathology. This strengthens the association between OA and metabolic syndrome. Cholesterol buildup in chondrocytes plays a significant role in the pathogenesis of OA and induces the chondrocyte OA phenotype. Chondrocytes with abnormal metabolism play a role in the pathophysiology of OA. Therefore, regulating cholesterol metabolism, particularly in articular cartilage, might be a potential target for treating OA. Cholesterol uptake, synthesis, and efflux are the key pathways of cholesterol metabolism that prevent cholesterol buildup in cells. Cholesterol metabolism is important for maintaining cellular homeostasis.

Silent information regulators are essential in gene sequencing, genome instability, and cell lifespan. Forkhead transcription factor (FoxO) 1 expression is highest in chondrocytes and significantly regulates growth, senescence, and inflammatory stress. The FoxO1 binding site lies in the promoter region of the cholesterol synthesis gene SREBP-2, which promotes the phosphorylation of FoxO1 and upregulates
SREBP-2 gene expression. FoxO1 expression was reduced in the hepatocytes of hyperlipidemic rats, which improved SREBP-2 gene expression and increased cholesterol synthesis. In contrast, after SIRT1 was activated, FoxO1 expression increased, SREBP-2 expression decreased, and hepatocyte cholesterol synthesis was inhibited. The inhibition of HCV replication significantly increased the expression of SIRT1 and enhanced FoxO1 activity in Huh-7.5 cells carrying HCV replicons, downregulating the transcription of SREBP-2 and other genes and lowering lipid synthesis. However, the relationship between cholesterol buildup in OA cartilage and the SIRT1/FoxO1 pathway has not been reported yet. Therefore, abnormalities in chondrocyte cholesterol metabolism may play a role in the pathophysiological development of OA. Accordingly, targeted modulation of the SIRT1/FoxO1 pathway is a potential approach to regulate cholesterol buildup in OA cartilage.

RES is associated with alterations in cholesterol metabolism. Given that cholesterol buildup in chondrocytes is highly correlated with OA development, we hypothesized that resveratrol might affect cholesterol metabolism in articular cartilage via the SIRT1/FoxO1 pathway, which would exert beneficial effects on OA. The study aimed to investigate the mitigating impact and mechanisms of RES on cholesterol buildup in OA chondrocytes.

Results

1 Influence of RES on chondrocyte viability

We tested the effects of RES on normal chondrocytes and IL-1β-induced chondrotoxicity using the CCK-8 kit. Cells were treated with different concentrations of RES for 24 hours. The maximum RES concentration that was not cytotoxic, i.e., cell survival > 90%, was selected for subsequent experiments, and the most suitable RES concentration was 50uM based on the experimental results (Fig. 1A).

2 Correlation between cholesterol buildup in OA chondrocytes and validation of the SIRT1-FoxO1 pathway based on clinical specimens

We collected cartilage tissues from three patients with OA and three with femoral neck fractures. After H&E staining, the fracture group’s four-layer cartilage structure was visible, and the chondrocytes were arranged neatly. The cartilage surface was continuously damaged and significantly lost and eroded in the OA group, leading to the formation of cracks (Fig. 2A). The p-FoxO1 protein expression was detected by immunofluorescence (Fig. 2E and F). Chondrocyte p-FoxO1 expression was significantly lower in the OA group than in the control group. We examined the expression levels of SIRT1, FoxO1, p-FoxO1/FoxO1, and cholesterol metabolism-related genes (HMGCR, SREBP2, ABCA1, ApoA1, and LXRα) (Fig. 2B, C, and D). SIRT1, FoxO1, p-FoxO1, ABCA1, ApoA1, and LXRα expression levels were lower in the chondrocytes of the OA group. HMGCR and SREBP2 had significantly higher expression levels. Further, total and free cholesterol kits were used to measure chondrocyte cholesterol levels. The OA group’s chondrocytes had significantly higher cholesterol levels (Fig. 2G and H). In summary, the SIRT1/FoxO1 pathway regulates...
cholesterol metabolism in OA chondrocytes, and there is a correlation between cartilage cholesterol buildup and the development of OA.

3 Resveratrol Improves The Phenotype Of Il-1β-induced Chondrocytes Via The Sirt1-foxo1 Pathway

The expression levels of SIRT1 and p-FoxO1/FoxO1 were significantly reduced in IL-1β-induced chondrocytes, while the addition of RES significantly increased the expression levels (Fig. 3A-D). This suggests that RES activates the SIRT1/FoxO1 pathway.

We investigated the expression levels of SOX6, COL-II, ADAMTS5, and MMP-13, which are associated with chondrocyte phenotype, to explore further the role of the SIRT1-FoxO1 pathway on chondrocyte phenotype. IL-1β significantly reduced SOX6 and COL-II levels while increasing MMP13 and ADAMTS5 levels (Fig. 3A-B and E-H). The addition of RES significantly reduced the effect of IL-1β on chondrocytes (Fig. 3A-B and E-H). The protective effect of RES on chondrocytes was significantly reduced when RES and EX-527 or AS were added (Fig. 3A-B and E-H). Therefore, RES can effectively alleviate the IL-1β-induced chondrocytes phenotype by activating the SIRT1/FoxO1 pathway.

4 Resveratrol Inhibits Il-1β-induced Chondrocytes Cholesterol Synthesis Via The Sirt1-foxo1 Pathway

We investigated the effects of RES on cholesterol metabolism in chondrocytes to determine the mechanisms underlying the protective effects of RES on OA chondrocytes. IL-1β significantly promoted the expression of cholesterol synthesis-related factors SREBP-2 and HMGCR (Fig. 4A-E). Following IL-1β-induced chondrocyte addition of RES, RES significantly reversed the effects of IL-1β on chondrocyte cholesterol synthesis (Fig. 4A-E). The reversal of IL-1β-induced chondrocytes cholesterol synthesis was significantly reduced by the addition of RES and EX-527 or AS (Fig. 4A-E). Therefore, RES can effectively ameliorate IL-1β-induced chondrocytes cholesterol synthesis via the SIRT1-FoxO1 pathway.

5. Resveratrol inhibits IL-1β-induced chondrocytes cholesterol efflux via the SIRT1-FoxO1 pathway

IL-1β significantly inhibited the expression of cholesterol efflux-related factors ABCA1, ApoA1 and LXRa (Fig. 5A-G). RES significantly reversed the effects of IL-1β on chondrocyte cholesterol efflux following IL-1β-induced chondrocyte addition of RES (Fig. 5A-G). The reversal effect of RES on IL-1-induced chondrocytes cholesterol efflux was significantly reduced by the addition of RES and EX-527 or AS (Fig. 5A-G). The above results were also verified by measuring the total cellular and free cholesterol (Fig. 5H and I) levels. Therefore, RES could reduce IL-1β-induced chondrocytes free and total cholesterol levels. In conclusion, RES can effectively ameliorate IL-1β-induced chondrocytes cholesterol accumulation via the SIRT1-FoxO1 pathway.
6.res Ameliorates Oa Development In Dmm Mice Models

According to the HE and SO staining experiments results, cartilage in the DMM group showed significant erosion, proteoglycan loss, and chondrocyte hypertrophy (Fig. 6A and B). The cartilage in the RES group had a smoother surface and experienced less proteoglycan loss than the DMM group (Fig. 6A and B). Additionally, the DMM experimental groups’ OARSI score decreased significantly after RES treatment (Fig. 6C). This was consistent with the results of H&E and SO staining.

To further verify the protective effect of RES in vivo, we detected the expression of COL-II, MMP13, SREBP-2 and SIRT1 by immunohistochemistry. The analysis of immunohistochemistry results indicated that the number of MMP13 and SREBP-2 positive cells was higher whereas the number of COL-II, LXRα and SIRT1 positive cells was lower in the DMM group compared with the Sham group (Fig. 7A- E). After treatment with RES, the number of MMP13 and SREBP-2 positive cells was lower, while the number of COL-II and LXRαpositive cells was higher (Fig. 7A- E). Additionally, the cholesterol content in chondrocytes was determined, and the results showed that the cholesterol content of cartilage in the RES group was significantly lower than that in the DMM group (Fig. 7F and G). The results showed that RES reduced the accumulation of cholesterol in the cartilage of OA mice via the SIRT1/FoxO1 pathway, thereby alleviating the progression of OA.

Discussion

The prevalence of OA is increasing every year, and it affects a significant portion of the elderly population globally. Serum cholesterol levels are associated with OA in humans. However, the role of cholesterol metabolism in the development of OA is unclear. Orthop et al. found that the pathogenesis of OA was not merely a localized lesion of the articular cartilage but a systemic metabolic abnormality. Furthermore, Clin et al. reported that cellular cholesterol buildup affects the function and structure of cell membranes and can even cause cell lysis.

In the present study, an in-depth investigation of the clinical specimens of OA showed that SIRT1 and p-FoxO1 were downregulated in OA chondrocytes and caused cholesterol buildup in OA chondrocytes, indicating that the SIRT1-FoxO1 pathway is involved in cholesterol buildup in chondrocytes. Notably, RES reduced IL-1β-induced cholesterol accumulation and OA phenotype in chondrocytes by activating the SIRT1-FoxO1 pathway. Therefore, RES can reverse cholesterol accumulation in OA chondrocytes by activating the SIRT1-FoxO1 pathway, thereby improving the OA degradation.

RES is a strong agonist of SIRT1, and RES has a positive effect on OA. SIRT1 regulates FoxO1 expression in the skeletal muscle and adipocytes, and FoxO1 is highly expressed in cartilage. We report SIRT1-FoxO1 to be closely associated with the progression of OA, given that its expression was significantly reduced in the cartilage tissue of patients with OA. Furthermore, RES promoted IL-1β-induced COL-II and SOX6 in chondrocytes and inhibited the expression of MMP13 and ADAMTS5. COL-II and SOX6 are the major genes involved in cartilage extracellular matrix synthesis, and MMP13 and ADAMTS5 are
the major genes involved in ECM decomposition\textsuperscript{24}. RES can maintain chondrocyte matrix synthesis and inhibit matrix degradation via the SIRT1-FoxO1 pathway, thereby alleviating the OA phenotype in IL-1\textbeta-induced chondrocytes. Meanwhile, the \textit{in vivo} study results showed that RES ameliorates OA development in DMM mice models.

With the significant scientific progress achieved in recent years, the potential therapeutic effects of RES on OA have gradually been uncovered. However, most current studies on RES in OA have demonstrated that RES attenuates the inflammatory response, reduces pain sensitivity in OA, and delays articular cartilage matrix degradation by regulating autophagy and apoptosis\textsuperscript{25,26}. RES can minimize cholesterol accumulation in vascular endothelial cells, thereby improving atherosclerosis\textsuperscript{27}. Sun et al. found that \textit{SIRT1} inhibits the expression of the hepatocyte cholesterol synthesis genes \textit{SREBP-2} and \textit{HMGCR} by promoting the phosphorylation of \textit{FoxO1}\textsuperscript{10}. \textit{LXRA} is a crucial gene involved in cholesterol efflux and regulates the expression of genes acting downstream of the cholesterol efflux pathway\textsuperscript{28}. RES plays a role in cholesterol metabolism and is also a \textit{SIRT1} agonist; therefore, we investigated the role of the SIRT1-FoxO1 pathway in regulating cholesterol accumulation in chondrocytes by RES.

As the accumulation of intracellular cholesterol causes cellular dysfunction, most cells have a strictly regulated cholesterol metabolism system to avoid increases in intracellular cholesterol levels\textsuperscript{29}. Tsezou et al. found a significant decrease in the expression levels of the cholesterol efflux genes in OA chondrocytes (\textit{LXRA}, \textit{ABCA1}, and \textit{ApoA1})\textsuperscript{6}, corroborating our results. The expression levels of cholesterol synthesis genes (\textit{SREBP-2} and \textit{HMGCR}) in OA chondrocytes increased, implicating the disruption of cholesterol metabolism as an important factor in the development of OA. To explore the protective mechanisms of RES in chondrocytes, we investigated the role of RES in gene expression related to cholesterol synthesis and efflux. With the addition of RES, cholesterol efflux was promoted via the activation of the SIRT1-FoxO1 signaling pathway, and cholesterol synthesis was inhibited. Moreover, the addition of inhibitors EX-527 and AS enhanced cholesterol synthesis and attenuated cholesterol efflux considerably. RES could regulate the expression of chondrocyte cholesterol synthesis and efflux-related genes and ameliorate cholesterol accumulation in IL-1\textbeta-induced chondrocytes. Additionally, we investigated the effects of RES on total and free cholesterol levels in chondrocytes to verify the above results. RES alleviated cholesterol buildup in IL-1\textbeta-induced chondrocytes via the SIRT1-FoxO1 pathway. In conclusion, our experimental results strongly indicated that RES ameliorates cholesterol accumulation in IL-1\textbeta-induced chondrocytes via the SIRT1-FoxO1 signaling pathway, thereby improving the OA phenotype of chondrocytes.

DMM is a commonly used \textit{in vivo} OA model. The DMM group showed articular cartilage erosion, cell hypertrophy, massive proteoglycan loss, and cholesterol buildup in our study. Nonetheless, all these phenomena significantly improved after RES treatment. These \textit{in vivo} and \textit{in vitro} findings suggest that RES can reduce cartilage cholesterol accumulation in OA cartilage through the SIRT1/FoxO1 pathway, thereby alleviating the progression of OA. (Fig. 8).
Conclusion

The results of this study suggest that RES improves IL-1β-induced chondrocyte cholesterol buildup in chondrocytes by activating the SIRT1-FoxO1 pathway, which attenuates the IL-1β-induced OA phenotype. Furthermore, our in vivo results suggest that RES attenuates cholesterol buildup in the cartilage of the DMM model, thereby delaying the progression of OA. These results provide a theoretical basis for RES treatment of OA.

Materials And Methods

Human cartilage tissue

Three male and three female patients with OA or femoral neck fractures (mean age 70.59 ± 2.57 years) who underwent arthroplasty for cartilage tissue were used as the control and OA groups, respectively. The cartilage tissue was fixed in 4% paraformaldehyde, embedded and sectioned in paraffin, and each section was stained with hematoxylin and eosin (H&E) to assess the degree of cartilage destruction. Articular cartilage tissue was cut into small pieces, digested, and the obtained chondrocytes were cultured. The first generation of cultured chondrocytes was used in all the experiments. The Ethics Committee approved the study at Wuhan University, and each participant provided informed consent.

Animal Models

Forty-five 10-week-old C57BL/6 female mice were obtained from Rat Leprechaun, China. The rats in this study had free access to normal food and water prior to any experimental procedure. The experimental animal procedures adhered to the National Institutes of Health guidelines for the use of laboratory animals and were approved by the Animal Use Committee of the Renmin Hospital of Wuhan University. Mice underwent surgery for medial meniscal instability (DMM), producing an animal OA model. The mice were anesthetized by injecting 30 mg/kg of 2% (w/v) pentobarbital. The medial meniscus was then removed after dissecting the medial capsule of the right knee. Incisions were made in the medial meniscus and tibial ligament. Microsurgical scissors were used. A sham operation, consisting of an arthroscopy without cutting the medial meniscus ligament, was performed simultaneously to serve as a control group. Mice were randomly divided into three groups. Sham group (n = 15), DMM group (n = 15), and RES + DMM group (n = 15). Mice in the DMM and Sham groups were injected with normal saline immediately after surgery, while mice in the RES + DMM group were intraperitoneally injected RES (50 mg/kg/day) in saline. Mice were killed at the end of week eight using an overdose of sodium pentobarbital.

Chondrocyte Culture
Mice (Shulb, Wuhan, China) were sacrificed, and bilateral knee cartilage tissue was extracted. The cartilage tissue was cut with scissors in a sterile laboratory environment, after which it was soaked in 0.1% trypsin (Beyotime, Shanghai, China) for 1 h and cultured in a cell culture chamber overnight. The digested cells were collected and resuspended in a DMEM/F-12 medium (BI, Kibbutz Beit Haemek, Israel). The chondrocytes were treated with the SIRT1 inhibitor EX-527 (10 µM/mL), the FoxO1 inhibitor AS (1 µM/mL), and resveratrol (50 µM) for 6 h to inhibit the SIRT1/FoxO1 signaling pathway.

**Histological Evaluation**

Cartilage tissue was fixed with 4% paraformaldehyde and decalcified with 10% ethylenediamine tetraacetic acid solution for a month before histological analysis. Decalcified tissue was embedded in paraffin, the paraffin was sectioned, and the sections were stained with H&E and saffron O. The cellular morphology of cartilage and subchondral bone was assessed using the Osteoarthritis Research Society International scoring system. In addition, COL-II, MMP-13, LXRα and SREBP-2 were detected by immunohistochemistry. The sections were de-paraffined and rehydrated. The sections were then incubated with primary antibodies for COL-II (1:200), MMP13 (1:200), LXRα and SREBP-2 overnight at 4°C. After washing, the sections were incubated with the corresponding secondary antibodies for 2 hours at room temperature. Positive cells showing brownish-yellow color in the cytoplasm or nucleus after DAB (Zsbio, China) and hematoxylin staining were observed. The number of positively stained cells and their percentage on the joint surface were counted. Fifteen mice per group were used for histological evaluation.

**Rt-qpcr**

The expression levels of COL-II, ADAMTS5, MMP13, SOX6, SIRT1, FoxO1, ABCA1, ApoA1, GAPDH, SREBP-2, and HMGCR were analyzed. Total cellular RNA was extracted using a TRizol reagent (Ambion, China). cDNA was synthesized using a cDNA synthesis kit (VAZYME, Nanjing, China). The target genes were multiplied using the SYBR Premix ExTaq kit (VAZYME, Nanjing, China). The mRNA levels were normalized to endogenous GAPDH, and the target gene expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method. The primers used are provided in Table 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat GAPDH</td>
<td>5′-ACAGCAACAGGGTGTTGAGC-3′</td>
<td>5′-TTTGAGGCTGCAGCGAACC-3′</td>
</tr>
<tr>
<td>Rat COL-II</td>
<td>5′-ATGTTACCCTCAGCCACC-3′</td>
<td>5′-GACAAGGCTTGAGGGCAGC-3′</td>
</tr>
<tr>
<td>Rat MMP13</td>
<td>5′-CCCGAGAGCTCATGTTATCTC-3′</td>
<td>5′-CTTCTTCTCATAGGCGGAGGGG-3′</td>
</tr>
<tr>
<td>Rat Foxo1</td>
<td>5′-CCATGCCTCACATCTGCG-3′</td>
<td>5′-TTTTATCCAGGTATCCTCCG-3′</td>
</tr>
<tr>
<td>Rat APOA1</td>
<td>5′-AACGCGGAGGATGCAAAAG-3′</td>
<td>5′-AGGGTGGTCTTGTCTGCTG-3′</td>
</tr>
<tr>
<td>Rat LXRα</td>
<td>5′-GTGCCCTGATGTTCCTCTCAG-3′</td>
<td>5′-ATACACTGCATAGCTCGTTCC-3′</td>
</tr>
<tr>
<td>Rat ABCA1</td>
<td>5′-GCAGCGGACCATGAAAGTGAC-3′</td>
<td>5′-GAGGCCGTCATCAATCTCCG-3′</td>
</tr>
<tr>
<td>Rat SREBP-2</td>
<td>5′-CGAAGTTGGCGATCGAGAAGA-3′</td>
<td>5′-TCTCCACTAGTTGCTGACA-3′</td>
</tr>
<tr>
<td>Rat HMGCR</td>
<td>5′-CCTCCATTGAATGATCCAGG-3′</td>
<td>5′-ACCCGTTATCGTGAGGATG-3′</td>
</tr>
<tr>
<td>Rat SIRT1</td>
<td>5′-TGCCATGATCAGCCAGAGA-3′</td>
<td>5′-CATCGCAGTCTCCAGAAGC-3′</td>
</tr>
<tr>
<td>Rat ADAMTS5</td>
<td>5′-CACCAGAGCCAGCATAGG-3′</td>
<td>5′-TTTAACTCAAGCTGCTGCGG-3′</td>
</tr>
<tr>
<td>Rat SOX6</td>
<td>5′-CTTCCAGTGATGCTCCTACCC-3′</td>
<td>5′-CTGCGTGGCATGTCCCACTA-3′</td>
</tr>
<tr>
<td>Homo GAPDH</td>
<td>5′-TCAGAAAGGTGGTGAGGAGG-3′</td>
<td>5′-ATCAAGGTGAGGTGGGCT-3′</td>
</tr>
<tr>
<td>Homo SIRT1</td>
<td>5′-AGCAGATTAGTAGGCGGCTT-3′</td>
<td>5′-GACTCTGGGATGTCCCCACTA-3′</td>
</tr>
<tr>
<td>Homo ABCA1</td>
<td>5′-TGCTCCCTCCACAAAGAAAAA-3′</td>
<td>5′-CCGCCCACATACCTAATCCATCAC-3′</td>
</tr>
<tr>
<td>Homo APOA1</td>
<td>5′-GACAGCTGAGCTCACCTCCTT-3′</td>
<td>5′-ATCTCCCTGCTGACCATTTCTC-3′</td>
</tr>
<tr>
<td>Homo LXRα</td>
<td>5′-CCTCGGGCTTCCACTACAATGTT-3′</td>
<td>5′-TCTCTGGCGCTGCTCCATCT-3′</td>
</tr>
<tr>
<td>Homo SREBP2</td>
<td>5′-GGTGAACATCTGAGCCAAGAAAGA-3′</td>
<td>5′-CCCACAGAGTTCCACAAAGGA-3′</td>
</tr>
<tr>
<td>Homo HMGCR</td>
<td>5′-GGTTTCCTGCTGCCAGTTTATCTC-3′</td>
<td>5′-TCTTCTGCTGCTTCCTGCTC-3′</td>
</tr>
<tr>
<td>Homo FOXO1</td>
<td>5′-TGAAACGAGCAACTTCAAGAC-3′</td>
<td>5′-ATAAAAGAGAAACAGAGTGAGACT-3′</td>
</tr>
</tbody>
</table>
Western Blotting

Western blot analysis was used to analyze the protein expression levels of COL-II, ADAMTS5, SOX6, MMP13, SIRT1, FoxO1, p-FoxO1, ABCA1, ApoA1, LXRx, SREBP-2, and HMGCR in the chondrocytes. The cells were cultured for 24 h, after which the chondrocytes were lysed using cell lysate to obtain proteins; the target protein content was analyzed using a bicinchoninic acid assay kit (Bain-marie, China). The collected proteins were stored at −20°C. The target proteins were electrophoresed separately, transferred, immersed in a warm blocking buffer for 1 h, incubated with a primary antibody for 6 h, and incubated with a secondary antibody (horseradish peroxidase-labeled goat anti-rabbit, Wuhan Bost Biotech Co., Ltd.) (1:50,000) for 1 h. The strip was developed after the final wash. GAPDH was used as the standard endogenous protein. ImageJ was used to convert the image to grayscale. The test was performed in triplicate. The antibodies used are provided in Table 2.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
<th>Catalog</th>
<th>Dilution ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL-II (142KD)</td>
<td>Affinity</td>
<td>AF0135</td>
<td>1:1500</td>
</tr>
<tr>
<td>ADAMTS5 (72KD)</td>
<td>Affinity</td>
<td>DF7470</td>
<td>1:1000</td>
</tr>
<tr>
<td>SREBP-2 (124KD)</td>
<td>Affinity</td>
<td>DF7601</td>
<td>1:2000</td>
</tr>
<tr>
<td>HMGCR (97KD)</td>
<td>Abcam</td>
<td>Ab174830</td>
<td>1:4000</td>
</tr>
<tr>
<td>SIRT1 (120KD)</td>
<td>PROTEINTECH GROUP</td>
<td>60303-1-Ig</td>
<td>1:5000</td>
</tr>
<tr>
<td>FoxO1 (74KD)</td>
<td>ABclonal</td>
<td>A2934</td>
<td>1:1000</td>
</tr>
<tr>
<td>p-FoxO1 (78KD)</td>
<td>Affinity</td>
<td>AF3416</td>
<td>1:1000</td>
</tr>
<tr>
<td>SOX6 (49KD)</td>
<td>Abcam</td>
<td>ab195966</td>
<td>1:1000</td>
</tr>
<tr>
<td>MMP-13 (54KD)</td>
<td>Abcam</td>
<td>Ab39012</td>
<td>1:3000</td>
</tr>
<tr>
<td>GAPDH (37KD)</td>
<td>GOODHERE</td>
<td>AB-P-R 001</td>
<td>1:1000</td>
</tr>
<tr>
<td>ABCA1 (254KD)</td>
<td>Abcam</td>
<td>Ab66217</td>
<td>1:1000</td>
</tr>
<tr>
<td>APOA1 (31KD)</td>
<td>Affinity</td>
<td>DF6264</td>
<td>1:1000</td>
</tr>
<tr>
<td>LXRx (50KD)</td>
<td>Affinity</td>
<td>DF6864</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Cell Counting Kit-8 (CCK-8) assay for cell activity

Normal chondrocytes and IL-1β-induced chondrocytes were placed in 96-well plates overnight. They were treated with various RES concentrations and incubated at 37°C for 24 h. The cell viability was then
determined after the CCK-8 solution was added to the cell culture wells, and the incubation was continued for 4 h. The test was performed in triplicate.

**Measurement Of Cellular Free And Total Cholesterol Levels**

Kits for detecting free and total cholesterol in cells were purchased from Solarbio Bioscience &Technology (Shanghai, China) and Applygen Ltd. (Beijing, China). The specific procedures were carried out following the instructions provided with the kits.

**Immunofluorescence Staining**

The chondrocytes were first fixed in 4% paraformaldehyde for 20 min and then permeabilized with phosphate-buffered saline with Tween for 15 min. Diluted target protein \( p\text{-FoxO1} \) primary antibody (Affinity, AF3416) (1:1000) was added drop by drop, and the cells were incubated at 4°C overnight. Goat anti-rabbit immunoglobulin (Ig)G antibody (Affinity, AF0135) (1:5000) was added drop by drop, and the cells were incubated at 37°C for 1 h. Further, a DAPI solution was added, and an inverted fluorescence microscope was used to capture the images. The test was performed in triplicate.

**Reagents**

Resveratrol (RES, No. R107315, 99% purity), Selisistat (EX-527, No. S1541, 99% concentration), and AS1842856 (AS, No. 88222, 99% concentration) were obtained from Maclean Biotech (Shanghai, China).

**Statistical analysis**

Each test was repeated three or more times. Data are presented as mean ± standard error of the mean. The data were processed with GraphPad Prism 8.0. A one-way analysis of factors was used to analyze differences between multiple groups, and a \( p\)-value < 0.05 was considered statistically significant.

**Declarations**

**Acknowledgments**

This research was supported by the “National Natural Science Foundation of China (81071494)” and “Science and Technology Department of Hubei Province (2015BCA316).”

**Ethics Approval**

The Ethics Committee approved this study of the Wuhan University Renmin's Hospital. Our experiment was conducted following the guidelines mentioned in the Declaration of Helsinki. The ethical clearance number is WDRY2020-K226.
All animals were kept in a sterile environment. The Ethics Committee approved this study of the Wuhan University Renmin's Hospital. The license number is WDRM20190517.

Author Contributions

CCL wrote the manuscript text. HTX and CYW collected samples and prepared figures. XFX analysed the data. BQ and YRH designed the experiment and revised the manuscript. All the authors contributed to the article and approved the submitted version.

Disclosure

The authors have no conflicts of interest to declare.

References


**Figures**

![Figure 1](image_url)

**Figure 1**

Influence of RES on chondrocyte viability (A) Effect of resveratrol on chondrocyte proliferation determined by the Cell Counting Kit-8 assay to select the optimal drug concentration for subsequent in vitro experiments.
Correlation between cholesterol buildup in osteoarthritis (OA) chondrocytes and the SIRT1/FoxO1 pathway based on validation of clinical specimens. (A) The extent of OA pathology was assessed by hematoxylin and eosin staining clinical OA cartilage tissue. (B-C) Protein expression of SIRT1, p-FoxO1/FoxO1, HMGCR, SREBP2, ABCA1, APOA1, and LXRα in cartilage tissues were detected. (D) Reverse transcription-quantitative polymerase chain reaction analysis of the expression of SIRT1, p-FoxO1/FoxO1,
**HMGCR**, **SREBP2**, **ABCA1**, and **LXRα**. (E, F) Immunofluorescence detection of p-FoxO1 protein content (x200) and quantitative analysis using ImageJ software. (G, H) Free and total cholesterol levels in cartilage tissues were quantified using the respective cholesterol assay kits. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.
Resveratrol ameliorates the phenotype of interleukin-1β-induced chondrocytes via the SIRT1-FoxO1 pathway. (A-B) Western blot to detect the expression levels of the chondrocyte phenotype-related genes MMP13, ADAMS5, COL-II, and SOX6. (C-H) The mRNA levels of SIRT1, p-FoxO1/FoxO1, MMP13, ADAMTS5, COL-II, and SOX6 were detected by reverse transcription-quantitative polymerase chain reaction. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, and ns: not significant.

Figure 4

Resveratrol ameliorates IL-1β-induced chondrocytes cholesterol synthesis via the SIRT1-FoxO1 pathway. (A-C) Western blot analysis of protein expression levels of SREBP-2, HMGCR. (D-E) Levels of SREBP-2 and
**HMGCR** in each group of chondrocytes were quantified by qRT-PCR. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

**Figure 5**

Resveratrol ameliorates IL-1β-induced chondrocytes cholesterol efflux via the SIRT1-FoxO1 pathway. (A-D) Western blot analysis of protein expression levels of **LXRα**, **ABCA1** and **ApoA1**. (E-G) Levels of **ABCA1**, **ApoA1** and **LXRα** in each group of chondrocytes were quantified by qRT-PCR. (H, I) Free and total cholesterol profiles of chondrocytes in each group were analyzed using the respective cholesterol assay.
kits. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

Figure 6

RES ameliorates OA development in DMM mice models. (A, B) Hematoxylin and eosin and safranin-O staining of different groups (scale bar: 300 µm). (C) OA score for cartilage. (D, E) The respective cholesterol assay kits quantified cartilage tissues’ free and total cholesterol levels. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 7

RES reverses cartilage OA phenotype by improving cholesterol accumulation in articular cartilage of DMM mice. (A) Immunohistochemical staining of COL-II, MMP13, SREBP-2 and LXRα expressions in the cartilage samples (scale bar: 20 μm). (B–E) The percentage of positive chondrocytes. (F, G) The respective cholesterol assay kits quantified cartilage tissues' free and total cholesterol levels. The values represent the mean ± standard deviation of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Resveratrol attenuates the degeneration of osteoarthritic chondrocytes by regulating the SIRT1/FoxO1-mediated cholesterol metabolism in osteoarthritic chondrocytes.