

Original Research

Effect of luteolin on apoptosis, MAPK and JNK signaling pathways in guinea pig chondrocyte with osteoarthritis

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Abstract: Osteoarthritis (OA) is a degenerative joint disease usually seen in the elderly, which incidence increases with age. Its pathogenesis and underlying mechanism are still unclear. The disease severely affects the physical health and life quality of patients, thereby constituting a huge economic burden to family and society. Luteolin (LUT) is a natural flavonoid with multiple pharmacological properties. Many plants containing LUT have been applied in the treatment of several inflammation-related diseases due to the relatively strong anti-inflammatory effects of LUT. The present study investigated the influence of LUT on cell apoptosis and inflammatory reactions in cartilage of OA guinea pigs, and its underlying mechanism. It was found that LUT effectively inhibited proliferation of OA cartilage cells, down-regulated the expressions of JNK and p38MAPK in cartilage cells of OA, and downregulated NO, TNF- α and IL-6. Thus, it alleviated inflammatory reactions, protected cartilage cells, and delayed cartilage degeneration.

Key words: Luteolin; Osteoarthritis; Chondrocytes; apoptosis; MAPK; JNK.

Introduction

Osteoarthritis (OA) is a chronic joint disease characterized by degenerative changes in joint tissue components, structure and function (1). It manifests as joint cartilage injury, with impacts on subchondral bone and surrounding structure in form of lesions of subchondral bone, osteophyte and arthromeningitis. Osteoarthritis (OA) manifests as joint pain, joint dysfunction or joint malformation. According to WHO statistics, in elderly persons aged over 60 years, about 9.6 % of males and 18 % of females suffer from OA all over the world. In the developed countries, 25 % OA patients have disabilities (2). Currently, it is believed that OA is associated with the gene s, aging and obesity. The pathogenesis and mechanism involved in OA are complicated (3). The search for effective strategies for treatment and prevention of OA, as well as the mechanism involved in its pathogenesis have continued to engage the attention of researchers. Presently, the treatment methods for OA include aerobic exercises, weight loss, and drug and surgical treatments, all aimed at alleviating pain, reducing stiffness, and improving joint function. Most of the lead drugs used are natural chemical compounds. Indeed, at present, derived drugs from over 100 natural products are on clinical trial (4). The anti-inflammatory properties of flavonoid compounds, especially LUT from plants with multiple traditional therapeutic functions have attracted a lot of attention. Luteolin (LUT, Fig.1) is a naturally occurring polyphenolic flavonoid present in many vegetables, fruits and medicinal plants in the form of glucoside. These plants are broccoli, onion leaves,

carrot, pepper, cabbage, apple peel, and chrysanthemum (5). Studies have revealed that LUT has good medicinal potential, with multiple pharmacological effects such as anti-tumor, antioxidant, anti-inflammation and anti-vascular properties (6-8). These pharmacological properties are related to its anti-inflammation effects. In Chinese *Materia Medica*, plants containing LUT are usually applied in treating inflammation-related diseases (9). A recent study showed that LUT at micromolar concentrations, produced strong anti-inflammation effects (10). However, studies on the use of LUT for treatment of osteoarthritis are scanty. The present study was aimed at exploring the influence of LUT on LPS-induced cell apoptosis and inflammatory reactions in cartilage osteoarthritis, and the mechanism involved, using LPS-induced osteoarthritis in cartilage cells in guinea pigs.

Materials and Methods

Drugs and main reagents

The drugs and reagents used, and their sources were: LUT (99% pure, Chengdu Puruifa Tech & Development

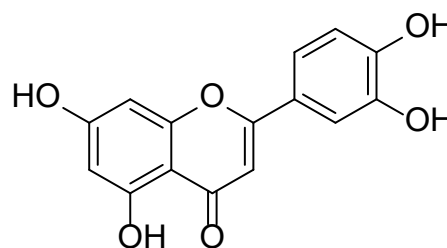


Figure 1. Chemical structure of luteolin.

Company); PLS (Sigma company), 10% fetal serum, DMEM culture medium and 0.25% trypsin (American Gibo Company); MMP-13, JNK, and P38 antibodies (Abcam). Interleukin (IL-6) kits, TNF- α kits, and nitric oxide (NO) kits were products of Beyoptime.

Establishment of in vitro culture system of cartilage cells of bone joint of guinea pigs

A guinea pig was sacrificed through decapitation according to NIH guidelines (NIH Pub No 85-23, revised 1996), and the protocol was approved by the Ethics Committee of the Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, Xi'an, China. The site of surgery was disinfected using 75% alcohol. The bilateral knee joints were excised using aseptic technique to avoid opening the joint cavity. The knee joints were soaked in alcohol for 5 min, and then transferred into PBS. Soft tissues surrounding the joint were removed on a benchtop, and the joint cavity was opened up. The soft tissues were cut and excised using aseptic surgical blade, and placed in petri dish containing PBS to avoid air-drying. Small bits of cartilage were washed three times using PBS and cut into sizes of approximately 1mm³, and then washed again three times using PBS prior to drying. The cartilage slices were put into a centrifuge tube with 0.2% type II collagenase and then placed in a shaking water bath at a temperature of 37°C for 60 min. After removing cartilage cells, the liquid was filtered using a 60 mesh filter screen. The filtrate was put into sterile centrifuge tube and centrifuged at 800 rpm for 5min at 37°C. The supernatant was discarded after centrifugation, and the turbid lower layer was collected. Then, 4ml of DMEM containing 10% fetal serum was added, blew and beat in order to acquire cell suspension of cartilage of osteoarthritis in guinea pigs. The concentration was regulated to 2×10⁵piece/ml. The suspension was transferred to 25mL culture bottle and placed in culture box with 5% CO₂ at 37°C. The culture medium was changed every 3 to 5 h. The small residual bits of cartilage were digested, centrifuged and heavily suspended until which basically disappeared using above processes continuously.

Cell grouping and drug treatment

The guinea pig cartilage cells were divided into four groups: control group, drug group, LPS + drug group, and LPS group. Then, LPS (10ng/ml) was used to stimulate the cartilage cells for 8 h to induce osteoarthritis, after which different concentrations of LUT were used to treat the cells for 24h. The control group was treated using DMSO .

Effect of LUT on cartilage cell proliferation of guinea pigs

The second generation of cartilage cells treated with PBS (2×10⁴ piece/mL, 100 μ L/well) were seeded in 96-well plates and cultured for 24 h in serum-free DMEM. The cells were incubated with different doses of LUT (0, 5, 10, 20 and 40 μ M) for 48 h. The control group contained normal cartilage cells. After 48 h, 100 μ L of 0.5% MTT was added to each well. The wells were incubated for 4 h in an atmosphere of 5% CO₂. Thereafter, the medium in each well was discarded, and replaced with 150 μ L DMSO to dissolve the formazan crystals

formed. The absorbance of each well was read at 490 nm) in an ELISA microplate reader.

Determination of cartilage cell apoptosis of guinea pigs using flow cytometry

Cell apoptosis was assayed using Annexin V-PE/7-AAD cell apoptosis test kits (Beijing Baiaolaibo Tech Co., Ltd). Cartilage cells containing 1×10⁶ piece/mL were inoculated into 6-well cell culture plate for 24h. At adherence, the culture medium was discarded and LUT was added, followed by incubation for 48h. The experimental groups and drug (LUT) doses were same as in MTT assay above. Three double-wells were set in each group. The cells were washed twice and were collected by centrifugation. Then, 0.25% Trypsin was used to digest the cells, and the digest was washed twice using PBS and suspended in 200 μ l combined buffer solution. Then, 10 μ l of 7-AAD and 5 μ l Annexin V-PE were added. After mixing for reaction to occur for 15 min away from light at room temperature, 300 μ l of combined buffer solution was added. The percentage cell apoptosis (%) was determined using flow cytometry.

Cell protein extraction and Western blot detection

Cells in various groups were washed using PBS, and then cell lysis solution containing protease inhibitors were added to extract total protein with degeneration for 5 min at 100°C. Then, equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis, followed by transfer to PVDF membrane. Non-specific binding was blocked with 5% BSA. Incubation with the corresponding primary antibodies was carried out overnight at 4°C, followed by incubation with secondary antibody labeled with horseradish peroxidase at room temperature for 1.5 h. Finally, luminescent solution was added, and gel imager was used to take exposure photos. Gray value was counted and the relative expressions of proteins were calculated.

Enzyme-linked Immunosorbent assay (ELISA)

The supernatant of cell culture to be detected was collected, and the levels of NO, TNF- α and IL-6 were determined with ELISA kits in accordance with the kit instructions.

Statistical analysis

Measurement data are expressed as mean \pm standard deviation (SD). Two-sample comparison was done with *t*-test, while comparison between multiple groups was carried out using single-factor ANOVA. Graphpad Prism 7.0 statistical software was used for statistical analysis. Statistical significance was assumed at *p* < 0.05.

Results

Influence of LUT on cartilage cell proliferation of bone joints

This study used MTT method to detect the effect of LUT on proliferation of cartilage cells. The results show that LUT inhibited the proliferation of OA cartilage cells dose-independently, but had no obvious effect on normal cartilage cells (Figure 2).

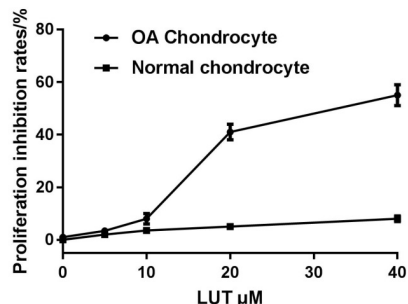


Figure 2. Inhibition of OA chondrocyte proliferation by different concentrations of LUT. OA chondrocytes or normal chondrocytes were treated with different concentrations of LUT for 48 h. Data are shown as mean \pm SD from three independent experiments.

Effect of LUT on apoptosis of osteoarthritic cartilage cells

The influence of LUT on cartilage cell apoptosis was determined using flow cytometry. The results are shown in Figure 3. Cell apoptosis in LPS group was higher than that in the control group ($p < 0.05$). Compared with the LPS group, cell apoptosis in LPS+LUT groups were significantly decreased ($p < 0.05$). These results show that LUT reduced the increase in cartilage cell apoptosis due to LPS-induced OA.

Effect of LUT on MAPK and JNK-related protein expression in osteoarthritic cartilage cells

The effect of LUT on the expressions of JNK, P38 and MMP-13 cartilage cells of OA were assayed using Western blot. The results shown in Figure 4 indicate that JNK, P38 and MMP-13 expression levels in LPS group were significantly higher than that in the control group ($p < 0.001$). The expression levels of JNK, P38 and MMP-13 were significantly decreased in the LPS+LUT, when compared with LPS group ($p < 0.01$).

LUT lowered cartilage cell inflammatory reactions in LPS-induced osteoarthritis

The effect of LUT on levels of the Inflammatory factors NO, TNF- α and IL-6 levels was determined in LPS-induced OA cartilage cell using ELISA. As seen in Figure 5, NO, TNF- α and IL-6 levels in LPS group were significantly higher than those in the control group, while NO, TNF- α and IL-6 levels in LPS+LUT groups were significantly lower than those of LPS group ($p < 0.01$ or $p < 0.001$). These results show that LUT lowered inflammatory reactions in LPS-induced osteoarthritic cartilage cells.

Discussion

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by joint cartilage damage, remodeling of subchondral bone, osteophyte formation, and inflammatory changes in surrounding tissues of the joints, leading to chronic pain (11). The disease severely affects the physical health and life quality of patients, thereby bringing huge economic burden to family and society (12). The pathogenesis of the disease is still unclear. However, it is generally believed that it may be linked to a combination of factors such as age, obesity, trauma, inflammation, and genetic susceptibility (3). In

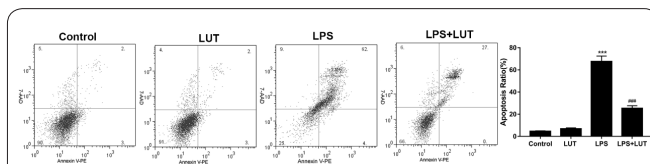


Figure 3. Effect of LUT on chondrocyte apoptosis in osteoarthritis. The experimental groups were: Control group, 20 μ M LUT group, 10 ng/ml LPS group and 10 ng/ml LPS + 20 μ M LUT group. Data represent mean \pm SD of at least three independent experiments. *** $p < 0.001$, relative to control group; ### $p < 0.001$, relative to LPS group.

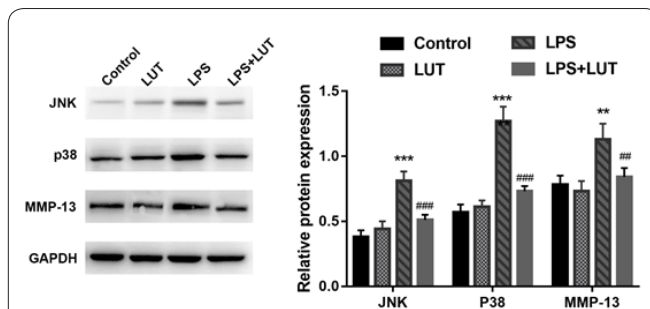


Figure 4. Effect of LUT on the MAPK and JNK signaling pathway-related protein expressions in osteoarthritic chondrocytes. The experimental groups were: Control group, 20 μ M LUT group, 10 ng/ml LPS group, and 10 ng/ml LPS + 20 μ M LUT group. ** $p < 0.01$, *** $p < 0.001$, compared to control group; ### $p < 0.01$, compared to LPS group.

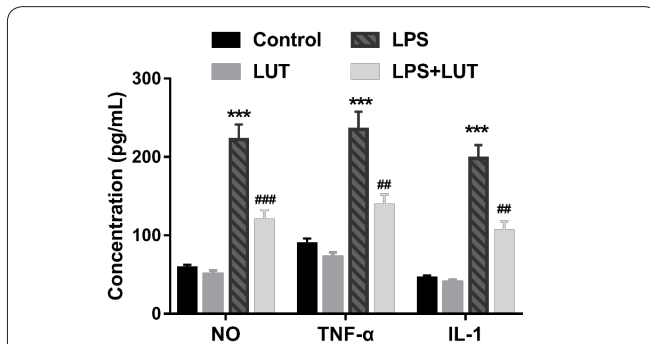


Figure 5. Effect of LUT on levels of inflammatory factors, as measured using ELISA. *** $p < 0.001$, compared with control group; ## $p < 0.01$, ### $p < 0.001$, compared to LPS group.

addition, it may be due to biological factors which increase degradation of cartilage and imbalance in synthesis and coupling of cartilage cells, extra-cellular matrix, and subchondral bone (3). Luteolin (LUT), a flavonoid compound present in many medicinal plants, is one of the most plentiful secondary metabolites in plants. Multiple pharmacological and clinical trials have shown that LUT has multiple pharmacological effects such as anti-tumor, bacteriostatic, anti-virales, and analgesic effects (7, 13-15). Cartilage cell apoptosis is positively correlated with severity of osteoarthritis. Cartilage cells significantly decrease with age (16). Therefore, cartilage cell apoptosis plays an important role in osteoarthritic lesion characterized by joint cartilage degeneration and extra-cellular matrix degradation. Thus, arrest and reduction of cartilage cell apoptosis are the effective methods for treating osteoarthritis (17). In this study, cartilage cells were treated with LUT of different concentrations and its effect on cell proliferation was determined using MTT. The results showed that LUT inhibited the pro-

liferation of OA cartilage cells in a dose-independent manner. Cell apoptosis assay using flow cytometry showed that LUT promoted OA cell apoptosis, but had no significant effect on normal cartilage cells.

The MAPKs signal pathway is the most important signal transduction system that mediates cartilage damage in osteoarthritis (18). Activation of p38 MAPK signal is closely linked to multiple biological effects such as apoptosis, hypertrophy and calcification (19). Studies show that the main pathological change in osteoarthritis is degenerative degradation of extra-cellular matrix which causes progressive loss of cartilage components and destruction of chondrocyte structure and function (20). These changes are manifested in the joints. Inflammatory cytokines (e.g. interleukin-6 and tumor necrosis factor - α), growth factor activate the MAPKs signal transduction pathway in cells by combining with receptors on the cell membrane, leading to a series reactions such as increase in expressions of matrix metalloproteinases (MMPs), cartilage cell apoptosis and cartilage damage (21).

In the process of inflammation, p38 MAPK, an important signal pathway that induces MMP-13 expression, causes progressive degradation of type II collagen and promotes cartilage damage. The P38 signal pathway is one of the upstream NO-induced cartilage cell apoptosis signal pathways (22). Studies have shown that JNK leads to matrix degradation of cartilage cells by transcription factors in the downstream participating in regulation of MMP-3 and MMP-13 increment expression. Thus, inhibition of JNK pathway blocks NO-induced cartilage cell apoptosis (23). In this study, it was found that LUT lowered protein expression in OA cartilage cells, and reduced the increases in levels of NO, TNF- α and IL-6 as well as inflammatory reactions; protected cartilage cells, and delayed cartilage degeneration.

Cartilage cell proliferation in osteoarthritis in guinea pigs is closely associated with the expressions of JNK and genes. The less the gene expression of JNK and p38 MAPK, the less the proliferation of OA cartilage cells. Luteolin (LUT) effectively down-regulates JNK and p38 MAPK gene expressions in osteoarthritic cartilage cells in guinea pigs, and reduces the expressions of NO, TNF- α and IL-6. Thus, LUT has promising potential as an effective therapy for osteoarthritis.

Acknowledgements

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Conflict of Interest

There are no conflict of interest in this study.

Author's contribution

All work was done by the author s 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 Jianli Xue; Jianli Xue, Jintao Ye, Zhuqing Xia, Bin Cheng collected and analysed the data; Jianli Xue wrote the text and all authors have read and approved the text prior to publication.

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