Icariin interferes with TDP43-induced inflammatory factor secretion and inhibits the JNK and p38 MAPK signaling pathway *in vitro*

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Abstract

Introduction: To investigate the molecular mechanism of icariin (ICA) intervention in TAR DNA binding protein 43 (TDP-43) mediated chondrocyte lesions of osteoarthritis.

Material and methods: Human articular chondrocytes (HC-α) were transfected with TDP-43 lentiviruses to generate TDP-43-overexpressing chondrocytes and treated with 5 μg/ml icariin. The levels of TDP-43, JNK, p38 MAPK and relative factors were detected by Western blotting assays. Tumor necrosis factor α (TNF-α) and interleukin (IL)-1β in the supernatant were determined by ELISA.

Results: Compared with the HC-α group, TDP-43 expression was significantly higher in the TDP-43-HC-α group and was not significantly different that of the HC-α + ICA group. However, TDP-43 expression in the TDP-43-HC-α + ICA group was significantly lower than that in the TDP-43-HC-α group. ELISA showed that the secretion of TNF-α and IL-1β in the supernatant of the TDP-43-HC-α group was significantly higher ($p < 0.01$) compared with the HC-α group, but was significantly lower in the supernatant of the TDP-43-HC-α + ICA group than that of the TDP-43-HC-α group ($p < 0.01$). ICA treatment reduced the expression of TDP-43 in chondrocytes and inhibited the elevation of inflammatory cytokines caused by TDP-43. ICA processing can also inhibit the activation of JNK/p38 MAPK related signaling pathways caused by TDP-43. Overexpression of TDP-43 reduced the formation of stress granules (SGs) in chondrocytes, and increased the receptor for activated protein kinase C1 (RACK1) level. ICA could reverse these changes.

Conclusions: Icariin could interfere with TDP-43-induced secretion of inflammatory factors and inhibit JNK/p38 MAPK signaling. Our findings provided a new theoretical basis for the treatment of osteoarthritis.

Key words: TDP-43, JNK, p38 MAPK, icariin.

Introduction

Osteoarthritis (OA) is a common chronic degenerative disease and a main contributor to joint pain and disability in the elderly [1]. The occurrence and development of OA are closely related to inflammatory cells and cytokines [2]. In OA, the main cause for the secretion of inflammatory factors is mechanical wear of cartilage and instability of intra-articular structure, which can cause inflammatory pain in the joints. When inflammation develops in the subchondral bone, ligament, synovium and other

tissues rich in nerve endings, the peripheral nerve around the knee joint is damaged [3]. When the nerve is damaged, the glial cells are activated and secrete inflammatory factors, thus aggravating inflammation and forming a vicious cycle [4].

Inflammation is a cellular stress response, in which endoplasmic reticulum stress promotes the synthesis of TAR DNA binding protein 43 (TDP-43) [5]. TDP-43 is a common nucleoprotein and is the binding protein of type I TAR DNA elements. When cells are stimulated, TDP-43 accumulates in the cytoplasm and leads to apoptosis. When TDP-43 is disabled, it will affect apoptosis and nuclear membrane stability through phosphorylation of pRb [6]. However, the relationship between the expression of TDP-43 and chondrocyte lesions in OA has been relatively little studied.

When the cell is stimulated by the external environment, it can survive through the activation of the defense mechanism. It also may potentially cause apoptosis, which mainly depends on the type of external environmental stimulus [7]. When cells are stimulated by genotoxic drugs or X-rays, apoptosis is triggered by activation of the JNK and p38 MAPK signaling pathways [8]. The inflammatory response is an emergency factor of the cell to the external stimulus, and may lead to apoptosis.

OA is called "bi syndrome" in Chinese medicine, and its main cause is deficiency of kidney essence. Therefore, the pathological manifestations of OA mainly include deficiency of kidney qi, absence of blood flow, and stagnation of blood stasis [9]. In the pathogenesis of OA, kidney deficiency and blood stasis are the main factors. Therefore, traditional Chinese medicine (TCM) is often used to treat OA by supplementing the kidney and promoting blood circulation. The combination of blood circulation and kidney tonification can make kidney gas flourish and the whole body's channels and collaterals open, thus preventing the occurrence and development of lesions [10]. Among many drugs for invigorating the kidney and activating blood, icariin has been widely studied for its functions of nourishing the kidney and strengthening Yang, removing wind and dampness, and treating OA.

Icariin, a flavonoid monomer, is an effective chemical component with strong physiological activity in epimedium. Li *et al*. confirmed that epimedium can increase the secretion of osteoblast growth factor, thus promoting the proliferation and differentiation of osteoblasts, and also inhibit osteoclasts [11]. Recent studies have also confirmed that icariin can promote osteoblast differentiation of bone marrow stem cells (BMSCs) in primary culture to some extent [12]. However, in different research reports, the mechanism of icariin promoting bone formation is still very different. Zhang *et al*. found that icariin can promote the expression of nuclear transcription factor Cbfa1 through activation of the ERK/MAPK and p38/MAPK signaling pathways in osteoblasts [13]. In the experiment of ICA intervention on MT3T3-E1 osteoblasts, it was found that the promotion of proliferation and differentiation of osteoblasts by ICA was related to the estrogen-mediated JNK and ERK signaling pathway [14].

The treatment of OA in TCM is mainly based on kidney tonifying and blood circulation, but the specific mechanism is not clear. The therapeutic effect of invigorating the kidney and activating blood on OA may be achieved by regulating the signaling pathway of pathological changes of OA and inhibiting the degree of chondrocyte lesions. In this study, after TDP-43 lentivirus transfection of chondrocytes, icariin was used to treat the chondrocytes. Moreover, the protein expression of TDP-43 was determined, and the secretion of tumor necrosis factor α (TNF-α) and interleukin (IL)-1β was analyzed, as well as the proteins of the JNK and p38 MAPK signaling pathways. To explore the molecular mechanism of Chinese medicine icariin intervention in TDP-43-mediated inflammatory factor secretion and inhibition of the JNK and p38 MAPK signaling pathway, it is beneficial to elucidate the molecular mechanism of ICA to reverse OA, so as to provide a basis for ICA in the treatment of OA.

Material and methods

Construction of high expression TDP-43 chondrocytes

We built green fluorescence carrying (green fluorescent protein – GFP) report gene TDP-43 lentivirus vectors: the obtained TDP-43 gene is a full-length cloned plasmid and helper plasmid. A four-plasmid lentivirus packaging system was constructed. Transfection reagent Lipofectamine 2000 (Invitrogen, USA) was added to transfect a total of 293 T cells. The gene via a selective enzyme was connected to a slow virus vector, 48 h after collecting supernatant on cells that were rich in slow virus particles; after purification, enrichment of cloning sequencing identification was performed. Our project team has successfully constructed and validated TDP-43 high-expression chondrocytes (TDP-43-HC-α).

Cell culture and grouping

Human articular chondrocytes (HC-α) were purchased from the Cell Bank of the Institute of Biochemistry and Cell Biology (Shanghai, China). The untransfected HC-α and TDP-43 lentivirus transfected TDP-43-HC-α were added into the culture vials, and the cell density was adjusted to 2×10^5/ml. DMEM hyperglycemia culture medium (United States, Gibco) containing 10% fetal bovine serum

was used to incubate cells. We added 100 mg/ml penicillin and 100 mg/ml streptomycin if necessary (Gibco, USA). Icariin (ICA) was extracted from epimedium with a purity of more than 96%. The HC-α and TDP-43-HC-α of human chondrocytes were treated with 5 μg/ml of ICA each. In other words, the cells were divided into the HC-α group, TDP-43-HC-α group, HC-α + ICA group and TDP-43-HC-α + ICA group. We put cells in a 37°C and 5% CO_2 incubator, for 12 days' continuous culture, and changed the culture solution every 4 days.

ELISA

The supernatant was obtained by centrifugation on the 12th day of culture in each group of cells and maintained at –80°C. The contents of TNF-α and IL-1β in supernatant of each group were determined using ELISA kits (Beijing Zhongshan Jinqiao biotechnology co., LTD). The specific operation procedures were strictly in accordance with the kit instructions. Experiments were repeated three times.

Western blot

The protein expression levels of JNK, phosphor-JNK (p-JNK), p38 MAPK, phosphor-p38 MAPK (p-p38 MAPK), nuclear factor κB (NF-κB), phosphor-NF-κB (p-NF-κB), stress granules (SGs), activator protein-1 (AP-1), activating transcription factors 2 (ATF2) and receptor for activated protein kinase C1 (RACK1) in each group were detected by Western blot assay.

The cells of each group were taken out on the 12th day of culture. Cells were removed from the wells with a cell scraper, centrifuged, washed and centrifuged with Hanks buffer. The cells were added to a volume of RIPA protein lysate (Beyotime institute of biotechnology, China), 30 times with a pipette. After cracking in the ice for 30 min, it was centrifuged for 10 min under 4°C, 12,000 g. The supernatant which was the total protein in the pancreatic tissue of each group of mice was absorbed. The BCA protein concentration kit (Beyotime) was used to quantify the protein, and the SDS-PAGE electrophoresis samples were prepared with bromo-phenol blue indicator. 5% concentrated adhesive and 10% separated adhesive SDS-PAGE were selected, and 20 μg protein samples were subjected to electrophoresis. The gel was made into a "sandwich" structure, with a constant flow of 200 mA for 90 min, and the protein was transferred to the PVDF membrane (Millipore, USA). PVDF membranes were closed for 2 h with 10% degreased milk powder solution, and were incubated by the primary antibody, anti-TDP-43, anti-SGs, anti-RACK1, anti-JNK, anti-p-JNK, anti-p38 MAPK, anti-p-p38 MAPK, anti-ATF2, anti-NF-κB, anti-p-NF-κB, anti-AP-1 and anti-actin (1 : 1000 dilution, Cell Signaling Technology LTD, USA; Abcam and Affinity Biosciences LTD, USA) at 4°C overnight. We washed the PVDF membrane three times with TBST for 15 min each. The PVDF membrane was incubated by the second antibody (1 : 4000, Abcam) at room temperature for 2 h. After the TBST washing of the PVDF membrane three times, the protein was imaged using ECL chemiluminescence solution (Thermo Fisher LTD., USA). Each sample should be repeated at least 3 times.

Statistical analysis

The experimental results are expressed as mean ± standard deviation (± s). Statistical software SPSS 17.0 was used for data analysis. One-way ANOVA was used in the comparison between groups, $p < 0.05$ was considered to be statistically significant.

Results

ICA treatment reduced the level of TDP-43 in HC-α cells

As shown in Figure 1, compared with the HC-α group, the protein expression of TDP-43 was significantly higher in the TDP-43-HC-α group ($p < 0.01$) and was not significantly different from that of the HC-α + ICA group. However, the protein expression of TDP-43 in the TDP-43-HC-α + ICA group was significantly lower than that of the TDP-43-HC-α group. This indicated that the TDP-43 lentivirus vector transfected human chondrocytes successfully, and made TDP-43 protein highly expressed.

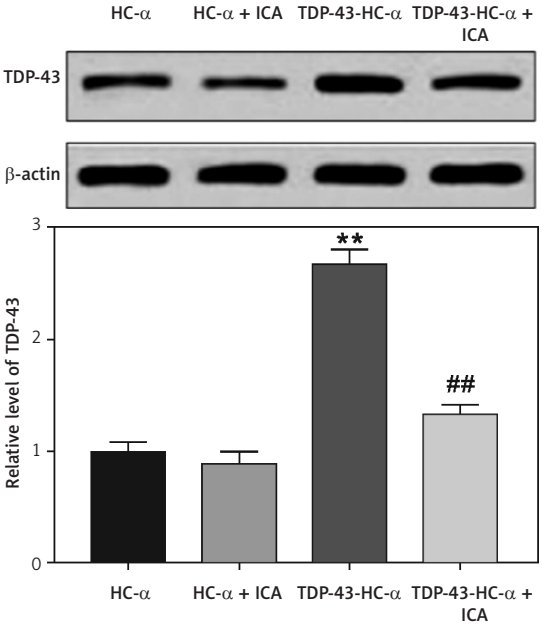

Figure 1. Relative level of TDP-43 in each group of cells

***p < 0.01, compared with HC-α; ##p < 0.01, compared with TDP-43-HC-α.*

Table I. Secretion of TNF-α and IL-1β in each group of cells ($\bar{x}$±s)

Groups	TNF-α [μmol/l]	IL-1β [μmol/l]
HC-α	211.02 ±27.46	116.56 ±28.35
HC-α + ICA	206.36 ±29.41	119.57 ±24.38
TDP-43-HC-α	435.02 ±44.73**	346.35 ±38.04**
TDP-43-HC-α + ICA	303.23 ±23.88##	227.74 ±29.54##

***p < 0.01, compared with HC-α group; ##p < 0.01, compared with TDP-43-HC-α group.*

ICA treatment reversed the elevated levels of inflammatory factors caused by TDP-43

In this study, the secretion of TNF-α and IL-1β in the supernatant of cells in each group was detected by ELISA. As shown in Table I, the secretion of TNF-α and IL-1β in the supernatant of the TDP-43-HC-α group was significantly higher ($p < 0.01$) compared with the HC-α group. However, TNF-α and IL-1β secretion in the supernatant of the TDP-43-HC-α + ICA group was significantly lower than that of the TDP-43-HC-α group ($p < 0.01$), but still higher than that of the HC-α group. This indicated that ICA could reduce the chondrocyte inflammation caused by TDP-43 overexpression.

ICA treatment could reverse the activation of the apoptotic pathway caused by TDP-43

The protein levels of ATF2 and AP-1 and phosphorylation levels of JNK, p38 MAPK and NF-κB in each group of chondrocytes was determined by Western blotting assays, and showed the same trends. As shown in Figure 2, the phosphorylation levels of JNK, p38 MAPK and NF-κB and expression levels of ATF2 and AP-1 in the TDP-43-HC-α group were significantly higher ($p < 0.01$) compared with the HC-α group. However, relative levels in the TDP-43-HC-α + ICA group were significantly lower than those in the TDP-43-HC-α group ($p < 0.01$). It indicated that ICA could inhibit TDP-43-induced activation of the JNK/MAPK related apoptotic signaling pathway.

ICA treatment can reverse the decrease in SG formation and increase in RACK1 caused by TDP-43

Western blotting was used to determine the formation of cytoplasmic SGs and RACK1 level in each group of chondrocytes. As shown in Figure 3, the formation of cytoplasmic SGs in cells in the TDP-43-HC-α group was significantly lower compared with the HC-α group ($p < 0.01$). However,

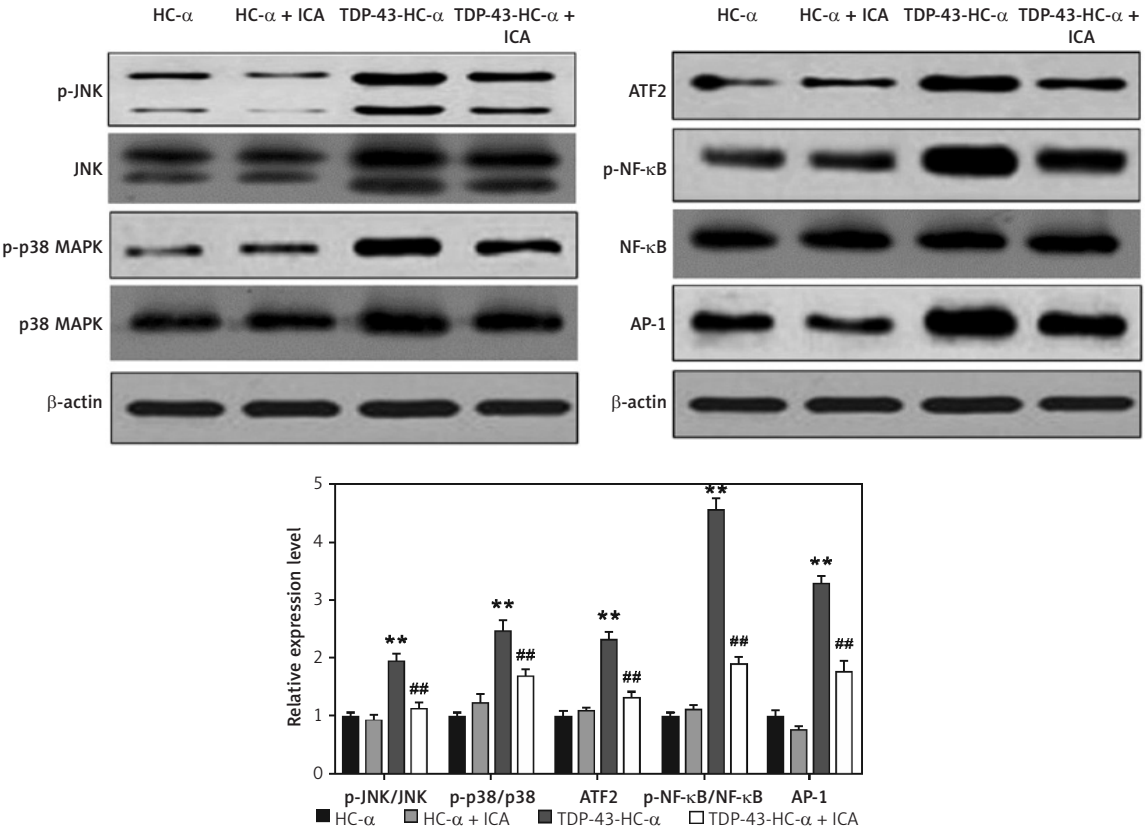

Figure 2. Phosphorylation level of JNK/MAPK pathway and expression level of related proteins in each group of cells

***p < 0.01, compared with HC-α; ##p < 0.01, compared with TDP-43-HC-α.*

the formation of cytoplasmic SGs in the TDP-43-HC-α + ICA group was significantly higher than that in the TDP-43-HC-α group ($p < 0.01$), which indicated that ICA promoted the formation of cytoplasmic SGs inhibited by TDP-43. The expression level of RACK1 tends to be the opposite; the level of RACK1 in the TDP-43-HC-α group was significantly higher compared with the HC-α group ($p < 0.01$). However, the protein expression of RACK1 in the TDP-43-HC-α+ICA group was significantly lower than that in the TDP-43-HC-α group ($p < 0.01$), which indicated that ICA could inhibit TDP-43-induced activation of RACK1.

Discussion

Traditional Chinese medicine believes that OA is a type of arthralgia syndrome, and the cause of the disease is deficiency of kidney essence. Icariin has physiological functions such as activating blood circulation, removing blood stasis, relieving collaterals and pain, removing wind and dampness, and nourishing the liver and kidney [15]. In this study, human chondrocytes HC-α were transfected with TDP-43 lentiviruses, and chondrocytes overexpressing TDP-43 (TDP-43-HC-α) were constructed. The high expression of TDP-43 protein in transfected chondrocytes was confirmed, indicating the success of transfection of human chondrocytes with TDP-43 lentivirus vector. Icariin has been shown to increase chondrocyte viability by promoting hypoxia-inducible factor-1α expression and anaerobic glycolysis [16] and inhibits chondrocyte apoptosis and angiogenesis by regulating the TDP-43 signaling pathway [17]. Zuo *et al.* demonstrated that icariin alleviated IL-1β-induced matrix degradation and eliminated reactive oxygen species by activating the Nrf2/ARE pathway in human chondrocytes in vitro [18]. Icariin is also implicated in regulating autophagy and apoptosis in chondrocytes and could protect against OA by suppressing inflammatory cytokines and apoptosis [19]. Our results confirmed that icariin could inhibit the expression of TDP-43 in human chondrocytes transfected with TDP-43 lentivirus. These findings together demonstrate that icariin may be a promising compound with therapeutic potential for OA.

The JNK signaling pathway is closely related to the inflammatory response and apoptosis [20]. Studies have shown that blocking the p38 MAPK signaling pathway can inhibit the inflammatory response and apoptosis [21]. Our study showed that the expression of JNK and p38 MAPK in the TDP-43-HC-α+ICA group was significantly lower than that in the TDP-43-HC-α group. This indicated that icariin could inhibit activation of the JNK and p38 MAPK signaling pathways induced by TDP-43. JNK is involved in regulating the transcription of

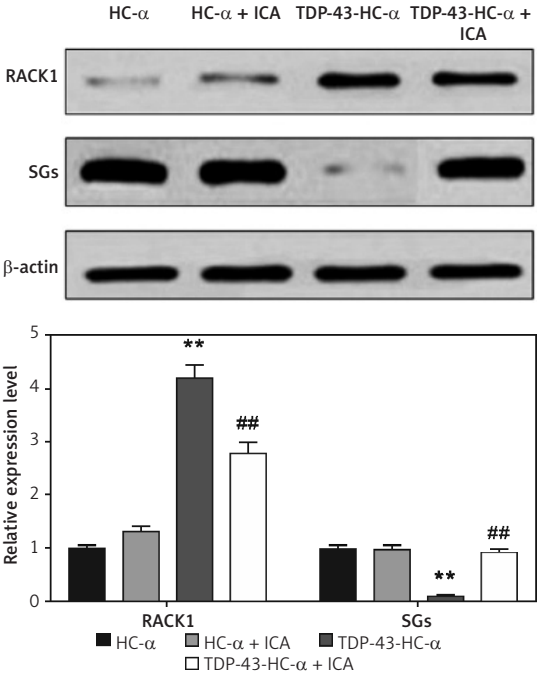

Figure 3. Formation of cytoplasmic SGs and RACK1 levels in each group of cells

***p < 0.01, compared with HC-α; ##p < 0.01, compared with TDP-43-HC-α.*

downstream apoptosis-related target genes and the expression of apoptosis-related proteins, thus initiating apoptosis-related signaling pathways and causing apoptosis [22]. Hwang *et al*. confirmed that activation of the p38 MAPK signaling pathway regulated the expression of NF-κB, thereby regulating the expression of epoxy-synthase 2 [23]. Apoptosis of chondrocytes is the main cause of OA. Studies have suggested that blocking the p38 MAPK signaling pathway could inhibit the apoptosis of chondrocytes in OA. In this study, the expression levels of transcription factors ATF2, NF-κB and AP-1 related to the JNK and p38 MAPK signaling pathway were determined. The results showed that the levels of ATF2, NF-κB and AP-1 were significantly lower in the TDP-43-HC-α + ICA group than in the TDP-43-HC-α group. It was speculated that ICA could also inhibit the expression of TDP-43 mediated ATF2, NF-κB and AP-1, thus blocking chondrocyte apoptosis. This is consistent with the literature.

Studies have confirmed that the apoptosis mechanism of chondrocytes induced by TDP-43 may be related to SGs. RACK1 is the regulator of SG and JNK. Under hypoxic stress, RACK1 interacts with SGs to inhibit activation of JNK and p38 MAPK signals, thereby inhibiting the occurrence of apoptosis [24]. Therefore, the expression of SGs and RACK1 in human chondrocytes transfected with TDP-43 lentivirus vector by icariin was determined in this study. The study showed that the formation

of SGs in the TDP-43-HC-α+ICA group was significantly higher than that in the TDP-43-HC-α group. The expression of RACK1 in TDP-43-HC-α+ICA cells was significantly lower than that in the TDP-43-HC-α group. This indicated that icariin could down-regulate the overexpression of RACK1 caused by TDP-43, and up-regulate the low expression of SGs caused by TDP-43, thus preventing the occurrence of chondrocyte apoptosis caused by TDP-43.

In conclusion, icariin can interfere with TDP-43-mediated secretion of inflammatory factors, inhibit the activation of the JNK and p38 MAPK signaling pathways, thereby reversing the molecular mechanism of chondrocyte lesions, and providing ideas for further studies in the future.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

References

1. Yamato TP, Deveza LA, Maher CG. Exercise for osteoarthritis of the knee (PEDro synthesis). Br J Sports Med 2016; 50: 1013-4.
2. Qiao C, Ye W, Li S, Wang H, Ding X. Icariin modulates mitochondrial function and apoptosis in high glucose-induced glomerular podocytes through G protein-coupled estrogen receptors. Mol Cell Endocrinol 2018; 473: 146-55.
3. Mathiessen A, Conaghan PG. Synovitis in osteoarthritis: current understanding with therapeutic implications. Arthritis Res Ther 2017; 19: 18.
4. Berenbaum F, Griffin TM, Liu-Bryan R. Review: Metabolic regulation of inflammation in osteoarthritis. Arthritis Rheumatol 2017; 69: 9-21.
5. Hochberg MC, Martel-Pelletier J, Monfort J, et al. Combined chondroitin sulfate and glucosamine for painful knee osteoarthritis: a multicentre, randomised, double-blind, non-inferiority trial versus celecoxib. Ann Rheum Dis 2016; 75: 37-44.
6. Wei CY, Sun HL, Yang ML, et al. Protective effect of wogonin on endotoxin-induced acute lung injury via reduction of p38 MAPK and JNK phosphorylation. Environ Toxicol 2017; 32: 397-403.
7. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. Nat Rev Rheumatol 2016; 12: 632-44.
8. Reyes C, Leyland KM, Peat G, Cooper C, Arden NK, Prieto-Alhambra D. Association between overweight and obesity and risk of clinically diagnosed knee, hip, and hand osteoarthritis: a population-based cohort study. Arthritis Rheumatol 2016; 68: 1869-75
9. Wang W, Wang L, Lu J, et al. The inhibition of TDP-43 mitochondrial localization blocks its neuronal toxicity. Nat Med 2016; 22: 869-78.
10. Conicella AE, Zerze GH, Mittal J, Fawzi NL. ALS mutations disrupt phase separation mediated by alpha-helical structure in the TDP-43 low-complexity C-terminal domain. Structure 2016; 24: 1537-49.
11. Junttila A, Kuvaja M, Hartikainen P, et al. Cerebrospinal fluid TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis patients with and without the C9ORF72 hexanucleotide expansion. Dement Geriatr Cogn Dis Extra 2016; 6: 142-9.
12. Su YS, Fan ZX, Xiao SE, et al. Icariin promotes mouse hair follicle growth by increasing insulin-like growth factor 1 expression in dermal papillary cells. Clin Exp Dermatol 2017; 42: 287-94.
13. Xia Q, Wang H, Hao Z, et al. TDP-43 loss of function increases TFEB activity and blocks autophagosome-lysosome fusion. EMBO J 2016; 35: 121-42.
14. Song L, Zhao J, Zhang X, Li H, Zhou Y. Icariin induces osteoblast proliferation, differentiation and mineralization through estrogen receptor-mediated ERK and JNK signal activation. Eur J Pharmacol 2013; 714: 15-22.
15. Chen MC, Lee NH, Hsu HH, et al. Inhibition of NF-kappaB and metastasis in irinotecan (CPT-11)-resistant LoVo colon cancer cells by thymoquinone via JNK and p38. Environ Toxicol 2017; 32: 669-78.
16. Wang P, Xiong X, Zhang J, Qin S, Wang W, Liu Z. Icariin increases chondrocyte vitality by promoting hypoxia-inducible factor-1alpha expression and anaerobic glycolysis. Knee 2020; 27: 18-25.
17. Huang H, Zhang ZF, Qin FW, et al. Icariin inhibits chondrocyte apoptosis and angiogenesis by regulating the TDP-43 signaling pathway. Mol Genet Genomic Med 2019; 7: e00586.
18. Zuo S, Zou W, Wu R-M, et al. Icariin alleviates IL-1β-induced matrix degradation by activating the Nrf2/ARE pathway in human chondrocytes. Drug Des Devel Ther 2019; 13: 3949-61.
19. Mi B, Wang J, Liu Y, et al. Icariin activates autophagy via down-regulation of the NF-κB signaling-mediated apoptosis in chondrocytes. Front Pharmacol 2018; 9: 605.
20. Zhuang RJ, Ma J, Shi X, et al. Cold-inducible protein RBM3 protects UV irradiation-induced apoptosis in neuroblastoma cells by affecting p38 and JNK pathways and Bcl2 family proteins. J Mol Neurosci 2017; 63: 142-51.
21. Liu J, Lv L, Gong J, et al. Overexpression of F-box only protein 31 predicts poor prognosis and deregulates p38alpha- and JNK-mediated apoptosis in esophageal squamous cell carcinoma. Int J Cancer 2018; 142: 145-55.
22. Ruangsuriya J, Budprom P, Viriyakhasem N, et al. Suppression of cartilage degradation by zingerone involving the p38 and JNK MAPK signaling pathway. Planta Med 2017; 83: 268-76.
23. Zheng Y, Lin L, Yu T. Advanced glycation end products induce colonic smooth muscle cells of rats apoptosis through rage and activation of JNK and P38 MAPK pathways. Gastroenterology 2017; 152: S513.
24. Guo J, Qiu X, Zhang L, Wei R. Smurf1 regulates macrophage proliferation, apoptosis and migration via JNK and p38 MAPK signaling pathways. Mol Immunol 2018; 97: 20-6.