Original Article
Crocin alleviates the steroid-induced osteonecrosis of the femoral head injuries via ROS-mediated JNK/c-Jun signaling pathway

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Abstract: Objective: To explore the role and functional mechanism of crocin in glucocorticoids-induced osteonecrosis of the femoral head (ONFH). Methods: Primary osteoblasts from rat were stimulated with dexamethasone and treated with crocin in different doses and reactive oxygen species (ROS) inhibitor in vitro. Cell viability and cell apoptosis rate were assayed by cell counting kit-8 and flow cytometry, respectively. The level of alkaline phosphatase activity, ROS and mitochondrial membrane potential were detected by ELISA. The expression of related proteins was detected via Western blot. As for in vivo study, 30 rats were equally grouped into control group, ONFH model group (steroid-induced ONFH) and crocin treatment group. The occurrence and histopathological changes of ONFH, bone tissue cell apoptosis, and bone microstructure and loss were analyzed. Results: In vitro experiments, crocin and ROS inhibitor could reverse the reduction of cell viability, differentiation and mitochondrial membrane potential content, as well as the increase of ROS production, apoptosis rate and the expression levels of p-JNK and p-c-Jun in primary osteoblasts caused by dexamethasone. In vivo experiments, compared with rats in the control group, ONFH model rats had decreased amount of femoral head bone and the integrity of bone tissues, but aggravated apoptosis rate of femoral head cells and empty trabecular; however, crocin treatment significantly improved these microstructures of femoral head bone. Conclusion: Crocin had a favorable therapeutic effect on steroid-induced ONFH in rats by ROS/JNK/c-Jun pathway.

Keywords: Crocin, steroid-induced osteonecrosis of the femoral head, reactive oxygen species, JNK/c-Jun signaling pathway

Introduction
Steroid-induced osteonecrosis of the femoral head (ONFH) is clinically a common and frequently-occurring disease, usually with a high disability rate [1]. Glucocorticoids (GCs) with high dose for short-term or low dose for long-term is the most common steroid that causes ONFH [2-4]. However, the exact pathogenesis of steroid-induced ONFH remains unclear, and thus, its clinical treatment efficacy is unsatisfactory.

JNK, a pivotal member of the MAPK family, is ubiquitous in multitudinous organizations. It has been proved that JNK participated in the cellular processes, including oxidative stress, apoptosis, proliferation, etc. [5-7]. Oxidative stress activates the JNK, induces C-Jun phosphorylation and accelerates the release and expression of caspase-3, and finally induces apoptosis [8-10].

Crocin (dicarboxylic acid polyene monosaccharide ester), is a chemical component extracted from dried stigmas of Crocus sativus L. (saffron) [11]. Previous studies have reported that crocin has anti-oxidation and anti-inflammation functions [12-14]. Crocin could inhibit the progression of breast cancer, colorectal cancer, etc [15, 16]. Recently, crocin has been also reported to attenuate GCs-induced apoptosis of MC3T3-E1 osteoblasts [17].

In the current study, we aimed to further investigate the role and underlying mechanism of crocin in GCs-induced ONFH, with the hope to afford novel evidence for the pathogenesis and therapeutic strategy of steroid-induced ONFH.
Materials and methods

Main materials

Lipopolysaccharide and methylprednisolone sodium succinate, Ham’s F-12 culture media were purchased from Gibco, Carlsbad, CA, USA. Fetal bovine serum (FBS) was purchased from Serapio, Naila, Germany. Phosphate Buffer solution (PBS) and trypsin were purchased from Hyclone, Logan, UT, USA. Penicillin and gentamicin were purchased from Solarbio science & technology Co., Ltd., Beijing, China. Crocin, lipopolysaccharide (LPS), N-acetylcysteine (NAC) and dexamethasone (Dex) were purchased from Sigma, St. Louis, MO, USA. Methylprednisolone sodium succinate (MPS) was purchased from Pfizer, New York, NY, USA. A Lkaline Phosphatase (ALP) kit was purchased from KeyGen Biotech Co., Ltd., Nanjing, China. Reactive oxygen species (ROS) detection kit and cell apoptosis detection kit were purchased from KeyGen Biotech Co., Ltd., Nanjing, China. Primary antibodies including anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-cytochrome C and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as well as HRP-Goat anti Rabbit secondary antibody were purchased from Abcam, Cambridge, United Kingdom. RIPA lysis buffer and primary antibodies against C-Jun, p-c-Jun, c-Jun N-terminal kinase (JNK), p-JNK and β-Actin were purchased from CST, Boston, MA, USA.

Cell model establishment and grouping

The skull and periosteum were taken out from rats and the blood vessels and connective tissues on them were removed. Head of femur tissues were cut into pieces and digested with 4 mL of 0.25% trypsin for 20 min in a 10 cm culture flask. Then 4 mL of complete medium was added into the flask and the supernatant was discarded after centrifugation (500×g, 5 min). The sample was resuspended with 10 mL of 0.1% collagenase solution and incubated at 37°C, 5% CO₂ for 30 min. Primary osteoblasts were collected by centrifugation (500×g, 10 min) and cultivated in Ham’s F-12 medium containing 10% FBS and 1% antibiotics (penicillin and gentamicin). Isolated osteoblasts were passaged and the third-generation osteoblasts were used for the following experiment. Cells were divided into five groups: the control group, the Dex group, the Dex + crocin low dose group, the Dex + crocin high dose group, the Dex + NAC group. For the Dex treatment, cells were treated with 1 μM Dex for 24 h [18]. NAC was an ROS scavenger [19, 20]. All operations were implemented under aseptic condition.

Animal model establishment and grouping

Thirty-three male Sprague-Dawley (SD) rats (six-eight weeks old, 220±10 g) were bought from Vital River Laboratory Animal Technology (Beijing, China). Rats were randomly divided into 3 groups: the control group (n=12): rats were intravenously injected with 0.9% saline; the ONFH model group (n=12): rats were intravenously injected with 2 mg/kg LPS in the first and second days, then intramuscularly injected with 20 mg/kg MPS in three-seven days; the crocin treatment group (n=12): rats were intraperitoneal injection with 15 mg/kg crocin per day for 7 days, and 2 hours later, they were intravenous injection with LPS (1.8 mg/kg) in the first and second days and intramuscularly injected with 20 mg/kg MPS in three-seven days. On the 30th day, all the rats were anaesthetized and euthanatized by quick cervical dislocation, and then the bone samples were collected for further analysis. This study has been approved by the Ethics Committee of Wuhan University (No. 20180920).

Cell viability detection

The cell viability of isolated primary osteoblasts was detected by CCK-8 assay. Cells were inoculated into 96-well plates with 1×10³ cells in per well. After treatment with Dex for 24 h, 10 μL CCK-8 reagent was added into each well and the plates were placed in a 37°C incubator for 1 h. Eventually, the absorbance of each well at 450 nm was measured by a microplate reader. Cell viability (%) = the average value of the absorbance of the wells in the experimental group/the average value of the absorbance of the wells in the control group ×100%.

ALP viability detection

Osteoblasts were inoculated into 12-well plates with 5×10⁴ cells in per well, and treated with...
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different conditions. After treatment for 7 d, the plates were washed and administrated with 100 μL lysis buffer, and then the level of ALP viability (U/mL) was examined according to the manufacture's instruction of the ALP kit.

ROS level assessment

The intracellular ROS level was detected by ROS detection kit. Osteoblasts were inoculated into 24-well plates with 3*10³ cells in per well. Cells were exposed to different treatment conditions, followed by washing with PBS for three times. Cell suspension was incubated with 10 μmol/L of 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) for 30 min at room temperature under dark surroundings. Ultimately, ROS level was detected by flow cytometry.

Cell apoptosis rate detection

Osteoblasts were inoculated into 24-well plates with 3*10³ cells in per well. After treatment, cells were washed by pre-cooled PBS and centrifuged at 500xg for 5 min, and resuspended by 500 μL of Binding buffer. Then 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) were added to the sample and the sample was incubated at 4°C in the dark for 30 min. Apoptotic cells were detected by a flow cytometer (BD Biosciences, San Diego, CA, USA).

Mitochondrial membrane potential (MMP) detection

MMP was detected by JC-1 method to evaluate the loss of MMP. Osteoblasts were inoculated into 6-well plates. After treatment, cells were incubated with 500 μL of JC-1 solution in the dark for 20 min. After centrifugation at 500xg for 5 min, cells were resuspended in the buffer solution for 3 times. Finally, MMP was detected on flow cytometry.

Western blot

Proteins were extracted using RIPA lysis buffer and separated by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred onto Polyvinylidene Fluoride membranes (Millipore, Bedford, MA, USA) and blocked with 5% skimmed milk. Primary antibodies (1:1,000) were added onto the membranes, which were incubated at 4°C overnight. After washing with PBST, the membranes were incubated with secondary antibody (1:5,000) for 2 h at room temperature. The membranes were developed by ECL reagent (Millipore) and analyzed by Image J software (NIH, Bethesda, MD, USA). GAPDH or β-Actin were used as internal references.

Hematoxylin-eosin (HE) staining

Fresh head of femur tissues of rats were fixed in 4% paraformaldehyde for 48 h and soaked in 10% Ethylene Diamine Tetraacetic Acid (EDTA) for decalcification for 4 weeks. Then the tissues were embedding in paraffin and sliced into 5 μm thickness sections. The routine HE staining experiment was performed to observe the tissue morphology.

Terminal dUTP nick-end labeling (TUNEL) staining

The apoptotic cells in the tissues slices of the femur head was detected by using the One-Step TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) according to the kit instruction. Cells were observed under a microscope and brown cells were defined as positive apoptotic cells.

Micro CT scanning analysis

The head of femur tissues of rat were scanned using Micro-CT (Scanco, Zurich, Switzerland). The Micro-CT can clearly observe the trabecular microstructure, the integrity of bone tissue structure and change of bone volume [21, 22]. We set the scanner at a resolution of 20 μm with the electric current of 114 μA and voltage of 70 kV, then defined the region of interest (ROI) to cover the whole subchondral bone in bearing area of tibial plateaus. The histomorphometric parameters measured were listed as below: bone volume/total tissue volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp).

Statistical analysis

All experiments were repeated three times. SPSS 15.0 software was used for data analysis and GraphPad Prism 5 was used for drawing figures. All the data were expressed as mean ± standard deviation (±sd) and the comparison among groups adopted Student’s t-test or one-way analysis of variance combined with
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Dunnett's test. P<0.05 indicates a significant difference.

**Results**

**Crocin ameliorated the inhibition effect of Dex on the cell viability and differentiation in primary osteoblasts from rats**

We first analyzed the effect of crocin on cell toxicity and viability of primary osteoblasts. After treatment with crocin at different concentrations for 24 h, the cell viability was detected by CCK-8 assay, which showed no significant changes, suggesting crocin had no cytotoxicity for primary osteoblasts with concentration less than 500 μM (Figure 1A).

It has been confirmed that 1 μM of Dex could significantly impact the cell viability of osteogenic cells [18]. Herein, we used 1 μM of Dex to establish ONFH cell model. Subsequently, cells were treated with crocin at the concentration of 10 μM (low dose, Dex + Cro10 group) and 25 μM (high dose, Dex + Cro25 group). The results showed that crocin alleviated the inhibition effect of Dex on cell viability in a dose-dependent manner (Figure 1B).

ALP is a significant marker for the evaluation of osteoblast differentiation and bone formation. Dex reduced the ALP activity of osteoblasts, while crocin could reverse the reduction (Figure 1C). These results demonstrated that crocin ameliorated the Dex-induced inhibition of cell viability and differentiation of primary osteoblasts from rats in a dose-dependent manner (Figure 1B).

**Crocin protected Dex-treated primary osteoblasts by inhibiting oxidation and apoptosis and the loss of MMP**

After Dex treatment, the ROS level, cell apoptosis rate and the loss of MMP in primary osteoblasts were detected by flow cytometry. Dexam treatment would induce the increase of ROS level, cell apoptosis rate and MMP loss in osteoblasts, however, crocin treatment and NAC (a scavenger of ROS) treatment, could reverse these changes caused by Dex (Figure 1D-I), indicating that crocin could protect Dex-treated primary osteoblasts by inhibiting oxidation and apoptosis and the MMP loss.

**Crocin suppressed cell apoptosis through inhibiting ROS-mediated JNK/c-Jun signaling pathway in Dex-treated primary osteoblasts**

The expression of apoptosis related proteins and JNK/c-Jun signaling pathway were detected Western blot (Figure 2). The results showed that the expressions levels of Bax and Cleaved caspase-3, p-JNK and p-c-Jun were increased, while Bcl-2 was decreased in the Dex group, suggesting that the activation of JNK/c-Jun signaling pathway might be involved in the Dex-induced osteoblast apoptosis. Interestingly, compared with the the Dex group, the crocin and NAC groups had significantly decreased expression levels of Bax and Cleaved caspase-3, p-JNK and p-c-Jun, but significantly increased expression level of Bcl-2, indicating that crocin could inhibit cell apoptosis through inhibiting JNK/c-Jun signaling pathway and reducing the excessive production of ROS in Dex-treated primary osteoblasts.

**Crocin alleviated GCs-induced ONFH in SD rats**

We established ONFH rat model to further validate the preventive effect of crocin on GCs-induced ONFH in vivo. As shown in Figure 3A, the pathological change of rats in the control group was not obvious as HE staining results showed that the trabeculae in the control group was complete and regular, and osteocytes were found in the bone lacunae. Conversely, in the ONFH group, all the rats had significant pathological changes as the trabecular structure was disorganized with necking or fracture and empty lacunae were ubiquitous (Figure 3B). In the crocin group, the necrosis was less than that in the ONFH group as the trabeculae shape was better with no obvious stenosis or fracture, and the number of empty lacunae was fewer (Figure 3C). The percentages of empty lacunae in the control group, the ONFH model group and the crocin treatment group were 7.3%, 38.1% and 12.8%, respectively (Figure 3D). Hence, crocin treatment relieved GCs-induced ONFH in SD rats.

**Crocin attenuated GCs-induced cell apoptosis in osteonecrosis area of SD rats**

The effect of crocin on GCs-induced apoptosis of femoral head tissues in vivo was analyzed by TUNEL staining. The results showed that the
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Figure 1. Crocin ameliorated the inhibition of cell viability, differentiation and MMP loss but the promotion of oxidization and apoptosis induced by Dex in primary osteoblasts of rats. A: Structure of crocin; B, C: CCK-8 was applied for detecting cell viability in primary osteoblasts after treatment with crocin at different concentrations (0 μM, 1 μM, 10 μM, 25 μM, 100 μM and 500 μM) for 24 h or cells treated with examined in control, Dex, Dex + Cro10 and Dex + Cro25 groups; D, E: Cell oxidization and differentiation were analyzed by detecting ROS generation level and ALP activity, respectively; F, G: Cell apoptosis was assayed through flow cytometry; H, I: The loss of MMP was determined via JC-1 staining method through flow cytometry. Compared with the control group or the 0 μM group, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
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Figure 2. Crocin suppressed cell apoptosis through inhibiting ROS-mediated JNK/c-Jun signaling pathway in Dex-treated primary osteoblasts. A-E: Respective bands and quantification results of the protein expression of Bcl, Bax, cleaved caspase 3 and cytochrome C; F-J: Respective bands and quantification results of the protein expression of p-c-JUN, c-JUN, p-JNK and JNK.
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Femoral head tissues from model rats had significantly more TUNEL positive cells than those from normal rats. However, the crocin group had significantly less TUNEL positive cells than the model group, indicating that GCs-induced cell apoptosis in femoral head tissues of rats can be partly reduced by crocin treatment (Figure 4D).

**Crocin improved the microstructure of femoral head in SD rats**

The microstructure of femoral head of rats was detected by Micro CT scan. Rats in the control group had visibly compact in trabeculae and the surface of femoral head was smooth with no collapse (Figure 5A). Rats in the ONFH group had sparse trabeculae, enlarged trabecular spacing and the abundance of trabecular microfracture (Figure 5B). However, model rats with crocin treatment had less damage than those without treatment (Figure 5C). In addition, crocin improved bone parameters (BV/TV, Tb.Th, Tb.Sp and Tb.N) of femoral head of model rats (Figure 5D-G), indicating that crocin could benefit rats with GCs-induced osteonecrosis.
Discussion

ONFH is a refractory orthopedic disease with a high incidence and disability rate. The blood supply of the femoral head is interrupted or damaged in ONFH, which results in the death of bone cells and bone marrow components and subsequent repair difficulty, and causes the structural change of the femoral head, collapse of the femoral head, and joint dysfunction [23]. Hip replacement is the common surgery treatment of ONFH, which usually causes severe financial burden to patients and society [1]. However, there is no effective non-surgical treatment strategy in the early stage of ONFH. Currently, the pathogenesis of ONFH has not been fully elucidated. Previous studies had proved that GCs could induce osteoblast apoptosis, which plays a crucial role in the onset of ONFH as 9% to 40% of patients used GCs were diagnosed with ONFH [24-26]. Therefore, it is of importance to study the pathogenesis of steroid-induced ONFH and to explore non-surgical therapies.

Previous studies have shown that the ROS mediated JNK signaling pathway plays an important role in inducing apoptosis or necrosis of mammalian cells [27, 28]. ROS is mainly generated in mitochondria and can produce free radicals through redox reaction [29]. Excessive ROS can cause oxidative damage and aggravate oxidative stress and cell apoptosis via mitogen-activated protein kinase and P53 signaling pathways [29-31]. In our study, we constructed the Dex-induced primary osteoblast cell model and found that Dex conspicuously caused cell apoptosis with excessive ROS generation, whereas osteoblast apoptosis was reduced after ROS inhibition treatment, suggesting that ROS could mediate the Dex-induced primary osteoblast apoptosis. Moreover, crocin could significantly alleviate Dex induced osteoblast apoptosis and overexpression of apoptosis-related proteins, the ROS level increase, and the loss of MMP in a dose-dependent manner. Therefore, crocin could protect primary osteoblasts by inhibiting ROS generation.

JNK was reported to play a critical role in the osteoblastic formation [32, 33]. However, whether the JNK/C-Jun signaling pathway influences the occurrence and development of GCs-induced ONFH has not been reported. In our study, we found that Dex treatment could increase the expression of Bax, Cleaved caspase3, p-JNK and p-c-Jun but descend Bcl-2 expression level, which were all apoptosis related proteins [33]. Dex activated the ROS-mediated JNK/c-Jun pathway to promote the expression of pro-apoptosis protein Bax, which was transferred into mitochondria to combine with anti-apoptotic protein Bcl-2, forming
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dimers to antagonize the inhibitory effect of Bcl-2 [34]. Furthermore, the permeability of mitochondrial membrane to Cytochrome C was enhanced to trigger the MMP loss, activating the mitochondrial apoptotic pathway to induce osteoblast apoptosis [35]. Therefore, the anti-apoptosis effects of crocin and NAC in osteoblast treated with Dex might be associated with the inhibition of JNK/c-Jun signaling pathway.

To further investigate the effects of crocin on GCs-induced ONFH in vivo, the GCs-induced ONFH rat model was established. The microstructure of bone tissue was evaluated by Micro CT and the results showed that ONFH mice had severe damaged microstructure and apoptosis in the femoral head tissues cells and severe trabecular lacunae in HE staining, indicating the model establishment was successful. Crocin treated rats had better trabecular morphology and bone tissue integrity, as well as less empty bone lacunae and apoptotic cells than rats in the ONFH model group. Therefore, crocin had great therapeutic effect on steroid-induced ONFH in rats.

In summary, this study firstly illuminates that crocin could ameliorate GCs-induced ONFH injuries via inhibition of the ROS mediated JNK/c-Jun signaling pathway. These findings may provide some new insights for the treatment of GCs-induced ONFH.

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Disclosure of conflict of interest

None.

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References

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