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Celecoxib Combined with Diacerein Effectively Alleviates Osteoarthritis in Rats *via* Regulating JNK and p38MAPK Signaling Pathways

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Abstract—Osteoarthritis (OA) has long been a difficult to overcome joint disease for medical workers. However, there is still a lack of effective treatments for OA. In the present study, we aimed to evaluate the treatment effect of celecoxib (CLX) combined with diacerein (DC) on OA and delineate the underlying molecular mechanism. The OA model was established by using rats, and OA rats were treated with either CLX alone, DC alone, and CLX combined with DC. The results showed that, as compared with a single treatment of CLX or DC, CLX combined with DC markedly attenuated OA and inhibited the levels of inflammatory mediators interleukin-1β and nitric oxide, improved bone cartilage metabolism, and suppressed chondrocyte apoptosis. Most importantly, CLX combined with DC significantly inactivated the c-Jun N-terminal kinases (JNK) signaling pathway by the inhibition of MEKK1 and MKK7, as detected by Western blot analysis. Furthermore, the protein expression of downstream genes of JNK, including activating-transcription factor (Atf-2), matrix metalloproteinase-13 (MMP-13), and cyclooxygenase (COX-2), were also significantly inhibited by CLX combined with DC as compared with single treatments. Furthermore, CLX combined with DC also effectively inhibits p38 mitogen-activated protein kinase and nuclear factor-κB signaling pathways. Taken together, our study suggests that CLX combined with DC has satisfactory treatment effects on OA *via* a stronger inhibitory effect on inflammatory signaling pathway.

KEY WORDS: celecoxib; diacerein; JNK signaling pathway; osteoarthritis.

INTRODUCTION

Osteoarthritis (OA) is a degenerative joint disease in elderly populations subjected to the impact of mechanical factors and biological factors. These harmful factors cause metabolic imbalance between chondrocyte, extracellular matrix, and subchondral bone. Metabolic imbalance leads to inflammatory mediators and matrix components combining with special receptors, then transmitting signals into the nucleus to activate gene transcription, that eventually leads to matrix degradation and cell apoptosis [1, 2]. The pathological nature of OA is the destruction of articular cartilage such as subchondral bone sclerosis, cartilage degradation, meniscal degeneration, and osteophytes and synovial hyperplasia [3]. Currently, OA has brought considerable damage to human health. However, there is still a lack of effective treatment for OA. Therefore, the development of a novel and effective therapy for OA is of great importance.

Increasing studies have suggested that inflammation is involved in OA. It has been reported that pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) released from inflammatory synovial tissue inhibit the matrix synthesis and

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Abbreviations: OA, Osteoarthritis; MMP-13, Matrix metalloproteinase-13; IL-1β, Interleukin-1β; TNF-α, Tumor necrosis factor α; CTX-II, C-telopeptide fragments of type II collagen; MAPKs, Mitogen-activated protein kinases; JNK, c-Jun N-terminal kinases; Atf-2, Activating-transcription factor; COX-2, Cyclooxygenase; MAPK, Mitogen-activated protein kinase; NSAID, Non-steroidal anti-inflammatory drug; CLX, Celecoxib; DC, Diacerein; ELISA, Enzyme-linked immune sorbent assay; BMD, Bone mineral density

chondrocytes proliferation [4, 5]. Under the stimulation of IL-1β, the cartilage cells can produce NO, which can participate in some protein modifications followed by induction the of cartilage matrix degradation leading to cartilage destruction. In addition, NO can produce peroxynitrate by combining with some superoxide, which is able to mediate chondrocytes apoptosis [6]. Chondrocytes are the only cells in mature cartilage, but many previous studies about OA have focused on the enzymatic degradation and synthesis of the extracellular matrix. However, there is little research focused on apoptosis or survival of the chondrocytes in cartilage degradation [7]. Mitogen-activated protein kinases (MAPKs) including c-Jun N-terminal kinases (JNK) and p38MAPK signaling pathways have been suggested to be extensively involved in regulating OA [8, 9]. JNK can activate transcription factors and regulate gene expression through three conserved enzymatic cascades (MAPKKK → MAPKK → MAPK). MEKK1 was found to be the first member of the MAPK KK family which is involved in regulating pro-apoptotic effects by the JNK signaling pathway and could induce double phosphorylated JNK activation by activating MKK4 or MKK7. However, the difference is that MKK4 can activate protein kinase p38MAPK and JNK, whereas MKK7 is specifically involved in only JNK activation [10]. A large number of experiments suggest that the JNK signaling pathway plays a key role in cell differentiation, apoptosis, and proliferation and joint injury mediated by interleukin-1 (IL-1), interleukin-17 (IL-17), TNF-α, and other inflammatory factors [11]. Through the JNK pathway, IL-1 not only induces the expression of MMP-13 resulting in cartilage matrix degradation and chondrocyte apoptosis but also activates activating-transcription factor (Atf-2), which initiates transcription of the cyclooxygenase (COX-2) gene, leading to inflammation and chondrocyte apoptosis [12]. Activation of p38MAPK has been reported to be associated with cartilage collagen degradation, chondrocyte apoptosis, and inflammation process in OA [13–15].

Currently, the drugs used to treat OA are mainly divided into two categories: (1) drugs for symptom control, including non-steroidal anti-inflammatory drugs (NSAIDs), antipyretic analgesics, *etc.* and (2) drugs for improving the condition and protective cartilage reagents. Although many OA-related drugs have been applied, there is no clear conclusion on which drugs can effectively improve OA symptoms and slow the progression of the disease. OA is accompanied by local inflammation and inflammatory mediators including TNF-α and IL-1β, and NO induces the synthesis and accumulation of inflammatory prostaglandins *via* the action of COX-2. Therefore, COX-2 inhibitors have a protective effect on OA by reducing the production of inflammatory mediators [16]. Celecoxib (CLX) is a novel selective COX-2 inhibitor belonging to the class of NSAIDs and has been widely used in the treatment of OA, as it can inhibit the generation of prostaglandin D2 and reduce the inflammation associated with OA. CLX has been suggested to be a "Disease-Modifying Drug" for human OA [17, 18]. Ashkavand *et al.* reported that CLX combined with silymarin had an anti-inflammatory effect on MIA-induced OA [19]. It was also reported that the liposomal CLX–hyaluronic acid combination had shown better effects at relieving OA symptoms compared with a single drug [20]. Diacerein (DC) is an important IL-1β inhibitor on OA, which has been reported to suppress cartilage degradation and promote cartilage synthesis and inhibition of synovitis [21]. However, studies have also been reported for knee joint OA patients; DC had very minimal effects at relieving symptoms, but had lots of side effects, such as diarrhea [22]. In the current research about OA, a single drug treatment was found to have a limited effect on OA, as it fails to relieve OA symptoms essentially and has a high possibility of recurrence in the short-term. In the current study, we aimed to evaluate the treatment effects of combination therapy of CLX and DC on OA and investigate the underlying molecular basis.

MATERIALS AND METHODS

Experimental Animals

Thirty 6-week-old male Sprague–Dawley (SD) rats (weighing 240±20 g) were purchased from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). All SD rats were raised under the conditions of 25 ±2 °C and 70 % humidity with a 12-h light/dark cycle, with free access to water and food. All experimental operations on animals followed the rules of Institutional Animal Care and Use Committee of Zhengzhou University.

Materials and Reagents

Celecoxib (99.65 %) was purchased from Excel Pharmaceutical Company (Zhejiang, China). Diacerein was purchased from Kunming Jida Pharmaceutical Company (Yunnan, China); Rat IL-1β and C-telopeptide fragments of type II collagen (CTX-II) ELISA kit were purchased from Cell Signaling Technology (Danvers, MA, USA). Caspase-3 activity assay kit was from Beyotime Institute of Biotechnology (Shanghai, China).

Animal Model and Experimental Groups

After adaptive feeding for a week, all animals were randomly assigned into five groups ($n=6$): control, OA, OA + CLX, OA + DC, and OA + CLX + DC. OA rats were established according to previously described procedures [23]. Briefly, the right knee joint of rats was injected with 0.3 ml II collagenase twice on the first and fourth day, respectively. The control group received 0.3 ml (9 %) normal saline. A successful OA model was built successfully after 6 weeks from the first injection of II collagenase. The test drugs were injected continuously for 4 weeks into the experimental groups at a frequency of once a day. The OA + CLX group was treated with CLX (0.5 mg/ml), the OA + DC group was treated with DC (0.045 mg/ml), and the OA + CLX + DC group was treated with CLX and DC (0.5 and 0.045 mg/ml, respectively); and the control group and OA group were treated with the same dose of normal saline. The injected volumes were 0.5 ml. The pharmacological dose was calculated according to the conversion formula of adult and experimental animals.

Samples Collection

The rats were anesthetized with intraperitoneal injection of 10 % chloral hydrate solution (0.4 ml/100 g) on day 28. The blood from arteria femoralis was centrifuged at 3000*g* for 10 min to gain serum. The serum was then stored at −20 °C. After blood collection, all animals were euthanized by air embolism and the tibiofemoral joints were dissected to acquire tissue samples followed by immediate placement into liquid nitrogen, before being stored at −70 °C for further biological and molecular analysis. For histologic analysis, the joint cartilage of the medial femoral condyle was dissected, fixed, and embedded in paraffin. Paraffin-embedded tissues were cut into 5-μm thick sections and stained with safranin O following the standard protocols. Histologic scores of cartilage degeneration were assessed according to a previously reported scoring system [24].

Determination of IL-1β and CTX-II in Serum

The levels of IL-1β and CTX-II in serum were measured by ELISA kits according to the manufacturer's instructions. Experimental data are expressed as nanogram per deciliter (ng/dl). Absorbance was measured using a microplate reader at 450 nm (Sigma, St. Louis, MO, USA).

Determination of NO Content

The determination of total NO content in the knee joint samples homogenate was measured according to the Griess reaction [25]. NO is easily oxidized to generate NO_2^- *in vivo* or in an aqueous solution. Under acidic conditions, NO_2^- reacts with sulfonamides to generate diazonium, which further couples with naphthyl ethylenediamine to form an azodye. The concentration of the reaction product and the NO concentration maintained a linear relationship, and the maximum absorption peak was detected at a wavelength of 550 nm. The NO content was expressed as nanomoles per milligram of protein in samples.

Detection of Caspase-3 Activity

The joint tissue samples previously stored at −70 °C were removed and mashed to prepare tissue homogenate. The protein concentrations were quantitated using the method of Bradford protein quantification. Caspase-3 activity was detected according to the instructions provided with the caspase-3 activity assay kit. Absorbance at 405 nm was detected using a microplate reader.

Nuclear Proteins Extraction

Nuclear proteins were isolated using an extraction kit (Sangon, Shanghai, China) as per the supplier's instructions. Briefly, the tissues were homogenate in cytoplasmic buffer containing protease inhibitors mixed followed by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was collected as cytosolic fraction. Nuclear pellets were collected and were resuspended in nucleus buffer for 10 min at 4 °C. Then, the sample was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected as nuclear proteins.

Western Blot Analysis

Proteins were extracted from bone tissue homogenates, and the concentrations were measured using Bradford protein quantification. A total of 15 μg of protein samples was separated by 12 % SDS-PAGE electrophoresis followed by electronic blotting on a nitrocellulose membrane (Amersham, Little Chalfont, UK). Then, the membrane was incubated in Tris-HCl/Tween-20 (TBST) containing 5 % non-fat dry milk to block non-specific binding at room temperature for 1 h. Then, the membrane was cut in accordance with the relative molecular mass of the unknown-protein followed by incubation with primary antibodies (against JNK, MMP-13, Atf-2, COX-2, MEKK1, MKK7, p38MAPK, and NF-κB obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA); β-actin and histone from Boaosen Biotechnology Company

(Beijing, China); p-MEKK1, p-MKK7, p-JNK, and phosphorylation of p38MAPK (p-p38MAPK) from Cell Signaling Technology (Danvers, MA, USA). All antibodies were diluted (1:1000) in the TBST buffer (containing 5 % non-fat dry milk) overnight at 4 °C. Next, the membrane was washed once every 15 min three times with TBST buffer. Subsequently, the membrane was incubated in horseradish peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, China) diluted (1:2000) in TBST buffer for 2 h. Then, the NC membrane was washed in accordance with the above washing method. Finally, ECL blotting substrate was added to the membrane to react for 30 s followed by exposure to X-ray film for 20 min, which was used for protein visualization.

Statistical Analysis

All measured data were expressed as mean±standard deviation (SD). The statistical significance of differences between the two groups was determined by Student's *t* test, and among multiple groups was determined by ANOVA. A p value of less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

CLX Combined with DC Effectively Attenuates OA in the Rat Model

To investigate the effect of CLX combined with DC on OA, we analyzed their effects on pathologic changes of in the joint cartilage of the medial femoral condyle using safranin O staining method. The results showed that CLX combined with DC exhibited a better performance on superficial layer with decreased cartilage surface erosion and increased safranin O staining in the superficial layer (Fig. 1a), as compared with a single treatment by CLX or DC. The histologic scores of the different treated groups were shown in Fig. 1b.

CLX Combined with DC Effectively Reduces the Inflammatory Mediators in the Rat OA Model

To investigate the effects of CLX combined with DC on inflammatory mediators, we depicted the content of IL-1β and NO. The results showed that the levels of IL-1β and NO were significantly increased in the OA group compared with the control group. DC treatment was capable of decreasing IL-1β and NO levels, whereas CLX treatment had no effect on IL-1β but was capable of decreasing NO levels. Notably, CLX combined with DC significantly decreased IL-1β and NO levels compared to a single treatment by CLX or DC (Fig. 2a, b).

CLX Combined with Diacerein Effectively Improves Cartilage Metabolism Rate

To investigate the effect of CLX combined with DC on cartilage metabolism and cartilage degradation, we detected the level of CTX-II, a key indicator in cartilage metabolism [26]. The results showed that CLX and DC treatment alone significantly elevated the CTX-II levels which were decreased in OA rats. As expected, CLX combined with DC treatment more effectively increased CTX-II levels than CLX and DC treatment alone (Fig. 3).

CLX Combined with DC Effectively Inhibits Chondrocyte Apoptosis

To examine the effect of CLX combined with DC on chondrocyte apoptosis in knee cartilage of OA rats, the activity of caspase-3 in knee cartilage was measured. The results showed that caspase-3 activity was significantly increased in OA rats and a single treatment of CLX or DC markedly decreased caspase-3 activity. Moreover, compared with a single treatment with CLX or DC, CLX combined with DC further decreased caspase-3 activity (Fig. 4).

CLX Combined with DC Effectively Suppresses JNK Signaling Pathway

To further investigate the underlying molecular mechanism of CLX combined with DC in the treatment of OA, the effect of CLX combined with DC on the JNK signaling pathway was examined by Western blot analysis. The results showed that JNK signaling was activated in OA rats in which the protein levels of MEKK1 (Fig. 5a), MKK7 (Fig. 5b), JNK (Fig. 5c), and their phosphorylated forms as well as the ratio between total and phosphorylated forms were all highly upregulated. A single treatment with CLX or DC could decrease the expression of these proteins and inhibited JNK signaling pathway activation, whereas CLX combined with DC decreased the expression of these proteins and inhibited JNK signaling pathway activation more remarkably (Fig. 5a–c).

CLX Combined with DC Effectively Decreases JNK Downstream Gene Expression

To further validate the effect of CLX combined with DC on JNK signaling inactivation, the

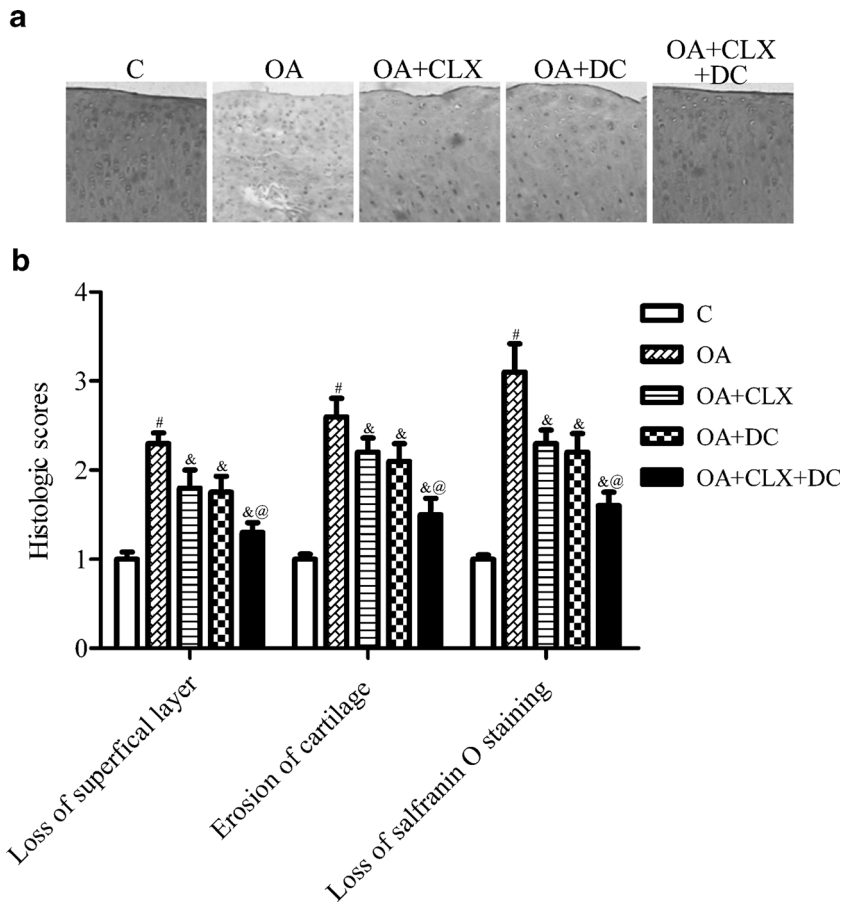

Fig. 1. Treatment effects of CLX combined with DC on OA. **a** Histologic sections of medial femoral condyle cartilage stained by safranin O from different treated groups. *C* control health rats, *OA* OA rats without treatment, *OA + CLX* OA rats treated with CLX, *OA + DC* OA rats treated with DC, *OA + CLX + DC* OA rats treated with CLX in combined with DC. **b** The histologic scores of medial femoral condyle cartilage in different groups. $N=3$; $^{\#}p<0.05$, *vs.* control; $\&p<0.05$, *vs.* OA group; $^{@}p<0.05$, *vs.* OA + CLX or OA + DC.

downstream protein levels of JNK were analyzed by Western blot analysis. The results showed that the protein expressions of MMP-13 (Fig. 6a), Atf-2 (Fig. 6b), and COX-2 (Fig. 6c) were highly upregulated in OA rats which were significantly downregulated by a single treatment with CLX or DC, whereas CLX

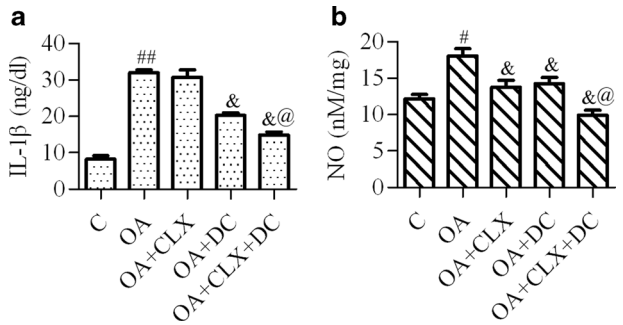

Fig. 2. Effects of CLX combined with DC on inflammatory mediators in a rat OA model. Detection of levels of **a** IL-1β in serum and **b** NO in cartilage tissue in different treated groups. $N=3$; $^{\#\#}p<0.01$, *vs.* control; $^{\#}p<0.05$, *vs.* control; $\&p<0.05$, *vs.* OA group; $^{@}p<0.05$, *vs.* OA + CLX or OA + DC.

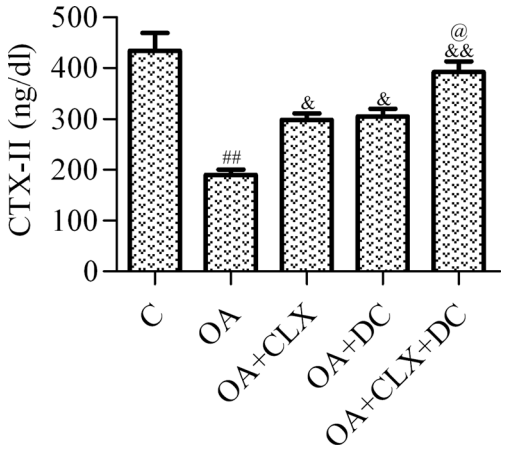

Fig. 3. Effects CLX combined with DC on cartilage metabolism rate in different treated groups. N=3; $^{\#\#}p$<0.01, *vs.* control; &p<0.05, *vs.* OA group; &&p<0.01, *vs.* OA group; $^{@}p$<0.05, *vs.* OA + CLX or OA + DC denotes.

combined with DC had a stronger inhibitory effect on the expression of these proteins.

CLX Combined with DC Effectively Inhibits p38MAPK and NF-κB Signaling Pathways

Moreover, we further investigated the effect of CLX combined with DC on the activation of p38MAPK and NF-κB signaling pathways. Western blot analysis exhibited that the phosphorylation of p38MAPK (p-p38MAPK) which was upregulated in the OA groups were significantly decreased by a single treatment with CLX or DC and further decreased by CLX combined with DC (Fig. 7a, b). In addition, the nuclear accumulation of NF-κB p65 was significantly increased in OA groups as compared with the control. And the increased accumulation of NF-κB p65 in the nucleus was markedly inhibited by a single treatment with CLX or DC, whereas CLX combined with DC exhibited a stronger inhibitory effect (Fig. 7c, d).

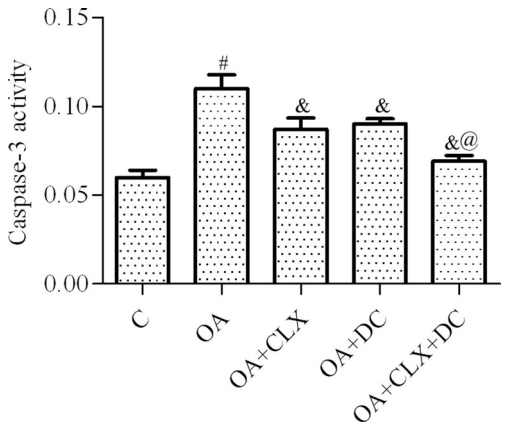

Fig. 4. Effects of CLX combined with DC on chondrocyte apoptosis. Caspase-3 activity was determined by the caspase-3 activity assay kit in the knee joint tissue. N=3; $^{\#}p$<0.05, *vs.* control; &p<0.05, *vs.* OA group; $^{@}p$<0.05, *vs.* OA + CLX or OA + DC.

DISCUSSION

Osteoarthritis (OA) is one of the most common joint diseases affecting human health. Depending on the potential risk factors, OA is divided into two kinds: generalized osteoarthritis, associated with age, sex, *etc.* and localized osteoarthritis, which is associated with obesity, joint damage, and deformities. Regardless of the potential risk factors, the etiology and molecular mechanisms of osteoarthritis are still not known. Currently, most of the research is only for extracellular matrix synthesis and degradation, with little focus on aspects of chondrocyte apoptosis. Studies found that IL-1β can induce the synthesis of NO by inducible NO synthase (iNOS), followed by activating MMPs to promote matrix degradation. In addition, NO plays an important role in chondrocyte apoptosis, leading to cell death [27]. As the primary enzyme involved in apoptosis, caspase-3 plays an important role in chondrocyte apoptosis. In this study, we found that CLX and DC can significantly rival increasing caspase-3 activity, and NO and IL-1β content induced by II collagenase in an OA model; more satisfying results were found in the group of CLX combined with DC. Thus, our results indicate that CLX and DC may be involved in inhibiting apoptosis of cartilage and clearing of inflammatory mediators NO and IL-1β. Our findings have an important significance for the early prevention and treatment of OA.

The JNK pathway plays an important role in cell apoptosis, differentiation, and proliferation. Studies have reported that TNF-α-induced apoptosis was related to the sustained activation of JNK [11]. Activated JNK cannot only increase the expression of MMP-13 but can also activate the transcription factor Atf-2, which starts the process of gene transcription of COX-2 [28]. Also, application of the JNK inhibitor can inhibit cartilage destruction [29]. Legendre *et al.* considered that Rhein, a diacerein-derived metabolite, can regulate chondrocyte proliferation and the expression of some matrix-degrading enzymes (MMP-3 and MMP-13) through inhibiting the JNK signaling pathway [30]. Our study demonstrated that the expression of three proteases MEKK1, MKK7, JNK, and their phosphorylation in the JNK signaling pathway, as well as the expression of the downstream transcription factors and

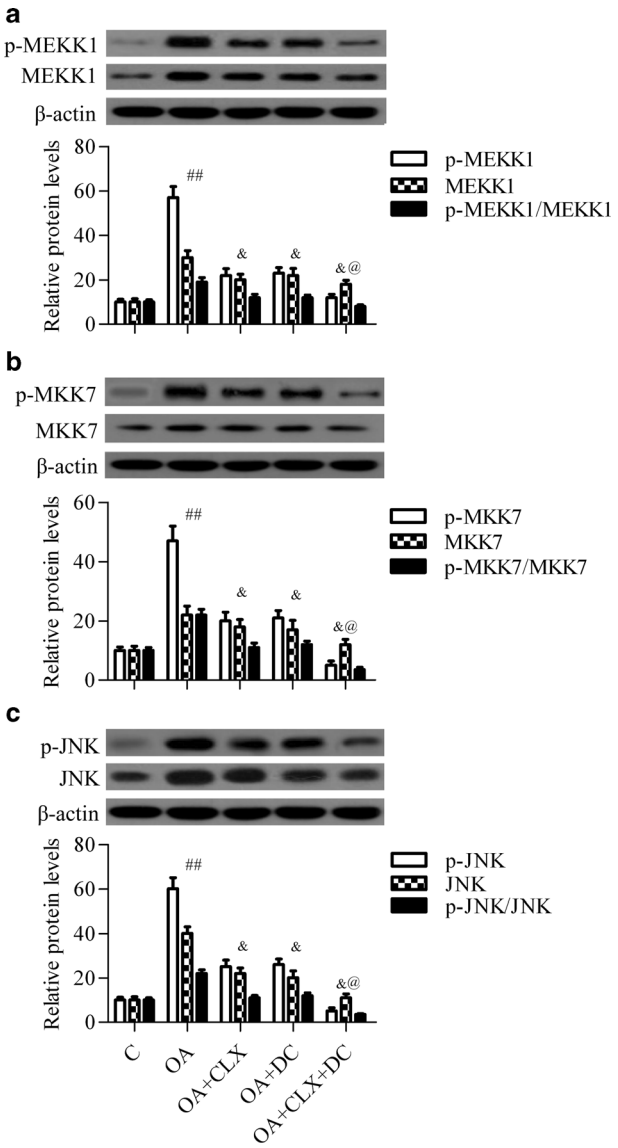

Fig. 5. Effects of CLX combined with DC on JNK signaling pathway. Western blot analysis was performed to detect the protein levels of **a** MEKK1, **b** MKK7, **c** JNK, and their phosphorylated forms in the knee joint tissues of different treated groups. The relative protein expression was quantitated by Quantity One software and normalized to β-actin. $N=3$; $^{\#\#}p<0.01$, *vs.* control; $\&p<0.05$, *vs.* OA group; $^{@}p<0.05$, *vs.* OA + CLX or OA + DC.

matrix metalloproteinases, were remarkably decreased by CLX combined with DC. The results showed that protein expression of JNK signaling pathway and its downstream protein in the groups of CLX and/or DC individually or in combination were significantly lower than that in the OA group. These results implied that high levels of inflammatory mediators induced by the injection of II collagenase possibly activate the transcription factor Atf-2 by activation of the JNK signaling pathway, which initiate transcription of inflammatory gene COX-2, resulting in apoptosis and inflammatory reactions. On the other hand, the activation of JNK may induce the gene expression of MMP-13, promoting the degradation of cartilage matrix, resulting in cartilage damage. Our results indicated that CLX combined with DC effectively protected chondrocytes and maintained homeostasis of extracellular matrix through inhibition of inflammatory mediators and related signaling pathways, such as JNK.

Furthermore, we found that CLX combined with DC also effectively inhibited the p38MAPK and NF-κB

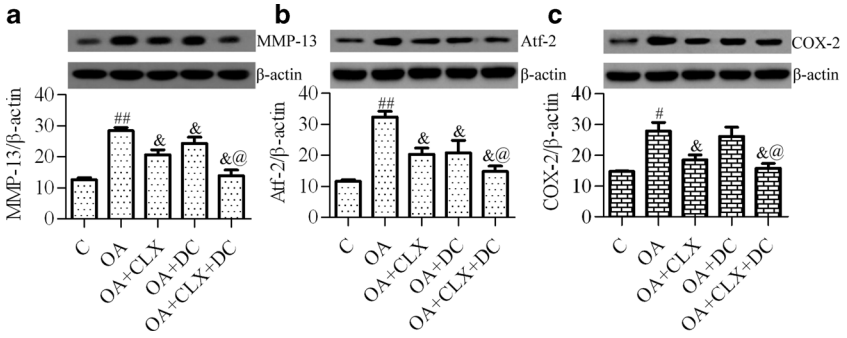

Fig. 6. Effects of CLX combined with DC on the down stream gene expression of JNK signaling. Western blot analysis was performed to detect the protein expression of **a** MMP-13, **b** Atf-2, and **c** COX-2 in the knee joint tissues of different treated groups. The relative protein expression was quantitated by Quantity One software and normalized to β-actin; N=3; $^{\#\#}p$<0.01, *vs.* control; $^{\#}p$<0.05, *vs.* control; &p<0.05, *vs.* OA group; $^{@}p$<0.05, *vs.* OA + CLX or OA + DC.

signaling pathways. Activation of p38MAPK signaling pathway is associated with cartilage collagen degradation, chondrocyte apoptosis, and inflammation process in OA [13–15]. It has reported that p38MAPK activation induced by oleanolic acid is inhibited and prevented by CLX in human coronary smooth muscle cells [31]. Hypoxia mimetic cobalt chloride-induced activation of p38MAPK is significantly blocked by CLX renal tubular cells [32]. In mouse skin, CLX abrogated the phorbol ester-induced activation of p38MAPK [33]. CLX has been demonstrated to inhibit MMP production *via* downregulation of NF-κB and JNK signaling pathways [34]. CLX has been found to sensitize *Staphylococcus aureus* to antibiotics through inhibiting JNK activation and thereby stabilizing and activating SIRT1 protein [35]. In addition, DC has also been shown to inhibit the IL-1-activated MAPK and NF-κB signaling pathways [36]. In human chondrocytes and synoviocytes, DC modulates the expression of matrix-degrading enzymes and the cell proliferation by inhibiting JNK and NF-κB signaling pathways [30]. DC exhibited a protective effect against subchondral bone remodeling through the inhibition of extracellular signal-regulated kinase-1/2 and p38MAPK signaling pathways [37]. In the present study, we have demonstrated that CLX combined with DC showed a stronger inhibitory effect on JNK, p38MAPK, and NF-κB signaling pathways than applied alone due to their inhibitory effects on widely inflammatory signaling pathways.

In summary, our study provided evidence that CLX combined with DC had a more satisfactory treatment effect on OA as compared with a single treatment by CLX or DC. However, further evaluation of CLX combined with DC on treatment of OA and the underlying mechanism remain to be validated.

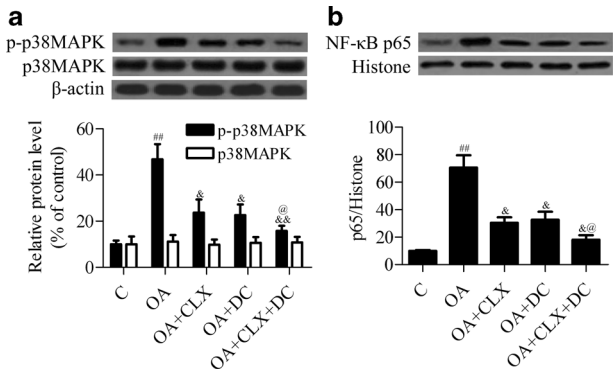

Fig. 7. Effects of CLX combined with DC on p38MAPK and NF-κB signaling pathways. **a** The protein levels of p-p38MAPK and total p38MAPK in the knee joint tissues of different treated groups were detected by Western blot analysis, and quantitated by using Quantity One software and normalized to β-actin. **b** Western blot analysis of NF-κB p65 nuclear protein expression level in different treated groups. The relative protein expression was quantitated by Quantity One software and normalized to histone. N=3; $^{\#\#}p$<0.01, *vs.* control; &p<0.05, *vs.* OA group; &&p<0.01, *vs.* OA group; $^{@}p$<0.05, *vs.* OA + CLX or OA + DC denotes.

Conflict of Interest. The authors declare that there are no conflicts of interest.

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