Original Research

Oxymatrine suppresses IL-1β-induced degradation of the nucleus pulposus cell and extracellular matrix through the TLR4/NF-κB signaling pathway

Kang Wei[1,*], Jun Dai[2,*], Zhenggang Wang[1], Yaping Pei[1], Yan Chen[1], Yifan Ding[1], Qing Ding[1], Paerxiati Ahati[1], Xiaozhong Zhou[2], Huan Wang[1] and Huang Fang[1]

[1]Department of Orthopaedics, Biological Engineering and Regenerative Medicine Center, Tongji Hospital, Tongji Medical College, HuaZhong University of Science and Technology, Wuhan 430030, China; [2]Department of Orthopaedics, The Second Affiliated Hospital of Soochow University, Suzhou 215004, China

Corresponding authors: Huan Wang. Email: wanghuan@tjh.tjmu.edu.cn; Huang Fang. Email: fanghuangtjh@hust.edu.cn

*These authors contributed equally to this article.

Impact statement

Currently, drug therapy is a potential treatment for patients with intervertebral disc degeneration. In the present research, oxymatrine intervenes in intervertebral disc degeneration effectively via regulating inflammation in intervertebral disc degeneration rats. Our research highlights the therapeutic potential of oxymatrine in the treatment of intervertebral disc degeneration.

Abstract

Intervertebral disc degeneration is the main cause of low back pain. However, its pathomechanism has not been fully clarified yet. Previous studies have indicated that inflammation may lead to apoptosis of nucleus pulposus cells and break the balance between anabolism and catabolism of the nucleus pulposus extracellular matrix. The purpose of this study is to explore the mitigative effect of oxymatrine on extracellular matrix degradation and apoptosis of nucleus pulposus cells after interleukin-1 beta-induced inflammation, and its possible signaling pathway. We examined the gene and protein levels of collagen II, aggrecan, and MMPs (MMP2/3/9/13) and interleukin 6 in nucleus pulposus cells. The results demonstrated that oxymatrine could reduce extracellular matrix degradation and apoptosis of nucleus pulposus cells; interleukin-1 beta prompted the expression of MMPs and interleukin 6 through TLR4/NF-κB axis, while oxymatrine reduced the expression of MMPs and TNF-α induced by interleukin-1 beta. Moreover, TAK 242, as a small molecule inhibitor of TLR4 signaling, was used to detect the effect of oxymatrine on the TLR4/NF-κB signaling. The final experimental results show that oxymatrine could reduce the inflammatory response of nucleus pulposus cells and degradation of nucleus pulposus tissue. Oxymatrine may be a potential medicine to reduce disc inflammation and relieve intervertebral disc degeneration by inhibiting the TLR4/NF-κB signal pathway.

Keywords: Inflammation, TLR4/NF-κB signaling pathway, oxymatrine, intervertebral disc degeneration

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Introduction

Intervertebral disc degeneration (IDD) is the major pathogenesis of low back pain, which causes severe physical pain and mental stress and reduces the quality of life of patients.[1,2] However, as a progressive and irreversible process, the mechanism of IDD still remains unclear. Currently, drug therapy of IDD mainly focuses on the relief of low back pain, for example through the use of non-steroidal anti-inflammatory drugs (NSAIDs). Although NSAIDs are effective in relieving pain, their side effects should not be neglected. Representing a safer way for using NSAIDs, natural plant medicine has been introduced to treat IDD.[3] This research explored the interventional effects of oxymatrine (OMT), a traditional Chinese medicine, on the pathological process and inflammatory signaling pathway of IDD.

Intervertebral disc (IVD) degeneration is closely associated with the disorders between anabolism and catabolism in the extracellular matrix (ECM) and the apoptosis of the nucleus pulposus (NP) cells.[4] Furthermore, inflammation is

a significant factor leading to decomposition of ECM and apoptosis of NP cells, and is involved in the pathogenesis of IDD.[5] It has been reported that the activation of the NF-κB signaling pathway can aggravate the division of apoptotic protein and the production of multitudinous inflammatory molecules, leading to typical apoptosis of cells and synthesis of fibrotic matrix, and ultimately causing a more severe progressive inflammatory response around the IVD tissue.[6,7] In addition, matrix metalloproteinases (MMPs) play important roles in the inflammation response by regulating pro-inflammatory cytokines and chemokines.[2,8] It has been found that MMPs (MMP2/3/9/13) promoted the release of inflammatory factors and the decomposition of ECM in patients with IDD.[9]

Up to now, a variety of inflammatory factors related to the development of IDD have been studied, and a highly conserved nuclear protein named high mobility group box1 (HMGB1) has been reported to play a significant role in the pathogenesis of osteoarthritis and arthritic diseases.[10] In addition, toll-like receptor 4 (TLR4) is a key immune recognition receptor that mediates the transcription of inflammatory cytokines and promotes an inflammatory response.[11] HMGB1 combines and interacts with TLR4 to activate the nuclear factor kappa B (NF-κB), resulting in the release of inflammatory factors. This process indicates that the utilization of elements regulating the HMGB1/TLR4/NF-κB signaling pathway is available as a potential method to relieve IDD.[12]

In the process of exploring natural medicine for the treatment of IDD, it has been found that OMT inhibits the inflammatory response of IDD. OMT is a natural alkaloid extracted from the medicinal herb of *Sophora flavescens* Ait.[13] Previous research has shown that OMT has antioxidant, anti-inflammatory, antiviral, hepatoprotective, and immunosuppressive effects. At present, OMT is widely used to treat viral hepatitis, traumatic brain injury, and acute pancreatitis in clinical practice.[14,15] In previous research, OMT has been reported to reduce inflammation of influenza A virus and hepatic carcinoma.[16] However, there are so far no reports that OMT inhibits the inflammation of IDD through the HMGB1/TLR4/IkBα/NF-κB signaling pathway. This research therefore proposes to investigate the effect of OMT on inflammation of IDD, and identifies the mechanism of OMT intervening in NP cell inflammation, mainly focusing on the HMGB1, TLR4, IkBα, and NF-κB signaling pathways.

Materials and methods

Experimental animals

Sprague-Dawley rats were used in all experiments in this study, including Sprague-Dawley rats of 200 g–250 g for cell experiments and Sprague-Dawley rats of 350 g for animal experiments. All animal experiments were performed in accordance with the International Guiding Principles for Animal Research and approved by the Animal Experimentation Committee of Huazhong University of Science and Technology.

Isolation and culture of NP cells

The NP cells were collected from the disc tissue of Sprague-Dawley rats (220 g–250g). Firstly, the disc tissue was cut and separated using 0.25% trypsin (Biothink Biological Technology, China) and 0.01% EDTA at 37°C for about 45 min, and then digested at 37°C for about 3 h with 0.25% type II collagenase (Biothink Biological Technology, China). Finally, the NP cells were cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS, Gibico, South America) and 1% penicillin/streptomycin (Biothink Biological Technology, China) in a humidified atmosphere of 5% CO_2 at 37°C. During culture, NP cells were employed in the first three generations, and no significant morphological changes were observed between the primary and the progeny. The cellular inflammation model was induced using 5 ng/mL interleukin-1 beta (IL-1β).[17]

Cell viability assay

The Cell Counting Kit-8 (CCK8, Vazyme Biotech, USA) was used to detect the activity of NP cells. In this experiment, cells were seeded into three replicating wells with a density of 1000 cells/well and pre-incubated in 96-well plates for 24 h. Different concentrations of OMT (0, 0.25, 0.5, 1, or 2 mg/mL) and IL-1β (0, 2.5, 5, 10, or 20 ng/mL) (R&D systems, USA) were added to each well and incubated in an incubator for 72 h. Ten microliters of CCK-8 reagent diluted in DMEM/F12 (10% FBS) were added to each well. The plates were incubated in darkness at 37°C for 2 h, and the absorbance of each well was then measured at a wavelength of 450 nm with a miniature tablet reader (BioTek, USA). Following the CCK8 instructions, the cell viability of each well was calculated as (OD OMT/IL-OD blank)/(OD con-OD blank).

Western blot analysis

Briefly, the total protein extracts of NP cells were homogenized in cold radio immunoprecipitation assay (RIPA) lysis buffer containing 1% Phenylmethanesulfonyl fluoride (PMSF) (Biothink Biological Technology, China). Bicinchoninic acid (BCA) Protein Assay Kit (Biothink Biological Technology, China) was used to determine protein concentration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Biothink Biological Technology, China) was used to isolate the protein extract and electrotransfer to Polyvinylidene fluoride (PVDF) membrane (Millipore, America). After being blocked with tris-buffered saline tween-20 (TBST) containing 5% skimmed milk at 25°C for 1 h, the membrane was incubated with primary antibody diluent of the target protein on a shaker at 4°C overnight. Subsequently, the membrane was rinsed with 1 × TBST five times for 5 min and incubated with the corresponding secondary antibody at 25°C for 1 h. Enhanced chemiluminescence (ECL, Biothink Biological Technology, China) visualization of target proteins was performed by imager Chemidoc XRS Imaging System (BIO-RAD, USA). The major antibodies used in

the research are shown in Table 1. GAPDH was used as the loading control.

RNA extraction and real-time RT-PCR analysis

Total RNAs were extracted from NP cells by RNAiso Plus (Takara Bio Inc., Japan), and cDNA was prepared using reverse transcriptase (Toyobo, Japan) according to the manufacturer's specifications. Then, the cDNA was amplified with specific primers and SYBR Premix Ex Tap (Tli RNaseH Plus) (2×) (Toyobo, Japan). The sequences of primers used in the research are shown in Table 2. Fluorescence detection was performed using the real-time PCR detection system (Bio-Rad Laboratories, USA). The mRNA expression levels of the target genes were standardized by the mRNA level of GAPDH based on the $2^{-\triangle\triangle Ct}$ method and compared with the control group.

Apoptosis analysis

Apoptosis in NP cells was induced by serum deprivation. The cells were seeded on aseptic cover slides on the six-well plates. Upon reaching 90% confluence, the cells were treated for 72 h, and analyzed by two methods as follows: (1) Hoechst 33258 staining: after NP cell intervention, cells were washed three times with 1 × PBS and stained with the Hoechst 33258 staining solution (Beyotime, China) according to the instructions; (2) Flow cytometry: NP cells were isolated and harvested with trypsin (0.25%) and then rinsed three times with 1 × PBS for 10 min. After being treated with the FITC Annexin V Apoptosis Detection Kit according to the manufacturer's instructions (BD Pharmingen, USA), the NP cells were detected by flow cytometry.

Table 1. Primary antibodies and drug list.

Name	Source	Brand	Catalog number	Dilution
GAPDH	Mouse	Proteintech	60004-1-Ig	1:5000
MMP2	Rabbit	Proteintech	66366-1-Ig	1:1000
MMP3	Mouse	Proteintech	66338-1-Ig	1:5000
MMP9	Rabbit	Proteintech	10375-2-AP	1:500
MMP13	Rabbit	Proteintech	18165-1-AP	1:500
HMGB1	Rabbit	Proteintech	10829-1-AP	1:1000
TLR4	Mouse	Proteintech	66350-1-Ig	1:5000
NF-κB p65	Rabbit	Proteintech	10745-1-AP	1:2000
Caspase 3	Mouse	Proteintech	66470-2-Ig	1:3000
Collagen 2	Rabbit	Proteintech	15943-1-AP	1:500
Aggrecan	Rabbit	Proteintech	13880-1-AP	1:500
TAK 242		MCE	243984-11-4	

NF-κB: nuclear factor kappa B; TLR4: toll-like receptor 4; HMGB1: high mobility group box1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP.2: matrix metalloproteinase2; MMP3: matrix metalloproteinase3; MMP9: matrix metalloproteinase9; MMP13: matrix metalloproteinase13.

Surgical technique and magnetic resonance imaging

The rats were anesthetized with isoflurane, and the surgical field was prepared aseptically. The normal group received no treatment, and after palpation determined the disc levels, a small cross incision in the skin (about 2 mm in length) was made to help locate the position of the nucleus of a needle. The needle (18-gauge outer diameter 1.270 mm) was used to stab the center of the IVD. Each needle was kept in the disc for 10 s, and 10 μL of OMT (100 μg/μL) was injected simultaneously in the treatment group and the same amount of normal saline was injected in the degenerate group. Then, magnetic resonance imaging (MRI) was performed on the animals two weeks after surgery. The rats were anesthetized with 2% isoflurane/oxygen mixture throughout the MRI examination. The animals were placed prone with tail straight in a 1.5-TMR scanner (Siemens, Germany). Eight consecutive sagittal T2-weighted images were obtained by scanning the tail region of rat with a double-tuned volume radio frequency coil, and all disc regions were covered with a spin echo sequence.

Histological evaluation

Spinal cord tissue was fixed with 4% paraformaldehyde (Biothink Biological Technology, China), which was washed with 0.9% normal saline for three times. After 48 h, the spine was decalcified with 10% EDTA (Biothink Biological Technology, China) for 45 days and embedded in paraffin wax. The paraffin blocks of IVD were cut into 3 μm coronal sections containing the endplate, annulus fibrosus (AF), and coronal section of NP and then stained with hematoxylin-eosin (H&E).

Statistical analysis

Most research in the figure legends were studied by paired Student's t-test and ANOVA with *post hoc* test in GraphPad prism version 5. All of the experiments were repeated at least three times, and the data were expressed as

Table 2. Primer sequences list.

Gene name	Forward (5′-3′)	Reverse (5′-3′)
MMP2	CTGGGCCACGCCATCGCTGC	GCTTGCGGGGAAAGAAGTTG
MMP3	GCTCATCCTACCCATTGCAT	GCTTCCCTGTCATCTTCAGC
MMP9	TCCTTGCAATGTGGATGTTT	CGTCCTTGAAGAAATGCAGA
MMP13	ACCATCCTGTGACTCTTGCG	TTCACCCACATCAGGCACTC
IL-6	GAGGATACCACTCCCAACAGACC	AAGTGCATCATCGTTGTTCATACA
Col2	CTGGTGGAGCAGCAAGAGC	GTGGACAGTAGACGGAGGAAAG
Aggrecan	CTTCCCAACTATCCAGCCAT	TCACACCGATAGATCCCAGA
GAPDH	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG

IL-6: interleukin-6; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP2: matrix metalloproteinase2; MMP9: matrix metalloproteinase9; MMP13: matrix metalloproteinase13; Col2: collagen II.

means ± SEM. p Values <0.05 were considered as statistically significant.

Results

OMT had no obvious toxicity on NP cells at low concentration

NP cells were collected from the IVD as shown in Figure 1 (a). The cytotoxicity of OMT in NP cells was detected by CCK-8 assay. The NP cells were treated with OMT (0, 0.25, 0.5, 1.0, and 2.0 mg/mL) and IL-1β (0, 2.5, 5, 10, and 20 ng/mL) for 48 h. The cytotoxicity of OMT was determined by CCK-8 assay. The results showed that OMT exhibited no conspicuous cytotoxicity on NP cells at the concentration of 0–2 mg/mL (Figure 1(b)). The viability of NP cells had no significant alteration when NP cells were incubated with IL-1β of the concentrations of 0–20 ng/mL (Figure 1(c)). The survival rate of NP cells incubated with IL-1β (10 ng/mL) and OMT (0–2 mg/mL) was determined by CCK-8 assay. The survival rates of NP cells also showed no significant changes after NP cells with different concentrations of OMT combined with IL-1β (Figure 1(d)).

OMT inhibited mRNA levels of MMPs in NP cells induced by IL-1β

The transcriptional expression levels of MMPs (MMP2, MMP3, MMP9, MMP13) and IL-6 in NP cells were detected by RT-qPCR after IL-1β (5 ng/mL) addition and OMT regulation. As shown in Figure 2, the mRNA expression levels of MMP3 were significantly upregulated after the addition of IL-1β compared with that in the normal group. This result was consistent with previous research that has reported the relationship between these metabolic enzymes and NP cells' ECM.[18] Nevertheless, the mRNA expression levels of MMP3 decreased significantly with the increase of OMT concentration (0, 0.25, 0.5, 1.0, and 2.0 mg/mL, respectively) (Figure 2(a)). Consistent results were also observed when the mRNA levels of other MMPs (MMP2, MMP9, and MMP13) were detected after treatment with OMT of 1.0 mg/mL (Figure 2(b)). In addition, relevant expression levels of MMPs were also tested. Compared with the normal group, both the protein and mRNA expression levels of MMPs (MMP2, MMP3, MMP9, and MMP13) and IL-6 were upregulated markedly after IL-1β treatment. However, the expression levels of MMPs and IL-6 were downregulated significantly after subsequent OMT treatment, which returned to almost normal (Figure 2(c) and (d)).

OMT prevented the decrease of ECM collagen II and aggrecan levels induced by IL-1β in NP cells

To evaluate the effects of OMT on the production of NP ECM in IDD, the gene and protein levels of matrix, including collagen II (Col2) and aggrecan were detected (Figure 3). Compared with the normal group, IL-1β treatment (10 ng/mL) could significantly reduce the mRNA levels of Col2 and aggrecan. However, the mRNA levels of Col2 and aggrecan increased with the addition of OMT (1 mg/mL) compared with the IL-1β-treated group (Figure 3(a)). Furthermore, OMT treatment also increased the protein expression levels of Col2 and

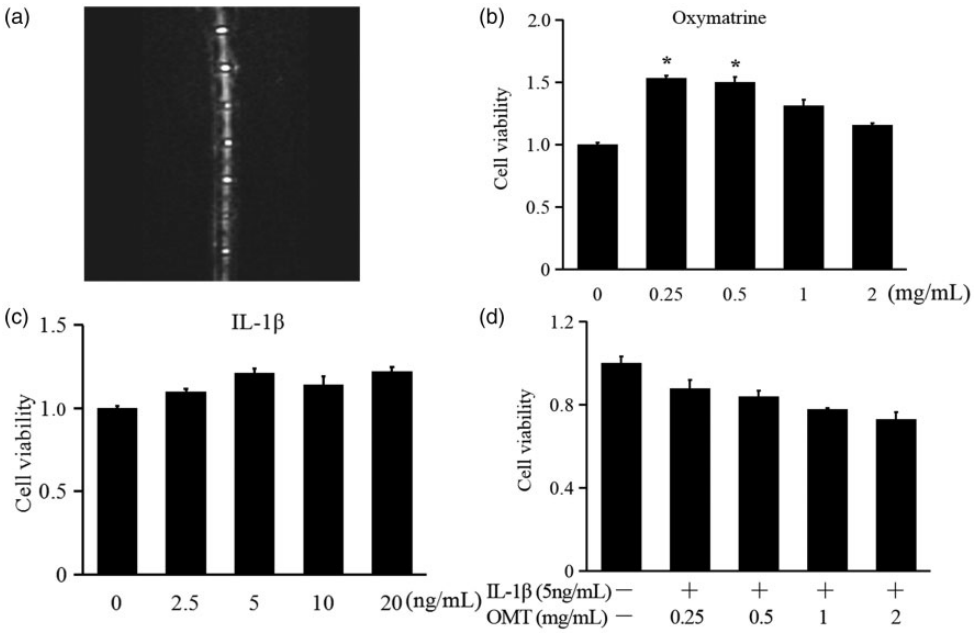

Figure 1. The effect of OMT and IL-1β on NP cells viability. (a) The NP cells were collected from the coccyx of Sprague-Dawley rats. (b) Oxymatrine (0, 0.25, 0.5, 1 and 2 mg/mL). (c) IL-1β (0, 2.5, 5, 10 and 20 ng/mL) for 48 h, followed by the CCK8 analysis for cell viability. (d) NP cells were pretreated for 2 h with various concentrations of oxymatrine (0, 0.25, 0.5, 1, and 2 mg/mL) and then stimulated with IL-1β (5 ng/mL) for 48 h, followed by CCK8 analysis of cell viability. The values are presented as the mean ± SD. The values are presented as the mean ± SD. *$p < 0.05$ vs. control group, $n = 3$.
IL-1β: interleukin-1 beta.

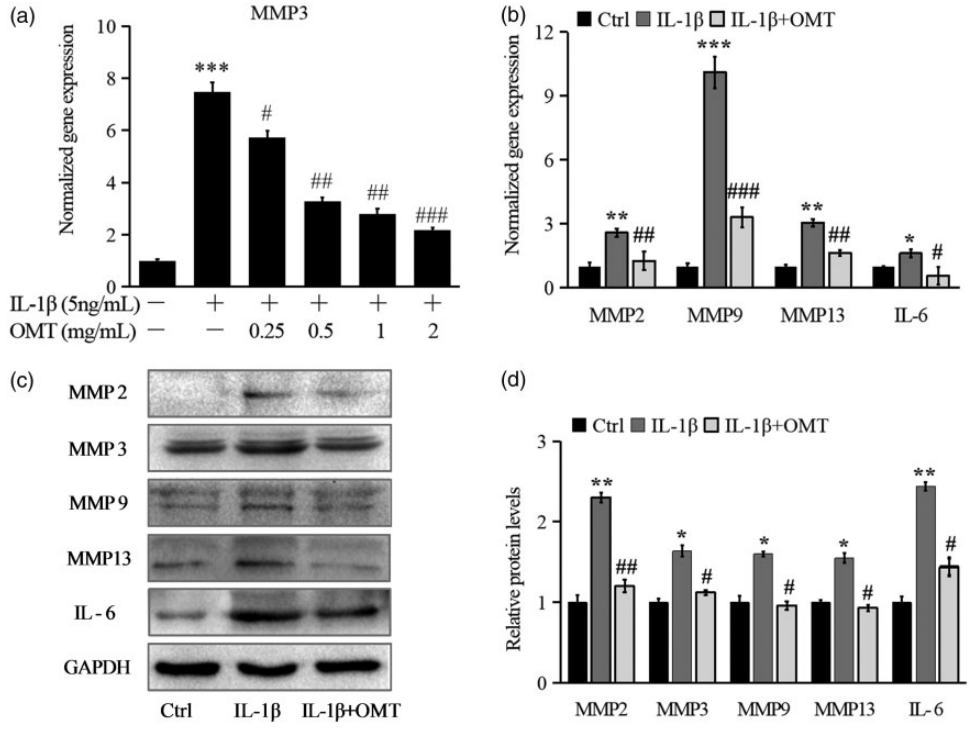

Figure 2. Effects of OMT on MMPs expression in NP cells. Cells were stimulated by IL-1β with or without OMT treatment. (a) The mRNA levels of MMP3 with different concentrations of OMT (0. 0.25 mg/mL, 0.50 mg/mL, 1.0 mg/mL, 2.0 mg/mL, respectively) were assayed by RT-qPCR. (b) The mRNA levels of MMP2/9/13 and IL-6 were assayed by RT-qPCR. (c) The protein expression levels of MMP2/3/9/13 and IL-6 were determined by Western blot analysis. (d) Quantification of protein levels of MMP2/3/9/13 and IL-6. The values were presented as mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control group, $n = 5$; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ vs. IL-1β group, $n = 5$.
IL-6: interleukin-6; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; MMP2: matrix metalloproteinase2; MMP9: matrix metalloproteinase9; MMP13: matrix metalloproteinase13; IL-1β: interleukin-1 beta; OMT: oxymatrine.

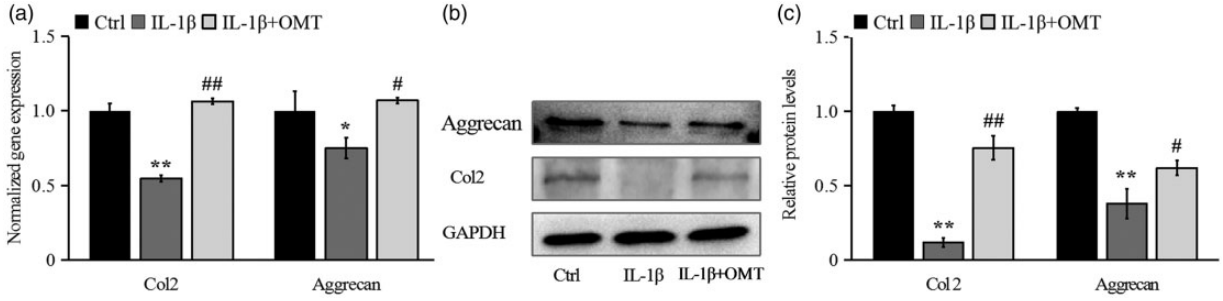

Figure 3. Effects of OMT on Col 2 and aggrecan expression in NP cells. Cells were stimulated by IL-1β with or without OMT treatment. (a) The mRNA levels of Col 2 and aggrecan were assayed by RT-qPCR; (b) The protein expression of Col 2 and aggrecan was determined by Western blot analysis. (c) Quantification of protein levels of Col 2 and aggrecan. The values were presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ vs. control group, $n = 4$; #$p < 0.05$, ##$p < 0.01$ vs. IL-1β group, $n = 4$.
Col2: collagen II; OMT: oxymatrine; IL-1β: interleukin-1 beta; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

aggrecan in NP cells previously treated with IL-1β (Figure 3 (b) and (c)).

OMT reduced the protein expression levels of HMGB1, TLR4, IkBα, and NF-κB p65 after IL-1β-induced inflammation in NP cells

To explore the influence of OMT on the TLR4/NF-κB signaling pathway, the expression levels of HMGB1, TLR4, IkBα, and NF-κB p65 in NP cells treated with IL-1β were detected. We found that HMGB1 was upregulated after treatment by IL-1β. However, after incubation with OMT (1 mg/mL) for 72 h, both the protein and mRNA expression levels of HMGB1 in NP cells were decreased significantly (Figure 4(a) and (b)). Moreover, we also found that the protein expression levels of TLR4, IkBα, and NF-κB p65 in OMT treatments were significantly decreased compared with IL-1β treatment group (Figure 4(a) and (b)).

OMT regulated inflammation in NP cells after IL-1β stimulation through HMGB1, TLR4, and NF-κB signaling

To investigate the mechanism of OMT during the repair process after IL-1β-induced inflammation, a small molecule inhibitor TAK 242 (10 μM) was used to inhibit the TLR4 signaling pathway. Then, the protein expression levels of

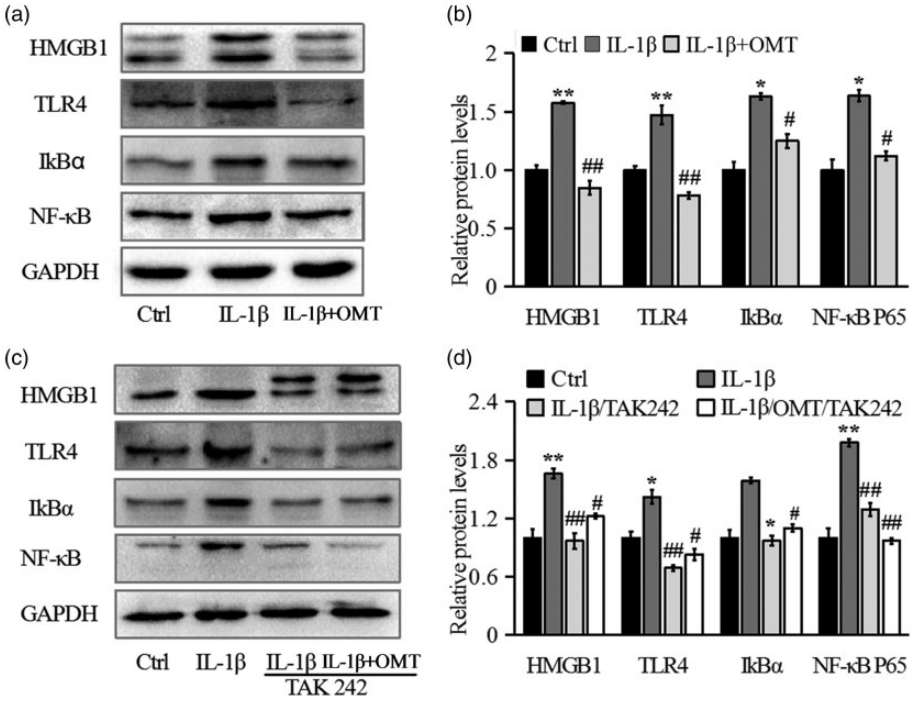

Figure 4. Effects of OMT or TAK 242 on HMGB1, TLR4, IkBα, and NF-κB p65 expression in NP cells. Cells were stimulated by IL-1β with or without OMT and TAK 242 treatment. (a and c) The protein expression levels of HMGB1, TLR4, IkBα, and NF-κB p65 were determined by Western blot analysis. (b and d) Quantification of protein levels of HMGB1/TLR4/IkBα/NF-κB p65. The values are presented as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group. $n = 3$. $\#p < 0.05$, $\#\#p < 0.01$ vs. IL-1β group. $n = 3$.
HMGB1: high mobility group box1; TLR4: toll-like receptor 4; NF-κB: nuclear factor kappa B; IL-1β: interleukin-1 beta; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; OMT: oxymatrine.

genes related to the TLR4/NF-κB signaling pathway in NP cells after IL-1β-induced inflammation were compared. Interestingly, it was found that TAK 242 treatment could markedly decrease the protein expression levels of these genes compared with the normal group, including HMGB1, TLR4, IkBα, and NF-κB p65 (Figure 4(c)). Consistently, the levels of HMGB1, TLR4, IkBα, and NF-κB p65 were markedly decreased in the OMT group after IL-1β-induced inflammation (Figure 4(d)).

OMT alleviated serum deprivation and IL-1β-induced apoptosis of NP cells

To explore the influence of OMT on serum deprivation and IL-1β-induced apoptosis of NP cells, the morphology and apoptotic rate of NP cells were observed by Hoechst 33258 staining and flow cytometry, respectively. After serum deprivation, a higher rate of nuclear fragmentation was observed (Figure 5(a) and (c), 0% FBS). With the stimulation of IL-1β, the fragmentation of nuclei was further increased (Figure 5(a) and (c), 0% FBS + IL-1β). However, the data showed significant morphological changes of NP cells and indicated that the fragmentation of nuclei was upregulated significantly following serum deprivation and IL-1β (10 ng/mL) stimulation compared with the normal group (Figure 5(a) and (c)). In contrast, OMT (1 mg/mL) significantly reduced this phenomenon induced by serum deprivation and IL-1β. Coincidently, the incidence of NP cell apoptosis under different treatment conditions was monitored by flow cytometry (Figure 5(b) and (d)). The results indicated that the apoptosis rate of NP cells after serum deprivation and IL-1β stimulation increased to an average of 26.4% compared with the untreated control group. However, after incubation with OMT, the apoptosis rate of NP cells decreased to 9.2% on average (Figure 5(b) and (d)).

OMT improved IL-1β-induced inflammation in caudal vertebra of rats

Finally, to investigate the effects of OMT on IVD degeneration of rats, we observed the efficacy of OMT on the IDD rat model by MRI. With the loss of NP in IDD, the MRI showed water content of IVD. Compared with the normal group, the water content of IVD after being needle-tabbed was significantly reduced in rats. This demonstrated that IVD was clearly inhibited in the needle-tabbed group, suggesting that the disc had degenerated to different degrees (stage IV/V) (Figure 6(a)). However, the degree of degeneration of IVD after treatment with OMT was significant and similar to that of the normal group (Figure 6(a)). H&E staining showed that ECM decreased significantly after needle-tabbed treatment, and fibrosis was more serious than that in the normal group at two and four weeks. However, the fibrosis of IVD was significantly relieved after injections of OMT, and the structure of IVD in the OMT group was more integrated than that in the needle-tabbed group (Figure 6(b)).

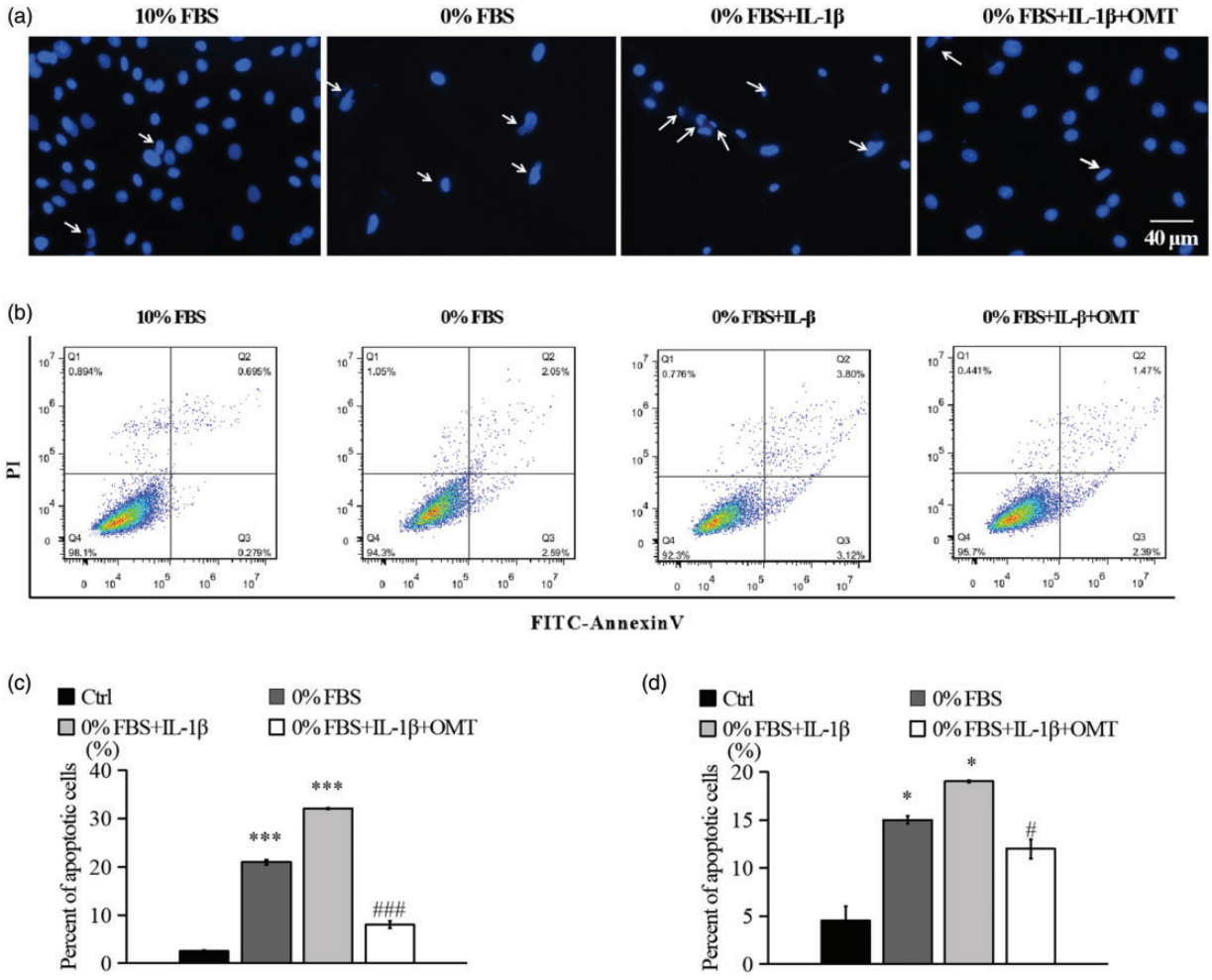

Figure 5. Protective effects of OMT on serum deprivation-induced apoptosis in NP cells. (a and c) Morphological changes of NP cells after different treatments by Hoechst 33258 staining, observed with phase contrast microscope. Bar = 40 μm. (b and d) NP cells stained with Annexin V-FITC/PI were analyzed by flow cytometry. (A color version of this figure is available in the online journal.)
IL-1β: interleukin-1 beta; OMT: oxymatrine; FBS: fetal bovine serum.

Discussion

At present, the primary treatments for diseases resulting from IDD include non-drug treatment, surgery, and drug therapy.[19] The first two methods are intended only to alleviate clinical symptoms, not to interfere with pathological process of IDD. Inflammation is supposed to be the major molecular mechanism of IDD.[19] The previous research has reported that NF-κB signaling pathway regulates the inflammation response, accelerates ECM decomposition and NP cell apoptosis.[20] In addition, MMPs decompose multifunctional ECM proteins such as proteoglycans, laminin, fibronectin, gelatin, and collagens.[21] Therefore, these mechanisms may be the potential targets for drug therapy of IDD.

This research is the first to report that OMT can reduce ECM decomposition and apoptosis in NP cells by regulating the HMGB1/TLR4/NF-κB signaling pathway (Figure 3). OMT is a monosomic alkaloid with anti-inflammatory and protective effects on diseases such as arthritis.[22,23] OMT inhibits RANKL-induced osteoclast generation by downregulating the RANKL-induced NFATc1 and c-fos signaling pathway *in vitro* and has anti-inflammatory and anti-osteoclastic properties through mediating the NF-κB and MAPK pathways.[23] Moreover, OMT can improve the survival ability of cells by suppressing the TLR4/PI3K/Akt/GSK-3β signaling pathway.[24,25] In the IL-1β-induced inflammatory model, TLR4, IkBα, and NF-κB are activated successively.[12] Subsequently, IL-6, TNF-a, and Cox2 are released. In the present research, the same results were found in the IL-1β group without OMT treatment.[16] In contrast, in the OMT group, the expression of NF-κB, IL-6, TNF-α, Cox2 was significantly inhibited, which is better than the TAK 242 inhibitor group (Figure 4). Furthermore, the TLR4/IkBα/NF-κB p65 signaling pathway was inhibited in OMT treatments compared with that in the IL-1β treatment group, and the TLR4 signaling pathway was significantly blocked after adding TAK 242 inhibitor. The animal results of MRI and H&E have shown that IDD was clearly presented in the needle-tabbed group, but pathologic features were relieved after OMT treatment. OMT has potential therapeutic effects on IL-1β-induced NP inflammation, including alleviating symptoms, mitigating inflammation, and decreasing apoptosis. All of these effects are closely related to the unique

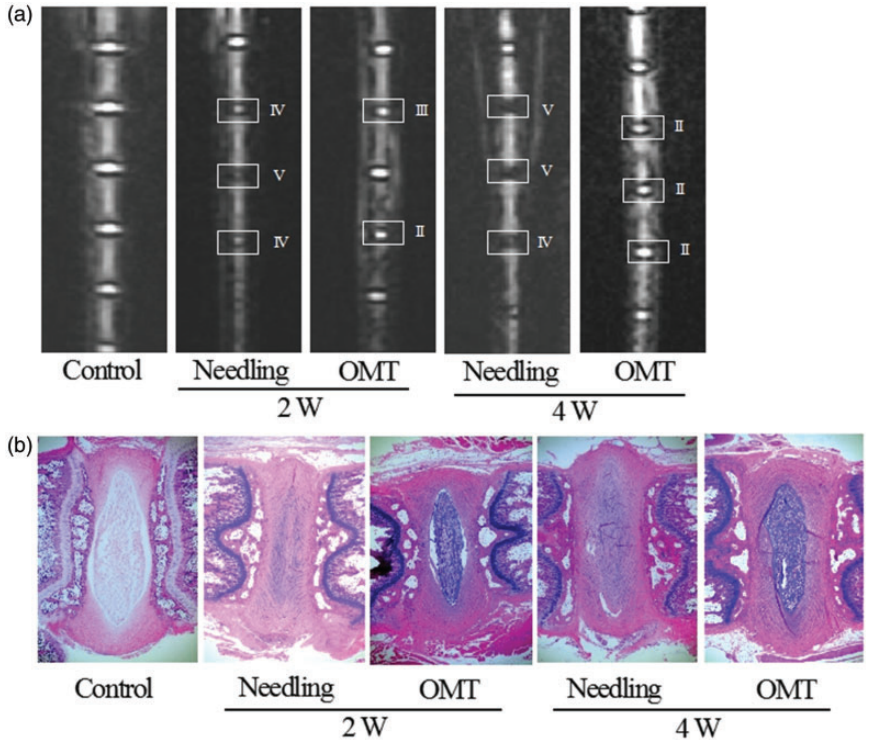

Figure 6. Therapeutic effect of OMT on rat caudal intervertebral disc degeneration. (a) Representative serial MR images of a rat caudal spine showing T2-weighted MR studies at stabbed and intact disc levels at 2/4 weeks after stab injury. (b) Safranin O staining of the intervertebral disc degeneration at 2/4 weeks after stab injury. (A color version of this figure is available in the online journal.)
OMT: oxymatrine.

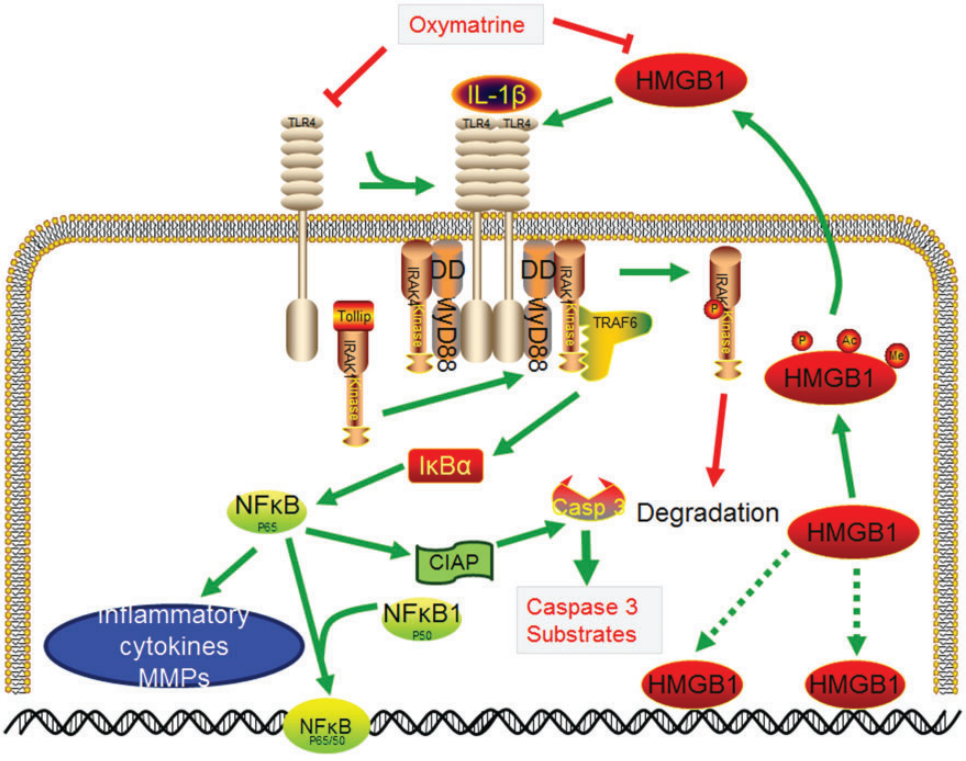

Figure 7. Mechanism of the functions of OMT on inhibiting NP cells inflammation. (A color version of this figure is available in the online journal.)
HMGB1: high mobility group box1; MMP: matrix metalloproteinase; NF-κB: nuclear factor kappa B; NF-κB: nuclear factor kappa B.

mechanism of inhibition of TLR4/NF-κB signaling, which has not been mentioned in previous research. Hence, it can be concluded that the TLR4/NF-κB signaling pathway is involved in the pathogenesis of IDD, and OMT can inhibit the inflammatory response by inhibiting the TLR4/NF-κB signaling pathway (Figure 7).

Moreover, OMT can inhibit serum deprivation and IL-1β-induced apoptosis of NP cells and promote cell growth, which is consistent with previous reports. Additionally, the activity of NP cells grown in the culture environment with low OMT concentrations (0–2 mg/mL) shows very limited differences, indicating that OMT had no significant cytotoxicity at low concentrations (Figure 1). We also detected the mRNA and protein levels of MMP2/3/9/13 in NP cells after IL-1β and OMT treatment. These results have shown that OMT could inhibit the expression of MMPs and IL-6. This supports our hypothesis that OMT could delay the progression of IDD through inhibiting NP cells apoptosis and ECM degradation (Figure 2).

However, there are still some limitations of the present research. Firstly, in the research, we simply confirmed that the presence of OMT was closely associated with the suppression of TLR4/NF-κB signaling pathway. However, we have no indication as to how TLR4 could be activated in these cells following IL-1β treatment, and whether or not OMT treatment could intervene in the other signaling pathways. The underlying molecular mechanisms therefore still need to be further studied.

In summary, OMT inhibits the HMGB1/TLR4/NF-κB signaling pathway and reduces the production of inflammatory factors like IL-6 and TNF-a.[26,27] Meanwhile, OMT decreases the expression of MMPs (MMP2/3/9/13) and increases the expression of aggrecan and Col2.[8] Accordingly, it is believed that OMT, particularly as it is a natural plant medicine, is likely to be a promising candidate for intervening in the progress of IDD and treating IDD diseases in clinical practice.

Authors' contributions

Experiments conceived and designed by: HF, HW, XZ and performed by KW, YP, YC. Experimental data analyzed by: HF, JD, ZW. Reagents/materials/analysis tools contributed by: YD, QD, PA. Paper written by: HF, HW. Equal contribution: HF, HW.

DECLARATION OF CONFLICTING INTERESTS

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ORCID IDS

Jun Dai https://orcid.org/0000-0001-5155-8838
Huang Fang https://orcid.org/0000-0002-6204-6552

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