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Original Article

Effect of warming moxibustion on expression of MMP1/13 by JNK pathway of cartilage cells in rabbit knee osteoarthritis

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Abstract: Knee osteoarthritis (KOA) is one of the most common joint disease and causes the extensive concern in the international community. Up to now, effective treatment for KOA is deficient. Warming moxibustion (WM) which is an approach of curing body pain by burning moxa at specific spots on the skin is found to possess the ability to treat osteoarthritis. This study aims to preliminarily explore the mechanism of how WM influences the disease. Forty New Zealand White rabbits were bound with plaster cast at extension position to create the model of KOA. Of these rabbits, 10 rabbits served as normal controls (NC). 30 rabbits were randomly divided into warming moxibustion group (WM), diclofenac sodium group (DS) and model group (MG). HE staining and Mankin scoring were used to judge the pathological changes. Immunohistochemistry was used to investigate the expression of MMP1 and MMP13. Western blotting was utilized to further detect the protein expression of MMP1, MMP13, JNK1 and JNK2 after the intervention of WM therapy. Mankin's score in model group compared with control group was significantly higher ($P < 0.05$), and Mankin's scores of WM group and DS group were decreased significantly compared with the model group ($P < 0.05$). The experiment of immunohistochemistry indicated that MMP1 and MMP13 were significantly reduced by therapy of WM. Moreover, western blot assay has shown that expression of MMP-1/13 was also reduced ($P < 0.05$). And the expression of JNK1/2 was correspondingly reduced after the therapy of WM. In conclusion, JNK1 and 2 serving as the important member of JNK pathway might be obstructed by the approach of WM. WM might reduce the expression of MMP1 and MMP13 by JNK pathway. WM probably relives knee osteoarthritis by this way.

Keywords: Warming moxibustion, KOA, JNK pathway, MMP-1, MMP-13

Introduction

Knee osteoarthritis (KOA), which is characterized by the progressive loss of articular cartilage that leads to chronic pain and functional limitations, is one of the most common joint diseases and causes the extensive concern in the international community. More than 10% people aged > 60 experience the KOA symptoms [1] and the disability rate of KOA is up to 53% [2]. The typical symptoms of KOA is the long-term chronic pain and the prevalence of KOA is increasing tendency in future because of rising life expectancy [3] and increasing obesity population [4]. By far, the surgical treatment can only deal with end-stage symptoms, but early-to-mid of KOA are lack of effective treatments. Currently the regimes for the treatment are pharmacological interventions aiming to relive symptom and recover function. However, numerous side effects of medicine on patients obstruct usage of these therapies. Hence, the discovery of effectiveness and safety of non-pharmacological interventions is necessary for the vulnerable patients who need long term treatment for KOA.

Moxibustion, consisted of *Artemisia vulgaris*, is a traditional oriental therapy that treats diseases through thermal stimulation by burning moxa at specific spots on the skin. This therapeutic means of burning moxibustion has been used

as an analgesic method for thousands of years in China and other Asian countries, and it is thought that moxibustion acquiring the efficacy of preventing and treating disease is by means of affecting the function of the meridians and acupoints [5]. Over the past few years, it was reported that thermal infrared imaging was used to investigate the distribution of infrared radiation on the surface of the human body along the meridian channel after treatment with moxibustion. Researchers have found that infrared radiation spectra of acupuncture points corresponded to relevant diseases was changed. These studies fully prove the ability of moxibustion in treatment for pain symptoms. Moreover, Moxibustion is still frequently used in the present clinical practice in that its advantages of safety, effectiveness and no side effects [6, 7].

According to different way of burning moxibustion, this therapeutic means can be divided into scarring moxibustion (the way of directly burning small moxa on the skin), warming moxibustion (also named suspended moxibustion, the way of moving smoky moxibustion above the skin) and herb-partition moxibustion (the way of indirectly burning mixed materials). Of these, warming moxibustion is the most practical and convenient one from the perspective of clinical application.

Clinical studies have found that warming moxibustion possesses better clinical curative effect of treating knee osteoarthritis, but the accurate mechanism of how warming moxibustion influencing the disease is unclear. Many studies reported that the different pathogeny can cause a series of changes and injury, which subsequently induces the generation of cytokines in cartilage and synovial fluid by approach of stimulating JNK pathway [8]. And these inflammatory factors can increase the expression of matrix metalloproteinases, free radical generation and apoptosis and so on by cell signal transduction through JNK pathway [9-11]. By this way, the synthesis of proteoglycan and II type collagen will be inhibited, the degradation of extracellular matrix (ECM) will be promoted and the aggravation of KOA will be induced. Based on that early research, scientific studies are now shown that MMPs and JNK signaling pathways play an important role in the cartilage degeneration [12, 13], and the treatment of warming moxibustion can inhibit the expression of MMPs in chondrocytes [14]. Hence, there is certain contact between MMPs expression and JNK signaling pathways. The aim of this research is to detect the relationship among them and mechanism of how warming moxibustion influencing the function of JNK signal pathway and the expression change of MMP-1\13 in KOA.

Materials and methods

Animals and grouping

Forty healthy female New Zealand white rabbits aged 6 months weighing 2.54 ± 0.51 kg were provided by Ningxia Medical University, and the permit number of animal is SCXK2014-005. These rabbits were randomly divided into warming moxibustion group (WM), diclofenac sodium group (DS), model group (MG) and normal controls (NC). According to the reference, the rabbit model of KOA was established by extension position with plaster cast. The model can complete simulate the degeneration of knee joint companied with less effect on knee joint internal environment, and this method is simple and easy to be controlled. After model established 3 days later, rabbits of WM group were treated by warming moxibustion for 15 minute each time, and one time a day for 2 weeks. Similarly, after model established 3 days later, rabbits of DS group were treated with diclofenac sodium (15 mg/kg) by oral administration for one time a day for 2 weeks. In addition, rabbits of MG group were fixed holder for 15 minute every day for 2 weeks. The rabbits belonged to normal group were fed without any treatments. All rabbits which were accepted with corresponding treatment were feed 3 days and a portable X-ray unit was used to confirm that rabbit models have been successfully established. Images were captured on digital X-ray plates (Fuji CR Cassette; Fuji Photo Film Co Ltd, Tokyo, Japan). This study was approved by the Ethics Committee of the General Hospital of Ningxia Medical University.

HE staining and Immunohistochemistry

HE stained sections were routine accompanied with OLYMPUS BX-50 biological microscope at 100 times, 200 times and 400 times. The microscope was used to observe the structure of the damaged portion of cartilage tissue, cell number and arrangement of the integrity of the tide line in according with Mankin's standards

Table 1. RT-PCR Primer sequences

Gene		Sequence (5'-3')	Length
MMP-1	F	ATGTGGCTCAGTTCGTCCTC	20
	R	CATCTGCCCTTGACAGGTCT	20
MMP-13	F	TGGTGGCAAAGTAGATGCTG	20
	R	GGGAATTTGTTGGCATGACT	20
JNK1	F	AAACGAGTCAAGCCAGGGATC	22
	R	GGCATCGTAAATCTGAGGTGGT	22
JNK	F	ACACCGTCCGCAGAGTTCAT	20
	R	GATAACAGATCCCTGGCTTGACTC	25
GAPDH	F	CATGTTTGTGATGGGCGTGAA	21
	R	CCCTCCACAATGCCGAAGT	19

score, which is one of the histologic and histochemical grading systems used to quantify the degree of OA [15].

Sections were incubated at 60°C overnight and deparaffinized. Antigen retrieval was performed with 0.1% trypsin for 20 min. Then sections were incubated with 3% H_2O_2 for 10 min, blocked with goat serum for 10 min, and treated at 37°C for 75 min with rabbit Atin-MMP1/13 antibody (1:100 or 1:50; BeacomBio, USA) in Phosphate-buffered saline containing Tween (PBST). After three washes with PBST (5 min), sections were incubated with HRP-conjugated goat anti-rabbit IgG (1:2000; Beijing Zhongshan Goldenbridge Biotech Co., Ltd.) at 37°C for 40 min. Visualization was carried out with 3, 3'-diaminobenzidine (DAB) and hematoxylin used for counterstaining. Routine dehydration and mounting were performed. In negative controls, the primary antibody was replaced with PBST. Results analysis with IPP image analysis software, measured its OD value.

RT-PCR

In brief, subchondral bone samples were thawed, weighed and homogenized in liquid nitrogen. Then, total RNA was extracted using RNA Pure Tissue Kit (Beijing Kangwei Shiji Biotech Co., Ltd.) according to the manufacturer's instructions. Total RNA amounts and purity were determined by UV spectrophotometry. Primers for MMP-1/13 and JNK1/2 were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (**Table 1**). Total RNA was reverse-transcribed into cDNA using the SuperRT cDNA kit (Beijing Kangwei Shiji Biotech Co., Ltd.) according to the manufacturer's instructions. Quantitative PCR reactions (25 μL) were composed of cDNA (0.5 μL), SYBR Green ER qPCR Super Mix Universal (Beijing Kangwei Shiji Biotech Co., Ltd.) (12.5 μL), forward and reverse primers (0.5 μL each), and diethyl-pyrocarbonate-treated water (11 μL). PCR was carried out at 50°C (20 min), 95°C (10 min) and 40 cycles at 95°C (15 s) and 60°C (60 s). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Bio-Rad, USA) was used as an internal reference; Ct in early OA served as a control. Finally, relative mRNA levels of target genes were assessed by the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Five samples from each group were assessed for protein expression levels by Western blotting. In brief, subchondral bone specimens were thawed, washed with pre-chilled distilled water (to remove bone marrow cavity contents), weighed and homogenized in liquid nitrogen. Total protein was extracted with Total Protein Sample Kit (Sigma, USA) according to the manufacturer's instructions and concentration determined by the bicinchoninic acid (BCA) method. Subsequently, proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into a polyvinylidene fluoride membrane at 300 mA for 90 min. After three washes in TBST (5 min each time), the membrane was blocked in Tris-buffered saline-Tween (TBST) containing 5% nonfat milk for 1 h and treated with monoclonal antibodies raised against MMP-1 (1:100), MMP-13 (1:100), JNK (1:100), JNK2 (1:100), and GAPDH (1:200) in TBST at 4°C overnight. After three washes in TBST, the membrane was incubated with HRP-conjugated goat anti-rabbit IgG (1:10000) at room temperature for 1 h. Visualization was carried out by electro-chemiluminescence, with the protein bands revealed on an ALS4000 gel image analysis system (GE, USA). The Quantity One software (Bio-Rad) was employed to assess the protein bands, with target protein expression normalized to cytokines levels.

Statistical analysis

Statistical analysis was performed with SPSS version 20.0 (IBM; Chicago, IL, USA). Data are mean ± standard deviation (SD), and were compared by t-test and one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

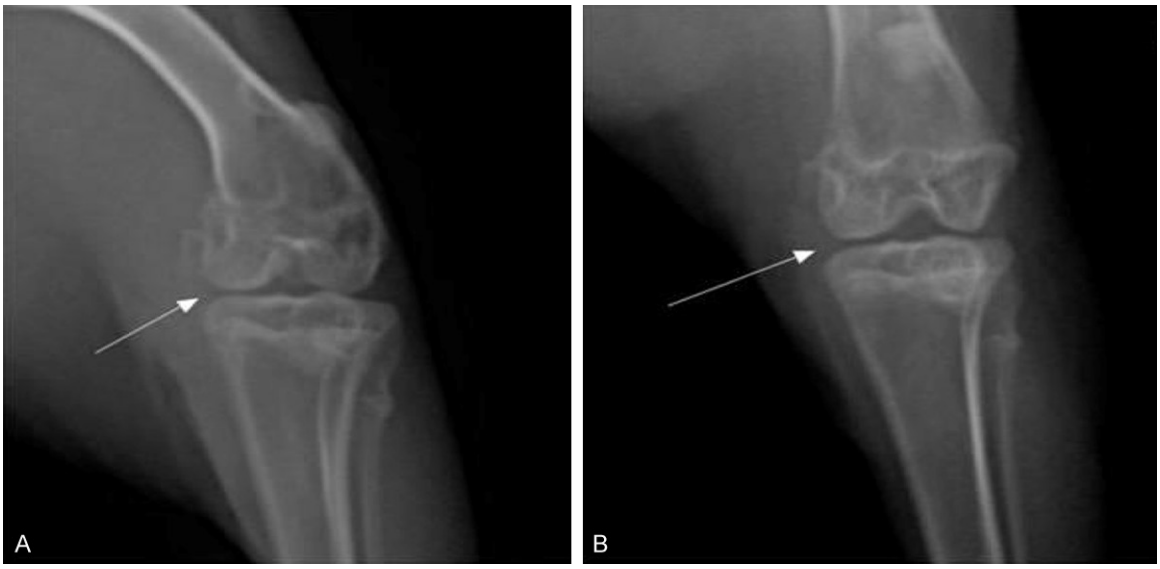

Figure 1. Images were captured on digital X-ray plates. A. Model group; B. Normal group.

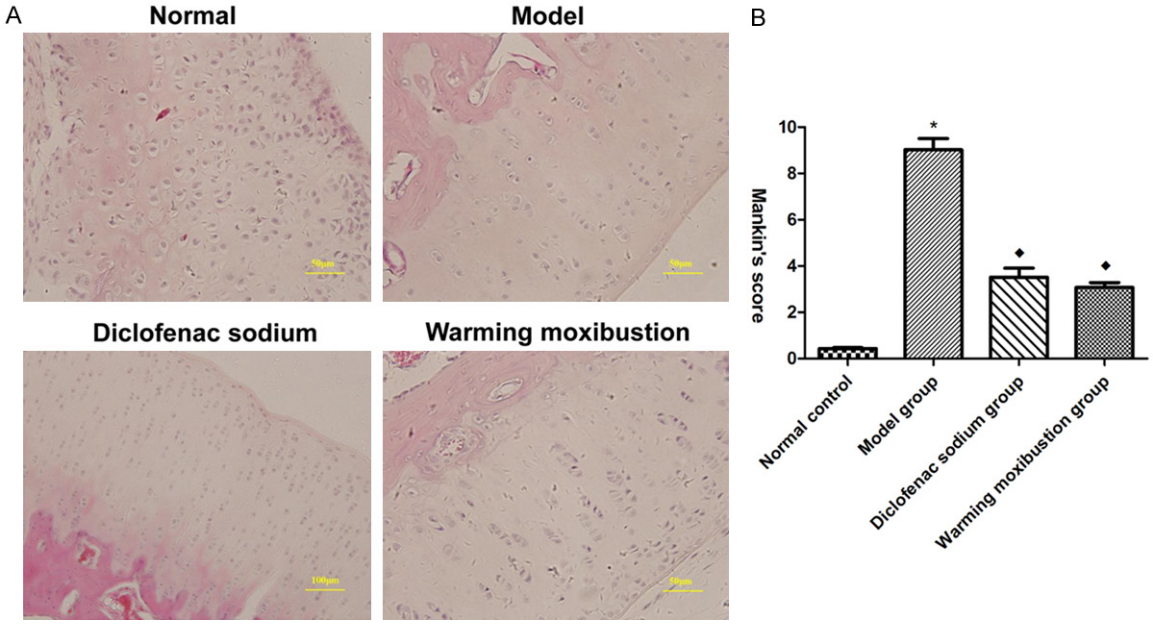

Figure 2. In comparison with model group, the histomorphology of cartilage tissue in DS and WM group were found to be more intact and health. A. The results of HE staining for normal, model DS and WM groups. B. The Mankin's score for four groups. $^{*}P < 0.05$ vs normal control; $^{\blacklozenge}P < 0.05$ vs model group.

Results

The successful establishment of KOA model by detection of X-ray plates

As shown in **Figure 1B**, rabbit joint of normal control group manifested itself as smooth articular surface, normal joint space and neatly edge joints. By contrast, rabbit joint of model group is found to be osteophyte formation, narrowed joint space and less smooth edge joint (as shown in **Figure 1A**).

Warming moxibustion possesses similar effect to diclofenac sodium by detection of HE staining and Mankin's score

Cartilage tissue changes in normal group were observed at cartilage surface smooth. Uniform

distribution of cartilage cells was found to be neat rows and clearly structured hierarchy without cells cluster. By contrast, cartilage tissue of model group was observed to be disorder in the cartilage tangent layer, damage in the transitional layer and difficulty in identification for the hierarchy. The entire thin layer of cartilage and cartilage cell disordered were detected to be clustered. In addition, a lot of disintegration necrotic cells, capillary invasion subchondral bone and partially broken tide line were able to be observed in two groups. But there is no crack, and more smooth cartilage surface in the WM group and DS groups, just as shown in **Figure 2A**.

The method of warming moxibustion can influence the expression levels of MMP-1 and MMP-13

MMP-1 and MMP-13 levels were expressed mainly in the cytoplasm and nucleus of osteocytes. In normal control, weak MMP-1\MMP-13 signals were found in cartilage cells (**Figure 3**). In model group, strong positive sclerostin signals were obtained in cartilage cells. Moreover, cytoplasm and nucleus of osteocytes are visible in comparison with normal control (**Figure 3**). In warming moxibustion group, MMP-1\ MMP-13 showed weaker expression compared with model group, and these proteins can only be detected in nucleus of cartilage cell and pale (**Figure 3**). In diclofenac sodium group, the expression of MMP-1\MMP-13 was detected in local where MMP-1\MMP-13 were found in cytoplasm and nucleus of osteocytes (**Figure 3**).

Warming moxibustion has reduced the expression of JNK1, JNK2, MMP-1 and MMP-13 mRNA levels in cartilage tissue

Compared with normal control values, the expression of mRNA correlated to JNK1, JNK2, MMP-1 and MMP-13 were found to be increased in model group ($P < 0.05$). The mRNA expression of JNK1, JNK2, MMP-1 and MMP-13 was decreased in warming moxibustion group and diclofenac sodium group ($P < 0.05$); and the reduction of mRNA expression is more significant in warming moxibustion grouo ($P < 0.05$, just as shown in **Figure 4**).

The expression of JNK1 and JNK2 was decreased in the WM group

Compared with normal control group, the protein expression of JNK1 and JNK2 were increased in model group ($P < 0.05$). However, the expression of JNK1 and JNK2 were found to be significantly decreased in warming moxibustion group in comparison with model group ($P < 0.05$). No significant difference between WM group and DS group in the expression of JNK1 and JNK2 was detected.

Discussion

Moxibustion is a traditional therapy for body ache by burning moxa in many oriental countries [16, 17]. Knee osteoarthritis is a disease characterized with structural and biochemical changes in chondrocytes and cartilages, and insufficient synthesis of extracellular matrix [18]. In previous researches, warming moxibustion has been reported to possess the ability of cure the knee osteoarthritis [19, 20]. Hence, we performed experiments to preliminarily explore the potential mechanism of warming moxibustion curing knee osteoarthritis.

In this study, we found that the therapy of warming moxibustion was able to protect the correct order of cartilage tissue free from the damage induced by osteoarthritis. Further investigations indicated that mRNA expression of MMP1 and MMP13 was significantly reduced in comparison with that in the model group. Even significant difference between diclofenac sodium and warming moxibustion, which were found to down-regulate the mRNA expression, was detected. In addition, the mRNA expression level of JNK1 and JNK2 was also down-regulated by diclofenac sodium as well as warming moxibustion, and significant difference between diclofenac sodium and warming moxibustion has also been observed. These results indicate that warming moxibustion might be more effective approach in the treatment for knee osteoarthritis by influencing the expression of JNKs and MMPs. Subsequently, western blot experiment has further defined that therapy of warming moxibustion might aim to influence protein expression of JNK1 and JNK2, which were significantly reduced by warming moxibustion. Although, there is no significant difference between the treatment of warming moxibustion and diclofenac sodium in the protein expression of JNK1 and 2, the reduction of JNKs expression in the WM group is more obvious. Immunohistochemistry apparently demonstrate that the signals were vastly cut down by warming moxibustion which was found to suc-

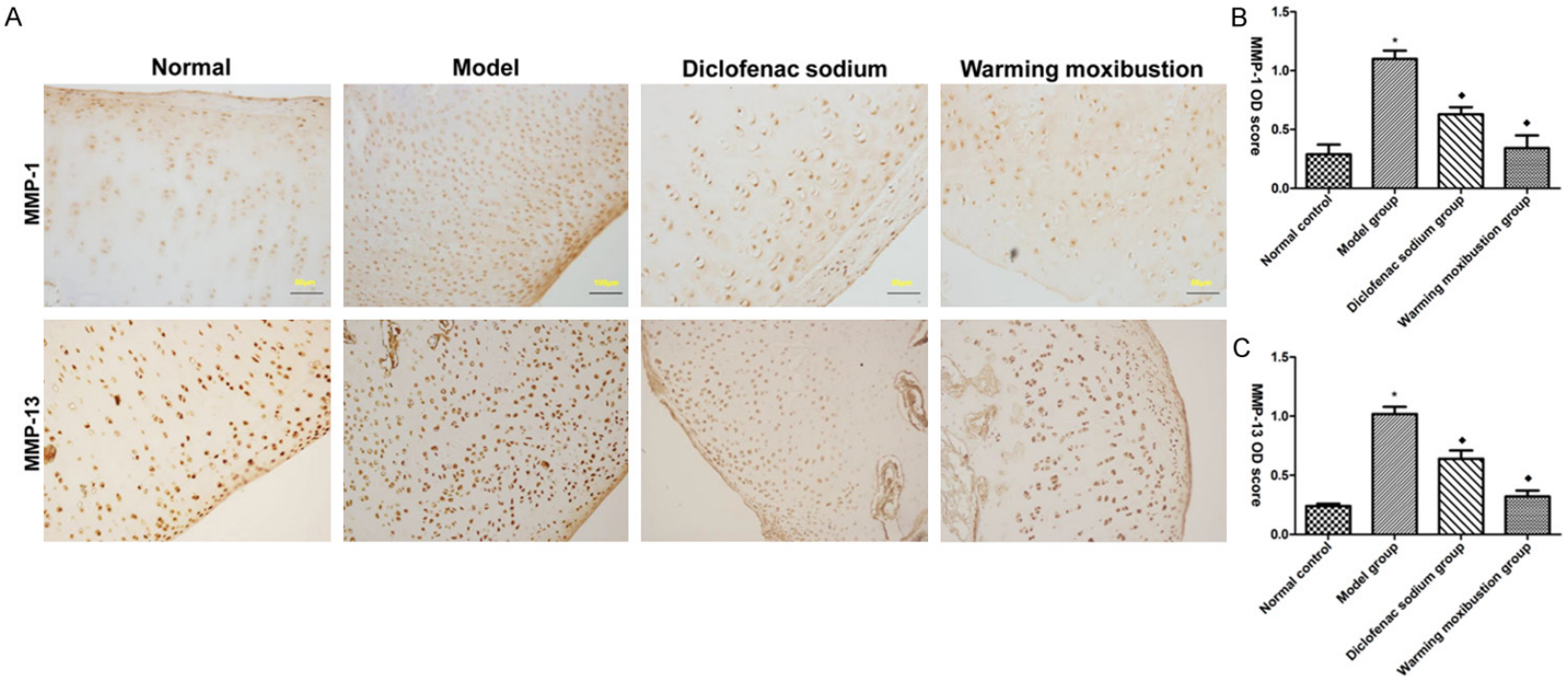

Figure 3. The results of immunohistochemical expression corresponding to four groups. A. The expression of MMP-1\MMP-13 respectively in the normal, model, DS and WM group. B and C. The corresponding expression value of MMP1 and MMP1 in four groups. $^{*}P < 0.05$ vs normal control; $^{\blacklozenge}P < 0.05$ vs model group; $^{※}P < 0.05$ vs diclofenac sodium group.

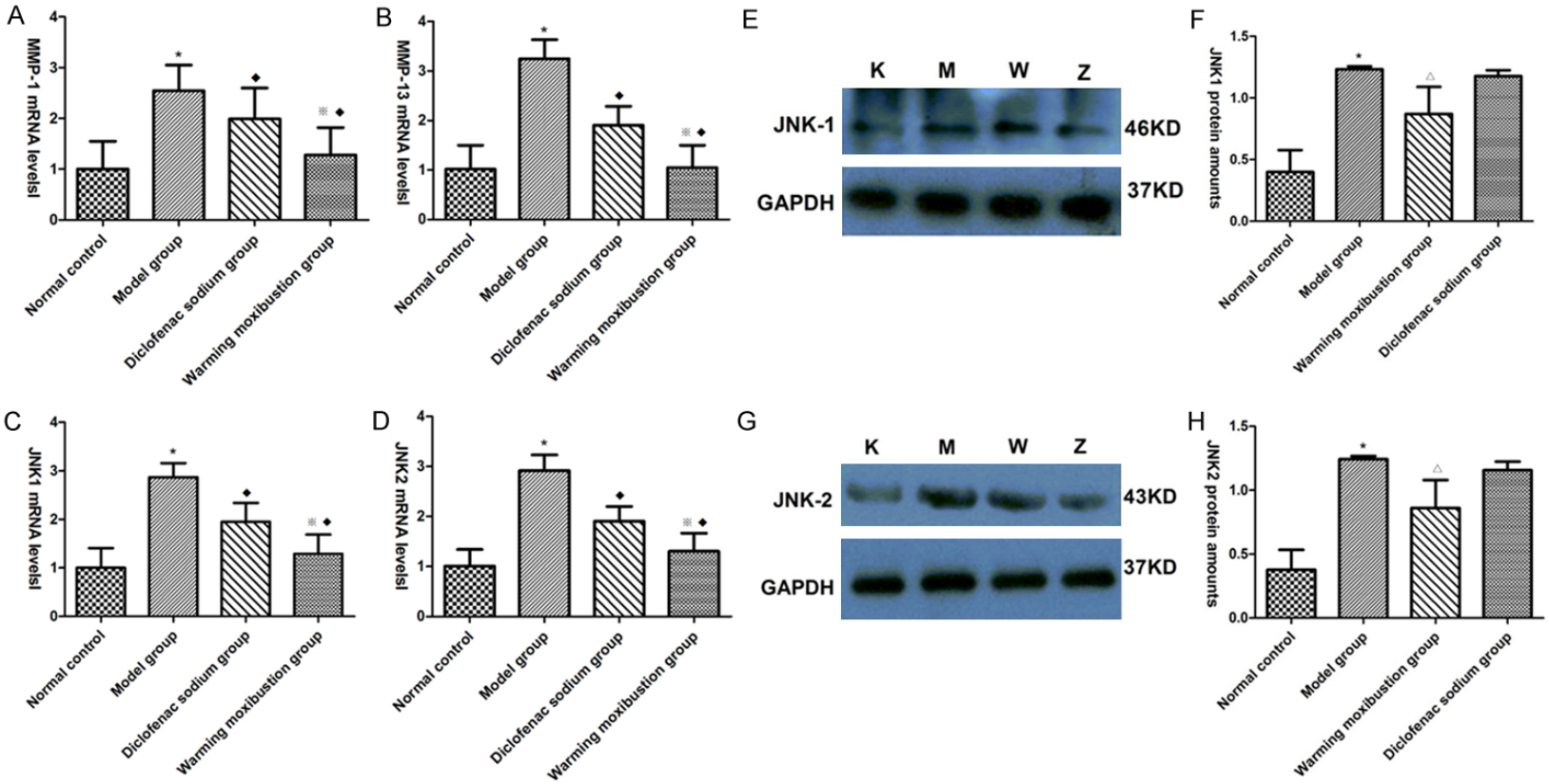

Figure 4. A-D. The mRNA expression levels of MMP1/13 and JNK1/2. E-H. The results of western blot corresponding to JNK1 and 2, and the relevant gray-scale value of JNK1 and 2. $^{*}P < 0.05$ vs normal control; $^{\blacklozenge}P < 0.05$ vs model group; $^{※}P < 0.05$ vs diclofenac sodium group; $^{\Delta}P < 0.05$ vs model group.

cessfully weaken the expression of MMP1 and 13 in cytoplasm and nucleus of osteocytes.

Chondrocytes, which are essential for physiological cartilage homeostasis, maintain stable and albeit low-level equilibrium between matrix synthesis and degradation of extracellular matrix molecules [21]. MMP1 and MMP13, serve as the main member of matrix metalloproteinase (MMP) family, is the principle matrix-degrading enzymes which participates in the regulation of homeostatic cartilage [22, 23]. However, MMP1 and MMP13, induced by IL-1β and TNFα, were detected to be overexpression and accelerate degradation of extracellular matrix in KOA [24, 25]. In this study, the experiment of immunohistochemistry displayed the effect of WM therapy on the expression MMP1 and MMP13. Moreover, western blot has further confirmed the therapy of WM which was able to significantly reduce the expression of MMP1 and MMP13. These results indicated that therapy of WM might be able to regulate unbalance of matrix synthesis and degradation of extracellular matrix molecules by inhibiting mRNA and protein expression of MMP1 and MMP13.

In order to further investigate the mechanism of WM influencing the expression of MMP1 and MMP13, we detected the mRNA and protein expression of JNK1 and JNK2. JNK signaling pathway involved in inflammatory reaction is activated by phosphorylation [26, 27]. And the switch can be triggered by a variety of stimulus such as cytokines, mechanical stress and fluid shear stress [28, 29]. Moreover, mechanical stress is an important factor in the pathogenesis of OA [30]. JNK1 and JNK2 exist extensively in most cells, and JNK pathway mainly including JNK1 and JNK2 has become the focus of exploring the mechanism of WM therapy in depth [31]. Up to now, JNK pathway together with p38 MAPK and NF-κB signaling pathways is found to predominate in the regulation of IL-1β and TNFα-induced catabolic responses in chondrocytes [12, 32]. And MMP1 and MMP13 can be induced to enhance the expression in cells by simulation of IL-1β. Therefore, JNK pathway might regulate the expression of MMP1 and MMP13 during the process of KOA. Indeed, we have found that mRNA and protein of JNK1 and JNK2 were reduced by the therapy of WM, and the expression of MMP1 and MMP13 were also found to be reduced after the implement of WM. In consequence, we conclude that WM might be a potent method of reducing the expression of MMP1 and MMP13 by inhibiting the JNK pathway, and the moxa burned might produce some volatile substance of percutaneous absorption, which can be absorbed to exert the function of obstructing the JNK pathway. This phenomenon should be further investigated in the future research.

In our present research, we found that expression levels of MMP-1, MMP-13, JNK1 and JNK2 are reduced after treatment of WM. This cascade of signaling events is able to result in overwhelming cartilage degeneration and promotion of KOA progression, just as previously reported. In view of the effect of WM on these protein expression, we speculate that warming moxibustion might inhibit JNK signaling pathway, reduce the expression of MMP1 and MMP13, delay the degeneration of cartilage cells and finally relieve the extracellular matrix degradation. However, there is no relevant inhibitors applicated in this study, insufficient evidences fail to prove that the decreasing expression of MMP1 and MMP13 is directly induced through the JNK signaling pathway in articular cartilage cells. Whether the therapy of warming moxibustion can influence other pathways associated with KOA is still unable to confirm, and these researches about the effect of warming moxibustion on other cytokines and pathways should be further studied.

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Disclosure of conflict of interest

None.

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