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Basic Study

Effect of acupuncture plus thunder-fire moxibustion on MMP-3, TIMP-1 and TGF-β1 in rats with knee osteoarthritis

针刺结合雷火灸对膝骨关节炎大鼠 MMP-3、TIMP-1 及 TGF-β1 的影响

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Abstract

Objective: To observe the effect of acupuncture plus thunder-fire moxibustion on the expressions of matrix metalloproteinase-3 (MMP-3), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β1 (TGF-β1) in cartilage of knee osteoarthritis (KOA) rats, and to explore the mechanism of acupuncture plus thunder-fire moxibustion in the treatment of KOA.

Methods: Thirty Sprague-Dawley (SD) rats were randomly divided into a blank control group, a model group and an acupuncture-moxibustion group by random digits table, 10 rats in each group. Rats in the model group and the acupuncture-moxibustion group were injected with papain in the right posterior knee joint to prepare the models. The levels of MMP-3 and TIMP-1 in rat synovium of each group were measured by enzyme-linked immunosorbent assay (ELISA) after 2 weeks of treatment. The level of TGF-β1 was determined by Motic B5 Micro-camera system.

Results: The levels of MMP-3 and TIMP-1 in the cartilage of the model group were significantly higher than those in the blank control group (all $P<0.01$); the levels of MMP-3 and TIMP-1 in the acupuncture-moxibustion group were lower than those in the model group, and the between-group differences were statistically significant (all $P<0.05$). The levels of MMP-3 and TIMP-1 in the acupuncture-moxibustion group were higher than those in the blank control group, and the differences were statistically significant (all $P<0.05$). The level of TGF-β1 in cartilage tissues of the model group was significantly lower than that in the blank control group ($P<0.01$); the level of TGF-β1 in the acupuncture-moxibustion group was higher than that in the model group ($P<0.05$), but it was lower than that in the blank control group, and the between-group difference was statistically significant ($P<0.05$).

Conclusion: Acupuncture plus thunder-fire moxibustion can effectively recover the abnormal expressions of MMP-3 and TIMP-1 in KOA model rats and somewhat up-regulate TGF-β1, which may be one of its mechanisms of acupuncture plus thunder-fire for KOA.

Keywords: Acupuncture Therapy; Moxibustion Therapy; Acupuncture-moxibustion Therapy; Thunder-fire Moxibustion; Osteoarthritis, Knee; Metalloproteases; Transforming Growth Factors; Rats

【摘要】目的：观察针刺结合雷火灸对膝骨关节炎(KOA)大鼠软骨组织中基质金属蛋白酶-3 (MMP-3)、基质金属蛋白酶抑制剂-1 (TIMP-1)和转化生长因子-β1 (TGF-β1)表达的影响，探讨针刺结合雷火灸治疗KOA的作用机制。**方法：**Sprague-Dawley (SD)大鼠30只，根据随机数字表法随机分成空白组、模型组和针灸组，每组10只大鼠。模型组和针灸组予木瓜蛋白酶右后膝关节注射造模。治疗2星期后，采用酶联免疫吸附法测定各组大鼠滑膜MMP-3、TIMP-1的含量，采用Motic B5显微摄像系统测定TGF-β1的含量。**结果：**模型组软骨组织中MMP-3和TIMP-1的含量较空白组显著增加(均$P<0.01$)；针灸组MMP-3和TIMP-1含量低于模型组，组间差异有统计学意义(均$P<0.05$)；针灸组MMP-3和TIMP-1含量高于空白组，组间差异有统计学意义(均$P<0.05$)。模型组软骨组织中TGF-β1的含量较空白组显著降低($P<0.01$)，针灸组TGF-β1含量较模型组升高($P<0.05$)，但低于空白组，组间差异有统计学意义($P<0.05$)。**结论：**针刺结合雷火灸能有效平衡KOA模型大鼠中MMP-3和TIMP-1的异常表达，并且对TGF-β1具有一定的上调功能，这可能是针刺结合雷火灸治疗KOA的作用机制之一。

【关键词】针刺疗法；灸法；针灸疗法；雷火灸；膝骨关节炎；金属蛋白酶；转化生长因子；大鼠
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Knee osteoarthritis (KOA) is a common chronic knee joint disease characterized by degeneration and destruction of the knee articular cartilage and hyperosteogeny. Although the pathogenesis of KOA is not clear[1], more and more evidences have confirmed that cartilage lesions play an important role in the pathogenesis of KOA. Acupuncture therapy is based on the theory of traditional Chinese medicine and aims to prevent disease. Thunder-fire moxibustion is a suspended moxibustion method with an open fire, which comes from the traditional pressing moxibustion method of thunder-fire miraculous moxa roll. Thunder-fire moxibustion can warm and dredge the meridians, and regulate the functions of human body by propagating sensation of the heat, infrared radiation and drug, chemical and physical factors produced by moxa and drug when burning[2]. This extended the range of fire-heat treatment in Chinese medicine.

Acupuncture can dredge the meridians and collaterals and harmonize yin and yang to adjust the imbalance of the body. In this study, expressions of matrix metalloproteinase-3 (MMP-3), tissue inhibitor of metalloproteinase-1 (TIMP-1) and transforming growth factor-β1 (TGF-β1) in cartilage tissues of experimental KOA rat model were detected by immune-histochemistry to evaluate the therapeutic effect of acupuncture plus thunder-fire moxibustion. The purpose is to study the regulatory effect of acupuncture plus thunder-fire moxibustion on MMP-3, TIMP-1 and TGF-β1, and provide experimental evidences for acupuncture combined with thunder-fire moxibustion in the treatment of KOA.

1 Materials and Methods

1.1 Materials

SPF grade healthy male Sprague-Dawley (SD) rats, body weight (180±10) g, were provided by the Experimental Center of Hunan University of Chinese Medicine; Hwato Brand sterile acupuncture needles (specifications: 0.35 mm in diameter, 40 mm in length, Suzhou Medical Products Factory Co., Ltd., China); thunder-fire moxibustion sticks (25 g, Chongqing Zhao's Thunder-fire Moxibustion Traditional Medicine Research Institute, China); papain (Merck, Germany); MMP-3 and TIMP-1 enzyme-linked immunosorbent assay kits, ready-to-use SABC kit, DAB color kit (Wuhan BOSTER Biological Technology Co., Ltd., China); anti-mouse TGF-β1 monoclonal antibody (Santa, USA). Other involved reagents and instruments were provided by the Experimental Center of Hunan University of Chinese Medicine.

1.2 Modeling methods

After 7 d adaptive feeding, 30 SPF grade SD rats were randomly divided into a blank control group, a model group and an acupuncture-moxibustion group according to the random number table method, 10 rats in each group. Rats in the model group and the acupuncture-moxibustion group were subjected to KOA modeling as follows[3]. Skin around the knee joints of the right hind limbs was prepared. After routine sterilization with ethanol, 4% papain solution (0.2 mL) was injected into the rat knee joint cavity of the right hind limb on the 1st day, the 4th day and the 7th day during modeling. The expressions of MMP-3, TIMP-1 and TGF-β1 were detected to evaluate whether the model was successful on the second day after the third injection (i.e., the 8th day during modeling). Same amount of saline was injected into knee joint cavity of right hind limb at the same time point for rats in the blank control group. The results showed that expressions of MMP-3 and TIMP-1 in 20 rats were higher than those in the blank control group, while the level of TGF-β1 was lower than that in the blank control group. This indicated that all the modeling was successful.

1.3 Statistical processing

The SPSS 19.0 version statistical software was used for data analysis. Measurement data of normal distribution were expressed as mean ± standard deviation ($\bar{x} \pm s$). One-way analysis of variance was used to compare the data among groups. $P<0.05$ indicated that the difference was statistically significant.

2 Methods

2.1 Treatment methods

2.1.1 Acupuncture-moxibustion group

Rats in the acupuncture-moxibustion group were treated with acupuncture and thunder-fire moxibustion after successful modeling.

Acupoints: Dubi (ST 35), Zusanli (ST 36) and Yanglingquan (GB 34).

Methods: Rats were fixed on the special rat frame, and the acupoints were positioned according to the *Experimental Acupuncture Science*[4]. After routine disinfection, needles (0.35 mm in diameter and 40 mm in length) were inserted into above-mentioned acupoints on the right hind limb to 5-7 mm. Even reinforcing-reducing manipulation was performed after arrival of needling sensation. Needles were retained for 30 min. Needling manipulation was performed once every 10 min. During needle retaining, thunder-fire moxibustion was performed simultaneously on the same acupoint until the skin became red and hot. Pay attention to avoid burns. Moxibustion was done 20 min for each time. Acupuncture and thunder-fire moxibustion were conducted once a day for 2 weeks in total.

2.1.2 Blank control group and model group

Rats in the blank control group and the model group did not receive any treatment but had the normal diet for two consecutive weeks.

2.2 Observation items and evaluation methods

2.2.1 Lequesne MG score[5]

Local pain stimulus response: Pushed the rat knee joint with fingers to assess the pain response degree to local stimulus. The reaction degree was divided into four levels. Level I: no abnormal reaction (0 point); level II: contraction of affected limbs (1 point); level III: contraction and spasm of affected limbs with mild systemic reactions, such as whole body trembling, turning the head around and sucking (2 points); level IV: severe contraction and spasm of affected limbs, whole body trembling, scurrying and struggling (3 points).

There were four levels according to the changes of running gait. Level I: limbs showed no limp but normal running and strong pedal (0 point); level II: limbs showed slight limp when running and strong pedal (1 point); level III: affected limbs still involved in walking but with obvious limp (2 points); level IV: affected limbs could not participate in walking, touch the ground, or pedal the ground (3 points).

There were four levels according to the movement angles of knee joints. Level I: 90 ° or more (0 point); level II: 45-90 ° (1 point); level III: 15-45 ° (2 points); level IV: 15 ° or less (3 points).

There were three levels according to the degree of joint swelling. Level I: no swelling, the bony landmark was clear (0 point); level II: mild swelling, the bony landmark became shallow (1 point); level III: significantly swollen, the bony landmark disappeared (2 points).

The Lequesne MG score can be used to assess the severity and functional status of patients with knee osteoarthritis. The total Leguesne MG score ranges from 0 to 11 points, the higher score indicates more serious disease and functional status[6]. The scores were performed according to the 4 aspects of the local pain degree (0 to 3 points), the gait (0 to 3 points), the movement angle of knee joint (0 to 3 points) and the joint swelling degree (0 to 2 points). One week after the last intervention, rat behavioral assessment was performed again for each group by the same person who was blind to the experimental design.

2.2.2 MMP-3 and TIMP-1

At the end of the experiment, rats were sacrificed by dislocation and fixed on the operating table with joint straightened; about 50 mg of the cartilage in the middle of the knee joint was sampled; removed the surrounding adipose tissues and cut it into small pieces to place in a centrifuge tube; ground in 10% saline solution and centrifuged at 30 000 r/min for 20 min to collect the supernatant and stored in 4 ℃ refrigerator. After all the specimens were collected, enzyme-linked immunosorbent assay (ELISA) was used to detect the levels of MMP-3 and TIMP-1 in synovial membrane, strictly following the instructions of the kit.

2.2.3 TGF-β1

At the end of the experiment, rats were sacrificed by dislocation. The femoral end cartilage of the right-hind knee joint was collected and fixed with 4% paraformaldehyde. The cartilage was dehydrated with gradient ethanol. The paraffin-embedded sections were sliced at about 5 μm. Paraffin slices were dewaxed with ethanol, and rinsed with PBS for 3 times; incubated with 3% H_2O_2 at room temperature for 10 min, washed 3 times with PBS; incubated with BSA blocking solution for 20 min at room temperature; incubated with diluted primary antibody for 60 min at room temperature and rinsed with PBS for 3 times; incubated with secondary antibody at room temperature for 10 min, and washed with PBS for 3 times; incubated with SABC for 30 min at room temperature, and washed with PBS for 3 times; colored with DAB solution, washed with distilled water; counterstained with hematoxylin; dehydrated with different levels of ethanol (60%-100%); incubated in xylene and sealed with neutral gum; observed different fields (the positive cells were brownish with more regular shape and clear nuclei) under microscope by Motic B5 microscopic imaging system. The mean optical density of cartilage TGF-β1 was measured.

3 Results

3.1 Pathological observation

In the blank control group, the cartilage matrix was pink with uniform coloring; the cartilage surface was smooth with neatly arranged chondrocytes, clearly visible four layers of structure and integrity tidal line. Cartilage destruction was most severe in the model group with roughness and erosion of cartilage surface, even complete exfoliation to form defect area. In the acupuncture-moxibustion group, the articular cartilage showed little inflammatory cell infiltration, the articular chondrocytes had mild degeneration, and the tidal line was basically complete (Figure 1, hematoxylin-eosin staining, HE).

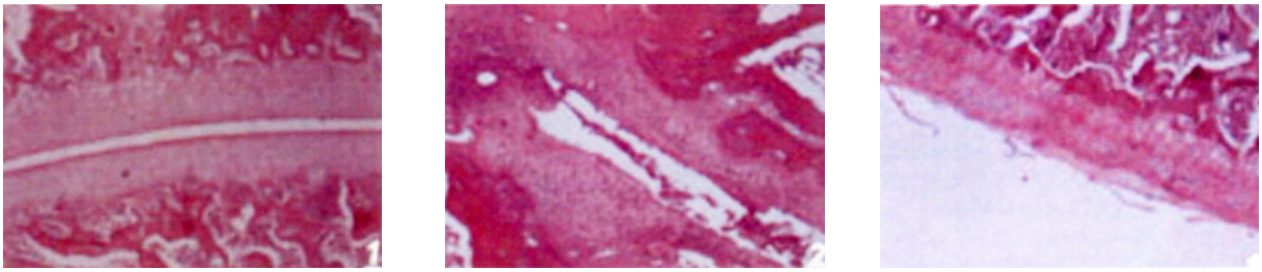
3.2 Comparison of Lequesne MG score

Before intervention, the Lequesne MG scores of the model group and the acupuncture-moxibustion group were significantly different from that in the blank control group (both $P<0.05$), indicating successful modeling. After intervention, the Lequesne MG score of the acupuncture-moxibustion group was significantly

lower than that before intervention, the difference was statistically significant ($P<0.05$). Compared with the model group, the post-intervention score and the difference-value before and after intervention in the acupuncture-moxibustion group were all statistically different (both $P<0.05$), suggesting that acupuncture plus thunder-fire moxibustion can relieve the symptoms of KOA rats and improve their behavioral status (Table 1).

3.3 Comparison of MMP-3 and TIMP-1 levels

The differences of MMP-3 and TIMP-1 among groups were statistically significant ($P<0.01$). The levels of MMP-3 and TIMP-1 in the synovial tissues of the model group were significantly higher than those in the blank control group (all $P<0.01$). The levels of MMP-3 and TIMP-1 in the acupuncture-moxibustion group were significantly lower than those in the model group ($P<0.05$), but still higher than those in the blank control group ($P<0.05$). These results suggested that acupuncture plus thunder-fire moxibustion can reduce the levels of MMP-3 and TIMP-1 in KOA model rats (Table 2).



Blank control group　　Model group　　Acupuncture-moxibustion group

Figure 1. Pathological observation of each group (HE, ×100)

Table 1. Comparison of Lequesne MG score in each group ($\bar{x}\pm s$, point)

Group	*n*	Before intervention	After intervention	Difference-value before and after intervention
Blank control	10	0	0	0
Model	10	8.38±1.57[1)]	8.32±1.55	0.02±0.06
Acupuncture-moxbustion	10	8.40±1.58[1)]	3.87±0.90[2)]	4.53±0.68[2)]

Note: Compared with the blank control group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$

Table 2. Comparison of MMP-3 and TIMP-1 levels in cartilages of each group ($\bar{x}\pm s$, pg/mL)

Group	*n*	MMP-3	TIMP-1
Blank control	10	72.53±2.43	71.38±2.26
Model	10	87.16±2.18[1)]	81.42±2.07[1)]
Acupuncture-moxibustion	10	80.87±2.54[2)3)]	74.63±2.14[2)3)]

Note: Compared with the blank control group, 1) $P<0.01$, 2) $P<0.05$; compared with the model group, 3) $P<0.05$

3.4 Comparison of TGF-β1 optical density

The differences of TGF-β1 among groups were statistically significant ($P<0.01$). The level of TGF-β1 in synovial tissue of the model group was significantly lower than that in the blank control group ($P<0.01$). The level of TGF-β1 in the acupuncture-moxibustion group was higher than that in the model group ($P<0.05$), however still lower than that in the blank control group ($P<0.01$). These results suggested that acupuncture plus thunder-fire moxibustion can effectively increase the level of TGF-β1 in KOA model rats (Table 3).

Table 3. Comparison of average optical density of cartilage TGF-β1 in each group ($\bar{x}\pm s$)

Group	*n*	TGF-β1
Blank control	10	39.41±3.28
Model	10	20.55±3.14[1)]
Acupuncture-moxibustion	10	28.42±2.71[1)2)]

Note: Compared with the blank control group, 1) $P<0.01$; compared with the model group, 2) $P<0.05$

4 Discussion

KOA belongs to bone Bi-impediment in traditional Chinese medicine, mostly caused by the deficiency of healthy qi coupled with contraction of external wind, cold and dampness[7]. Physical therapy can improve the blood circulation with certain anti-inflammatory and analgesic effects[8]. Many studies have confirmed that acupuncture can effectively relieve joint pain and swelling and improve the restricted movement in patients with KOA[9-14]. Materials used in thunder-fire moxibustion are pure Chinese herbal medicine formula.

The physical and chemical factors, produced by drug burning during moxibustion, together with the function of acupoints and meridians, produce a comprehensive effect. The heat, generated by the drug burning, stimulates the acupoints via suspended moxibustion. This opens the local striae and interstitial space, thus the drugs can reach the relative acupoints to play the role in dredging the channels and activating the collaterals, therefore, to improve the blood circulation in surrounding tissues. Yuan QD, *et al*[15] observed the clinical efficacy of thunder-fire combined with heat-sensitive moxibustion in the treatment of KOA, and found that the total effective rate was 95.9% and 95.6% respectively immediately after treatment and 3 month after treatment. As a result, it was concluded that thunder-fire plus heat-sensitive moxibustion could be used as an effective therapy for KOA. Dubi (ST 35) and Yanglingquan (GB 34) act to dredge the local meridian qi and blood, thus harmonize the Ying-nutrient and Wei-defense and remove pathogenic factors. For the characteristics of deficiency in the origin of bone Bi-impediment, moxibustion at Zusanli (ST 36) can regulate Yangming meridians, and tonify qi to promote production of blood. This can effectively improve the local inflammation of the knee joint, circulate blood, therefore, be conducive to the recovery of joint function[16]. In this study, Dubi (ST 35), Yanglingquan (GB 34) and Zusanli (ST 36) were selected for acupuncture and thunder-fire moxibustion treatment, according to the *Experimental Acupuncture and Moxibustion*, so that both the effects of drugs and warmth could go deep into the knee joints[17], resulting in a comprehensive therapeutic effect[18].

The pathogenesis and development of KOA is closely related to the degradation of cartilage. The normal coupling of degeneration and synthesis among the cartilage, matrix outside cartilage and subchondral bone is imbalance due to the external factors[19], which further causes the imbalance of apoptosis and proliferation in chondrocytes, as well as production of a series of inflammatory mediators, thus resulting in inflammation and destruction of bone and joint.

Matrix metalloproteinases (MMPs) are a class of proteolytic enzymes that present outside the chondrocytes, which can degrade most of the extracellular matrix proteins. Under normal circumstances, MMPs and tissue inhibitors of metalloproteinase (TIMPs) maintain a dynamic balance to stable the cartilage structure. The cartilage and synovial cells secrete excessive MMPs during the pathogenesis of KOA. Meanwhile, the balance of cartilage and synovial cells was broken under the effect of the mechanical load and inflammatory factors, thus the matrix outside the knee cartilage was degraded and eventually led to destruction of articular cartilage[20]. In present study, the levels of MMP-3 and TIMP-1 in the synovial tissues of the model group were significantly higher than those in the blank control group ($P<0.01$), while the increase of MMP-3 was more obvious than that of TIMP-1. This indicated that rats with KOA have a protective response to the damage, however, this natural protective ability was limited, which is consistent with the literature report[21]. The levels of MMP-3 and TIMP-1 in the acupuncture-moxibustion group were significantly lower than those in the model group ($P<0.05$), while still higher than those in the blank control group. This indicated that acupuncture combined with thunder-fire moxibustion could effectively recover the abnormal expression of MMP-1.

Transforming growth factor-beta (TGF-β) is a multifunctional cytokine that regulates cell growth and differentiation, and plays an important role in the formation of extracellular matrix, wound repair and bone remodeling. During the pathogenesis of KOA, excessive apoptosis of chondrocytes causes imbalance between the proliferation and destruction in vivo, thus initiates the compensation of chondrocytes[22]. TGF-β1 can antagonize the inflammatory effect of inflammatory factors, mediate cartilage synthesis and inhibit collagen and proteoglycan decomposition, which is a protective factor to promote tissue repair[23]. In the present study, the level of TGF-β1 in the synovial tissues of the model group was significantly lower than that in the blank control group ($P<0.01$); the level of TGF-β1 in the acupuncture-moxibustion group was higher than that in the model group ($P<0.05$), but still slightly lower than that in the blank control group. This indicated that acupuncture combined with thunder-fire moxibustion has a certain up-regulation effect on TGF-β1.

Our current study confirmed that acupuncture plus thunder-fire moxibustion can effectively recover the abnormal expressions of MMP-3 and TIMP-1 in cartilages of KOA rats, while up-regulate TGF-β1 expression. This indicated that acupuncture plus thunder-fire moxibustion could promote repair of the damaged joints, which may be one of the treatment mechanisms of acupuncture and thunder-fire moxibustion.

Conflict of Interest

There was no potential conflict of interest in this article.

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Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria in this experiment.

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