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Basic Study

Effect of moxibustion on tumor necrosis factor-α and nuclear transcription factor kappa B in ankle joints of rats with rheumatoid arthritis

艾灸对类风湿关节炎大鼠踝关节肿瘤坏死因子-α 和核转录因子 kappa B 的影响

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Abstract

Objective: To observe the effect of moxibustion on the expressions of tumor necrosis factor-α (TNF-α) and nuclear transcription factor kappa B (NF-κB) proteins in ankle joints of rats with rheumatoid arthritis (RA), and to explore the anti-inflammatory mechanism of moxibustion in the treatment of RA.

Methods: Adjuvant arthritis (AA) rat models were induced and used as rat models of RA. Rats were randomly divided into a normal group, a model group, a moxibustion group and a saline group. Rats in the normal and model groups were not treated; rats in the moxibustion group accepted treatment by moxibustion at Zusanli (ST 36) and Shenshu (BL 23) after modeling; rats in the saline group were injected with 0.15 mL saline to the bottom of the left hind paw and had no other treatments. Hematoxylin-eosin (HE) staining was used to observe the histopathological changes of rats' ankle joints under light microscope. Immunohistochemistry was used to observe the expressions of TNF-α and NF-κB in ankle joints of rats.

Results: Compared with the normal group, the rats' ankle joints in the model group showed disorganization, joint surface defect, and significantly increased mean optical density (MOD) of TNF-α and NF-κB (P<0.05). After moxibustion treatment, rats in moxibustion group showed repaired ankle tissues, smooth joint surface without defects, decreased MOD of TNF-α and NF-κB than those in the model group (all P<0.05). Rats in the saline group had no damage to ankle joints, while TNF-α and NF-κB were significantly different from those in the model group (P<0.05).

Conclusion: Moxibustion could down-regulate the expression of NF-κB and TNF-α protein in ankle joints of RA rats, and produce an anti-inflammatory effect to promote tissue repair.

Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Arthritis, Rheumatoid; Tumor Necrosis Factor-alpha; NF-kappa B; Rats

【摘要】目的：通过观察艾灸对类风湿关节炎(RA)大鼠踝关节组织肿瘤坏死因子-α (TNF-α)和核转录因子kappa B (NF-κB)蛋白表达的影响，探讨艾灸治疗类风湿关节炎的抗炎机制。**方法：**以佐剂性关节炎(AA)作为类风湿关节炎大鼠模型，复制RA大鼠模型。将大鼠随机分为正常组、模型组、艾灸组和盐水组。正常组和模型组不做治疗;艾灸组造模后予以艾灸足三里、肾俞穴治疗；盐水组予左后足趾底部注入生理盐水0.15 mL，不做其他处理。应用苏木精-伊红(HE)染色，光镜下观察大鼠踝关节组织病理学变化；采用免疫组化法观察大鼠踝关节组织TNF-α和NF-κB蛋白表达。**结果：**与正常组比较，模型组大鼠踝关节组织结构破坏，关节表面有缺损，TNF-α和NF-κB平均光密度(MOD)明显增高(均P＜0.05)；艾灸组大鼠经艾灸治疗后，踝关节组织修复，关节表面光滑未见缺损，TNF-α和NF-κB的MOD较模型组降低(均P＜0.05)；盐水组大鼠踝关节组织正常无损害，TNF-α和NF-κB与模型组比较有显著性差异(P＜0.05)。**结论：**艾灸能够下调RA大鼠踝关节组织NF-κB和TNF-α蛋白表达，以发挥抗炎作用，促进组织修复。

【关键词】灸法；艾条灸；关节炎，类风湿；肿瘤坏死因子-α；核转录因子 kappa B；大鼠

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Rheumatoid arthritis (RA) is a chronic, inflammatory synovitis-based systemic disease with unknown cause. It is one of the major cause of disability and productivity loss. Therapeutic efficacy of moxibustion for RA has been confirmed by a large number of clinical and experimental studies[1-4]. In recent years, studies have shown that tumor necrosis factor-α (TNF-α), as an important proinflammatory cytokine, plays an important role in the pathogenesis of RA[5-6]; nuclear factor kappa B (NF-κB) plays an important regulatory role in the development of RA by regulating inflammatory factors, immune-related receptors and cytokines. Our previous study confirmed that the anti-inflammatory effect of moxibustion on RA was closely related to its ability to down-regulate the levels of interleukin (IL)-1β, IL-2[7], and the intercellular adhesion molecule (ICAM-1)[8]. Based on these results, we investigated the effect of moxibustion on the expression of TNF-α and NF-κB protein in ankle joints of RA rats, and explored the anti-inflammatory mechanism of moxibustion in the treatment of RA in order to further promote the application and generalization of moxibustion in the treatment of RA in clinical practice.

1 Materials and Methods

1.1 Animals and grouping

Sixty healthy clean male Sprague-Dawley (SD) rats [license number: SCXX (Shanghai) 2012-0002, Shanghai Slack Experimental Animal Co., Ltd., China], body weight (180±20) g, were divided into a normal group, a model group, a moxibustion group and a saline group (n=15), by completely randomized method.

1.2 Major reagents

Freund's complete adjuvant (FCA) (batch number: 06K8761, Sigma, USA); rabbit anti-rat TNF-α polyclonal antibody (batch number: AF01114144), rabbit anti-rat IKK polyclonal antibody (batch number: 00980787) and rabbit anti-rat NF-κB p65 polyclonal antibody (batch number: AF02165755), (Beijing Bioss Biotechnology Co., Ltd., China); general purpose secondary antibody kit (batch number: 16GD1010) and DAB chromogenic agent (batch number: K162418A), (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China); specially made fine moxa (Suzhou Dongfang Moxa Factory, China).

1.3 Modeling method[9-10]

Rats in the normal group and the saline group did not undergo modeling; rats in the model group and the moxibustion group were used to prepare adjuvant arthritis models. A 0.15 mL FCA was subcutaneously injected into the left hind paw of rats, with 1 mL sterile syringe, to induce inflammation. The rats were then observed for 3 d.

Appearance of acute inflammatory swelling in rat ankles after 24 h, and secondary systemic polyarthritis after 48 h with manifestation of swelling or inflammatory nodules in forelimbs, contralateral forelimbs, even ears or tails, suggested that the model replication was successful.

1.4 Intervention methods

Normal group: Rats did not receive any treatment from the first day of the experiment with regular feeding.

Saline group (iso-solvent control group): Three days after the routine feeding, rats in the saline group were injected with 0.15 mL saline to the bottom of left hind paw without other treatments, and continued routine feeding.

Model group: Modeling was conducted after 3-day routine feeding. Rats were continued routine feeding after successful modeling without other treatments.

Moxibustion group: Modeling was conducted after 3-day routine feeding; bilateral Zusanli (ST 36) and Shenshu (BL 23) were selected for the treatment after successful modeling. Aucpoints were located according to the 'Laboratory Animal Acupoint Atlas' in *Experimental Acupuncture Science*[11]. On the 5th day of modeling, the hair covering the rat acupoints was shaved and marked with colors, then the suspended moxibustion was conducted at 2 cm away from each acupoint with the specially made cigarette-type pure moxa (0.8 cm in diameter, 11.8 cm in length) for 20 min, once a day, an acupoint for a day with bilateral points used alternately and continuously for 14 d.

1.5 Observation items and testing methods

1.5.1 General states of health

Rat fur, consciousness, activity, diet, and defecation status were observed.

1.5.2 Morphological observation of rats' ankle joints under light microscope

Rats were sacrificed by dislocation, then the tibiofibula and metatarsal were dissected horizontally immediately by 1 cm upper and 1 cm lower the ankle joint of the right hind limb; fixed in 10% neutral formaldehyde for 24 h after numbering, embedded in paraffin and prepared paraffin sections for hematoxylin-eosin (HE) staining. The pathological observation was then performed under light microscope.

1.5.3 The expressions of TNF-α and NF-κB evaluated by immunohistochemistry

After decalcification using Jenkins mixed acid decalcifying solution (4 mL of concentrated hydrochloric acid, 3 mL of glacial acetic acid, 10 mL of chloroform, 73 mL of pure ethanol and 10 mL of distilled water) in microwave, the bone tissues were routinely dehydrated, dipped in wax, embedded and sliced. The prepared paraffin sections were routinely dewaxed to water and washed with H_2O; washed with 0.01 mol/L PBS (pH: 7.4) for 3 min × 3 times; treated with 1% H_2O_2 for 20 min; washed with PBS for 3 min × 3 times. Antigens were

repaired by high temperature in microwave for 10 min × 2 times and naturally cooled to room temperature. Slices were washed with PBS for 3 min × 3 times; blocked with 1% normal serum for 20 min at room temperature and then incubated with appropriately diluted (1:200) primary antibody of TNF-α or NF-κB at 4 ℃ overnight; washed with PBS for 3 min × 3 times; colored with 0.04% DAB + 0.03% H_2O_2 for 8 min; washed with H_2O; stained with hematoxylin for 30 s; washed with H_2O; differentiated for 2 s with hydrochloric acid ethanol; washed with H_2O; to be blue in microwave; routinely mounted with resin. Three fields of view in each slice were randomly selected under 400 times amplification and took pictures for preservation. Images were analyzed using MOTIC image analysis system.

1.6 Statistical methods

The SPSS 17.0 version statistical software was used to process the data. The measurement data in normal distribution were expressed as mean ± standard deviation ($\bar{x} \pm s$). Comparison among groups was performed using one-way ANOVA, the least significant difference (LSD) was used when the variance was homogeneous; the Games-Howell test was use for data with heterogeneity of variance. $P<0.05$ indicated that the difference was statistically significant.

2 Results

2.1 General state of rat's health

Fur was white, mental state was excellent and active with normal diet in rats in the normal group.

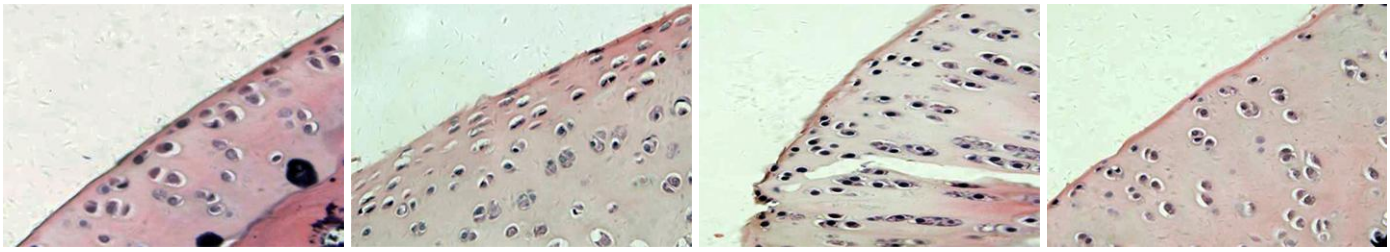
Rats in the saline group had a glossy white color fur, with a better and active mental status, and normal diet.

In the model group, rat's fur was dull; some rats showed difficult crawling and reduced diet.

In the moxibustion group, rat's fur was bright; rats were active with improved diet and normal defecation.

2.2 Histomorphological observation of rats' ankle joints

After HE staining, the slices in each group were observed under light microscope, the results showed that the joint structure was complete and the articular surface was smooth without defect in the normal group; joint tissue was normal without damage in the saline group; the articular surface of the model group was damaged and the articular surface was defective; the articular surface of the moxibustion group was smooth without defect (Figure 1).



Normal group Saline group Model group Moxibustion group

Figure 1. Histomorphological comparison in rats' ankle joints of each group (HE, ×400)

2.3 Expressions of TNF-α and NF-κB in rats' ankle joint tissues

2.3.1 The expression of TNF-α in rats' ankle joints of each group

The mean optical density (MOD) of TNF-α in the model group was significantly higher than that in the normal group ($P<0.05$); there was no significant difference between the saline group and normal group ($P>0.05$); compared with the model group, the MOD of TNF-α in the moxibustion group was significantly decreased ($P<0.05$); the MOD of TNF-α in the saline group was significantly different from that in the model group ($P<0.05$), (Figure 2 and Figure 3).

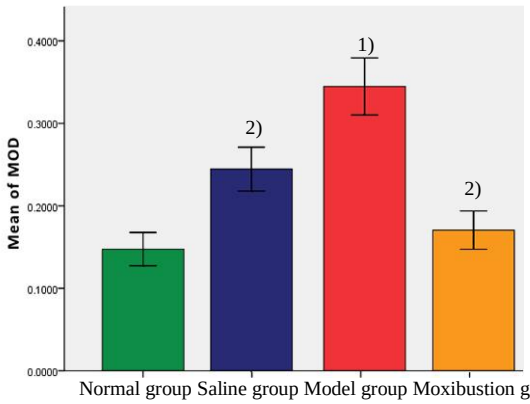
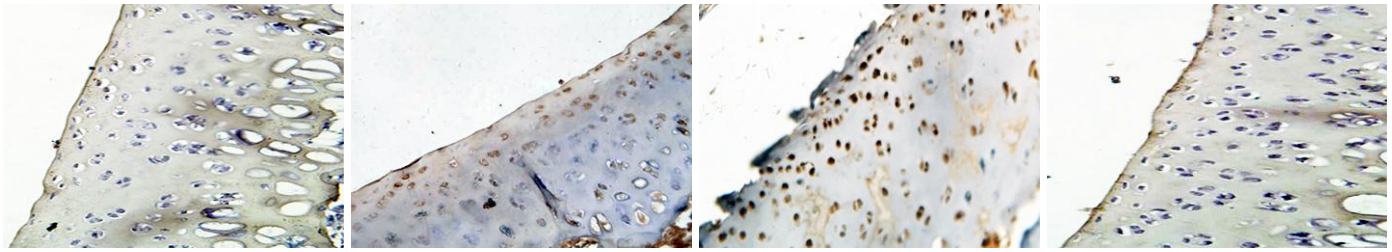


Figure 2. Comparison of TNF-α expression in the rats' ankle joints

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



Normal group Saline group Model group Moxibustion group

Figure 3. Immunohistochemistry of TNF-α in rats' ankle tissues of each group (immunohistochemistry, ×400)

2.3.2 The expression of NF-κB in rats' ankle joints of each group

Compared with the normal group, the MOD of NF-κB in the model group was significantly increased ($P<0.05$); there was no significant difference between the saline group and normal group ($P>0.05$); compared with the model group, the MOD of NF-κB in the moxibustion group was significantly decreased ($P<0.05$); the MOD of NF-κB in the saline group was significantly different from that in the model group ($P<0.05$), (Figure 4 and Figure 5).

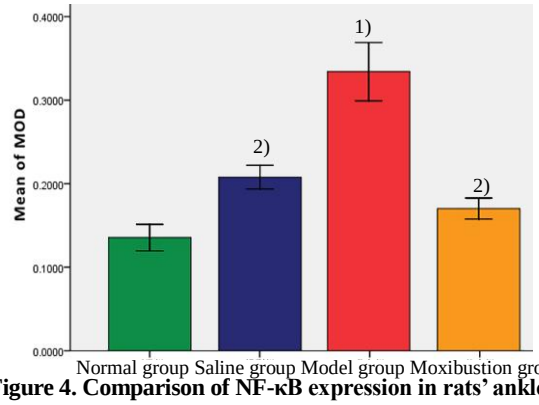
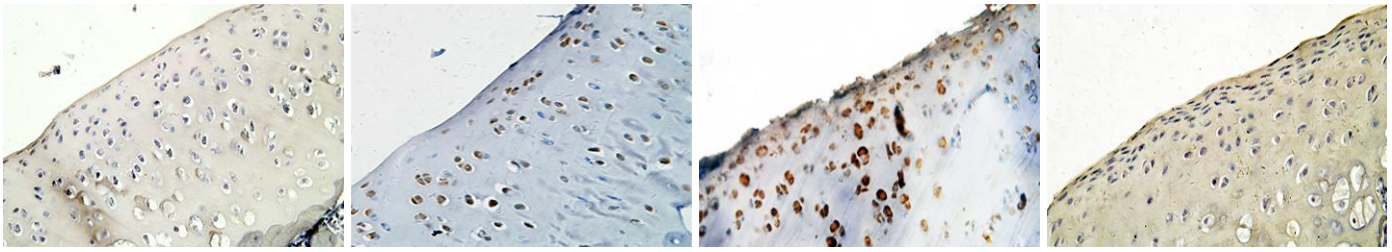


Figure 4. Comparison of NF-κB expression in rats' ankle joints of each group

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



Normal group Saline group Model group Moxibustion group

Figure 5. Immunohistochemistry of NF-κB in ankle joints of each group (immunohistochemistry, ×400)

3 Discussion

RA is an unexplained autoimmune disease characterized by symmetry, erosive synovitis, most involvement of facet joints and multiple peripheral joints all over the body. RA also has the characteristics of persistence, relapse and high disability rate because of the difficulty to control its pathological process, which seriously impairs the physical and mental health, and the patient's quality of life. RA has become a tough problem in medical field, and urgently needs to be solved.

Recent studies have shown that TNF-α and NF-κB play important roles in the development of RA[12-18]. TNF is a cytokine with a wide range of biological effects, which is separated into 2 types of TNF-α and TNF-β according to its structure.

TNF-α is produced by activated macrophages, monocytes and T lymphocytes, and an important proinflammatory cytokine in the pathogenesis of RA. It participates in various pathogenic mechanisms of RA, including activation of endothelial cells, induction of cytokines, leukocyte aggregation, osteoclast activation, and cartilage destruction[19], leading to persistent inflammatory responses and progressive destruction of cartilage and bone. NF-κB is known to bind to the enhancer kappa B of the B cell immunoglobulin kappa light chain gene and to promote the expression of kappa gene. Therefore, it is called nuclear factor κB, which is the most important transcription factor found in recent years. NF-κB is present in a variety of types of cells and an important transcription factor in the transcription and expression of a wide range of genes. It is closely related to the important physiological and pathological processes, such as inflammatory response,

immune response and cell proliferation, differentiation and apoptosis.

NF-κB plays an important regulatory role in RA and involves in arthritis and injury response, and other pathological processes mainly through regulating the cytokines (TNF-α, IL-1β), matrix metalloproteinases, vascular endothelial growth factor (VEGF), cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and other genes[20].

Moxibustion therapy has been a commonly used therapy like acupuncture and Chinese medication as early as in Spring and Autumn period. Moxibustion has important therapeutic effects including warming channel for dispelling cold, promoting blood circulation to remove meridian obstruction, dispersing blood stasis and removing stasis. RA belongs to the category of arthromyodynia in traditional Chinese medicine, closely related to wind, cold and dampness during the development of RA. Zusanli (ST 36) has the therapeutic efficacy of dispelling wind and removing dampness, benefiting qi and nourishing blood, and stretching tendon and un-obstructing collaterals. As the He-Sea point of the Stomach Meridian, it is the important acupoint for the treatment of lower limb paralysis.

Shenshu (BL 23) is the Back-Shu point of the kidney and located at the place where to infuse and coagulate kidney qi, and has the function of reinforcing deficiency and cultivating Yuan-Primary qi to regulate the disorders of organs, meridians, qi, blood, and body fluid. Moxibustion at Zusanli (ST 36) and Shenshu (BL 23) has the effect on promoting qi to activate blood, dispel cold and remove dampness, strengthen body resistance, therefore, to eliminate pathogenic factors and promote disease recovery.

This study found that rats in the saline group had no damage in ankle joints, and the protein expressions of TNF-α and NF-κB were not statistically different from those of the normal group, but significantly different from those of the model group. It was suggested that the saline solution had no effect on the swelling and inflammatory reaction of rat's toes under the physiological temperature. The structure of ankle joints in the model group was damaged and the articular surface was defective, and the TNF-α and NF-κB protein expressions were significantly higher than those in the normal group and the saline group, which confirmed the success of FCA modeling. It's also suggested that TNF-α and NF-κB were induced and inflammatory cytokines were produced in large quantities in the RA animal models, leading to the continuous occurrence of inflammatory responses and progressive destruction of cartilage and bone. After treatment with moxibustion, the inflammatory response of ankle joints of RA model rats was improved obviously, and TNF-α and NF-κB protein expressions were significantly reduced. This indicated that moxibustion therapy could reduce the inflammatory response and repair rat's ankle joint injury.

Li XH, *et al* found that reverse moxibustion could reduce foot swelling rate in the early stage and the secondary stage of adjuvant arthritis (AA) rats, which may be related to the regulation of serum inflammatory cytokines of IL-1β and TNF-α[21]. Tang ZL, *et al* found that moxibustion could significantly reduce the level of upregulated inflammatory cytokines such as IL-6, IL-1β and TNF-α in AA animals. With the functions of anti-inflammatory, regulating and improving immune disorders, promoting absorption and dissipation of inflammatory swelling, reducing or slowing inflammatory response, moxibustion could prevent or block the delayed systemic polyarthritis[22]. This indicated that moxibustion could effectively regulate the inflammatory cytokines in inflammation site and plasma, thereby alleviating the inflammatory response.

In this study, we examined the expressions of TNF-α and NF-κB proteins in local ankle joint tissues, the results showed that moxibustion could repair ankle joint injury in rats, down-regulate the expression of TNF-α protein in ankle joints of RA rats and decrease the level of NF-κB. The anti-inflammatory mechanism of moxibustion on RA is more complicated. This study only confirmed that the anti-inflammatory effect of moxibustion on RA is related to the decrease of TNF-α and NF-κB protein expressions in rats' ankle joints.

Because NF-KB can regulate a variety of inflammation and immune gene expression, whether moxibustion plays the anti-inflammatory effect by regulating NF-κB signaling pathway in RA remains to be further studied.

Conflict of Interest

The authors declared that 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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