Zhang CY, Hu L, Wu ZJ, *et al*. Effects of moxibustion on serum IL-6, IL-8 and TNF-α in rats with experimental rheumatoid arthritis. J Acupunct Tuina Sci, 2019, 17(5): 305-311
DOI: https://doi.org/10.1007/s11726-019-1129-4

Special Topic for 973 Program

Effects of moxibustion on serum IL-6, IL-8 and TNF-α in rats with experimental rheumatoid arthritis

艾灸对实验性类风湿性关节炎大鼠血清 IL-6、IL-8 及 TNF-α 的影响

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Abstract

Objective: To observe the effects of moxibustion on serum levels of interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α), and to explore the effects of moxibustion on inflammatory damaging factors in experimental rheumatoid arthritis (RA) model rats; the relationship between the therapeutic effect of moxibustion on RA and the change in the Toll-like receptor (TLR) signaling pathway was analyzed using Toll-like receptor 4 (TLR4) antagonists and agonists.

Methods: Fifty Sprague-Dawley (SD) rats were divided into a normal group, a model group, a moxibustion group, a moxibustion plus TLR4 agonist group (agonist group) and a moxibustion plus TLR4 antagonist group (antagonist group) according to the random number table, with 10 rats in each group. Except the normal group, rats in the other four groups were subjected to model preparation with the wind, cold and wet environmental factors plus Freund's complete adjuvant (FCA). Rats in the normal and model groups were not treated; rats in the moxibustion, agonist and antagonist groups started to be treated with the moxibustion (cigarette-type moxa) at bilateral Shenshu (BL 23) and Zusanli (ST 36) from the 4th day after the successful modeling, for 20 min each time with a total of 10 d. Rats in the agonist and the antagonist groups were injected with TLR4 agonist or antagonist [0.1 mg/(kg·bw)] via the tail vein 30 min before moxibustion. The concentrations of serum IL-6, IL-8 and TNF-α in each group were determined by enzyme-linked immunosorbent assay (ELISA).

Results: Compared with the normal group, in the model group, the rat's right hind paw swelling was significantly obvious (P<0.01), there was a lot of inflammatory infiltration in the synovial tissues, the surface of the synovial membrane was unsmooth, the synovial membrane was hyperplasia and thicker, and the serum IL-6, IL-8 and TNF-α concentrations increased significantly (P<0.05). Compared with the model group, the paw swelling degrees of the rats in the moxibustion, the agonist and the antagonist groups reduced significantly (all P<0.01); the swelling degree in the antagonist group was milder than that in the agonist group, but the between-group difference was not statistically significant (P>0.05); inflammatory infiltration and synovial membrane hyperplasia in the synovial tissues of the moxibustion group and the antagonist group were all relieved differently; the decrease of synovial layer number in the moxibustion group was more obvious, and there were no obvious improvements in inflammatory infiltration and synovial thickness in the agonist group; the concentrations of IL-6, IL-8 and TNF-α in the moxibustion group were decreased, and the differences in the IL-6 and TNF-α concentrations were statistically significant (all P<0.01); there was no significant between-group difference in the IL-8 concentration (P>0.05); the concentrations of serum IL-8 and TNF-α in the agonist group increased significantly (both P<0.01), while the IL-6 concentration decreased without significant difference (P>0.05); the concentrations of IL-6 and IL-8 in the antagonist group decreased but the between-group differences were statistically insignificant (both P>0.05), and the TNF-α concentration significantly increased (P<0.05). Compared with the moxibustion group, IL-6, IL-8 and TNF-α concentrations increased in the agonist group, and the differences in the IL-8 and TNF-α concentrations were statistically significant (both P<0.01); the concentrations of IL-6, IL-8 and TNF-α increased in the antagonist group, and the differences in the IL-6 and TNF-α concentrations were statistically significant (both P<0.01); there was no significant difference in the IL-8 concentration between the groups (P>0.05). The serum levels of IL-6, IL-8 and TNF-α in the antagonist group were lower than those in the agonist group (all P<0.05).

Conclusion: Moxibustion at Shenshu (BL 23) and Zusanli (ST 36) can reduce the joint swelling degree and inflammation in synovial tissue of RA model rats, decrease the serum levels of IL-6, IL-8 and TNF-α in RA model rats; the decreases of IL-6 and TNF-α are more significant than the decrease of IL-8; TLR4 agonist and antagonist can significantly attenuate the effect of moxibustion in inhibiting releases of IL-6, IL-8 and TNF-α, so that the change in TLR signaling pathway affects the effect of moxibustion in inhibiting the releases of IL-6, IL-8 and TNF-α.

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Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Suspended Moxibustion; Arthritis, Rheumatoid; Point, Shenshu (BL 23); Point, Zusanli (ST 36); Tumor Necrosis Factor-α; Rats

【摘要】目的：观察艾灸对实验性类风湿性关节炎(RA)模型大鼠血清中白细胞介素(IL)-6、IL-8和肿瘤坏死因子α(TNF-α)含量的变化，探讨艾灸对RA模型大鼠相关炎性致损因子的干预作用；通过应用Toll样受体4(TLR4)拮抗剂和激动剂，分析艾灸对RA的治疗作用与Toll样受体信号通路变化之间的关系。**方法：**将50只Sprague-Dawley(SD)大鼠根据随机数字表分为正常组、模型组、艾灸组、艾灸加TLR4激动剂组(激动剂组)和艾灸加TLR4拮抗剂组(拮抗剂组)，每组10只。除正常组外，其余4组大鼠采用风、寒、湿环境因素+弗氏完全佐剂(FCA)复合造模。正常组和模型组不作治疗；艾灸组、激动剂组和拮抗剂组于造模成功后第4天开始用香烟型艾条灸双侧肾俞和足三里，每穴每次灸20 min，共灸10 d。激动剂组和拮抗剂组分别在艾灸前30 min经尾静脉注射TLR4激动剂或拮抗剂 [0.1 mg/(kg·bw)]。采用酶联免疫吸附(ELISA)法测定各组大鼠血清IL-6、IL-8和TNF-α的浓度。**结果：**与正常组比较，模型组大鼠右后肢肿胀明显($P<0.01$)，滑膜组织中有大量炎性浸润表现，滑膜表面不整齐，滑膜增生变厚；血清IL-6、IL-8和TNF-α浓度均显著升高(均$P<0.05$)。与模型组比较，艾灸组、激动剂组和拮抗剂组大鼠的足趾肿胀程度均明显减轻($P<0.01$)，拮抗剂组肿胀程度轻于激动剂组，但组间差异无统计学意义($P>0.05$)；艾灸组和拮抗剂组滑膜组织中炎性浸润和滑膜增生变厚现象不同程度缓解，其中艾灸组滑膜层数减少最明显，激动剂组的炎性浸润和滑膜厚度缓解不明显。艾灸组IL-6、IL-8和TNF-α浓度均下降，其中IL-6和TNF-α浓度的组间差异有统计学意义(均$P<0.01$)，IL-8浓度的组间差异无统计学意义($P>0.05$)；激动剂组大鼠血清IL-8和TNF-α的浓度均显著上升(均$P<0.01$)，而IL-6下降，但组间差异无统计学意义($P>0.05$)；拮抗剂组IL-6和IL-8浓度均降低，但组间差异无统计学意义($P>0.05$)，TNF-α浓度升高，组间差异有统计学意义($P<0.05$)。与艾灸组比较，激动剂组IL-6、IL-8和TNF-α均升高，其中IL-8和TNF-α浓度的组间差异有统计学意义(均$P<0.01$)；拮抗剂组IL-6、IL-8和TNF-α浓度均升高，其中IL-6和TNF-α浓度的组间差异有统计学意义(均$P<0.01$)，IL-8浓度的组间差异无统计学意义($P>0.05$)。拮抗剂组大鼠血清IL-6、IL-8和TNF-α浓度均低于激动剂组(均$P<0.05$)。**结论：**艾灸肾俞和足三里可减轻RA模型大鼠足趾肿胀和滑膜炎症，降低血清中IL-6、IL-8和TNF-α浓度，且IL-6和TNF-α的浓度下降较IL-8更为显著；TLR4激动剂和拮抗剂介入艾灸治疗能显著减弱艾灸抑制IL-6、IL-8和TNF-α释放的作用，TLR信号通路的变化影响艾灸抑制IL-6、IL-8和TNF-α释放的作用。

【关键词】灸法；艾条灸；悬灸；关节炎，类风湿；穴，肾俞；穴，足三里；肿瘤坏死因子α；大鼠

【中图分类号】R2-03　　**【文献标志码】**A

Rheumatoid arthritis (RA) is an autoimmune disease characterized by inflammation and injury of the joint synovial membrane. The pathogenesis of RA is not fully understood. In recent years, studies have found that interleukin (IL)-6, IL-8 and tumor necrosis factor-α (TNF-α) are important inflammatory cytokines in the pathogenesis of RA, which are regulated by the Toll-like receptor 4 (TLR4) signaling pathway[1-2]. In addition, the TRL4 signaling pathway is involved in RA development, arthrosynovitis and osteoclast differentiation, resulting in joint damage. The serum levels of IL-6, IL-8 and TNF-α were are significantly higher in RA patients than those in normal population[3-4]. It has been reported that the worldwide RA incidence is about 0.5%-1.0%[5]. The cause of RA has not yet been fully elucidated by the modern medicine. There is no unified treatment regimen and specific drugs for RA treatment. Therefore, researches on the mechanisms of RA pathogenesis, prevention and treatment, and related therapeutics therapies have been become the focushot topics in this field.

As one of the commonly used external therapies in traditional Chinese medicine (TCM), moxibustion has a notable effect on RA and can significantly improve the clinical symptoms and signs of active RA patients, which has been widely used in the clinical practice[6].

In this study, the effects of moxibustion on serum levels of IL-6, IL-8 and TNF-α in experimental RA model rats were observed to explore the effects of moxibustion on inflammatory cytokines in RA rats. Whether the therapeutic effect of moxibustion on RA is related to the changes in TLR4 signaling pathway were analyzed by applying TLR4 antagonist and agonist, thus to reveal the mechanism of moxibustion in treating RA model rats from the molecular biological level, and to provide the experimental basis for improving the clinical efficacy.

1 Materials and Methods

1.1 Laboratory animals and groups

Fifty healthy male SPF Sprague-Dawley (SD) rats, body weight (250±20) g, were provided by Anhui Experimental Animal Center [license number: scxk (Wan) 2017-001]. After 1-week adaptive feeding, according to the random number table of *Health Statistics*[7], the rats were randomly divided into a normal group, a model group, a moxibustion group, a moxibustion plus TLR4 agonist group (agonist group) and a moxibustion plus TLR4 antagonist group (antagonist group), with 10 rats in each group. Eight rats were randomly selected from each group for the final experimental test due to rat

death during the experiment. The experimental process strictly followed the relevant regulations in the *Instructive Notions with Respect to Caring for Laboratory Animals* promulgated by the Ministry of Science and Technology of the People's Republic of China in 2006.

1.2 Main instruments and reagents

1.2.1 Main instruments

RT-6000 microplate reader (Rayto Life and Analytical Sciences Co., Ltd., China); JW3021HR centrifuge (Anhui Jiawen Instrument Equipment Co., Ltd., China); GL-88B vortex mixer (Qilin Beier Instrument Manufacturing Co., China); DNP-9052BS-Ⅲ electric thermostated container (Shanghai Sanfa Scientific Instrument Co., Ltd., China); micropipettes (specifications: 0.5-10 μL, 200 μL and 1 000 μL, Eppendorf, Germany).

1.2.2 Primary reagents

Rat IL-8 enzyme-linked immunosorbent assay (ELISA) kit (batch number: E20170901A), rat IL-6 ELISA kit (batch number: E20170901A) and rat TNF-α ELISA kit (batch number: E20170901A) (Shanghai Yuanye Biotechnology Co., Ltd., China); Resatorvid (TAK-242) (batch number: 10568, MCE, China); lipopolysaccharide (LPS) (batch number: 421J031, Beijing Solarbio Science & Technology Co., Ltd., China).

1.3 Model establishment and evaluation

The model was prepared by the wind, cold and wet environmental factors combined with the Freund's complete adjuvant (FCA), referring to the modeling method of 'combination of disease and syndrome'[8]. Rats in each group were weighed, numbered, and randomly housed in cages.

The experiment was carried out in December at a room temperature of about 10 ℃. Rats in the normal group were placed in an air-conditioned room for 20 d with controlled room temperature at about 23 ℃. Rats in the other 4 groups were placed beside the window and blown directly with a fan. Water was sprinkled and ice cubes were placed into the rat cages to maintain the wind, cold and wet experimental environment for 20 d. Then, the right hind feet of the model rats were disinfected with 75% alcohol and injected intradermally with FCA (0.1 mL/rat), resulting in an acute swelling of the feet and ankles. The rats were observed for 3 d. The immune-mediated pathological phenomenon of the secondary systemic polyarthritis, including red swelling or inflammatory nodules in the front and rear limbs, ears and tails, indicated the successful model replication.

1.4 Acupoint selection and stimulation

1.4.1 Normal group

As in the moxibustion group, rats in the normal group were similarly grasped and fixed on a special suspended wood frame without other intervention. The rats were fixed for 20 min each time, once a day for consecutive 10 d.

1.4.2 Model group

As in the moxibustion group, rats in the model group were similarly grasped and fixed on the special suspended wood frame without other intervention. The rats were fixed for 20 min each time, once a day for consecutive 10 d.

1.4.3 Moxibustion group

Acupoints: Bilateral Shenshu (BL 23) and Zusanli (ST 36).

Methods: Acupoint localization was according to the animal acupoint map described in *Experimental Acupuncture Science*[9]. Moxibustion was initiated on the 4th day after modeling. The rats were placed on a special suspended wood frame and fixed naturally. Cigarette type moxa sticks were used for moxibustion at 2 cm above the acupoint. The moxibustion treatment was applied simultaneously to bilateral acupoints. Moxibustion was performed for 20 min for each point, once a day for 10 d.

1.4.4 Agonist group

Rats in the agonist group received TLR4 agonist (LPS) via the tail vein injection 30 min before moxibustion. LPS was dissolved in dimethyisulfoxide (DMSO) at a concentration of 1 mg/mL and injected at 0.1 mg/(kg·bw). Moxibustion treatment was performed same as in the moxibustion group.

1.4.5 Antagonist group

Rats in the antagonist group were injected with TLR4 antagonist (TAK-242) via the tail vein injection 30 min before moxibustion. TAK-242 was dissolved in DMSO at a concentration of 1 mg/mL and injected at 0.1 mg/(kg·bw). Moxibustion treatment was performed same as in the moxibustion group.

1.5 Tested items

On the next day of the last treatment, the foot circumference of the right hind limb in each group was measured after anesthetization. The abdominal aorta blood was collected, standed and centrifuged at 3 000 r/min for 15 min to separate the serum, and stored in a refrigerator at –40 ℃ for later use. Then the rats were sacrificed. The ankle synovial tissues of the right hind limbs were fixed in formalin, embedded in paraffin, and sectioned for hematoxylin-eosin (HE) staining.

Serum samples were pre-formed at room temperature and used to analyze the concentrations of serum IL-6, IL-8 and TNF-α by ELISA according to the instruction of the kits. Briefly, the kits were equilibrated for 20 min at room temperature. The 20× wash buffer in the ELISA kit was diluted with the distilled water. The standard sample and test sample wells were set in the selected strips, 50 μL of each standard sample with the designated concentration was added into each designated well, 10 μL of the test sample and 40 μL of

the sample diluents were added into each of the test sample well. Then, 100 μL of the horseradish peroxidase (HRP)-labeled detection antibody was added into each well, and the reaction well was sealed with a sealing membrane, and incubated at 37 ℃ for 60 min in an incubator. Then the wells were washed 5 times, dried with the absorbent paper and filled with the washing solution and dried with the absorbent paper after 1 min. Added 50 μL of substrate A and 50 μL of substrate B into each well and incubated at 37 ℃ for 15 min in the shade. Added 50 μL of stop solution into each well, and measured the absorbance [optical density (OD) value] at 450 nm with a microplate reader within 15 min to prepare a standard curve. In the Microsoft Excel worksheet, the concentrations of the standard samples were used as the abscissa, and the OD values were plotted as the ordinate. The linear regression curve of the standard samples was drawn, and the concentration of each sample was calculated according to the curve equation.

1.6 Statistical processing

All statistical data in this experiment were processed with SPSS version 17.0. The measurement data were first tested for normal distribution and homogeneity of variance, and one-way ANOVA was used for statistical analysis. Data with homogeneity of variance were expressed as mean ± standard deviation ($\bar{x} \pm s$) and analyzed by the least significant difference (LSD) method. Data with heterogeneity of variance were analyzed by Games-Howell. $P<0.05$ indicated a statistical significant difference.

2 Results

2.1 Changes in swelling degree in rat's right hind limb

Compared with the normal group, the rat's right hind paw swelling was more significant in the model group ($P<0.01$). Compared with the model group, the paw swelling degrees of the rats in the moxibustion, the agonist and the antagonist groups reduced significantly (all $P<0.01$); the swelling degree in the antagonist group was milder than that in the agonist group, but the difference between the groups was statistically insignificant ($P>0.05$). The right hind paw swelling degree in the agonist group was severer than that in the moxibustion group ($P<0.05$). The right hind paw swelling degree in the antagonist group was severer than that in the moxibustion group, but the difference between the groups was not statistically significant ($P>0.05$). These results suggested that moxibustion significantly reduced the joint swelling degree in RA model rats; TLR4 agonists reduced the therapeutic effect of moxibustion; TLR4 antagonists had no significant effect on the efficacy of moxibustion (Figure 1).

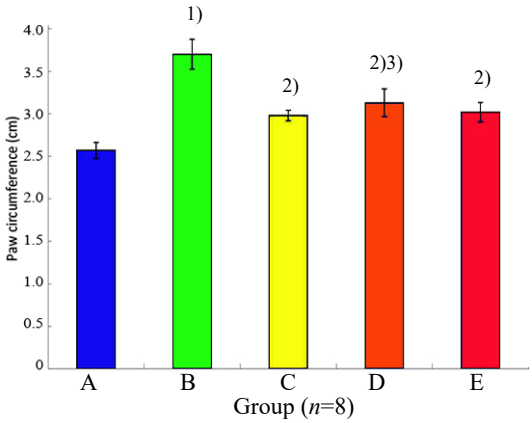

Figure 1. Comparison of the right hind paw circumference

Note: A=Normal group; B=Model group; C=Moxibustion group; D=Agonist group; E=Antagonist group; compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$; compared with the moxibustion group, 3) $P<0.05$

2.2 Pathological changes of synovial tissues in the right hind paw of rats

In the normal group, the lining cells of the joint synovium were mostly monolayer and arranged regularly, and the surface of the synovial membrane was smooth and tidy without inflammatory infiltration. In the model group, there was a lot of inflammatory infiltration in the synovial tissues; the surface of the synovial membrane was untidily; the synovial membrane was hyperplasia and became thicker. Inflammatory infiltration and synovial membrane hyperplasia in the synovial tissues of the moxibustion and the antagonist groups were all relieved differently. Among them, the decrease in synovial layer number in the moxibustion group was the most obvious, and there were no obvious inflammatory infiltration and synovial thickness alleviation in the agonist group (Figure 2).

2.3 Changes of serum IL-6, IL-8 and TNF-α concentrations

Compared with the normal group, the serum IL-6, IL-8 and TNF-α concentrations in the model group increased significantly (all $P<0.05$), which was consistent with the previous report[4]. Compared with the model group, the concentrations of IL-6, IL-8 and TNF-α in the moxibustion group decreased, and there were inter-group significant differences in the IL-6 and TNF-α concentrations (both $P<0.01$), but there was no inter-group significant difference in the IL-8 concentration ($P>0.05$), suggesting that moxibustion could reduce the concentrations of the serum pro-inflammatory factors in the experimental RA model rats, and the effects on IL-6 and TNF-α were stronger than that on IL-8; rat serum IL-8 and TNF-α were all significantly increased in the agonist group (both $P<0.01$), and IL-6 was decreased but there was no significant difference between the groups ($P>0.05$), indicating TLR4 agonist

intervention led to increased IL-8 and TNF-α concentrations, and could antagonize the efficacy of moxibustion; the concentrations of IL-6 and IL-8 in the antagonist group were decreased, but there was no significant difference between the groups (both $P>0.05$), and the TNF-α concentration increased significantly ($P<0.05$); suggesting that TLR4 antagonists had an inhibitory effect on the moxibustion efficacy. Compared with the moxibustion group, IL-6, IL-8 and TNF-α in the agonist group were all increased significantly (all $P<0.05$), and the differences in the IL-8 and TNF-α concentrations were significant (both $P<0.01$); the concentrations of IL-6 and TNF-α in the antagonist group increased significantly (both $P<0.01$), and the IL-8 concentration increased without significant difference ($P>0.05$), suggesting that moxibustion only had the better inhibitory effect on IL-6, IL-8 and TNF-α than TLR4 agonist or antagonist intervention combined with moxibustion (Figure 3-Figure 5).

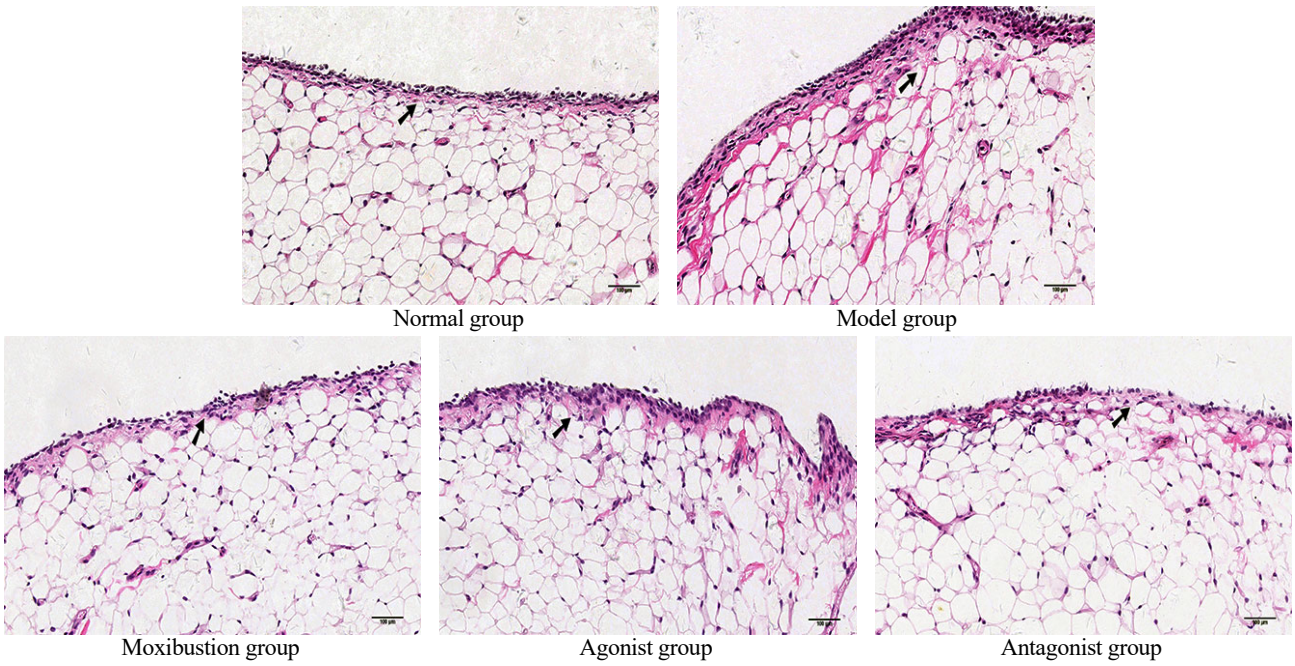

Figure 2. Pathological comparison of rat synovial tissues in each group (HE, ×400)

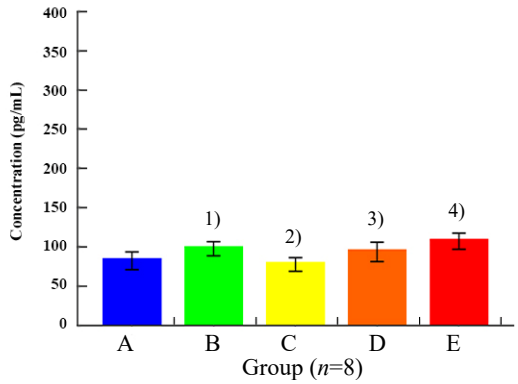

Figure 3. Comparison of serum IL-6 level in each group

Note: A=Normal group; B=Model group; C=Moxibustion group; D=Agonist group; E=Antagonist group; compared with the normal group, 1) $P<0.05$; compared with the model group, 2) $P<0.01$; compared with the moxibustion group, 3) $P<0.05$, 4) $P<0.01$

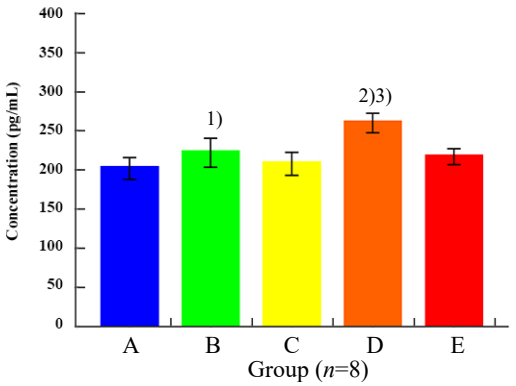

Figure 4. Comparison of serum IL-8 level in each group

Note: A=Normal group; B=Model group; C=Moxibustion group; D=Agonist group; E=Antagonist group; compared with the normal group, 1) $P<0.05$; compared with the model group, 2) $P<0.01$; compared with the moxibustion group, 3) $P<0.01$

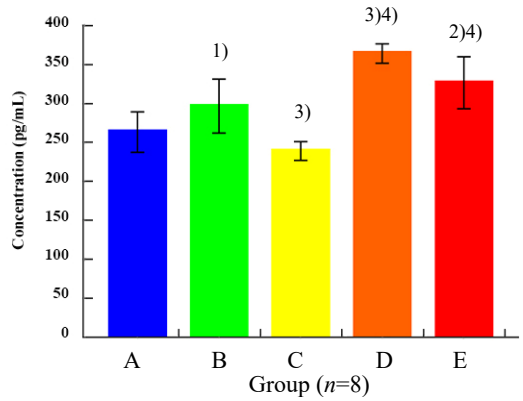

Figure 5. Comparison of serum TNF-α level in each group

Note: A=Normal group; B=Model group; C=Moxibustion group; D=Agonist group; E=Antagonist group; compared with the normal group, 1) $P<0.05$; compared with the model group, 2) $P<0.05$, 3) $P<0.01$; compared with the moxibustion group, 4) $P<0.01$

3 Discussion

RA belongs to Bi-impediment syndrome in Chinese medicine. Contributing factors include cold, dampness, fatigue, trauma or stress. Moxibustion has the functions of warming meridians and dispelling cold, promoting qi circulation to remove meridian obstruction, assisting yang to prevent collapse and diseases, it is one of the commonly used and effective methods to treat Bi-impediment syndrome. Studies have shown that moxibustion has anti-inflammatory, analgesic and immune regulation effects, which can significantly reduce inflammatory swelling and increase pain threshold[10-11], effectively improve clinical symptoms and related physical and chemical indicators (such as rheumatoid factor and erythrocyte sedimentation rate); and the effect is better than non-steroidal anti-inflammatory drugs[12-14]. Clinical observations have found that the symptoms of joint pain and swelling in RA patients are positively correlated with the levels of related pro-inflammatory cytokines[15]. Our previous experimental studies have found that moxibustion at Shenshu (BL 23) alleviated paw swelling in RA rats, inhibited the release of serum inflammatory factor IL-1, increased the immunoregulatory factor IL-2, and corrected the imbalance of anti-inflammatory/ proinflammatory cytokines.

In this study, we found that moxibustion at Shenshu (BL 23) and Zusanli (ST 36) reduced the serum levels of IL-6, IL-8 and TNF-α in RA model rats, and the concentrations of IL-6 and TNF-α were decreased more significant than that of IL-8. These results suggested that reducing the pro-inflammatory factor levels (IL-6 and TNF-α) may be one mechanism of moxibustion in treating RA. At the same time, this experiment compared the impact of TLR4 agonist and antagonist on the moxibustion efficacy. The results showed that TLR4 agonist and antagonist significantly reduced the effects of moxibustion on IL-6, IL-8 and TNF-α; TLR4 antagonist and moxibustion did not synergistically work in RA rat model; instead, it inhibited moxibustion in reducing the IL-6, IL-8 and TNF-α. It may be that moxibustion reduced TLR4 antagonist activity, thus to inhibit its function. The specific mechanism needs further study.

TLR4 is a member of the Toll-like receptor (TLR) family. TLR is a specific transmembrane receptor and pathogen recognition receptor in the innate immune system. The TLR signaling pathway is the key pathway in the innate immunity and acquired immunity. The RA patients initiated the body's inflammatory response by TLR to synthesize and release pro-inflammatory cytokines and inflammatory chemokines, and involve in inflammatory damage, thereby inducing infectious and non-infectious inflammation[16-19]. As a major ligand of TLR4, LPS activates the TLR4 signaling pathway, which leads to the activation of the downstream nuclear factor kappa B, thereby promoting the transcription of the related genes, resulting in the massive release of inflammatory cytokines such as IL-6, IL-8 and TNF-α[20-21]. Raymond SL, *et al*[22] pointed out that exogenous LPS activated TLR4 signaling pathway and increased plasma IL-6, IL-8 and TNF-α levels in premature neonates, term newborns and adults. TAK-242 has been shown to be a novel small molecule inhibitor of the TLR4 signaling. It inhibits the production of cytokines such as IL-6, IL-8 and TNF-α by selectively inhibiting intracellular signal transduction of TLR4, and is a promising therapeutic drug for inflammatory diseases[23].

Based on this, we believe that changes in the TLR signaling pathway can affect the effect of moxibustion in inhibiting the release of anti-inflammatory factors (IL-6, IL-8, and TNF-α), while the function and the possible targets of the TLR signaling pathway in the treatment of RA by moxibustion are worthy of further study and discussion.

In this study, rats in the agonist and antagonist groups were treated with moxibustion combined with TLR4 agonist or antagonist. However, without sole use of TLR4 agonist or antagonist, it is difficult to fully explain their effect on moxibustion in lowering the IL-6, IL-8 and TNF-α levels during simultaneous intervention of TLR4 antagonist combined with moxibustion. This is the limitation of this study. Therefore, the future study should investigate the effects of TLR4 agonist or antagonist alone on proinflammatory cytokines and downstream nuclear factor kappa B of TLR signaling pathway in RA model rats, thus to explore the role of TLR signaling pathway in RA treatment with moxibustion more deeply and the possible targets.

Conflict of Interest

The authors declared that there was no potential conflict of interest in this article.

Acknowledgments

This work was supported by Project of Anhui Provincial Natural Science Foundation of China (安徽省自然科学基金项目, No. 1608085QH219); National Basic Research Program of China (973 Program, 国家重点基础研究发展计划项目, No. 2015CB554504).

Statement of Human and Animal Rights

The treatment of animals conformed to the ethical criteria.

Received: 28 January 2019/Accepted: 25 February 2019

References

[1] He W, Rebello O, Savino R, Terracciano R, Schuster-Klein C, Guardiola B, Maedler K. TLR4 triggered complex inflammation in human pancreatic islets. Biochim Biophys Acta Mol Basis Dis, 2019, 1865(1): 86-97.

[2] Huffman KM, Jessee R, Andonian B, Davis BN, Narowski R, Huebner JL, Kraus VB, McCracken J, Gilmore BF, Tune KN, Campbell M, Koves TR, Muoio DM, Hubal MJ, Kraus WE. Molecular alterations in skeletal muscle in rheumatoid arthritis are related to disease activity, physical inactivity, and disability. Arthritis Res Ther, 2017, 19(1): 12-29.

[3] Li YY, Zhang WT. IL-6: the next key target for rheumatoid arthritis after TNF-α. Shengwu Gongcheng Xuebao, 2017, 33(1): 36-43.

[4] Li P, Cao JP, Zhao LJ. Serum levels of IL-6, IL-8 and TNF-α in patients with rheumatoid arthritis and their significance. Zhonghua Fengshibingxue Zazhi, 1998, 2(4): 59-61.

[5] Ye RG, Lu ZY. Internal Medicine. 6th Edition. Beijing: People's Medical Publishing House, 2004: 885.

[6] Hu L, Hao F, Zhong F, Qin LH, Xia XH, Chu HR, Luo L, Cai RL. Multicenter randomized controlled clinical study on rheumatoid arthritis with moxibustion. Huanqiu Zhongyiyao, 2011, 4(6): 401-405.

[7] Ni ZZ. Health Statistics. 4th Edition. Beijing: People's Medical Publishing House, 2002: 267.

[8] Hu L, Lars DH, Song XG, Tang ZL, Cai RL, He L, Luo L. Effect of moxibustion on IL-1β and IL-2 in rat models of rheumatoid arthritis. J Acupunct Tuina Sci, 2010, 8(3): 149-153.

[9] Yu SG, Guo Y. Experimental Acupuncture Science. Shanghai: Shanghai Scientific & Technical Publishers, 2014: 147-148.

[10] Ding F. Moxibustion alone in treatment of rheumatoid arthritis. Zhenjiu Linchuang Zazhi, 2007, 23(10): 40-41.

[11] Zhang H, Ma XP, Wu HG, Wu SB, Su SS, Hu YC, Li ZF, Zhang L, Xie MY. Effect of moxibustion on tumor necrosis factor-α and nuclear transcription factor kappa B in ankle joints of rats with rheumatoid arthritis. J Acupunct Tuina Sci, 2017, 15(3): 171-176.

[12] Cai RL, Hu L, Song XG, Xia XH, Li M. A survey of clinical and experimental research on moxibustion for arthritis. Zhongyiyao Linchuang Zazhi, 2008, 20(4): 427-429.

[13] Zhang LX, Wu J, Lan Y, Lu P, Liu M, Zhang YH, Hu YP. Research progress in the clinical and mechanism of acupuncture treatment of rheumatoid arthritis. Heilongjiang Zhongyiyao, 2013, 42(1): 51-52.

[14] Luo L, Hu L, Song XG, Tang ZL, He L, Cai RL, Hao F. Effect of moxibustion on the metatarsal joint cytokine in rheumatoid arthritis rats. Huanqiu Zhongyiyao, 2011, 4(6): 416-419.

[15] Chen KH, Li RB, Li K, Qin L, Qin SJ, Lai YH, Zhong QN, Liang TW. Clinical effects of moxibustion treatment of rheumatoid arthritis and a study of its blood IL-6 and CRP levels. Zhongyao Yaoli Yu Linchuang, 2015, 31(1): 303-304.

[16] Can CX, Yang QW, Lü FL, Cui J, Fu HB, Wang JZ. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. Biochem Biophys Res Commun, 2007, 353(2): 509-514.

[17] Teng JY. Study on the Correlation of TLR4 Gene Polymorphism with the Pathogenesis of Rheumatoid Arthritis. Xiamen: Master Thesis of Huaqiao University, 2017.

[18] Zhu XK, Yang RF, Meng YQ. Research progress in ligands of Toll-like receptor 4. Zhongguo Yaolixue Yu Dulixue Zazhi, 2016, 30(4): 389-396.

[19] Sun W, Xiong Y, Fu YY. Research progress in Toll-like receptor. Shiyan Yu Jianyan Yixue, 2008, 26(4): 413-416.

[20] Ding RR, Chen W, Guo CY, Liao WT, Yang X, Liao FE, Lin JM, Mei HF, Zeng Y. Danggui Shaoyao San attenuates LPS-induced neuroinflammation *via* the TLRs/NF-κB signaling pathway. Biomed Pharmacother, 2018, 105: 187-194.

[21] Xu JJ, Lu CJ, Liu ZQ, Zhang P, Guo HL, Wang TT. Schizandrin B protects LPS-induced sepsis via TLR4/NF-κB/MyD88 signaling pathway. Am J Transl Res, 2018, 10(4): 1155-1163.

[22] Raymond SL, Hawkins RB, Murphy TJ, Rincon JC, Stortz JA, López MC, Ungaro R, Ellett F, Baker HV, Wynn JL, Moldawer LL, Irimia D, Larson SD. Impact of Toll-like receptor 4 stimulation on human neonatal neutrophil spontaneous migration, transcriptomics, and cytokine production. J Mol Med (Berl), 2018, 96(7): 673-684.

[23] Ii M, Matsunaga N, Hazeki K, Nakamura K, Takashima K, Seya T, Hazeki O, Kitazaki T, Iizawa Y. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl) sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits Toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. Mol Pharmacol, 2006, 69(4): 1288-1295.

Translator: Yang Yan-ping (杨燕萍)