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Basic Study

Experimental study on the effect of different moxibustion durations on rats with rheumatoid arthritis

不同艾灸持续时间对类风湿关节炎大鼠影响的实验研究

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Abstract

Objective: To observe the effect of different moxibustion durations on rats with rheumatoid arthritis (RA) and to evaluate the relationship between moxibustion amount and moxibustion efficacy.

Methods: Eight rats were randomly selected as a normal group from the 40 male Sprague-Dawley (SD) rats, and the other 32 rats were used to establish type Ⅱ collagen-induced RA models. After successful modeling, the 32 rats were randomly divided into a model group, a moxibustion for 20 min group, a moxibustion for 40 min group and a moxibustion for 60 min group, with 8 rats in each group. Rats in the normal group did not receive modeling and moxibustion intervention; rats in the model group did not receive moxibustion after modeling; rats in the moxibustion for 20 min group, the moxibustion for 40 min group and the moxibustion for 60 min group were treated with moxibustion at Shenshu (BL 23) and Zusanli (ST 36) for 20 min, 40 min and 60 min, respectively. Six days were a course of treatment, with a total of 3-course treatments and a 1-day rest between the courses of treatment. After treatment, the serum levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α, arthritis index (AI) scores, toe volumes and pathological score of synovitis were evaluated in the rats.

Results: Compared with the normal group, the serum IL-1β and TNF-α levels, and the toe volumes in the model group were increased, and the differences were statistically significant (P<0.01), before the treatment. Compared with the model group, the serum IL-1β and TNF-α levels, toe volumes and arthritis index (AI) scores were significantly decreased in the moxibustion for 20 min group, the moxibustion for 40 min group and the moxibustion for 60 min group (P<0.05 or P<0.01). Compared with the moxibustion for 20 min group and the moxibustion for 60 min group, serum IL-1β and TNF-α levels, toe volumes and AI scores were decreased more significantly in moxibustion for 40 min group, and the differences were statistically significant (P<0.05 or P<0.01). There were no significant differences in serum IL-1β and TNF-α levels, AI scores and toe volumes between the moxibustion for 20 min group and the moxibustion for 60 min group (all P>0.05). The synovial histopathological improvement was the most obvious in the moxibustion for 40 min group, when the synovial histopathological changes were compared among the moxibustion for 20 min group, moxibustion for 40 min group and moxibustion for 60 min group.

Conclusion: The therapeutic efficacy of moxibustion for 40 min in RA rats was more significant than that of moxibustion for 20 min and moxibustion for 60 min, indicating that the duration of moxibustion is the main factor affecting its therapeutic efficacy.

Keywords: Moxibustion Therapy; Moxa Stick Moxibustion; Arthritis, Rheumatoid; Rat

【摘要】目的：观察不同艾灸持续时间对类风湿关节炎(RA)大鼠影响的差异，评价灸量与灸效的关系。**方法**：将40只雄性Sprague-Dawley (SD)大鼠随机选取8只作为正常组，其他32只建立Ⅱ型胶原诱导的RA模型。造模成功后，将32只大鼠随机分为模型组、艾灸20 min组、艾灸40 min组和艾灸60 min组，每组8只。正常组不造模，不进行艾灸干预；模型组大鼠造模后不予艾灸干预；艾灸20 min组、艾灸40 min组和艾灸60 min组大鼠分别接受艾灸肾俞和足三里20 min、40 min和60 min治疗。6 d为1个疗程，疗程间休息1 d，共治疗3个疗程。治疗结束后，观察大鼠血清中白介素(IL)-1β和肿瘤坏死因子(TNF)-α水平、关节炎指数(AI)评分、足趾容积和滑膜炎症病理学评分。**结果**：治疗前，与正常组比较，模型组大鼠血清IL-1β和TNF-α水平升高，足趾容积增加，组间差异均

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有统计学意义(均 $P<0.01$)。与模型组比较，艾灸 20 min 组、艾灸 40 min 组和艾灸 60 min 组血清 IL-1β、TNF-α 水平、足趾容积和 AI 评分均显著下降($P<0.05$ or $P<0.01$)。与艾灸 20 min 组和艾灸 60 min 组比较，艾灸 40 min 组血清 IL-1β 和 TNF-α 水平、足趾容积和 AI 评分下降更明显，组间有统计学差异($P<0.05$ or $P<0.01$)。艾灸 20 min 组和艾灸 60 min 组比较，血清 IL-1β、TNF-α 水平、AI 评分和足趾容积差异均无统计学意义(均 $P>0.05$)。艾灸 20 min 组、艾灸 40 min 组和艾灸 60 min 组的滑膜组织病理的改变比较，艾灸 40 min 组滑膜组织病理改善最明显。**结论：**艾灸 40 min 治疗 RA 大鼠的作用效果比艾灸 20 min 及艾灸 60 min 更明显，说明艾灸治疗持续时间是影响艾灸治疗效果的主要因素。

【关键词】灸法；艾条灸；关节炎，类风湿；大鼠

【中图分类号】R2-03　　**【文献标志码】**A

Rheumatoid arthritis (RA) is a symmetrical and multiple joint inflammation, mainly involving the small joints of the hands and feet. At present, the adult RA incidence in the world is 1%. RA is the most common inflammatory arthritis and the main cause of disability[1]. Inflammation-induced bone destruction is the main pathological feature of RA, mediated by osteoclasts[2-3], and characterized by vascular proliferation and cartilage destruction, leading to body function damage[4]. Anti-rheumatic drugs, such as methotrexate, sulfasalazine and other drugs, are the main strategy for RA treatment in Western medicine[5]. However, these drugs have serious side effects such as liver damage, myelosuppression and gastrointestinal reactions[6]. Traditional Chinese medicine has a special advantage in the treatment of RA, and moxibustion therapy is one of the Chinese medicine therapies[7]. Moxibustion has been applied in the treatment of RA to inhibit arthritis, relieve pain and improve the patients' daily living. Confirmed by the bibliometrics statistics, moxibustion therapy is an important external therapeutic method of traditional Chinese medicine and can treat 364 types of diseases, while RA, as a dominant disease, is recommended to use moxibustion therapy. By statistical analysis, each disease has a best moxibustion time, that is, the best moxibustion duration for most patients (the best moxibustion amount)[8]. The purpose of this study is to explore the best moxibustion amount in treatment of RA, to simplify the clinical procedures, reduce the error and improve efficacy via the standardization of moxibustion amount research[9].

1 Materials and Methods

1.1 Experimental animals

Healthy male Sprague-Dawley (SD) rats, 7-8 weeks old, were provided by Zhejiang Experimental Animal Research Center [SCXK (Zhejiang) 2014-0001] and housed in the SPF Animal Experimental Center of Nanjing University of Traditional Chinese Medicine. The rats were adaptively fed for one week with free access to food and water. Feeding conditions: free sterile diet; light/dark period of 12 h/12 h (illumination period 06:00-18:00); background noise (40±10) dB; temperature (20±3) ℃; relative humidity 40% to 50%.

1.2 Modeling method

Eight rats were randomly selected as normal group from the 40 male SD rats, and the other 32 rats were used to establish type Ⅱ collagen-induced arthritis (CIA) model. According to the literature[10], the bovine type Ⅱ collagen was dissolved in 0.05 mol/L acetic acid, and stirred at 4 ℃ overnight, to prepare 2 mg/mL collagen solution. Type II collagen emulsion (final concentration of 1 mg/mL) was prepared by adding an equal volume of complete Freund's adjuvant under low-speed mixture on ice with sample homogenizer. A 200 μL type II collagen emulsion was subcutaneously injected at the end of each rat's tail to immunize, after 7 d, another 100 μL of type II collagen emulsion was injected intradermally at the end of each rat's tail for the secondary immunization.

The arthritis index (AI) was evaluated using the scoring method after observation of the condition of each toe.

Limb lesion of each rat was evaluated with the following criteria[11]. 0 point: normal; 1 point: mild inflammation of single toe joint; 2 points: moderate arthritis involving the toe and ankle joints or toes; 3 points: severe arthritis involving the ankle joint or forepaws; 4 points: extreme inflammatory arthritis leading to joint rigidity and movement disorders. The AI score was the sum of the scores of the 4 toes. AI score ＞4 points indicated a successful CIA model[11].

1.3 Grouping and intervention

After successful modeling, the 32 rats were randomly divided into a model group, a moxibustion for 20 min group, a moxibustion for 40 min group and a moxibustion for 60 min group, with 8 rats in each group. Rats in the normal group and the model group did not receive moxibustion treatment[11]. Rats in moxibustion for 20 min group, moxibustion for 40 min group and moxibustion for 60 min group began to receive moxibustion intervenion on the 7th day after modeling.

Suspended moxibustion was performed with smoke-free moxa stick at 2-3 cm away from the skin. Shenshu (BL 23, 7 mm from both the left and right sides under the second lumbar spinous process) and Zusanli (ST 36, posterolateral knee joint and about 5 mm from the capitulum fibulae) were selected according to the published literature[12]. The acupoints were located

according to the 'Laboratory Animal Acupoint Atlas' in *Experimental Acupuncture Science*[13]. Moxibustion was performed at the two acupuncture points in turns, following the order of Shenshu (BL 23)-contralateral Shenshu (BL 23)-Zusanli (ST 36)-contralateral Zusanli (ST 36). Rats in the moxibustion for 20 min group received moxibustion at each point for 5 min, with a total of moxibustion intervention time of 20 min; rats in moxibustion for 40 min group received moxibustion at each point for 10 min, with a total of moxibustion intervention time of 40 min; rats in moxibustion for 60 min group received moxibustion at each point for 15 min, with a total of moxibustion intervention time of 60 min. Six days were a course of treatment, with a total of 3 courses of treatment (21 d) and a 1-day rest between courses of treatment.

1.4 Observation items

1.4.1 Serum interleukin (IL)-1β and tumor necrosis factor (TNF)-α levels

At the end of the treatment, serum IL-1β and TNF-α levels were measured by enzyme-linked immunosorbent assay (ELISA). 2-4 mL of blood was taken from each rat's orbit after anesthesia and put into the test tube. The blood was allowed to keep at room temperature for 20 min and then centrifuged at 15 ℃, 3 000 rpm for 30 min. The serum was then extracted from the test tube, sealed and stored at –70 ℃. The detection operation should strictly follow the kit instruction.

1.4.2 Changes of rat's toe volumes

Three weeks after the moxibustion intervention, the rat ankle joints were marked, and the right toe volumes of rats were measured three times using the toe volume measuring instrument. The average values were calculated and the changes in the toe volumes were compared among groups.

1.4.3 AI score

The AI score was calculated according to the erythema, degree of swelling and joint deformity around rat's joints[14-15].

1.4.4 Histopathological changes of the synovial tissues

After 3 weeks of intervention, the rats were sacrificed after anesthesia, and the pathological specimens of single side ankle joints were collected from rats in each group. Paraffin sections were prepared after formalin fixation. Histomorphological observation was performed after hematoxylin-eosin (HE) staining. The pathological sections and HE staining were completed by the professional technicians in pathological experimental center of Nanjing Medical University. The main detection indexes included vasocongestion, synovial membrane swelling, inflammatory cell infiltration and tissue necrosis. The pathological changes of the rat's ankle joints were also observed.

1.5 Statistical analysis

Data were analyzed using SPSS 16.0 version software. The measurement data were expressed as mean ± standard deviation ($\bar{X} \pm s$). The measurement data fit normal distribution and homogeneity of variance confirmed by the normal distribution test and homogeneity test for variance. Comparison among multiple samples was performed using one-way ANOVA. $P<0.05$ indicated that the difference was statistically significant.

2 Results

2.1 Serum IL-1β and TNF-α

Before treatment, compared with the normal group, serum IL-1β and TNF-α levels were significantly increased in the model group, and the differences were statistically significant (all $P<0.01$). After treatment, compared with the model group, the levels of serum IL-1β and TNF-α were significantly decreased in moxibustion for 20 min group, moxibustion for 40 min group and moxibustion for 60 min group, and the differences were statistically significant ($P < 0.05$). Compared with moxibustion for 20 min group and moxibustion for 60 min group, the serum IL-1β and TNF-α levels were decreased more significantly in moxibustion 40 min group, and the differences were statistically significant among groups ($P<0.05$).

It indicated that moxibustion for 40 min can effectively reduce serum IL-1β and TNF-α levels. There was no statistically significant differences in serum levels of IL-1β and TNF-α between moxibustion for 20 min group and moxibustion for 60 min group ($P>0.05$), (Table 1).

Table 1. Changes in serum IL-1β and TNF-α levels ($\bar{X} \pm s$)

Group	*n*	IL-1β	TNF-α
Normal	8	6.17±0.13	16.69±3.53
Model	8	9.24±0.86[1)]	27.16±1.66[1)]
Moxibustion for 20 min	8	8.13±0.91[2)]	24.38±1.55[2)]
Moxibustion for 40 min	8	7.13±0.39[2)3)]	22.06±2.16[2)3)]
Moxibustion for 60 min	8	8.26±1.24[2)]	24.82±1.55[2)]

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.05$; compared with moxibustion for 20 min group and moxibustion for 60 min group, 3) $P<0.05$

2.2 Changes of rats' toe volumes

There were no statistically significant differences in the toe volumes of rats before modeling.

Before intervention, compared with the normal group, the toe volumes were significantly increased in the model group, moxibustion for 20 min group, moxibustion for 40 min group and moxibustion for 60 min group (all $P<0.01$), suggesting that the models

were successfully prepared. Before the intervention, compared with the model group, toes volumes showed no statistically significant differences among the moxibustion for 20 min group, the moxibustion for 40 min group and the moxibustion for 60 min group (all $P>0.05$). Compared with the model group, the toe volumes were significantly reduced in the moxibustion for 20 min group, the moxibustion for 40 min group and the moxibustion for 60 min group after 3 courses of moxibustion treatment (all $P<0.01$); compared with the moxibustion for 20 min group and moxibustion for 60 min group, the toe volumes were decreased more significantly in the moxibustion for 40 min group ($P<0.05$), however, there was no statistically significant difference between the moxibustion for 20 min group and the moxibustion for 60 min group ($P>0.05$). There was no statistically significant difference between the moxibustion for 40 min group and the normal group ($P>0.05$). These results suggested that the efficacy to reduce the toe swelling in moxibustion for 40 min group was better than that in moxibustion for 20 min group and moxibustion for 60 min group (Table 2).

Table 2. Comparison of toe volumes among rats of each group ($\bar{X}$ ±s, mL)

Group	*n*	Before modeling	Before intervention	After intervention
Normal	8	1.95±0.12	1.83±0.18	1.98±0.12
Model	8	1.93±0.17	2.32±0.23[1)]	3.17±0.21
Moxibustion for 20 min	8	2.00±0.17	2.23±0.28[1)]	2.43±0.25[2)]
Moxibustion for 40 min	8	2.09±0.21	2.21±0.20[1)]	2.12±0.24[2)3)]
Moxibustion for 60 min	8	2.08±0.14	2.22±0.26[1)]	2.5±0.22[2)]

Note: Compared with the normal group, 1) $P<0.01$; compared with the model group, 2) $P<0.01$; compared with moxibustion for 20 min group and moxibustion for 60 min, 3) $P<0.05$

2.3 AI score

Except for the normal group, there were no statistically significant differences in the other 4 groups before intervention (all $P>0.05$). After 3 courses of moxibustion treatment, the AI scores in all 3 moxibustion treatment groups were significantly lower than those in the model group (all $P<0.01$). Compared with the moxibustion for 20 min group and the moxibustion for 60 min group, the arthritis score of the moxibustion for 40 min group was reduced more significantly ($P<0.01$), while no statistically significant between the moxibustion for 20 min group and the moxibustion for 60 min group ($P>0.05$). The results suggested that moxibustion for 40 min was better in reducing the AI score than moxibustion for 20 min and moxibustion for 60 min (Table 3).

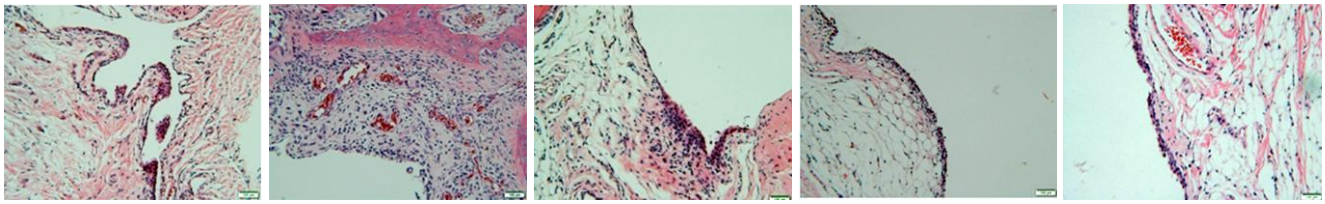
Table 3. AI score comparison ($\bar{X}$ ±s, point)

Group	*n*	After modeling	After intervention
Model	8	4.88±0.84	10.5±1.20
Moxibustion for 20 min	8	4.89±0.84	7.13±0.83[1)]
Moxibustion for 40 min	8	4.88±0.84	5.00±1.07[1)2)]
Moxibustion for 60 min	8	5.00±0.76	7.25±1.28[1)]

Note: Compared with the model group, 1) $P<0.01$; compared with moxibustion for 20 min group and moxibustion for 60 min group, 2) $P<0.01$

2.4 Pathological change of synovial inflammation

The articular surface was covered with transparent cartilage, joint capsule was smooth, joint synovial membrane cells were continuous and temper rolling with clear structure and layers, and without synovial membrane swelling, tissue necrosis and inflammatory cell infiltration in the normal group. In the model group, synovial membrane inflammation showed severe congestive edema, severe synovial membrane swelling, severe inflammatory cell infiltration and moderate tissue necrosis. Synovial membrane inflammation pathology showed, mild synovial membrane swelling, mild congestion and edema, mild proliferation of synovial membrane cells, and a small amount of inflammatory cell infiltration, in moxibustion for 20 min group; joint synovial membrane cells were continuous and temper rolling with clear structure and layers, mild congestion and edema of joint synovium, while no tissue necrosis or inflammatory cell infiltration, in moxibustion for 40 min group; mild hyperemia and edema of joint synovial membrane, mild hyperplasia of synovial membrane cells, and mild synovial membrane swelling, in moxibustion for 60 min group. The synovial membrane inflammation was improved more significantly in moxibustion for 20 min group, moxibustion 40 min group and moxibustion 60 min group than in the model group, and was the most significant in the moxibustion for 40 min group (Figure 1).



Normal group | Model group | Moxibustion for 20 min group | Moxibustion for 40 min group | Moxibustion for 60 min group

Figure 1. Synovial membrane inflammation (HE, ×200)

3 Discussion

It has been reported that moxibustion at Shenshu (BL 23) and Zusanli (ST 36) has a significant anti-inflammatory effect for RA[16-19]. Moxibustion in treatment of rat RA can inhibit IL-1β and TNF-α, and regulate immune function[20]. IL-1β and TNF-α are key inflammatory cytokines in the pathogenesis of RA. IL-1β is mainly produced by synovial membrane macrophages and an important proinflammatory factor in the pathogenesis of RA[21]. TNF-α is also an important inflammatory cytokine in the pathogenesis of RA[22]. Previous studies have showed that stimulation of acupoints by moxibustion can reduce the inflammatory response, however, different durations of moxibustion on the efficacy have not been evaluated[20]. Our current study revealed that moxibustion at Shenshu (BL 23) and Zusanli (ST 36) could reduce serum IL-1β and TNF-α levels and the inflammatory response of CIA rats, which was consistent with the results reported in the previous study[23]. Our current study was mainly focued on the therapeutic effects of different moxibustion durations on CIA rats.

Zheng BZ, *et al* compared the impacts of moxibustion for 20 min and moxibustion for 40 min on RA rats, and found that the analgesic effect in moxibustion for 40 min group was better than in moxibustion for 20 min group[24]. Xu JF, *et al* investigated the effect of different moxibustion stimulation time on low back pain, and found that moxibustion stimulation for 60 min was more effective to relieve lower lumbar pain after comparing moxibustion at Guanyuan for 15 min, 30 min or 60 min, respectively[25]. Therefore, the two moxibustion durations (40 min and 60 min) with the best therapeutic effects reported in the literature were used in this study to explore the optimal moxibustion treatment time for RA rats.

Moxibustion exerting therapeutic effects via stimulation of acupoints requests a certain stimulation time. In moxibustion treatment, the treatment efficacy can only appear after 20-30 min stimulation[26], therefore, moxibustion intervention for 20 min was selected in this study to compare the therapeutic effects with other moxibustion intervention times. The therapeutic efficacies of moxibustion for 20 min, moxibustion for 40 min and moxibustion for 60 min on CIA rats were compared in this study, the results showed that moxibustion for 40 min had the best therapeutic efficacy for RA. Thus, duration of moxibustion treatment is the main factor affecting the therapeutic efficacy of moxibustion[27]. Zhang W, *et al* investigated the effect of different moxibustion durations on asthma rats. The effect of moxibustion for 15 min, 30 min, 60 min and 120 min were compared, which showed that the best treatment time was moxibustion for 60 min, indicating that long moxibustion treatment time ($>$ 60 min) is not necessary[9]. It takes time for the moxibustion to produce a therapeutic effect, however, it is noteworthy that moxibustion is not a simple linear relationship between the duration and the efficacy, which indicated that not the longer the moxibustion duration, the better the therapeutic effects. This study revealed that the best duration of moxibustion stimulation was 40 min in the treatment of CIA rats, indicating that the best moxibustion time is not same for different diseases.

This study showed that moxibustion at Shenshu (BL 23) and Zusanli (ST 36) could reduce the local inflammatory cell infiltration, synovial membrane cell proliferation and tissue necrosis in RA rats. Improvement of synovial membrane inflammation pathology and other indicators was most significant in moxibustion for 40 min group. In summary, the therapeutic efficacy of moxibustion treatment for RA rats was closely related to the moxibustion duration, therefore, we should pay attention to the duration of moxibustion in clinical practice. To understand the relationship between duration and efficacy of moxibustion has a very important significance in the clinical practice and experimental research.

This study also has some limitations. The first limitation is that only 3 time points of moxibustion duration were observed. We should increase the time point numbers of moxibustion duration in future research. The second limitation is that our study object is CIA rats, the results obtained may not be suitable for extrapolation to clinical patients, and whether moxibustion for 40 min in RA patients is the best needs to be further validated in clinical trials. Finally, the best duration of moxibustion evaluated in this study is for RA

rats, and the optimal moxibustion duration for other diseases remains to be explored. Further exploration of how the inflammatory cytokine levels of IL-1β and TNF-α are regulated by moxibustion and the therapeutic mechanism of moxibustion in RA is also worthy of our further study.

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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