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Influence of moxibustion on collagen-induced arthritis in mice

JIAN-QIAO FANG, ERI AOKI, AKIRA SETO, YING YU, TAKAKO KASAHARA and TADASHI HISAMITSU

Department of Physiology, School of Medicine, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo, 142-8555, Japan

ABSTRACT

The influence of moxibustion, a traditional Chinese medical treatment, on type II collagen-induced arthritis (CIA) was examined in DBA/1J mice in vivo. Mice were immunized intradermally twice at the 3-week interval with bovine type II collagen (C II). The main incidence of arthritis started about on day 30 and lasted to day 60 after the first immunization. Moxibustion with three different regimens, was applied at the acupoint equivalent to GV4 every other day. Moxibustion, from day 0 to day 30 after the first immunization, suppressed the onset and development of arthritis, as well as anti-collagen antibody level. Treatment with moxibustion, from the day 31 to day 60, also resulted in a significant inhibition of progression of arthritis and production of anti-C II antibody.

Thirdly we examined the influence of moxibustion on the established arthritis. Moxibustion given from day 61 to day 120, significantly but mildly decreased the anti-C II antibody level in diseased mice, while the bone erosion and joint destruction were not affected. These results indicate that moxibustion could prevent the incidence and attenuates the development of murine CIA.

Key words: Moxibustion, type II collagen, arthritis, DBA/1J mouse.

Introduction

Moxibustion, a traditional Chinese medical treatment, has been widely used, particularly in China, in the treatment of some immune related diseases (1, 2). Governer Vessle (GV) meridian described in Chinese acupuncture, located at the posterior midline of the back, is thought to possess the functions of invigorating the body resistance and eliminating cold-damp sensation (3). Mingmen (GV4) acupoint, one of the commonly-used points from GV meridian, has been applied in treating human immunological disorders and inducing immune modulation in animals (3-5). Moxibustion treatment, especially at the GV meridian, has been shown to treat human rheumatoid arthritis (hRA) with satisfactory results (6, 7). However, little is known about the mode of action of moxibustion on hRA.

Immunization with type II collagen (C II) is well known to be able to induce inflammatory polyarthritis in rats and susceptible strains of mice (8, 9). Although immune mechanisms that include both humoral and cellular immunity to C II have been implicated in the pathogenesis of the disease (10, 11), there is much evidence that anti-C II antibodies play an important role in the initiation of the disease (12, 13). Collagen-induced arthritis (CIA) in mice is characterized by a severe swelling of the paws associated with a massive infiltration of inflammatory cells into the joints, and the progression of the disease results in destruction of joints and severe deformities. Since CIA has both clinical and histological similarities to hRA (8, 9), it is widly used to understand the mechanisms of disease and to evaluate anti-arthritic agents.

In the present study, we used this experimental model and investigated the influence of moxibustion, applied directly and mainly at the acupoint equivalent to GV4 with three different regimens, on the incidence and development of arthritis in murine CIA.

Materials and Methods

Animals. Male DBA/1J mice, 7 weeks of age, were purchased from Nihon SLC Co., Ltd. (Hamamatsu, Japan). They were fed standard rodent chow and water ad libitum.

Induction of arthritis. C II (Collagen Research Center, Tokyo, Japan) isolated and purified from bovine articular cartilage was solubilized at 4_C in 0.01 M acetic acid at 4.0 mg/ml, after which the solution was emulsified in an equal volume of complete Freund's adjuvant (CFA, Difco Lab., Detroit, MI, USA) in an ice cold water bath. Each mice was immunized by an intradermal injection of 0.1 ml (200 _g C II) of the cold emulsion into the base of the tail. The booster immunization with the same dose of collagen emulsified in incomplete Freund's adjuvant (IFA, Difco Lab.) was given 21 days later.

Moxibustion application. Moxibustion was directly conducted with small pieces of moxa (Mugwort wool) cones. The moxa cone, weighing 2 mg, was placed on the skin surface of the acupoint and then ignited. Five moxa cones were used per treatment and this was repeated every other day. The point equivalent to GV4, located below the spinal process of the third lumber vertebra, was cauterized in all treatment groups. Treatment was given in three different periods: 1) started on the same day with the first immunization and stopped on day 30 (described as pre-moxibustion), 2) started on day 31 after the primary immunization and stopped on day 60 (described as post-moxibustion), and 3) started on day 61 after the first immunization and ceased on day 120 (described as delayed-moxibuston). In the delayed-moxibustion treatment, the points equivalent to GV9 (Zhiyang) located between the 7th and 8th thoracic processes and GV14 (Dazhui) located between the 7th cervical process and the 1st thoracic process, were applied alternatively with GV4.

Assessment of arthritis. The clinical symptoms of arthritis were evaluated with a visual scoring system, based on the degree of periarticular erythema, swelling and joint deformity. Mice were checked three times per week. Each lesion of the four paws was graded on a scale of 0 to 4 and scores for all four extremities were summed, with a maximum possible score of 16: 0 = normal; 1 = swelling and erythema of one digit; 2 = swelling and erythema of more than two digits or mild swelling and erythema of the entire paw; 3 = progressively more severe swelling and erythema of the paw; 4 = severe

swelling and erythema, lack of flexibility. The data were expressed as the percentage of arthritic limbs per group of mice and compared at various time points. The incidence and day of onset of arthritis were also recorded.

Measurement of anti-CII antibody. Blood samples were obtained from the mice by retro-orbital or cardiac puncture under ether anesthesia. Serum antibody levels to C II were measured using the commercially prepared Mouse IgG Anti-type II Collagen Antibody ELISA Kit containing standard, sufficient reagents and materials (Chondrex, Redmond, WA, USA). Briefly, type II collagen coated 96-well microtiter plates were firstly washed. Wells were blocked with 100 _l of blocking buffer for 1 h at room temperature, and then washed three times. Diluted solution (1:1000) of mouse test serum were added to each well (100 _l/well) in duplicate, and incubated for 2 h at room temperature. After washing, 100 _l of peroxidase-conjugated goat anti-mouse IgG was dispensed into each well. After incubation for 1 h, 100 _l of substrate, orth-phenylene diamine solution, was added to each well at a volume of 100 _l/well. The reaction was stopped by adding 50 _l of 2.5N sulfuric acid approximately 30 min later. The absorbance was read at 490 nm and 650 nm used as reference with an ELISA reader (ImmunoMini-NJ2300, Inter Med Corp., Tokyo, Japan). The titers were calculated by reference to a standard curve and expressed as U (unit) per ml of serum.

Histopathological examination. All the paws in immunized control, pre-moxibustion and post-moxibustion groups were removed post-mortem, fixed in 10% buffered formalin, then decalcified in EDTA. The paws were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic evaluation. The severity of arthritis in each was classified as mild, moderate, or severe based on the following criteria: mild = detectable inflammation, tissue damage, and cartilage loss; moderate = synovitis, and erosions present but joint architecture intact; severe = synovitis, extensive erosions, and joint architecture disrupted.

Radiological evaluation. To investigate the influence of moxibustion on the established arthritis, the diseased mice

were sacrificed on day 120 and arthritic limbs were amputated. Using a cabinet soft X-ray apparatus (SOFRON type SRO-M40, Soken Co., Ltd., Tokyo, Japan), radiography was performed using Fuji FR X-ray film under the following conditions: 45 cm distance; 30 kV tube voltage; 5 mA tube current; and 50 s irradiation time. Radiological assessment of each diseased paw was made in two stages: stage I = bone destruction in limited paw joints; stage II = bone destruction in all the joints of entire paw or with ankylosis.

Statistical analysis. Data presented are mean ± SE. The incidence of arthritis and radiological changes in different groups was compared using x2 analysis. Statistical analysis for the data of serum anti-CII antibody levels, anthritic index and pathological index was performed using ANOVA (analysis of variance) followed by Fisher's LSD test.

Results

Influence of moxibustion before or during the onset of arthritis

Regimen 1 and 2 were arranged to examine the influence of moxibustion on the incidence and development of arthritis. Mice were treated with direct moxibustion every other day for one month starting on the day of the first immunization with CII or 10 days after the second immunization. As shown in Table 1, 12 out of 14 mice immunized with type II collagen in non-treated control group developed arthritis (85.7%). The two groups of mice treated with moxibustion produced lower incidence of arthritis (42.9% in pre-moxibustion group and 57.1% in post-moxibustion group). There was a significant difference ($p<0.05$) in the incidence of arthritis between immunized control group and pre-moxibustion group. No significant difference could be detected among three groups in the time of onset of disease.

Although the incidence of arthritis in a limb seemed to be random and not to be related to whether or not other limbs in the same mouse were involved, the number of limbs involved has been shown to be another useful parameter for evaluating the activity of arthritis. By comparison with the immunized control mice (74.0% limbs affected), a statistically significant reduction in the number of arthritic limbs was observed in mice with moxibustion from day 0 to

30 (28.6% limbs affected) and moxibustion from day 31 to 60 (38.3% limbs affected) (Table 1).

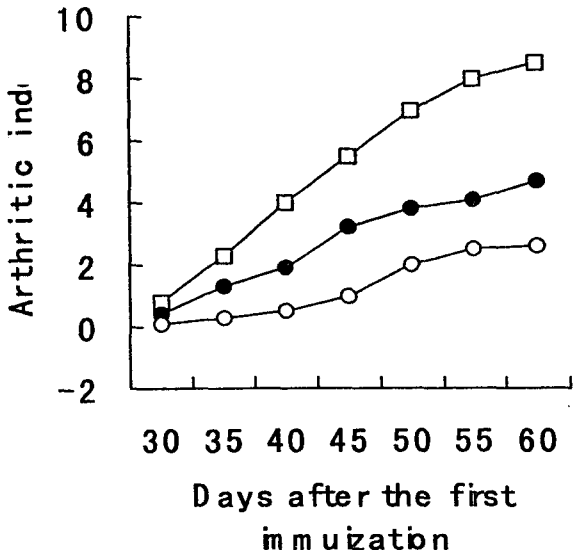
Table 1 Effect of moxibustion on clinical serverity of collagen arthritis.

Clinical serverity	Immunized control	Moxibustion (0-30d)	Moxibustion (31-60d)
Incidence of arthritis	12/14	6/14*	8/14
Incidence of arthritic Limbs	37/56	16/56**	22/56**
Day of onset	32.3±2.1	36.5±2.6	35.2±2.0
Arthritic index	8.4±1.4	2.7±1.0**	4.6±1.3*

Incidence of arthritis and arthritic limbs was observed in 14mice (56paws) each group. Data for day of onset and arthritic index checked on day 60 are the mean ± SE. * $p<0.05$, ** $p<0.01$ vs corresponding immuized group.

The average arthritic index for all mice in three groups are shown in Fig. 1. Disease severity increased progressively in immunized control mice from day 30 onward, and began to be significantly greater from day 35 compared to that in the pre-moxibustion group. From day 40 onward, the difference in arthritic index was very significant ($p<0.01$) between

Figure 1 Effect of moxibustion on the arthritic index of CIA mice.

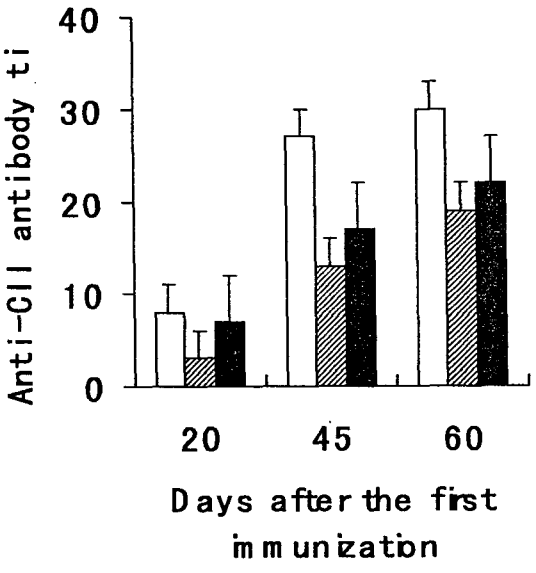


Data points are mean ± SE for 14 mice in each group. □:immunized control group; ○:moxibustion treatment from day 0 to day 30; ●:moxibustion treatment from day 31 to day 60. *$p<0.05$, **$p<0.01$:significant difference vs immunized control group

pre-moxibustion group vs. immunized control and significant ($p<0.05$) between post-moxibustion group vs. immunized control group. On day 60, the disease severity in mice treated with moxibustons fron day 0 to 30 or from day 31 to 60 reached a maximum scores of only 2.7±1.0 and 4.6±1.3 respectively, representing 68% and 45% decrease in clinical severity of the disease.

An important consequence of immunization with CII is a rapid rise in serum IgG level to CII. Therefore, we next examined the serum IgG anti-CII titers in mice. As shown in Fig. 2, the anti-collagen antibodies on day 20 just before the second immunization were 8.9 $\pm 2.2 \times 10^4$ U/ml in immunized control mice, $1.8 \pm 0.5 \times 10^4$ U/ml in pre-moxibustion mice, and $6.2 \pm 2.2 \times 10^4$ U/ml in post-moxibustion mice.

Figure2 Effect of moxibustion on anti-CII antibody levels of mice immunized with collagen.



Each bar represents mean ± SE for 14 mice of each group. □: immunized control mice; ▨: mice treated with moxibustion from day 0 to day 30; ■: mice treated with moxibustion from day 31 to day 60. *$p<0.05$ and **$p<0.01$ compared with immunized control group.

Significant reduction in anti-CII antibody level was obtained in pre-moxibustion group as compared with immunized control group. On the day 45 after the primary immunization, the control mice developed the high level of serum anti-CII antibody, which was much significantly ($p<0.01$) elevated than that in the mice treated with moxibustion from day 0 to 30 or from day 31 to 60. The anti-collagen antibody titers still kept high level in control mice on day 60, while that was lower in mice treated with direct moxibustion at different periods.

All paws of mice from each group observed in this part of experiment were examined microscopically for evidence of inflammatory cell infiltrates and cartilage and bone erosions typical of CIA. The histopathological assessments are summarized in Table 2. The data demonstrated the low frequency of pathological changes in paws from pre-moxibustion and post-moxibustion groups. The diseased mice in two moxibustion-treated groups also developed less severe lesions than the mice in collagen-immunized control

Table 2. Histopathological chamges in individual paws of mice immunized with collagen

Group	No. of paws assessed	No. of normal	Severity, No			Average index of histopatho-logic lesions
			Mild	Moderate	Severe	
Immunzed control	56	13 (30)	17 (30)	9 (16)	17 (30)	1.54±0.16
Moxibustion (0-30d)	56	23 (41)	20 (36)	5 (9)	8 (14)	0.96±0.14**
Moxibustion (31-60d)	56	19 (34)	20 (36)	7 (12)	10 (18)	1.14±0.15

14 mice immunized with CII collagen in each group were examined histopathologically. Data presented are numbers of paws and data in paretheses are percent. Average index of histopathologic lesions is calculated according to the different severity of arthritis and numbers of normal paws. **p<0.01 compared to immunized control group.

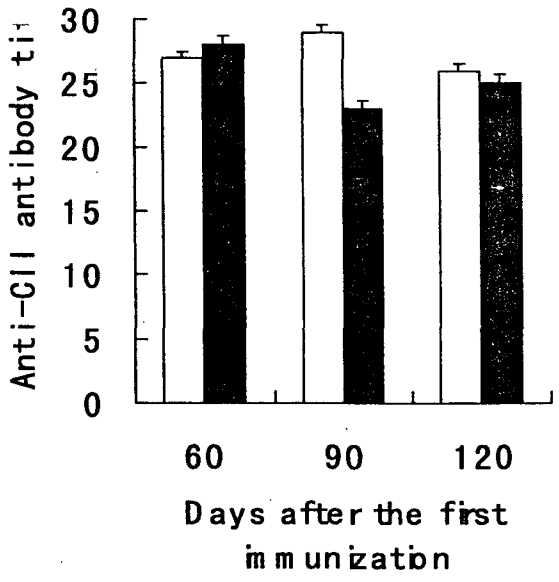
mice. To statistically compare the difference in pathological changes, the severity of arthritis: mild, moderate and severe, was graded as 1, 2, and 3, and normal as 0, and then calculated to yield an average pathological score in all paws from different groups. As shown in Table 2, the paws of pre-moxibustion mice showed very significantly less severe score than paws of immunized control mice, and no significant difference was obtained between post-moxibustion group and immunized control group. These microscopic findings correlate basically with the low incidence and severity of disease identified during clinical observation of the mice treated with moxibustion, especially given at the early stage.

Influence of moxibustion on the established arthritis

The regimen 3 was designed to observe the influence of moxibustion on the established disease of arthritis. On day 60 after the first immunization, the diseased mice were divided into two groups sharing the equal numbers of arthritic joints and indexes: one as arthritic control group without any treatment and the other as delayed -moxibustion group, to be treated with moxibustion for two months at acupoints GV4, GV9 and GV14.

The serum anti-CII antiboby titers were examined on days 60, 90 and 120 after the first immunization. As shown in Fig. 3, high levels of anti-C II antibody were detected in the mice of both groups at the start of experiment (d 60), and these levels were maintained throughout the experiment. However, treatment of mice with moxibustion presented a significant suppression of the antibody levels as compared to th the appropriate controls.

Fig. 3. Influence of moxibustion on anti-CII antibody level of arthritic mice.



Each bar represents mean ± SE for 6 mice in each group. □: arthritic control; ■: moxibustion treatment for arthritic mice from day 61 to day 120. *p<0.05 and **p<0.01: significant difference vs arthritic control group. #p<0.05 and ##p<0.01: significant difference vs corresponding mice on day 20.

All diseased paws in each group were examined radiologically to observe the chronic pathological change of joints. Table 3 shows the radiological stage in two groups 120 days after the first immunization. The incidence of radiological Stage II changes in arthritic non-treated control group was higher than that of delayed-moxibustion (61-120 d) group, however, statistical analysis showed no significant difference between the two groups.

Table 3. Comparison of radiological changes between arthritic control and moxibustion (61-120d) group

	Arthritic control	Moxibustion (61-120d)
Stage I	11 (47.8)	13 (59.1)
Stage II	12 (52.2)	9 (40.9)

Incidence of the radiological stage in the arthritic control mice and mice treated with moxibustion from day 61 to day 120. Data presented are numbers of paws and data in paretheses are percent. No significant difference was observed between two groups by using x2 analysis.

Discussion

Application of Moxibustion results in humoral or immune reactions (1, 2). It has been also suggested that moxibustion acts as a preventive means and strengthens physical resistance of the body, resulting in impediment of the development of disease (14). In China, there have been a number of clinical reports about the treatment of chronic arthritis with moxibustion (6, 7, 15), but the mechanisms of moxibustion on chronic arthritis are poorly understood.

This is the first report of the influence of moxibustion in CIA model, which is quitely similar to hRA. The present results clearly showed that the most effective protocol for the treatment of CIA was to apply moxibustion before the onset of disease. The late administration of moxibustion (31-60 d) during the onset of arthritis also inhibited the development of arthritis, but this treatment was weaker than that from day 0 to 30. In contrast, delayed treatment (61-120 d), besides slighly affecting the anti-C II antibody levels, did not extend the strong suppression of CIA activity. These results are interpretable that moxibustion might be a good prophylactic method in treating hRA. Peterman, et al. (16) reported that early injections of anti-TCR___ mAb completely prevented the development of arthritis in C II-immunized mice, but delayed treatment could not, or even exacerbated the arthritis. Administration of rmIL-10 to mice inhibited the development of CIA, and the most pronounced suppression was observed when IL-10 was given from day 0 to 21 (17). These data, together with ours, indicate that a period around the second immunization of C II is a critical point in the development of CIA.

The mechanism by which the early administration of moxibustion suppressed the incidence and development of CIA is not clear. It is reported that moxibustion inhibits the imflammatory reactions and decreases IL-1 concentration in rats with adjuvant arthritis (18, 19). Many reports has shown that treatment with moxibustion promoted the production of IL-2 in both patients with RA and arthritic animals (15, 19, 20). Kasahara, et al. (21) reported that pretreatment of mice with moxibustion modulated lipopolisacharide (LPS)-induced endogenous cytotoxic

factor and interferon production in serum. It is also observed that the moxibustion applied to the GV4 equivalent point in mice modulated delayed type hypersensitivity to picryl chloride (22). Taken together, there is a possibility that moxibustion has a modulatory effect on various aspects of T cell-mediated immune responses and resulted in attenuation of the severity of CIA.

However, the functions and network of cytokines in CIA are not well understood and influence of moxibustion on cytokines has not been widely investigated. Furthermore, moxibustion often possesses multiple or two-way effects (14) and various causative factors may be involved in the incidence and development of CIA (23). Therefore, the precise mechanisms that moxibustion inhibits the development of murine CIA is needed to be explained in many further studies.

Besides the autoimmune disorders, arthralgia is accepted to be another main suffering in RA patient. In animal texpements, moxibustion has been shown to accelerate the relief of arthralgia and maintain Substance P and leu-enkephalin at a higher level in the spinal cord (24, 25). Therefore, moxibustion can be a means in the treatment of arthritis, particularly applied at the early stage of the disease.

In summary, moxibustion treatment was capable of suppressing the incidence and development of collagen arthritis in mice when the treatment was started on the same day as the initial immunization or soon after the booster challenge. The other means of moxibustion used for chronic arthritis needs to be further studied.

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