Analgesic effect of different moxibustion durations in rheumatoid arthritis rats

Baozhu Zheng, Ling Hu, Xiaoge Song, Zijian Wu, Ronglin Cai, Lu He, Cheng Zhang, Qing Yu

OBJECTIVE: To observe the influence of different moxibustion durations on hypothalamic pro-opiomelanocortin (POMC) and prodynorphin (PDYN) mRNA expressions and plasma β-endorphin (β-EP) content in rheumatoid arthritis (RA) rats, to understand the mechanism of moxibustion analgesia and its dose-effect relationship.

METHODS: Twelve male Wistar rats were randomly selected from 48 male Wistar rats as a normal control group. The RA model was created by raising rats in a windy (blowing with electric fan), cold (6°C±2°C), and wet (80%-90% humidity) environment for 20 days, 12 h each day. This was followed by injection of Freund’s complete adjuvant (0.15 mL) into the ankle. Then, rats were randomly divided into a model group, moxibustion group I, and moxibustion group II, with 12 rats in each group. In moxibustion groups I and II, moxibustion was given at Shenshu (BL 23) and Zusanli (ST 36) for 20 and 40 min, respectively, once daily for 15 days. Hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content were determined.

RESULTS: Compared with the normal group, the pressure pain threshold decreased, while the hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content increased in the moxibustion groups (P<0.01). Compared with the model group, the pressure pain threshold, hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content in the moxibustion groups increased significantly (P<0.01). Compared the moxibustion group I, the pain threshold, hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content in moxibustion group II significantly increased (P<0.01).

CONCLUSION: Moxibustion has an analgesic effect and increases hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content in RA rats. The analgesic effect in moxibustion group II is better than that in moxibustion group I.

INTRODUCTION

Study indicates that the mechanisms of acupuncture analgesia are varied. The release of endogenous opioid peptide (EOP) plays a role in acupuncture analgesia, and the hypothalamus is the center of recent study for moxibustion analgesia. There is a relationship between analgesic effect and duration of acupuncture. Rheumatoid arthritis (RA) is currently studied via cytokines and inflammation. However, there is less study...
on EOP and the immunoregulatory action of moxibus-
tion on adjuvant arthritis rats. Moreover, there is no
study on the time-effect relationship of moxibus-
tion. Therefore, in this study, we explored if moxibus-
tion has an analgesic effect, whether EOP is involved in this
effect, and if the analgesia is related to length of moxi-
bustion.

MATERIALS AND METHODS

Experimental animals
Healthy male Wistar rats, SPF grade, aged 3-4 months,
weighing (200 ± 20) g, were supplied by Beijing Wei-
tong Lihua Experimental Animal Research Center
Rats were raised alone in a cage for 1 week, with a 12-h
light-dark cycle and free access to food and water. After
detection of analgesic threshold, 48 rats with a pressure
pain threshold of (250±25) g were used for the experi-
mental study. Twelve rats were randomly selected as the
normal group reference to Random number table and
were routinely raised. The other 36 rats were placed in-
to a modeling box for imitating a windy, cold, and wet
environment for 20 days. All procedures were ap-
proved by the Committee of Experimental Animal Ad-
ministration and Ethics and conformed to the ethics
guiding policy of the International Pain Research Asso-
ciation.

Experimental materials and instruments
Reagents and instruments used included: specially
made pure cigarette type moxa roll (Φ = 5 mm, Nan-
yang Wolong Han Medical Moxa Wool Factory, Henan,
China, 061205); Freund’s complete adjuvant (Sigma,
St. Louis., MO, USA, 10 mL, 068K8561); TRizol Re-
agent (Life Technologies, Carlsbad, CA, USA,
15596026); reverse transcription kits (Fermentas, Bei-
jing, China, 00106559); Polymerase Chain Reactio-
ne (PCR) kit (Fermentas, Beijing, China, 0077926); ELI-
SA kit (Boaoshen, Beijing, China, 760557); YLS-3
Electronic pressure pain detector (Equipment Station,
Shandong Provincial Academy of Medical Sciences,
China); and Biometra PCR instrument (TGradient 96,
Göttingen, Germany).

Model establishment
In reference to the literature,7 the RA rat model of
“wind, cold, damp, bi-syndrome type” conforms to a
combination of modern medicine with TCM disease
cause and pathogenesis. Each rat was placed into a
self-made modeling box with a fan in a high position
at a temperature of 6°C±2°C and relative humidity of
80%-90%, for 12 h per day (8:00-20:00), for 20 days.
On the 21st day, after light anesthesia with ether,
0.15 mL Freund’s complete adjuvant was injected sub-
cutaneously between the 2nd and 3rd toe webs of the
right foot. Rats were observed for 3 days, and within
24 h the right ankle showed acute inflammatory
swelling. Within 48 h, secondary general multi-arthritis
occurred, which manifested as red swelling or in-
flammatory nodes in the forelimbs or contra-lateral
limbs, and even the ear and tail, indicating successful
modeling.

Grouping and treatment
After successful modeling, model rats were randomly
divided into a model group, moxibus-tion group I, and
moxibus-tion group II, with 12 rats in each group.
The normal group and the model group were not treat-
ed. Rats in moxibus-tion group I and moxibus-tion

group II began treatment 3 days after injection of
Freund’s complete adjuvant. The specially made pure
moxa roll was used and suspending moxibus-tion was
given 2-3 cm over Shenshu (BL 23) and Zusanli (ST
36) with both points alternated. Points of the same
name on both sides were used each day, 20 min/session
daily for moxibus-tion group I and 40 min/session
daily for moxibus-tion group II. The therapeutic course
each group was 15 days.

Selection and location of acupoints
In reference to the literature,7 Shenshu (BL 23) and Zu-
sanli (ST 36) were selected. The points were located in
reference to a standard point atlas for the rat and per-
sonification control method in the teaching materials,
the Experimental Acupuncture & Moxibus-tion Sci-
ence in “the tenth 5-years” national plan.

Detection of pressure pain threshold
In reference to the literature,7 the pressure pain thresh-
old (g) was detected with an electronic pressure pain
detector. The pain threshold was detected for the same
length of time in the same location before modeling. 3
days after injection of Freund’s complete adjuvant (the
23rd day of the experiment), and 15 days after treat-
ment (the 38th day of the experiment), three times
each session with an interval of 20 min between ses-
sions. The mean value was used as the pressure pain
threshold.

Determination of hypothalamic POMC and PDYN
mRNA expressions
After treatment for 15 days, the rats in each group
were sacrificed under anesthesia and the hypothalamus
was removed, placed on ice, and then stored in liquid
nitrogen until detection.
About 100 mg hypothalamic tissue for each rat was
added to 1 mL preliminary cooling TRizol and fully
ground in a tissue grinder. Then, total RNA was ex-
tacted according to the manufacturer’s protocol. 5 μL
RNA was added to 495 μL DEPC-H₂O, and concen-
tration and purity of RNA were determined with a
spectrophotometer. RNA with a concentration 4-fold
of A260, and a purity (A260/A280) between 1.8 and
2.0, was kept.
PCR primers were designed by Primer Premier 5.0 software and synthesized by Shanghai Shenggong Technic Co. Ltd. (Shanghai, China). The following are sequences of β-actin, POMC, and PDYN primers: β-actin: Forward 5′-GAAATCGTGCCGTACATTTAGG-3′, Reverse 5′-CAGTCACACTTCTATGAGGATTAG-3′, product length 242 bp; POMC: Forward 5′-TAGGATCCCAGGAATGACGCTCAGT-3′, Reverse 5′-ATAAGGCTGAAGCCGTACAGGGG-3′, product length 487 bp; PDYN: Forward 5′-AGACGCGGTTTCTAC-3′, Reverse 5′-ATGTGCACCAGATTTCTAC-3′, product length 669 bp. For the real-time reaction, the following procedure was used. In a 0.2 mL eppendorf tube, 8 μL total RNA, 1 μL 10 μmol/L Oligo (dT), and 3 μL diethylpyrocarbonate (DEPC)-H₂O were added, mixed lightly and centrifuged at 4°C, 3000 xg, for 5 min. The tube was heated in the PCR instrument at 65°C for 5 min, and immediately placed in an ice bath for 3 min. 4.0 μL 5× Moloney Murine Leukemia Virus (M-MLV) Buffer, 2.0 μL M-MLV were added to the tube. After mixture and centrifugation at 4°C, 3000 xg, for 5 min. the following manipulations were made on the PCR instrument: 42°C for 60 min, 70°C for 5 min.

The 25 μL PCR reaction system included: 5 μL template, 2.5 μL 10× Taq Buffer, 1.5 μL 25 mmol/L MgCl₂, 0.5 μL 10 mmol/L deoxy-ribonucleoside triphosphate (dNTP), 0.5 μL upstream primer, 0.5 μL downstream primer, 1 μL Taq enzyme and 13.5 μL DEPC-H₂O. After a preliminary test, β-actin reaction conditions were: 94°C 30 s, 55°C 30 s, and 72°C 30 s, for 35 cycles. POMC reaction conditions were: 94°C 30 s, 53°C 30 s, and 72°C 30 s, for 35 cycles, PDYN reaction conditions were: 94°C 30 s, 55°C 30 s, and 72°C 30 s, for 35 cycles. For detection of the target gene, 10 μL PCR reaction solution was added to 2 μL 6× loading buffer and mixed. 5 μL marker and 8 μL of the mixed solution were added in order into 1.5% agar gel wells for electrophoresis at 120 V for 40 min, to fully separate the products and observe the size of target DNA. For semi-quantitative analysis, the gel for electrophoresis at 120 V for 40 min, to fully separate the products and observe the size of target DNA. For semi-quantitative analysis of PCR products, the gel imaging system Quantity One was used. In a 0.2 mL eppendorf tube, 8 μL total RNA, 1 μL 10 μmol/L Oligo (dT), and 3 μL diethylpyrocarbonate (DEPC)-H₂O were added, mixed lightly and centrifuged at 4°C, 3000 xg, for 5 min. After, 1 mL plasma was poured into a digitaliform tube with a thin membrane, sealed, and kept at ~70°C until use.

**Statistical methods**

Data are expressed as mean±standard deviation ( ¯x ±s). The comparison between the multiple sample mean using single factor analysis of variance, multiple pairwise comparisons between the sample mean using multiple comparison. Comparison between two groups was done with independent sample t-test and correlation analysis. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) software was used, and P<0.05 was used as a standard of statistical difference.

**RESULTS**

**Comparison of right foot pain thresholds among groups (Table 1)**

Table 1 shows that there were no significant differences in the pressure pain threshold of the right foot before modeling among the groups. There was acute inflammation in the right sole of the foot and ankle 3 days after injection of Freund’s complete adjuvant, and the pressure pain threshold in the model group, moxibustion group I, and moxibustion group II significantly decreased (P<0.01). After treatment for 15 days, compared with the model group, moxibustion significantly increased the pressure pain threshold of the right foot (P<0.01), almost reaching the level of the normal group. There was a significant difference in the pressure pain threshold between moxibustion group II and moxibustion group I (P<0.01), suggesting that moxibustion group II is superior to moxibustion group I in the improvement of pain.

**Hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content**

Compared with the normal group, hypothalamic

| Table 1 Comparison of right foot pain threshold among groups ( ¯x ±s) |
|----------------|------|----------------|----------------|
| Group          | n    | Before modeling | After modeling |
| Normal         | 12   | 254±10          | 270±19         |
| Model          | 12   | 267±18          | 131±25<sup>b</sup> |
| Moxibustion I  | 12   | 258±19          | 141±25         |
| Moxibustion II | 12   | 252±17          | 140±8          |

Notes: the normal group and the model group were not treated. 20 min/session daily for moxibustion group I and 40 min/session daily for moxibustion group II. The therapeutic course in each group was 15 days. Compared with the normal group, <sup>b</sup>P<0.01; compared with the model group, <sup>c</sup>P<0.01; compared with moxibustion group I, <sup>d</sup>P<0.01.

**Determination of plasma β-EP content with ELISA**

Under anesthesia, 3-4 mL blood in each rat was taken from the abdominal aorta and poured into a tube with anticoagulant. Blood was mixed and incubated for 20 min, and then centrifuged at 4°C, 3000 xg, for 5 min. After, 1 mL plasma was poured into a digitaliform tube with a thin membrane, sealed, and kept at ~70°C until use.

**Hypothalamic POMC and PDYN mRNA expression levels and plasma β-EP content**

Compared with the normal group, hypothalamic
POMC and PDYN mRNA expression levels significantly increased in the model group ($P < 0.01$). Compared with the model group, moxibustion could significantly increase hypothalamic POMC and PDYN mRNA expression levels ($P < 0.01$). There were also significant differences between moxibustion group I and moxibustion group II ($P < 0.01$). The effect of moxibustion group II was superior to that of moxibustion group I in improving hypothalamic POMC and PDYN mRNA expression levels (Figure 1). Compared with the normal group, plasma β-EP content in the model group increased ($P < 0.01$). Compared with the model group, moxibustion induced significantly higher plasma β-EP content ($P < 0.01$), with a significant difference between moxibustion group I and moxibustion group II ($P < 0.01$). Moxibustion group II had a superior effect to that of moxibustion group I in increasing plasma β-EP content (Table 2).

### DISCUSSION

In this experiment, we used RA rats as a chronic inflammation model, and investigated the therapeutic effect of moxibustion on the analgesic ability of the endogenous opioid system. The results showed that the analgesic effect of moxibustion was positive, and moxibustion had a superior effect to that of moxibustion group II ($P < 0.01$). The therapeutic course in each group was 15 days. POMC: pro-opiomelanocortin; PDYN: prodynorphin; β-EP: β-endorphin. Compared with normal control group, $P < 0.01$; compared with model group, $P < 0.01$; compared with moxibustion group I, $P < 0.01$.

### Table 2 Comparison of hypothalamic POMC and PDYN mRNA and plasma β-EP levels among groups ( ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>POMC mRNA</th>
<th>PDYN mRNA</th>
<th>Plasma β-EP (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>12</td>
<td>0.45±0.09</td>
<td>1.04±0.10</td>
<td>60.88±3.78</td>
</tr>
<tr>
<td>Model</td>
<td>12</td>
<td>1.05±0.24</td>
<td>1.84±0.14</td>
<td>82.79±6.71</td>
</tr>
<tr>
<td>Moxibustion I</td>
<td>12</td>
<td>1.44±0.15</td>
<td>2.53±0.76</td>
<td>118.29±9.42</td>
</tr>
<tr>
<td>Moxibustion II</td>
<td>12</td>
<td>1.96±0.64</td>
<td>3.31±0.12</td>
<td>161.53±17.08</td>
</tr>
</tbody>
</table>

Notes: the normal group and the model group were not treated. 20 min/session daily for moxibustion group I and 40 min/session daily for moxibustion group II. The therapeutic course in each group was 15 days. POMC: pro-opiomelanocortin; PDYN: prodynorphin; β-EP: β-endorphin. Compared with normal control group, $P < 0.01$; compared with model group, $P < 0.01$; compared with moxibustion group I, $P < 0.01$.

### Figure 1 Comparison of hypothalamic POMC mRNA, PDYN mRNA expression among rats of the groups

A: β-actin PCR results (product length 242 bp); B: POMC PCR results (product length 487 bp); C: PDYN PCR results (product length 669 bp). POMC: pro-opiomelanocortin; PDYN: prodynorphin; PCR: Polymerase Chain Reactione. M: DNA Marker (The molecular weight 100, 250, 500, 750, 1000, 2000bp); 1: Normal control; 2: Model group; 3: Moxibustion group I; 4: Moxibustion group II.

### Table 3 Correlation coefficient matrix between each index (r)

<table>
<thead>
<tr>
<th>Index</th>
<th>Pressure pain threshold</th>
<th>POMC mRNA</th>
<th>PDYN mRNA</th>
<th>β-EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure pain threshold</td>
<td>1.000</td>
<td>0.812</td>
<td>0.844</td>
<td>0.778</td>
</tr>
<tr>
<td>POMC mRNA</td>
<td>0.812</td>
<td>1.000</td>
<td>0.905</td>
<td>0.883</td>
</tr>
<tr>
<td>PDYN mRNA</td>
<td>0.844</td>
<td>0.905</td>
<td>1.000</td>
<td>0.881</td>
</tr>
<tr>
<td>β-EP</td>
<td>0.778</td>
<td>0.883</td>
<td>0.881</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Notes: the normal group and the model group were not treated. 20 min/session daily for moxibustion group I and 40 min/session daily for moxibustion group II. The therapeutic course in each group was 15 days. POMC: pro-opiomelanocortin; PDYN: prodynorphin; β-EP: β-endorphin. Correlation is significant at the 0.01 level (two-tailed); Correlation is significant at the 0.05 level (two-tailed).
flammatory pain model to examine the analgesic effect of moxibustion. Pain threshold is an index that is widely used for judgment of analgesic effect. Pain threshold is the tolerant threshold of an organism to pain, which can indicate the time needed for the pain response induced by the same stimulation intensity. After model establishment in this experiment, the tolerance of rats to pain decreased and pressure pain threshold significantly decreased, indicating that the rats in the groups were sensitive to pain. After moxibustion, the pressure pain threshold of the sole of the right foot increased, returning almost to the normal level, suggesting that moxibustion has a significant analgesic effect. Many studies indicate that the release of EOP plays a role in acupuncture analgesia. \( \beta \)-EP and dynorphins (Dyn) are main members of EOP, which have strong morphine-like activity and analgesic effects and are widely involved in the regulation of pain. POMC is a precursor protein of \( \beta \)-EP, and PDYN is a common precursor protein of Dyn-A, Dyn-B, \( \alpha \)-Neo-Endorphin, and \( \beta \)-Neo-Endorphin. Increases in POMC and PDYN mRNA expression levels represent levels of gene expression of endorphin and dynorphin systems, respectively. Studies indicate that inflammatory pain can stimulate the release of EOP-nergic neurons, increasing the activity of neurons in the main nuclei in the descending inhibiting system, and inhibiting transmission of pain sense. However, the EOP released is insufficient to antagonize inflammatory pain. Nevertheless, acupuncture and moxibustion can increase the release of EOP from various levels of the central nervous system, to better antagonize inflammatory pain. This experiment also showed that moxibustion could further increase hypothalamic POMC and PDYN mRNA expression levels and plasma \( \beta \)-EP content in RA rats. This can antagonize inflammatory pain from the central and peripheral levels, increasing the analgesic intensity. Correlative analysis indicated that the analgesic effect of moxibustion was correlated with hypothalamic POMC mRNA, PDYN mRNA expression levels and plasma \( \beta \)-EP content, and hypothalamic POMC mRNA was positively correlated with plasma \( \beta \)-EP, indicating that increases in the precursor protein of \( \beta \)-EP possibly lead to increases in \( \beta \)-EP released into peripheral blood.

The generation and development of acupuncture and moxibustion effects need time, and the relationship between effect and time of acupuncture is called the time-effect relationship. The generation and development of the acupuncture time-effect relationship includes three stages: the latency period, the effect stage, and the after later-effect stage. Understanding the time-effect relationship of acupuncture and moxibustion is of important significance for clinical and experimental acupuncture and moxibustion studies. The time factor includes the time of each moxibustion treatment, the time interval between two treatments, and total treatment time. Therefore, the time factor is a main influence in stimulation amount and effects of acupuncture and moxibustion. In this study, it was found that the analgesic effect of moxibustion for 40 min is superior to that of moxibustion for 20 min, which is due to the latency period of moxibustion’s analgesic effect of about 20-30 min. Therefore, only after the latency period of 20-30 min can the effect of acupuncture and moxibustion be observed. However, the relationship between time and effect of acupuncture and moxibustion is not only a linear relationship. When the organism produces tolerance to pain, the best effect of acupuncture and moxibustion will be influenced. Therefore, finding the “platform stage” in the time-effect curve and determining each therapeutic course and time interval between courses still requires further study.

REFERENCES


