


The preemptive analgesia of pre-electroacupuncture in rats with formalin-induced acute inflammatory pain

Molecular Pain
Volume 15: 1–9
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DOI: 10.1177/1744806919866529
journals.sagepub.com/home/mpx


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Abstract

Background: Electroacupuncture has been elicited to effectively alleviate the pain sensation. Preemptive analgesic effect of pre-electroacupuncture has also been suggested in recent studies, while the underlying analgesic mechanism of pre-electroacupuncture requires further investigation. This study aimed to explore the preemptive analgesia of pre-electroacupuncture in formalin-induced acute inflammatory pain model.

Methods: Forty rats were randomly divided into control, model, pre-electroacupuncture, and post-electroacupuncture group. Inflammatory pain model was induced via injecting 50 μ l 5% formalin into the plantar surface of right hind paw, while the equal volume of saline injection in the control group. Rats in the pre-electroacupuncture group were treated with electroacupuncture at ipsilateral Zusanli (ST36) and Weizhong (BL40) acupoints (2 Hz, 1 mA) for 30 min before formalin injection, while received the same electroacupuncture treatment immediately after formalin injection in the post-electroacupuncture group. Flinching number and licking time were recorded during 60 min after formalin injection. Immunofluorescence and Western blot were used to detect the expression of ionized calcium binding adapter molecule 1 (Iba1) and c-fos in spinal cord. Moreover, enzyme-linked immunosorbent assay was applied to measure the secretion of IL-6, IFN- γ , IL-4, substance P, and calcitonin gene-related peptide in spinal cord.

Results: Paw flinching and licking were obviously induced by formalin injection. Iba1, c-fos, proinflammatory cytokines (IL-6 and IFN- γ), and pain neurotransmitters (substance P and calcitonin gene-related peptide) were dramatically increased in the L4-5 spinal cord after formalin injection, while anti-inflammatory cytokine IL-4 was decreased. Pre-electroacupuncture and post-electroacupuncture administration significantly attenuated formalin-induced nociceptive effects, spinal microglia and neurons activation, proinflammatory cytokines and pain neurotransmitters upregulation, and upregulated the anti-inflammatory cytokine. Furthermore, these effects of pre-electroacupuncture were more significant than that of post-electroacupuncture.

Conclusions: This study illustrates the potential therapeutic effect of pre-electroacupuncture against acute inflammatory pain and reveals the mechanism underlying pre-electroacupuncture mediated analgesia, thus providing a novel preemptive analgesic treatment.

Keywords

Preemptive analgesia, pre-electroacupuncture, formalin-induced acute inflammatory pain, Iba1, c-fos, inflammatory cytokines, neurotransmitters

Date Received: 19 February 2019; revised: 14 May 2019; accepted: 4 June 2019

Introduction

Preemptive analgesia is an intervention that is conducted before initiating painful stimuli, which may alleviate or inhibit subsequent pain.¹ Due to the “preventative” effect on nociceptive amplification, preemptive analgesia possesses the potential to be more effective than the similar analgesic therapy initiated after injury, as it

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alleviates following postoperative pain and forestalls the development of persistent pain by suppressing the altered central sensory processing.^{2,3} Notably, emerging studies confirmed that preemptive analgesia treatment prior to surgery can decrease postoperative analgesics requirements and improve the postoperative pain assessment and satisfaction scores.^{4,5} As is known, the present preemptive analgesic approaches are mainly consisted of pharmacological strategy, such as non-steroidal anti-inflammatory drugs and cyclo-oxygenase-2 inhibitors, local anesthetics (e.g., epidural), opioids, antidepressants, and anticonvulsants.⁶ Nevertheless, patients with analgesic needs may prefer non-pharmacological options because preemptive analgesia also comes with considerable risks for adverse effects. So, it is urgent for us to explore a valuable non-pharmacological preemptive analgesic approach. Electroacupuncture (EA), a combination of traditional acupuncture and modern medicine that apply various levels of stimulating currents to acupoints through acupuncture needles, represents a potentially useful adjunct to available pain relief strategies.^{7–9} Although the exact mechanisms in EA analgesia are unclear, research works have been well illustrated that the analgesic effect of EA is mainly associated with its function in regulating endogenous opioid peptides, cytokines, and other central neurotransmitter release.^{10–13}

Interestingly, there is an evidence that pre-EA exerts a satisfactory analgesic effect against postincision pain in rats.^{14–16} Although there was a lack of clarification, these results showed us a novel strategy for non-pharmacological preemptive analgesic option. Thus, one purpose of our study is to confirm the preemptive analgesic effect of EA.

It is well established that the crosstalk between microglia and sensory neurons plays an indispensable role in the maintenance of persistent pain. During this pathological state, the microglial proinflammatory cytokines and the neurotransmitters released from nociceptive neurons not only drive the pain sensitization but also serve as mediators in the communication between microglia and sensory neurons.^{17–19} Furthermore, recent developments in the research of EA have heightened the role of microglia in EA analgesia, which inhibited the activation of microglia and improved the neuroinflammation.²⁰

This study aimed to confirm the analgesic effect of pre-EA on inflammatory pain induced by formalin. In addition, this study also aimed to observe the inhibitory effect of pre-EA on the activation of microglia and nociceptive neurons in spinal cord in order to evaluate the preemptive analgesic ability of EA and explore its possible mechanisms.

Materials and methods

Animals

Forty male Sprague Dawley rats weighed 220 to 250 g were obtained from Experimental Animal Center of Southwest Medical University (Luzhou, China). Four rats were housed per cage at a temperature of 22°C to 24°C and with a 12-h reversed light-dark cycle. All animals were provided free access to food and water. All animal experimental procedures and protocols were approved by Animal Care and Ethics Committee of the Affiliated Traditional Chinese Medicine Hospital of Southwest Medical University, and the study was performed in accordance with the guidelines published in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85–23, revised 1996). The numbers of animals used and their suffering were minimized by all efforts.

Formalin test

Rats were moved to the test room at least 30 min before starting the experiment. The animals were randomly divided into four groups: control group, model group, pre-EA group, and post-EA group. In this study, rats were first bound for 30 min. In model group, the rats were subcutaneously injected with 50 μ l of 5% formalin into the plantar surface of the right hind paw, while with the same volume of saline solution (0.9%) in control group. In pre-EA group, EA treatment was applied for 30 min before formalin injection, and in post-EA group, EA was conducted for 30 min immediately after formalin injection. Flinching number and licking time of pain-related behaviors were recorded continuously every 5 min immediately after formalin injection up to 60 min post-injection. At 2 h after formalin injection, animals were sacrificed for the followed experiments: immunofluorescence, Western blot, and enzyme-linked immunosorbent assay (ELISA).

EA treatment

For EA treatment, the rats were bound without anesthesia. A pair of stainless steel acupuncture needles (diameter 0.35 mm, length 40 mm, Huatuo; Suzhou Medical Appliance Manufacturing, Jiangsu, China) was inserted 2 mm deep into the rats' ipsilateral Zusanli (ST36) and Weizhong (BL40) acupoints. Zusanli acupoint (ST36) is located 5 mm beneath the capitulum fibulae and lateral posterior to the knee-joint,⁷ and Weizhong acupoint (BL40) is located on the medial aspect of knee, at the midpoint of the joint between os femoris and tibia²¹ (Figure 5). EA stimulation was delivered using an electrical stimulation device (6805-D; Kangmai Medical Technology, Co., Ltd., Guangzhou, China) at 2 Hz

and 1 mA, and a slight shrinkage in the muscle was observed. In pre-EA group, EA was performed for 30 min before formalin injection, while conducted after formalin injection for 30 min in post-EA group.

Sample preparation and immunofluorescence

Rats were deeply anesthetized with 10% chloral hydrate (3 ml/kg) and were perfused intracardially with 0.9% saline followed by 4% paraformaldehyde. The L4-5 spinal cord segments were dissected out and post-fixed in the same fixative for 4 h at 4°C. Tissue was then maintained in 30% sucrose in 0.1 M phosphate-buffered saline (PBS) at 4°C overnight. Dissected tissue was embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) frozen on dry ice. The transverse spinal cord was cut at a thickness of 20 µm in a cryostat (Microm HM 525). For immunofluorescence analysis, the sections were fixed for 10 min with the same fixative, and then were washed in 0.01 M PBS three times (5 min each). After blocking in 10% normal goat serum in 0.01 M PBS with 0.3% Triton X-100 for 1 h at room temperature, the sections were incubated overnight at 4°C with rabbit anti-ionized calcium binding adapter molecule 1 (Iba1) polyclonal antibody (1:500; Abcam) and rabbit anti-c-fos polyclonal antibody (1:250, Proteintech) dissolved in 10% normal goat serum. After rinsing in 0.01 M PBS three times 5 min each, the sections were incubated with fluorescein isothiocyanate-conjugated secondary antibody (1:600, Abcam) dissolved in 10% normal goat serum for 1 h at room temperature in the dark. The sections were rinsed again and were subsequently stained with diaminido-2-phenylindole dihydrochloride (1:1,000; Solarbio Science and Technology Ltd., Beijing, China). Finally, the sections were rinsed with PBS, and the coverslips were mounted with antifade solution (Solarbio Science and Technology Ltd., Beijing, China). Fluorescence images of Iba1 and c-fos were determined with a digital upright microscope (Olympus BX53, Anbiping Meditech Ltd., Guangzhou, China).

Western blot analysis

To examine protein expression of Iba1 and c-fos, the rats were deeply anesthetized as previously described at 2 h after formalin injection. The L4-5 segments were quickly excised and stored at -80°C for further analysis. The tissue was subjected to homogenize in the radioimmuno-precipitation assay (RIPA, Beyotime, Shanghai, China). The target proteins were harvested from the obtained supernatant after centrifugation (12,000 r/min for 30 min at 4°C). The total protein concentration was determined by using the bicinchoninic acid assay method (Beyotime, Beijing, China). In sum, 20 µg of

protein was loaded in each lane and was separated by using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime, Shanghai, China), followed by transferring it onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% skim milk for 2 h at room temperature, the membranes were incubated with rabbit anti-c-fos (1:250, Proteintech), rabbit anti Iba1 (1:500, Abcam), and anti-β-actin (1:2000, Cell Signaling, MA, USA) for loading control overnight at 4°C. After three washes with Tris-buffered saline with Tween 20 (TBST), these blots were subsequently incubated with horseradish peroxidase anti-rabbit secondary antibody (1:2000, ZSGB-Bio, Origene, Beijing, China) for 2 h at room temperature. Images were developed by enhanced chemiluminescence plus reagent, and Gel-Pol analyzer was used to analyze the intensity of bands.

ELISA for substance P, calcitonin gene-related peptide, IFN-γ, IL-6, and IL-4

After the EA experiments were conducted, the supernatants of L4-5 were collected. An ELISA was conducted by using these supernatants. The levels of substance P (SP), calcitonin gene-related peptide (CGRP), IFN-γ, IL-6, and IL-4 in the supernatants were detected using an ELISA kit (Shanghai bridge club, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

The obtained data in this study were expressed as the mean ± standard deviation (SD). SPSS 22.0 software was used for the statistical analysis. A statistical evaluation of the data was analyzed using a one-way analysis of variance ($P < 0.05$), followed by post hoc comparisons using the least significant difference or Dunnett's post hoc test. $P < 0.05$ was considered statistically significant.

Results

EA attenuated formalin-induced pain-related behavior in rats

Formalin injection into the rat's right hind paw induced a significant pain-related behavior characterized by flinching and licking the injected paw, as shown in Figure 1. To further validate the role of EA, EA was conducted at ST36 and BL40 for 30 min before and after the formalin injection. Both pre-EA and post-EA significantly reduced the paw flinching number and lifting time in the two phases following the injection, compared with the Model group ($P < 0.05$). Moreover, the administration of pre-EA dramatically reduced the

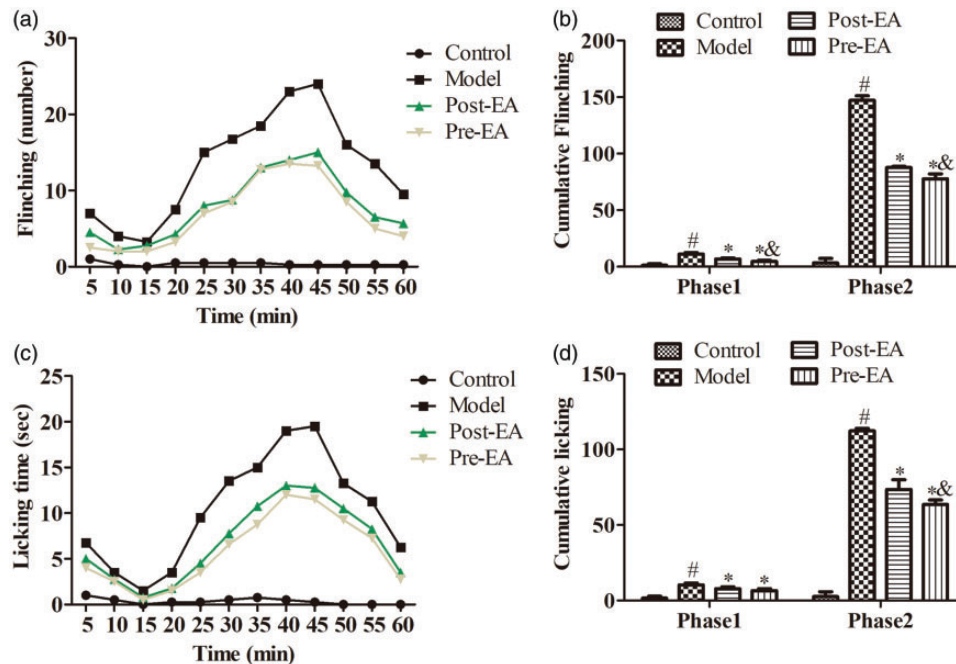


Figure 1. Effects of EA on formalin-induced pain-related behavior ($n = 16$). Pain-related behaviors paw flinching number (a) and licking time (c), cumulative flinching (b) and cumulative licking (d) were recorded for 60 min immediately after formalin injection. Paw flinching and licking were observed immediately after formalin injection, and that behaviors were characterized by two distinct phases, respectively. EA dramatically inhibited the increased cumulative flinching and cumulative licking. Data were expressed as the Mean \pm SD. # $P < 0.05$, compared with control group; * $P < 0.05$ compared with model group; & $P < 0.05$ compared with post-EA group. EA: electroacupuncture.

flinching number in the two phases and licking time in phase 2 in comparison to post-EA, respectively ($P < 0.05$), while there was no significant difference in phase 1 of licking time between pre-EA and post-EA group ($P > 0.05$).

EA inhibited spinal microglia and neurons activation in the L4-5 spinal cord of formalin-treated rats

To determine whether glial-neuron activation was associated with formalin-induced acute inflammatory pain and the effect of EA treatment on glial-neuron activation, we measured the protein expression of Iba1 and c-fos at the L4-5 segments by immunofluorescence and Western blot. As is shown in Figure 2(c) and (d), microglia and neurons activation, represented by the Iba1 and c-fos fluorescence density, was increased in formalin-injected rats compared to pre-EA, post-EA, and control rats. Pre-EA or post-EA treatment for 30 min before or after formalin injection resulted in a remarkable decrease in the fluorescence density of Iba1 and c-fos compared to model rats. Western blot analysis validated that both treatment with EA significantly decreased microglia and neurons activation as reflected by the Iba1 and c-fos protein expression in the L4-5 spinal cord sections ($P < 0.05$) (Figure 2(a) and (b)). Furthermore, there was a significant change between pre-EA and post-EA group

in Iba1 and c-fos protein expression, respectively ($P < 0.05$).

EA altered formalin-induced production of cytokines in the L4-5 spinal cord

To further detect the effect of EA treatment on the spinal inflammatory response after formalin injection, ELISA was performed to measure the levels of inflammatory cytokines. Pre-EA or post-EA was conducted for 30 min before or after formalin injection, and the L4-5 spinal cord segments were separated at the 2 h after formalin injection or saline. ELISA analysis demonstrated that the levels of IL-6 and IFN- γ in formalin-injected rats were significantly increased compared with pre-EA, post-EA, and control rats, while the level of IL-4 was remarkably decreased ($P < 0.05$) (Figure 3). Pre-EA before formalin injection for 30 min significantly inhibited the increased levels of IL-6 and IFN- γ in the spinal cord and upregulated the level of IL-4 in comparison to post-EA ($P < 0.05$).

EA altered formalin-induced production of neurotransmitters in the L4-5 spinal cord

To further detect the effect of EA treatment on the spinal pain neurotransmitters after formalin injection, ELISA was also performed to measure the levels of SP

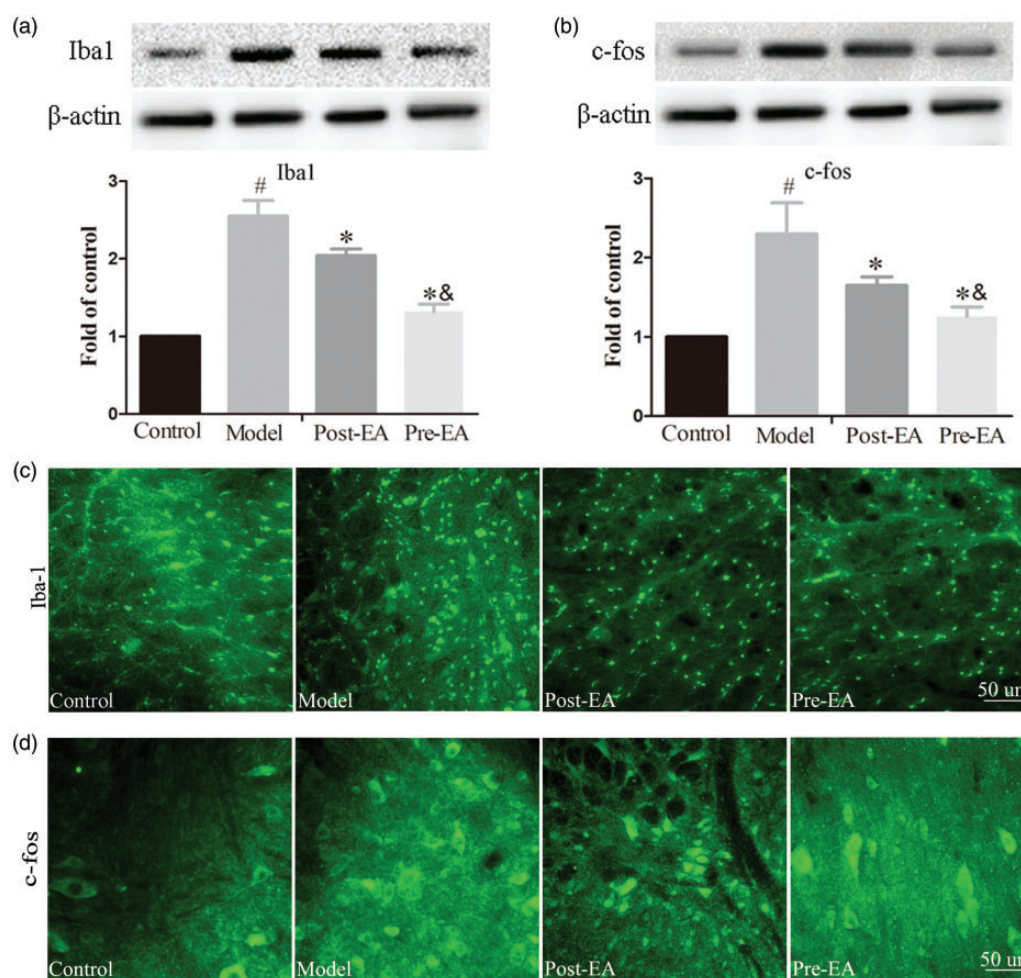


Figure 2. Effects of EA on the increased expression of Iba1 and c-fos in the L4–L5 spinal cord induced by formalin injection ($n = 12$). (a) Representative bands and quantification of the Western blot analysis revealed that EA treatment dramatically reduced the increased Iba1 protein level. (b) Representative bands and quantification of Western blot analysis revealed that EA significantly reduced the increased c-fos protein level. (c) Representative immunostaining pictures revealed the change in Iba1 expression in the L4–L5 spinal cord in control, model, pre-EA, and post-EA group. (d) Representative immunostaining images revealed the change in c-fos expression in the L4–L5 spinal cord in control, model, pre-EA, and post-EA group. # $P < 0.05$, compared with control group; * $P < 0.05$, compared with model group, & $P < 0.05$, compared with post-EA group; scale bars = 50 μm . EA: electroacupuncture; Iba1: ionized calcium binding adapter molecule 1.

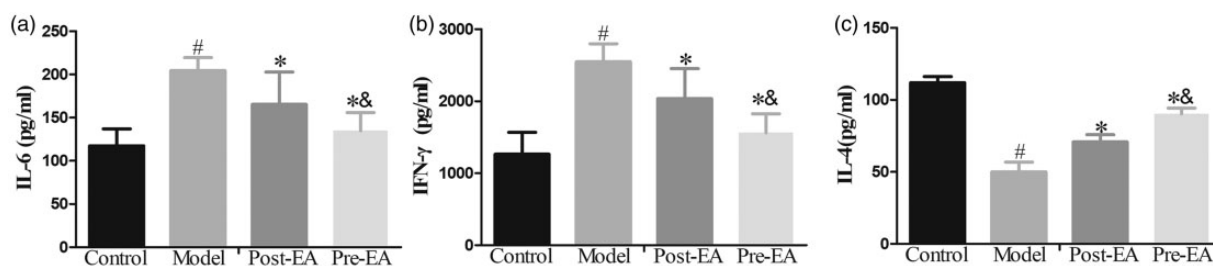


Figure 3. Effects of EA on the levels of IL-6, IFN- γ , and IL-4 in the L4–L5 spinal cord of formalin-treated rats as determined by ELISA ($n = 16$). Compared with control group, the levels of IL-6 and IFN- γ were significantly increased in model group, while the level of IL-4 was remarkably reduced. Pre-EA and post-EA effectively blocked the increased levels of IL-6 and IFN- γ induced by formalin injection, upregulated the level of IL-4. Data were expressed as Mean \pm SD. # $P < 0.05$ compared with control group; * $P < 0.05$ compared with model group, & $P < 0.05$ compared with post-EA group. EA: electroacupuncture.

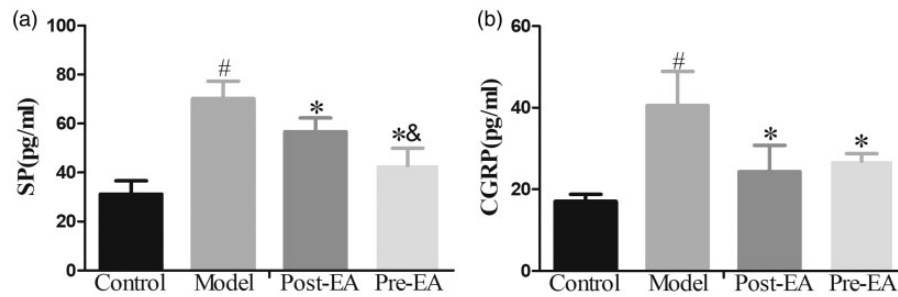


Figure 4. Effects of EA on the levels of SP and CGRP in the L4–L5 spinal cord of formalin-treated rats as determined by ELISA. Compared with control group, the levels of SP and CGRP were dramatically increased in model group. Pre-EA and post-EA effectively inhibited the increased levels of SP and CGRP induced by formalin injection, and pre-EA treatment significantly reduced the level of SP as compared to post-EA. Data were expressed as Mean \pm SD. [#] $P < 0.05$ compared with control group; ^{*} $P < 0.05$ compared with model group; [&] $P < 0.05$ compared with post-EA group. EA: electroacupuncture; SP: substance P; CGRP: calcitonin gene-regulated peptide.

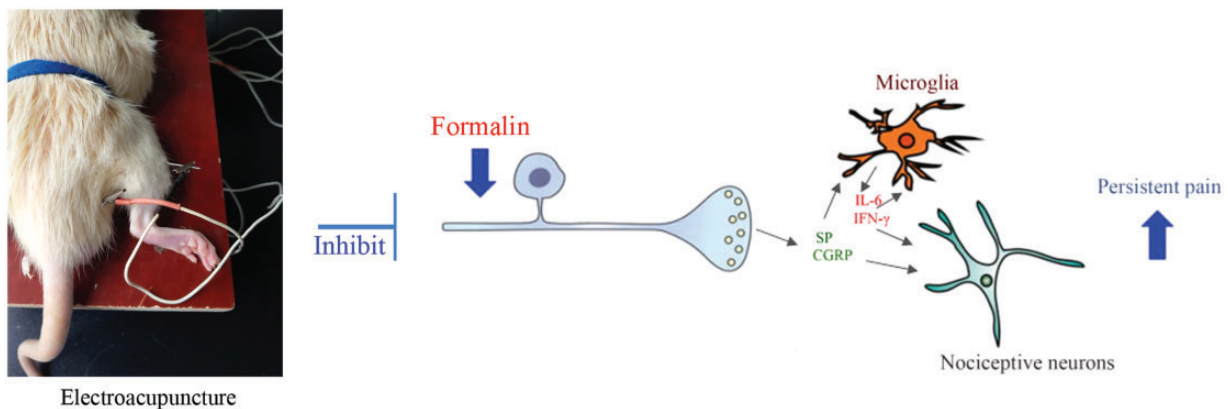


Figure 5. EA inhibited the development of persistent pain in formalin-injected rats. Pre-EA treatment inhibited formalin induced the release of SP and CGRP from primary afferent neurons, thus suppressed the activation of spinal microglia, reduced the secretion of IL-6 and IFN- γ , and further inhibited the activation of nociceptive neurons, accordingly, inhibited the development of persistent pain. EA: electroacupuncture; SP: substance P; CGRP: calcitonin gene-regulated peptide.

and CGRP. ELISA analysis showed that the levels of SP and CGRP in formalin-injected rats were dramatically increased compared to that in pre-EA, post-EA, and control rats ($P < 0.05$) (Figure 4). Moreover, pre-EA treatment significantly inhibited the increased level of SP in L4-5 spinal cord compared with post-EA, respectively ($P < 0.05$), while there was no significant difference in CGRP level between the two groups ($P > 0.05$).

Discussion

This study was designed to determine the preemptive analgesic effects of pre-EA on acute inflammatory pain. Our data demonstrated that formalin intraplantar injection-induced inflammatory pain can be attenuated by pre-EA at ipsilateral Zusanli (ST36) and Weizhong (BL40) acupoints. The related mechanisms of pre-EA may attribute to inhibit the activation of spinal microglia and sensory neurons. Accordingly, proinflammatory cytokines and nociceptive neurotransmitters in spinal

cord, released from activated microglia and sensory neurons, were dramatically reduced by pre-EA. Besides, the analgesic effects of pre-EA on inflammatory pain are better than those of post-EA.

EA inhibits pain by activating numerous bioactive chemicals via peripheral, spinal, and supraspinal mechanisms. These include opioids, which inhibit the sensitization of peripheral nociceptors and decrease proinflammatory cytokines peripherally and in the spinal cord and activating supraspinal serotonergic and noradrenergic neurons that project to the spinal cord.^{9,22} Pre-EA is the treatment before initiating painful stimuli, while post-EA is initiated after injury. As the “preventative” effect on nociceptive amplification and the “gate control theory” of pain, pre-EA could effectively forestall the transmission of nociceptive information via blocking the altered peripheral and central sensory processing,^{23,24} thus may alleviate or inhibit subsequent pain.

In this study, right hind paw plantar injection of formalin was used to mimic the acute inflammatory pain.

As is known, there is a biphasic pain response involved in the inflammatory injury.²⁵ This phenomenon also occurred in our formalin model rats, and the underlying mechanisms of two phases are likely to be different: tissue injury contributes to the first phase (0–5 min) of pain response, representing nociceptive pain, while the second phase (10–45 min) attributes to the peripheral inflammation and central sensitization.²⁶ Furthermore, paw flinching and paw licking were monitored and considered to be the parameters of pain intensity, as similar as previous studies.²⁷ This study found that EA therapy significantly alleviated formalin-induced pain. Interestingly, our data also showed that 2-Hz pre-EA had better analgesic effects than post-EA, these findings indicated that EA might be considered to be a promising preemptive analgesic strategy. Consistent with our results, recent studies had demonstrated the expected outcome of EA and its preemptive analgesia.^{14,16} However, one of the previous study insisted that 100-Hz EA, instead of 2-Hz EA, was preemptive against incision pain in rats, whereas 2-Hz EA was more effective for postincision analgesia.¹⁶ A possible explanation for this contradiction might be the difference of EA acupoints and stimulating spontaneous time between two studies. Accordingly, ipsilateral Zusanli (ST36) and Weizhong (BL40) were stimulated for 30 min with 2-Hz/1 mA EA in our experiment, and based on the “JingMai” theory of traditional Chinese medicine, ST36 and BL40 are the most valuable acupoints for treating the lower limbs pain. Furthermore, the accumulative effect of EA on analgesia reveals that the stimulating time may act as a key factor for EA preemptive analgesia.²⁸ Meanwhile, future studies are needed to design active control group for EA (e.g., acupuncture needle insertions without electrical stimulation or non-acupoint EA stimulation).

It is well established that microglia derive from primary macrophages.²⁹ In almost all conditions, microglia are specifically distributed in the spinal cord and brain, maintaining the local environment.³⁰ Once received the pathophysiological stimulus, microglia are activated, alter morphologically, increase the cell number, and change the expression of genes, including proinflammatory cytokines, such as IL-6 and IL-1 β in the spinal cord.³¹ As mentioned in the previous literature, the proinflammatory cytokines, which works as the mediators in the progress of “crosstalk” with nociceptive neurons, finally promote pain processing and maintenance.³² This study demonstrated that the expression of microglial marker Iba1 was dramatically increased in L4-5 of spinal cord at 2 h after formalin injection, suggesting that microglia have already been activated by nociceptive stimuli in the early period of inflammation injury. As hypothesized, in the model group, ELISA analysis demonstrated that IL-6 and IFN- γ expression

levels of L4-5 area of spinal cord were increased, while anti-inflammatory cytokine IL-4 decreased. Notably, pre-EA treatment significantly suppressed the activation of microglia and inhibited the expression levels of inflammatory cytokines, as compared with the post-EA group. These results indicated that microglia might be the target for EA preemptive analgesia, thus inhibited the progress of inflammation in central nervous system. Prior studies have noted the important role of microglia in EA analgesia both in chronic pain and acute pain model, via suppressing its activation to reverse the central sensitization of pain.^{7,33} However, there is no attention to the change in microglia and neurons in spinal cord by EA stimulation at different times in this study, which may provide the correlation between microglia and neuron activation.

Meanwhile, we also detected that the expression of c-fos was augmented in L4-5 after formalin injection; historically, c-fos has been used as the representative marker for neuronal activation following noxious stimulation.^{34,35} Coinciding with microglial activation, we found that neurons were polarized in this situation. As mentioned before, the crosstalk between microglia and sensory neurons in spinal cord is crucial for pain amplification. Microglia could communicate with neurons via releasing or receiving extracellular molecules, which initiate the intracellular activation signaling.³⁶ For example, the upregulated IFN- γ and IL-6 in present research were confirmed that contributed to neurons polarization.^{17,37,38} SP and CGRP, the nociceptive neurotransmitters which were synthesized by small diameter sensory nerve fibers almost are C-fibers, also increased in L4-5 of spinal cord by formalin injection. Previous studies showed that the SP and CGRP were involved in pain transmission and contributed to the nociceptive synaptic strengthen.³⁹ Moreover, a strong relationship between neurotransmitters and microglial activation has been reported in the literature, which indicated that SP and CGRP may act as the promoters of microglia activation.^{40,41} Following treatment with pre-EA, the expression levels of SP and CGRP as well as c-fos were significantly decreased, suggesting that pre-EA was able to inhibit the activation of nociceptive neurons in rat models of inflammatory pain. Therefore, as a promising preemptive analgesic strategy, EA could reduce the pain signal transmission from primary afferent neurons to spinal secondary sensory neurons in the early stage of inflammatory pain.

In conclusion, this study demonstrated that treatment with pre-EA significantly alleviated the formalin-induced acute inflammatory pain. Meanwhile, pre-EA dramatically decreased the activation of microglia and neurons by decreasing the expression of IFN- γ , IL-6, SP, and CGRP, thus interrupted the crosstalk between microglia and sensory neurons in spinal cord and, furthermore,

inhibited spinal neuroinflammation and central nociceptive sensitization. Taken together, these finds provide a novel preemptive analgesic strategy for the clinical treatment of pain and give a clarification of the potential mechanisms involved in pre-EA analgesia.

Author contributions

Q Liu and YZ conceived and designed the experiments; YZ, YL, and Q Li performed the experiments; JB and Q Li analyzed the data; Q Liu and YL wrote the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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