https://doi.org/10.1590/0004-282X20200078

Electroacupuncture alleviates inflammatory pain via adenosine suppression and its mediated substance P expression

A eletroacupuntura alivia a dor inflamatória por meio da supressão de adenosina e da expressão mediada de substância P

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ABSTRACT

Background: Acupuncture has been widely used for alleviating pain. However, its mechanisms remain largely enigmatic. **Objective:** In the present study, we focused on whether the analgesic effect of electroacupuncture is related to its regulation on adenosine and substance P expression. **Methods:** We established chronic inflammatory pain model in rats through a single injection of Complete Freund's Adjuvant, and then we treated animals using daily electroacupuncture. We applied seven bilateral sessions of electroacupuncture (ST36 and BL60, 0.5 to 1.5 mA, initial strength of 0.5 mA, increased by 0.5 mA every 10 minutes, for 30 minutes per session, one section per day) to Complete Freund's Adjuvant rats for seven days. The analgesic effect of electroacupuncture was evaluated by measuring paw withdrawal threshold in rats that received mechanical and thermal stimulation. **Results:** Daily electroacupuncture stimulation effectively increased paw withdrawal threshold in Complete Freund's Adjuvant rats. Electroacupuncture increased the adenosine level in zusanli. A further study showed that electroacupuncture could decrease substance P, neurokinin-1 receptor, tumor necrosis factor-alpha, interleukin-1β, interleukin-6 and CD68 levels in dorsal root ganglion. Interestingly, direct injection of adenosine A1 or substance P receptor antagonists, or dorsal nerve root transection could significantly impair electroacupuncture induced analgesic actions in Complete Freund's Adjuvant rats could and reduce the levels of substance P, neurokinin-1 receptor, tumor necrosis factor-alpha, interleukin-1β, interleukin-6 and CD68. Finally, we confirmed that direct injection of adenosine A1 receptor agonist replicated the analgesic effect of electroacupuncture. **Conclusion:** Our results indicate regulation of adenosine-mediated substance P secretion. Substance P-mediated pathway may be involved in the analgesia process by electroacupuncture in rats.

Keywords: Electroacupuncture; Chronic Pain; Adenosine; Substance P.

RESUMO

Introdução: A acupuntura tem sido amplamente utilizada para alívio de dor. No entanto, seus mecanismos são muito pouco conhecidos. **Objetivo:** Investigar a relação entre o efeito analgésico da eletroacupuntura e a regulação da expressão de adenosina e de substância P. **Métodos:** Utilizou-se um modelo de dor inflamatória crônica em ratos por injeção única do Adjuvante Completo de Freund e, em seguida, os animais foram tratados com eletroacupuntura diariamente. Foram aplicadas sete sessões bilaterais de eletroacupuntura (ST36 e BL60, 0,5 a 1,5 mA, força inicial de 0,5 mA, aumentada em 0,5 mA a cada 10 minutos, 30 minutos por sessão, uma sessão por dia) em ratos com Adjuvante Completo de Freund, por sete dias. O efeito analgésico da eletroacupuntura foi avaliado pela medida do limiar de retirada da pata em ratos que receberam estimulações mecânica e térmica. **Resultados:** A estimulação diária com eletroacupuntura aumentou efetivamente o limiar de retirada da pata em ratos com Adjuvante Completo de Freund. A eletroacupuntura aumentou o nível de adenosina na região zusanli. Estudos posteriores mostraram que a eletroacupuntura poderia diminuir os níveis de substância P, receptor de neurocinina-1, fator de necrose tumoral-alpha, interleucina-1β, interleucina-6 e CD68 nos gânglios da raiz dorsal. Curiosamente, a injeção direta de antagonistas do receptor de adenosina A1 ou de substância P, ou a transecção da raiz do nervo dorsal, podem prejudicar significativamente as ações analgésicas induzidas pela eletroacupuntura em ratos com Adjuvante Completo de Freund e reduzir os níveis de substância P, receptor de neurocinina-1, fator de necrose tumoral-alfa, interleucina-1β, interleucina-6 e CD68. Por fim, confirmamos que a injeção direta de um agonista do receptor da adenosina A1 reproduziu os efeitos analgésicos da eletroacupuntura. **Conclusão:** Nossos resultados indicam a regulação da secreção da substância P mediada pela adenosina. A via mediada pela substância P pode estar envolvida no processo de analgesia por eletroacupuntura em ratos.

Palavras-chave: Eletroacupuntura; Dor Crônica; Adenosina; Substância P.

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Conflict of interest: There is no conflict of interest to declare.

Support: This work was supported by the National Natural Science Foundation of Anhui province (No. 1808085MH310) and by Grants of the Scientific Research of BoshiKeYan (BSKY) (No. 3101005001249) from the First Affiliated Hospital of Anhui Medical University.

Authors' contributions: Rong yi ZHANG contributed to the conception of the study; Rong yi ZHANG, Ben fan ZHU, Yang SONG, Yan GUO performed the experiment; Li kui WANG, Jia gui ZHAO, Long ZHAO contributed significantly to manuscript preparation; Rong yi ZHANG, Yan GUO, Shi CHEN performed the data analyses and wrote the manuscript.

Received on November 02, 2019; Received in its final form on April 07, 2020; Accepted on May 04, 2020.

Chronic pain (CP) is defined as pain that typically lasts over six months. It can be initiated with injury or disease and may persist after the triggering injury has been healed[1]. Chronic inflammatory pain (CIP) is a subtype of CP, which is usually caused by trauma, bacterial and viral infection, chemical stimulation, and surgery[2]. The prevalence of CIP in adults is estimated to range between 5 and 25%. CIP not only offers great pain and burden to patients, but it also leads to limited labor capacity and reduced work efficiency[3]. Currently, several options are available for clinicians and patients to attenuate CIP. The first choice is using medicines that target the central nervous system to suppress pain, such as opioid receptor agonist morphine. These drugs lead to strong analgesia; however, they also produce lots of side effects, such as respiratory depression, drug resistance, reduction of gastrointestinal motility, and drug addiction[4]. The second option is application of nonsteroidal anti-inflammatory drugs, which usually induce a weak analgesic effect[4,5]. Finally, the third choice is the acupuncture therapy for analgesia. It is well known that acupuncture is a famous analgesic therapy with remarkable curative effect and a long history in the Asian medicine[6,7]. Currently, acupuncture has been considered to have a superiority of almost no side effect compared with drug analgesia. Therefore, it has been widely used around the world[6,7]. Nevertheless, the exact analgesia mechanism of acupuncture is still poorly understood. Therefore, it is urgent to uncover the analgesia mechanism of acupuncture, which not only provides theoretical basis for clinical application, but also promotes its application.

A previous study reported that adenosine mediates analgesia effect of acupuncture. Interfering with adenosine metabolism may prolong the clinical benefit of acupuncture[8]. However, little is known about how adenosine contributes to the analgesic effect of electroacupuncture (EA). Tissue injury causes local releases of inflammatory mediators, such as substance P (SP), histamine, and prostaglandins E[9]. These mediators activate immune cells, which in turn release many pro-inflammatory factors, such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α). Finally, pro-inflammatory cytokines induce peripheral nociceptor cells to produce action potentials, resulting in pain[9,10]. We have recently noticed adenosine inhibits the release of SP and calcitonin gene-related peptide from afferent nerve endings[11]. Therefore, we suspect EA improves pain threshold via increasing adenosine levels, which acts on adenosine receptor and inhibits the release of SP from the dorsal root ganglion (DRG). In the present study, we used Complete Freund's Adjuvant (CFA) induced pain model to investigate the hypothesis that adenosine and its mediated SP release are involved in EA analgesia.

METHODS

Animals

Male Sprague-Dawley rats (180–220 g) were purchased from the experimental animal center of Anhui Medical University. All rats were housed in a controlled environment (temperature — 25±2°C, humidity — 55±5%, and light — 12-hour light/dark cycle) and fed with standard rodent food and allowed distilled water *ad libitum*. All procedures were approved by the Animal Care and Use Committee of Anhui Medical University (Hefei, China, LLSC20183042).

Inflammatory pain induction

Inflammatory pain was induced in rats through intra-plantar injection of 100 μL of CFA (Sigma-Aldrich Corporation, St. Louis, MO, USA) on the plantar surface of the left hind paw[12]. Each milliliter of the injection contained 1 mg heat-killed and dried mycobacterium tuberculosis, 0.85 mL paraffin oil and 0.15 mL mannide monooleate. Control animals were injected with the same volume of 0.9% saline.

Treatment

All animals were separated randomly into seven groups: regular Control Group, received 0.1 mL of saline injection; CFA Group received 0.1 mL of CFA injection; CFA+EA Group received 0.1 mL of CFA injection together with daily EA stimulation for seven days; CFA+EA+Dorsal Nerve Root Transection Group (DNRR) received 0.1 mL of CFA injection, daily EA stimulation and DNRR surgery performed at 24 hours after immunization; CFA+EA+adenosine A1 receptor antagonist (ANTAG, rolofylline, Sigma-Aldrich Corporation, St. Louis, MO, USA) Group received 0.1 mL of CFA injection, daily EA stimulation and injection of rolofylline at a dose of 3 mg/kg into acupoint ("Zusanli"); CFA+EA+adenosine A1 receptor agonist (AG, N6-cyclopentyladenosine: CPA; Sigma, St. Louis, MO, USA) Group received 0.1 mL of CFA injection, daily EA stimulation and injection of CPA at a dose of 0.1 mg/kg into acupoint ("Zusanli"); CFA+SP receptor antagonist (CP96345; Sigma, St. Louis, MO, USA) received 0.1 mL of CFA injection, daily EA stimulation and subarachnoid administration of CP96345 at a dose of 2.5 mg/kg.

The EA procedure was conducted based on a previous published method with minor modifications[12]. In brief, the rats received EA treatment with stimulator parameters as follows: 2/100 Hz, 0.5 to 1.5 mA (initial strength of 0.5 mA, increased by 0.5 mA every 10 minutes) for a total of 30 minutes. We applied a total of seven bilateral sessions of EA therapy (30 minutes per session, one section per day) to CFA rats for seven days. Electro-stimulation was performed with constant square wave current output (pulse width: 0.6 ms at 2 Hz, 0.2 ms at 100 Hz). Rats were loosely immobilized. Four stainless steel acupuncture needles of 0.25 mm in diameter were inserted at a depth of 5 mm into bilateral "Zusanli" (ST36, 5 mm lateral to anterior tubercle of the tibia) and "Kunlun" (BL60, at ankle joint level and between the tip of external malleolus and tendon calcaneus) acupoints. Electrical stimulation was produced by a Trio 300 electrical stimulator (Trio 300, ITO Corporation, Germany).

Paw withdrawal threshold

The paw withdrawal threshold (PWT) was determined by von Frey behavioral test and Hargraves' test according to previous methods[12,13]. In brief, all stimuli were performed at room temperature and applied when animals were calm. Mechanical sensitivity was measured by testing the force of responses to stimulation with three applications of electronic von Frey filaments (North Coast Medical, Gilroy, CA, USA), while thermal pain was assessed with three applications using Hargraves' test IITC analgesiometer (IITC Life Sciences., Model 390 G, CA, USA).

Analysis of adenosine levels

Microdialysis probes were implanted on the left side of the foot in rats of the Control Group, CFA Group and CFA+EA Group before the study. The microdialysis solution was collected at different time points during the experiment (one, three, five and seven days after immunization). Adenosine levels were assessed by a High Performance Liquid Chromatography (HPLC) method as already described[8].

Real-time quantitative polymerase chain reactions

Total RNA was extracted from tissue homogenization using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using 1 μg total RNA by Prime Script WRT reagent kit (TaKaRa, Japan). The expression of genes was analyzed by real-time quantitative polymerase chain reactions (qPCR) using CFX96™ real-time PCR detection system (Bio-Rad, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for target genes, and the relative expression level was calculated through the $2^{-\Delta\Delta CT}$ method. The primers described in Table 1 were designed and synthesized.

Western blotting analysis

The animals were sacrificed after behavioral testing seven days after immunization. Ipsilateral spinal dorsal horns (L4-L6) were removed and stored at -80°C. The dorsal horns were homogenized with a mechanical rotary cutter in strong radioimmunoprecipitation assay (RIPA) buffer (Sigma, St. Louis, MO, USA) containing a cocktail of phosphatase and proteinase inhibitors. The protein concentration of tissue lysates was detected with a bicinchoninic acid protein assay kit (Thermo Scientific, Grand Island, NY, USA). All samples were separated on 10% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis gels and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were blocked with 5% low-fat milk in Tris Buffered saline Tween for one hour and then incubated with NK-1R and CD68 antibodies (Santa Cruz, CA, USA). Afterwards, the bolts were incubated for one hour at 37°C with HRP-conjugated secondary antibody and visualized in enhanced chemiluminecence solution (Thermo Scientific, Grand Island, NY, USA). β-actin was used as an internal control.

Table 1. Primers used in the current study.

Gene names	Primers
TNF-α	Forward: 5'-TACTGAACTTCGGG GTGATTGGTCC-3' Reverse: 5'-CAGCCTTGTCCCTTGAAGA GAACC-3'
IL-1β	Forward: 5'-TG AAGCAGCTATGGCAACTG-3' Reverse: 5'-CTGCCTTCCTGAAGCTCTTG-3'
IL-6	Forward: 5′-GCCAGAGTCATTCAGAGCAATA-3′ Reverse: 5′-TTAGGAGAGCATTGG AAGTTGG-3′
Neurokinin-1 receptor (NK-1R)	Forward: 5′-CACACT ATGGGCCAGTGAGATC-3′ Reverse: 5′-GCACACCACGACAATCATCATT-3′
CD68	Forward: 5'-ctcAcAaaAaGgctgccact-3′ Reverse: 5′-ttccggTggttGtAggtgtc-3′
GAPDH	Forward: 5′-GGTGGTCTCCTCTGACTTCAACA-3′ Reverse: 5′-GTTGCTGTAGCCA-AATTCGTTGT-3′

TNF-α: tumor necrosis factor-alpha; IL-1β: interleukin-1beta; NK-1R: neurokinin-1 receptor; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Measurement of cytokines

Spinal dorsal horns of rats were homogenized, and a homogeneous section has been collected. The levels of TNF-α, IL-1β, IL-6 and SP were assessed by using ELISA kits offered by the manufacturing company (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All statistical tests were conducted using SPSS 15.0 (SPSS Inc., Chicago, IL, USA) software. Data were presented as mean±standard deviation. Statistical analysis was conducted through one-way analysis of variance (ANOVA) followed by individual *post hoc* multiple comparisons. A statistical difference was considered as significant at $p<0.05$.

RESULTS

Electroacupuncture stimulation reduced Complete Freund's Adjuvant-induced pain in rats

Rats were injected with CFA into the surface of the left hind paw to induce persistent inflammatory pain. As seen in Figure 1, both mechanical and thermal hyperalgesia were measured and used to evaluate the analgesia activity of the animals. No significant difference was observed in mechanical sensitivity and thermal hyperalgesia among the seven groups at the baseline (before CFA injection). Moreover, the pain threshold was significantly lower in CFA Groups in days three, five, and seven after immunization compared with the Control Group ($p<0.01$). In contrast, mechanical and thermal hyperalgesias were significantly improved in the CFA+EA Group ($p<0.01$) compared with CFA group, suggesting well analgesic effects induced by EA

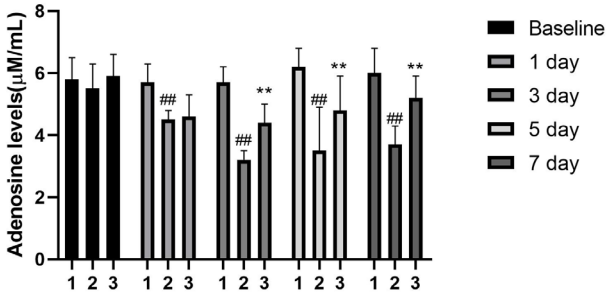
stimulation. Furthermore, to study whether the analgesic effects of EA therapy were based on adenosine-regulated SP release, we applied different "tool reagents" together with EA to treat CFA rats. As shown in Figure 1, we found that CFA rats who were given DNRR and EA showed increased mechanical and thermal hyperalgesia compared with those that only received EA ($p<0.01$). In addition, CFA rats who were given ANTAG and EA showed an impaired pain tolerance compared with those that only received EA ($p<0.01$). As expected, similarly to EA treatment, daily AG injection could significantly increase pain threshold in CFA rats. Finally, we noted SP receptor antagonist (CP96345) treatment could undermine the analgesia effects induced by EA stimulation in rats ($p<0.01$).

Electroacupuncture stimulation increased adenosine level in the Complete Freund's Adjuvant-treated rats

We investigated whether EA therapy could increase adenosine levels in CFA rats. As shown in Figure 2, compared with the animals in the Control Group, CFA rats showed decreased level of adenosine at 24 hours after CFA immunization ($p<0.01$). However, EA treatment could significantly increase adenosine levels in CFA rats that began at day three after stimulation ($p<0.01$).

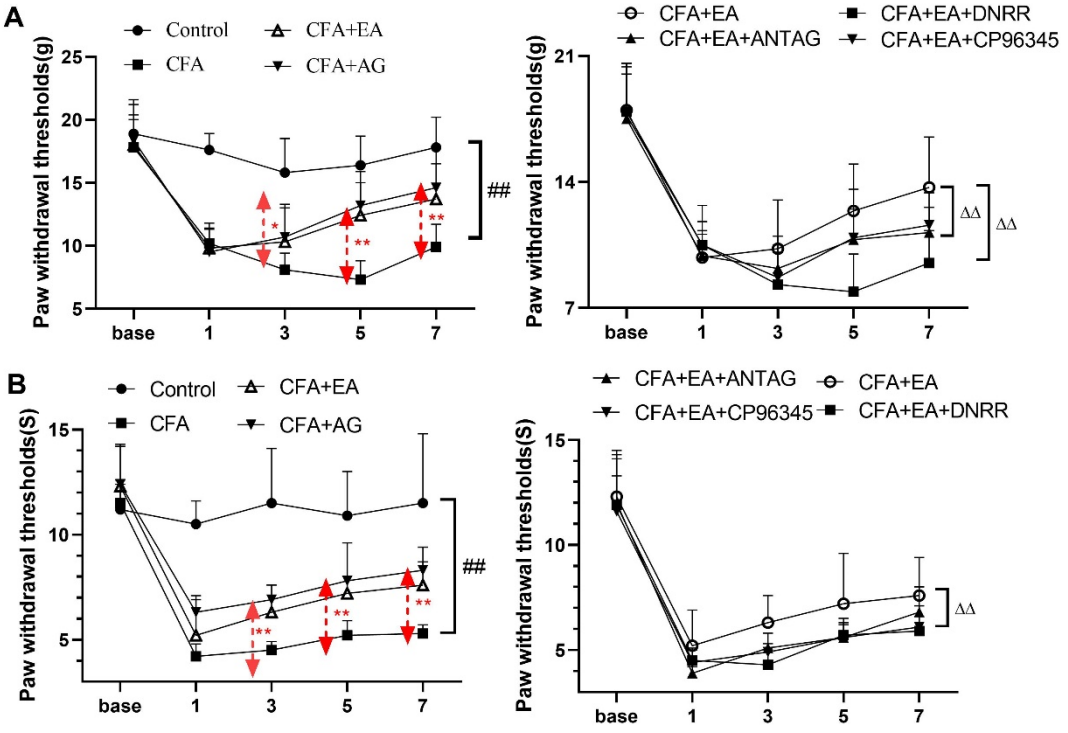
Electroacupuncture stimulation decreased mRNA levels of inflammatory pain-related genes in Complete Freund's Adjuvant-treated rats

As shown in Figure 3, we further investigated mRNA levels of inflammatory pain associated genes in response to EA stimulation. The results charted in Figure 3 indicate CFA injection significantly increased SP, NK-1R, CD68, TNF-α, IL-1β and IL-6 mRNA expression in the DRG of rats ($p<0.01$). However, EA significantly downregulated the expression of SP, NK-1R, CD68, TNF-α, IL-1β, and IL-6 mRNA, compared with the CFA



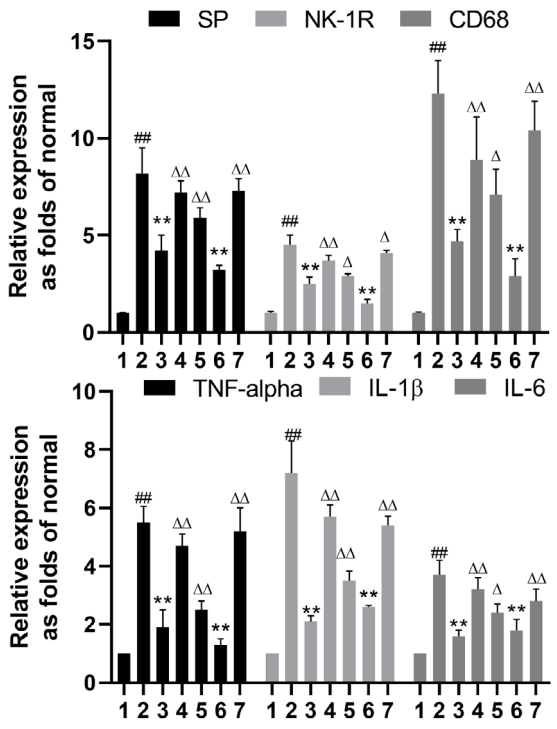
EA: electroacupuncture; CFA: Complete Freund's Adjuvant. 1: Control Group; 2: CFA Group; 3: CFA+EA Group. Values represent mean±standard deviation; n=8 per group; ##p<0.01 *versus* Control Group; **p<0.01 *versus* CFA Group.

Figure 2. Adenosine levels in rats treated by Complete Freund's Adjuvant together with electroacupuncture.



EA: electroacupuncture; CFA: Complete Freund's Adjuvant; AG: antagonist receptor; DNRR: Dorsal Nerve Root Transection Group; CP: chronic pain; ANTAG: adenosine A1 receptor antagonist. Values represent mean±standard deviation; n=8 per group for each time point; ##p<0.01 *versus* Control Group; *p<0.05; **p<0.01 *versus* Complete Freund's Adjuvant group; ΔΔp<0.01 *versus* CFA+EA Group.

Figure 1. Effects of electroacupuncture stimulation on paw withdrawal threshold in rats. (A) Mechanical sensitivity of rats was measured by assessing the force of responses to stimulation with electronic von Frey filaments. (B) Thermal sensitivity of rats was measured using Hargraves' test IITC analgesiometer.



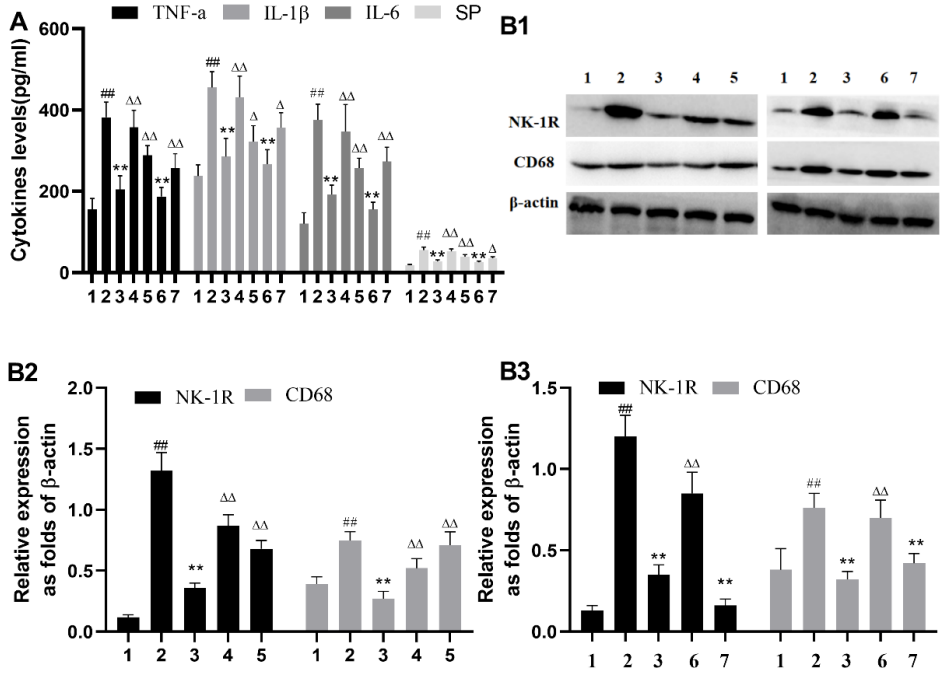
EA: electroacupuncture; CFA: Complete Freund's Adjuvant; AG: antagonist receptor; DNRR: Dorsal Nerve Root Transection Group; CP: chronic pain; ANTAG: adenosine A1 receptor antagonist. 1: Control Group; 2: CFA Group; 3: CFA+EA; 4: CFA+EA+DNRR; 5: CFA+EA+ANTAG; 6: CFA+AG; 7: CFA+EA+CP 96345. Values represent mean±standard deviation; n=8 per group; ##p<0.01 *versus* Control Group; *p<0.05; **p<0.01 *versus* CFA Group; ΔΔp<0.01 *versus* CFA+EA Group.

Figure 3. mRNA levels of inflammatory pain-related genes in dorsal root ganglion of Complete Freund's Adjuvant rats that received electroacupuncture stimulation.

Group (p<0.01). Moreover, we found CFA rats that were given ANTAG and EA showed decreased levels of SP, NK-1R, CD68, TNF-α, IL-1β and IL-6 mRNA compared with those that only received EA (p<0.05). AG treatment significantly decreased the mRNA levels of these genes compared with the CFA Group (p<0.01). In addition, compared with the EA Group, both blockade of DRG by DNRR and inhibition SP-mediated pathway by SP receptor antagonist could increase the mRNA levels of SP, NK-1R, CD68, TNF-α, IL-1β and IL-6 (p<0.01).

Electroacupuncture decreased the expression of inflammatory pain-related protein in Complete Freund's Adjuvant-treated rats

To investigate changes in the molecule expression of associated inflammatory pain in DRG following EA stimulation, we used ELISA and western blotting to measure these protein levels in each group. As shown in Figure 4, CFA injection significantly increased the levels of SP, NK-1R, CD68, TNF-α, IL-1β, and IL-6 in DRG (p<0.01). However, after EA stimulation, these molecule levels were markedly reduced compared with the CFA Group (p<0.01). Moreover, we found that in rats that were given EA together with DNRR, ATANG or SP receptor antagonist, expression of SP, NK-1R, CD68, TNF-α, IL-1β, IL-6 were significantly enhanced, compared to those in rats that only received EA (p<0.05). Finally, we observed AG treatment could significantly decrease the expression of SP, NK-1R, CD68, TNF-α, IL-1β, IL-6 in DRG of CFA rats (p<0.01), suggesting the important role of AG in the development of CFA-induced CIP.



EA: electroacupuncture; CFA: Complete Freund's Adjuvant; AG: antagonist receptor; DNRR: Dorsal Nerve Root Transection Group; CP: chronic pain; ANTAG: adenosine A1 receptor antagonist. 1: Control Group; 2: CFA Group; 3: CFA+EA; 4: CFA+EA+DNRR; 5: CFA+EA+ANTAG; 6: CFA+AG; 7: CFA+EA+CP 96345. Values represent mean±standard deviation; n=8 per group. ##p<0.01 *versus* Control Group; **p<0.01 *versus* CFA Group; *p<0.05; ΔΔp<0.01 *versus* CFA+EA Group.

Figure 4. Expression of inflammatory pain-related protein in dorsal root ganglion of rats that received electroacupuncture stimulation. (A) Pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and SP were detected by ELISA kits; (B) expression of NK-1R and CD68 was assessed by Western blotting analysis.

DISCUSSION

EA has well analgesia activity and has been proved to be effective in treating various kinds of pain. In 2004, Huang et al.[14] reported that EA therapy could attenuate mechanical hyperalgesia in a rat model of CIP. Afterwards, EA was proved to inhibit inflammatory pain elicited by carrageenan and cold allodynia in a rat model of neuropathic pain[15,16]. EA, therefore, has been widely used to treat different kinds of pain. However, it is still not trustworthy due to incomplete decryption of its molecular mechanism. In the present study, we have observed that EA stimulation provided analgesia in a rat model of CFA-induced inflammatory pain. In agreement with previous studies, our data support the conclusion that EA therapy induces well anti-nociceptive effect.

Several clinical and basic studies have been performed to disclose the biological basis of EA-induced analgesia. At present, several molecules and their mediated pathways have been found to be involved in EA-induced analgesia. For instance, it has been previously demonstrated that EA attenuates inflammatory pain by targeting CB2 receptors, transient receptor potential vanilloid 1 and p38 MAPK pathway[17,18,19]. Just like opioids, adenosine plays an important role in CP[8,20]. In previous studies, direct injection of adenosine A1 receptor agonist could replicate the analgesic effect of acupuncture, while selective A1 and A2A antagonists completely prevented antinociception[21,22].

How does adenosine participate in the analgesic effect induced by EA? Some studies[16,23] showed EA stimulation that induced analgesia in animal models may target Nav1.8, COX-2 and pPKCε via regulating adenosine and its mediated pathway. In addition, there are no other reports focused on the role of adenosine in EA-induced analgesia. SP is a peptide neurotransmitter from sensory nerve endings and is the main mediator of neurogenic inflammation[9]. SP plays an important role in the development of pain, tissue damage, and inflammatory reactions. Interestingly, it has been reported that SP release in rat spinal cord was mediated by adenosine. Intrathecal adenosine analog administration reduced SP in cerebrospinal fluid along with antinociception behavioral effects[11,24]. Therefore, EA treatment may exert analgesia action via inhibiting adenosine mediated release of SP from DRG neurons.

In the present research, we found EA treatment could increase the level of adenosine, SP, and NK-1R. It is well known that SP binding with NK-1R could increase the secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6[25], which directly promote inflammatory pain. Intramuscular injection of TNF-α induces muscle hyperalgesia in rats[26]. Neutralizing antibodies to IL-1 receptor reduce pain-associated behavior in mice with experimental neuropathy[27]. Therefore, we have further investigated the genetic and protein levels of TNF-α, IL-1β and IL-6 in rats that received EA. The results showed that EA treatment reduced these cytokines at transcription and protein levels. Moreover, we have also observed the effects of DNRR on the efficacy of EA. Our results showed that DNRR not only blocked EA-induced analgesia in CFA rats, but also increased the expression of SP, NK-1R and their downstream inflammatory molecules. These findings strongly suggest that EA-induced analgesia in CFA rats is dependent on the participation of DRG neurons.

Furthermore, to test whether EA-induced analgesia requires involvement of SP, we used SP receptor antagonist CP96345 to interfere in the treatment of EA. As expected, similarly to DNRR, we found that CP96345 almost completely impaired EA-induced analgesia in CFA rats, implying SP mediated pathway is involved in the analgesia action of EA. Moreover, we have also treated CFA rats using EA together with ANTAG. Interestingly, ANTAG weakened EA-induced analgesia and correspondingly increased SP and NK-1R levels, which suggest that regulation of SP secretion is an important mechanism of adenosine to exert biological activity in the EA treatment.

In summary, our results suggest that the upregulation of adenosine level and enhancement of adenosine induced inhibitory effects on SP are both necessary and sufficient for the clinical benefits of EA. To the best of our knowledge, SP and its relationship with adenosine have not been previously implicated in the antinociceptive actions of EA. Therefore, our findings further broaden the current understanding of EA analgesia mechanism.

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