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Research report

Synergistic anti-hyperalgesia of electroacupuncture and low dose of celecoxib in monoarthritic rats: Involvement of the cyclooxygenase activity in the spinal cord

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ABSTRACT

Electroacupuncture (EA) can effectively control the exaggerated pain in humans with inflammatory disease and animals with experimental inflammatory pain. However, there have been few investigations on the effect of co-administration of EA and analgesics and the underlying synergistic mechanism. Using behavioral test, RT-PCR analysis, enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA), the present study demonstrated that (1) Unilateral intra-articular injection of complete Freund's adjuvant (CFA) produced a constant hyperalgesia and an up-regulation of the prostaglandin E_2 (PGE_2) level as well as the tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 levels in the spinal cord; (2) Celecoxib, a selective inhibitor of cyclooxygenase-2 (COX-2), at a dose of 2, 10, and 20 mg/kg (twice daily, p.o.), presented a dose-dependent anti-hyperalgesic effect; (3) Repeated EA stimulation of ipsilateral 'Huan-Tiao' (GB30) and 'Yang-Ling-Quan' (GB34) acupoints significantly suppressed CFA-induced hyperalgesia, and markedly inhibited the CFA-induced increase of the level of PGE_2 as well as IL-1β, IL-6, and TNF-α in the spinal cord; (4) EA combined with low dose of celecoxib (2 mg/kg, twice daily, p.o.) greatly enhanced the anti-hyperalgesic effects of EA, with a synergistic reversing effect on CFA-induced up-regulation of spinal PGE_2, but not on the IL-1β, IL-6, or TNF-α. These data indicated that repeated EA combined with low dose of celecoxib produced synergistic anti-hyperalgesic effect in the CFA-induced monoarthritic rats, which could be made possible by regulating the activity of spinal COX, hence the spinal PGE_2 level. Thus, this combination may provide an effective strategy for pain management.

1. Introduction

Arthritis is a painful and disabling disease that affects millions of patients [44]. Although effective in relieving pain and inflammation, traditional nonsteroidal anti-inflammatory drugs (NSAIDs), one of the most widely used medications, are associated with a significant increase in the risk for gastrointestinal adverse events, because of the non-selective inhibition on both cyclooxygenase (COX)-1 and -2 (COX-2) [46]. And the selective inhibitors of COX-2, proved to induce fewer gastrointestinal toxicities compared to traditional NSAIDs, have been reported to raise a high risk for cardiovascular events that are associated with chronic use and higher doses [9,35,47]. These studies suggest that the use of COX-2 inhibitors as the sole strategy for gastroprotection in patients with arthritis and other pain syndromes should be reconsidered [1].

Acupuncture, an important part of Traditional Chinese Medicine (TCM), has been confirmed effective in pain relief by numerous clinical observations and experimental studies [2,3]. And it has also been shown effective to treat human rheumatoid arthritis and osteoarthritis [7,16]. Recent large randomized, controlled trials showed that acupuncture was safe and effective for reducing pain in patients with symptomatic knee osteoarthritis who have moderate or greater pain despite background therapy with analgesic

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or anti-inflammatory therapy (including COX-2 inhibitors) [6,41]. At the same time, these investigators further pointed out that acupuncture is still an adjunctive or complementary therapy with moderate analgesic effect. Thus, the integration of acupuncture and analgesics seems to be a promising strategy in pain management.

Prostaglandins are synthesized from arachidonic acid by the action of two enzymes, COX-1 and COX-2. There is increasing evidences to show that prostaglandin E_2 (PGE_2) is produced in the spinal cord after peripheral tissue injury and inflammation [40], and also behavioral studies suggested that PGE_2 facilitates the nociceptive transmission in the spinal cord, thus contributing to central sensitization, which has been shown to be an essential mechanism underlying the hypersensitivity of pain [25]. The previous study showed that electroacupuncture (EA)'s anti-hyperalgesic effects might be attributed to the regulation of peripheral COX-2 expression and PGE_2 synthesis in the carrageenan-induced acute peripheral inflammation in rats [27]. However, little is known about the relationship between EA analgesia and spinal COX-2 expression or spinal PGE_2 production in peripheral inflammatory pain.

Furthermore, it is well known that spinal cord glia (especially microglia and astrocytes) importantly contribute to the development and maintenance of central sensitization in chronic pain [13,43,45]. Peripheral nerve or tissue inflammation produces hyperalgesia and allodynia, as well as activation of glia in the spinal cord [10,26,36,37]. Upon activation, glia was found to be capable of releasing a variety of algesic substances enhancing pain transmission. Of these glial products, proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 were shown to be common mediators of allodynia and hyperalgesia [13,42,43]. It was found that EA could inhibit the Complete Freund's adjuvant (CFA)-induced glia activation and the up-regulation of the proinflammatory cytokines [38].

Celecoxib, one of the selective COX-2 inhibitors, is commonly administered in the treatment of arthritis with fewer gastrointestinal toxicities [14]. Using an inflammatory pain model induced by intra-articular injection of CFA in rats [11,31], the present study was intended to examine (1) whether combined treatment of EA with low dose of celecoxib could produce a synergistic analgesic effect; (2) whether this synergistic analgesic effect was mediated by the regulation of spinal COX-2 expression or PGE_2 production; and (3) whether this effect was related to the regulation of the expression of spinal proinflammatory cytokines.

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats weighing 200–220 g, supplied by the Experimental Animal Center, Chinese Academy of Sciences, Shanghai. Prior to experimental manipulation, the rats were allowed to acclimate for 1 week in groups of four to six rats per cage, and maintained under controlled conditions (22 ± 1 °C, 6 a.m. to 6 p.m. alternate light–dark cycles) with food pallets and water *ad libitum*. All experiments were conducted strictly in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the guidelines of the International Association for the Study of Pain [53]. All efforts were made to minimize the number of animals used and their suffering.

2.2. Induction of monoarthritis (MA)

Monoarthritis (MA) was induced by an intra-articular injection of CFA (Sigma, St. Louis, USA; 1 mg/ml). The rat was briefly anesthetized with 1.2% isoflurane. The skin around the site of injection was sterilized with 75% alcohol, and then the left foot of the rat was held and the fossa of the lateral malleolus of the fibula was located. A 28-gauge needle was inserted vertically to penetrate the skin, and turned distally to insert into the articular cavity from the gap between the tibiofibular and tarsus bone until a distinct loss of resistance was felt. A volume of 50 μl CFA was then injected. Sham MA control animals were similarly injected with sterile normal saline. After injection, rats were housed in the cages covered with sawdust in order to minimize the possibility of painful mechanical stimulation. Rats were able to eat and drink unaided.

2.3. Electroacupuncture treatment

The detailed EA procedure has been described previously [17]. In brief, during EA treatment, the trunk of the rat was kept motionless while the head and four limbs kept freedom of movement in a specially designed holder. Restrained in the holders, the rats were underlying brief anesthesia by inhaling ether. Rats were allowed to acclimate for 30 min before EA treatment. The skin cleaned with alcohol swabs, a pair of stainless steel needles of 0.3 mm diameter were inserted into the ipsilateral acupoints 'Huan-Tiao' (GB-30, located near the hip joint, on the inferior borders of muscle gluteus maximus and muscle piriformis; the inferior gluteal cutaneous nerve, the inferior nerve; deeper, the sciatic nerve) and 'Yang-Ling-Quan' (GB-34, located near the knee joint, anterior and inferior to the small head of the fibula, in muscle peroneus longus and brevis, where the common peroneal nerve bifurcates into the superficial and deep peroneal nerves) at a depth of 7 and 5 mm, respectively. The two needles were connected with the output terminals of an EA apparatus (Model G-6805-1A, Shanghai Huayi Medical Electronic Apparatus Company, China). Alternating strains of dense-sparse frequencies (60 Hz for 1.05 s and 4 Hz for 2.85 s alternately, bidirectional asymmetric pulse, 0.6 ms pulse width) were selected. The intensity of stimulation was approximately 1 mA, and mild muscle twitching was observed. The stimulation lasted for 30 min each time.

In order to exclude the possibility of analgesia induced by stress such as animal fixation, sham EA group animals were given the same manipulation as the EA group, except electrical current or manual needle manipulation. This form of sham EA showed little anti-hyperalgesia and seemed to be an appropriate control for non-specific needling effects.

GB30 and GB34 were chosen based on TCM meridian theory and their successful use in previous studies in the treatment of inflammatory pain and arthritis both in clinical and basic research [6,36,38,41].

2.4. Behavioral test

The paw withdrawal latency (PWL) to radiant heat was examined as previously described [21]. All the experiments were carried out every other day at the same time of the day between 8 a.m. and 12 a.m. to avoid diurnal variation in behavioral tests. Rats were placed into an inverted, clear plastic cage upon an elevated floor of window glass. After an accommodation period of 30 min, using IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, USA), a constant intensity radiant heat source (50 W, 8 V bulb) was aimed at the ankle joint until the rat lifted its paw. The time from onset of radiant heat application to paw withdrawal was defined as PWL. The intensity of radiant heat was adjusted to elicit the response around 12 s in normal rats, and a cut-off time was set at 20 s in order to avoid tissue injury. Both hind paws were tested independently with a 15 min interval between tests. The degree of hyperalgesia was expressed as difference score in PWLs and was determined as follows. Negative difference scores indicated a hyperalgesic response on the ipsilateral side.

Difference scores in PWLs (%) = [(PWL on the ipsilateral side − PWL on the contralateral side) / PWL on the contralateral side] × 100.

2.5. Reverse transcription polymerase chain reaction (RT-PCR)

For RT-PCR analysis, rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4–L6 spinal cords were collected in dry ice. Total RNA extraction was performed using the Trizol reagent (Invitrogen, USA), following the instructions of the manufacturer. RNA was further purified using the RNeasy kit according to the RNA clean-up protocol, and eluted in 20 μl of RNase-free distilled H_2O. The amount of RNA was measured spectrophotometrically. Total RNA (1 μg) was used for the synthesis of the first strand of cDNA using the M-MLV Reverse Transcriptase (Promega, USA). Briefly, RNA, oligo (dT) 15 primers (0.5 μg/μl) were first denatured for 5 min at 70 °C, chilled on ice for 1 min, and then incubated for 60 min at 37 °C, 5 min at 95 °C in 25 μl of a reaction mixture containing M-MLV 5 × Reaction Buffer, 10 mM dNTP mix, Recombinant RNasin Ribonuclease Inhibitor and 200 units of M-MLV Reverse Transcriptase. Primers used (Sangon, Shanghai, China) were as follows [5]: COX-1: 5′-CATGGATCCGGATTGGTGGGGGTAG-3′ (sense) and 5′-ATCTCGAGGGGC AGGTCTTGGTGTTG-3′ (antisense); COX-2: 5′-CTGTATCCCGCCCTGCTGGTG-3′ (sense) and 5′-ACTTGCGTTGATGGTGGCTGTCTT-3′ (antisense); β-actin: 5′-TCAGGTCATCACT ATCGGCAAT-3′ (sense) and 5′-AAAGAAAGGGTGTAAAACGCA-3′ (antisense). cDNA (1 μl) was amplified with Taq DNA polymerase in a 20 μl reaction mixture (MBI Fermentas, USA). PCR reaction was performed as follows: 5 min at 95 °C to activate the Taq polymerase, followed by 30 cycles of 30 s at 95 °C, 45 s at 57 °C (COX-1/β-actin), 68 °C (COX-2), and 45 s at 72 °C. A final elongation step at 72 °C for 10 min completed the PCR reaction. Each PCR production (10 μl) was electrophoresed in 2% agarose gel, visualized by ethidium bromide staining and scanned with ultraviolet transilluminator (GDS 8000, Gene Tools from Syngene Software, UK). The PCR quantitative method takes advantage of the fact that β-actin was employed as internal standard in the same condition. All the results were expressed as ratios of the intensity of the COXs bands to that of β-actin band.

2.6. Prostaglandin E_2 determination

Prostaglandin from spinal cord fragments was compared in different times and different groups. Rats were sacrificed with an overdose of urethane (1.5 g/kg, i.p.) and the L4–L6 segments of spinal cord were collected in dry ice and stored at −80 °C until sonication. Total protein was dissociated mechanically from tissue using an ultrasonic cell disruptor, and then centrifuged at 3000 × *g* for 15 min. Supernatant was removed and stored at −20 °C until analysis. Release of prostaglandin E_2 from the tissue fragments into the incubation medium was determined by commercially available enzyme immunoassay kits (Adlitteram Diagnostic Laboratories, USA). Measurement was completed using an enzyme-linked immunosorbent assay with an absorbency maximum at 450 nm.

2.7. Cytokine protein estimation by ELISA

Concentrations of IL-1β, IL-6, and TNF-α in the spinal cord were detected by ELISA. Tissue homogenization was prepared in the same way as the PGE_2 determination. IL-1β, IL-6, and TNF-α protein concentrations were determined utilizing the quantitative sandwich enzyme immunoassay according to the manufacturer's protocol (RapidBio Lab, California, USA).

2.8. Experimental design

Two experiments were conducted. Experiment 1 was designed to evaluate the arthritic pain model induced by CFA injection as well as the anti-hyperalgesic effects of different doses of celecoxib. Rats were randomly divided into six groups (n = 7–11 per group): (1) MA (2) Sham MA, (3) Vehicle (0.5% methylcellulose, p.o.), (4) Celecoxib (2 mg/kg p.o.), (5) Celecoxib (10 mg/kg p.o.), (6) Celecoxib (20 mg/kg p.o.). Celecoxib (marketed as Celebrex, Searle and Co. Caguas, Puerto Rico, USA) was used as their free base and was suspended in 0.5% methylcellulose and administered to the MA rats twice daily by gavage for 10 days starting from day 4 after CFA injection.

Experiment 2 was designed to test the anti-hyperalgesic effect of EA as well as co-application of EA and low dose of celecoxib. After CFA injection for 4 days, all the MA rats were randomly divided into the following five groups (n = 11 per group): (1) MA, (2) EA, (3) Sham EA, (4) Celecoxib (2 mg/kg, twice daily, p.o.) (5) EA plus low dose of Celecoxib (2 mg/kg, twice daily, p.o.). EA and sham EA were administered once every other day from day 4 after CFA injection till the end of the experiment, and the anti-hyperalgesic effects were tested the day after EA treatment to avoid the disturbance of immediate influence of EA on pain behavior.

On days 4, 10 and 14 after CFA injection and on day 14 after EA and/or low dose of celecoxib treatment, were rapidly sacrificed and the lumbar spinal cord tissues were collected for RT-PCR, EIA and ELISA analysis. All the testing (including behavior, RT-PCR, EIA and ELISA) was performed blinded.

2.9. Statistical analysis

Data are presented as mean ± S.E.M. and analyzed by statistical software SPSS 11.5. Repeated measures analysis of variance (ANOVA) followed by Student–Newman–Keuls test was used for post hoc analysis for differences between groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Chronic thermal hyperalgesia induced by intra-articular CFA injection in rats

Before CFA injection, the basal withdrawal latencies of all rats, as well as the PWLs between the left and right hind paws, were not distinctly different. Two days after injection, the monoarthritic rats presented hypersensitivity to the heat stimulation of the hind paw ($P < 0.05$). And the difference score in PWLs of the monoarthritic rats decreased to minus values, whereas there was no significant change of the sham MA. The hyperalgesia in monoarthritic rats was stable and lasted for 14 days, the whole period of the experiment. Meanwhile, no significant differences were observed at different time points from day 2 to day 14 among monoarthritic rats (Fig. 1).

3.2. Dose-dependent anti-hyperalgesic effects of celecoxib on monoarthritic rats

There was a dose-dependent anti-hyperalgesic effect of celecoxib on the thermal hyperalgesia in arthritic rats (Fig. 2). The control rats administered with 0.5% methylcellulose (twice daily, p.o.) showed constant thermal hyperalgesia after CFA injection. A lower dose of celecoxib (2 mg/kg, twice daily, p.o.) produced

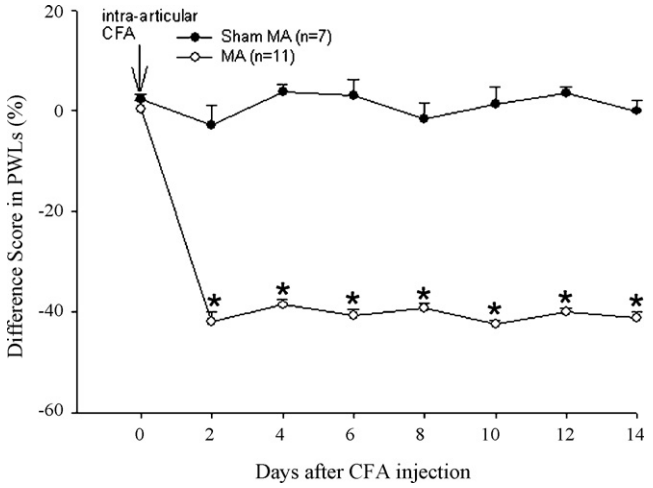

Fig. 1. Thermal hyperalgesia induced by unilateral intra-articular injection of complete Freund's adjuvant (CFA). The symbol ↓ indicates the time point of the CFA injection. The time from onset of radiant heat application to paw withdrawal was defined as paw withdrawal latency (PWL). Hyperalgesia indicated by PWL difference score is calculated as follows: difference scores (%) = [(PWL on the ipsilateral side − PWL on the contralateral side)/PWL on the contralateral side] × 100. Data were presented as mean ± S.E.M. $^*P < 0.05$ vs. sham MA group.

no anti-hyperalgesic effect, since no significant change of the difference score in PWLs was found between celecoxib (2 mg/kg) and vehicle groups ($P > 0.05$). In comparison with vehicle group, administration of 10 mg/kg celecoxib (twice daily, p.o.) markedly increased the difference score from day 10 to day 14 after CFA injection ($P < 0.05$). Celecoxib at the dose of 20 mg/kg (twice daily, p.o.) significantly enhanced the difference score in PWLs on day 8, compared with vehicle group ($P < 0.05$). From that day on, the difference score increased gradually and reached the peak on the day 14.

3.3. Anti-hyperalgesic effect of repeated EA and/or low dose of celecoxib on monoarthritic rats

To observe the anti-hyperalgesic effect of EA, EA was applied to GB-30 and GB-34 for 30 min every other day from the 4th day after CFA injection. Three treatments latter, difference score in PWLs

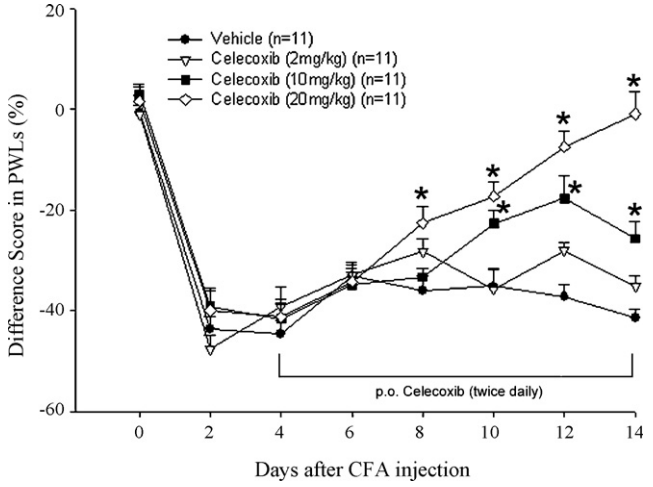

Fig. 2. Celecoxib administration showed dose-dependent anti-hyperalgesic effects on complete Freund's adjuvant (CFA)-induced monoarthritic rats. The symbol ⌊_____⌋ indicates the time course (from the 4th day to the 14th day) of the Celebrex (p.o., twice daily) treatments. Data were presented as mean ± S.E.M. $^*P < 0.05$ vs. vehicle group.

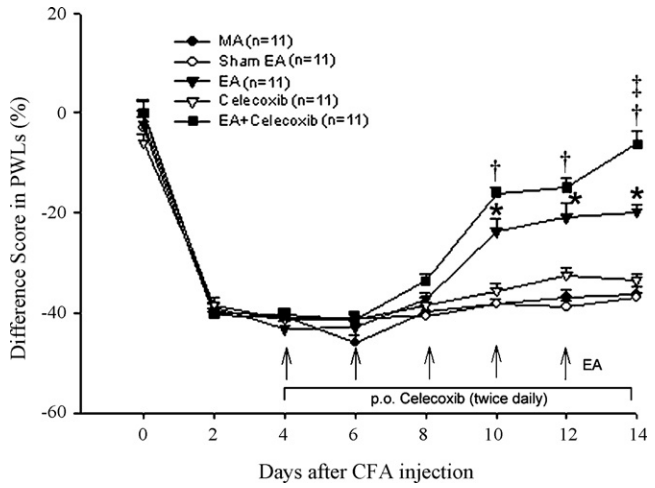

Fig. 3. Co-administration of electroacupuncture (EA) and low dose of celecoxib (2 mg/kg) synergistically inhibited thermal hyperalgesia in monoarthritis (MA). The symbol ⌊——⌋ indicates the time course (from the 4th day to the 14th day) of the Celebrex (p.o., twice daily) treatments. The symbol ↑ indicates the five EA treatments from the 4th day to the 12th day (once every other day), each treatment lasted for 30 min. Data were presented as mean ± S.E.M. $^{*}P < 0.05$ vs. MA group, $^{\dagger}P < 0.05$ vs. celecoxib (2 mg/kg) group, $^{\ddagger}P < 0.05$ vs. EA group.

increased markedly, and this increase was still observed on the day 14 ($P < 0.05$), indicating a therapeutic anti-hyperalgesic effect of repeated EA on monoarthritic rats. In contrast, sham EA showed no significant anti-hyperalgesic effect in comparison with MA group (Fig. 3).

When a combination of EA and sub-effective celecoxib was given repeatedly, difference score in PWLs remarkably elevated from day 10 to day 14, compared with 2 mg/kg celecoxib alone ($P < 0.05$). Furthermore, the group of EA plus low dose of celecoxib showed significant increase there when compared with EA group on day 14 ($P < 0.05$), indicating an synergistic anti-hyperalgesic effect of repeated EA and low dose of celecoxib on monoarthritic rats (Fig. 3).

3.4. Effect of EA and/or low dose of celecoxib on the level of COX and PGE_2 in the lumbar spinal cord in monoarthritic rats

To determine the effect of EA and/or low dose of celecoxib on the gene expression of spinal COXs, the mRNA levels of COX-1 and COX-2 in the spinal cord were assessed using semi-quantitative RT-PCR. Constitutive expression of both COX-1 and COX-2 mRNA were presented in the spinal cord of normal rats. However, the mRNA levels of neither COX-1 nor COX-2 were significantly increased from day 4 to day 14, the period we observed, and repeated EA and/or low dose of celecoxib treatment showed no significant effect to the COX-1 or COX-2 gene expression ($P > 0.05$) (Fig. 4A–D).

Spinal PGE_2 levels were measured by EIA. The spinal PGE_2 levels remarkably increased compared with the normal group 10 or 14 days following the CFA injection ($P < 0.05$). EA treatment repeated for 10 days significantly reduced the MA-induced up-regulation of PGE_2 level ($P < 0.05$), whereas low dose of celecoxib alone showed no significant effect. The group EA plus celecoxib demonstrated a stronger inhibition to the increased PGE_2 level in comparison with EA alone ($P < 0.05$) (Fig. 4E).

3.5. Suppression of EA and/or celecoxib on MA-induced up-regulation of IL-1β, IL-6, and TNF-α expression in the spinal cord

Using ELISA, protein concentration of spinal IL-1β, IL-6, and TNF-α were assessed (Fig. 5). Low levels of proinflammatory cytokines were presented in the normal rat spinal cord. Intra-articular injection of CFA produced a marked elevation in the levels of spinal proinflammatory cytokines during the chronic phases of inflammation ($P < 0.05$). EA repeated every other day for 5 times markedly reduced MA-induced up-regulated expression of proinflammatory cytokines ($P < 0.05$). Administration of EA combined with celecoxib showed a remarkable suppression of the increased expression of proinflammatory cytokines ($P < 0.05$), but no significant difference when compared with EA alone.

4. Discussion

Consistent with previous reports [11,36], the present study demonstrated that following intra-articular injection of CFA, thermal hyperalgesia developed within hours and lasted for more than 2 weeks in the injected paw. This exaggerated pain is thought to result from peripheral sensitization and central sensitization [13,25]. It is reported that the deep spinal nociceptive-specific neurons may play a crucial role in the development of central sensitization in pathological nociception [48]. Furthermore, there is increasing recognition that central neuroinflammation may mediate central sensitization. In the spinal cord, immunelike glia is often attractive candidates as mediators of central sensitization [42,43]. Following inflammation and damage of peripheral tissues, peripheral nerves and spinal nerves, spinal glia become activated, and then release a variety of algesic substances that enhance pain transmission, such as proinflammatory cytokines, ATP, nitric oxide (NO), prostaglandins and excitatory amino acids [28,30,33,39,42].

Prostaglandins have been shown to play a crucial role in the hypersensitivity of pain [25]. The increase in prostaglandin synthesis in spinal cord has been reported to be accompanied by a rapid enhancement of COX-2 gene expression in peripheral inflammation, and the increase is rapid and transient (from 3 to 24 h, at the acute state of inflammation) [5,32]. The COX-2 activity has been reported to parallel well with PGE_2 level and usually COX-2 activity was estimated by measuring the level of PGE_2 with the PGE_2 ELISA kit [52]. In the present study, we observed the elevated spinal PGE_2 levels from the 4th day after CFA injection (a chronic state of inflammation), but no significant change of COX-2 expression. The data suggested that the activity of COX-2, but not its levels, may play a key role in maintaining central sensitization in the peripheral hyperalgesia of the CFA-induced MA model [22].

Electroacupuncture has been investigated extensively with normal (uninjured) or acute hyperalgesic animal models [18], which cannot completely resemble clinical pathological chronic pain conditions. Furthermore, previous studies on chronic pain animal model using single EA treatment showed only brief (20–60 min) analgesia [3,37]. However, in a proportion of patients with nociceptive pain, acupuncture is often applied repeatedly so as to produce a long-term therapeutic pain relief for days rather than minutes or hours [8]. Previous studies demonstrated that repeated EA produced a therapeutic analgesic effect in different chronic pain models, including inflammatory, neuropathic, cancer and chronic visceral pain [12,15,17,29]. In the present study, we further proved the therapeutic anti-hyperalgesic effect of repeated EA treatment for once every other day, which was also consistent with considerable clinical and basic reports showing that acupuncture displayed notable analgesic effects in peripheral inflammatory and arthritic pain [6,36].

However, it is reported that chronic tolerance to 100 Hz EA was developed when 100 Hz EA was applied once daily for 6 days consecutively and the analgesic effects decreased with the prolonged treatment [20,23]. On the other hand, in the present study, dense-sparse frequencies (60 Hz for 1.05 s and 4 Hz for 2.85 s alternately)

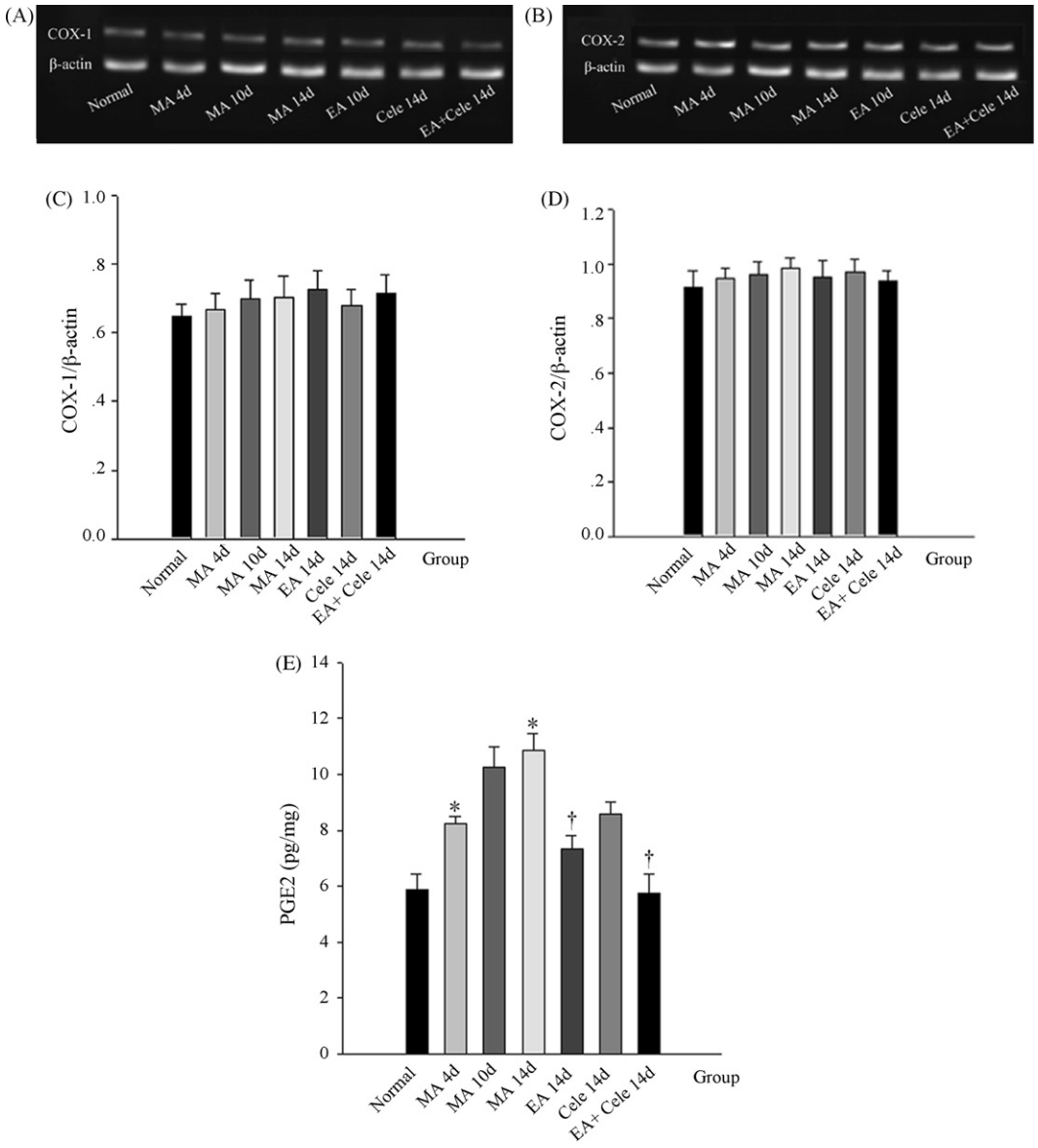

Fig. 4. Effects of electroacupuncture (EA) and/or celecoxib on the levels of cyclooxygenase (COX) (A–D) as well as prostaglandin E_2 (PGE_2) (E) in lumbar spinal cord in monoarthritis (MA). The expected PCR products of COX-1 (A) and COX-2 (B) were quantified and demonstrated, and the mRNA level was expressed as a ratio to that of corresponding β-actin (C, D). Data were presented as mean ± S.E.M. ($n = 6$ in each group at each time point). $^{*}P < 0.05$ vs. normal group; $^{\dagger}P < 0.05$, vs. MA 14 d group.

were selected. And we did not observe the decrease of the analgesic effects along with the treatment, which is in agreement with the previous studies using the same EA parameters with dense-sparse alternating frequencies [15,29,36].

The present study showed that although low dose of celecoxib (2 mg/kg) alone did not affect PWLs in MA rats, co-application of EA and low dose of celecoxib significantly intensified the anti-hyperalgesia of EA. Since synergy occurs when the effect provided by combination between two or more treatments administered simultaneously is superior to that produced by each treatment separately, the enhanced anti-hyperalgesia of combined treatment of EA and celecoxib indicating a synergistic anti-hyperalgesic effect. The outcomes were in parallel with a series of previous studies, both clinical and basic, that EA plus drug made additive [50] and/or synergistic [24,37,51] analgesic effects.

EA has been proved to have modulatory effect on the expression and release of various endogenous bioactive substances in the nervous system including opioids, monoamines, oxytocin and so on, which are important in the transmission and modulation of nociception [19,49]. However, the mechanism of EA analgesia has not been fully understood. Here we observed that EA could inhibit MA-induced up-regulation of PGE_2 level in the lumbar spinal cord, without affecting the gene expression of COX-2. These results indicated that EA might suppress spinal PGE_2 production through inhibiting COX-2 activity. It is found that increased COX activity with age is due to the activation of COX catalytic reaction by reactive oxygen species (ROS) without increased gene expression of COX-2 [4]. Previous study also showed that EA could minimize the oxidative modifications in ischemia-reperfused rats [34]. Therefore, it is likely that the regulation of EA on COX-2 activity might be mediated at least partly by inhibiting ROS activity. However, this part of work needs to be further investigated to be confirmed. In addition, in accordance with the recent report that repeated EA markedly reduced MA-induced up-regulation of spinal IL-1β, IL-6, and TNF-α mRNA level [38], we observed that EA remarkably inhibited the pro-inflammatory cytokines levels, suggesting that

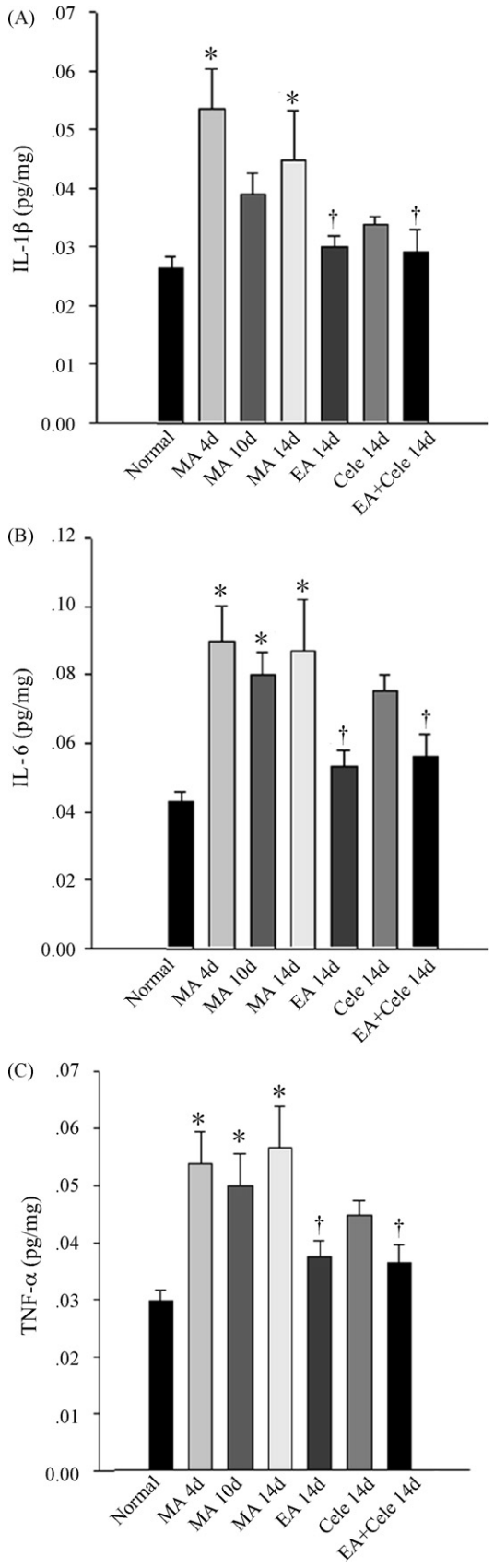

Fig. 5. Suppression of electroacupuncture (EA) and/or celecoxib on monoarthritis (MA)-induced up-regulation of proinflammatory cytokine IL-1β (A), IL-6 (B), and TNF-α (C) expression in the spinal cord. Data were presented as mean ± S.E.M. ($n = 6$ in each group at each time point) $^{*}P < 0.05$ vs. normal group; $^{\dagger}P < 0.05$ vs. MA 14 d group.

regulating the activation of glia could be another mechanism of EA analgesia.

The mechanism of the synergistic analgesic effect of EA combined with drug is probably more complicated, which has not been widely investigated. With the results of the synergistic analgesic effect on CFA-induced peripheral inflammation of the EA–indomethacin combination, it was considered that EA and indomethacin might act through different mechanisms, via the central release of endogenous opioids and inhibition of COX-2, respectively, to alleviate hyperalgesia [50]. In addition, it was reported that fluorocitrate, a glia metabolic inhibitor, synergized electroacupuncture analgesia, whose effect was probably due to block of the activation of spinal glia to reduce the release of pain enhancing substances in the spinal dorsal horn, and disrupt neuron-to-glia-to-neuron excitatory circuit [36,38]. It is clear that celecoxib selectively suppresses PGE_2 production through inhibiting COX-2 activity. According to the result of the intensified inhibition of co-application of EA and celecoxib to the spinal PGE_2, without affecting the gene expression of COX-2, we hypothesized that the synergistic anti-hyperalgesia may be mediated at least partly by modulating the same target, i.e., inhibiting the spinal COX-2 activity. Besides, no stronger inhibition to the spinal pro-inflammatory cytokines levels was observed, indicating that the synergistic effect of EA and celecoxib might have nothing to do with the spinal pro-inflammatory cytokines expression.

In conclusion, the present study demonstrated that repeated co-administration of EA and low dose of COX-2 inhibitor celecoxib produced greater anti-hyperalgesic effects than either agent alone in monoarthritic rats. The possible explanations of this reinforcing action are likely that they both affecting the activity of spinal COX-2 to inhibit the production of PGE_2 to attenuate the central hyperalgesia. This combination could not only increase the therapeutic analgesic effects of repeated EA, but also decrease the drug dose as well as the chronic side effects. Moreover, it would provide an experimental evidence for the clinical use of repeated-EA–COX-2 inhibitor combination for pain relief in arthritic patients.

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