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Inhibitory Effect of Electroacupuncture on Murine Collagen Arthritis and its Possible Mechanisms

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ABSTRACT

The influence of electroacupuncture (EA), a traditional Chinese medical treatment, on type II collagen-induced arthritis (CIA) was examined in DBA/1J mice in vivo. Mice were immunized intradermally twice at the 3-week interval with bovine type II collagen (C II). EA stimulation, begun on day 21 simultaneously with the second immunization, was applied at the acupoint equivalent to GV4 three times a week for 3 weeks. The results showed that EA delayed the onset, attenuated the severity of arthritis, and reduced the anti-collagen antibody level. Furthermore, we investigated the impact of EA on the productions of endogenous interleukin-1β (IL-1 beta) and prostaglandin E2 (PGE2), and the levels of IL-1 beta mRNA in splenocytes and synovial tissues from C II immunized mice on day 45 and cyclooxygenase-2 (COX-2) mRNA in lipopolysaccharide (LPS)-stimulated macrophages of normal mice by using reverse transcriptase-polymerase chain reaction (RT-PCR). EA stimulation significantly inhibited the concentrations of splenic endogenous IL-1 beta and serum PGE2. The expression of IL-1 beta mRNA in spleen cells was obviously down-regulated and that in synovial tissues was modestly affected by EA. COX-2 mRNA was highly expressed in cultured

peritoneal macrophages when stimulated with LPS. Previous treatment with EA also reduced LPS-stimulated induction of COX-2 mRNA. These data suggest that EA has an inhibitory effect on murine CIA, and the partial mechanism of its therapeutic result may be attributed to inhibiting the productions of IL-1 beta and PGE2 by suppressing the IL-1 beta and COX-2 gene activations.

Key words: Electroacupuncture, collagen arthritis, IL-1 beta, PGE2, COX-2, mRNA expression.

Acupuncture, a traditional Chinese medical therapy, has been used in China for several thousands of years, in the treatment of many acute and chronic disorders. It is reported that electroacupuncture (EA), electrostimulation through needles at acupoints, is widely used for pain relief and is shown good at checking arthralgia (1, 2, 3). Recently published data showed that EA could be applied for the treatment of immune related diseases, which did not only up-regulate immune functions, but could also down-regulate immune responses in some immunoinvigorated objects (4, 5, 6, 7). EA therapy has been shown to treat human rheumatoid arthritis (hRA) with satisfactory results(8, 9). However, little is known about the mode of action of EA on hRA.

Type II collagen-induced arthritis (CIA) in mouse has been proven to be a useful model of hRA. Although immune mechanisms that include both humoral and cellular immunity to C II have been implicated in the pathogenesis of the disease (10, 11), there is much evidence that anti-C II antibodies play an important role in the initiation of the disease (12, 13). In this animal model of arthritis, the systemic administration of interleukin-1β (IL-1 beta) increases the incidence and severity of murine CIA (14). Wooley et al. found that injection of antibodies to IL-1 beta and IL-1 receptor significantly reduced the incidence of CIA in mice in a dose-related manner (15, 16). These data may suggest that interleukin-1β (IL-1 beta) is involved in onset of the disease and joint destruction (17).

It is known that excessive production of

prostaglandin E2 (PGE2) is associated with many pathologic processes and plays a critical role in eliciting inflammation including rheumatoid synovium(18). PGE2 has been shown to trigger osteoclastic bone resorption and is detected in the culture supernatants of synovial cells from collagen immunized mice (19, 20). It is reported that administration of IL-1 beta increases the PGE2 synthesis (21). Our previous work found that EA could suppressed the IL-1 beta or LPS-induced production of PGE2 in rats (22).

Cyclooxygenase (COX) is a rate -limiting enzyme in prostanoids (PGs) biosynthesis. Recent reports that COX-2 is induced in cells exposed to proinflammatory agents such as cytokines and endotoxin, especially IL-1(23, 24), and that it is responsible for the production of PGs at the site of inflammation (25). Crofford, et al. demonstrated that IL-1 beta enhanced synthesis of COX-2 mRNA and protein in rheumatoid synthesis synovial explants and curtured rheumatoid synoviocytes (26). Several data have shown that selective inhibition of COX-2 induction or activity exerts a potent antiinflammatory effect (27, 28) and COX-2 is increased in parallel with the augmentation of PG production in inflamed tissues (29). Moreover, non-steroidal antiinflammatory drugs (NSAIDs) exert their antiinflammatory effect through reducing PGE2 contents via inhibition of COX activity (15). These observations suggest that COX-2 may play an important part in the over-production of PGE2 in RA.

The present work was undertaken to investigate the influence of electroacupuncture on the incidence and development of arthritis in murine CIA. EA was applied at a point anatomically equivalent to the acupoint GV4, since GV4 point has been reported to treat human immunological disorders and to exert immune modulation in rodents (30, 31). We also examined the possible mechanism of therapeutic effect of EA treatment on CIA via measuring the productions of IL-1 beta and PGE2, mRNA levels of IL-1 beta_and COX-2.

Materials and Methods

Animals.

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

Induction of arthritis.

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

Electroacupuncture treatment

The point equivalent to GV4, located below the spinal process of the second lumber vertebra, was selected in all EA treated rats. The treatment, begun on day 21 simultaneously with the second injection of collagen, was given 3 times per week for 3 weeks. Electrostimulation was carried out by a pulse generator NC-707 (Kimura Med. Ins. Co., Ltd, Tokyo, Japan). Briefly, mouse was fixed in a mouse holder (KN-330, Natsume Seisakusho Co., Ltd., Tokyo, Japan).

Two sterilized acupuncture needles were inserted into the point GV4 and 5 mm inferior to it. Electrical stimulation pulse with voltage ranging from 2 to 4 V and frequency of 1 Hz, was applied using two outlets via two needles. The intensity of stimulation was determined to be the minimum voltage to cause moderate muscle constraction. EA stimulation lasted for 15 min at each treatment. Holder control mice were restrained for 15 min in the same mice-holders with no special treatment.

Assessment of arthritis.

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

percentage of arthritic limbs per group of mice and compared at various time points. The incidence and day of onset of arthritis were also recorded.

*Measurement of anti-*CII *antibody.*

Blood samples were obtained from the mice by retro-orbital or cardiac puncture under ether anesthesia. Serum antibody levels to C II were measured using the commercially prepared Mouse IgG Anti-type II Collagen Antibody ELISA Kit (Chondrex, Redmond, WA, USA). The assay was done in duplicate according to the manufacturer's recommendations. Briefly, type II collagen coated 96-well microtiter plates were firstly washed with washing buffer. Wells were blocked with 100 μl of blocking buffer for 1 h at room temperature, and then washed three times. Test serum samples diluted at 1:1000 were added to each well (100 μl/well), and incubated for 2 h at room temperature. After washing, 100 μl of peroxidase-conjugated goat anti-mouse IgG was dispensed into each well. After incubation for 1 h, 100 μl of substrate, orth-phenylene diamine solution, was added to each well at a volume of 100 μl/well. The reaction was stopped by adding 50 μl of 2.5N sulfuric acid approximately 30 min later. The absorbance was read at 490 nm and the results were expressed as U (unit) per ml of serum.

Radiological evaluation.

All the mice were sacrificed on day 45 and limbs were amputated for radiological examination. Using a cabinet soft X-ray apparatus (SOFRON type SRO-M40, Soken Co., Ltd., Tokyo, Japan), radiography was performed with Fuji FR X-ray film under the following conditions: 45 cm distance; 30 kV tube voltage; 5 mA tube current; and 55 s irradiation time. Radiological assessment of each paw was made in three stages: normal = no obvious abnormal finding; mild = inflammatory sign of tissues, bone destruction in limited paw joints; severe = bone destruction of general paw joints. The condition of joints were graded as 0 (normal), 1 (mild) and 2 (severe), and then calculated to yield an average index of pathological changes in all paws from different groups.

Assay for IL-1 *beta and* PGE2 *production*

The concentrations of IL-1 beta in spleen and serum was measured using

commercial enzyme-linked immunosorbent assay (ELISA) kit for murine IL-1 beta_(Genzyme), and the concentration of splenic PGE2 was assesed by PGE2 enzyme immunoassay (EIA) kit (Cayman). To prepare splenic aqueous tissue extract for cytokine and PGE2 assays, organs were removed from mice, weighed, immersed in RPMI-1640 medium with the ratio of 100 mg spleen weight in 300 μl medium, and then homogenized by glass tissue homogenizer in an ice cold water bath. The homogenates were then contrifuged at 8000 g for 30 min at 4 ℃ and the supernatants were collected and stored at - 80 ℃ until use. The assays were performed in duplicate according to the manufacturer's recommendations.

Cell preparation for COX-2 mRNA *assay*

For investigating the influence of EA on COX-2 mRNA level, murine peritoneal cells were firstly primed by an intraperitoneal injection of OK-432 (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan), an inactivated and lyophilized low-virulence strain of streptococcus pyogenes preparation, at a dose of 0.1 mg per mouse in isotonic sodium chloride solution. Just after the priming injection, a single EA stimulation for 20 min at GV4 point was applied in 3 mice and other 3 as control group. After 48 hours, elicited peritoneal cells were harvested from the peritoneal cavity using ice cold Hank's Balanced Salt Solution (HBSS). The cells were suspended in RPMI 1640 supplemented with heat-inactivated 10% FBS after twice washings.

Equal numbers of macrophages (1×10^7 cells) were taken from each group and resuspended in the medium and cultured in a humidified, 5% CO_2 atmosphere at 37℃. After an adherence period of 2 hours, nonadherent cells were removed by washing HBSS and the remaining cells consisted of > 95% macrophages as judged by phagocytosis of latex particles and Diff Quik stain.

The macrophage monolayers of control group and EA treated group were incubated for 1-3 hours with culture medium in the presence of LPS (1 μg/ml, Escherichia Coli 026:B6, Sigma).

By the end of incubation at each time point, culture plates were centrifuged and cells were collected by trypsin-EDTA for COX-2 mRNA assay.

Detection of IL-1 *beta and* COX-2 mRNA *by* RT-PCR

For determination of splenic and synovial IL-1 beta and COX-2 mRNA levels, spleens were made into single cell suspensions ($1x10^7$/ml cells) as our previous report (32). The synovial biopsy tissues from ankle and carpal joints were immediately dissected from mice of each group and frozen in liquid nitrogen. Total RNA was isolated from the spleen cells, squashed synovium samples and LPS -stimulated peritoneal macrophages with chloroform and precipitated with ethanol. 2 μg of total RNA were reverse transcribed into cDNA (Superscript Preamplications System, GIBCO BRL) and the final volume of this mixture was adjusted to 200 μl with diethyprocarbonate (DEPC)-treated water for PCR assay.

The polymerase chain reaction was performed in a 20 μl reaction mixture containing 2 μl cDNA solution, 2 μl 10 × Ex Tag buffer, 1.6 μl (0.2 mM) dNTP, 13.3 μl DPEC-water, 0.1 μl (0.5 unit) Tag polymerase and 1 μl (5 pmol) β2m, IL-1 beta and COX-2 primers respectively.

The oligo-nucleotide used were as follows:

β2m sense primer
5'TGACCG -CTTGTATGCTATC3'
and β2m antisense primer
5'CAGTGTGAGC-CAGGATATAG3',
IL-1 beta sense primer
5'TTGACGGACCCCAAAAG-ATG3'
and its antisense primer
5'AGAAGGTCTCATGTCCTCA3',
COX-2 sense primer
5'TTCAAAAGAAGTGCTGGAAAAGGT3'
and COX-2 antisense primer
5'GATCATCTCTACCTGAGTGTCTTT3'.

Reactions were incubated in a thermal cycler for 35 cycles: denaturation 30 sec, 94℃; annealing 1 min, 55℃; extension 1 min, 72℃. The negative and position control cDNA was amplified in each PCR experiment. PCR products were run on 2% agarose gel in TAE buffer, and visualized using ultraviolet transillumination.

Statistical analysis.

Data presented are mean ± SE. The incidence of arthritis and radiological changes in different groups was compared using χ^2 analysis. Statistical analysis for the data of serum anti-CII antibody levels, anthritic index and concentrations of IL-1 beta and PGE2 were performed using ANOVA (analysis of variance) followed by Fisher's LSD test.

Results

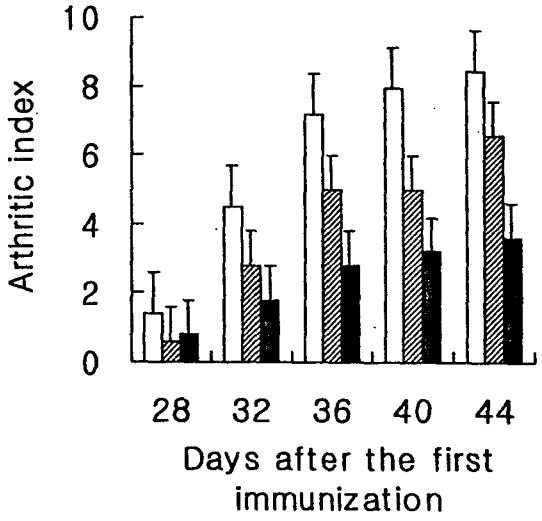
Influence of EA on the onset and severity of arthritis. The first experiment of present study was arranged to examine the influence of EA stimulation on the incidence and development of arthritis. Mice were treated with EA 3 time a week for 3 weeks starting on the day of the second immunization. As shown in Table 1, seven mice immunized with type II collagen alone all developed arthritis (100%) up to day 45.

Table 1 Influence of EA on clinical severity of cillagen arthritis.

Clinical severity	CII control	CII + Holder	CII + EA
Incidence of arthritis	7/7	5/6	5/6
Incidence of arthritic limbs	16/28	15/24	11/24
Day of onset	29.1±2.5	29.0±2.5	34.4±2.5#*
Average arthritic index	5.2±0.5	3.5±0.4**	2.1±0.4***

Data for incidence, arthritic limbs and day of onset were checked on day 45. Average arthritic index presents the average change of total arthritic indexes observed at all time points of each group. *p<0.05, **p<0.01 and ***p<0.001 compared to CII immunized control group; #p<0.05 vs holder control group

Figure1 effect of EA onarthritic index(AI) of collagen immunized mice



Bars are mean AI±SE at each time point for 6-7 mice in each group □:CII immunized control group; ▨:CII immunized plus holder treatment; ■:CII immunized plus EA treatment. *p<0.05, **p<0.01: significant difference vs immunized control group.

The immunized mice treated with EA and holder also produced high incidence of arthritis. No statistical significant reduction in the number of arthritic limbs was observed in EA-treated mice with non-treated and holder-treated control groups.

However, the significant difference was found between EA group and the two control groups in the time of onset of the disease.

The average arthritic indexes of whole

observed periods were significantly different among the three groups (see Table 1). The mice treated with EA reached an average score of only 2.1?.4, representing 60% and 40% decrease in clinical severity of the disease compared with immunized control and holder control groups. As shown in Figure 1, the disease severity increased progressively in immunized non-treated mice from day 28 onward, and began to be significantly severe from day 32 compared with that in EA-treated group.

An important consequence of immunization with CII is a rapid rise in serum IgG level to CII. Therefore, we next examined the serum IgG anti-CII titers in mice. As shown in Figure 2, the anti-collagen antibodies on day 21 just before the second immunization were at the similar levels among the three groups of mice. Significant reduction in anti-CII antibody level on day 45 was obtained in EA group as compared with immunized control group.

Holder treatment also affected the antibody level, but it was elevated than that in EA group and not different from that in control group immunized alone.

All diseased paws in each group were examined radiologically to observe the pathological change of joints. Table 2 shows the radiological stage in three groups 45 days after the first immunization.

Table 2 Comparision of radiological changes between control groups and EA group.

	Normal	Mild	Severe	Average index of radiological changes
CII control	11	2	15	1.00±0.16
CII + Holder	10	6	8	0.91±0.25
CII+ EA	17	5	2#**	0.38±0.20*

Data presented are numbers of paws in the immunized control mice and mice treated with electroacupuncture and holder. Significant difference was examined by using χ^2 analysis. *$p<0.05$ and **$p<0.01$ compared with immunized control group, #$p<0.05$ compared with holder group.

The incidences of severe radiological changes in arthritic non-treated control group and holder-treated group were much higher than that in EA-treated group. The average index of radiological changes for each paw of mice showed the joints in EA group were less pathologically affected. Effect of EA on IL-1 beta and PGE2 production The

concentration of IL-1 beta in splenic aqueous extract and serum of all collagen immunized mice was examined on day 45. As shown in Figure 3, the splenic IL-1 beta content was in a high level (7.11±.30 ng/g) in immunized control group. In contrast, C II immunized mice stimulated with EA produced remarkably lower concentration of IL-1 beta ($p<0.001$). The splenic IL-1 beta_production was only modestly inhibited by holder treatment and its increase showed significant difference with that in EA-treated group. The serum IL-1 beta in all experimental mice was not obviously detectable (data not shown).

Since PGE2 is associated with the severity of arthritis and its synthesis is partly regulatetd by IL-1 beta, the production of serum PGE2 in C II immunized mice and influence of EA on it were also examined. As seen in Figure 4, highly increased PGE2 production was detected in sera of control mice immunized alone, compared with non-immunized normal mice. The mice in holder control group also produced high content of serum PGE2. In contrast, EA strongly suppressed the collagen-induced PGE2 production, to the level of 60% of that in immunized control mice, and also showed much significant reduction than that in holder control mice.

Effect of EA on IL-1 beta and COX-2 mRNA expression. To determine whether EA stimulation affects mRNA level of IL-1 beta in CIA of mice, the expression of IL-1 beta mRNA in both splenocytes and synovial tissues was examined by RT-PCR analysis. Figure 5 showed that IL-1 beta mRNA was expressed in both splenic and synovial samples of collagen immunized mice of each group, but not in non-immunized normal mice. IL-1 beta mRNA in spleen cells of immunized non-treated mice was strongly expressed, and that in holder-treated and EA-treated mice was less expressed. The hold treatment had only mild effect on the down-regulation of IL-1 beta expression while EA treatment could obviously down-regulate IL-1 beta expression. Collagen also induced the expression of IL-1 beta mRNA in synovial tissue on day 45. EA treatment caused moderate decrease in synovial COX-2 mRNA expression, but holder treatment had less inhibition on collagen-induced synovial COX-2 mRNA level.

Since PGE2 synthesis is regulated by COX-2, the next step of experiments was arranged to examine the regulatory

effect of EA on this enzyme. Initial studies examined the expression of COX-2 mRNA in all collagen immunized mice, however its expression was not detectable in either spleen cells or synovial tissues. Therefore, we performed the extra experiment.

Three OK-432-injected mice were treated by EA once for 20 min two day before sacrificied and other three injected mice as control group. The peritoneal macrophages from these mice were taken, seperated and then cultured for 1, 2 and 3 h with the stimulation of LPS. As shown in Figure 6, LPS induced COX-2 mRNA expression from 1 h onwards and the maximal expression was reached by 3h of stimulation. It should be noted that pre-treatment of EA could dramatically down-regulate the LPS-induced expression of COX-2 mRNA. COX-2 gene expression in macrophages was not expressed at 2 h and its expression level was decreased 3 h after LPS stimulation in EA pre-treated mice.

Discussion

Application of acupuncture results in modulation of humoral or immune reactions (33, 34). It has been also suggested that acupuncture acts as a preventive means and strengthens physical resistance of the body, thus resisting the development of disease (4). In China, the treatment of chronic arthritis with acupuncture showed the attenuateing effects on the diseaset (8, 35, 36), but the mechanisms of acupuncture on chronic arthritis are poorly understood.

In our knowledge, this is the first report showing the influence of electroacupuncture on the CIA model, which is similar to hRA. The present results clearly show that the administration of EA, started on the day of the first immunization, did not significantly affect the incidence of arthritis but delayed the onset of disease, compared with the immunized control group and holder treated group. EA significantly inhibited the severity of arthritis from day 32 to day 44 with the average arthritic index 2.1?.4. Holder treatment also modestly inhibited the development of arhritis, but its average arthritic index was significantly higher than that in EA group. EA remarkably inhibited the anti-C II antibody levels vs. that in immunized control mice and

holder treatment did not show significant suppression on it. These results are interpretable that electroacupuncture might be a good prophylactic method in treating hRA. The fact that holder treatment affects the development of CIA on some aspects is needed to be further observed.

The precise mechanisms by which the administration of EA delayed the onset and suppressed the development of CIA is not clear. It is reported that administration of exogenous IL-1 beta can accelerate collegen-induced disease and IL-1 beta_mRNA has also been found in the spleens of collagen -immunized mice (14, 37). Now it is generally considered that IL-1 beta is implicated in the incidence of arthritis and joint destruction.

We have previously reported that treatment with EA suppressed the fever induced by LPS or IL-1 beta_in rats (22) and our unpublished data found that pretreatment of mice with EA modulated LPS-induced IL-1 beta production derived from macrophages. These reports and unpublished data may suggest that EA has a modulatory effect on macrophage-mediated immune responses and resulted in attenuation of the severity of CIA. Therefore, the next experiments were designed to examine the influence of EA on both protein level and gene expression of IL-1 beta induced by collagen. The present results conformed with our prediction that EA administration did not only remarkably inhibited the production of splenic IL-1 beta, but also notably down-regulated the expression of IL-1 beta mRNA in spleen cells and modestly decreased synovial IL-1 beta mRNA level. These results suggest that the therapeutic effect of EA on CIA may be related to the suppression of the production and gene activity of IL-1 beta.

It is generally accepted that PGE2 is involved in the inflammatory process and its synthesis is upregulated by IL-1 beta. Our previous study documented that EA exerts potent suppressive effect on the production of PGE2 in serum and brain tissue of LPS or IL-1 beta-stimulated rats (22).

Results from this study showed that EA stimulation was able to reduce the production of PGE2 induced by type II collagen in DBA/1J mice. The data confirmed the inhibitory effect of EA on the synthesis of PGE2 in mice, which may be attributed to the attenuation of

severity of arthritis, and also raised the question that if the inhibition of PGE2 production by EA is obtained through other means in addition to possible mechanism of via suppressing IL-1 beta level.

COX-2 is an inducible enzyme present especially in inflammatory tissues and mitogen-stimulated cells. It has been reported that COX-2 mRNA is expressed in cultured synovial fibroblasts in RA (26). An increasing number of studies demonstrate that IL-1 beta enhances the COX-2 mRNA level, COX-2 is participated in the synthesis of PGE2, and PGE2 production by IL-1 beta is mediated partly at the level of COX-2 (38, 39, 40). Therefore, we further detected the gene activity of COX-2 in LPS-stimulated peritoneal macrophages and effect of EA on it. RT-PCR analysis showed that LPS induced the expression of COX-2 mRNA, which was markedly down-regulated by pre-treatment of EA. The results that EA inhibited both the expression of COX-2 mRNA and the production of PGE2, indicated possibility that EA inhibited inducible COX-2 gene expression and consequently reduced the production of PGE2.

However, many other cytokines besides IL-1 beta_are involved in the onset and development of CIA (17), their functions and network in arthritis are not well understood.

The influence of electroacupucnture on cytokines has not been widely investigated at present. Furthermore, EA often possesses multiple effects (30) and various causative factors may be implicated in the incidence and development of CIA (17). Therefore, the precise mechanisms that EA inhibits the development of murine CIA is needed to be explained in many further studies.

In conclusion, electroacupuncture treatment was capable of delaying the onset and attenuating the severity of collagen arthritis in mice. It is suggested that at least some of the inhibitory effect of EA on murine CIA are mediated by the inhibition of production and activity of IL-1 beta, and the production of PGE2 via blocking the up-regulation of COX-2.

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