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원 저

# Effect of Low Frequency Electroacupuncture on the Chronic Monoarthritis and the Abundance of mRNA Encoding Substance P and Trk A mRNA levels at the Spinal Level in Rats

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## 초 록

## 저빈도 전침이 만성 단발성 관절염 흰쥐의 관절염 치료효과 및 척수에서의 P 물질과 trk A mRNA 발현조절에 미치는 영향

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**목적** : 본 연구는 (a) 저빈도 전침의 만성 단발성 관절염에 대한 치료 효과와 (b) 척수에서 동통과 관련되는 지표인 P 물질과 trk A mRNA의 발현조절에 미치는 영향을 연구하기 위해 실행되었다.

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***방법*** : Complete Freund's adjuvant를 실험동물의 우측 경족근관절에 주입하여 관절염을 유발시키고 9주 동안 관찰하였다. 만성 관절염의 최적의 치료조건을 찾기 위하여 각각 세 종류의 경혈(환도, 용천, 족삼리)과 전침자극강도(0.5, 1, 2 mA)를 사용하여, 행동학적 지표로서 동통을 측정하였다. 측정 결과 가장 큰 효과를 나타낸 군을 선택하여 RT-PCR 방법을 사용하여 P 물질 및 trk A mRNA의 변화를 척수 후각과 후근신경절에서 측정하였다.

***결과*** : 관절의 굴신을 통한 통증 검사와 족과관절 둘레 길이 측정을 통하여 관찰한 결과 원위부에 위치한 환도에 저강도의 자극을 시행한 경우(환도, 0.5 mA)가 가장 우수한 치료 효과를 나타내었다. RT-PCR을 시행한 결과, 환측 후근신경절의 P 물질이 관절염 유발 대조군의 경우 정상군에 비해 증가하였고 환도-0.5mA 군에서 다시 감소하는 것을 관찰하였으며, trk A는 관절염 유발군의 경우 척수 후각에서 양측성 증가를 나타내었고 이는 전침치료에 의해 다시 감소되는 것을 관찰하였다.

***결론*** : 환도 0.5mA의 저빈도 전침은 관절염을 효과적으로 감소시킬 뿐 아니라 관절염으로 증가된 후근신경절의 P 물질과 척수후각의 trkA mRNA 발현을 효과적으로 감소시켰으며, 이는 원위취혈로 선혈된 환도혈의 만성관절염에 대한 치료효과를 분명히 보여준다고 할 수 있다.

***Key words*** : Chronic Inflammatory Pain, Electroacupuncture, Spinal Cord, Substance P, Trk A

# I. INTRODUCTION

Chronic arthritic pain, though seldom life threatening, has been regarded as a serious medical problem due to its long persistence, slow recovery, and difficulty in medical treatment[1]. Chronic arthritis has been reported to respond favorably to acupuncture treatment both in human and animals[1~3], although the mechanisms underlying the therapeutic effects are not yet understood fully.

In animal models, complete Freund's adjuvant (CFA)-induced monoarthritis has been used to induce an arthritic disease that shows many pathological similarities with those of human arthritis[4,5]. Monoarthritis raises the expression level of various kinds of immediate-early genes[5,6] and also changes the expressions of many neuropeptides such as calcitonin gene related peptide(CGRP) and substance P(SP)[7,8] in the central nervous system. These observations suggest the existence of long-term functional adaptive changes in the first central relay of nociception during the development of the chronic pain states. Neurotrophins, including molecules of the nerve growth factor(NGF) family, are found to promote the survival of sensory neurons during development, and have various pharmacological effects.[9,10] Certain neurotrophins appear to play a part in these a daptive changes.[11,13] Recently it was reported that trk A, nerve growth factor(NGF) receptor, might participate in the adaptive mechanism of central nociceptive pathways observed in chronic pain states.[14,15]

The present study was designed (a) to identify the effect of electroacupuncture(EA) to relieve experimental inflammatory pain in the rat,

and (b) to assess the expression of SP and trk A at the mRNA level in the spinal cord and dorsal root ganglia(DRG) during CFA-induced monoarthritis for long-term adaptive response. And finally (c) to observe whether the adaptive changes mentioned above could be modified by optimized EA.

# Ⅱ. MATERIALS AND METHODS

## 1. General procedures

Female Wistar rats(Center for Animal Breeding, Beijing Medical University) weighing 150 ~180g were used throughout the experiment. Animals were housed under a 12h light/dark cycle, and water and food were given ad libitum. Animals were cared according to the guidelines of NIH and Korean Academy of Medical Sciences. All assays were conducted under the ethical guidelines set forth by the International Association for the Study of Pain.[16]

CFA was prepared by mixing incomplete adjuvant(Gibco BRL) and a suspension of killed mycobacterium tuberculosis(Human strain, 20 mg/ml, from the Chinese Inspection Institute for Biological Materials) in equal volume until a thick water-in-oil emulsion was formed.[17] Under light anesthesia by 10% chlorohydrate(300mg/kg, i.p.) rats were given injection on the right tibio-tarsal joint.

The skin around the site of injection was sterilized with 75% alcohol. Holding the right foot of the rat, the lateral malleolar fossa of the fibula was located. A 26 1/2-gauge needle was vertically applied into the skin, and was turned distal so as to inject into the articular cavity from the gap between the tibiofibular and tarsal bone until a distinct loss of resistance was felt. A volume of 40㎕ CFA was injected. Then animals which received CFA injection were randomly divided into each group.

## 2. Joint edema and pain assessment

The ankle circumference and the ankle flexion pain scores were observed 30min after the rats were held in specially designed holders with the hind limbs protruding and tail hanging freely. The circumferences of the ankles were measured around the lower edge of lateral and medial malleolus with a scaled soft ruler without elasticity to a precision of 1mm.

The circumferential difference in mm(CD) between the arthritic(ipsilateral) and the normal(contralate- ral) leg was calculated(CD = ipsilateral-contralateral)[18]. And for the ankle extension and flexion pain score, one ankle joint was gently extended to the plantar direction and flexed to dorsal direction for 5 times respectively with an inter-test interval of 5 seconds. The squeaking and leg-withdrawal scores were measured respectively[5]<Table 1>.

## 3. Electroacupuncture(EA)

We chose each three acupoints(① GB 30, located at the point of 5mm inside to the greater trochanter, ② ST 36, located at the lower

Table 1. Evaluation scale for extension and flexion test

| | |
|---|---|
| No squeaking or quick leg-withdrawal | 0 |
| Only one of squeaking or quick leg-withdrawal | 1 |
| Both squeaking and quick leg-withdrawal | 2 |

Responses to five flexions and extensions of ankle with an inter-test interval of 5 seconds.
Maximum score : 10

lateral of the knee joint, and ③ KI 1, on the sole of the border between the anterior and middle thirds of the foot, proximal to the 2nd and 3rd metatarsalphalangeal joint) with the three kinds of intensities(① 0.5, ② 1 and ③ 2mA) for deciding the optimal condition on chronic monoarthritis using 2Hz EA(totally 10 groups including control group). For EA stimulation, one pair of stainless steel needles were inserted into both the left and the right acupoints.

The shaft of the needle was 2mm for KI 1, 5mm for ST 36 and 10mm for GB 30 in length, so that the proper stimulation was provided by EA stimulation. The two pins were connected to the output terminals of the Hans Acupoint Nerve Stimulator(HANS, Beijing Hua-Wei Medical Instruments, China) that delivered square waves of 0.6ms pulse width, at a frequency of 2Hz, lasting for 30min[19].

The rats of the CFA group experienced the same procedure with EA group except for the electrical stimulation. The EA treatment was administered once a week on the day after the pain assessment from 1:00 PM to 6:00 PM. The room temperature was kept at 20℃.

## 4. RT-PCR

After the termination of the behavioral tests, rats of normal, GB 30~0.5mA and control groups were sacrificed by decapitation, and the DRGs of the lumbar(L) 4~6 were rapidly dissected. The lumbar spinal cord was isolated as quickly as possible and divided into the left and right hemicords, which were then further cut along the middle line into the ventral and dorsal parts. Female Wistar rats of the same age were also sacrificed in the same manner as the control group.

The DRGs and dorsal spinal cords were immediately homogenized in 0.5ml of pre-cooled Trizol reagent(Gibco, BRL) and the total RNA was extracted according to the manufacturers instruction. The total RNA was then subjected to electrophoresis in 1% agarose gel for confirmation of the structural integrity.

The absorbance at 260nm was measured for estimation of the RNA concentration. 2 $\mu$g of total RNA was reverse transcribed(RT) with oligo-$dT_{12-18}$ primers by using MMLV reverse transcriptase(Promega) in a total volume of 25 $\mu$l. PCRs were performed in 15 $\mu$l reaction volume containing 10mM Tris, pH 8.3, 50mM KCl, 100 M of each dNTP, 0.75 unit *Taq* polymerase(Promega), 15pmol of primer pairs, optimal amount of $MgCl_2$(3.0mM for trk A and 1.5mM for SP and GAPDH) and 1 $\mu$l cDNA.

The amplification was then performed in a thermo-cycler(Robocycler, Stratagene), beginning with a 5min pre-incubation at 94℃, followed by optimal cycles of 45s at 94℃, 60s at optimal annealing temperature and 80s at 72℃,

Table 2. Sequences of primers, fragment sizes, and annealing temperatures.

| cDNA | | Sequence of primers | Fragment size (bp) | Annealing Tm (℃) |
|---|---|---|---|---|
| GAPDH | S | 5-TCCCTCAAGATTGTCAGCAA 3 | 309 | 57 |
| | A | 5-AGATCCACAACGGATACATT 3 | | |
| Substance P | S | 5-TGAGCATCTTCTTCAGAGAATCGC-3 | 468 | 59 |
| | A | 5-ATCGCTGGCAAACTTGTACAACTC-3 | | |
| Trk A | S | 5-GCTGACCAATGAGACCATGCGGCAT-3 | 690 | 61 |
| | A | 5-GTGAGCAGCTCTGCCTCACGATGG-3 | | |

S : sense, AS : anti-sense

ending with a 5min incubation at 72℃[13]. The amplified products were electrophoresed on 2.0 % agarose gel to be visualized with ethidium bromide for fragment size estimation<Table 2>. For quantification of the band intensities, the images were scanned and analyzed by PHORETIX 1D gel analysis software.

### 5. Data analysis and statistics

For behavioral tests, the observation periods were divided into four sessions, the early acute (one day after inducing arthritis), late acute(1st week-3rd week), early chronic(4th week-6th week), and the late chronic period(7th week-9th week). The data in each session were averaged.

Continuous data were expressed in the form of "means±SEM", and tested for statistical significance with two way repeated measures ANOVA, followed by Dunns multiple comparison test. Discrete data were expressed as median± median-derived absolute deviation(MAD). Friedman ANOVA(repeated measures ANOVA on ranks) was used to analyze the change of pain scores with time, and Krus-ka-Wallis test was used to compare the groups at the same time point. With RT-PCR data, all values were normalized to GAPDH, which was used as an internal control to correct for subtle sample-to-sample differences in the amounts of starting material. Differences between groups were analyzed by ANOVA, followed by *post hoc* Newman-Keuls test for multiple comparisons as needed. A $p$ value less than 0.05 was considered statistically significant.

## Ⅲ. RESULTS

### 1. Development of arthritis and behavioral effect of EA

Observation of ankle circumference showed

a noticeable inflammation on the ankle, which appeared as early as 4h after the intra-articular injection of CFA and became substantial after 24h. The condition of arthritis remained stable at least up to the 9th week<Fig 1>.

The ankle flexion pain score soon reached over grade 8 at 24h after the injection, and changed in a concomitant manner with the increase of ankle perimeter. These signs were observed only in the injected(right) joint till the end of the observation period(the 9th week). Each group gained weight and remained normal except for the induced arthritis over the entire observation period.

It can be seen in <Fig. 1> that there was a tendency of gradual decrease of the joint swelling. We chose each three acupoints(GB 30, ST 36 and KI 1) and intensities(0.5, 1 and 2mA) for deciding the optimal condition on chronic monoarthritis using 2 Hz EA. In acute and primary chronic periods, no group showed a significant decrease compared to the control group. After the secondary chronic period, the significant differences appeared. GB 30~0.5mA and KI 1~2mA showed improvement in CD(each $p < 0.01$ and $p < 0.05$) <Fig 1>.

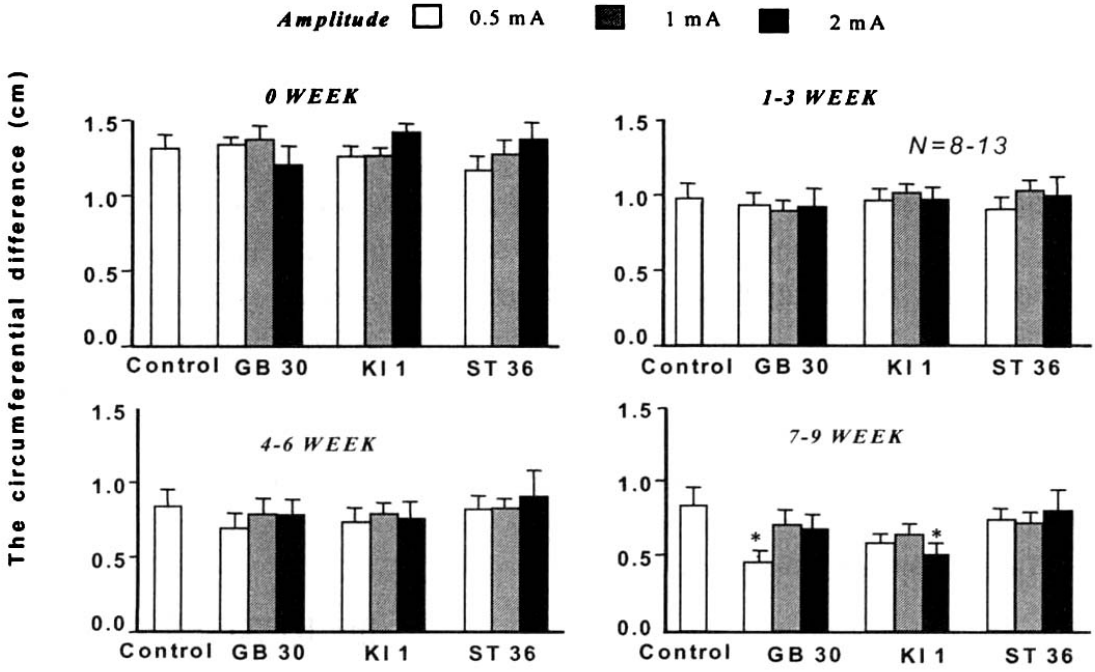


Fig 1. Evaluation of circumferential differences between ipsilateral and contralateral ankle joint at different time points after inoculation of complete Freund's adjuvant(CFA). Each column represents the means ± SEM.

* $p < 0.05$ compared with arthritic rats without electroacupuncture(control group) (Two way ANOVA followed by Dunn's multiple comparison test).

In the plantar extension pain test, only GB 30~0.5mA and KI 1~0.5mA showed significant difference comparing to control group(each $p < 0.05$). In the dorsal flexion pain reflex, there were differences in GB 30~0.5mA($p < 0.05$) <Fig 2 and 3>. The results showed that acupoint GB 30 with 0.5mA is the optimal condition for this arthritic model this parameter alleviated the arthritic pain most effectively among these conditions.

## 2. Changes of SP mRNA in DRG and DH

Electrophoresis of RT-PCR products on agarose gel after 35 cycles showed the presence of SP mRNAs in the DRG and lumbar dorsal horns. Amplification with the specific primers yielded products of expected size<Table 2>. The abundance of SP mRNA of L4-6 DRG showed an increase by 100% in the control group(CFA without EA) at the ipsilateral side ($p < 0.01$ compared with the contralateral side). This over expression of SP mRNA was not observed in rats receiving 2 Hz EA<Fig 4>. No such findings were observed at the lumbar spinal cord (data not shown).

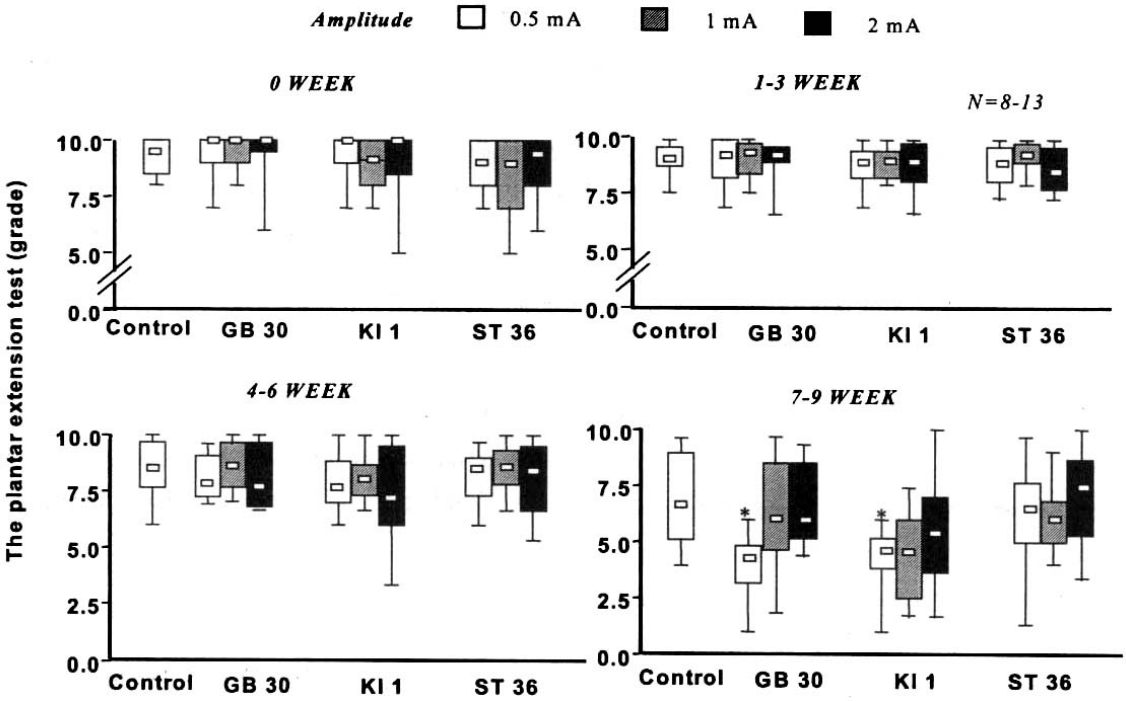


Fig 2. Ankle extension pain score based on the number of vocalization and withdrawal of the legs in the rat. The data are illustrated as notched box and whiskers plot, depicting the median(small square), 25th and 75th percentile line below and above the median. Whiskers extended down to the smallest value and up to the largest.

* $p < 0.05$ compared with control group by nonparametric Kruskal-Wallis test.

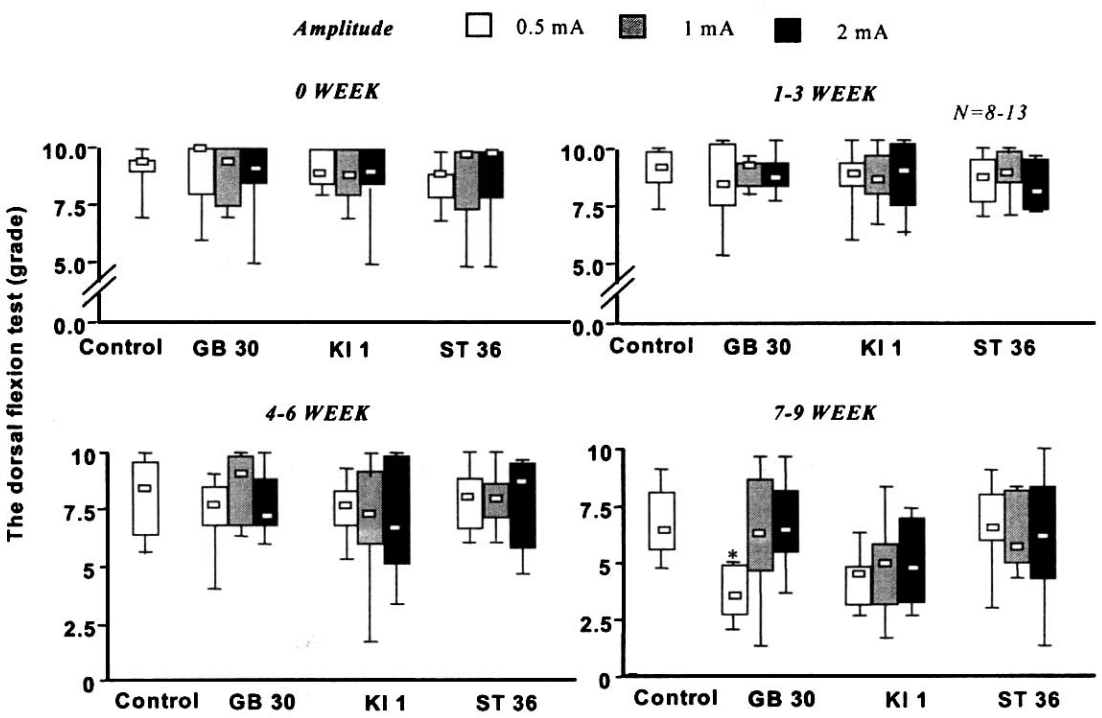


Fig 3. Ankle extension pain score based on the number of vocalization and withdrawal of the legs in the rat. The data are illustrated as notched box and whiskers plot, depicting the median(small square), 25th and 75th percentile line below and above the median. Whiskers extended down to the smallest value and up to the largest.

* $p < 0.05$ compared with control group by nonparametric Kruskal-Wallis test.

### 3. Changes of trk A mRNA in DRG and DH

RT-PCR analysis of RNA purified from lumbar spinal hemicords and DRG after 35 cycles showed that trk A transcripts were detected at the positions corresponding to the predicted size<Table 2>. Trk A mRNA levels increased significantly in both ipsilateral(+146%) and contralateral(+85%) lumbar spinal cord after C FA injection compared with the control group. These changes were not observed in rats receiving EA treatment(Fig. 5) The abundance of trk A mRNA in lumbar DRG, however, showed no significant differences among groups(data not shown).

## Ⅳ. DISCUSSION

Our results show that CFA injection into the right ankle joint evoked joint edema, and the

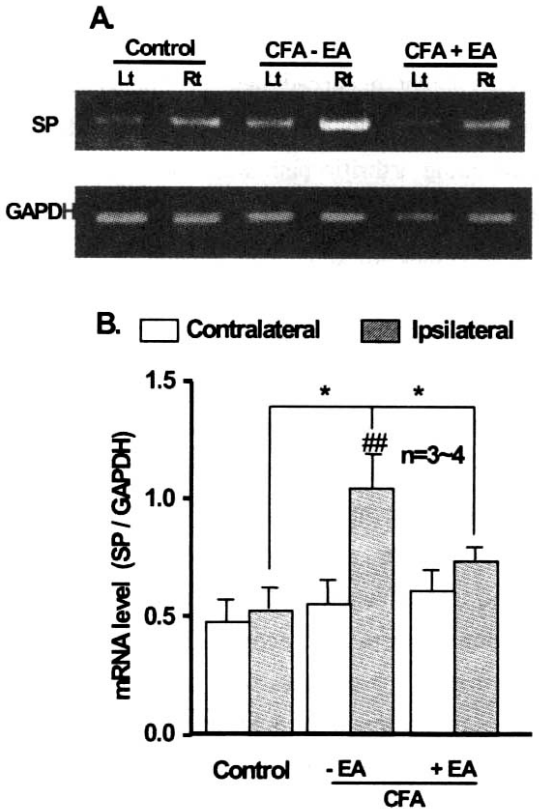


Fig 4. RT-PCR analysis of substance P(SP) mRNA expression in the DRG of Lumbar 4-6. Electrophoresis of RT-PCR products showed the presence of SP and glycealdehyde 3-phosphate dehydrogenase(GAPDH) mRNAs at the expected sizes. GAPDH was used a s an internal control. (A) and (B) SP mRNA levels of DRG(Lumbar 4 -6) increased at the 9th week after injecti on of complete Freund's adjuvant(CFA) at the ipsilateral side, which was suppressed and returned to control status by 2 Hz electroacupuncture(EA) at GB 30. Each column represents the means± SEM per group.

* $p < 0.05$ compared with arthritic rats without EA, ## $p < 0.01$ compared with contralateral side of the same group.

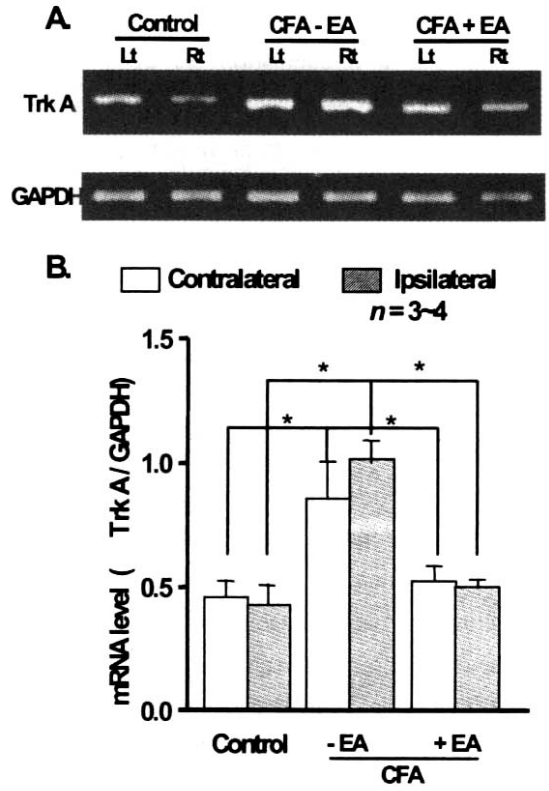


Fig 5. RT-PCR analysis of trk A mRNA expression in the dorsal horn of lumbar dorsal cord. Electrophoresis of RT-PCR products showed the presence of trk A and GAPDH mRNAs at the expected sizes. GAPDH was used as an internal control. (A) and (B) trk A mRNA levels of lumbar dorsal cord increased of 9th week of complete Freund's adjuvant(CFA) injection at both sides. This overexpression was suppressed and returned to control status by 2 Hz electroacupuncture(EA). Each column represents the means± SEM.

* $p < 0.05$ compared with arthritic rats without EA.

condition of arthritis remained stable at least up to the 9th week. Since the development and persistence of inflammation and pain found in the present study parallels with reports from other researchers, we think this model is useful for evaluating the therapeutic effects of EA and investigating the mechanism underlying this treatment.

## 1. Effects of EA on chronic arthritic pain in behavioral tests

2 Hz EA once a week at acupoint GB 30 for 8 weeks produced a cumulative therapeutic effect on CFA-induced monoarthritis. There are two main principles to select acupoints ; one is selecting the close acupoint(SCA) and the other is selecting remote acupoint(SRA) from the foci. The main reason causing arthritis is the factor of Wind-Damp(風濕 Poong-Sup, Feng-Shi) in oriental medical theory. GB 30 and ST 36 are known as the acupoints to treat arthritis by eliminating Wind-Damp according to SRA principle. KI 1 is comparatively near from ankle joint which can be used in ankle joint arthritis by SCA principle. Among the groups of three acupoints and intensities used here, as demonstrated, the group with 0.5mA at GB 30 alleviated the arthritic pain very effectively, but higher intensities(1mA, 2mA) were not so effective. The high amplitude seems to provide too excessive stress because of the anatomical character of GB 30 : sciatic nerve surpasses under GB 30 directly. The group of KI 1 improved to a degree, but didn't do so much as GB 30~0.5mA. ST 36 did not show any effects<Fig 1, 2, and 3>. The significant differences appeared only after the late chronic period, which might occurred because the dose of injection was too excessive or the indices of evaluating arthritic pain was not so sensitive to show differences until primary chronic period.

According to the experimental protocol, EA was administered once a week and pain assessment was performed one day prior to the administration of EA. Therefore the analgesic effect of EA should have been lasting as long as one week in order to be visible in the next pain assessment. This design was based on two considerations. In clinical practice, acupuncture treatment administered once a week can show a cumulative pain relieving effect after multiple treatments for patients with osteoarthritis[3] and for the treatment of migraine headache[20]. Liu *et al.*[17] using the same animal model of CFA-induced arthritis reported that the arthritic pain score could be suppressed by 100 Hz EA administered once a week for 9 weeks, with the pain-relieving effect fully manifesting during the period of 6~9 weeks. It is thus obvious that multiple treatments are needed in order to demonstrate the therapeutic effect of EA for chronic inflammatory arthritis, although the possible neurochemical mechanisms underlying this phenomenon remain to be elucidated.

## 2. The possible role of substance P in the DRG in mediating monoarthritic pain and the suppression of SP gene transcription by 2 Hz EA

The sustained activity of primary afferent

fibers that occurs after peripheral sensitization induces an increase in the efficacy of synaptic transmission between primary afferent fibers and dorsal horn neurons, a process referred to as central sensitization[21]. The specific mechanisms that underlie central sensitization are not fully understood. However, several in vivo and in vitro pharmacological studies have implicated synergistic interaction between SP-and NMDA-mediated events in the development and maintenance of inflammation-induced central sensitization[22]. Results obtained in the present study showed that the development of arthritic pain was accompanied by an increase in the abundance of SP mRNA in ipsilateral, but not in contralateral DRG. Interestingly, EA stimulation at GB 30 produced a reduction of arthritic pain, together with an abolishment of the increase in SP expression. These results support those observed by Liu[17] : that repeated TENS treatment for 9 weeks produced a progressive reduction of the arthritic pain as well as a significant decrease in the concentration of SP-immunoreactivity(ir) in spinal perfusate, representing a reduction in the amount of SP released from SPergic neurons.

Concerning the activity of SP in spinal dorsal horn, our study displayed that although the SP mRNA in the dorsal horn showed a tendency of increase during the development of arthritis, the change was not statistically significant. Honore et *al.*[22] also reported that long-term inflammation did not produce any significant change in spinal SP immunofluorescence. However, they found a significant increase in the same spinal sections of the substance P receptor(SPR) immunofluorescence in laminae I-II of the dorsal horn, as compared with control group rats. This suggested an increased release of SP from the afferent terminals that facilitated the mediation of arthritic pain. It would be interesting to observe whether these plastic changes would be blocked by EA stimulation.

### 3. The possible role of trk A in the dorsal horn in mediating monoarthritic pain and the suppression of trk A gene transcription by EA

In chronic pain states, several neurotrophins are considered to relate with central adaptive mechanism[13]. NGF is selectively expressed in nociceptive sensory neurons particularly containing sensory neuropeptides such as substance P and CGRP[10]. While the early component of NGF-induced hyperalgesia depends on peripheral events that lead to sensitization of nociceptors[9,11], the later phase of NGF effects likely involves central plasticity.[14] Trk A also seems to be involved in adaptive changes linked to long-lasting pain in deep layers of the spinal dorsal horn.[14,15] We have found in the present study that the abundance of trk A mRNA increase d in both ipsilateral and contralateral lumbar spinal cords after CFA injection. However no changes were found in DRGs. The contralateral expression of the trk A might have been influenced by the activation of supraspinal pathways. Several studies also observed bilateral neuronal activation in several hindbrain structures[6] and bilateral increase in neu-

ropeptide FF binding in the superficial layers of the dorsal horn[23], by activation of supraspinal pathways after induction of arthritis. Several investigators suggested that the interruption of NGF transduction in spinal cord may help to alleviate chronic pain.[14,24] EA administered to GB 30 might accelerate the decrease of trk A according to the decrease of arthritic pain.

In conclusion, this study showed that 2Hz EA treatments applied to GB 30 with the amplitude of 0.5mA alleviated arthritic pain and suppressed the expression of SP mRNA in lumbar DRGs and trk A receptor mRNA in the dorsal cord induced by CFA injection. These changes may serve as part of the mechanisms for the therapeutic effect of EA observed in the present study.

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