

Original Article

The Analgesic Effect and the Mechanism of Electroacupuncture on Thermal Hyperalgesia in the Rat Model of Collagenase-induced Arthritis: Mediation by Adrenergic Receptors

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국문초록

Collagenase-induced Arthritis Rat Model에서 Thermal Hyperalgesia에 대한 電鍼의 鎮痛效果 및 기전연구: Adrenergic Mechanism에 對한 研究

서병관 · 박동석 · 백용현

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목적 : Collagenase-induced osteoarthritis(OA) 동물 모델에서 전침의 adrenergic mechanism을 연구하고자 한다.

방법 : Collagenase-induced arthritis(CIA)를 유발하기 위하여 5주령의 male Sprague-Dawley rat의 뒷다리 좌측 무릎 관절에 0.05ml의 4mg/ml collagenase solution을 intra-articular 주입하고, 다시 4일 후에 같은 부위에 같은 농도의 collagenase solution을 intra-articular boosting injection 시행한 뒤, gross, histopathological features 및 biomarker activity 변화를 관찰하였다. 예비실험을 통하여 CIA rat model에서 진통효과를 발휘하는 것으로 확인한, 足三里 (ST₃₆)에 대한 저빈도 train pulse EA stimulation (2Hz, 0.07 mA, 0.3ms)을 침치료 방법으로 적용하였다. 전침의 진통기전을 확인하기 위하여, α 1-adrenergic antagonist (prazosin, 1 mg/kg, *i.p.*), α 2-adrenergic receptor antagonist (yohimbine, 2mg/kg, *i.p.*), α 1-adrenergic

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receptor agonist(phenylephrine, 2mg/kg, *i.p.*), α_2 -adrenergic receptor agonist(clonidine, 40 μ g/kg, *i.p.*)을 전침시행 20분 전에 복강 내로 전처치하였다. Tail flick unit(Ugo Basile Model 7360)을 이용하여 열자극에 대한 통증역치를 측정하였다.

결과 : 퇴행성관절염 징후(gross, histopathological features)와 통증역치의 변화가 최대값을 나타내는 CIA 유발 4주차에 저빈도 전침자극(train pulse, 2Hz, 0.07mA, 0.3ms)을 족삼리(ST₃₆)에 적용하였으며, 족삼리 전침의 진통효과는 α_2 -adrenergic receptor antagonist(yohimbine, 2mg/kg, *i.p.*)전처치에 의해 억제되었으나, α_1 -adrenergic antagonist(prazosin, 1 mg/kg, *i.p.*)전처치에는 억제되지 않았다. 또 α_2 -adrenergic receptor agonist(clonidine, 40 μ g/kg, *i.p.*)의 전처치를 통하여 유의한 synergistic analgesic effect가 관찰되었으나, α_1 -adrenergic receptor agonist(phenylephrine, 2mg/kg, *i.p.*)의 전처치는 전침의 진통효과에 synergistic effect를 미치지 않는 것으로 나타났다.

결론 : 저빈도 족삼리 전침은 CIA로 유발된 염증성 통증에 대하여 진통효과를 발휘하며, 이는 α_2 -adrenergic receptor에 의하여 매개되는 것으로 보이며 α_1 -adrenergic receptor는 영향을 미치지 않는 것으로 사료된다.

핵심 단어 : electroacupuncture, collagenase-induced arthritis, adrenergic receptor, antinociceptive mechanism

I. Introduction

Osteoarthritis(OA) is a progressive disease that affects joint cartilage and causes pain and disability. With an alteration of tissue homeostasis, an imbalance of anabolic and catabolic processes consequently leads to the loss of articular cartilage^{1,2)}. In the pathogenesis of OA, prostaglandin E2 (PGE2) plays an important role in joint inflammation, tissue destruction, and inflammatory pain³⁾. PGE2 is associated with the breakdown of cartilage extracellular matrix via upregulation of metalloproteinases(MMPs), bone resorption, osteophyte formation, and regulation of other proinflammatory cytokines, nitric oxide(NO), and connective tissue-degrading enzymes^{4,5)}. Prostaglandin E2(PGE2) is produced via cyclooxygenase(COX) enzymes from arachidonic acid, following the stimulation of proinflammatory cytokines, such as interleukin (IL)-1 β and tumor necrosis factor(TNF)- α , which are secreted from activated synoviocytes, mononuclear cells, or by articular cartilage itself and are related to the inflammatory process in OA⁶⁻⁸⁾.

Previous researches have been elucidated thera-

peutic properties of various modalities of acupuncture which alleviate pain, reduce inflammation and pathogenesis of OA⁹⁻¹¹⁾. Electroacupuncture (EA) improves OA of knee either on its own or as an adjunctive therapy¹²⁾, and acupuncture is significantly superior to non-penetrating sham acupuncture¹³⁾. EA can treat more effectively OA, relieve joint pain and improve joint function than conventional treatment such as oral administration of Diclofenac Sodium and injection of hyaluronic acid^{11,14,15)}. And the therapeutic effect of EA on OA partially achieved by modulation of parameters such as IL-1beta, IL-6, TNF- α , PGE(2a) and MMP-3^{15,16)}.

Repeated application of EA suppresses the peripheral inflammation response with acupoint specificity¹⁷⁻¹⁹⁾, and the analgesic effect of EA was partially mediated by the descending modulation of nociceptive processing correlated with various neural substrates, such as adrenergic systems in the neuropathic pain model and ankle sprain model²⁰⁻²²⁾.

The current study attempts to establish rat model of OA with pathophysiological similarity to human OA properties and investigate the antinociceptive effect of EA and its adrenergic mechanisms.

II. Materials and Methods

A. Subjects

Five-week-old male Sprague Dawley rats (Samtaco, Osan, Korea, 200g) were housed under specific pathogen-free conditions under controlled temperature ($22 \pm 1^\circ\text{C}$), humidity ($55 \pm 5\%$), and a 12:12 light-dark cycle (light on 6:00 AM to 6:00 PM) with food and water available *ad libitum*. Rats were randomly assigned to each experimental group with the initial injection of collagenase. All of the experimental procedures were conducted according to National Institute of Health guide for the Care and Use of Laboratory Animals and the ethical guidelines of the International Association for the Study of Pain (IASP). For the adaptation to the restraint, the experimental rats were properly fitted into a plastic holder ($5.3 \times 15, 6.3 \times 18$ cm in diameter \times length) with their tails protruding outside for 60 min/day for 7 days. Adaptation was conducted before TFL assessment and EA treatment.

B. The induction of collagenase-induced arthritis rat model

After one week of adaptation to the experimental environment, Male Sprague-Dawley rats were intra-articularly (i.a.) injected with 0.05 ml of 4 mg/ml type II collagenase solution (*Clostridium histolyticum*, type II; enzyme activity 425U/mg) in the left knee, followed by a boost injection 4 days after first injection.

C. Electroacupuncture

Followed by acclimation, EA treatment was performed at the end of the fourth week after initiation of CIA. The *Zusanli* (ST₃₆) acupoint was selected based on a human equivalent method of bone-length measurement at the anterior tibialis muscle of left hind limb. Two disposable sterile stainless-steel acupuncture needles (0.25mm in

diameter and 40mm in length) were inserted 5mm deep into the *Zusanli* acupoint and into another point about 5 mm distally and caudally from the *Zusanli* acupoint. For EA, negative electrode was connected to the *Zusanli* acupoint, and train pulses (2Hz, 0.07mA, 0.3ms) generated by an electrical stimulator (Nihon Kohden) were applied to the inserted needle for 30 minutes.

D. Pretreatment with agonists and antagonists

The α_1 -adrenergic antagonist (prazosin, 1mg/kg, i.p.), α_2 -adrenergic receptor antagonist (yohimbine, 2mg/kg, i.p.), α_1 -adrenergic receptor agonist (phenylephrine, 2mg/kg, i.p.), α_2 -adrenergic receptor agonist (clonidine, 40 $\mu\text{g}/\text{kg}$, i.p.) were dissolved in sterile 0.9% NaCl (normal saline, NS) and 10% DMSO (dimethyl sulfoxide) and were injected intraperitoneally 20 minutes before EA treatment.

E. Histopathological Analysis & Determination of PGE2 and COX-1 and -2 activity

At the end of the fourth week, the rats were sacrificed for histopathological analysis. The left knee joints were dissected out and fixed in 10% phosphate-buffered formalin for 2 days, decalcified in Calci-Clear Rapid solution (National Diagnostics, Atlanta, GA, USA) for 10 days, and then embedded in paraffin wax. A standard frontal section of 5 μm was prepared and stained with hematoxylin and eosin (H & E staining). The following six parameters were evaluated in the histological analysis: loss of the superficial layer, erosion of cartilage, fibrillation and/or fissures, disorganization of chondrocytes, loss of chondrocytes, and cluster formation. All sections were graded using a Biocom microscope with an Axio Cam camera (Carl Zeiss, Germany) by two independent observers who were blinded to the treatment groups.

After sacrifice, the serum from each subject was pre-diluted to 1 : 10, 1 : 50, and 1 : 200, and then

COX-2 inhibitor(DuP-697) was added in the test samples for the measurement of COX-1 activity. For the measurement of COX-2 activity, the serum was serially diluted to 1:10, 1:50, 1:200, and then COX-1 inhibitor(SC-560) was added. COX-1 and COX-2 concentrations in the diluted serum samples were analyzed by colorimetric assay(Cayman kit, Ann Arbor, MI, USA).

The activity of PGE2 was determined by enzyme-immunoassay kit(Cayman, Ann Arbor, MI, USA). Briefly, cells were grown in 6-well tissue culture plates until they were 80~90% confluent. The growth medium was replaced with serum-free medium M199. After 24 hours, culture supernatant was individually collected, filtered through 0.22 mm fiber, and frozen until the time of assay. Concentrations of PGE2 were measured at 405 nm by an ELISA reader.

F. Assessment of pain threshold

The pain threshold to thermal stimuli was assessed at the tail of a rat restrained in a plastic holder by the tail flick unit(Ugo Basile Model 7360, Comrio, Italy), according to D'Amour and Smith, 1941. The mean reaction time was assessed as the time lapse between the initiation of irradiation of infrared bulb of tail flick unit and the evasion of tail from three consecutive measurements at baseline, 10, 20, 30, 45 and 60 minutes. Preliminary exploratory experiments set the intensity as basal latency of 10 seconds in normal rats and the maximum cut-off latency as 20 seconds to avoid thermal injury. All the assessment was performed after acclimation to the laboratory room environment and restraint. Tail flick latency was expressed in seconds. The change of TFL was calculated as a percentage of change of tail flick latency :

$$\text{Acquired TFL change(\%)} = \frac{\text{post ATx TFL} - \text{baseline TFL}}{\text{baseline TFL}} \times 100$$

G. Statistical analysis

The results are presented as the mean \pm S.D. The significance of the statistical differences was determined using the non-parametric Friedman's rank test followed by Dunnett's post hoc test in a group, non-parametric Mann-Whitney U test between two groups, and non-parametric Kruskal-Wallis ANOVA followed by Dunnett's post hoc test among groups. $p < 0.05$ was considered statistically significant.

III. Results

A. Gross and histological arthritic features

The severity of joint edema and movement impairment and the change of pain threshold increased continuously and reached the maximum arthritic features from the fourth week. From the cartilage surfaces of the femoral condyle and tibial plateau of collagenase injected joints obtained sacrificed animals at the fourth week from first injection, the irregularity with superficial and deep fibrillation, loss of superficial-layer cells, erosion, and disorganization of chondrocytes were observed (data not shown).

B. Serological examinations

The activities of PGE2, COX-1, and COX-2 were evaluated from serum samples of the CIA group and normal control group at the end of the fourth week, and the results are shown in Fig. 1 & 2. In the CIA group, PGE2 production was significantly increased, as compared to the control group. COX-1 and COX-2 activities were also both significantly increased, as compared to the control group.

C. Inhibition of thermal hyperalgesia of electroacupuncture

The low frequency *Zusanli* EA treatment

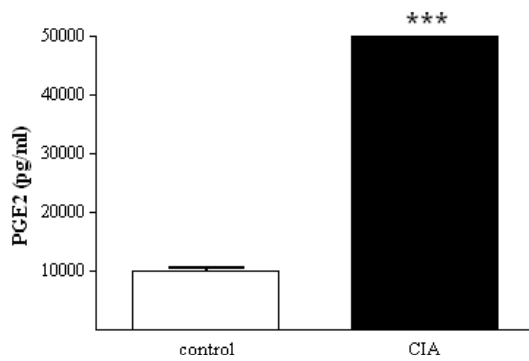


Fig. 1. Activity of PGE2 assayed by ELISA in serum from rats that received an intra-articular injection of normal saline(control) or 0.05ml of 4mg/ml collagenase solution(CIA)

PGE2 production was significantly elevated at 4weeks after CIA induction.

*** : $p < 0.001$. Mann-Whitney U test.

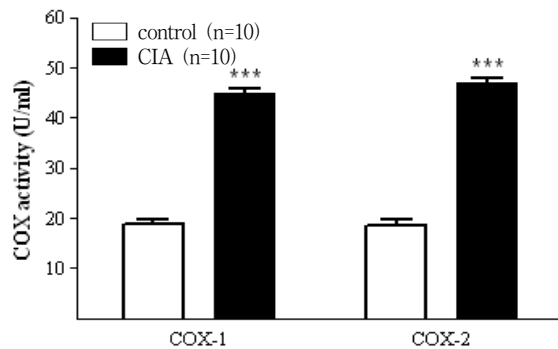


Fig. 2. Activity of COX-1 and COX-2, assayed by colorimetric assay in serum from the rats with intra-articular injection with normal saline(control) or 0.05ml of 4mg/ml collagenase solution(CIA)

COX-1 and COX-2 activities were significantly elevated at 4weeks after CIA induction.

*** : $p < 0.001$. Mann-Whitney U test.

significantly prolonged the decrement in TFL caused by collagenase injection and the increment in TFL was observed from the beginning of EA stimulation, reached the maximum value at 30 minutes and diminished at the end of the EA stimulation, but the latency difference between EA treated rats and non-treated control rats lasted for 60minutes after initiation(data not shown).

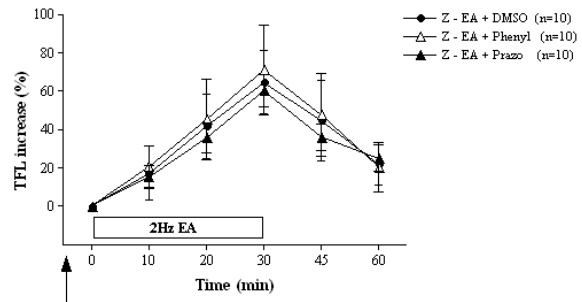


Fig. 3. Intraperitoneal pretreatment(arrow) of $\alpha 1$ -adren-ergic antagonist prazosin (Prazo, 1 mg/kg, *i.p.*, $n=10$) and $\alpha 1$ -adrenergic receptor agonist phenylephrine (Phenyl, 2mg/kg, *i.p.*, $n=10$) did not showed any significant changes of increment of tail flick latency(TFL) caused by low frequency(2-Hz) *Zusanli*(ST₃₆) electroacupuncture. DMSO, prazosin, phenylephrine were administered 20minutes before the initiation of EA stimulation.

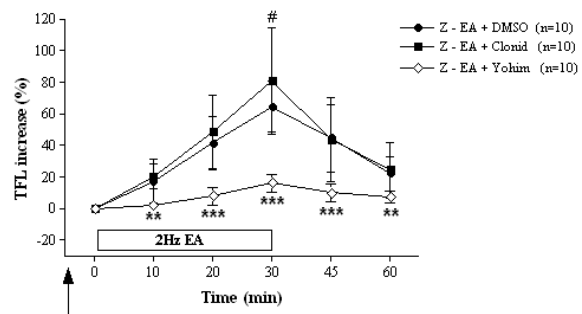


Fig. 4. Intraperitoneal pretreatment(arrow) of $\alpha 2$ -ad-nergic receptor antagonist yohimbine(Yohim, 2mg/kg, *i.p.*, $n=10$) significantly inhibited the analgesic effects of 2-Hz *Zusanli*(ST₃₆) electroacupuncture(Z-EA+DMSO, $n=10$)

There were significant differences in TFL at 10, 20, 30, 45, 60min after the initiation of EA stimulation between the Z-EA+Yohim and the Z-EA+DMSO group. And the synergistic increment in TFL was observed by pretreatment of $\alpha 2$ -adrenergic receptor agonist clonidine(Clonid, 40 μ g/kg, *i.p.*, $n=10$), and it is significantly differentat 30minutes comparing the Z-EA+DMSO.DMSO, yohim-bine, clonidine were administered 20minutes before the initiation of EAstimulation.

** : $p < 0.01$; *** : $p < 0.001$ indicate the significant differences between Z-EA+Yohim and vehicle control.

: $p < 0.05$ indicates the significant difference between Z-EA+Clonid and vehicle control(Kruskal-Wallis ANOVA followed by Dunnett's *post hoc* test).

D. Adrenergic mechanism study

The effects of adrenergic receptor agonists and antagonists on EA-induced analgesia in CIA are shown in Fig. 3, 4. The antinociceptive effect of *Zusanli* EA was significantly blocked by intraperitoneal(*i.p.*) pretreatment of α_2 -adrenergic receptor antagonist yohimbine(Yohim, 2mg/kg, *i.p.*, n=10), but not by α_1 -adrenergic antagonist prazosin (Prazo, 1 mg/kg, *i.p.*, n=10). There were significant differences in TFL at 10, 20, 30, 45, 60min after the initiation of EA stimulation between the Z-EA + Yohim and the Z-EA + DMSO group. The synergistic increment in TFL was observed by pretreatment of α_2 -adrenergic receptor agonist clonidine(Clonid, 40 μ g/kg, *i.p.*, n=10) with statistical significance, but not observed by α_1 -adrenergic receptor agonist phenylephrine(Phenyl, 2mg/kg, *i.p.*, n=10) respectively. Comparing between the Z-EA + DMSO and Z-EA + Clonid, there was significant synergistic increase in TFL at 30minutes.

IV. Discussion

Performing wide variety of studies on the therapeutic application of EA in human and animals have elucidated the analgesic effect and its mechanism of action for treating musculoskeletal disorders. Diverse modalities of acupuncture have been used extensively to treat OA of knee because of its safety and efficacy^{13,23}. It is reported that EA is more effective modalities than manual acupuncture²⁴ because EA is easily standardized by various stimulation parameters²⁵. EA is significantly more effective in treating OA of knee than placebo and diclofenac in a single-blind, placebo controlled trial¹¹ and can be a therapeutic options superior to oral administration of Diclofenac Sodium for OA patients needed to improve joint functions¹⁴ and intra-articular injection of hyaluronic acid¹⁵. In addition to alleviate pain in OA of knee(visual analogue scale(VAS) and Western Ontario and

McMaster osteoarthritis index(WOMAC)), EA also reduces stiffness(WOMAC) in patients with OA of knee, either on its own or as an adjunct therapy, with beneficial maintenance after one month from treatment¹². EA not only improves the intensity of pain and the ability of movement but also inhibits activity of IL-1beta, IL-6, PGE(2a), MMP-3 and TNF- α ^{15,16} and raises synovia SOD activity and lower MDA and NO content, which may contribute to their effect in relieving knee OA²⁶. However, in spite of the fluent investigations proving the effectiveness of EA for treating OA, there are some controversies, limitations and shortcomings about the antinociceptive properties of EA in rigid RCTs^{27,28}, and the need to investigate the analgesic effect of EA and its therapeutic mechanisms using more sophisticated animal models reflecting histology and pathophysiology of OA have been raised.

Numerous studies have observed the analgesic effect of EA and its mechanisms in various animal models such as neuropathic pain, inflammatory pain, post-herpetic neuralgia, and referred pain²⁹, these animal models have some limitations which do not reflect the pathogenesis of OA. Degeneration of articular cartilage plays a most important role in the pathogenesis of OA; therefore, the evaluation of histopathological changes of cartilage with pain-relieving efficacy is crucial for assessing the therapeutic effect and exploring its mechanisms. Transgenic modifications³⁰ and surgical procedures with load-bearing³¹ are complicated and take a significant amount of time to induce OA-like changes. Intra-articular injection of chemical substances, like papain³², needs a large injection volume, and its mechanism related to cartilage degeneration is unknown. CIA models have been reported to result in rapid, convenient and reproducible human OA-like changes within a short period with articular cartilage degeneration by direct digestion of cartilage and inflammatory reaction of joint tissues at an early stage³³. Gross, histopathological features and biomarker analysis changes consistent with human OA characteristics, including cartilage destruction

and related clinical features of pain, swelling, and movement impairment^{34,35} have been demonstrated in various species such as rabbits and mice³⁶. In the present study, we chose the rats for the precise assessment of pain-related behavior and the convenient application of EA.

In current study, the intra-articular injection of collagenase demonstrated the disease characteristics of OA not only such gross features as joint stiffness, swelling, movement impairment, pain but also histological features as the irregularity with superficial and deep fibrillation, loss of superficial-layer cells, erosion, and disorganization of chondrocytes. Also the activities of PGE₂, COX-1, and COX-2 were significantly increased. From these results we could postulate the success of manifestation of osteoarthritic features at 4 week after intra-articular collagenase injections in rats, in accordance with the previous studies³⁷.

Our preliminary experiments showed inhibition of thermal hyperalgesia of EA on ST₃₆ in a short term follow up. It is generally accepted that repeated application of EA suppresses the peripheral inflammation response with acupoint specificity¹⁷⁻¹⁹, and the analgesic effect of EA was partially mediated by the descending modulation of nociceptive processing correlated with various neural substrates²⁰⁻²², such as adrenergic systems in the neuropathic pain model and ankle sprain model^{38,39}. Local administration of EA to adjunctive points may be sufficient to treat knee OA. Taechaarpornkul and colleagues reported that the two point group received low-frequency (3Hz) of EA treatment at ST₃₅ and EX-LE₄ (*Neixiyan*) and the six point group received the same EA treatment at ST₃₅, EX-LE₄, ST₃₆, SP₉, SP₁₀ and ST₃₄ have been showed no significant difference statistically in mean WOMAC score, number of celecoxib capsule taken as rescue medicine, global assessment of global change and body weight change⁴⁰. Huang and colleagues reported that EA on *Zusanli* (ST₃₆), *Liangqiu* (ST₃₄) can raise synovial SOD activity and lower MDA and NO content, which may contribute to their effect in relieving knee OA in the rabbit²⁶. Also previous

researches have shown that EA into *Zusanli* (ST₃₆) elicits anti-hyperalgesia more effectively through the peripheral nerve receptors and the analgesic signals relayed to the cerebro-cerebellar and limbic systems^{41,42}.

The results of the present study demonstrate that the antinociceptive effect of *Zusanli* EA was blocked by intraperitoneal (*i.p.*) pretreatment of α 2-adrenergic receptor antagonist (yohimbine, 2mg/kg, *i.p.*), but not by α 1-adrenergic antagonist (prazosin, 1 mg/kg, *i.p.*). Synergistic effect was observed by pretreatment of α 2-adrenergic receptor agonist (clonidine, 40 μ g/kg, *i.p.*) with statistical significance, but not observed by α 1-adrenergic receptor agonist (phenylephrine, 2mg/kg, *i.p.*).

Our results confirmed those of Baek⁴³, Kim³⁸, Kim⁴⁴ and Koo⁴⁵. Kim and colleagues reported that intrathecal pretreatment of yohimbine (α 2-adrenergic receptor antagonist, 30 μ g) significantly blocked the relieving effect of 2 Hz EA on cold allodynia but not by prazosin (α 1-adrenergic receptor antagonist, 30 μ g)³⁸. Koo and colleagues reported that intrathecal administration of yohimbine, an α (2)-adrenergic antagonist, reduced the EA-induced analgesia in a dose-dependent manner, whereas terazosin, an α (1)-adrenergic antagonist, did not produce any effect³⁹. Baek and colleagues reported that intraperitoneal pretreatment of yohimbine blocked the analgesic effect of bee venom pharmacopuncture into *Zusanli* (ST₃₆) in the adjuvant-induced arthritis model⁴⁶. Kim and colleagues reported that intrathecal pretreatment of prazosin did not modify the antinociceptive effect of apipuncture but intrathecal pretreatment of idazoxan (an α 2 adrenergic receptor antagonist) reversed apipuncture-induced antinociception⁴⁴. On the contrary, Su and colleagues⁴⁷ reported that an activation of α 1- or α 2-adrenergic receptors would decrease the analgesic effect of EA, which injection into the lateral cerebral ventricles with α 1-(prazosin) and α 2-(Yohimbine) adrenergic antagonist enhanced the analgesic effect of EA and with the α 1-(Clonidine) and α 2-(methoxamine) adrenergic agonist decreased the analgesic effect of EA.

The role of adrenergic system in EA analgesia is

postulated due to the complication of adrenergic receptors, the acupoint being applied and the condition being treated. And the inhibition of peripheral adrenergic mechanisms is proportional to the effectiveness of the acupuncture analgesia induction⁴⁸⁾. And the frequency selected and the roles of post-ganglionic sympathetic nervous system are postulated as another major factors in acupuncture analgesia, the suppressive effects of low frequency EA on carrageenan-induced paw inflammation are mediated by sympathetic post-ganglionic neurons, while the suppressive effects of high frequency EA are mediated by the sympatho-adrenal medullary axis⁴⁹⁾. Another study suggests that EA produces anti-inflammatory effect in the release of catecholamines from post-ganglionic nerveterminals, which act on beta-adrenoceptors on immune cells to inhibit their migration⁵⁰⁾. Collectively speaking, further research on adrenergic mechanisms in acupuncture analgesia will be needed in the variety of stimulation parameters, acupoints, diseases, administration of antagonists and agonists, and observation of long-term effect and repeated treatments.

In result, we observed that maximal osteoarthritic features(gross, histopathological features) and maximal altered pain threshold were achieved in the fourth week after induction of arthritis by intra-articular injection of collagenase and low-frequency EA stimulation delivered into *Zusanli*(ST₃₆) acupoint reduced the raised pain threshold. And the antinociceptive effect of *Zusanli* EA was blocked by intraperitoneal(i.p.) pretreatment of α_2 -adrenergic receptor antagonist yohimbine, but not by a α_1 -adrenergic antagonist prazosin. Synergistic effect was observed by pretreatment of α_2 -adrenergic receptor agonist clonidine, but not by α_1 -adrenergic receptor agonist phenylephrine.

V. Conclusion

The results of the present study demonstrate

that low frequency EA(2-Hz) can relieve inflammatory pain in rats with collagenase-induced arthritis and the analgesic effect of EA can be mediated by α_2 -adrenergic receptor, but not a α_1 -adrenergic receptor.

VI. References

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