

Experimental Research

A COMPARATIVE STUDY ON HYPOTHALAMIC MECHANISMS OF ANALGESIA INDUCED BY FOUR KINDS OF ACUPUNCTURE THERAPIES IN ADJUVANT ARTHRITIS RATS

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ABSTRACT Objective: To compare the mechanisms of analgesia induced by four kinds of acupuncture therapies at the hypothalamic level in adjuvant arthritic rats. Methods: Forty-eight SD rats were randomized into normal, model, electroacupuncture (EA), filiform needle (FN), pricking blood-letting (BL) and point injection (PI) groups, with 8 cases in each. EA (20 - 100 Hz, 2 - 4 V and duration of 20 min), FN, BL, PI were respectively applied to "Kunlun" (昆仑 BL 60). Arthritis model was established by injecting complete Freund's adjuvant (0.1 mL) into the rat's right foot pad. Behavioral reactions, pain threshold (latency of tail flick to heat stimulation) and local swelling severity (foot volume) were detected; the contents of β -endorphin (β -EP) and adrenocorticotropin (ACTH) were assayed with radioimmunoassay; and the expression of pro-opiomelanocortin (POMC) mRNA in hypothalamus were determined with hybridization method. Results: The pain threshold was significantly enhanced by all the four kinds of acupuncture therapies, and the effects of EA and PI were more obvious ($P < 0.05$, $P < 0.01$). The severity of local swelling was markedly alleviated by all the four kinds of acupuncture therapies ($P < 0.01$) without significant differences among them ($P > 0.05$). The content of β -EP in the hypothalamus was obviously elevated by EA and FN ($P < 0.05$, $P < 0.01$), and the effect of EA was more obvious, but no marked effect of BL or PI on β -EP was found ($P > 0.05$). The content of ACTH in hypothalamus was considerably elevated by PI ($P < 0.05$), but not by the other three therapies. The expression of POMC mRNA in hypothalamus was significantly strengthened by EA and FN ($P < 0.01$), but not by the other two therapies. Conclusion: EA, filiform needle, blood-letting and point-injection all can produce analgesic effect in adjuvant arthritis rats, the effect of EA and filiform needle may be related to their resultant increase of hypothalamic β -EP, and that of point-injection related to the increase of hypothalamic ACTH level.

KEY WORDS Electroacupuncture Filiform needle Blood-letting Point Injection Hypothalamic β -EP and ACTH POMC mRNA expression

Pain is one of the symptoms frequently encountered in most diseases. As defined by Merskey, the president of the Terminology Committee of International Pain Association, pain is a kind of unpleasant experience or feeling in somatic perception or psychology, which occurs simultaneously with tissue injury, sometimes, is described as "an injury" but actually without any tissue injuries. In a word, it is a kind of subjective unpleasant sensation. According to traditional Chinese medicine (TCM), pain is induced by obstruction of meridians and dysfunction of *Zang-fu* organs, obstructed flow of blood, or insufficiency of *qi*, blood, *yin* and *yang* and the resultant malnutrition of *Zang-fu* organs and meridians due to invasion of "six kinds of climatic conditions (exogenous

pathogenic factors)", or internal damage by "seven emotions", overstrain, traumatic injury, or injury by insects, etc. In TCM terms, it is called, "obstruction gives rise to pain" and "insufficient nourishment also gives rise to pain". Since the sixty's of the 20th century, the mechanisms of acupuncture analgesia have been widely investigated. By means of considerable clinical and experimental studies, remarkable achievements in physiological and biochemical mechanisms of acupuncture analgesia and meridians have been gained. However, most of them are related to electroacupuncture (EA), but comparative studies on different acupuncture and moxibustion therapies have been hardly seen. In the present study, by means of radioimmunoassay and in situ hybridization and by

taking pain threshold, local swelling severity in the inflammatory area, the contents of β -endorphin (β -EP) and adrenocorticotropin (ACTH), and the expression of pro-opiomelanocortin (POMC) mRNA in the hypothalamus as the indices, the effects of four kinds of acupuncture therapies and their analgesic mechanisms are investigated in adjuvant arthritic rats, so as to optimize clinical analgesic measures and provide experimental basis for raising clinical therapeutic effects.

1 INFLUENCE OF FOUR KINDS OF ACUPUNCTURE THERAPIES ON PAIN THRESHOLD AND LOCAL SWELLING SEVERITY

1.1 Experimental animals

Forty-eight adult SD rats weighing (200 \pm 20) g with both sexes, half in each, were supplied by the Experimental Animal Center of Chengdu TCM University. The animals were fed in our lab for one week before the experiments for adaptation, and their pain thresholds were measured for selection. The animals with pain threshold of 2 - 10 s in tail flick latency were chosen as the subjects in this study, and the others were excluded.

1.2 Reagents experimental apparatus

1) Complete Freund adjuvant was supplied by the Sigma Chemical Company, and beetroxin injection supplied by Fuyu Pharmaceutical Factory, Jilin Province.

2) 125 I-EP and 125 I-ACTH radio-immunoassay kits were supplied separately by Beijing Haikerui Biotechnological Center and Tianjin Depu Biotechnological and Medical Product Company Limited; DF-110 electronic analytical balance was supplied by Changshu Scale Apparatus Industry Company of Chinese Light Industry Mechanical General Company, and 5417R low-temperature centrifuge supplied by German Eppendorf Company.

3) Antisense RNA probe kit for digoxigenin (Dig)-labeling POMC was supplied by Neurophysiological Laboratory of Second Military Medical University; DEPE supplied by Sigma Chemical Co., PHS-

3C PH Meter supplied by Shanghai Leilin Equipment Factory; and DZ-9556 Horizontal Rocking Bed and HZ2258 Thermostatic Water Bath Shaker were supplied by Taicang Scientific and Teaching Apparatus Factory.

1.3 Grouping and treatment methods

The animals were randomly divided into 6 groups with 8 in each. The treatments given in different groups were as follows. Normal group: Normal animals without modeling were fixed once for 20 min in the same way as that in the other groups. Model group: Complete Freund's adjuvant (0.1 mL) was injected into the right foot pad in the direction towards the ankle joint one day before experiments, and other treatments were the same as those given in the normal group. Electroacupuncture (EA) group: Modeling was made one day before experiments in the same way as that in the model group, and EA was applied in the following way. The rats were fixed, and a filiform needle with 13 mm in length was perpendicularly inserted into the right "Kunlun" (昆仑 BL 60) to the depth of about 3 - 5 mm. One of the paired electrical leads of a WQ-10C multiple electrical apparatus was connected to the needle, and the other connected to the root of tail wrapped by cotton soaked with normal saline. Sparse-dense waves with 20 - 100 Hz in frequency and 2 - 4 V in strength, which was gradually intensified to cause the right lower limb to twitch slightly, were applied for 20 min. Filiform needle group (FN): Modeling was made one day before the experiments in the same way as that in model group, and the animals were treated in the following way. Animal fixation and point selection were the same as those in EA group, and a filiform needle with 13 mm in length was perpendicularly inserted into the right "Kunlun" (BL 60) to the depth of about 3 - 5 mm, and rotation rightward and leftward to 90 degrees was alternatively used nine times for 2 min, and the needle remained for 20 min. Blood-letting group (BL): Modeling was made one day before the experiments in the same way as that in model group, and blood-letting was made in the following way. Animal fixation and point selection were the same as those in EA group, and fast pricking for blood-letting was

made with a three-edged needle to cause 2 drops of blood out. Point injection group (PI): Modeling was made one day before the experiments in the same way as that in model group, and point injection was made in the following way. Animal fixation and point selection were the same as those in EA group, and 0.1 mL of beetroxin injection was injected into the point.

1.4 Indices and measurements

1) Pain threshold: Pain threshold was determined by using tail flick latency induced by the tail being immersed in a thermostatic water bath of 60 in temperature. Pain threshold was measured three times with an interval of 3 min between two measurements, and the mean value was taken as the pain threshold. The swelling severity of articulationes digitorum pedis was estimated by the volume of the digiti pedis according to a related reference^[1].

2) -EP content: Following termination of the experiments, 30 mg of the hypothalamic tissue was sampled to be put into 1 mL of normal saline at 100, and boiled for 5 min, which was then cooled rapidly, and processed into homogenate by adding 0.1 N of HCL. After being maintained for 1 - 2 h, the homogenate was neutralized by adding 0.1 N of NaOH, which was centrifuged, and the supernatant fluid was collected and stored at -20 until analysis. According to the indications of the kit, -EP content was assayed by radioimmunoassay in the Department of Isotope, Basic Medical College, Huaxi Medical University.

3) ACTH content: Following termination of the experiments, 400 mg of the hypothalamic tissue was collected, to which 1 mL of normal saline was added to be processed into homogenate, which was stored in refrigerator at 4 for 1 - 2 h and then centrifuged, and the supernatant liquid was stored at -20 until analysis. According to the instructions in the kit, ACTH content of hypothalamic tissue was assayed by using radioimmunoassay.

4) Hypothalamic POMCmRNA expression: a) After killing the animals, the hypothalamus tissue was taken out and put into 4% paraformaldehyde/0.1 M PBS (containing 1/1 000 DEPC, pH 7.4) to be fixed for 2 h. b) Conventional anhydration, soak-

ing in wax and embedding were made, and the tissue sample was sliced at 6 μm onto microscope slides. c) Following conventional deparaffin and anhydration, the sections were put into pepsin-newly diluted by 3% H₂O₂ citric acid (2 drops of concentrated pepsin were added into 1 mL 3% citric acid in misce bene) and flashy at 30 for 40 min, which was washed with 0.5 M PBS three times, 5 min every time, and washed with distilled water once. d) Exposure of mRNA nucleate segments: Drops of pepsin newly diluted by 3% H₂O₂ citric acid were added onto the sections (2 drops of concentrated pepsin were added into 1 mL 3% citric acid in misce bene), which was digested at 37 for 40 min, washed with 0.5 M PBS three times, 5 min each time, and washed with distilled water once. e) Prehybridization: 20 mL of 20% glycerin was added to the bottom of a dry box for hybridization for maintaining the humidity. The prehybridization liquid, 20 μL for each section, was added, and stored in a thermostat container at 37 for 3 h. The excrescent liquid was extracted without washing. f) Hybridization: The hybridization liquid, 20 μL for each section, was added onto the slices, and a cover slip specifically for in situ hybridization was put on the sample slice, which was stored in a thermostat container at 40 overnight. g) Washing after hybridization: After removal of the cover slip, the tissue sections were washed with 2 ×SSC at 37 for 5 min, twice, with 0.5 ×SSC for 15 min once, and 0.2 ×SSC for 15 min once separately. h) Confining liquid was added at 37 for 30 min, and the excrescent liquid flung off without washing. i) Biotinylation rat antispectacle digoxin was dropped to the sample sections which were stored at 37 for 60 min, and washed with 0.5 M PBS for 5 min, 4 times. j) Application of SABC to the samples at 37 for 20 min, followed by washing with 0.5 M PBS for 5 min, 3 times. k) Biotinylation peroxidase was applied to the samples at 37 for 20 min, followed by washing with 0.5 M PBS for 5 min, 4 times. l) DAB coloration was made by using a DAB kit in the way that 1 mL of distilled water was added with one drop of developers A, B, and C, respectively, mixed evenly and added onto the samples for coloration for 30 min,

and then washed adequately by water. m) Counter-stain was made by hematoxylin, and then washed adequately by water. n) Anhydration was made by alcohol, followed by transparency with xylene and mounting.

Computer-aided image analysis: The optical density (OD), integral OD, and mean OD values of the samples were observed and recorded respectively.

1.5 Statistical analysis

All the data collected were shown by mean \pm standard deviation ($\bar{x} \pm s$), and analyzed with one-way ANOVA and t -test by using SPSS10.0 software package.

2 RESULTS

2.1 Formation of inflammatory pain

Redness and swelling were found in the ankle and digiti pedis of the right lower limb with reduced movement, limping or avoiding touching the ground while walking, which occurred the next day after injection of the complete Freund adjuvant. The apparently decreased local pain threshold, augmented swelling and elevated cutaneous temperature were the typical indications of the formation of inflammatory pain.

2.2 Influence of the four acupuncture therapies on pain threshold (see Table 1)

Table 1 Influence of four kinds of acupuncture therapies on pain threshold and local swelling ($\bar{x} \pm s$)

Groups	n	Pain threshold (s)	Local swelling severity (mL)
Normal	8	5.73 \pm 1.55	0.25 \pm 0.04
Model	8	1.96 \pm 0.47	0.49 \pm 0.09
EA	8	4.06 \pm 1.55	0.41 \pm 0.02
FN	8	3.72 \pm 0.50	0.38 \pm 0.01
BL	8	3.38 \pm 0.76	0.41 \pm 0.03
PI	8	4.03 \pm 1.13	0.41 \pm 0.02

$P < 0.05$, $P < 0.01$ vs normal group; $P < 0.05$, $P < 0.01$ vs model group;
 $P < 0.05$ vs EA group; $P < 0.05$ vs BL group

It is shown in Table 1 that the pain threshold in model group was obviously lower than that in normal group ($P < 0.01$), and those in EA, FN, BL and PI groups were all raised after treatment. Compared with model group, the difference was significant in EA, PI, FN and BL groups ($P < 0.05$, $P < 0.01$). Compared with normal group, no difference was found in EA and PI groups ($P > 0.05$), but significant differences were found in BL and FN groups ($P < 0.05$). It is indicated that all the 4 therapies could raise the pain threshold in arthritis rats, especially, the effects of EA and point injection were apparent.

Compared with normal group, the difference in alleviating local swelling was very significant ($P < 0.01$) in all the 4 treatment groups, and compared

with model group, the difference was also very significant in the 4 treatment groups ($P < 0.01$). No differences were found among the 4 treatment groups ($P > 0.05$), indicating that all the 4 therapies could obviously improve local swelling induced by the inflammation in arthritis rats but had no considerable differences among them.

2.3 Influence of the four kinds of acupuncture therapies on the contents of hypothalamic -EP and ACTH (see Table 2)

It is shown in Table 2 that the -EP content in the hypothalamus in model group was significantly higher than that in normal group ($P < 0.05$), indicating that inflammatory pain itself could activate the endogenous opiate peptide (EOP) system. The -EP

contents in EA and FN groups were obviously higher than that in model group ($P < 0.05$, $P < 0.01$). The β -EP content in EA group was obviously higher than those in BL, PI and FN groups ($P < 0.05$, $P < 0.01$). No significant differences were found among FN, BL and PI groups ($P > 0.05$). The results indicated that both handle needle and EA could elevate the β -EP content in the hypothalamus, of which the effect of EA was more remarkable, but the effects of blood letting and point injection were not of significance.

Regarding hypothalamic ACTH content, results showed that the ACTH content in the hypothalamus in Group PI was higher than that in model group ($P < 0.05$), and no differences were found among the other groups, indicating that point injection could elevate the ACTH content in the hypothalamus, while the effects of EA, blood-letting and filiform needle were not significant.

2.4 Influence of the four kinds of acupuncture therapies on Expression of POMC mRNA in the hypothalamus (see Table 3)

It is shown in Table 3 that the expression of POMC mRNA in hypothalamus in model group was significantly higher than that in normal group ($P < 0.05$), indicating that inflammatory pain itself could increase POMC mRNA expression in hypothalamus. POMC mRNA expression in EA and FN groups was significantly stronger than that of model, BL and PI groups ($P < 0.01$, $P < 0.05$). No differences were found between BL and model groups and between PI and model groups ($P > 0.05$). It indicated that EA and filiform needle could strengthen POMC mRNA expression in hypothalamus, while the effects of blood letting and point injection were not of significance ($P > 0.05$).

3 DISCUSSIONS

3.1 Significance of the four acupuncture therapies in the content of β -EP in hypothalamus

In the central nervous system, almost all the nerve fibers containing β -EP origin from the arcuate nucleus and its surrounding area, and some others from the nucleus solitarius. Hypothalamus contains

Table 2 Influence of the four kinds of acupuncture therapies on hypothalamic β -EP and ACTH contents ($\bar{x} \pm s$, pg/mL)

Groups	n	β -EP	ACTH
Normal	8	338.67 \pm 48.84	23.72 \pm 8.73
Model	8	446.20 \pm 57.77	14.31 \pm 8.05
EA	8	718.49 \pm 213.95	18.54 \pm 13.55
FN	8	524.10 \pm 230.26	26.98 \pm 17.39
BL	8	483.26 \pm 131.77	19.25 \pm 10.45
PI	8	476.08 \pm 65.44	33.89 \pm 19.81

$P < 0.05$ vs normal group; $P < 0.05$, $P < 0.01$ vs model group; $P < 0.05$, $P < 0.01$ vs EA group

Table 3 Influence of four kinds of acupuncture therapies on POMC mRNA expression in the hypothalamus ($\bar{x} \pm s$)

Groups	n	OD values
Normal	8	2632394 \pm 129.76
Model	8	3811038 \pm 120.94
EA	8	7559485 \pm 122.73
FN	8	8226627 \pm 124.83
BL	8	4509305 \pm 117.66
PI	8	4017202 \pm 133.80

$P < 0.05$ vs normal group; $P < 0.01$ vs model group; $P < 0.05$ vs EA group; $P < 0.05$ vs BL group

β -EP in high concentration, and the nerve fibers containing β -EP widely project to various parts of the brain. β -EP involves various physiological functions, particularly in alleviating pain. A number of studies have shown that β -EP is involved in acupuncture analgesia. The arcuate nucleus and preoptic area of hypothalamus are the two main nuclei related to opioid-induced analgesia. The nerve fibers containing β -EP originating from the arcuate nucleus reach not only to the periaqueductal gray (PAG), but also to the septal area, nucleus accumbens septum, and nucleus ceruleus. The arcuate nucleus can activate neurons in PAG, but inhibit the nucleus ceruleus. Since the nucleus ceruleus has the antagonist function in acupuncture analgesia, the inhibition on the nucleus can enhance the analgesic effect of acupuncture. According to the related analyses, it is considered that β -EP can promote degeneration of 5-HT in hypothalamus, and

there exist physiological complementary mechanisms between them. The results obtained from the present study that hypothalamic β -EP content in arthritic rats was elevated following EA and filiform needle have indicated that EA and filiform needle fulfill their analgesic effects by way of enhancing the content of β -EP in hypothalamus, and the effect of EA is even more obvious. The results that no considerable effect on the content of β -EP was induced by blood letting and point injection indicated that the two therapies may fulfill their analgesic effects by way of some other approaches. Moreover, the result that the content of hypothalamic β -EP in the arthritic rats was higher than that in the normal rats indicated that inflammatory pain itself may activate the central EOP system to restrict pain reaction and hyperalgesia. By elevating the content of hypothalamic β -EP, EA and filiform needle can strengthen the anti-nociceptive ability of enhancing pain threshold.

3.2 Significance of the four acupuncture therapies in the content of ACTH in the hypothalamus

ACTH has various physiological functions, including initiating modulation on neurotransmitters and neuroendocrine and pain modulation through the endogenous analgesic system. The animals with insufficient ACTH are sensitive to pain. After intraperitoneal injection of ACTH in mice, the pain threshold was increased by 50% - 200% with a dose-dependent analgesic effect lasting at least for 80 min. Evidence has shown that ten min after microinjection of ACTH into PAG, pain threshold began to rise in rats. Removal of pituitary could attenuate the analgesic effect of EA, which was similar to the analgesic effect of opioid. Following electrolytic lesion of PAG, the analgesic effect of ACTH was decreased at least by 50%. Numerous studies have demonstrated that ACTH was closely related to 5-HTnergic neurons and 5-HT content, and produced an analgesic effect by way of elevating the content of central 5-HT. Intraperitoneal microinjection of ACTH could increase 5-HIAA, the metabolic product of 5-HT in the brain, accelerate the renewing rate of 5-HT, elevate the content of 5-HT, which was dose-dependent. The analgesic effect of acupuncture elevated by ACTH

may be fulfilled by ACTH activating the function of central 5-HT system and inhibiting central NE system^[2]. The results obtained from the present experiments that point injection could elevate ACTH content in the hypothalamus, but not by EA, filiform needle and blood letting, indicated that point injection may bring about its analgesic effect by way of raising ACTH content in hypothalamus. It is known that EA and filiform needle fulfill their analgesic effect by enhancing the content of hypothalamic β -EP. Since many kinds of bioactive substances are involved in analgesia, some other approaches other than β -EP and ACTH may be involved in blood letting-induced analgesia.

3.3 Significance of the four acupuncture therapies in POMC mRNA expression in hypothalamus

POMC mRNA is the antecedent substance of β -EP and ACTH. POMC neurons are mainly situated in the hypothalamus (arcuate nucleus), and their axons project to the median eminence, ventromedial nucleus and nucleus ceruleus. It has been demonstrated that there exist POMC-related peptides such as ACTH, β -EP, β -MSH in the brain, and they are rich in the hypothalamus. They also exist in the limbic system, but rarely in the midbrain, brain stem, striatum, and cortex. It was reported that during acupuncture stimulation, the release of central EOP was increased. However, the content of central EOP was not decreased, but increased^[3], for which it was assumed that the increase was due to the augment of dissection of EOP antecedent and gene transcription^[4]. In recent years, evidence has shown that the expression of central EOP was accelerated in acupuncture analgesia^[5]. It was considered that one of the functional significances for EOP gene expression was to compensate the reduction of the antecedent due to increased release of EOP, and the accelerated expression may be implicated in the long-term modulation produced by acupuncture^[4,5]. Evidence has shown that formalin-induced pain resulted in potentiation of POMC gene expression, and acupuncture could facilitate the process. The present experiments exhibited that EA and filiform needle could accelerate the expression of POMC mRNA, which might compensate

the reduction of POMC induced by the increased release of hypothalamic β -EP during the needling. However, the influences of blood letting and point injection on the expression of POMC mRNA in hypothalamus were not of significance. POMC is also an antecedent of ACTH. The mechanisms of the results that point injection could enhance the content of ACTH, but could not markedly influence the expression of POMC mRNA, are not yet clear, which remains to be studied further.

To sum up, EA, filiform needle, blood letting and point injection all can provide analgesic effects, and the effects of EA and point injection are better than those of the others. At hypothalamic level, EA and filiform needle fulfill their analgesic effects by way of elevating the concentration of β -EP and increasing the expression of POMC mRNA. Point injection maybe realize its analgesic effect by way of enhancing the content of ACTH. Since blood letting showed no marked effect on the contents of β -EP and ACTH and on the expression of POMC mRNA, it may exert its analgesic effect by some other approaches.

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Additional acupoints were used in combination according to pattern identification. The Back-*Shu* acupoints where the *qi* of *Zang-fu* organs converges, have functions of tonifying the heart and spleen, normalizing balance between the heart and kidney, nourishing *yin* to subdue the fire, harmonizing functional activities of the spleen and stomach, and regulating meridian *qi* flow of the five *zang*-organs, so that the mental activities resume normal state. Scalp massage, topical mechanical stimulation, may promote dilation of capillary vessels, increase the supply of blood to the brain and accelerate the cerebral blood circulation. In addition, rhythmic gentle massage may also regulate the function of the autonomic nerve system to potentiate the inhibitory process of the cerebral cortex.

Stimulation of the plantar reflex zones functions in nourishing *yin* to subdue *yang*, clearing heat from the head and eyes, and calming the mind. Listening light music during massage and acupuncture may help the insomnia person harmonize the psychological state, relieve anxiety, tension, and depression to induce sleep and increase the efficiency of sleep. A combination of these three modalities may rebalance *yin* and *yang*, free the flow of *qi* and blood within the internal organs, so that insomnia will disappear.

This integrated treatment has the advantages of satisfactory effects and absence of side-effects. It is recommended to treat the patient in the afternoon.

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