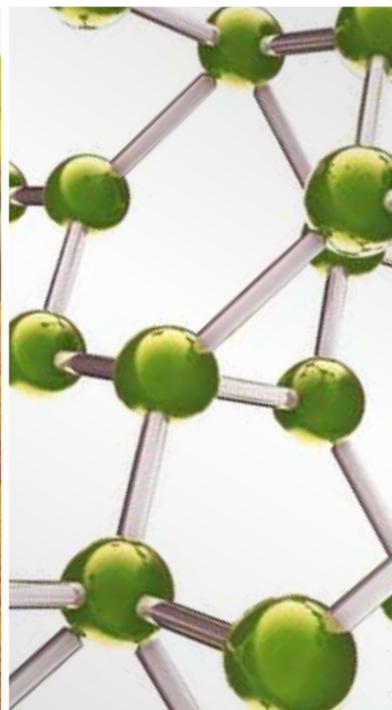


Clinical Efficacy, Mechanisms, AND SAFETY OF ACUPUNCTURE AND Moxibustion

GUEST EDITORS: JAUNG-GENG LIN, YI-HUNG CHEN, XIN-YAN CAO, LIXING LAO,
HYEJUNG LEE, AND GERHARD LITSCHER





Clinical Efficacy, Mechanisms, and Safety of Acupuncture and Moxibustion

Evidence-Based Complementary
and Alternative Medicine

Clinical Efficacy, Mechanisms, and Safety of Acupuncture and Moxibustion

Guest Editors: Jaung-Geng Lin, Yi-Hung Chen, Xin-Yan Gao,
Lixing Lao, Hyejung Lee, and Gerhard Litscher



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Editorial

Clinical Efficacy, Mechanisms, and Safety of Acupuncture and Moxibustion

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Acupuncture has recently increased in popularity and is widely used all over the world. It is described as one of the “complementary and alternative medicine/therapies,” showing promising efficacy in the treatment of many conditions and resulting in fewer adverse effects compared with some conventional medical treatments. Many studies in animals and humans have demonstrated that acupuncture results in multiple biological responses. Although the endorphin hypothesis is well established, the mechanisms underlying acupuncture treatments have not been extensively studied. Basic and clinical acupuncture studies are important and timely. Although acupuncture has a relatively sound safety profile, adverse effects after acupuncture have been reported. More information is needed on safe needling depths. Moxibustion is another traditional Chinese medical intervention that involves the burning of moxa above the body surface of the acupuncture points. The clinical efficacy and mechanism of moxibustion have not been extensively studied. Moreover, safety issues related to moxibustion safety need to be investigated, including concerns of potential tissue damage and adverse physical reactions.

In an attempt to summarize the current knowledge on clinical efficacy, mechanisms, and safety of acupuncture and moxibustion, this special issue contains 38 interesting publications: 14 describe the clinical effects of acupuncture; 15 describe the mechanisms of acupuncture; 3 concern the safety of acupuncture; 2 concern the clinical effects of acupressure; 2 detail clinical effects of moxibustion; and 2 describe the mechanisms of moxibustion. The investigations cover *in vitro* investigations, preclinical experiments, and studies in healthy volunteers and patients, as well as basic and clinical research.

The above-mentioned papers investigate the role of acupuncture in the following therapeutic areas: (1) pain, (2) the respiratory system, (3) microcirculation, (4) neurodegeneration, (5) the endocrine system, (6) gastric motility, (7) itch, (8) seizures, (9) neuroprotection, (10) opioid receptors, (11) diabetes mellitus, (12) public health issues of acupuncture, (13) the effects of acupuncture on infants, (14) adverse effects of acupuncture, (15) safe needling depth, (16) psychology during acupuncture, (17) acupressure and dysmenorrhea, (18) acupressure and sleep disturbance, (19) moxibustion and hyperlipidemia, and (20) a discussion of the mechanisms and safety of moxibustion.

Nowadays, modern technologies are being used to explore the effects of acupuncture and moxibustion. The results of these investigations enhance our knowledge of how acupuncture and moxibustion work and promote better insight into how best to use these tools.

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*Jaung-Geng Lin
Yi-Hung Chen
Xin-Yan Gao
Lixing Lao
Hyejung Lee
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Research Article

Electroacupuncture at Acupoints Reverses Plasma Glutamate, Lipid, and LDL/VLDL in an Acute Migraine Rat Model: A ¹H NMR-Based Metabolomic Study

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Background. The objective of this study was to identify potential biomarkers of electroacupuncture (EA) on relieving acute migraine through metabolomic study. **Methods.** EA treatments were performed on both acupoints and nonacupoints on the nitroglycerin (NTG)-induced migraine rat model. NMR experiments and multivariate analysis were used for metabolomic analysis. **Results.** The number of head-scratching, the main ethology index of migraine rat model, was significantly increased ($P < 0.01$) after NTG injection. The plasma metabolic profile of model group was distinct from that of the control group. Glutamate was significantly increased ($P < 0.01$), whereas lipids were significantly decreased ($P < 0.01$) in model rats. After EA at acupoints, the metabolic profile of model rats was normalized, with decreased glutamate ($P < 0.05$) and increased lipids ($P < 0.01$). In contrast, EA at nonacupoints did not restore the metabolic profile, but with six metabolites significantly different from acupoints group. Interestingly, the number of head-scratching and glutamate level were significantly decreased ($P < 0.05$) after receiving EA at both acupoints and nonacupoints. **Conclusions.** EA at acupoints may relieve acute migraine by restoring the plasma metabolic profile and plasma glutamate, while EA at nonacupoints may modestly relieve acute migraine by decreasing plasma glutamate.

1. Introduction

Migraine is one of the most common neurological disorders characterized by recurrent unilateral, throbbing headaches and neurological dysfunction, with or without aura [1]. Approximately 16%–18% of women and 6%–8% of men in the USA suffer from migraine during the most productive years of their professional lives [2–4]. Although enhanced excitatory neurotransmitters such as glutamate (Glu), which facilitate spontaneous cortical spreading depression (CSD), are valued as central mechanism of triggering migraine,

the pathophysiology of migraine is attributed to multiple factors, and many of these aspects are still not unraveled [5, 6]. Drug treatment with oral nonsteroidal anti-inflammatory drugs (NSAIDs) and triptans for relieving migraine usually have a modest effect and cause several side effects, such as gastrointestinal and cardiovascular disorders [7].

Acupuncture is a procedure whereby fine needles are inserted and manipulated into related acupoints of the individual, for the purpose of treating the disease. Although acupuncture has been widely used for migraine prophylaxis and treatment, little is understood about its biological

mechanism. A 2009 Cochrane meta-analysis proclaimed acupuncture as safe and effective for migraine prophylaxis compared to prophylactic drug treatment [8]. The US Headache Consortium has also deemed acupuncture to be an important therapy for management of migraine [9]. Recently, an increasing number of studies have confirmed that mechanistically, acupuncture analgesia works through the release of opioid peptides in the central nervous system (CNS), which occurs in response to long-lasting activation of ascending sensory tracks stimulated by acupuncture manipulation [10–13]. However, the primary mechanism by which acupuncture relieves migraine has not been well established. Further, lately a series of high quality trials have fuelled suspicions that acupuncture at acupoints is no more effective than acupuncture at nonacupoints or sham acupuncture in reducing migraine headaches [14–17]. Hence, two urgent questions in the field of acupuncture for migraine have been raised. First, are there any neurological or metabolic biomarkers that provide supporting evidence for acupuncture to relieve migraine? Second, are there true neurological or metabolic differences between acupuncture at acupoints and acupuncture at nonacupoints?

To address these two questions, nitroglycerin-treated (NTG) rats are employed in this study as acute migraine model to detect metabolic changes of acute migraine treated by electroacupuncture. NTG rat model is regarded as a reliable animal model for acute migraine. It replicates neurogenic inflammation and hyperalgesia of human migraines, and it has been successfully established in different migraine studies [18–22].

Metabolomics has rapidly emerged as a powerful tool for identification of new biomarkers and for monitoring the dynamic pathophysiological metabolic changes of whole organisms in disease states [23]. Recent advances in nuclear magnetic resonance (NMR) spectroscopy, aided by data-reduction techniques, have facilitated the use of metabolomics as a direct functional readout of the pathological state from biofluids and tissues [24–26]. The power of metabolomics lies in monitoring systemic metabolite changes and characterizing complete metabolic profiling, which properly meet the essence of Traditional Chinese Medicine (TCM) and acupuncture for its sensitivity and complexity. Therefore, there are increasing studies that employ metabolomics to detect metabolic evidence for Traditional Chinese Medicine and acupuncture [27–30].

In the current study, we employed a developed ^1H -NMR-based metabolomic technology to investigate metabolite changes in acute migraine evoked in NTG rat model and the effect of electroacupuncture on treating an acute migraine attack.

2. Methods

2.1. Ethics Statements. All experiments were conducted in strict accordance with the guidelines of International Association for the Study of Pain and Chinese national regulations for experimental animal use. The study was approved by the Sichuan Institutional Review Board for Animal Experiments

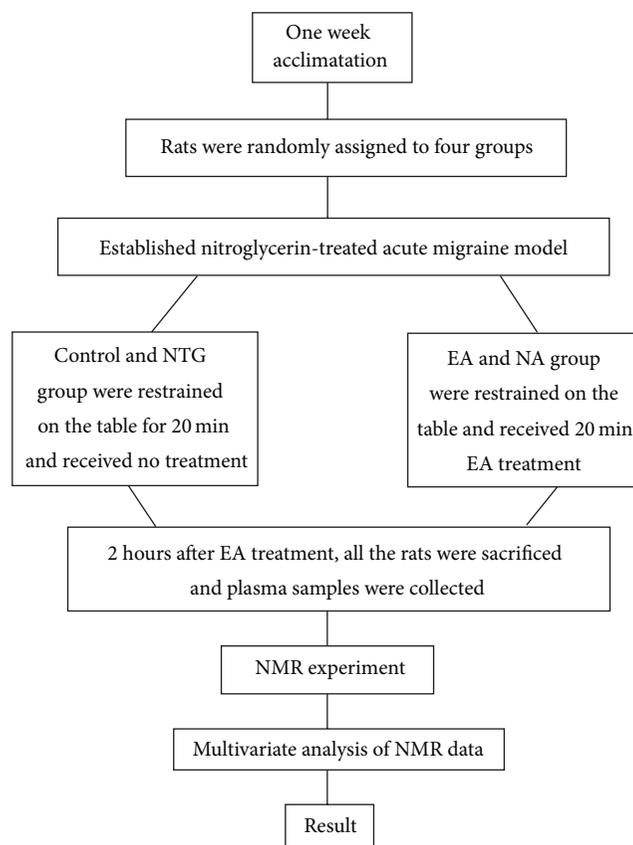


FIGURE 1: Trial fluidogram.

(permit number: Sichuan experimental animal institute (97) 6). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

2.2. Acute Migraine Model. The overall experimental approach is shown in Figure 1. Fifty female *Sprague Dawley* rats, 3 months old and weighing 160 ± 20 g, were purchased from the Experimental Animal Center of Chengdu University of Traditional Chinese Medicine. Rats were housed in a well-ventilated colony room at a temperature of $15\text{--}22^\circ\text{C}$ and a relative humidity of $50\text{--}70\%$ on a 12 h light/12 h dark cycle. Food and tap water were provided *ad libitum*. Rats were allowed to acclimatize for one week before experiments began and then were randomly divided into 4 groups using an SPSS randomization process: control (untreated, without any treatment), NTG (nitroglycerin-treated model), EA (electroacupuncture at acupoints following NTG-treatment), and NA (electroacupuncture at nonacupoints following NTG-treatment). Each group has 10 rats and was fed separately in two metabolic cages.

All rats were restrained on an operating table for further treatment. The rats in control group received no injection, while other three groups were injected intraperitoneally with NTG (10 mg/kg of 2 mg/mL solution [18]) according to previous study [22]. Thirty minutes after injection, three groups of NTG rats displayed red ear, frequent head-scratching, and climbing cage, which represent the anxiety and discomfort,

indicating the successful establishment of migraine model [18]. These typical symptoms lasted for 4 hours, which is consistent with previous reports. In contrast, the control rats did not show these symptoms.

Following NTG injection, the behavior of the rats was observed and documented. The total time of ethology observation lasted for 4 h, and each 30 min of observation was considered a time interval. The number of head-scratching was quantitatively measured as the main ethology index [18–22].

2.3. Electroacupuncture Treatment. One hour after injection, animals were restrained on an operating table for electroacupuncture treatment. The EA group received filiform needles that were inserted perpendicularly and bilaterally to a depth of 2–3 mm at the points of SJ5 (Waiguan, located on lateral side of forefoot, 3 mm away from wrist joint and in the middle of ulnar bone and radial bone) and GB34 (Yanglingquan, located on the postlateral side of knee joint, below capitulum fibulae) along the Shao Yang meridian of Hand and Foot (see Supplementary Figure 1 available online at <http://dx.doi.org/10.1155/2014/659268>). These acupoints are most frequently used to relieve migraine attacks [31]. Nonacupoints were located 5 mm above from each acupoint according to previous studies (Supplementary Figure 1) [14].

Transcutaneous electroacupuncture (EA) stimulation was then conducted at each acupoint for 20 min [32] using a Han's acupoint nerve stimulator (HNAS-200, Nanjing, China). Stimulation frequency was set at 14 Hz, and intensity was increased from 0.1 mA to a maximum of 1.0 mA until the rats' feet began to tremble slightly [32]. For the NA group, an identical electroacupuncture procedure was conducted, except that needles were placed 5 mm above from each acupoint [31]. Animals in the control and NTG groups were also fixed on the table for 20 min, but no electroacupuncture was performed. All rats were conscious during treatment.

2.4. Sample Collection and Pretreatment. To avoid potential acute effects of EA, rats were anesthetized with 3% sodium pentobarbital (40 mg/kg, i.p.) at 2 hours after electroacupuncture treatment [32] and then sacrificed at 4 hour after nitroglycerin injection by exsanguination from the femoral artery. Blood (~3 mL) from the femoral artery was collected into a heparin sodium tube, placed on ice for 30 minutes, and then centrifuged (3000 ×g, 4°C, 15 min). Plasma was collected and stored at –70°C.

Before NMR analysis, plasma samples were thawed, centrifuged (13000 ×g, 4°C, 10 min), and the supernatant extracted. In 5 mm NMR tubes, 300 μL of each supernatant sample was mixed with 250 μL of D₂O to lock the field frequency and 50 μL of 3-trimethylsilyl-2H₄-propionic acid sodium salt (TSP) in D₂O (1 mg/mL) as a chemical shift reference. All samples contained a final volume of 600 μL and were vibrated repeatedly [33, 34].

2.5. NMR Experiments. ¹H NMR spectra of plasma were acquired with a Varian INOVA 600 MHz NMR spectrometer

at 27°C using a Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence with a total spin-spin relaxation delay (2τ) of 320 ms. Water suppression was achieved with an irradiation of the water peak during the recycle delay (2 s) and mixing time (t_m) of 150 ms. Free induction decays (FIDs) were collected into 32,000 data points with a spectral width of 8,000 Hz over 64 scans. Presaturate frequency and central frequency were equally on the water peak. The FIDs were zero failed by a factor of two and multiplied by an exponential line-broadening factor of 0.5 Hz prior to Fourier transformation. In addition, diffusion-edited experiments were also performed with bipolar pulse pair-longitudinal eddy current delay (BPP-LED) pulse sequence. The gradient amplitude was set at 35.0 G/cm with a diffusion delay of 100 ms. A total of 128 transients and 16,000 data points were collected with a spectral width of 8,000 Hz. A line-broadening factor of 1 Hz was applied to FIDs before Fourier transformation.

¹H NMR spectra of plasma were manually phased and baseline-corrected using VNMR 6.1C software (Varian Inc.). For CPMG spectra, each spectrum over the range of δ 0.4–4.4 was data-reduced into integrated regions of equal width (0.01 ppm). For BPP-LED data, each spectrum over the range of δ 0.1–6.0 was segmented into regions of equal width (0.01 ppm). The regions containing the resonance from residual water (δ 4.6–5.1) were excluded. The integral values of each spectrum were normalized to a constant sum of all integrals in a spectrum to reduce any concentration plot variations between samples. Identification of metabolites in the spectra was accomplished based on information in the literature and the Chenomx NMR Suite 4.5 (Chenomx, Calgary, Canada).

Based on previous studies, the HMDB website (<http://www.hmdb.ca/>) and the ChenomxNMR Suite software, major metabolites in plasma were identified and shown in Figure 2. CPMG pulse sequence was used to emphasize the resonances of small metabolites in plasma, while resonances from macromolecules were attenuated (Figure 2(a)). Figure 2(b) shows diffusion-edited NMR spectra of plasma from each group, displaying the signals of lipids, N-acetyl glycoproteins (NAc), and O-acetyl glycoproteins (OAc) groups of glycoproteins. Subtle differences of these spectra were observed by visual inspection among the four groups. Further analysis was performed using multivariate statistical analysis to determine the metabolic changes among the four groups.

2.6. Pattern Recognition and Statistics. The resulting integral data were imported into SIMCA-P (version 10.04; Umetrics, Umeå, Sweden) for multivariate analysis. Prior to analysis, the CPMG data were mean-centered and Pareto-scaled, and the BPP-LED data were mean-centered. CPMG data and LED data were both subjected to principal component analysis (PCA) and partial least square discriminate analysis (PLS-DA) to discriminate differentiation in metabolites among the groups. PCA was firstly performed on the normalized ¹H NMR dataset after Pareto scaling in this study. Data were visualized by using the principal component (PC) score and loading plots. Each point on the scores plot represents an individual sample, and each point on the loadings plot

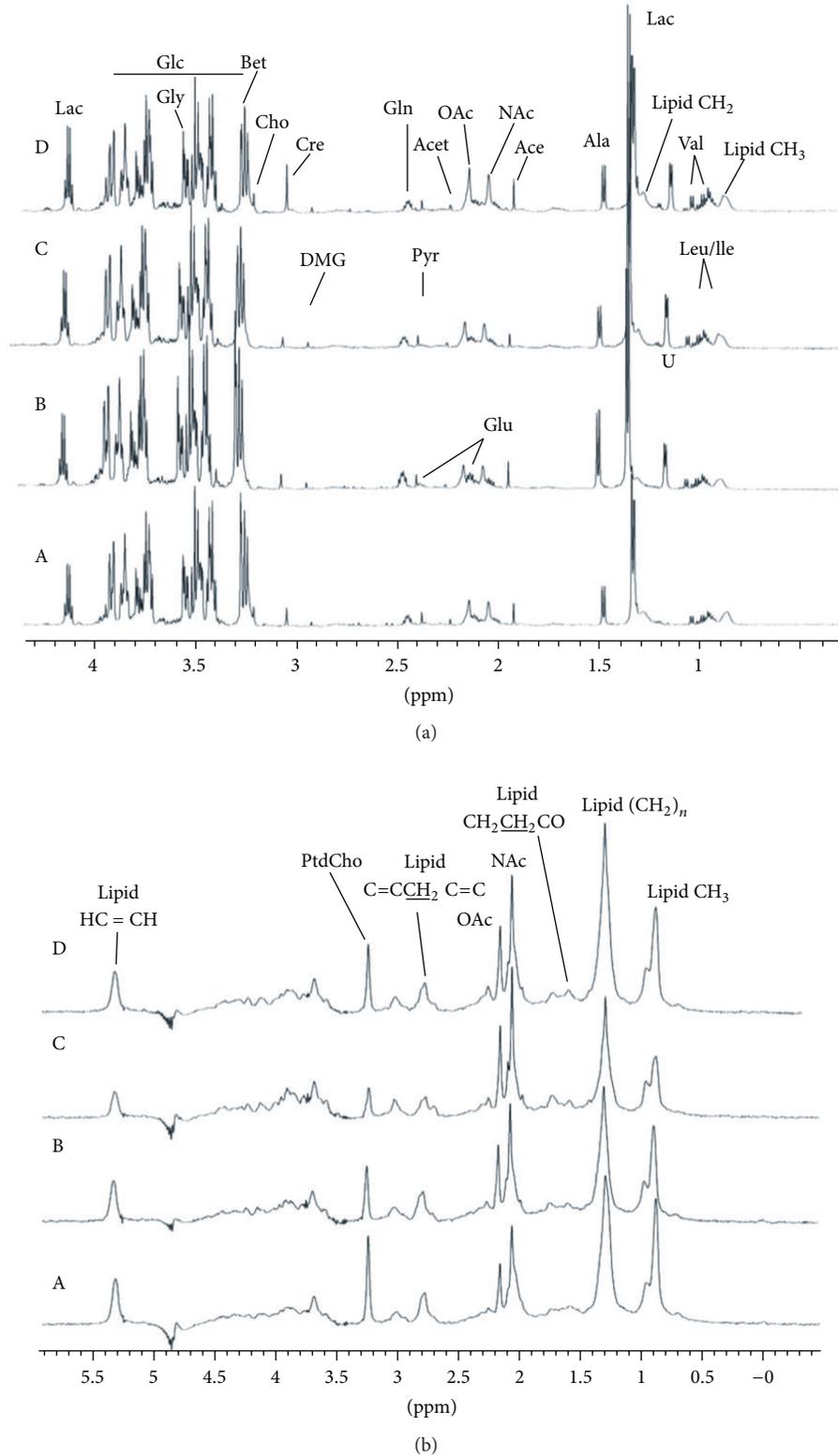


FIGURE 2: ^1H NMR spectra of rat plasma samples. (a) CPMG spectra; (b) LED spectra; A, control; B, NTG group; C, NA group; D, EA group ($n = 10$).

represents a single NMR spectral region. Orthogonal signal correction (OSC) was further used to remove the variations not correlated to group membership and to maximize the separation, followed by PLS analysis. The final lists of metabolites were chosen on the basis of the variables of importance parameter (VIP), which is a measure of each variable's relative influence on the model [35]. Because in this study we found series of metabolite changes and they were correlated with each other among metabolic systems, therefore multivariate analysis of variance was performed to discriminate significant changes of metabolites identified by SIMCA-P. Ethology data were calculated by repeated measures analysis of variance and multivariate analysis of variance.

3. Results

3.1. Electroacupuncture at Both Acupoints and Nonacupoints Reduce Head-Scratching Number in NTG Rats. Initially, the quantitative ethology observations, for the purpose of detecting NTG-induced acute migraine rat model and the effect of EA, were recorded and calculated. Following NTG injection, the number of head-scratching in the NTG group, EA group, and NA group increased significantly from NTG injection to 60 min compared to the control group ($P < 0.01$) (Figure 6(a)). Specially, the number of head-scratching in the NTG group significantly increased from NTG injection to 60 min and from 120 min to 240 min, indicating that a successful rat model of acute migraine had been established [21]. Secondly, 20 min of EA at acupoints and EA at nonacupoints treatments were performed accordingly on EA and NA groups from 60 min to 120 min after NTG injection. Subsequently, the number of head-scratching was obviously decreased ($P < 0.05$) in both EA and NA group from 120 min to 240 min (Figure 6(a)) compared to NTG group, suggesting that a typical symptom of acute migraine was relieved by performing EA at both acupoints and nonacupoints. In addition, the number of head-scratching was significantly decreased ($P < 0.05$) in the NA group from 210 min to 240 min following NTG administration (Figure 6(a)) compared to EA group. In conclusion, these data showed that the number of head-scratching in NTG group was significantly increased ($P < 0.01$) compared to the control group, while the number of head-scratching in EA and NA group was significantly decreased ($P < 0.05$) relative to NTG group after receiving EA at both acupoints and nonacupoints.

3.2. NMR Metabolic Profiling Can Differentiate Acute Migraine Rat Model. After NMR experiment, 22 metabolites in plasma of animal groups were identified by using Chenomx NMR Suite software and shown in Figure 2. To detect the role of electroacupuncture on metabolite changes in acute migraine rats, we first asked whether NMR metabolic profiling can differentiate between acute migraine rat model and untreated control rats. Thus, we performed PCA and PLS-DA with OSC pretreatment on the normalized ^1H NMR CPMG dataset. A clear separation was achieved predominately along PC1 (Figure 3(a); $R2X = 62.7\%$, $R2Y = 99.1\%$ and $Q2 = 97.5\%$)

between the control group and the NTG group. Based on the results of OSC-PLS-DA and multivariate analysis of variance, the dominant metabolites that influenced the differentiation between the control and NTG groups are displayed in the corresponding loadings plots (Figure 3(b)), the concentration plot (Figure 6(b)), and Table 1. Notably, NTG group demonstrated a significantly higher level of glutamate ($P = 0.009$), which is an important excitatory neurotransmitter for triggering migraine [36], and significantly lower levels of LDL/VLDL ($P = 0.001$) and lipid ($P = 0.008$) compared with the controls. Moreover, PCA, PLS analysis with OSC pretreatment were also performed on the ^1H NMR LED dataset, which is similar to the CPMG procedure. The results illustrated an obvious separation between the controls and the NTG group along PC1 (Figure 3(c); $R2X = 53.1\%$, $R2Y = 99\%$, $Q2 = 96.3\%$), as shown in Table 2 and Figure 6(b). Together, we extracted an enhanced anaerobic glycolysis and reduced gluconeogenesis from the metabolic profiling of nitroglycerin-treated acute migraine rat model, as displayed by decreased glucose, pyruvic acid and increased lactic acid, alanine, and 3-hydroxybutyric acid (3-HB) (Figure 6(b)). Meanwhile, the enhancement of lipid metabolism was also demonstrated in the NTG acute migraine rat model, as indicated by decreased levels of lipid, unsaturated lipid (UFA), choline, and phosphatidylcholine (Ptdcho) (Figure 6(b)). The metabolite change at 1.14 ppm remains unidentified, but it seems unlikely that this metabolite could be a component of the NTG solution that would have influenced any group differences.

3.3. Electroacupuncture at Acupoints Reverses Plasma Metabolite Changes in the Acute Migraine Rat Model. After establishing the metabolic profiling of NTG acute migraine rat model, we next determined whether EA reverses metabolite changes in the acute migraine rat model. We performed PLS analyses with OSC pretreatment on data from three groups: control, NTG, and rats who received EA at acupoints. After 20 min of EA at acupoints, we observed that the metabolic profiling of the EA group was similar to that of the control group, and both these groups were clearly separated from the NTG group along PC1 (Figure 4(a); $R2X = 77.4\%$, $R2Y = 98.3\%$, and $Q2 = 92.6\%$). Interestingly, the EA group illustrated a significant lower level of glutamate ($P = 0.048$) and significant higher levels of LDL/VLDL ($P = 0.002$) and lipid ($P = 0.002$) compared with the NTG group, which is the opposite of the metabolite changes in NTG group relative to controls (Figures 6(d) and 6(e)). Meanwhile, the concentration plot showed that the levels of lactic acid, OAc, pyruvic acid, alanine, LDL/VLDL, glutamate, glutamine, creatine, and lipid in EA group were restored to levels similar to that of the control group (Figures 6(c)–6(e)). Furthermore, the diffusion-edited NMR spectra was performed, which is similar to the CPMG procedure. The result demonstrated a clear differentiation among the control, NTG, and EA groups along PC1 (Figure 4(b); $R2X = 80.9\%$, $R2Y = 99.3\%$, $Q2 = 88.7\%$), as shown in Figure 4(b). In the EA group, we found significantly higher levels of lipid (CH_2), NAc and lower levels of choline ($P < 0.05$) compared with the NTG group

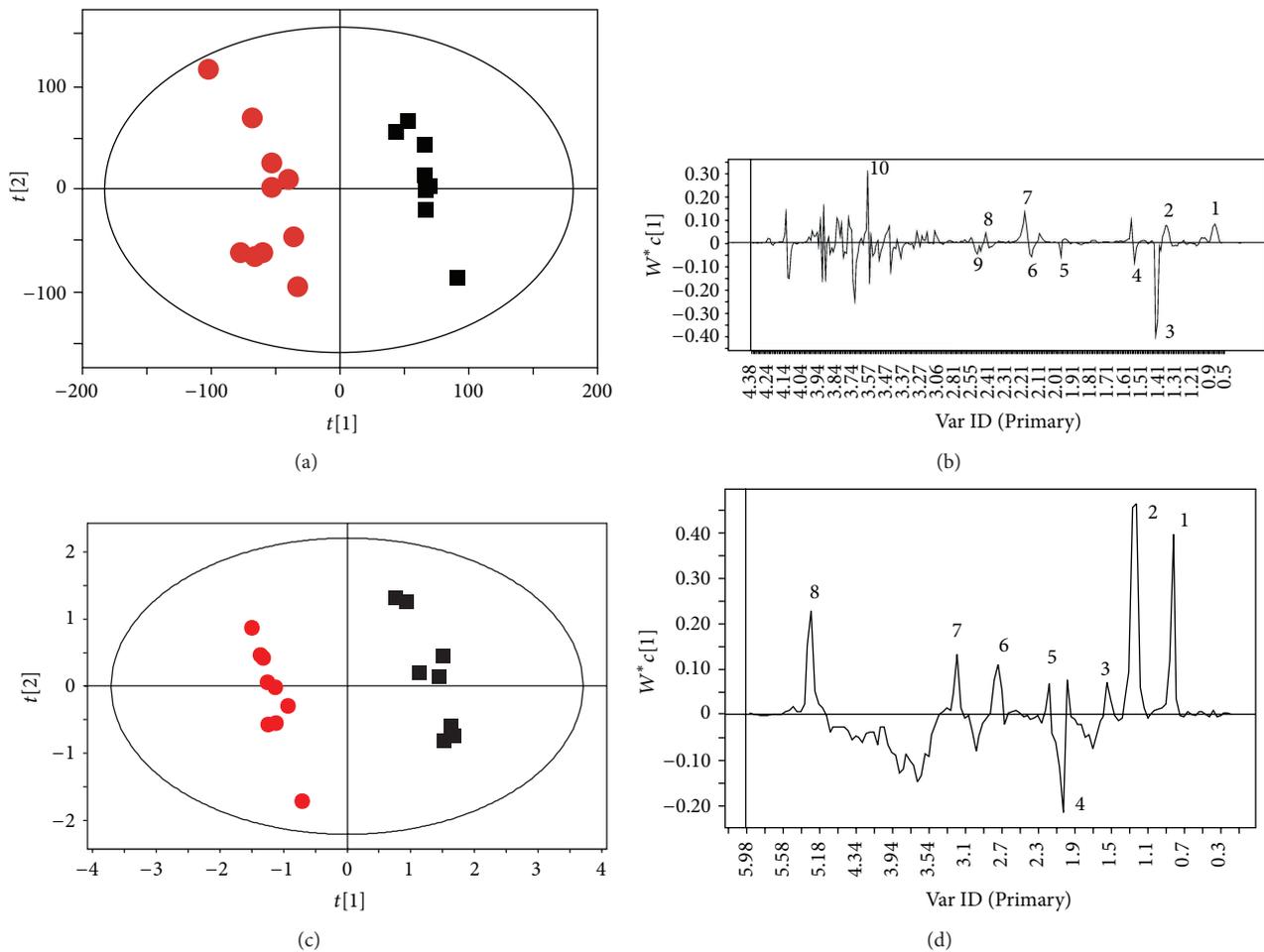


FIGURE 3: Clear separation of CPMG and LED spectra between NTG (red dots) and control (black boxes) groups. (a) Scores plot of OSC-PLS analysis of CPMG data; (b) corresponding loadings line plot. (c) Scores plot of OSC-PLS analysis of LED data; (d) corresponding loadings line plot. Keys to B: (1) lipid CH_3 ; (2) lipid CH_2 ; (3) lactate; (4) alanine; (5) acetate; (6) glutamate; (7) OAc; (8) pyruvate; (9) glutamine; (10), glycine. Keys to D: (1) lipid CH_3 ; (2) lipid CH_2 ; (3) lipid $\text{CH}_2\text{CH}_2\text{CO}$; (4) NAc; (5) lipid CH_2CO ; (6) PUFA; (7) PtdCho; (8) lipid $\text{CH}=\text{CH}$ ($n = 10$).

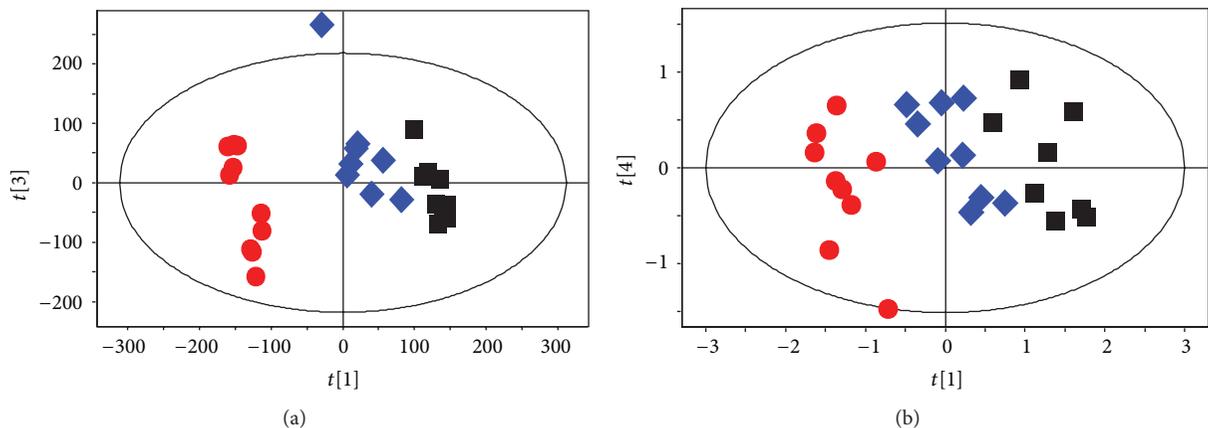


FIGURE 4: OSC-PLS analysis manifest EA at acupoints reverses plasma metabolite changes in the acute migraine rat model. NTG group (red dots), EA group (blue diamonds), and control group (black boxes). CPMG analyses were conducted (a) among three groups. LED analyses were conducted (b) among three groups ($n = 10$).

TABLE 1: Changes of plasma metabolites in CPMG NMR spectra induced by NTG and the effects of EA.

Metabolites	Peak regions (δ)	NTG versus untreated control	NTG versus EA	NTG versus NA
Unknown	1.14, 1.18	↑	↓	—
Lactic acid	1.34, 4.1	↑	↓	↓
Glutamate	2.11, 2.35	↑	↓	↓
Glutamine	2.42	↑	↓	↓
Glycerine	3.62, 3.66	↑	↓	↑
OAc	2.14, 2.18	↓	↑	↑
LDL/VLDL	0.86	↓	↑	—
Glycine	3.54	↓	↑	—
NAC	2.02, 2.06	↓	↑	—
Betaine	3.26	↓	↑	—
Lipid	1.26, 1.3	↓	↑	↑
	3.42, 3.38,			
D-Glucose	3.46, 3.5, 3.74, 3.82, 3.86,	↓	↑	↓
Alanine	1.46	↑	↑	↓
L-Isoleucine	0.94	↓	↑	—
Choline	3.22	↓	↑	↓
Pyruvic acid	2.38	↓	↑	—
Acetoacetic acid	1.9, 1.94	↑	↓	—
3-HB	1.22	↑	↓	↑
Creatine	3.94	↓	↑	↑

NAC: N-acetyl glycoproteins; OAc: O-acetyl glycoproteins; 3-HB: 3-hydroxybutyric acid. The arrows indicate the direction of the change (↑: increase; ↓: decrease) in the concentration among untreated control group of animals, EA group of animals, and NA group of animals relative to NTG group animals ($n = 10$).

TABLE 2: Changes of plasma metabolites in LED NMR spectra induced by NTG and the effects of EA.

Metabolites	Peak regions (δ)	NTG versus untreated control	NTG versus EA	NTG versus NA
NAC	2.02, 2.06	↑	↓	↑
OAc	2.14, 2.18	↑	↓	↑
Lipoid	1.3, 1.34, 1.26, 2.22, 2.26	↓	↑	↓
Unsaturated lipid	5.26, 5.34	↓	↑	↓
LDL/VDL	0.86, 0.9	↓	↑	↑
PUFA	2.74, 2.78, 2.82	↓	↑	—
PtdCho	3.22	↓	↑	↓
FA	1.58, 1.74, 1.78, 1.7, 1.82, 2.26	↓	↑	↑

NAC: N-acetyl glycoproteins; OAc: O-acetyl glycoproteins; PtdCho: phosphatidyl choline; UFA: unsaturated lipid; FA: fatty acid; PUFA: polyunsaturated fatty acid ($n = 10$).

(Figures 6(c) and 6(d)). Taken together, these data suggest that EA reverses NTG induced changes by restoring the metabolic profiling and reversing the levels of plasma metabolites, such as lactic acid, OAc, NAC, pyruvic acid, alanine, LDL/VLDL, glutamate, isoleucine, creatine, lipid (CH_2), and lipid (CH_3) in the EA groups relative to the NTG group.

3.4. Clear Separation of Metabolic Profiling between EA at Acupoints and EA at Nonacupoints. A remaining question is whether there are any metabolomic differences between EA at

acupoints and EA at nonacupoints. To address this question, we next executed PLS analysis with OSC pretreatment on both CPMG and LED data among the control, NTG, and the NA groups. After 20 min of EA at nonacupoints, we observed that the metabolic profiling of the NA group was clearly separated from that of the control and the NTG group (Figure 5(a), $R2X = 74.2\%$, $R2Y = 98.9\%$, and $Q2 = 97.6\%$). Meanwhile, of particular interest are data shown in Figure 5(b) ($R2X = 65.1\%$, $R2Y = 99.1\%$, $Q2 = 96.4\%$) and Figure 5(d) ($R2X = 72.7\%$, $R2Y = 98.8\%$, $Q2 = 88.7\%$), illustrating that the metabolic profile of the NA group was clearly distinct from controls, unlike EA group, which was

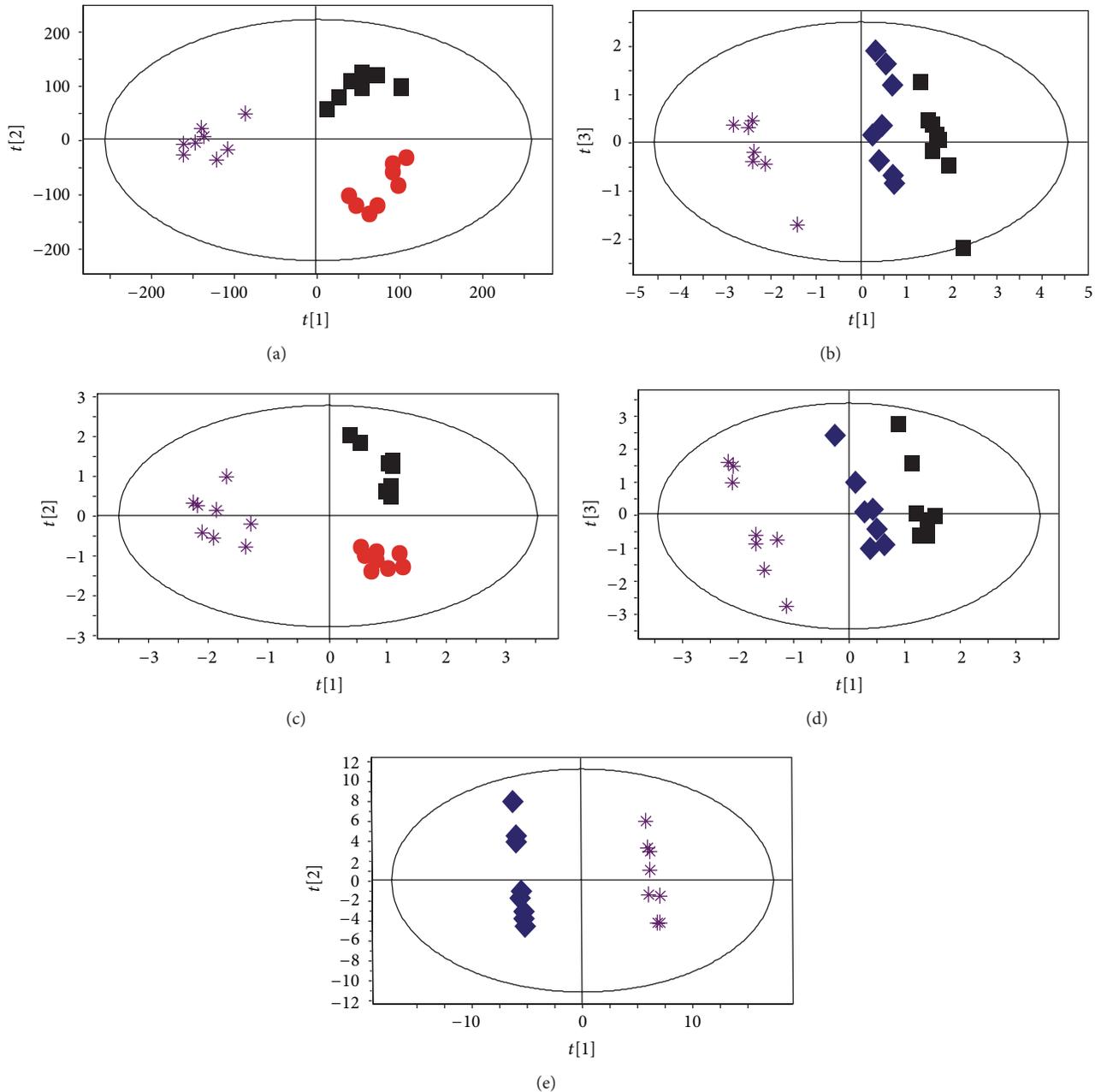
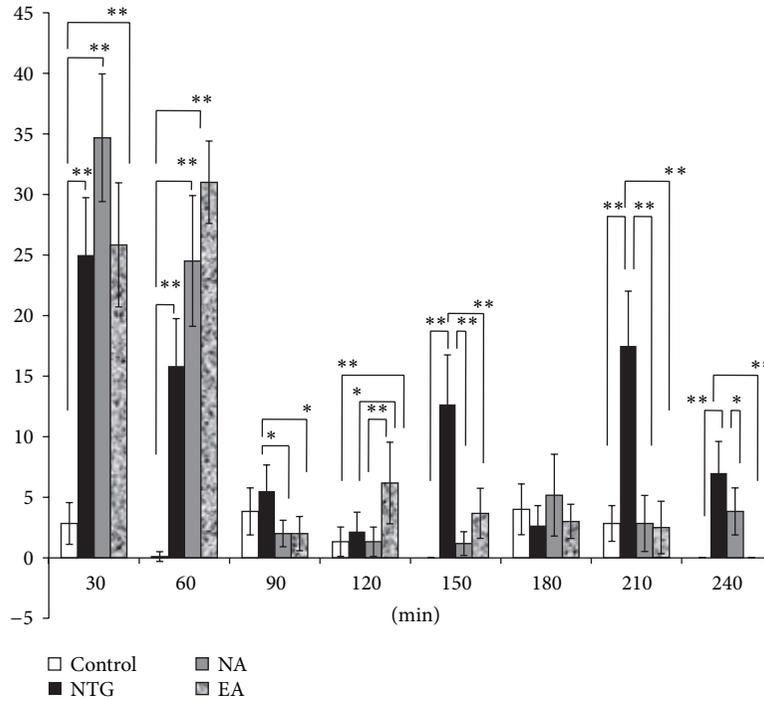


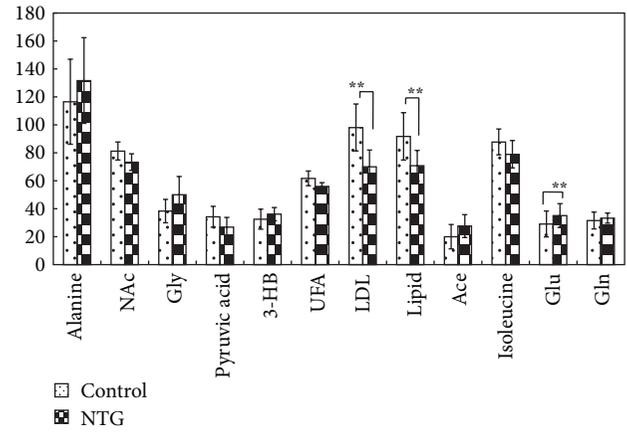
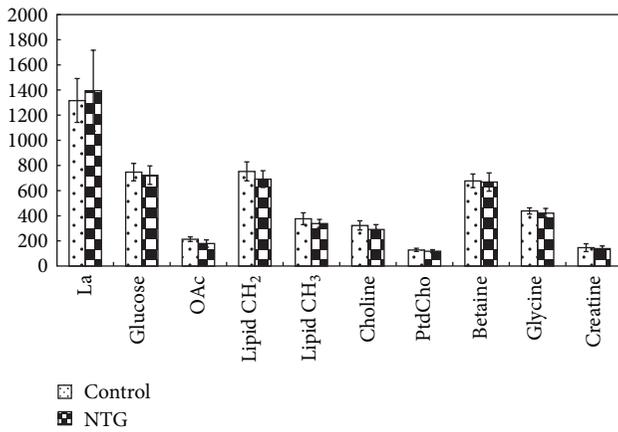
FIGURE 5: OSC-PLS analysis discriminates clear separation between EA at acupoints and EA at nonacupoints in the acute migraine rat model. NTG group (red dots), EA group (blue diamonds), NA group (purple stars), and control (black boxes) group. CPMG analyses were conducted (a) among three groups and (b) among EA, NA, and control group. LED analyses were conducted (c) among three groups and (d) among EA, NA, and control group and (e) between EA and NA group ($n = 10$).

very similar to controls. Furthermore, a clear separation was observed between the EA group and the NA group along PC1 (Figure 5(e), $R2X = 63.6\%$, $R2Y = 99.9\%$, $Q2 = 99.3\%$). The levels of six metabolites including lipid (CH_2), choline, alanine, isoleucine, LDL/VLDL, and NAc were significantly different in the EA group compared to the NA group (Figure 6(f); $P < 0.05$). Combined, these data suggest that EA at nonacupoints cannot reverse NTG-induced changes, but there are distinct differences compared to EA at acupoints,

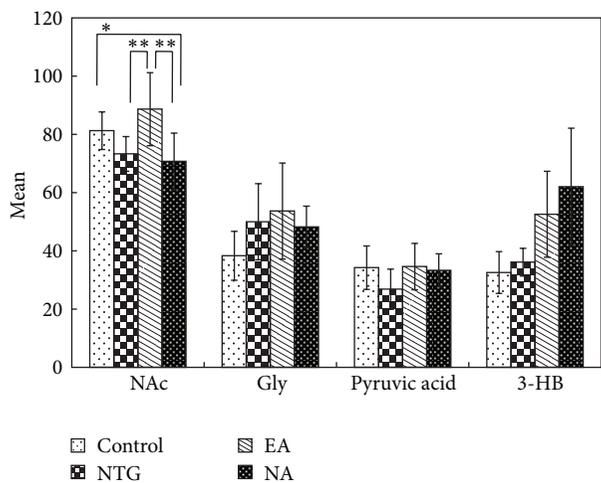
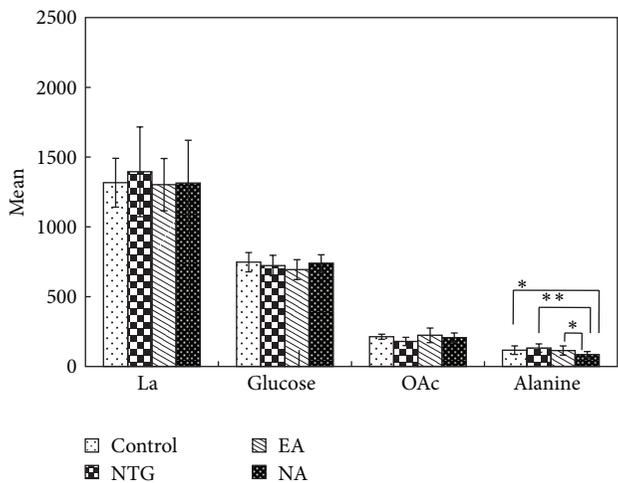
and this was characterized by different metabolic profiling and a series of significant changes of plasma metabolites in the NA groups relative to EA group. In contrast, we found that the level of glutamate, an important excitatory neurotransmitter that triggers migraine, was significantly decreased in both EA and NA groups compared with the NTG group. This observation indicates that glutamate may be crucial for electroacupuncture to relieve acute migraine, but it is not acupoint-dependent.



(a)



(b)



(c)

FIGURE 6: Continued.

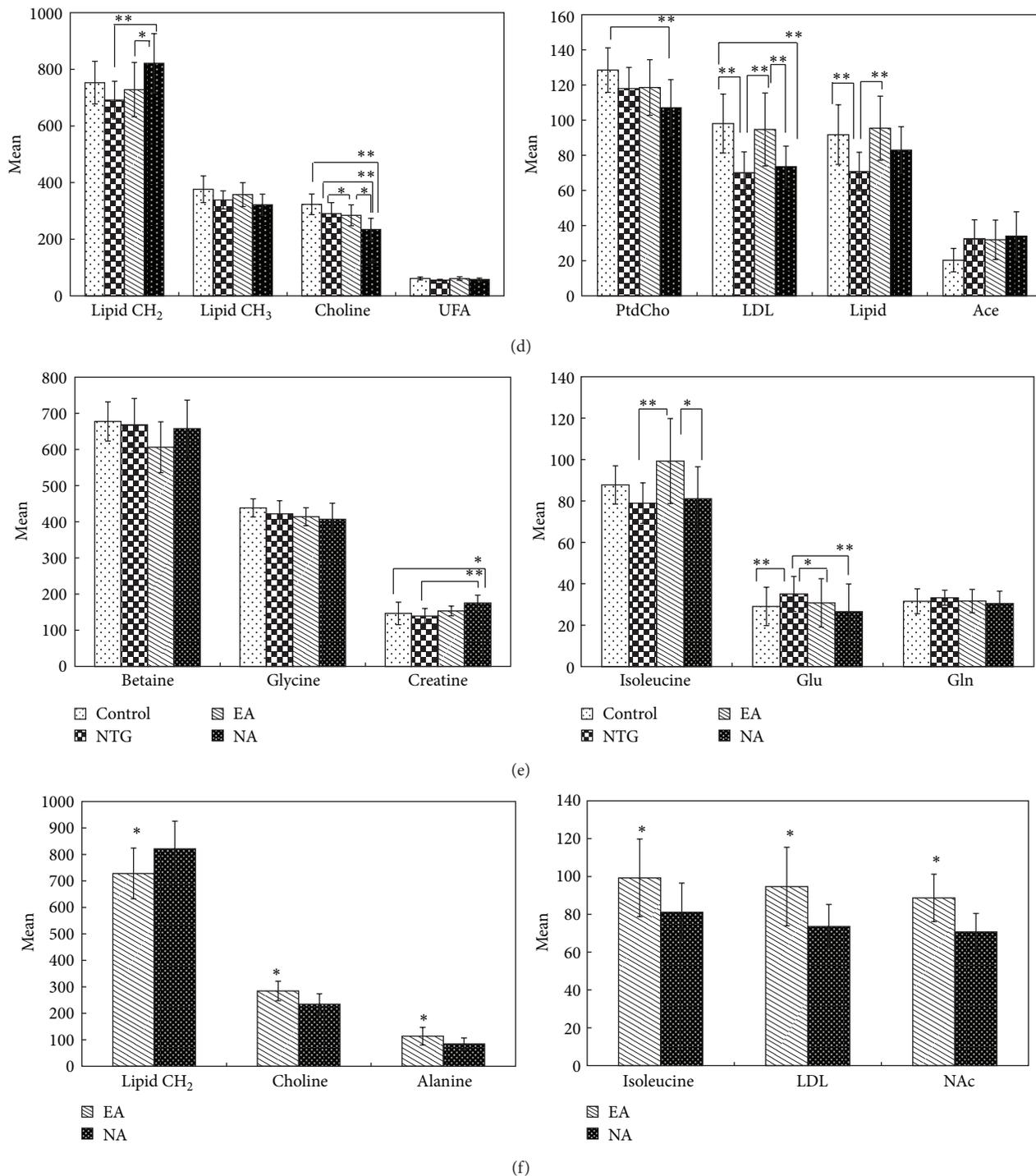


FIGURE 6: The number of head-scratching and the concentration of metabolites from control, NTG, EA, and NA groups. Repeated measures analysis of variance and multivariate analysis of variance were accordingly conducted among four groups. Each column represents the mean, with the SEM shown by error bars. (a) The number of head-scratching from control, NTG, EA, and NA groups after NTG injection ($n = 6$). $*P < 0.05$, $**P < 0.01$. (b) Differences in plasma levels of 22 metabolites were detected in nitroglycerin-induced rats compared with controls. LDL/VLDL, lipid were drastically decreased and glutamate was significantly increased in NTG group relative to control group ($n = 10$). $**P < 0.01$. (c) The metabolites changes of glycometabolism among control, NTG, EA, and NA group. EA group reversed plasma metabolite changes in NTG group ($n = 10$). $*P < 0.05$, $**P < 0.01$. (d) The metabolite changes of lipid metabolism among four groups ($n = 10$). $*P < 0.05$, $**P < 0.01$. (e) The metabolite changes of amino acids metabolism among four groups ($n = 10$). $*P < 0.05$, $**P < 0.01$. (f) Significant differences of metabolite changes between EA and NA group ($n = 10$). $*P < 0.05$.

4. Discussion

Our study found that EA at acupoints not only restores the metabolic profiling but also reverses a series of significant metabolite changes such as glutamate, LDL/VLDL, and lipid in the NTG-treated acute migraine model. In contrast, we found that EA at nonacupoints did not restore the metabolic profiling of NTG acute migraine rat. Six metabolites that might contribute to the specific effect of acupoints were identified by significant difference between EA at acupoints and EA at nonacupoints. Specially, we found that EA at both acupoints and nonacupoints may relieve acute migraine by decreasing the plasma intensity of glutamate. These data, to our knowledge, for the first time provide potential metabolomic evidence for the effect of EA on relieving acute migraine.

The NTG rat is a reliable animal model of acute migraine and was therefore employed in this study to determine the metabolic profile of acute migraine and the effect of electroacupuncture [18–22]. In the series of discomfort symptoms exhibited by NTG rats after being administered with nitroglycerin, “head-scratching,” which resembles the attack of human migraine, was quantitatively measured as an important observation index in acute migraine rat model [21]. The number of head-scratching obviously increased ($P < 0.01$) after NTG injection and lasted for 4 h, manifesting the key signal of success of acute migraine model.

The advantages of this study include the developed metabolomic strategy and advanced multivariate statistical analysis such as OSC-PLS-DA and MNOVA. Using a combination of these techniques, not only the whole plasma metabolic profiling was characterized, but also significant plasma metabolite changes were confirmed from all of small plasma metabolites changes of acute migraine rats. To investigate metabolic evidence of electroacupuncture relieving migraine, the metabolic profiling and the metabolic disturbances in NTG acute migraine rats were firstly presented in this study (Supplementary Figure 2). Glutamate, LDL/VLDL, and lipid were drawn out from all the metabolites changes and confirmed to be significantly reversed in the NTG-induced acute migraine animals.

As studies to date, glutamate plays a key role in the pathophysiology of migraine in both animal and human researches [36]. Ramadan found that glutamate facilitates cortical spreading depression (CSD), central sensitization, which are the central mechanisms for triggering migraine [37]. Peres et al. have shown that plasma glutamate levels are positively correlated with headache intensity in chronic migraine patients [38, 39]. Accordingly, our study further demonstrates that the elevation of glutamate in the plasma is a crucial contributory factor in acute migraine attack. On the other hand, recent migraine studies have revealed that normal weight migraine patients whose blood was collected on the eighth day after their last migraine attack presented an atherogenic lipid profile, including high density of LDL/VLDL [40]. In contrast, we found that LDL/VLDL and lipid were significantly lower in the acute migraine rat model. We hypothesize that the conflict between our results and those of the clinical study can be attributed

to the use of nitroglycerin in the rat model. Nitroglycerin liberates nitric oxide (NO), which is known as endogenous vasodilator, thereby causing dilation of meningeal blood vessels [41–43]. There are increasing evidences to support that migraine attack is associated with dilatation of both extra- and intracranial vessels. Accordingly, one of the advantages of NTG-induced acute migraine model just lies in this dilation of meningeal blood vessels which identically occurred during migraine attack [44–47]. These dilated vessels induced by NTG may quickly increase the blood volume and therefore dilute the level of lipid and LDL/VLDL. Thus, the significantly lower LDL/VLDL and lipid levels might be induced by nitroglycerin injection. Although the significant changes in the lipid and LDL/VLDL may not be a possible biomarker of migraine owing to the inconsistency between NTG-induced rat model and clinical migraine patients, little is known about the change in lipid metabolism during acute migraine attack. Our results may provide a useful clue for clinical studies of acute migraine.

While acupuncture has been proved to be safe and effective in various high quality trials, the analgesic effect of acupuncture in a clinical setting for migraine is still a hotly debated issue. Based on a series of clinic studies that showed that acupuncture at acupoints was no more effective than acupuncture at nonacupoints, some authors wondered if the effect of acupuncture in relieving migraine is only due to the placebo effect and bias [48, 49]. Recently, a meta-analysis including 18,000 randomized patients in high-quality randomized control trails (RCTs) manifested novel evidence that acupuncture is superior to sham acupuncture and placebo for managing chronic pain, including migraine [49]. Accordingly, the increased number of head-scratching in NTG-injected rats was significantly reduced after receiving EA treatment in our study, suggesting that EA relieved acute migraine. However, the scientific mechanism addressing the analgesic effect of acupuncture for migraine is still not well established. Our study is at the first to confirm the potential metabolic evidence of EA relieving migraine for the following two metabolic mechanisms: (i) EA at acupoints can reverse the metabolic profiling of acute migraine rats; (ii) we found that these metabolites change significantly in the plasma of acute migraine rat model, including glutamate, LDL/VLDL, and lipid, which were significantly reversed and restored in acute migraine rats after receiving EA at acupoints (Figures 6(d) and 6(e)). Noteworthy, glutamate, which is crucial in triggering migraine, was decreased after EA treatment, providing direct metabolic evidence that EA relieves acute migraine. To date, increasing numbers of neurological studies have noted that elevated plasma glutamate levels are positively correlated with cortical neuronal hyperexcitability and increased levels of glutamate in the central nervous system (CNS) [39]. Enhanced glutamate released in brain tissue, induced by brief pluses of high K^+ , was found to causatively facilitate CSD, thereby triggering migraine [37]. To conclude, the increased plasma level of glutamate has been proved to be crucial for triggering acute migraine attacks through stimulating cortical neuronal hyperexcitability, increasing glutamate levels in the CNS, and facilitating CSD. Thus, the depletion of glutamate stimulated by EA could inhibit

cortical neuronal hyperexcitability, lower glutamate levels in the CNS, depress CSD, and subsequently decrease acute migraine attack. In addition, acupuncture was illustrated to improve the blood flow and change blood perfusion in the previous studies [50, 51]. Hence, the significantly reversed LDL/VLDL and lipid after EA at acupoints in NTG rats may be attributed to the adjustment of blood flow and blood perfusion stimulated by EA at acupoints. In conclusion, the analgesic effect of EA for acute migraine may rely on substance metabolic basis rather than bias in this study. Therefore, the advantage underlying our results highlights the potential value of metabolomics for becoming surrogate outcome measure of further clinical acupuncture trials.

A series of high quality trials have found that acupuncture on acupoints was not superior to acupuncture on nonacupoints in reducing migraine headaches [14–17, 49, 52]. Consistent with these results, our study found that the number of head-scratching was significantly decreased after both EA at acupoints and EA at nonacupoints treatments. In particular, we found that plasma glutamate levels significantly decreased after performing EA at both acupoints and nonacupoints. It is important to note that this adjustment provides potential evidence that both EA at acupoints and EA at nonacupoints treatments may relieve acute migraine by decreasing the intensity of plasma glutamate. In contrast, Yang et al. reported that the different brain regions related to pain were evoked by performing EA at different acupoints through advanced PET-CT technology [53]. In comparison with previous studies, our findings firstly discriminate significant differences that may contribute to the specific effect of acupoints relative to nonacupoints by two mechanisms: (i) a clear difference between the metabolic profiling of EA and NA groups was successfully demonstrated (Figure 5(e)). (ii) Levels of six metabolites were found to be significantly different between EA and NA groups (Figure 6(f), $P < 0.05$). Furthermore, we found that the metabolic profile induced by EA at nonacupoints is significantly different from that induced in controls and NTG-induced rats. The significant change in lipid CH_2 and PtdCho compared to the control and NTG-induced rats may be an important contributor for this distinct metabolite profile. Whereas, the concentration plot and metabolic profile showed that EA at acupoints can reverse metabolites which were significantly changed in acute migraine model, but EA at nonacupoints did not show such an effect. In light of these facts, the effect of EA at acupoints, but not EA at nonacupoints, has been suggested to be more feasible for adjusting the metabolic disturbance in the plasma of acute migraine.

4.1. Limitations and Directions for Future Studies. The time-related metabolomic changes of electroacupuncture were not detected in this study. Acupuncture is usually performed in one session during the clinical treatment of acute migraine, and our study is consistent with such session. As numerous studies have granted the sensitivity and accuracy of metabolomics for assessing the pathological states of diseases, our study may provide direct and accurate readout of the metabolic changes for electroacupuncture relieving acute

migraine. We have already conducted a dynamic randomized control trial to investigate the effects of electroacupuncture on migraine induced metabolic changes in migraine patient (unpublished). Future studies will extend this work using a larger group of clinical migraine patients.

5. Conclusion

In summary, EA at acupoints may relieve acute migraine through restoring metabolic profile and reversing significant metabolite changes such as glutamate in acute migraine rats, while EA at nonacupoints might modestly relieve acute migraine by decreasing the intensity of plasma glutamate.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contributions

Zishan Gao, Xuguang Liu, and Shuguang Yu conceived and designed the experiments. Zishan Gao, Qi Zhang, QinChen, Qiaofeng Wu performed the experiments. Zishan Gao, Qi Zhang, Juan Liu, Jia Lin and Xianzhong Yan analyzed the data. Bo Sun, Juan Liu, and Xianzhong Yan contributed reagents/materials/analysis tools. Zishan Gao, Bing-Mei Zhu, Jia Lin, Li Fang, Xianzhong Yan and Xuguang Liu wrote the paper. All authors have read and approved the final paper. Zishan Gao and Xuguang Liu contributed equally to this work.

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Review Article

Moxibustion for the Correction of Nonvertex Presentation: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Objectives. This study aims to assess the effectiveness and safety of moxibustion for the correction of nonvertex presentation. **Methods.** Records without language restrictions were searched up to February 2013 for randomized controlled trials (RCTs) comparing moxibustion with other therapies in women with a singleton nonvertex presentation. Cochrane risk of bias criteria were used to assess the methodological quality of the trials. **Results.** Seven of 392 potentially relevant studies met the inclusion criteria. When moxibustion was compared with other interventions, a meta-analysis revealed a significant difference in favor of moxibustion on the correction of nonvertex presentation at delivery (risk ratio (RR) 1.29, 95% confidence interval (CI) 1.12 to 1.49, and $I^2 = 0$). The same findings applied to the cephalic presentation after cessation of treatment (RR 1.36, 95% CI 1.08 to 1.71, and $I^2 = 80\%$). A subgroup analysis that excluded two trials with a high risk of bias also indicated favorable effects (RR 1.63, 95% CI 1.42 to 1.86, and $I^2 = 0\%$). With respect to safety, moxibustion resulted in decreased use of oxytocin. **Conclusion.** Our systematic review and meta-analysis suggested that moxibustion may be an effective treatment for the correction of nonvertex presentation. Moreover, moxibustion might reduce the need for oxytocin.

1. Introduction

Moxibustion is a traditional Chinese medical intervention that utilizes the heat generated by burning herbal preparations containing *Artemisia vulgaris* (mugwort) to stimulate acupuncture points [1]. It is also believed to be effective in the treatment of stroke rehabilitation [2], pain [3], cancer care [4], ulcerative colitis [5], hypertension [6], osteoarthritis [7], constipation [8], child chronic cough [9], and breech presentation [10]. In China, moxibustion on the *Zhiyin* (BL67) point, located on the outer corner of the fifth toenail, has long been used to correct nonvertex presentation in obstetrics [11, 12]. Possible mechanisms of action attributed to moxibustion include stimulation of the production of

placental oestrogens, alterations in prostaglandin levels, and promotion of the uterine contractility, which leads to a stimulation of fetal movements and a higher probability of vertex presentation of the fetus [10, 12–14].

Before moxibustion can be recommended for routine clinical use for the correction of non-vertex presentation, evidence from randomized controlled trials is required. Unfortunately, most studies in which the moxibustion has been evaluated are open clinical trials, blinded to neither the practitioner nor the subjects. In moxibustion trials, sham treatments are conducted by adding insulation below the moxa pillar to prevent the transfer of heat from the pillar to the patient [15]. The sham treatment looks similar to the real moxibustion treatment in appearance and burning

procedure, and participants are able to smell the smoke or observe the burning moxa [15].

The efficacy of moxibustion for the correction non-vertex presentation has been evaluated in four clinical reviews [23–26]. All four studies failed to include all of the relevant articles published [23–26]. For example, none of these reviews included the study of Yang and colleagues [16], which met all of the inclusion criteria for each of the four reviews. Additionally, all of these reviews included interventions other than moxibustion including acupuncture [23–26]. Finally, some reviews included controlled clinical trials [23] and quasirandomised controlled trials [24–26] which were poorly executed and might have affected the conclusion of the reviews.

The objective of the current review and meta-analysis was to perform a comprehensive literature search to find and evaluate high-quality RCTs. Also, our study aim was to critically evaluate the clinical efficacy and safety of moxibustion therapy alone for the correction of non-vertex presentation (not combined with acupuncture or acupuncture alone).

2. Materials and Methods

2.1. Literature Search. The comprehensive literature search included the following electronic databases: MEDLINE (1950 to February 2013), EMBASE (1980 to February 2013), Cochrane Library (1980 to February 2013), CINAHL (1982 to February 2013), AMED (1985 to February 2013), British Nursing Index (1993 to February 2013), Chinese Biomedical Literature Database (CBM; 1980 to February 2013), China National Knowledge Infrastructure (which includes the database China Academic Journals) (CNKI; 1980 to February 2013), VIP Information (VIP; 1980 to February 2013), Wanfang Data (WAN FANG; 1980 to February 2013), Science paper Online (2006 to February 2013), and 28 major Chinese traditional medicine journals.

The following search terms were used: moxibustion OR moxa AND non-vertex presentation or labor presentation or abnormal foetal position or abnormal foetal presentation or podalic presentation or complementary medicine or alternative medicine. We also performed a hand search to identify any other articles. In an attempt to minimize the omission of potentially relevant trials, we also reviewed the reference lists of included articles and relevant reviews for additional eligible studies. Both published and unpublished studies were considered. No language restrictions were imposed.

2.2. Selection of Studies. Potentially relevant studies were independently evaluated by two reviewers (Y. J. H. and Z. Q. H.). Reviewers screened all titles and abstracts when available and they examined the full text if the study met the following inclusion criteria: (a) was a RCT; (b) included a comparison of moxibustion with nonmoxibustion therapy; and (c) included no restriction on the race or gestation of participants with a singleton non-vertex presentation. However, the study with following criteria was excluded: (a) duplication; (b) complex therapy that could not figure out the effect of

moxibustion for example, treatment group used moxibustion plus Chinese herbal ointment, while the control group used knee-chest therapy; (c) incomplete data (failed to provide basic characteristics of participants, such as age, gestational week, and duration of intervention); and (d) wrong intervention or comparator that could not evaluate the effect of moxibustion; for example, treatment group used moxibustion plus acupuncture intervention, while control group used moxibustion intervention. Disagreements between the two reviewers were resolved by discussion with a third author (S. Z. R.) to achieve consensus.

2.3. Outcome Measures. In this review, we present the results for the cephalic presentation at birth and after cessation of treatment. In addition, use of oxytocin, Apgar scores less than 7 at 5 minutes, cesarean section, preterm delivery, premature rupture of membranes, intrauterine fetal death, placental abruption, and cord blood pH less than 7.1 were also recorded.

2.4. Data Extraction. Two authors (S. Q. and H. C.) independently extracted data from eligible studies using a pre-designed extraction sheet and a third author (W. D.) verified the extracted data. Any discrepancies were settled through discussion. The third review author (W. D.) was consulted if a consensus could not be reached. The extracted data included demographic data, clinical characteristics of the study groups, quality of trial design, inclusion and exclusion criteria, interventions, results, and adverse events. If the required information was not available in the included studies, we contacted the original authors by email.

2.5. Quality of the Studies. The Cochrane risk of bias tool [27] was used to assess methodological quality of the trials. Two authors (Y. J. H. and Z. Q. H.) were independently involved in quality assessment. All discrepancies were resolved by consensus with the other author (L. M.).

2.6. Statistical Analysis. Data were pooled using the random-effects model. Treatment effect was expressed as a relative risk, and 95% confidence intervals (CIs) were calculated. Heterogeneity was evaluated using Cochran's Tau^2 , I^2 , and Chi^2 statistics, and high heterogeneity was assumed if the Tau^2 was greater than zero and either the I^2 was greater than 30% or P value was less than 0.10 in the Chi^2 test [27]. Subgroup analysis was conducted to identify and explain heterogeneity. Where possible, a funnel plot was used to assess publication bias. We also performed post hoc sensitivity analysis to test the robustness of the overall effect.

3. Results

3.1. Study Description. We identified 392 potentially relevant articles. Seven RCTs, including a total of 1387 participants, met our inclusion criteria [16–22] (Figure 1). The characteristics of the 7 trials are summarized in Tables 1 and 2. Of those 7 RCTs, four studies were from Western countries and

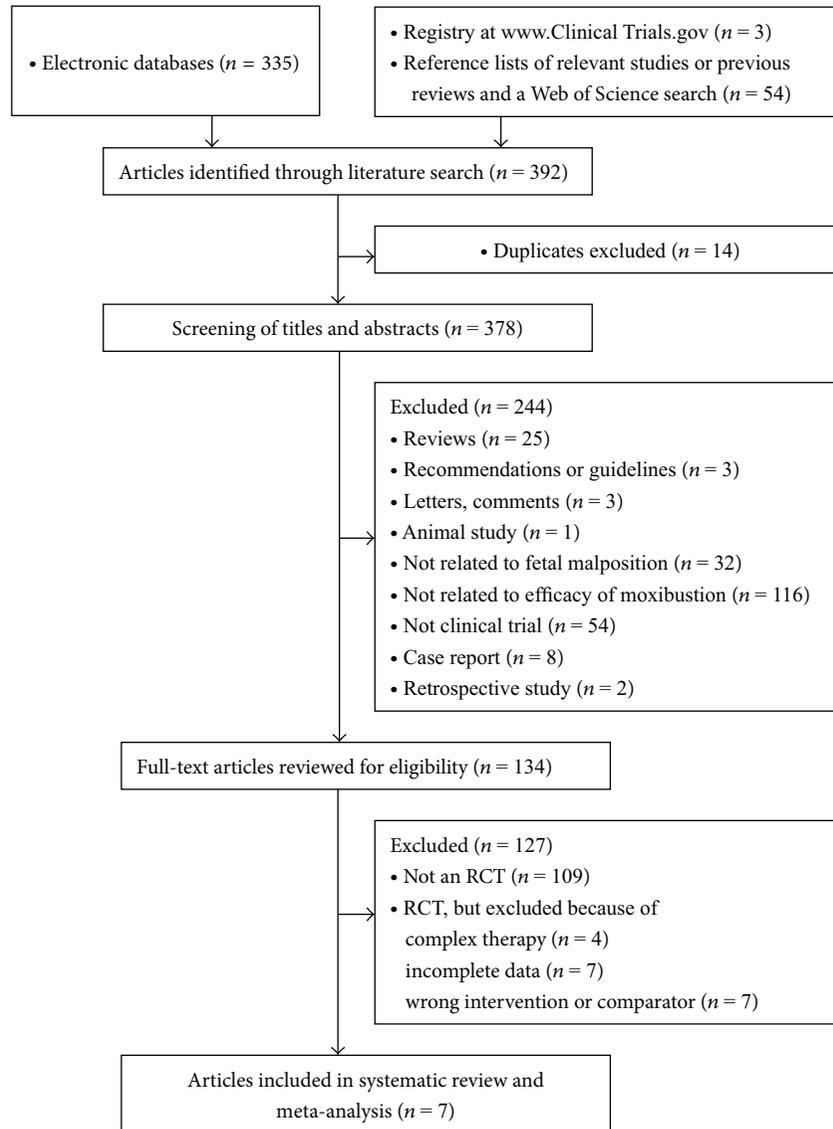


FIGURE 1: Flowchart of study selection.

published in English [18–21], while the other three trials were from China [16, 17, 22], one published in English [17] and two in Chinese [16, 22].

Four trials compared moxibustion therapy with observation [17, 18] and usual care [19, 20]. Two studies compared moxibustion therapy with postural techniques [16, 21], and one study compared moxibustion plus postural technique therapy with postural measures [22].

3.2. Study Quality. The Cochrane risk of bias was presented in Figures 2(a) and 2(b) and Table 3. All seven RCTs reported appropriate sequence generation [16–22]. Six studies conducted concealment of allocation by sealed envelopes [16–21], while one trial did report it [22]. In five studies, moxibustion was either applied at home by participants themselves [17–19, 21] or by practitioners in hospital [16, 20], while the remaining one study did not state who applied the

intervention [22]. In that study, it was not feasible to blind the participant or the therapist. Although the outcome assessor was blinded in only one study [18] and the analyst was blinded to groups in three studies [16, 19, 21], the review authors deemed that the outcomes and their measurements were not likely to be influenced by lack of blinding. Thus, all studies had a low risk of bias with the Cochrane risk of bias tool at blinding levels. Four studies reported complete followup of all subjects [17–21]. One study stated that 7 women from treatment group and 10 women from control group withdrew from the trial [16]. One trial reported that 1 woman was lost to followup in the control group, and 14 women discontinued treatment in the intervention group [18]. The other one did not provide any information of followup [22]. When it comes to selective reporting bias, the trial protocol was available for two trials [19, 21]; however, the other five studies failed to provide it [16–18, 20, 22]. Of those five trials, three studies

TABLE 1: Main characteristics of included RCTs.

Study	Study design	Patient population	Treatment group	Control group	Outcome measures
Yang et al. [16]	Parallel 2-arm	296 participants	Moxibustion at bilateral BL67; twice daily, 30 min each time, 15 min each side; 7 d course ($n = 147$)	Knee-chest therapy; twice daily, 15 min each time ($n = 149$)	NCPCT
Cardini and Weixin [17]	Parallel 2-arm	260 participants	Moxibustion at bilateral BL67; first 87 subjects once daily for 1 week, next 43 women twice daily for 7 d; 30 min each time, 15 min each side ($n = 130$)	Observation; once or twice daily for 30 min each time, 15 min each side ($n = 130$)	(i) NCPDE (ii) NCPCT (iii) CS (iv) UO (v) AS (vi) PD (vii) PRM (viii) IFD
Cardini et al. [18]	Parallel 2-arm	123 participants	Moxibustion at bilateral BL67; twice daily, 30 min each time, 15 min each side for 1 or 2 wk ($n = 65$)	Observation ($n = 58$)	(i) NCPCT (ii) PRM (iii) PA
Do et al. [19]	Parallel 2-arm	20 participants	Moxibustion at bilateral BL67; twice daily, 20 min each time, 10 min each side for 10 d ($n = 10$)	Usual antenatal care for 10 d ($n = 10$)	(i) NCPDE (ii) CS (iii) AS (iv) PD (v) PRM
Guittier et al. [20]	Parallel 2-arm	212 participants	Moxibustion at bilateral BL67; three times weekly; 20 min each time, 10 min each side for 2 wk ($n = 106$)	Expectant management care ($n = 106$)	(i) NCPDE (ii) CS (iii) AS (iv) CBPH
Vas et al. [21]	Parallel 3-arm	270 participants	Moxibustion at BL67; 20 min each time, 2 wk ($n = 136$)	Knee-chest therapy; 20 min each time, 2 wk ($n = 134$)	(i) NCPDE (ii) CS (iii) PD
Yang [22]	Parallel 2-arm	206 participants	Moxibustion at bilateral BL67 + knee-chest therapy; 15–20 min, twice daily, 7 d course for 1 wk ($n = 103$)	Knee-chest therapy, 15–20 min each time, twice daily, 7 d course for 1 wk ($n = 103$)	NCPCT

d: day, wk: week, NCPDE: number of cephalic presentations at delivery (excluding external cephalic version), NCPCT: number of cephalic presentations after cessation of treatment, CS: cesarean section, UO: use of oxytocin, AS: Apgar scores <7 at 5 min, PD: preterm delivery, PA: placental abruption, PRM: premature rupture of membranes, IFD: intrauterine fetal death, CBPH: cord blood pH less than 7.1.

included all expected outcomes [17, 18, 20], while the remaining two failed to state them, so the review authors were unable to determine whether all outcomes were prespecified [16, 22]. All seven trials conducted sample size calculations [16–21], except for one study that did not report it [22]. Five trials did not report imbalances at randomization, and they appeared free of other sources of bias [17, 19–22]. One study failed to provide sufficient information, so the review author did not determine whether the other bias is present [16]. The other one was interrupted when interim analysis revealed poor compliance and a high number of treatment interruptions [18].

3.3. Outcome Measures. Seven included trials assessed the effect of moxibustion (alone or in association with postural techniques) compared with observation alone or postural measures on cephalic presentation at delivery [17, 19–21] and after cessation of treatment [16–18, 22] (Figure 3). Five out of the seven studies involved the other outcomes of safety on the use of oxytocin [17], Apgar scores less than 7 at 5

minutes [17, 19, 20], cesarean section [17, 19–21], preterm delivery [17, 19, 21], premature rupture of membranes [17–19], intrauterine fetal death [17], placental abruption [18], and cord blood pH less than 7.1 [20] (Figure 4).

Our meta-analysis of four studies [17, 19–21], which included 737 participants, yielded encouraging effects in favor of moxibustion on cephalic presentation at delivery (excluding ECV) (RR 1.29, 95% CI 1.12 to 1.49, and $I^2 = 0$) (Figure 3). The same findings applied to the cephalic presentation after cessation of treatment, when moxibustion (alone or in combination with postural techniques) was compared with observation [17, 18] or postural techniques [16, 22] (RR 1.36, 95% CI 1.08 to 1.71, and $I^2 = 80\%$) (Figure 3). A subgroup analysis that excluded two studies with a high risk of bias [18, 22] showed significant effect of moxibustion (RR 1.63, 95% CI 1.42 to 1.86, and $I^2 = 0\%$) (Figure 3).

Five trials examined the safety of moxibustion for the correction of non-vertex presentation [17–21] (Figure 4). One study reported significant differences in favor of a reduced use of oxytocin in the treatment group [17] (RR 0.28, 95%

TABLE 2: Additional details of the included RCTs.

Study	Location (country)	Age (mean or range)	Duration	Gestational week	Inclusion	Exclusion
Yang et al. [16]	China	20–36 y	1–2 wk	30–36 wk	Meet the diagnostic criteria, 30 to 34 wk, informed consent, and voluntary acceptance of the experiment	Complicated with pregnancy-induced hypertension, gestational diabetes, merging genital tumor, contracted pelvis, polyhydramnios or oligohydramnios, cord around neck, and fetal biparietal diameter >8 cm, before placenta attach to uterine wall
Cardini and Weixin [17]	China	T: 25.5 ± 2.5 y C: 25.2 ± 3.0 y	1 wk	33 wk	Normal fetal biometry (biparietal and abdominal circumference between percentiles 10 and 90)	Pelvic anomalies, previous uterine surgery, pregnancy-related illness, fetal malformation, twin pregnancy, fibroma > 4 cm, uterine malformation, risk of premature delivery (hypercontractility, Bishop 4 or greater), and tocolysis during pregnancy
Cardini et al. [18]	Italy	T: 31 y C: 26.2 y	1–2 wk	32–33 wk plus 3 d	Normal fetal biometry	Nonacceptance of randomization, pelvic anomalies, previous uterine surgery, fetal malformation, uterine malformation, fibroma > 4 cm, twin pregnancy, previous or current tocolysis, and other pregnancy-related complications
Do et al. [19]	Australia	T: 30.36 ± 3.13 y C: 24.60 ± 5.23 y	10 d	34–36.5 wk	Women were aged greater than 18 years, at 34–36.5 wk of gestation with a singleton breech presentation (confirmed by ultrasound), and normal fetal biometry	Twin pregnancy, risk of premature birth, heart or kidney diseases affecting the mother, placenta previa, history of antepartum haemorrhage, intrauterine growth restriction, hypertensive disease, isoimmunisation, previous uterine operations, uterine anomaly, prelabour rupture of the membranes, multiple pregnancy, fetal congenital abnormality, contraindication to vaginal delivery, and fetal death in utero
Guittier et al. [20]	Switzerland	T: 32.0 ± 4.3 y C: 32.0 ± 4.2 y	2 wk	T: 35 ± 0.8 wk C: 34.8 ± 0.7 wk	Single fetus in breech presentation between 34 and 36 wk of gestation	Uterine malformation, placenta praevia, and transverse lie
Vas et al. [21]	Spain	T: 22.6–39.0 y C: 24.0–38.3 y S: 24.4–38.0 y	2 wk	33–35 wk	Diagnosed by physical examination and ultrasound; at least 18 years; 33–35 wk of gestation (confirmed by ultrasound); normal fetal biometry and no prior treatment with moxibustion to achieve version of the fetus	Multiple pregnancy, bone pelvic defects, previous uterine surgery, fetal malformation or chromosomal disorder, uterine malformations, risk of preterm birth (preterm uterine contractions and/or initial dilatation or shortening of the cervix with a score of 4 on the Bishop scale), uterine fibroids >4 cm, tocolytic therapy, and maternal heart or kidney disease
Yang [22]	China	T: 26–28 y C: 25–27 y	7 d	28–32 wk	Not stated	Not stated

T: treatment group, C: control group, S: sham group, y: year, wk: week, d: day.

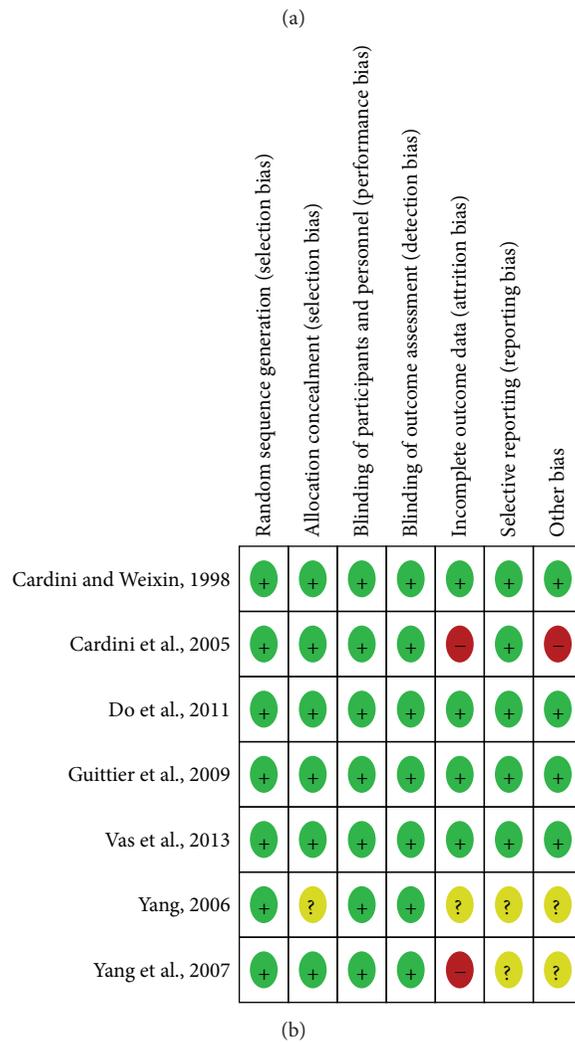
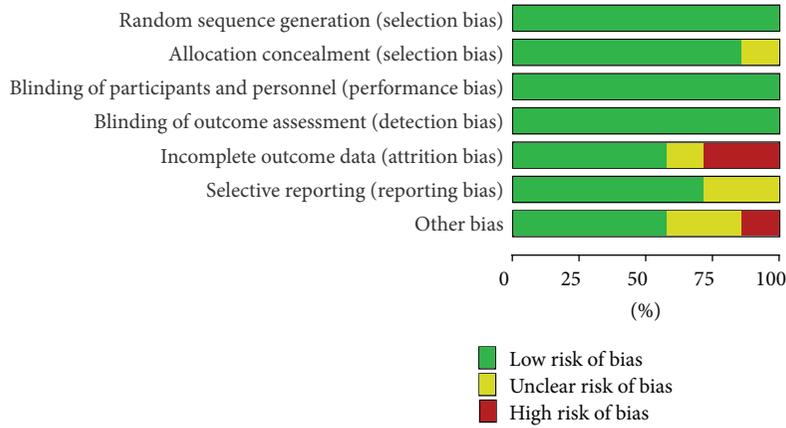


FIGURE 2: (a) Risk of bias graph: review authors’ judgments about each risk of bias item presented as percentages across all included studies. (b) Risk of bias summary: review authors’ judgements about each risk of bias item for each included study.

CI 0.13 to 0.60) (Figure 4). No other statistically significant differences were found in the comparison between moxibustion treatment group and nomoxibustion group on Apgar scores less than 7 at 5 minutes, cesarean section, preterm delivery, premature rupture of membranes, intrauterine fetal

death, placental abruption, and cord blood pH less than 7.1 (Figure 4).

3.4. Adverse Events. Three trials reported the adverse events in the moxibustion group [17–19]: two reported two and four

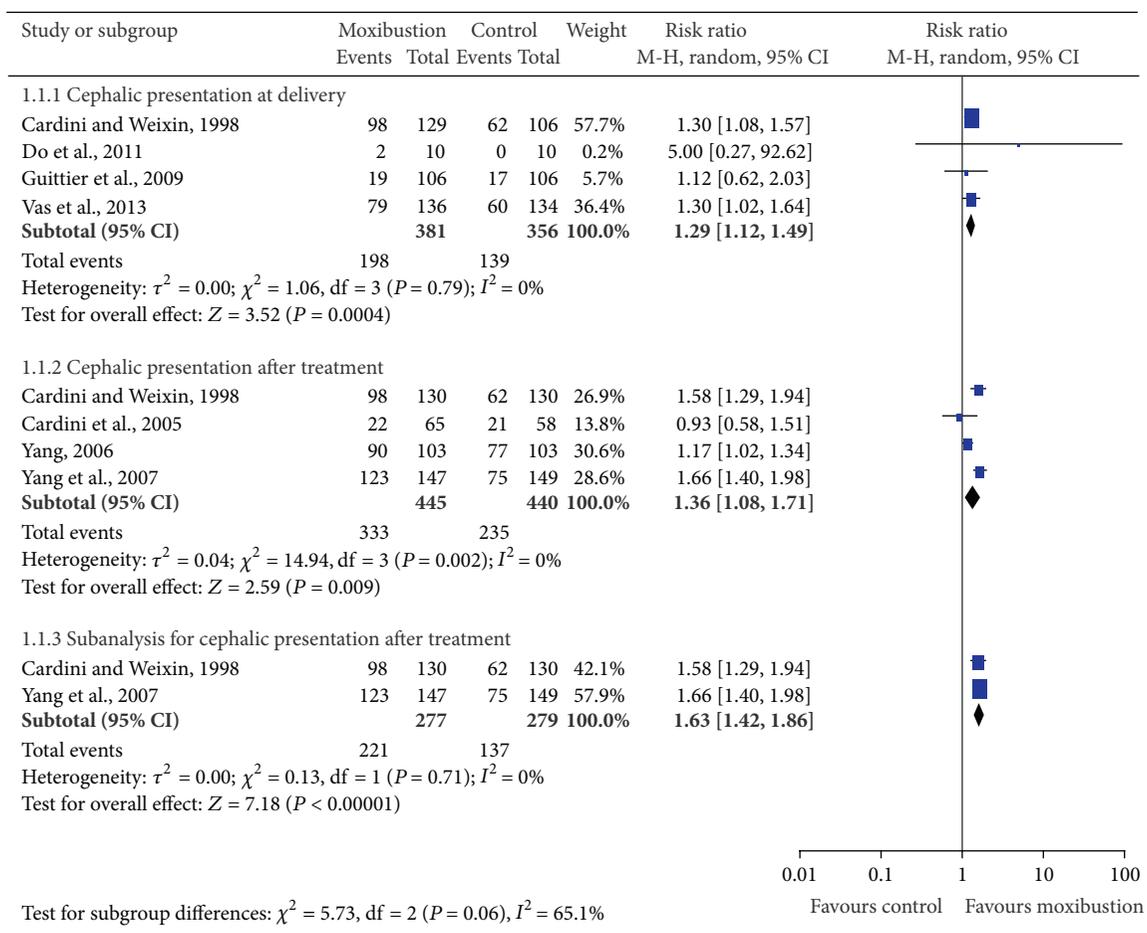


FIGURE 3: Effectiveness of moxibustion for the correction of non-vertex presentation.

cases of premature deliveries at 37 weeks, respectively. Four cases of premature rupture of the membranes after treatment were also reported [17]. Another trial noted two cases of premature deliveries and one case of bleeding at week 37 after ECV due to excessive pressure on the rear of the placenta [18]. The third trial recorded two cases of premature deliveries and three cases of prelabour rupture of the membranes [19].

4. Discussion

In this systematic review and meta-analysis, moxibustion at point *Zhiyin* (BL67) is found to be an effective intervention for correcting non-vertex presentation. With respect to safety, there was no significant difference between moxibustion and control group with outcomes of the use of oxytocin, Apgar scores less than 7 at 5 minutes, cesarean section, preterm delivery, premature rupture of membranes, intrauterine fetal death, placental abruption, and cord blood pH less than 7.1. In case of the use of oxytocin, moxibustion resulted in decreased use of it.

Previous reviews did not include all relevant trials [23–26]. For example, all four reviews failed to include the study of Yang and colleagues [16]. Although the newest Cochrane

review (from Coyle and colleagues in January 2012, updated to August 2011) was published within the last two years [26], two high-quality RCTs from Do and colleagues in 2011 and Vas and colleagues in 2013 were not included [19, 21]. Moreover, all these reviews included interventions other than moxibustion. For instance, all four studies included trials, which combined with acupuncture therapy [23–26] or even laser intervention [23]. As we know, moxibustion, acupuncture, and lasers are different interventions. Thus, it is difficult to determine what kind of intervention really works for the correction of non-vertex presentation.

We made an effort to identify all relevant trials and included high-quality RCTs. Although one study included in this analysis was of lower quality and resulted in high heterogeneity [22], the subgroup analysis that excluded it still showed a favorable effect of moxibustion for the correction of non-vertex presentation. Our study aims to evaluate the clinical efficacy and safety of moxibustion intervention for the correction of non-vertex presentation, so we only included trials comparing moxibustion with non-moxibustion therapy in participants with non-vertex presentation.

Our review has several limitations. Although great efforts were made to retrieve all trials on the subject, there may

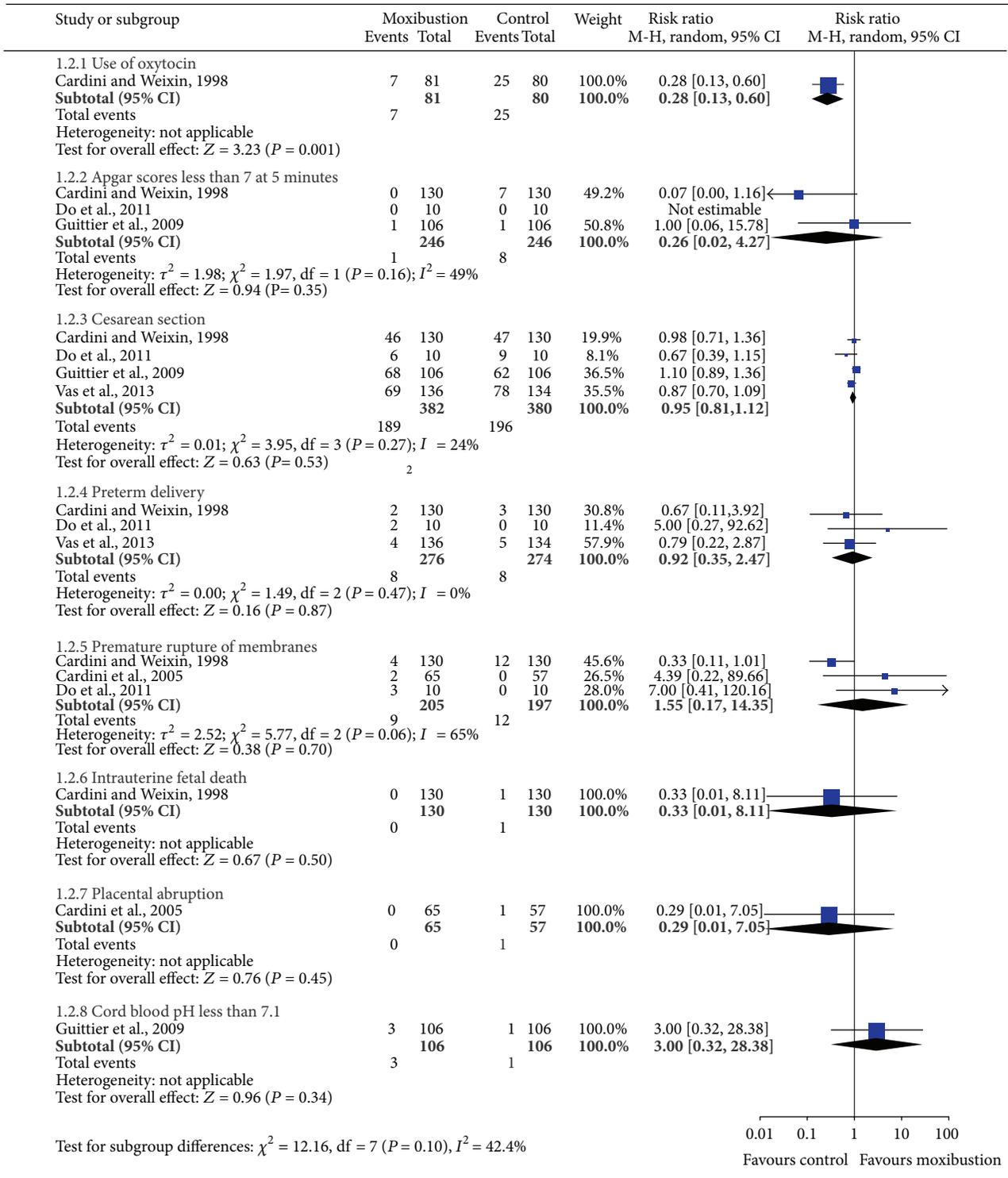


FIGURE 4: Safety of moxibustion for the correction of non-vertex presentation.

be still the possibility of missing studies. In addition, some incomplete information may affect the quality and validity of the results. Finally, a large degree of variability of frequency and duration from three times weekly to once or twice daily might be the possible source of bias.

5. Conclusion

The results of our systematic review and meta-analysis showed a positive effect of moxibustion on the correction of non-vertex presentation. In addition, moxibustion might

TABLE 3: Risk of bias of included RCTs.

Study	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other bias
Yang et al. [16]	Computer generated	Sealed envelopes	Not stated	Analyst was blinded	7 subjects from treatment group and 10 subjects from control group withdrew from the trial	SPUU	IID
Cardini and Weixin [17]	Computer generated	Sealed envelopes	Neither participants nor practitioner was blinded	Not stated	Complete followup of all subjects	SPUP	NIR; SAF
Cardini et al. [18]	Computer generated	Sealed envelopes	Neither participants nor practitioner was blinded	Assessor was blinded	1 subject in control group was lost to followup; 14 subjects in intervention group discontinued treatment	SPUP	TIIA
Do et al. [19]	Computer generated	Sealed envelopes	Not stated	Analyst was blinded	1 subject in control group was lost to followup, but less than 10%	Study protocol available	NIR; SAF
Guittier et al. [20]	Computer generated	Sealed envelopes	Not stated	Not stated	Complete followup of all subjects	SPUP	NIR; SAF
Vas et al. [21]	Computer generated	Sealed envelopes	Participants in true and sham moxibustion groups were blinded	Analyst was blinded	Complete followup of all subjects	Study protocol available	NIR; SAF
Yang [22]	Table of random numbers	Not stated	Not stated	Not stated	Followup of all subjects was not reported	SPUU	IID

SPUU: study protocol unavailable; unable to determine whether all outcomes were prespecified, SPUP: study protocol unavailable, but published report includes all expected outcomes, IID: insufficient information to determine whether the other bias is present, NIR: no imbalances at randomization, SAF: study appears free of other sources of bias, TIIA: trial was interrupted when interim analysis revealed poor compliance and a high number of treatment interruptions.

reduce the need for oxytocin. More rigorous high-quality RCTs are still needed to evaluate the efficacy as well as safety of moxibustion for the correction of non-vertex presentation in the future.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Qin-hong Zhang and Jin-huan Yue contributed equally to this paper.

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Review Article

The Mechanism of Moxibustion: Ancient Theory and Modern Research

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The moxibustion has a dual effect of tonification and purgation in TCM theories, which are based on two aspects: the actions of the meridian system and the roles of moxa and fire. Modern research works of the moxibustion mechanism mainly relate to the thermal effects, radiation effects, and pharmacological actions of moxa and its combustion products. Experimental results showed that moxibustion thermal stimulation affects both shallow and deep tissues of the skin, and the warm-heat effects of moxibustion have a close relation to the warm receptors or/and the polymodal receptor. The burning moxa radiation spectrum ranges from 0.8 to 5.6 μm ; peak is nearby 1.5 μm , lying within the near infrared portion. There is an amazing consistency in the infrared spectrums of three types of indirect moxibustion and the unified spectrum of acupoints; all have their peaks of radiation near 10 μm . Lots of ingredients had been identified from mugwort leaves and moxa smoke, which have a variety of biological activities; they were considered to participate in the comprehensive effects of moxibustion. Although lots of research works have been carried out and made some progress, there is still a great distance from fully understanding the mechanism of moxibustion.

1. Introduction

Moxibustion is a kind of external treatment; it is based on the theory of traditional Chinese medicine (TCM), and it usually bakes acupoints with burning moxa wool. Moxibustion can dredge meridians and regulate qi-blood and has been used to prevent and cure diseases for more than 2500 years. *Zuo zhuan* of the pre-Qin dynasty in China, which recorded a disease discussion occurred in 581 B.C., is considered to be the earliest literature of moxibustion. The silk books discovered in Mawangdui tomb of the Han dynasty (about 168 B.C.), *Moxibustion Classic of Eleven Foot-hand Meridians* and *Prescriptions for Fifty-two Diseases*, had documented the use of moxibustion to treat complex diseases. There are a lot of moxibustion contents in *Inner Canon of Huangdi*; it inferred that the origin of moxibustion is related to the living habits and disease characteristics of northern Alpine nation in the part of *Su wen, Yi fa fang yi lun*. Later doctors after Han dynasty had made considerable progress in theory and practice on moxibustion and promoted moxibustion to be a mature and widely used therapy.

Moxibustion has been applied in treating a great range of diseases. A bibliometric analysis on the papers published from 1954 to 2007 in China showed that up to 364 kinds of diseases can be treated with moxibustion. The most proper indications of moxibustion therapy are malposition, diarrhea, and colitis; the common proper indications are urinary incontinence and dysmenorrhea; the next common proper indications are knee osteoarthritis, temporomandibular joint disturbance syndrome, soft tissue injury, heel pain, asthma, urinary retention, and herpes zoster [1]. Moxibustion can also be used to treat weakness, fatigue, and aging related problems. Moxibustion can be classified as traditional moxibustion, drug moxibustion, and modern moxibustion. Traditional moxibustion therapy is the most commonly used in the ancient and contemporary moxibustion clinics; it is characterized by the use of moxa as burning material and can be divided into direct moxibustion and indirect moxibustion depending on whether moxa is directly in contact with the skin while operating. A moxa cone placed directly on the skin and ignited is called direct moxibustion, while the moxa kept at certain distance from the skin is called

indirect moxibustion. The insulating materials of indirect moxibustion can be air, garlic, ginger, aconite, salt, and so forth. Drug moxibustion, also named nature moxibustion, uses irritant drugs (such as cantharis, garlic, and semen sinapis) to coat the surface of acupoints and make local skin flushed and blistered to cure diseases. Modern moxibustions, such as microwave moxibustion, laser moxibustion, and electrothermal moxibustion, are used to simulate traditional moxibustion stimulation factors by physical or chemical methods to achieve therapeutic effects of moxibustion. Usually, narrow sense of moxibustion refers to the traditional moxibustion with moxa. This review will concentrate on the ancient theory and modern mechanism research of traditional moxibustion.

2. Traditional Moxibustion Theory

Ling Shu, Guan Neng says that where needle does not work, moxibustion does. TCM theory holds that moxibustion has a dual effect of tonification and purgation. Different from needles and drugs, characteristics of moxibustion in materials and using fire determine that its efficacy is inclined to warming and nourishing. So, moxibustion is often applied in deficiency-cold syndrome, though some excess-heat syndrome can also use it. The roles of moxibustion can be broadly grouped into warm nourishing, warm dredging, and warm melting. Warm nourishing refers to the benefits of warming Yang, tonifying qi, nurturing blood, and relieving depletion; warm dredging refers to the functions of activating blood, dissolving stasis, promoting qi, dredging channels, and relieving pain; warm melting refers to the roles of reducing phlegm, eliminating stagnation, removing wind, dispelling dampness, drawing out poison, and purging heat. Some people believe that warm dredging is the nature of moxibustion and is the key role of moxibustion effects. The functions of moxibustion, expelling cold, promoting the circulation in meridians and collaterals, clearing away heat, detoxification, and so forth, are dependant on the efficacy of moxibustion for circulating qi and blood flow [2].

In TCM basic theory, moxibustion effects are based on two aspects: the action of the meridian system and the role of moxa and fire.

2.1. Meridian System. TCM usually takes “needling” and “moxibustion” collectively, for both of them are similar therapeutics based on the same theory of meridian and acupoint. In other words, the moxibustion therapeutic effect is partly dependant on the body’s nonspecific system of meridians.

Moxibustion is closely related to meridians, cutaneous regions, and acupoints. Meridian system consists of channels and collaterals; they are pathways of communicating internal and external, contacting organs, running qi-blood, and regulating the whole body. *Ling Shu, Hai Lun* says that there are twelve regular channels, the inner ones belong to viscera and the outer ones connect with limbs. TCM believes that a person is as a whole. The organs and limbs communicate and interact through the meridian system, which plays a very important role in physiological functions and pathological

processes. The cutaneous regions are the surface part of the twelve regular channels, which are nourished by channel-qi. The cutaneous regions can show the status of qi-blood from meridians and organs, also it can receive treatment stimulation and then make effects. Acupoints are the sites on the body surface, in which the qi of organs and meridians assembled, that act as target points and response points of treatment.

In the moxibustion treatment process, the cutaneous regions and acupoints are the terminals of the meridian system, as the receivers, by which moxibustion stimulations can be transmitted into the body. Through the meridian system, moxibustion can reinforce insufficiency and reduce excessiveness and directly correct the disease state of the human body or activate the meridian system self-healing function and play a therapeutic role. For example, the different acupoints can cure different diseases in moxibustion, and the same acupoints can get similar results regardless of acupuncture or moxibustion; all of these proved that the body meridian and acupoint system play an important role in the treatment of moxibustion.

2.2. Moxa and Fire. *Elementary Medicine* believes that the diseases that cannot be cured by drugs and acupuncture should be treated with moxibustion. The unique therapeutic effects of moxibustion are closely related to the specificity of moxa and fire.

On moxibustion fire in TCM, there is a discussion in *Shen jiu jing lun* stating that moxibustion using fire, for being hot and rapid, with soft body can bear with that to eliminate the shadow; it can move instead of stay and always go into organs. Fire is hot, so it can warm back the Yang and eliminate cold of the Yin, even it can melt the poisoning things caused by damp, wind, phlegm, and so on; fire is speedy, so it can dredge the channels, remove the pain or numbness, and active blood and qi. So, the feature of moxa fire shows the main role of moxibustion.

Materials are very important to moxibustion. The choosing of materials of moxibustion in TCM is really harsh. *Pu ji fang, Acupuncture* cited the *Xiao pin fang* on eight kinds of fire: moxibustion with pine wood fire, hard to cure; cedar wood fire, ulcer and pus; orange wood fire, skin hurt; mulberry wood, muscle withered; jujube wood fire, body emaciated; bamboo fire, tendons injured, excessive lead tendons flabby; trifoliolate orange wood fire, veins “collapse”; elm wood fire, bone hurt, excessive lead bone withered; none of them can be used. But moxa fire is warm without dry, and it can ascend and descend with strong penetration ability into the viscera. *Compendium of Materia Medica* had said that moxa leaf are slightly bitter and over-spicy when raw, and slightly spicy and over-bitter when processed. Moxa with the nature of pure Yang, raw moxa is warm and become hot after processing. It can take the Tai-Yang fire and get back dying Yang. It can go through three Yin, get rid of all the cold and dampness, and turn the cold into warm after taking orally. Moxibustion with moxa leaf can get into the channels and cure hundreds of diseases. Its function is great. The drug properties of moxa leaves (raw) are that they turn warmer after being processed, become moxa wool (processed), which

are suitable for moxibustion, and the older the better. The ancients chose moxa as moxibustion material for it is easy to collect and more for its drug properties, and long-term clinical practices have proved that.

3. Mechanism Research of Moxibustion

Modern research of moxibustion started in the early of last century, Japanese scholars began to observe physical characteristics of moxibustion materials and the effects of moxibustion on blood pressure and intestinal peristalsis in 1912 [3, 4]. Up to this day, there have been more and more studies of effects of moxibustion on the human body or experimental animals, almost involving all major physiological systems, especially in the fields of analgesic, enhancing immunity and antiaging. At the same time, researching works on the mechanism of moxibustion also gradually developed, mainly related to the thermal effects, radiation effects, and pharmacological actions of moxa and its combustion products.

3.1. Thermal Effects. Burning moxa without flame can produce high temperature of about 548–890°C [5, 6]; it will give a warm feeling when it is close to the body, so some people think that this treatment is essentially a thermal physical effect [7]. Experiment confirmed that single Zhuang (a dose unit of moxibustion) of moxa cone (2 mg) moxibustion on mice abdomen can raise the temperature to 130°C outside the skin of the point and 56°C inside the skin; the same changes of temperature were not observed in the forelimb far away from the stimulation site [8]. By using 50 mg moxa cone direct moxibustion on the skin of mice with thermocouple implanted, the temperatures of epidermal, subcutaneous, and basal layers were different; the results suggested that moxibustion thermal stimulation affects both shallow and deep tissues of the skin [9]. The maximum temperature change by indirect moxibustion was about 65°C on the skin and 45°C in the subcutaneous layer [10]. The temperature-time curve of moxa cone can be characterized by slow rising, rapid rising, rapid decline, and slow decline phases, and ginger-separated moxibustion can “buffer” the temperature changes [11]. The actual temperature of indirect moxibustion is greatly affected by the texture, size, and the moisture content of the insulating material [12].

The thermal effects of different moxibustions are not the same. Some people used thermal resistor thermometer and computer online real-time processing to measure the skin temperature at the acupoints of different moxibustions: direct moxibustion, ginger-separated moxibustion, suspension moxibustion, light moxibustion, and He-Ne laser moxibustion. All of them except He-Ne laser moxibustion had significantly changed the temperature of the acupoints through the skin to the muscularis, and each had their own rules and characteristics. The results suggested that the effects on acupoint and even the efficacy of moxibustion depend on the temperature changing of acupoint caused by moxibustion [13]. Others observed the relationship between the moxibustion effect and the intensity of thermal stimulation through the change of pain threshold. In the 40~60 minutes of moxibustion, the pain threshold rose with the

operative time and increasing the burning moxa amount per unit time can significantly improve the immediate analgesic effect and lingering effects [14]. Experiment of activation of subnucleus reticularis dorsalis (SRD) neuron by variety intensities of moxibustion thermal stimulation shown that noxious thermal (44–52°C) stimulation can activate SRD neurons, which reaches a plateau when the stimulated area is increased to a certain range [15].

The warm-heat effect of moxibustion has a close relation to the warm receptors (WRs) or/and the polymodal receptor (PRs). The antipyretic and thermolytic effects of moxibustion are achieved by stimulating polymodal receptors of acupoints [16–18]. Effects of moxibustion on the skin can appear as hotness, flushing, pain, blisters, and other skin irritations and burns phenomena. Moxibustion can lead to vasoconstriction at the burning point while vasodilatation around the point and increase peripheral arterial blood flow and microvascular permeability [8, 19]. Another thermal effect of moxibustion is to induce heat shock proteins (HSPs) in local tissues. HSPs are a class of functionally related proteins involved in the folding and unfolding of other proteins. As an endogenous protective mechanism, HSPs can be synthesized in cells in response to hyperthermia and other environmental stresses. The HSPs induced by moxibustion may be an important factor of its mechanism of action [20].

3.2. Radiation Effects. By irradiating acupoints of pain model rats with radiogenic heat of 40–43°C, there are no significant changes in the tail-flick latency or vocalization threshold, suggesting that not any thermal stimulation can achieve moxibustion efficacy [21]. The burning moxa emits visible light and infrared (IR) radiation; therefore, besides the heat effects, nonthermal radiation effect may be an important role in the efficacy of moxibustion. Physics tells us that the radiation is a process of energy outward diffusion in the form of electromagnetic waves or particles; any object above absolute zero in temperature emits electromagnetic radiation. At present, the common view is that the ignited moxa radiation spectrum ranges from 0.8 to 5.6 μm; peak is nearby 1.5 μm, lying within the near infrared (NIR) portion [22]. But results are reported differently due to the measurement methods and the experimental conditions. Thermal radiation of burning moxa stick measured by indirect methods is mainly far infrared (FIR) near NIR, with spectrum peak at 2.8 μm [23]. Measured with visible-infrared monochromator, radiation spectrum of drug moxa sticks is distributed from red light through NIR to middle infrared (MIR), in which multipeaks especially at 2.4 μm are detected and without the parts of wavelength shorter than 0.6 μm [24].

By analyzing and comparing the infrared radiation spectrums of the moxibustion, the substitute moxibustion, and acupoints of human body, it was found that there was a surprising consistency in the spectrums of three types of indirect moxibustion, namely, separated with prepared monkshood, ginger, and garlic, and the unified spectrum of acupoints. Both had their peaks of radiation near 7.5 μm (after modification, this wavelength should be around 10 μm). However, the spectrum of the substitute moxibustion (separated with cucumber and carrot) was completely different

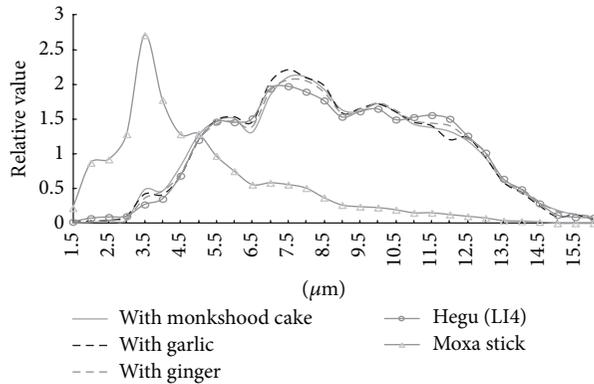


FIGURE 1: Unified infrared radiation spectrums of an acupuncture point, Hegu (LI 4), direct moxibustion with a traditional moxibustion stick, and indirect moxibustion with three traditional media.

from them. Its warming function was far less than the traditional moxibustion, and there was also a big difference between the infrared radiation spectrums of the moxa-stick (with a peak at $3.5 \mu\text{m}$) and acupoints (see also Figure 1 and Table 1). The results indicated that, in the therapeutic effect of traditional indirect moxibustion, the resonance vibrations of infrared radiation of indirect moxibustion and acupoints play an important role and the substitute moxibustion could not replace the traditional moxibustion in terms of the infrared characteristics of moxibustion [25–29].

Infrared acting on the body will produce thermal and nonthermal effects. Thermal effects are produced under the action of electromagnetic waves; the human body molecules absorb energy from IR and convert it into heat and therefore promote blood circulation and improve the cell and enzyme activities. The nonthermal effect is related to the interaction of electromagnetic waves and organism; it is more complex and with nonlinear characteristics. The actions of NIR and FIR on organism are different. NIR is generally believed to play a major role in the biological radiation effect of moxibustion. When NIR irradiates body, the light reflected by the skin is relatively low, the energy can be transmitted about 10 mm deep into the skin, reach the tissues, and be absorbed by them [30]. The NIR can induce some active substances produced within the tissues, after being absorbed by connective tissue, blood vessels, lymphatic vessels, and nerves under the irradiated skin, distribute to other parts of the body with the blood circulation, and enhance the metabolism and thermogenesis of organs they reached. NIR can also energize the metabolism of cells. The energy generated by the photoelectric effect and photochemical process and passed through the nerve-humoral system can provide the activation for the pathological cells lacking energy and then further adjust the body's immune and neurological functions [31, 32].

3.3. Pharmacological Actions. Moxa, *Artemisia argyi* Levl. et Vant., also known as mugwort, is a Compositae *Artemisia* perennial herb. Mugwort leaf can produce moxa wool after drying and grinding, which is a common moxibustion material. The ingredients of moxa are complicated; more

than 60 kinds of components had been identified [33]. The volatile oils of moxa include 1,8-Cineole, alkenes (alpha-thujene, pinene, sabinene, etc.), camphor, borneol, and little aldehydes, ketones, phenols, alkanes, and benzene series compounds. Heptatriacontane ($\text{C}_{37}\text{H}_{76}$) plays an important role in combustion [34]. The moxa also has tannins, flavonoids, sterols, polysaccharides, trace elements, and other ingredients.

The ingredients of moxa always change according to the place and season of production. The oil rate of QiAi in Hubei is obviously higher than in Hebei, Shangdong, and other places. Some people had measured the heat of combustion from different kinds of moxa: QiAi (from Hubei) was 18139 J/g, BeiAi was 17463.4 J/g, QiAi (from Hebei) was 17419.3 J/g, and ChuanAi was 16136.4 J/g [35]. The combustion heat of QiAi (from Hubei) was the biggest, and it has been considered to be the best moxibustion material since ancient times.

The volatile oil rate of moxa is 0.45%–1.00%. It has a variety of biological activities such as the expansion of airway smooth muscle, relieving cough, expectorant effect, and a strong antioxidant activity [36–38]. The moxa is rich in flavonoids and polysaccharides, which have strong antioxidant activity too [39, 40].

The moxa combustion test showed that the relative equilibrium moisture content of moxa was 13.51%, the relative ash content was 11.77%, and the relative smoke production rate was 126.42% [41]. Parts of the moxa combustion products are brown tar-like substances; they play a role by penetrating into the human body through the skin damaged by the burning. The moxa and the combustion products of moxa having been extracted with methanol, both extracts showed the actions of clearing the free radicals and lipid peroxidation, and the latter was stronger. The result indicated that the active ingredients of moxa were increased rather than being destroyed after burning. The methanol extracts of moxa combustion products, tars, can be divided with silica gel column chromatography, and the antioxidant components were found in band IV. Further divided by thin-layer chromatography, the antioxidant effect in band Rf 0.14 is better than the synthetic antioxidant BHT. Ginger and garlic, the important auxiliary materials for moxibustion, are commonly used in indirect moxibustion. The ginger and garlic had been put on the evaporating dish for experiment and had confirmed that gingerol and allicin, the active ingredients of them, could act on the body by heat to give the therapeutic effects [42–44]. The extracts of moxa combustion ashes also have the strong ability of antifree radical [45].

Another combustion product of moxa is smoke. The smoke of moxa contains a variety of complex components, and its volatile ingredients are ammonia, alcohols (ethylene glycol, pentyl butanol), aliphatic hydrocarbons, aromatic hydrocarbons, terpene compounds and their oxides, and so forth. They may come from the incomplete combustion products of moxa volatile oil of moxa and its oxidation products. Qualitative analysis of the smoke of burning moxa by solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) had isolated 61 peaks and identified 26 ingredients. The founded substances can be

TABLE 1: Intensities and peaks wavelengths of the infrared radiation of traditional moxibustion, moxibustion with controls, and Hegu (LI4).

	<i>n</i>	Intensity of radiation (mV)	Wavelength of the peak of radiation (μm)
Traditional moxa stick	4	43300.41 \pm 425.15	3.5
Smokeless moxa stick	4	31.15 \pm 3.49 [#]	7
555 cigarette	4	37.03 \pm 3.82 [#]	3.5
Indirect moxibustion with monkshood cake	4	681.87 \pm 47.52 ^{**$\Delta\Delta$}	8
Indirect moxibustion with ginger	4	520.27 \pm 68.22 ^{*Δ}	7.5
Indirect moxibustion with garlic	4	594.79 \pm 44.71 ^{**$\Delta\Delta$}	7.5
Indirect moxibustion with cucumber	4	274.47 \pm 19.61	5
Indirect moxibustion with carrot	4	50.53 \pm 4.68	5
LI4 (Hegu)	28	20.40 \pm 5.69	7.5

[#]Compared to the traditional moxa-stick, $P = 0.000$.

*Compared to indirect moxibustion with cucumber, $P = 0.004$.

**Compared to indirect moxibustion with cucumber, $P = 0.000$.

Δ Compared to indirect moxibustion with carrot, $P = 0.001$.

$\Delta\Delta$ Compared to indirect moxibustion with carrot, $P = 0.000$.

divided into 3 parts by time: the furan structure substances in 0–10 min, mainly aromatic compound in 10–40 min, and esters, alkanes, or hydroxyl-containing compounds in 40–70 min [46]. The smoke of moxa can be used in air disinfection and as antiviral and antifungal. It was also reported that it has applications in wound infections, vaginal itching, uterine prolapse, anal fistula, common warts, and so forth [47], and some studies showed that the smoke of moxa would make effects on the body through breathing [48].

There is still a debate on the safety of moxa smoke. Some reports showed that moxa smoke may be harmful to the human body, such as causing allergic reactions [49, 50]. The mugwort leaf contains terpenes; it may produce polycyclic aromatic carcinogens in the process of combustion, and during moxibustion, the concentration of air pollutants, such as nitrogen oxides, carbon monoxide, and particulates, is tenfold higher than the level of standard class II which was issued in the State Environmental Protection Act. They would do damage to the patients and staffs [51]. But a research giving consideration to short-term and long-term exposure showed that the volatile matter and carbon monoxide generated by the smoke of moxa under normal operating conditions did not exceed the safety level [52].

4. Conclusion

On the mechanism of moxibustion effects, there have been many viewpoints, such as thermal stimulation effect, non-specific autologous protein therapeutics, non-specific stress responses, and aromatherapy. The generally accepted view is that the meridian system combines with moxibustion physical and chemical effects to produce comprehensive effects. When physical and chemical factors act on the acupoint receptors, the signal enters the central nervous system through the peripheral pathways and outgoes after being integrated, adjusting the nerve-endocrine-immune network and circulatory system, so as to regulate the internal environment of the body, in order to achieve the effects of preventing and curing diseases [53]. Although lots of research works have

been carried out and made some progress, there is still a great distance from fully understanding the mechanism of moxibustion. Therefore, we will propose the following views on the study of mechanism of moxibustion in the future.

First, value the importance of whole, moxibustion cannot be separated from the theory of TCM. More than a simple stimulus, meridian and acupoint system of the human body is the key of efficacy of moxibustion. The studying of mechanism of moxibustion from the overall level, based on the further understanding of the meridian system or even of the TCM system, is indeed very difficult. But on the other hand, maybe the studies of moxibustion should be helpful to the understanding of acupoint, meridian, and TCM. For example, some people had reported the phenomenon of “heat-sensitive points” [54]; it is a useful exploration of extending the study perspective from the part to the whole with moxibustion as the breakthrough point.

Second, pay more attention to scarring moxibustion (suppurative moxibustion). Scarring moxibustion had been the favorite to ancient doctors, “where there is moxibustion sore, there is cure.” Modern clinical practice has also shown that scarring moxibustion, compared with other moxibustions, has advantage of curative effect in the treatment of some chronic refractory diseases.

Third, it is necessary to introduce more new technologies and disciplines into the mechanism research of moxibustion effect, such as bioheat transfer theory, the interdiscipline focus heat transfer phenomena in living organisms; its purpose is to reveal the rules of energy transport in the organisms by introducing the basic theory and research methods of the heat transfer into the field of biology and medicine. The application of the interdisciplinary approach will undoubtedly promote the research of moxibustion [55].

Fourth, study on the mechanism of moxibustion should be oriented to promote its clinical application. Many research achievements have already been applied in clinic, such as the applications of 650 nm–10.6 μm combined laser moxibustion on knee osteoarthritis and bradycardia [56, 57] and the multifunctional moxibustion instrument which simulate the

traditional moxibustion by heating artificial moxa (contains effective components of moxa) with electromagnetic-heating device [58]. There are enough reasons to believe that, with the progress of mechanism research, the new achievements will surely provide a larger space to improve the patient experience and the curative effect of moxibustion.

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Research Article

The Effects of Sa-Am Acupuncture Treatment on Respiratory Physiology Parameters in Amyotrophic Lateral Sclerosis Patients: A Pilot Study

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Respiratory dysfunction and complications are the most common causes of death in amyotrophic lateral sclerosis. This is a pilot study to observe the changes in respiratory physiology parameters after Sa-am acupuncture treatment. Eighteen ALS patients received Sa-am acupuncture treatment twice a day for 5 days. The EtCO₂, SpO₂, RR, and pulse rate were measured for 15 min before and during treatment, using capnography and oximetry. Correlation of K-ALSFRS-R scores against measured parameters showed that patients who had high K-ALSFRS-R scores had greater changes in pulse rate after acupuncture stimulation; they also showed a decrease in EtCO₂, RR, and pulse rate and an increase in SpO₂. A comparison of the mean values of these different parameters before and after Sa-am acupuncture stimulation revealed statistically significant differences ($P < 0.05$) in SpO₂ and pulse rate, but none in EtCO₂ and RR. Sa-am acupuncture treatment on ALS patients seems to be more effective in the early stages of the disease. In light of increased SpO₂ values, Sa-am acupuncture appears to have a greater effect on inspiration rather than on expiration. As a pilot study of acupuncture on ALS patients, this study could be used as a basis for future research.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a common progressive, neurodegenerative disorder of the voluntary motor system that affects motor neurons in the cerebral cortex, brain stem, and spinal cord [1]. It is characterized by progressive neuromuscular atrophy with early involvement of the respiratory system. The latter rapidly leads to pulmonary compromise requiring mechanical ventilation and represents the major cause of mortality [2]. Eighty-four percent of ALS patients die of respiratory complications and respiratory insufficiency in 2-3 years after diagnosis. On a retrospective chart review, it was found that 2.7% of patients with ALS had respiratory symptoms as their first symptom and that the average survival period of these patients was only 2 months [3, 4]. Hence, reduced ventilation results, in part, from progressive motor neuron degeneration, which leads to respiratory muscle weakness. Respiratory muscle weakness is defined as the

inability of the respiratory muscles to generate normal levels of pressure and air flow during inspiration and expiration [5]. This leads to respiratory insufficiency, which is defined as inadequate pulmonary ventilation to the point that gas exchange is impaired, resulting in carbon dioxide retention, hypoxemia, and respiratory failure [5, 6]. ALS patients are able to withstand respiratory muscle weakness by using other muscles at the initial stage, but the symptoms progress gradually to respiratory insufficiency and eventual respiratory failure. In the majority of cases, death is related to respiratory events. The time of progression and the degree of respiratory muscle weakness are, therefore, important prognostic factors for ALS patients. Hypoventilation in ALS patients is due to respiratory muscle weakness and is associated with poor survival, cognitive impairment, and a poor quality of life; this has led to an increase in concern about the respiratory problems faced by ALS patients and a dramatic increase of relevant knowledge [7].

Several studies have shown that treatment of ALS patients with noninvasive positive pressure ventilation (NIPPV) for respiratory insufficiency appears to prolong life expectancy and improves the quality of life; this is possibly attributable to the slower rate of decline of pulmonary function in these patients [8]. These recent studies have demonstrated reduced mortality in ALS patients with respiratory complications and prolonged the average survival period through an increase in the use of respiratory assisting devices for managing respiratory problems. However, there is no effective treatment for respiratory dysfunction in ALS patients so far.

Acupuncture is one of the oldest medical interventions in East Asian countries. It has been shown in a recent study to be a safe and potentially effective intervention in patients with dyspnea, which is a major symptom of chronic obstructive pulmonary disease (COPD). Acupuncture has also been shown to be effective for symptoms in an animal model of ALS [9, 10]. Korean Sa-am acupuncture methods with lung tonification effects were chosen for this study after a review of the Korean traditional literature. K-ALSFRS-R scores, which are the main assessment tools of ALS, were used to analyze the relationship between the status of ALS patients and respiratory physiology parameters. The aim of this study was to report changes in EtCO₂, SpO₂, RR, and pulse rate, after Sa-am acupuncture treatment on ALS patients.

2. Methods

2.1. Subjects. This study was conducted at the Wonkwang University ALS clinic from January through July, 2012. Eighteen eligible ALS patients were selected out of all ALS patients admitted during that period. This study was approved by the institutional review board (IRB), and written informed consent was obtained from all participants.

The inclusion criteria were as follows: patients who (1) satisfied El Escorial criteria and were diagnosed with ALS by EMG, (2) signed a consent, (3) cooperated with this study, (4) had not exercised within the previous 24 h, (5) had not smoked, drank alcohol, coffee, or green tea within the previous 8 h, (6) had eaten at least 1 h prior to testing, and (7) were not menstruating.

The exclusion criteria were as follows: patients who (1) needed intensive care for respiratory insufficiency, (2) were not able to give basic information owing to severe bulbar palsy, (3) had heart disease of ischemic or other etiology, (4) had endocrine disorders such as thyroid disease, (5) had renal diseases such as chronic renal failure, (6) had fever, (7) had a seizure disorder, (8) had mental illness, (9) were addicted to drugs such as alcohol, nicotine, or caffeine, and (10) were considered not eligible for this study at the discretion of the researcher.

2.2. Measurements

2.2.1. Measuring Devices: Capnography & Pulse Oximetry. Capnography & Pulse Oximetry (Nonin Medical, Japan) was chosen because it is easy to handle and is a useful measuring tool for observing changes in a patient's respiratory condition [7].

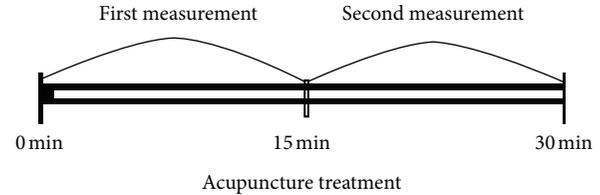


FIGURE 1: A pair of measurements in the trial. Respiratory parameters (EtCO₂, SpO₂, RR, and pulse rate) were measured using capnography and oximetry.

This study measured end-tidal carbon dioxide (EtCO₂), peripheral oxygen saturation (SpO₂), respiratory rate (RR), and pulse rate.

2.2.2. K-ALSFRS-R. The Amyotrophic Lateral Sclerosis Functional Rating Scale (ALSFRS) is a validated, questionnaire-based scale that measures physical functional status in terms of the ability to carry out activities of daily living in patients with ALS. It has been used in clinical trials, as well as in clinical practice, because of its ease of use and its correlation both with objective measures of disease status and levels of disability [11]. The score reflects the deterioration of function in the natural course of ALS, but may have a lower sensitivity in advanced disease stages [12]. The scale was developed primarily to assess outcomes in pharmaceutical clinical trials and does not rely upon physical examinations or instruments [11, 13]. An initial imbalance within the scale that minimized the importance of respiratory function was rectified by a revision (ALS Functioning Rating Scale, revised [ALSFRS-R]) to incorporate respiratory symptoms and assess the need for ventilation [14]. The K-ALSFRS-R was made to reflect its domestic applicability; a preliminary experiment reported that it had high reliability and validity [15]. It consists of 4 sections (verbal, detailed motor, gross motor, and respiration function) and 12 subsections; the maximum possible score is 48 points. The greater the decrease in muscle function, the lower the score; in other words, higher scores reflect better patient status.

2.3. Procedures. Experimental procedure was as follows: (1) subjects were advised to rest and not to undertake strenuous exercise before measurement, (2) respiratory parameters (EtCO₂, SpO₂, RR, and pulse rate) were measured using Capnography and Oximetry for 15 min, (3) Sa-am acupuncture was conducted at specific acupoints using 0.25 × 40 mm, sterile, disposable acupuncture needles made of stainless steel (DongBang Acupuncture Inc., Korea). The depth of insertion at each point was predetermined to be within the normal range of 8–20 mm, depending on the location of the point. The SP3 and LU9 acupoint needles were electrically stimulated at 100 Hz with the clinician adjusting the intensity so that the patients felt an uncomfortable sensation that was not painful; (4) needles were kept in place for 15 min while measuring EtCO₂, SpO₂, RR, and pulse rates simultaneously. The above process was considered as a pair of observations. Each patient received acupuncture treatment twice a day for 5 days (Figure 1).

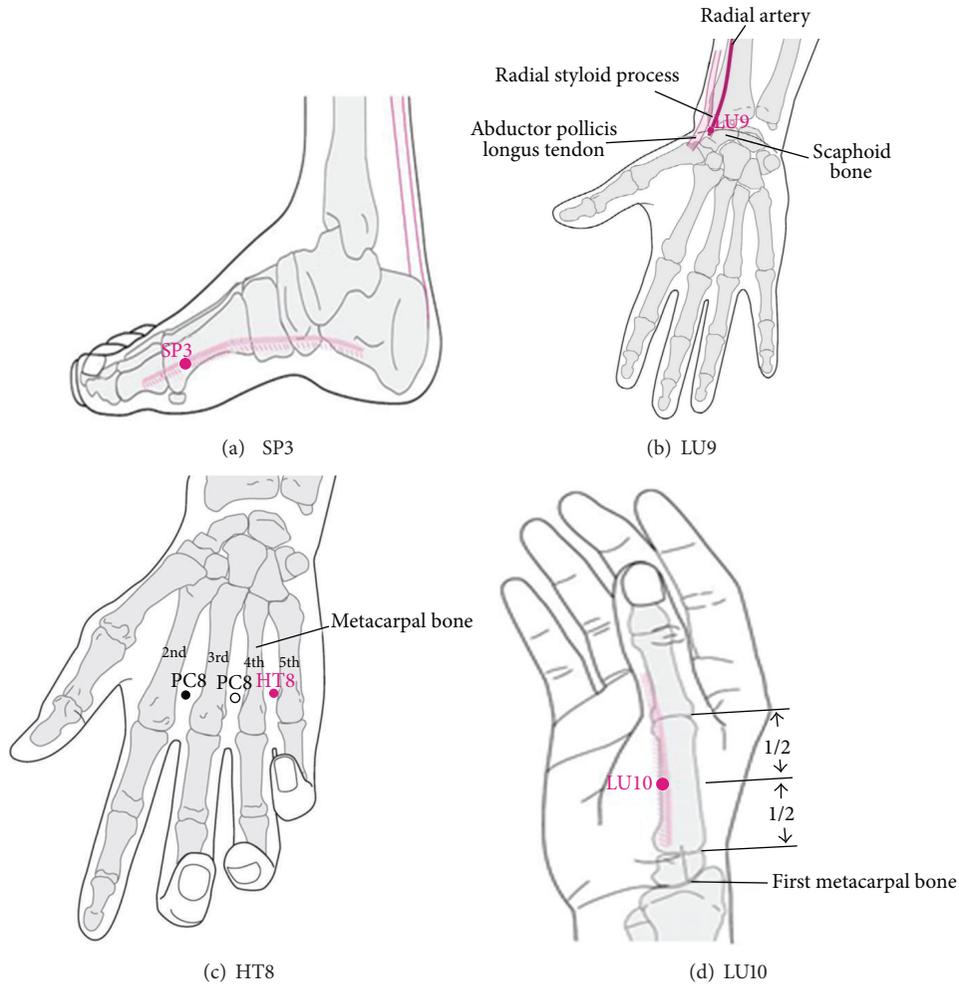


FIGURE 2: Locations of selected acupuncture points.

2.3.1. Selection of Acupuncture Points. Acupuncture points SP3, LU9, HT8, and LU10 (Figure 2) were selected on both sides of the body according to Sa-am Five-Element Acupuncture method found in the traditional Korean medical literature. These sets of acupuncture points are used for the tonification of lung functions. Certified practitioners with at least 6 years of experience in traditional Korean medicine, and an additional 3 years of clinical experience, performed the acupuncture treatment according to the WHO Standard Acupuncture Point Locations for the Western Pacific Region.

According to the Korean Sa-am acupuncture literature, SP3 (Figure 2(a)) and LU9 (Figure 2(b)) are used for the tonification of lung-qi, while HT8 (Figure 2(c)) and LU10 (Figure 2(d)) are used to clear lung fire. The selection of acupuncture points was based on the WHO standard acupuncture point guidelines [16].

2.4. Statistics. Statistical analysis using SPSS version 20.0 for Windows was used to compare changes in EtCO₂, SpO₂, RR, and pulse rate. The relationship between K-ALSFRS-R on one side and EtCO₂, SpO₂, RR, and pulse rate on the other was analyzed with Pearson's correlation analysis. To compare

the difference between parameters of respiratory function before and after acupuncture stimulation, a paired *t*-test was conducted using the mean value over a period of 15 min.

A significance level of $P < 0.05$ was used throughout.

3. Results

3.1. Demographic Characteristics. The sex, age, K-ALSFRS-R score, and site of onset of the included patients are given in Table 1.

3.2. Correlation Analysis with K-ALSFRS-R Score

3.2.1. The Analysis of the Values of the Differences of EtCO₂, SpO₂, RR, and Pulse Rate before and after Acupuncture Stimulation. Pearson's correlation analysis was used to analyze the relationship between K-ALSFRS-R score and differences in EtCO₂, SpO₂, RR, and pulse rate before and after acupuncture stimulation (Table 2, Figure 3). The results showed that there was a negative correlation between the K-ALSFRS-R score and the magnitude of increase in pulse rate after acupuncture stimulation ($r = -0.236$, $P < 0.01$).

TABLE 1: Baseline characteristics of amyotrophic lateral sclerosis (ALS) patients in the study.

ALS patients	N = 18
Sex (male : female)	14 : 4
Age (years)	56.06 (± 7.53) ^a
Age at onset	52.39 (± 9.29) ^a
K-ALSFRS-R score (maximum: 48)	33.24 (± 5.19) ^a
Respiration subscores in K-ALSFRS-R (total: 12)	9.71 (± 3.24) ^a
Site of onset	
Bulbar	3
Upper limb	5
Lower limb	10

a: mean \pm standard deviation.

K-ALSFRS-R: Korean-ALS Functional Rating Scale-Revised.

TABLE 2: The correlation between K-ALSFRS-R score and the difference of EtCO₂, SpO₂, RR, and pulse rate before and after acupuncture stimulation.

	EtCO ₂	SpO ₂	RR	Pulse
K-ALSFRS-R	0.104	0.046	-0.077	-0.236**

Analyzed by Pearson's correlation analysis (* $P < 0.05$, ** $P < 0.01$).

EtCO₂: end-tidal carbon dioxide, SpO₂: saturation of partial pressure arterial oxygen, and RR: respiratory rate.

TABLE 3: The correlation between K-ALSFRS-R score and the change in EtCO₂, SpO₂, RR, and pulse rate after acupuncture stimulation.

	EtCO ₂	SpO ₂	RR	Pulse
K-ALSFRS-R	-0.276**	0.173*	-0.254**	-0.420***

Analyzed by Pearson's correlation analysis (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

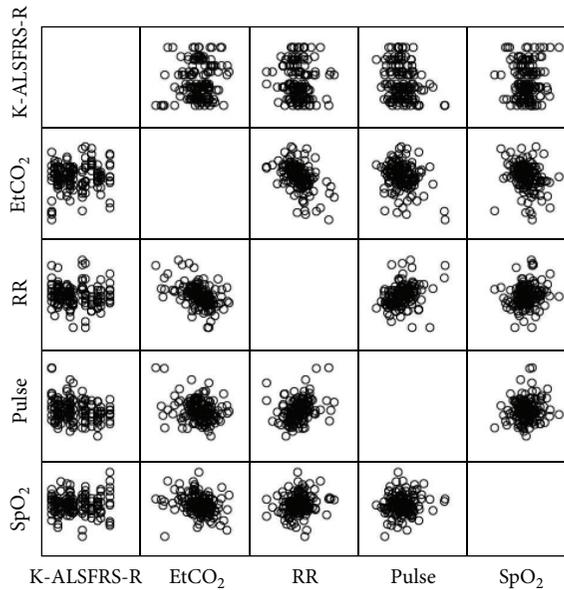


FIGURE 3: The correlation between K-ALSFRS-R score and the difference in EtCO₂, SpO₂, RR, and pulse before and after acupuncture stimulation.

3.2.2. Analysis of Respiratory Parameters after Acupuncture Stimulation. Pearson's correlation analysis was used to show the relationship between K-ALSFRS-R score on one hand and EtCO₂, SpO₂, RR, and pulse rate on the other. (Table 3, Figure 4). There was a negative correlation between K-ALSFRS-R score and each of EtCO₂ ($r = -0.276$, $P < 0.01$), RR ($r = -0.254$, $P < 0.01$), and pulse rate ($r = -0.420$, $P < 0.001$); that is, the greater the K-ALSFRS-R

score, the greater the decrease in EtCO₂, RR, and pulse rate after acupuncture stimulation. However, there was a positive correlation between the K-ALSFRS-R score and SpO₂ ($r = 0.173$, $P < 0.05$), so that the greater the K-ALSFRS-R score, the greater were the increase in SpO₂ after acupuncture stimulation.

3.3. Changes in Respiratory Parameters before and after Sa-Am Acupuncture. Results obtained by performing a paired t -test showed no significant change in EtCO₂ ($P = 0.702 > 0.05$) and respiratory rate ($P = 0.180 > 0.05$) after acupuncture stimulation. However, there was a significant difference between SpO₂ before and after acupuncture stimulation, with an increase from 95.42% to 95.58% ($P = 0.002 < 0.05$). There was a significant change in pulse rate after acupuncture stimulation, with a decrease from 82.49 bpm to 80.08 bpm ($P < 0.001$) (Table 4, Figure 5).

4. Discussion

ALS is a fatal and progressive neurodegenerative disease, leading to muscle weakness, paralysis, and death by respiratory failure. Although respiratory failure is generally the cause of death in ALS, little is known about the treatment and control of respiratory problems. Therefore, this pilot study was conducted to study the effect of acupuncture treatment on parameters of respiratory function with the help of capnography and oximetry to set up guidelines for preventing and managing respiratory problems in ALS.

ALS belongs to the category of Wei symptoms in traditional East Asian medicine. The earliest published literature about Wei symptoms is "Plain Question" [17]. It reports several causes of Wei symptoms and suggests a treatment, which is focused on making the digestive system healthy [18].

TABLE 4: The changes in EtCO₂, SpO₂, RR, and pulse rate before and after Sa-am acupuncture.

	Before		After		<i>t</i>	<i>P</i>
	M	SD	M	SD		
EtCO ₂	37.70	5.47	37.94	5.35	-0.383	0.702
SpO ₂	95.42	2.48	95.58	2.57	-3.097	0.002**
RR	20.64	3.31	20.47	3.02	1.359	0.180
Pulse rate	82.49	13.33	80.08	13.15	9.992	0.000***

Analyzed by paired *t*-test (**P* < 0.05, ***P* < 0.01, ****P* < 0.001).

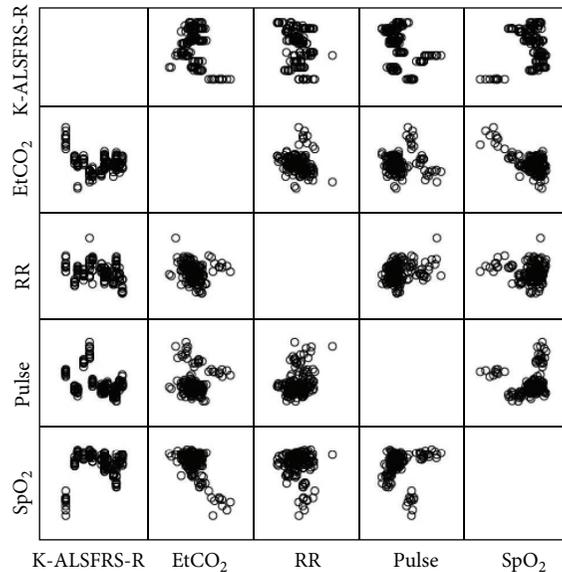


FIGURE 4: The correlation between K-ALSFRS-R score and change in EtCO₂, SpO₂, RR, and pulse rate after acupuncture stimulation.

Therefore, ALS patients have received acupuncture treatment specifically at the spleen meridian (SP) and stomach meridian (ST) in almost all case studies [19, 20].

Sa-am acupuncture, an original and traditional Korean acupuncture method, elicits a strong pain response when applied on the upper and lower extremities. Sa-am acupuncture is widely used in Korean clinical practice because it is considered safe and effective [21]; unfortunately, there has been little research on its use in ALS.

According to this theory, the 4 causes of imbalances in meridian energies are deficiency, excess, cold, and heat. Since there are 12 meridians with 4 possible imbalances, there are a total of 48 preestablished acupuncture prescriptions, each with 5 transport points to use to restore these imbalances. The 5 transport points are important acupoints of meridian that control the 5-phase *qi* of viscera and bowels.

The acupoints of SP3, LU9, HT8, and LU10, which are referred to as lung tonification prescriptions, were selected as intensive acupuncture treatment in this study because respiratory muscle weakness and respiratory symptoms such as sputum production and shallow respiration are thought to be related to lung dysfunction.

Several studies have been conducted on acupuncture treatment in ALS. Byun et al. reported that shortness of breath

improved by more than 50% after acupuncture treatment at HT8 and LU10 points on ALS patients [22]. Jiang et al. [10] reported that electroacupuncture could be an effective anti-inflammatory treatment for the respiratory impairment that occurs in animal models of ALS. However, almost all of these studies were conducted with small sample sizes, and the patients received not only acupuncture but also various traditional treatments. Moreover, some of these studies were performed on animal models of ALS and not on human patients. It is significant that the present study was conducted to observe the effect of acupuncture, with more patients recruited than ever before.

It is known that EtCO₂ exceeds 42 mm Hg when severe bronchial obstruction exists [23]. Therefore, the control of EtCO₂ was considered an appropriate marker of an improvement of respiratory function and the alleviation of respiratory symptoms. Hypoxemia is defined as an SpO₂ equal to or less than 93%, lasting for 15 s or longer; severe hypoxemia kills cells and suppresses mental activity, resulting in a comatose state and a reduction in the ability of muscles to perform work [24]. Dyspnea and hyperpnea are the most important symptoms of hypoxemia.

A pulse rate of 60–100 beats/min and respiratory rate of 16–20 breaths/min are considered to be within the normal range in adults [25]. Acupuncture is able to modulate various autonomic responses [26]. A number of studies have shown impaired cardiac autonomic control in patients with ALS together with parasympathetic dysfunction and sympathetic predominance [27]. Disturbances in autonomic cardiac control in respirator-dependent patients with ALS may significantly influence survival and may lead to hypertensive crisis, circulatory collapse, and sudden death [28]. Observing and regulating EtCO₂, SpO₂, respiratory rate, and pulse rate can, therefore, be an important process of care for ALS patients.

Analysis of K-ALSFRS-R score and parameters of respiratory function measured before and after acupuncture treatment showed that patients with high scores in K-ALSFRS-R had a greater difference in pulse rate before and after acupuncture stimulation. Patients who recorded high scores in K-ALSFRS-R showed a decrease in the values of EtCO₂, RR, and pulse rate and an increase in the values of SpO₂ after acupuncture stimulation. Overall results showed a high correlation with K-ALSFRS-R score and the therapeutic result of Sa-am acupuncture. There is rapid nerve degeneration as ALS progresses, therefore; acupuncture stimulation did not affect patients who were affected more severely. It is possible that Sa-am acupuncture would be more effective in the early stages of ALS.

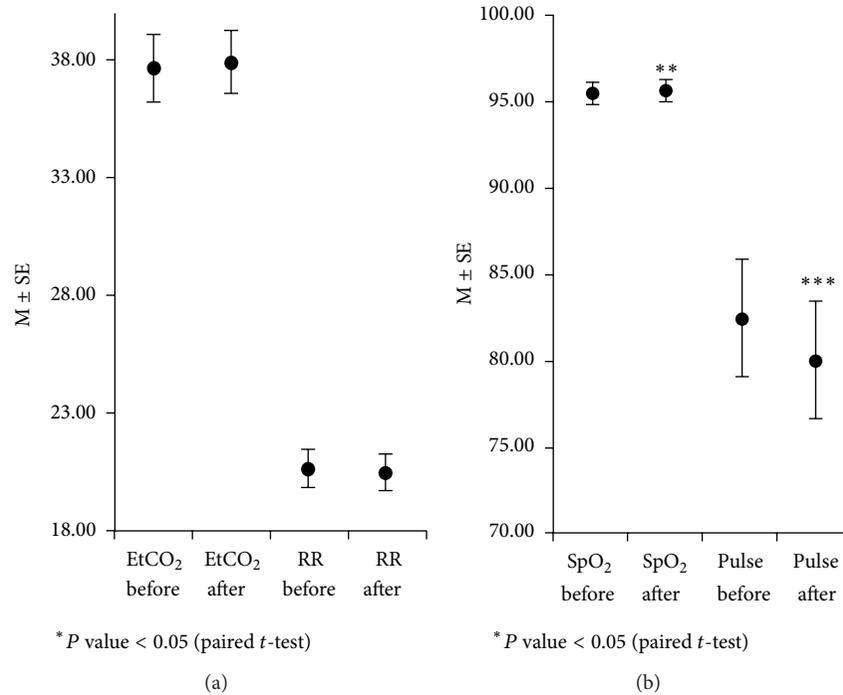


FIGURE 5: The changes in EtCO₂, SpO₂, RR, and pulse before and after Sa-am acupuncture.

There was a significant change in pulse and SpO₂ after lung tonification by Sa-am acupuncture. Unfortunately, EtCO₂ and RR showed no statistically significant changes despite a decrease at the time of measurement. Throughout this study, the results showed Sa-am lung tonification to be more effective in controlling inspiration rather than expiration. All patients who received Sa-am lung tonification showed a decrease in pulse rate; this suggests that Sa-am acupuncture treatment may play an important role in stabilizing sympathetic nerves and regulating the autonomic nervous system. However, acupuncture treatment achieved only a slight improvement in respiratory parameters; even then, it may help ALS patients to maintain respiratory function and retard the progression of respiratory muscle weakness.

There were no side effects during the study; nevertheless, the study had its limitations. As a pilot study, there was no control group. ALS is a progressive and incurable disease, so it was not possible to distinguish between the treatment group and the nontreatment group for ethical reasons. It was also impossible to measure the effect of acupuncture treatment over long periods because these patients experience difficulty in breathing in the supine position.

Research on the role of acupuncture in alleviating respiratory symptoms in ALS patients provides basic data for preventing respiratory complications, which generally lead to death in ALS patients.

Ongoing research with the development and validation of new acupuncture treatment should continue in order to extend the life of ALS patients and improve their quality of life.

5. Conclusion

Sa-am acupuncture led to a statistically significant difference in pulse rate and SpO₂ after acupuncture stimulation. Patients in the earlier stages of the disease with high K-ALSFERS-R scores responded better to acupuncture treatment than did patients with lower K-ALSFERS-R scores.

This study needs to be taken further with a larger sample size to obtain more valuable and meaningful data.

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Research Article

Blockade of Adrenal Medulla-Derived Epinephrine Potentiates Bee Venom-Induced Antinociception in the Mouse Formalin Test: Involvement of Peripheral β -Adrenoceptors

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The injection of diluted bee venom (DBV) into an acupoint has been used traditionally in eastern medicine to treat a variety of inflammatory chronic pain conditions. We have previously shown that DBV had a potent antinociceptive efficacy in several rodent pain models. However, the peripheral mechanisms underlying DBV-induced antinociception remain unclear. The present study was designed to investigate the role of peripheral epinephrine on the DBV-induced antinociceptive effect in the mouse formalin assay. Adrenalectomy significantly enhanced the antinociceptive effect of DBV during the late phase of the formalin test, while chemical sympathectomy had no effect. Intraperitoneal injection of epinephrine blocked this adrenalectomy-induced enhancement of the DBV-induced antinociceptive effect. Moreover, injection of a phenylethanolamine N-methyltransferase (PNMT) inhibitor enhanced the DBV-induced antinociceptive effect. Administration of nonselective β -adrenergic antagonists also significantly potentiated this DBV-induced antinociception, in a manner similar to adrenalectomy. These results demonstrate that the antinociceptive effect of DBV treatment can be significantly enhanced by modulation of adrenal medulla-derived epinephrine and this effect is mediated by peripheral β -adrenoceptors. Thus, DBV acupoint stimulation in combination with inhibition of peripheral β -adrenoceptors could be a potentially novel strategy for the management of inflammatory pain.

1. Introduction

In traditional eastern medicine, acupuncture therapy, including manual acupuncture, electroacupuncture, and chemical acupuncture, is commonly used to produce an analgesic effect and to reduce the severity of pain. With regard to chemical acupuncture, it is well established that subcutaneous injection of diluted bee venom (DBV) into an acupoint, termed *apipuncture*, can be used clinically to produce a potent analgesic effect in human patients [1]. Previous experimental studies in our laboratory have provided scientific support

for this alternative medicine approach by demonstrating that subcutaneous injection of DBV into the Zusanli (ST36) acupoint produces a robust antinociceptive effect in various animal models of pain including the formalin test [2, 3], the chronic constriction injury model [4, 5], the writhing test [6], and several models of arthritis [7]. We have also demonstrated that acupoint injection of DBV increases Fos expression in brainstem catecholaminergic neurons, including the locus coeruleus [8, 9], and that activation of spinal α 2-adrenoceptors, but not opioid receptors, is critically involved in this DBV-induced antinociceptive effect [10–12].

Collectively, these results indicate that the antinociceptive effect of DBV treatment is mediated by the activation of descending coeruleospinal noradrenergic pathways and α 2-adrenoceptors in the spinal cord dorsal horn.

The administration of DBV has also been reported to evoke a significant anti-inflammatory effect in human arthritic disease as well as in several animal models of inflammatory disease [9, 13, 14]. Cumulative studies from our laboratories indicate that the activation of sympathetic preganglionic neurons (SPNs) that innervate the adrenal gland partially underlie DBV's anti-inflammatory action. We also observed that DBV injection into an acupoint activates SPNs leading to release of adrenal medulla-derived epinephrine resulting in a dramatic suppression of neutrophil migration at sites of inflammation [15, 16]. We have also demonstrated that intrathecal injection of clonidine produces an anti-inflammatory effect via the activation of this SPN-adrenomedullary axis, which appears to mimic DBV's anti-inflammatory effect [17].

It has been widely accepted that adrenal medulla-derived epinephrine, an endogenous adrenergic receptor ligand, plays a critical role in the maintenance of hyperalgesia, that is, the increased sensitivity to painful stimuli. Khasar et al. reported that an increase in peripheral epinephrine produces cutaneous mechanical hyperalgesia and this nociceptive effect of epinephrine is mediated by both the protein kinase A and protein kinase C second-messenger pathways [18]. Moreover, it is reported that chronically elevated levels of epinephrine led to an enhancement of bradykinin-induced hyperalgesia [19] and that administration of epinephrine to adrenal medullectomized rats reconstituted these enhanced hyperalgesic responses [20].

Although we have reported that DBV can produce a significant antinociceptive effect in several pain models, it is not clear whether DBV-induced changes in peripheral epinephrine produce a positive or negative effect on DBV's antinociceptive action. Therefore, this study was designed to investigate the potential role of peripheral epinephrine on the DBV-induced antinociceptive effect in the mouse formalin assay. We first evaluated whether denervation of the adrenal gland or chemical sympathectomy alters the DBV-induced antinociceptive effect in the mouse formalin test using adrenalectomy and 6-hydroxydopamine injection, respectively. We next determined whether epinephrine itself might have an effect on DBV-induced antinociceptive effects in adrenalectomized animals. Finally, we investigated whether this DBV-induced antinociceptive effect could be potentiated by suppression of adrenal medulla-derived epinephrine using the following approaches: (1) inhibition of the PNMT enzyme or (2) antagonism of peripheral epinephrine receptors (α - and β -adrenoceptors).

2. Materials and Methods

2.1. Experimental Animals. Experiments were performed on male ICR mice (25 g to 30 g). All experimental animals were obtained from the Laboratory Animal Center of Seoul National University in Republic of Korea. They were housed in colony cages with free access to food and water and

maintained in temperature and light controlled rooms ($23 \pm 20^\circ\text{C}$, 12/12-hour light/dark cycle with lights on at 7 AM) for at least 1 week before the study. All of the methods used in the present study were approved by the Animal Care and Use Committee at Seoul National University and conform to NIH guidelines (NIH publication No. 86-23, revised 1985). All algometric assays were conducted under the ethical guidelines set forth by the International Association for the study of Pain (IASP).

2.2. Apipuncture with BV. DBV from *Apis mellifera* (Sigma) was dissolved in physiological saline. In the first experiment, we tested the following three doses of DBV: 0.8 mg/kg (1 K, diluted by saline with ratio of 1:1000); 0.08 mg/kg (10 K, diluted by saline with ratio of 1:10,000); and 0.008 mg/kg (100 K, diluted by saline with ratio of 1:100,000). In subsequent experiments, we used only the 0.8 mg/kg dose of DBV, since this dose produced a significant antinociceptive effect, while the other two doses did not. Each dose of DBV was subcutaneously injected into the left Zusanli acupoint (ST-36) located on the lateral side of the stifle joint adjacent to the anterior tubercle of the tibia as previously described [3]. Animals in the control group received an injection of vehicle into the same site. In all apipuncture experiments, DBV was injected 10 minutes after each drug injection.

2.3. Drug Treatment. 6-Hydroxydopamine, epinephrine, phenolamine, nadolol, atenolol (Sigma, St. Louis, MO, USA), DCMB, propranolol, and ICI-118,551 (Tocris, UK) were all dissolved in saline (0.9% NaCl) before use. All drugs were intraperitoneally administered in a volume of 100 μL and were injected 10 minutes before DBV injection (20 minutes before formalin injection).

2.4. Mice Adrenalectomy. Anesthesia was induced with a Zoletil-Rumpun mixture in saline, in a ratio of 2:1:2 total volume of anesthetic drug. To remove the adrenal glands, a dorsal midline incision was made. This incision was shifted to either side to expose the anterior pole of the kidney and the adrenal gland. The adrenal gland was removed by separating the gland from the surrounding tissue with tweezers and then gently pulling the gland through the flank incision; 4–0 chromic gut sutures were used to close the incisions in the muscle walls, and silk was used to close the flank incision. After adrenalectomy, 0.9% saline was given to adrenalectomized mice rather than water in order to maintain mineral balance and all animals were allowed to recover on a heating chamber immediately after surgery. All adrenalectomized mice recovered from surgery for a period of one week prior to further experimental use.

2.5. Chemical Sympathectomy. In order to examine the contribution of catecholamines released from postganglionic sympathetic nerve endings on DBV-induced antinociception, chemical sympathectomy was performed as previously described by Kohm and Sanders [21]. Experimental mice received a 200 mg/kg intraperitoneal injection of 6-hydroxydopamine (6-OHDA) in sterile saline containing 0.01%

ascorbic acid/saline, while control mice received an injection of vehicle. In previous studies, intraperitoneal injection of 6-OHDA has been shown to deplete norepinephrine in the spleen and lymph nodes. In the present study, we confirmed the depletion of catecholamines in sections of spleen using glyoxylic acid staining on day 6 following the injection of 6-OHDA (data not shown).

2.6. Formalin-Induced Nociceptive Behavioral Test. The formalin test is an analgesic behavioral observation assessment method that has 2 phases (an early phase and a late one) of nociceptive behavior representing 2 different types of pain. The early phase seems to be caused predominantly by C-fiber activation due to the peripheral stimulus, while the late phase appears to be dependent on the combination of an inflammatory reaction in the peripheral tissue and functional changes in the dorsal horn of the spinal cord. In the present study, mice were first acclimatized for 30 minutes in an acrylic observation chamber (30 cm in diameter and in height), and then 20 μ L of 1% formalin was injected subcutaneously into the plantar surface of the left hind paw with a 30 gauge needle. Following formalin injection, the animals were immediately placed in the test chamber, and nociceptive responses in each animal were recorded using a video camera for a period of 30 minutes. The summation of time (in seconds) spent licking the formalin-injected hind paw during each 5-minute block was measured as an indicator of the nociceptive response. Two experienced investigators, who were blinded to the experimental conditions, measured the formalin-induced behaviors. The duration of the responses during the first 10-minute period represented the early phase, while the duration of responses during the subsequent 20-minute period (from 10 to 30 minutes after injection) represented the late phase of the formalin test.

2.7. Statistical Analysis. All values are expressed as the mean \pm SEM. Statistical analysis was performed using Prism 5.0 (Graph Pad Software, San Diego, CA, USA). A one-way ANOVA was used to determine differences across all experiment groups. Post hoc analysis was performed using the Bonferroni's multiple comparison test in order to determine the P value among experiment groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of DBV Treatment on Formalin-Induced Nociceptive Behaviors in Normal and Adrenalectomized Mice. We first tested whether subcutaneously injection of DBV into the left Zusanli acupoint produced an antinociceptive effect during the early or late phases of the formalin test using several different doses (1 K, 10 K and 100 K) in normal and adrenalectomized mice. To accomplish this, we first injected DBV and then ten minutes later we injected formalin and subsequently measured licking time of the formalin-treated hind paw. In normal animals, administration of a high dose (1 K, $n = 7$) of DBV significantly decreased formalin-induced paw licking behaviors as compared with the vehicle-injected

group ($n = 7$) during the late phase of the formalin test ($***P < 0.001$, Figure 1(b)). However, DBV had no effect on paw licking time during the early phase of formalin-induced pain behaviors (Figure 1(a)). We also found that administration of lower doses (10 K and 100 K, $n = 7$, resp.) of DBV did not produce any significant antinociceptive effects during either the early or late phase of formalin-induced pain behaviors. In adrenalectomized mice, injection of formalin plus vehicle (rather than DBV) produced similar nociceptive responses to those observed in nonadrenalectomized animals receiving formalin plus vehicle during both the early and late phases of the formalin test. In adrenalectomized mice subcutaneous DBV injection (at 1 K and 10 K, $n = 7$, resp.) significantly reduced the paw licking time compared to that of vehicle-injected animal group ($n = 7$, $*P < 0.05$ and $***P < 0.001$), while the lowest dose (100 K, $n = 7$) of DBV failed to inhibit pain responses during the late phase. However, when compared to the same doses of DBV given to the non-adrenalectomized mice group, administration of DBV to adrenalectomized mice at all doses tested (1 K, 10 K and 100 K) significantly suppressed the paw licking time during the late phase of the formalin test ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, Figure 1(b)). Thus, adrenalectomy enhanced the antinociceptive effectiveness of DBV in the late phase of the formalin test. Conversely, DBV-injected into the ST-36 acupoint of adrenalectomized mice did not affect the paw licking time during the early phase of the formalin test.

3.2. Lack of Effect of Peripheral Chemical Sympathectomy on DBV-Induced Antinociception. We subsequently investigated whether catecholamines released from postganglionic sympathetic nerve endings alter the antinociceptive effects of DBV. To test this we performed a chemical sympathectomy using 6-OHDA. As illustrated in Figure 2, administration of 6-OHDA alone [(6-OHDA) + Veh + F, $n = 7$] did not alter formalin-induced paw licking time as compared to a vehicle-injected control group (Veh + Veh + F, $n = 7$) during either the early or late phases of the formalin test. Similarly, chemical sympathectomy combined with DBV treatment [(6-OHDA) + DBV + F, $n = 7$] did not have any suppressive effect on formalin-induced pain response as compared to a DBV-injected control group (Veh + DBV + F, $n = 7$, NS; no significance). These results indicate that postganglionic sympathetic nerve endings do not play a role in DBV-induced pain reduction during the formalin test.

3.3. Effect of Peripheral Epinephrine in DBV-Induced Antinociception. In order to investigate whether pure epinephrine might have an influence on DBV-induced antinociceptive effects, we performed adrenalectomy and measured the formalin-induced pain behaviors. As illustrated in Figure 3, the DBV-treated group (Veh + DBV + F, $n = 7$) significantly suppressed formalin-induced paw licking behaviors compared to that of vehicle-injected group (Veh + Veh + F, $n = 7$, $***P < 0.001$). However, administration of epinephrine (1 and 10 μ g/kg, Epi 1 + Veh + F and Epi 10 + Veh + F, $n = 7$, resp.) significantly increased paw licking behavior during the late phase of the formalin test as compared to the

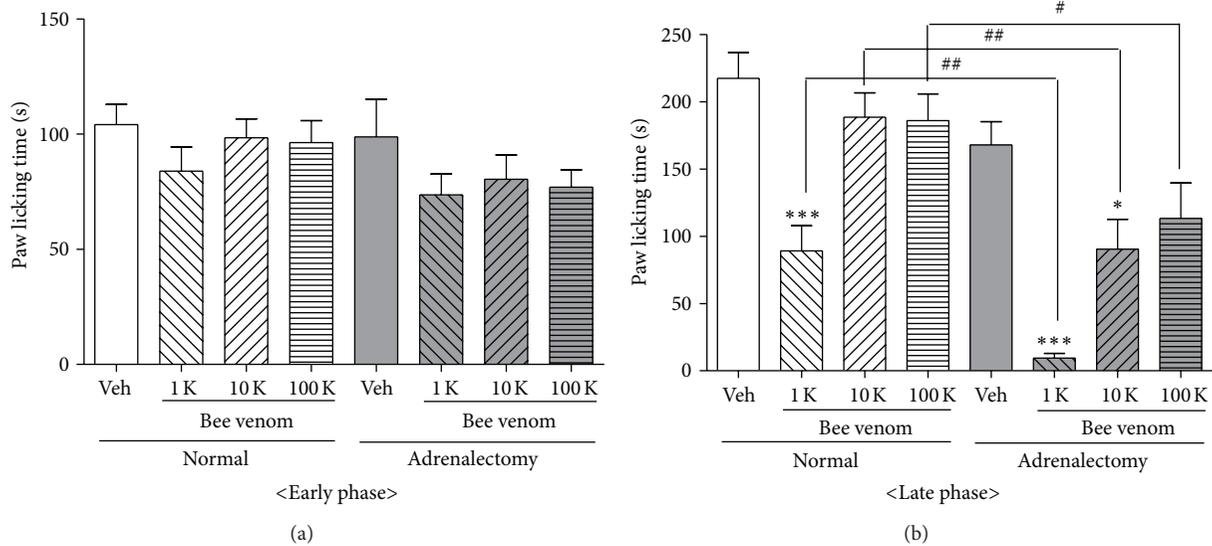


FIGURE 1: Graphs illustrating the antinociceptive effect of DBV administered at three different doses in normal and adrenalectomized mice, based on analysis of formalin-induced pain responses during the early (a) and late (b) phases of the formalin test. (a) During the early phase, there were no changes in licking behavior in either the normal or adrenalectomized groups treated with DBV at any of the doses tested. (b) During the late phase, administration of a high dose (1K) of DBV in normal mice significantly inhibited the pain behavior as compared with the vehicle-treated group ($***P < 0.001$). In adrenalectomized mice, formalin injection produced similar pain responses to those observed in nonadrenalectomized mice. Treatment with DBV (1K and 10K) significantly suppressed the paw licking time compared to the vehicle-injected animal group ($*P < 0.05$ and $***P < 0.001$), while the lowest dose (100K) of DBV had no effect on formalin-induced pain responses. Treatment with each of the three doses (1K, 10K, and 100K) of DBV suppressed pain behavior compared to the same dose of DBV administered to the normal mice (nonadrenalectomized) group ($#P < 0.05$ and $##P < 0.01$).

vehicle-injected group ($#P < 0.05$). Co-administration of epinephrine (10 $\mu\text{g}/\text{kg}$) with DBV (Epi 10 + DBV + F, $n = 7$) significantly decreased the reduction in paw licking time produced by DBV treatment alone during the late phase of the formalin test ($^{\&\&}P < 0.01$). Co-administration of epinephrine (10 $\mu\text{g}/\text{kg}$) with DBV (Epi 10 + DBV + F, $n = 7$) had no effect on licking behaviors during the early phase of the formalin test. These results demonstrated that epinephrine might be closely involved in both peripheral sensitization and DBV's antinociception in late phase but not early phase.

3.4. Effect of Co-administration of the PNMT Inhibitor: DCMB with DBV on Formalin-Induced Pain Behaviors. Phenylethanolamine N-methyltransferase (PNMT) is an enzyme that converts norepinephrine to epinephrine in the adrenal medulla. We examined whether administration of the PNMT inhibitor, DCMB, alone or in combination with DBV treatment had any effect on formalin-induced nociceptive responses. Administration of DBV (Veh + DBV + F, $n = 7$) significantly decreased pain behaviors compared to that of vehicle-injected group (Veh + Veh + F, $n = 7$, $***P < 0.01$). Systemic DCMB injection together with vehicle [DCMB (3, 10 mg) + Veh + F, $n = 7$, resp.] rather than DBV did not change formalin-induced pain behaviors as compared to the vehicle-vehicle-injected group in either the early or late phase of the formalin test (Figure 4). However, when a high dose of DCMB was co-administered with DBV [DCMB (10 mg) + DBV + F, $n = 7$], it significantly increased the antinociceptive effect of DBV on formalin-induced nociceptive responses

as compared with the vehicle-DBV-injected group during the late phase of the formalin test ($#P < 0.05$). This drug combination had no effect on the early phase of the formalin test. Moreover, administration of a low dose of DCMB given prior to DBV treatment [DCMB (3 mg) + DBV + F, $n = 7$] did not change formalin-induced pain responses.

3.5. Effect of Co-administration of Adrenoceptor Antagonists with DBV on Formalin-Induced Nociceptive Responses. The group of mice receiving vehicle plus DBV treatment (Veh + DBV + F, $n = 7$) significantly suppressed pain behaviors in comparison to vehicle-vehicle-injected group (Veh + Veh + F, $n = 7$, $**P < 0.01$, Figure 5(a)). Systemic injection of the α -adrenoceptor antagonist, phentolamine, plus DBV [Phento (5, 10 mg) + DBV + F, $n = 7$, resp.] had no significant effect on formalin-induced nociceptive behavior as compared to the control group during either the early or late phase of the formalin test at any of the doses tested. Similarly co-administration of phentolamine with vehicle [Phento (5, 10 mg) + Veh + F, $n = 7$, resp.] did not affect the paw licking time in either phase (Figure 5(a)). As illustrated in Figure 5(b), treatment of DBV (Veh + DBV + F, $n = 7$) significantly decreased formalin-evoked paw licking time compared to that of vehicle-injected group (Veh + Veh + F, $n = 7$, $**P < 0.01$), and administration of a β -adrenergic receptor antagonist, propranolol with vehicle [PRO (5 mg) + Veh + F, $n = 7$], had no effect on this formalin-induced pain behavior during either the early or late phase. On the other hand, co-administration of propranolol with DBV

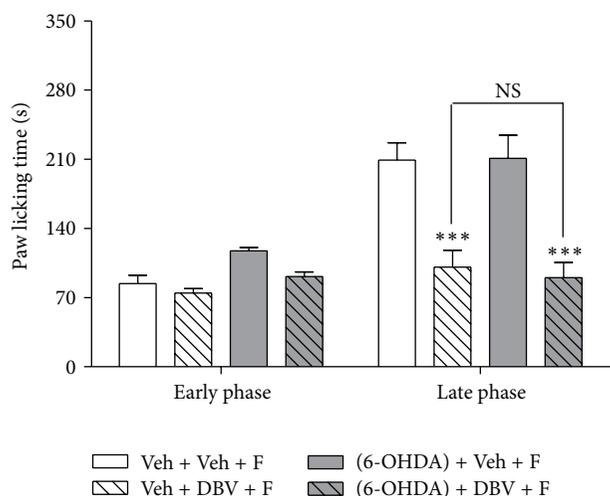


FIGURE 2: Graphs illustrating the effect of chemical sympathectomy with 6-OHDA in combination with DBV on the formalin test. This figure shows that chemical sympathectomy itself [(6-OHDA) + Veh + F] did not change formalin-induced pain behavior as compared to the vehicle-injected control group (Veh + Veh + F) during either the early or late phase of the formalin test. Injection of 6-OHDA in combination with DBV [(6-OHDA) + DBV + F] also did not affect formalin-induced pain behavior as compared to a DBV-injected control group (Veh + DBV + F, NS; no significance).

[PRO (5 mg) + DBV + F, $n = 7$] significantly decreased the paw licking time as compared to the DBV-treated group during the late phase, but not during the early phase of the formalin test ($^{\#}P < 0.05$). Additionally, administration of another β -adrenergic receptor antagonist, nadolol (which cannot cross the blood-brain barrier), with vehicle [Nadolol (20 mg) + Veh + F, $n = 7$], did not change formalin-induced pain behaviors as compared to vehicle-treated group in either the early or late phase of the formalin test. Similar to propranolol, injection of nadolol in combination with DBV treatment [Nadolol (20 mg) + DBV + F, $n = 7$] produced a significant antinociceptive effect as compared to the vehicle-DBV-treated group during the late phase of the formalin test ($^{\#\#}P < 0.01$).

We next coadministered either the β_1 -adrenergic receptor antagonist, atenolol (1, 5 mg), or the β_2 -adrenergic receptor antagonist, ICI-118,551 (2, 4 mg), with DBV, to determine the potential contribution of each β -adrenoceptor subtype to the antinociceptive effect of DBV on formalin-induced nociceptive behaviors. As shown in Figure 6, administration of DBV (Veh + DBV + F, $n = 7$) significantly decreased pain behaviors compared to that of vehicle-injected group (Veh + Veh + F, $n = 7$, $^{**}P < 0.01$ and $^{***}P < 0.001$), and intraperitoneal administration of either atenolol and ICI-118,551 with vehicle [Atenolol (1, 5 mg) + Veh + F, ICI-118,551 (2, 4 mg) + Veh + F, $n = 7$, resp.] did not change formalin-induced pain behaviors as compared to the vehicle-vehicle-injected group during either the early or late phase of the formalin test. Administration of a low dose of atenolol or ICI-118,551 in combination with administration of DBV [Atenolol (1 mg) + DBV + F, ICI-118,551 (2 mg) + DBV + F, $n = 7$, resp.]

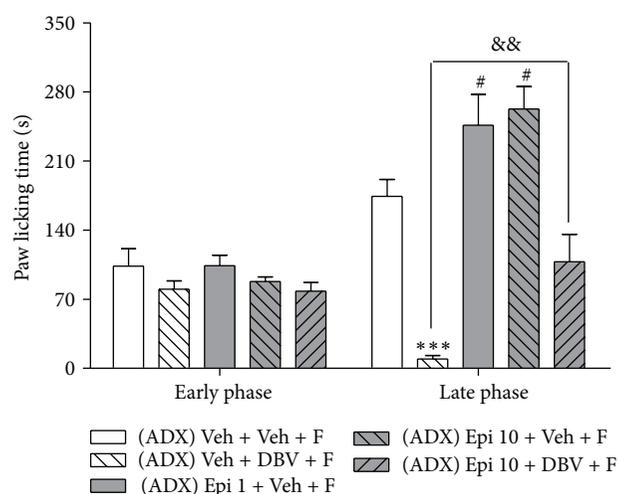


FIGURE 3: Graph illustrating the effect of administration of exogenous epinephrine in adrenalectomized mice on formalin-induced nociception. Treatment with DBV in adrenalectomized mice (Veh + DBV + F) significantly suppressed pain responses compared to that of the vehicle group (Veh + Veh + F, $^{***}P < 0.001$). Epinephrine (1 and 10 $\mu\text{g}/\text{kg}$) was intraperitoneally injected, and this significantly increased formalin-induced pain as compared with the vehicle group during the late phase ($^{\#}P < 0.05$). Pretreatment of epinephrine (10 $\mu\text{g}/\text{kg}$) before DBV (Epi 10 + DBV + F) significantly reversed the antinociceptive effect of DBV as compared to the DBV only treated group during the late phase of the formalin test ($^{\#\&}P < 0.01$).

had no effect on formalin-induced nociceptive responses as compared to the vehicle-DBV-treated group, whereas administration of a high dose of atenolol or ICI-118,551 with DBV [Atenolol (5 mg) + DBV + F, ICI-118,551 (4 mg) + DBV + F, $n = 7$, resp.] significantly decreased the paw licking time as compared to the vehicle-DBV-treated group during the late phase but not during the early phase of the formalin test ($^{\#}P < 0.05$, resp.).

4. Discussion

DBV injection into an acupoint has been reported to produce a powerful antinociceptive effect in a variety of animal models of acute and chronic pain. Several possible mechanisms have been proposed to explain the biological processes underlying this DBV-induced antinociceptive effect. For example, it has been shown that DBV injection can activate descending bulbospinal noradrenergic pathways, which in turn activate spinal α_2 -adrenoceptors [2, 11, 22]. On the other hand, it has also been demonstrated that DBV administration can also reduce peripheral leukocyte migration and inflammation-associated increases in TNF- α in a mouse air pouch model of inflammation. Thus, DBV injection can produce both antinociceptive and anti-inflammatory effects. It has also been shown that adrenalectomy significantly reduces this DBV-induced suppression of leukocyte migration and TNF- α expression [15]. In this regard, DBV stimulation causes increased release of acetylcholine in the spinal cord. This increased acetylcholine acts on spinal muscarinic type 2

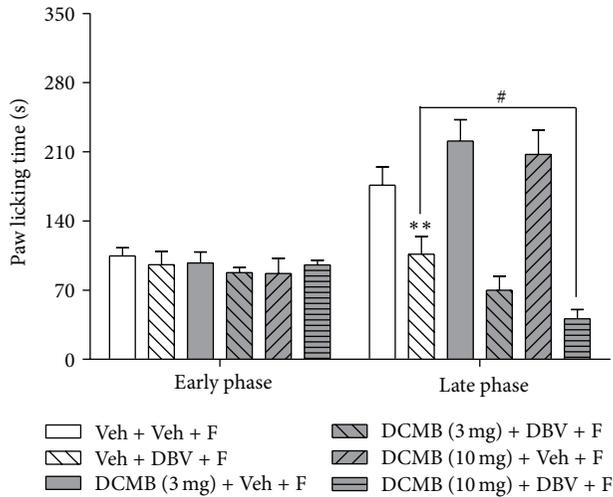


FIGURE 4: Graphs illustrating the antinociceptive effect of preinjection of the PNMT inhibitor, DCMB, prior to DBV treatment on the formalin test. DBV (Veh + DBV + F) significantly decreased pain behaviors compared to the vehicle group (Veh + Veh + F, $**P < 0.01$). DCMB-injected groups that were injected with vehicle [DCMB (3, 10 mg) + Veh + F] did not change formalin-induced pain responses during the early and late phases. While a low dose of DCMB given prior to DBV treatment [DCMB (3 mg) + DBV + F] did not change formalin-induced pain responses, a higher dose of DCMB given prior to DBV treatment [DCMB (10 mg) + DBV + F] significantly suppressed formalin-induced pain behaviors during the late phase of the formalin test as compared to that of the DBV-treated group ($#P < 0.05$).

receptors leading to a disinhibition mechanism that results in the activation of SPNs that project to the adrenal medulla, and this activation leads to the release of adrenal epinephrine [23]. While this DBV-induced increase of epinephrine from adrenal medulla is one of the mechanisms involved in DBV's anti-inflammatory effects, it is not clear whether a similar mechanism might be involved in DBV-mediated antinociceptive effects.

In the present study, we demonstrated that injection of a high dose of DBV significantly reduces pain behaviors during the late phase of the formalin test, and this DBV-induced antinociceptive effect can be dramatically enhanced by adrenalectomy. On the other hand, adrenalectomy itself had no influence on formalin-induced pain responses. Recently, several investigators have reported that adrenalectomy increases the antinociceptive effect of several analgesics and decreases the hyperalgesia resulting from vagotomy. In this regard, elimination of HPA axis function through adrenalectomy potentiates the antinociceptive effect of the calcium channel blocker, nifedipine, and attenuates its analgesic tolerance [24]. It has been reported that adrenalectomy potentiates morphine sensitivity at the level of the spinal cord and also potentiates the synergistic interaction of supraspinal and spinal morphine action [25]. Moreover, the adrenal medulla is also implicated in vagotomy's pain facilitatory mechanisms, and this hyperalgesic action is reversed by denervation of the

adrenal medulla [26]. Khasar et al. have also shown that the hyperalgesic action of the vagal nerve is decreased by suppression of adrenal medulla-derived epinephrine [19]. These findings suggest that an increased release of epinephrine from the adrenal medulla has an important role in peripheral nociception. Based on these reports, it is likely that the DBV-induced antinociceptive effect can be negatively modulated by increases in epinephrine release from adrenal gland.

Chemical sympathectomy has been reported to enhance the pain-attenuating effect produced by the alpha(2)-adrenoceptor agonist, MPV-2426 [27]. In this regard, we have investigated whether peripheral chemical sympathectomy has an effect on DBV-induced antinociceptive effects. In order to examine the role of sympathetic postganglionic neurons on DBV-induced antinociception, a chemical sympathectomy using intraperitoneal injection of 6-OHDA was performed. Because 6-OHDA cannot cross the blood-brain barrier, it is only able to denervate sympathetic postganglionic neurons and blocks the action of peripheral sympathetic nerve-derived catecholamine [28]. Interestingly, pretreatment with 6-OHDA did not alter DBV-induced antinociception in either phase of the formalin test. Moreover, 6-OHDA by itself did not affect formalin-induced pain responses. These results are similar to those reported in previous studies, which demonstrated that sympathectomy had no effect on baseline pain response and that the sympathetic nervous system has very little effect on pain sensation in healthy subjects [29]. Therefore, our results indicate that the DBV-induced antinociceptive effect is closely associated with adrenal gland-derived epinephrine but not with sympathetic postganglionic neuron derived catecholamines.

In order to evaluate whether increases in peripheral epinephrine can reverse the potentiation of DBV-induced antinociception in adrenalectomized mice, epinephrine was intraperitoneally injected in adrenalectomized mice. The injection of exogenous epinephrine increased peripheral nociception in late phase and restored the DBV-induced increase in antinociception in adrenalectomized mice. These results demonstrated that epinephrine injection is closely involved in peripheral sensitization in late phase and reversed the adrenalectomy-induced facilitation of DBV's antinociceptive effect in comparison to DBV's effect observed in normal mice. It is well established that increases in peripheral epinephrine can affect peripheral nociception. Khasar et al. determined that mechanical hypersensitivity induced by intraplantar injection of epinephrine in the rat was reduced by propranolol suggesting that peripheral β -adrenoceptors mediate epinephrine-induced hypersensitivity, and this β -adrenoceptor-mediated hyperalgesia is associated with both PKC and PKA second-messenger systems [18, 20]. Since it is also well established that the adrenal medulla is the major source of peripheral epinephrine release in rodents, adrenalectomy-induced facilitation of DBV's antinociceptive effect is likely associated with a decrease in adrenal medulla-derived epinephrine. This also implies that DBV's antinociceptive effect could be partially reduced by a DBV-induced increase in peripheral epinephrine.

Subsequently, we examined whether the suppression of adrenal medulla-derived epinephrine using a PNMT

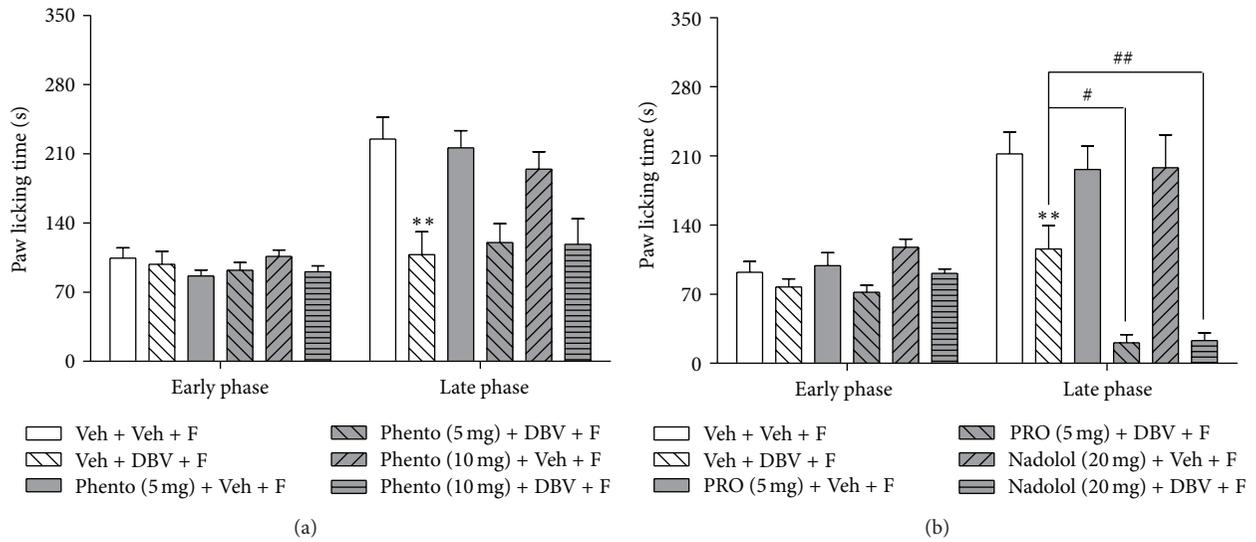


FIGURE 5: Graphs illustrating the antinociceptive effect of DBV administered together with adrenoceptor antagonists on formalin-induced pain responses during the early and late phases of the formalin test. (a) Injection of DBV (Veh + DBV + F) decreased pain responses compared to the vehicle-injected group (Veh + Veh + F, $**P < 0.01$). Coadministration of phentolamine with DBV or vehicle [Phento (5, 10 mg) + DBV + F, Phento (5, 10 mg) + Veh + F] did not change formalin-induced pain behaviors as compared to each control group during either the early or late phase. (b) The group receiving vehicle plus DBV and formalin (Veh + DBV + F) showed suppressed pain responses compared to the vehicle- plus-vehicle and formalin-injected group (Veh + Veh + F, $**P < 0.01$). Injection of propranolol and nadolol with vehicle [PRO (5 mg) + Veh + F, Nadolol (20 mg) + Veh + F] did not change formalin-induced pain responses as compared to the DBV-treated group during the early or late phase. Propranolol and nadolol with DBV treatment [PRO (5 mg) + DBV + F, Nadolol (20 mg) + DBV + F] significantly suppressed formalin-induced pain during the late phase ($^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ as compared with DBV-treated group) but not during the early phase.

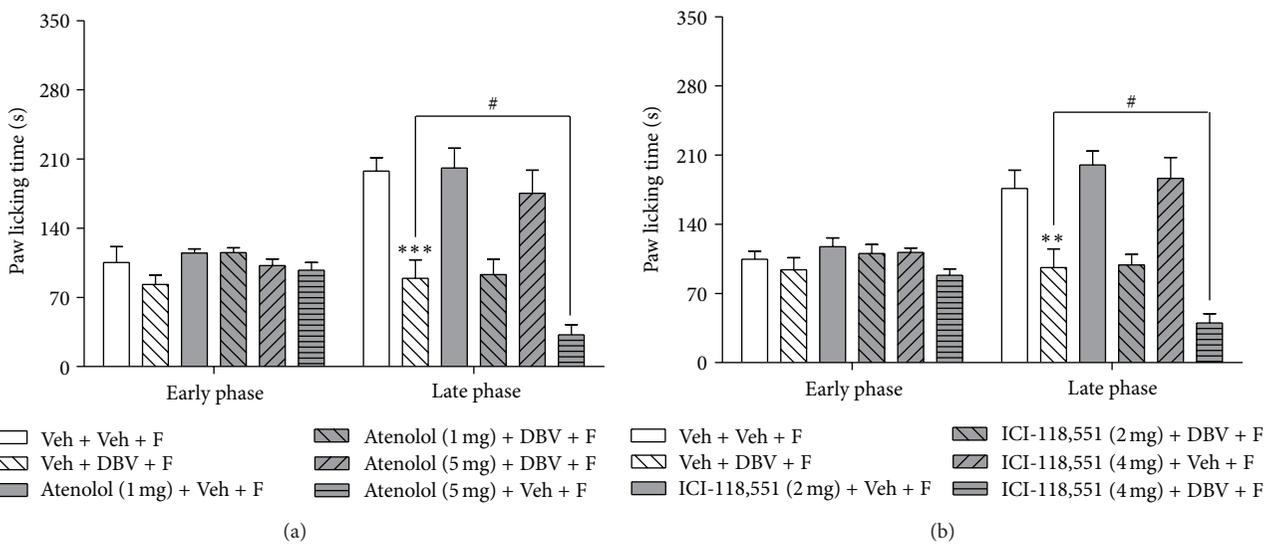


FIGURE 6: Graphs summarizing the effect of administration of β -adrenoceptor subtype antagonists on the antinociceptive effect of DBV on formalin-induced nociceptive behaviors. (a) DBV treatment (Veh + DBV + F) significantly decreased pain behaviors compared with vehicle group (Veh + Veh + F, $^{***}P < 0.001$). Coadministration of atenolol with vehicle [Atenolol (1, 5 mg) + Veh + F] did not change pain behaviors as compared to the vehicle-injected group during either the early or late phase. High dose of atenolol pretreatment with DBV [Atenolol (5 mg) + DBV + F] significantly decreased the formalin-induced pain responses as compared to DBV-treated group at the late phase ($^{\#}P < 0.05$). (b) Similarly, DBV (Veh + DBV + F) significantly decreased pain behaviors compared to the vehicle group (Veh + Veh + F, $^{***}P < 0.01$). Intraperitoneal injection of ICI-118,551 with vehicle [ICI-118,551 (2, 4 mg) + Veh + F] had no effect on pain behaviors as compared to the control group during either the early or late phase, whereas administration of a high dose of ICI-118,551 in combination with DBV [ICI-118,551 (4 mg) + DBV + F] significantly decreased the pain responses as compared to the DBV-treated group during the late phase ($^{\#}P < 0.05$) but not during the early phase of the formalin test.

enzyme inhibitor or a peripheral epinephrine receptor antagonist could also potentiate the DBV-induced antinociceptive effect. Epinephrine is synthesized by *N*-methylation of norepinephrine, a reaction catalyzed by PNMT, using a cosubstrate and methyl donor, *S*-adenosylmethionine (Ado-Met). As a regulator of epinephrine production, PNMT serves as a marker for adrenergic function [30]. Giordano et al. determined that blockade of epinephrine synthesis with intraperitoneal injection of the PNMT enzyme inhibitor, DCMB, resulted in a reduction of buspirone-elevated plasma epinephrine levels [31]. These findings imply that inhibition of the PNMT enzyme is able to reduce the production of endogenous epinephrine, which leads to hypersensitivity to nociceptive stimuli. The results of the present study showed that injection of DCMB alone does not produce an effect on either the early or late phase pain responses in the formalin test, whereas DCMB (10 mg/kg) coadministered with DBV significantly suppressed the formalin-induced pain responses during the late phase. Our data together with that of previous studies demonstrate that DBV-induced antinociception has an influence on the activity of adrenal gland PNMT, which ultimately contributes to a reduction in DBV's antinociceptive effect in the formalin test.

Finally, we examined which subtype of adrenergic receptor is involved in the negative modulation of this DBV-induced antinociceptive effect. It is well established that peripheral epinephrine serves as an endogenous ligand, which can act on both α - and β -adrenoceptors subtypes. The activation of these receptors via an increase in peripheral epinephrine can lead to peripheral hypersensitivity to noxious stimulus [32, 33]. In the present study, pretreatment with the α -adrenoceptor antagonist, phentolamine, was used to examine the role of α -adrenoceptors in DBV-induced antinociception. Since phentolamine pretreatment did not affect DBV-induced antinociception, it is reasonable to assume that α -adrenoceptors are not associated with DBV-induced antinociception. On the other hand, propranolol, a nonselective β -adrenergic antagonist, significantly potentiated DBV-induced antinociception. In order to examine the role of pure peripheral epinephrine, we also used another β -adrenergic antagonist, nadolol, which does not cross the blood-brain barrier. These results were similar to those in propranolol-treated mice or adrenalectomized mice, indicating that facilitation of DBV-induced antinociception was mediated by inhibition of peripheral β -adrenoceptors, not central nervous system associated β -adrenoceptors. Furthermore, we showed that coadministration of either the β_1 -selective adrenergic receptor antagonist, atenolol, or the β_2 -selective adrenergic receptor antagonist, ICI-118,551 with DBV, potentiated the antinociceptive effect of DBV alone. Collectively, these findings demonstrate that DBV treatment results in the release of adrenal medulla-derived epinephrine, which leads to increased peripheral sensitization via activation of β_1 - and β_2 -adrenoceptors, and ultimately leads to partial inhibition of the DBV-induced antinociceptive effect in the mouse formalin test. Thus, injection of DBV into an acupoint has an initial antinociceptive effect on the late phase of the formalin test but at the same time affects the

adrenal medulla to increase epinephrine secretion leading to a paradoxical reduction in DBV's antinociceptive effect.

5. Conclusion

The present study shows that adrenalectomy significantly potentiates DBV-induced antinociception, while chemical sympathectomy has no effect. In addition, administration of exogenous epinephrine reverses the enhanced antinociception produced by DBV in adrenalectomized animals. Pretreatment with α -adrenergic antagonists did not alter DBV-induced antinociception, whereas β -adrenergic antagonists significantly increase DBV-induced antinociception. Collectively, these results demonstrate that suppression of adrenal medulla-derived epinephrine, which acts on β -adrenoceptors, can potentiate DBV-induced antinociceptive in the mouse formalin test, suggesting that DBV acupoint stimulation performed in combination with administration of peripheral β -adrenoceptor antagonists would be a potential novel strategy for the pain management.

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Research Article

Effects of Laser Acupuncture on Longitudinal Bone Growth in Adolescent Rats

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Longitudinal bone growth is the results of chondrocyte proliferation and hypertrophy and subsequent endochondral ossification in the growth plate. Recently, laser acupuncture (LA), an intervention to stimulate acupoint with low-level laser irradiation, has been suggested as an intervention to improve the longitudinal bone growth. This study investigated the effects of laser acupuncture on growth, particularly longitudinal bone growth in adolescent male rats. Laser acupuncture was performed once every other day for a total of 9 treatments over 18 days to adolescent male rats. Morphometry of the growth plate, longitudinal bone growth rate, and the protein expression of BMP-2 and IGF-1 in growth plate were observed. The bone growth rate and the heights of growth plates were significantly increased by laser acupuncture. BMP-2 but not IGF-1 immunostaining in growth plate was increased as well. In conclusion, LA promotes longitudinal bone growth in adolescent rats, suggesting that laser acupuncture may be a promising intervention for improving the growth potential for children and adolescents.

1. Introduction

Short stature in childhood and adulthood is one important concern. Most of people desire to get taller due to the thought that long height may help to improve the physical appearance and attractive personality or that to be unusually short may have social disadvantages. In fact, short stature (height) is one of major causes of concern and anxiety to children, adolescents, and parents [1]. For this reason, interest in the growth promotion in childhood and adolescent periods is increasing.

An improvement in the height is significantly associated with the bone growth in length. Bone growth in the length occurs at the growth plate by endochondral ossification, a two-step process in which cartilage is first formed and then remodeled into hard calcified bone [2, 3]. The growth plates are composed of three functionally and structurally distinct layers: the resting, the proliferative, and the hypertrophic zones. At this site, growth plate chondrocytes proliferate,

differentiate into mature hypertrophic cells, synthesize the typical extracellular matrix, and form cartilage. The newly formed cartilage is then calcified and converted into hard bone, resulting in bone elongation [4].

Growth hormone (GH) therapy has been often considered as a treatment option to increase height in children. Its effectiveness is, however, still doubtful. Although GH therapy for short children seems effective in increasing growth and final height, individual responses in the therapy are highly variable and the mean final height in treated individuals remains relatively short when compared with normal stature [5, 6]; health-related quality of life in children treated with GH has been the same, or even worse [7]. Although GH treatment in children has been known to be generally safe, its significant side effects such as benign intracranial hypertension, slipped capital femoral epiphysis, scoliosis, features of acromegaly, pancreatitis, and the increase in cancers have been reported [8]. Also, the long-term risks of prolonged GH treatment of children remain still unknown. Hence, there is

a strong need for alternative therapies that are safe and effective for stimulating the growth plate and finally increasing height of short children.

According to traditional beliefs, disease results from stagnation of the flow of the body's vital energy, "Qi"; acupuncture stimulation at acupoint would help restore the flow and then promote the homeostasis of the Qi, reestablishing the body function to normal [9]. Being considered a relatively simple, inexpensive, and safe treatment compared to other conventional interventions, acupuncture is regarded as a nonpharmacological alternative to GH treatment; however, since the most children are afraid of needles, they would be unwilling to undergo acupuncture and the parents may hesitate to expose them to additional pain and stress [10]. For this reason, laser acupuncture is emerging as an alternative to needle acupuncture [11].

Laser acupuncture is one of interesting modalities of low-level laser therapy (LLLT), a noninvasive form of phototherapy, which is defined as the stimulation of traditional acupoints with low-intensity, nonthermal laser irradiation [12, 13]. The noninvasive and painless nature of treatment makes it useful for particularly providing children with holistic healthcare [12]. Although using low-level, nonthermal laser irradiation instead of needles, it shares the same principles of the traditional acupuncture and proves equal biological effect as needle acupuncture [14]. To our knowledge, studies demonstrating the effect of acupuncture including laser acupuncture on longitudinal bone growth in adolescent rats have previously not been reported.

The purpose of the present study was to investigate the effects of laser acupuncture on longitudinal bone growth in adolescent rat.

2. Materials and Methods

2.1. Animals. Male Sprague–Dawley rats aged 2 weeks were purchased with their mothers from Samtaco Co. (Osan, Korea). Animals were housed in an animal room maintained at $20^{\circ} \pm 2^{\circ}\text{C}$ with lighting (07:00–19:00). They had free access to water and food. After a 1-week acclimation period, the animals weighing approximately 40 g were used in the experiments. All studies were conducted in accordance with the National Institutes of Health (NIH) guidelines and were approved by the Institutional Animal Care and Use Committee of Kyung Hee University.

2.2. Experimental Schedules. Rats were randomly divided into two groups ($n = 10$ for each group): control group (CONT) and laser acupuncture treatment group (LA). The laser acupuncture was applied once every other day between 9:00 and 10:00 am, started on day 0, and continued until day 18. Body weight was recorded just before the acupuncture treatment. Nose-to-tail length was measured on day 18. For determining longitudinal bone growth rate, tetracycline (15 mg/kg body weight) was administered by intraperitoneal injection on days 13 and 16. On day 19, the rats were anaesthetized with an overdose of pentobarbital (100 mg/kg

body weight) and the tibias were removed and fixed in 10% buffered formalin (pH 7).

2.3. Laser Acupuncture. Laser acupuncture was performed by laser stimulation using Lapex-2000 (Meridian Medical Inc. Vancouver, Canada), a semiconductor-based low level laser therapy (LLLT) device emitting a cold red laser (635–680 nm/40 mW). Before laser acupuncture was applied, both hind limbs were shaved with an electric clipper, paying attention to not hurt the skin. Acupoints ST36 (Zusanli) and SP6 (Sanyinjiao) commonly used in the treatment of growth stimulation were selected in this study [15–17]. Each acupoint of rats lightly restrained by hands without anesthesia was stimulated, bilaterally, for 30 seconds (energy density of 1.2 W/cm^2) by holding a laser probe with a spot size of the laser of 3 mm in diameter in contact with, and perpendicular to, the acupoints; each treatment session lasted 120 seconds. Control rats were sham operated with the inactivated probe which was turned off. Acupuncture was performed by one of the authors who has a recognized training in traditional Chinese medicine.

2.4. Detection of Longitudinal Bone Growth Rate. The bones of each rat were labeled by administering tetracycline before rats were sacrificed (see "Section 2.2" in detail). Longitudinal bone growth rate was evaluated in undecalcified bone. The tibias fixed in 10% buffered formalin (pH 7) were cryopreserved in 30% sucrose solution and sectioned longitudinally at a thickness of $40 \mu\text{m}$ using a microtome (Leica Microsystems, Bensheim, Germany). The sections were mounted on collagen-coated glass slides and observed by a confocal fluorescence microscope (FLUOview FV10i; Olympus, Tokyo, Japan) to measure the gap distance between the fluorescent bands of the metaphysis of the proximal tibia. The distance between the bands of tetracycline-labeled bone was measured from the densest part of each band rather than from the edges, which were irregular and more difficult to define. The measurements were performed using FV10-ASW 2.0 microscopy software (Olympus). The distance between the bands is the amount that the tibia has grown by the activity of the proximal epiphyseal cartilage during the two days between injections. Longitudinal bone growth rate was calculated as the interlabel width divided by the number of days between the tetracycline injections.

2.5. Histomorphometrical Evaluation. The fixed tibia specimens were decalcified in a histologic decalcifying agent (Calci-Clear Rapid; National Diagnostics, Atlanta, USA), dehydrated through a graded ethanol series, cleared in xylene, and processed for embedding in paraffin with routine protocols. Four μm -thick sections were cut using a rotatory microtome (Finesse 325; Thermo Shandon, Cheshire, UK), mounted on collagen-coated glass slides, and subsequently stained with toluidine blue. Slides were viewed at a magnification of $100\times$, and images of the entire growth plate were taken with BX51 microscope (Olympus). Histomorphometry of growth plate was based on the zone definitions [18].

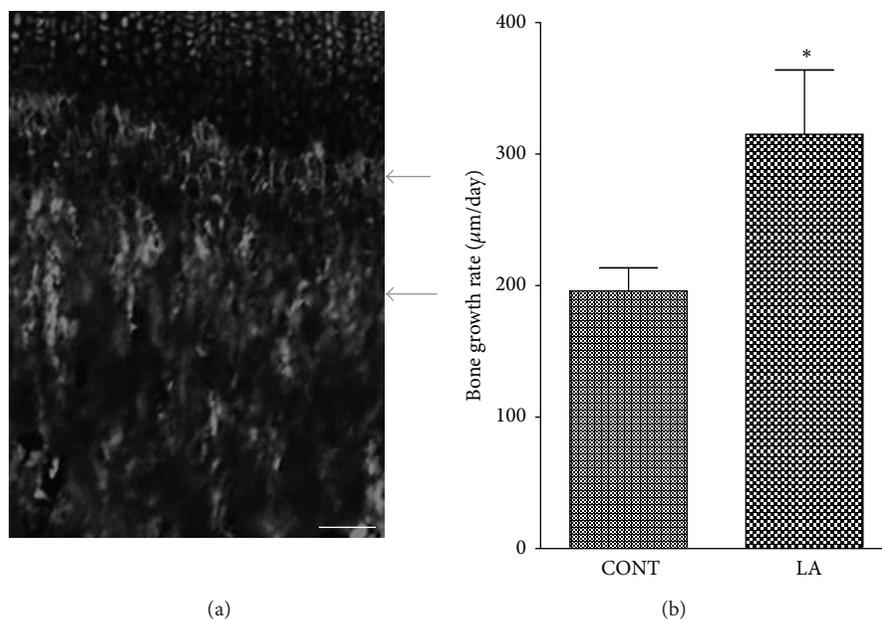


FIGURE 1: Effects of laser acupuncture on the longitudinal bone growth of the proximal tibia. Longitudinal bone growth rate was determined by tetracycline labeling. Representative fluorescent image of a longitudinal section of the proximal tibia showing tetracycline labeling (a) is shown. Arrows indicate two fluorescent lines representing the two injections of tetracycline. Scale bar = 100 μm . Longitudinal bone growth rate (b) was calculated by means of tetracycline labeling ($\mu\text{m}/\text{day}$). CONT, control group; LA, laser acupuncture group. Each value is the mean \pm SEM. * $P < 0.05$ versus control group.

For morphometry, the heights of the resting, proliferative, and hypertrophic zones in growth plate as well as the length of the growth plate were measured as indicated in Figure 2(a) using DP2-BSW software (Olympus). At least 5 measurements per sample were taken at three different locations by two observers blind to the experimental group. Each zone of the growth plate was also scored on a scale of 0–3 based on the number of chondrocytes within the same defined region: 0 = absent, 1 = moderate, 2 = discreet, and 3 = intense [19].

2.6. Immunohistochemistry. After decalcified and paraffin-embedded, the bones were sectioned at a thickness of 4 μm using a rotatory microtome Finesse 325 (Thermo Shandon). For the detection of bone morphogenetic protein-2 (BMP-2) and insulin-like growth factor-1 (IGF-1) in the growth plate, the deparaffinized sections were immersed in 0.01 M sodium citrate buffer (pH 6.0) for 40 min for antigen retrieval and incubated with rabbit anti-BMP-2 antibody (1:200 diluted; Novus Biologicals, Littleton, USA) or mouse anti-IGF-1 antibody (1:200 diluted; Novus Biologicals) at 4°C overnight. After washing, sections were processed with the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Labs, Burlingame, USA) including the appropriated secondary anti-rabbit or anti-mouse IgG (biotinylated, 1:200 diluted; Vector Labs) and developed with the DAB peroxidase substrate kit (Vector Labs). Slides were counterstained with hematoxylin. Staining was completely absent in identical tissue sections in which the primary antibody was omitted (data not shown). Images were taken with BX51 microscope (Olympus).

2.7. Statistical Analysis. All data were presented as the means \pm standard error of the mean (SEM) for each group. Statistical significance was calculated with Student's *t*-test using Graphpad Prism 5 software package (GraphPad Software, San Diego, USA). Differences were considered significant when $P < 0.05$.

3. Results and Discussion

Neither Body weight nor the nose to tail length was different between control and LA-treated groups (data not shown). To evaluate the longitudinal bone growth rate, tetracycline labeling was used to stain newly formed bone. Tetracycline binds to free calcium and gets deposited in newly deposited bone, causing staining and fluorescence under ultraviolet illumination. The tetracycline administered to rats formed two fluorescent lines corresponding to the two injections (Figure 1(a)). The longitudinal bone growth rate in normal adolescent rats was $195.9 \pm 17.5 \mu\text{m}/\text{day}$ and laser acupuncture was shown to promote bone growth, increasing the rate to $315.1 \pm 48.8 \mu\text{m}/\text{day}$ (Figure 1(b)). About 200 $\mu\text{m}/\text{day}$ of the longitudinal bone growth rate in control group is in accordance with the results reported previously [20].

The increase in body length is mostly due to the longitudinal bone growth, which is a reflection of the synchronized processes of chondrogenesis (the proliferation and differentiation of chondrocytes in the growth plates) and cartilage ossification (calcification at the metaphysis) [21]. Interestingly, laser acupuncture significantly increased longitudinal bone growth. As the height of growth plate was

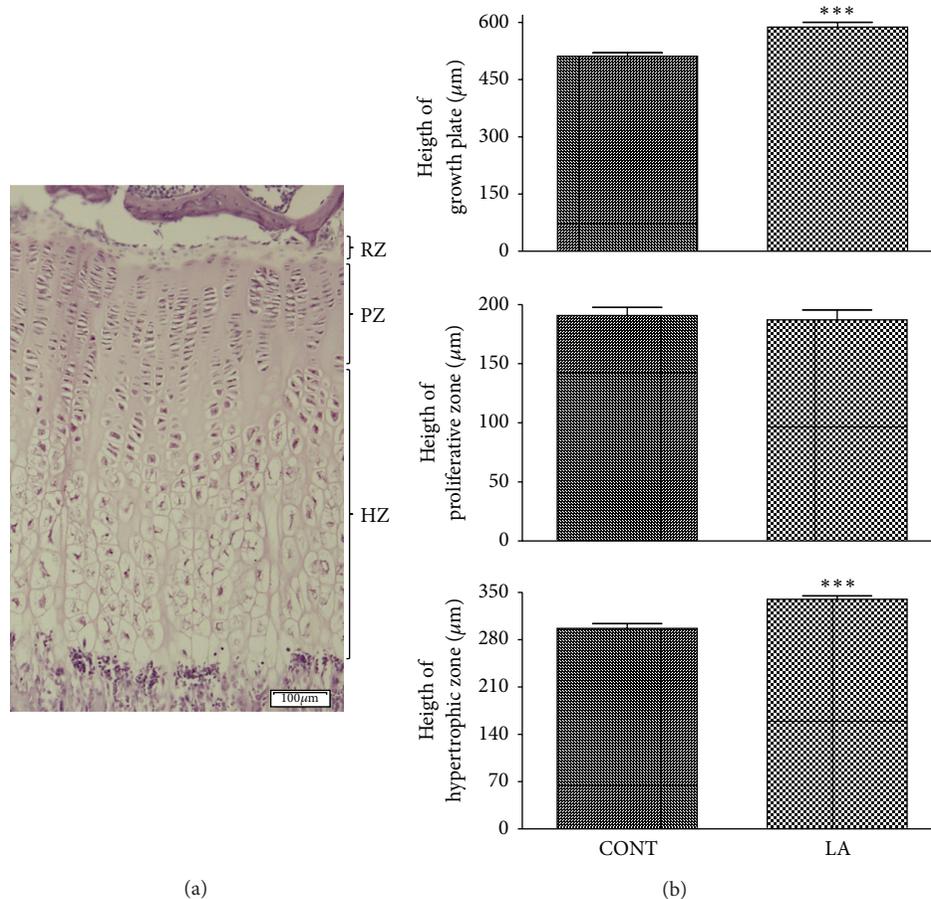


FIGURE 2: Effects of laser acupuncture on zone-specific growth within the growth plate. Representative proximal tibia section from normal rat (a) is shown. The tissues were stained with toluidine blue in which cartilage is pink. Resting zone (RZ), proliferative zone (PZ), and hypertrophic zone (HZ) are designated. Scale bar = 100 μm . The length of the entire growth plate and the length of the proliferation zone and the hypertrophic zone were measured separately using image processing software (b). CONT, control group; LA, laser acupuncture group. Each value is the mean \pm SEM. *** $P < 0.005$ versus control group.

correlated with the body growth rate [22], the heights of the proximal tibia growth plate were measured. The height of proximal tibia growth plate in normal adolescent rats was $511.3 \pm 9.2 \mu\text{m}$. Following laser acupuncture treatment, growth plate height increased to $587.5 \pm 13.0 \mu\text{m}$ (Figure 2(b) top). The growth plate is composed of three layers: the resting, proliferative, and hypertrophic zones [18].

The heights of proliferative and hypertrophic zones were also measured since the resting zone was almost absent in the vast majority of animals. Contrary to our expectation that LA may increase the height of both proliferative and hypertrophic zones, it only affected the hypertrophic zone; the height of hypertrophic zone was slightly but significantly higher in LA group as compared to control group (Figure 2(b) bottom), but that of proliferative zone was not different (Figure 2(b) middle). The proportion (%) of each zone in the growth plate was similar in control and LA-treated rats (data not shown). In addition, the scores were determined for the cell density in the proliferative and hypertrophic zones of the growth plate, which was in accordance with the morphometric analysis. The cell density in the hypertrophic

zone was significantly higher in laser acupuncture-treated group compared to control group (Figure 3(b)), but not different in the proliferative zone (Figure 3(a)). Although the rate of new cell production and matrix production in the proliferative zone is an important factor in bone formation, the hypertrophic zone plays a key role as well. Hypertrophic chondrocytes generated by terminal differentiation of chondrocytes in the proliferative zone cease dividing and then enlarge, contributing substantially to the growth process such as the initiation of ossification [23]. The amount of enlargement of the hypertrophic cells in the direction of growth together with a change in the height of the hypertrophic zone is important factors in the difference in the growth rate. Because chondrocytes no longer proliferate after early hypertrophy, the number of cell layers in the hypertrophic zone only reflects the time spent by the cells in this region. Therefore, the increase of chondrocyte numbers in the hypertrophic zone might, however, be not sufficient to affect the total length of the body.

Next, the expression of BMP-2 and IGF-1 was investigated in the growth plate using immunohistochemistry. BMPs play

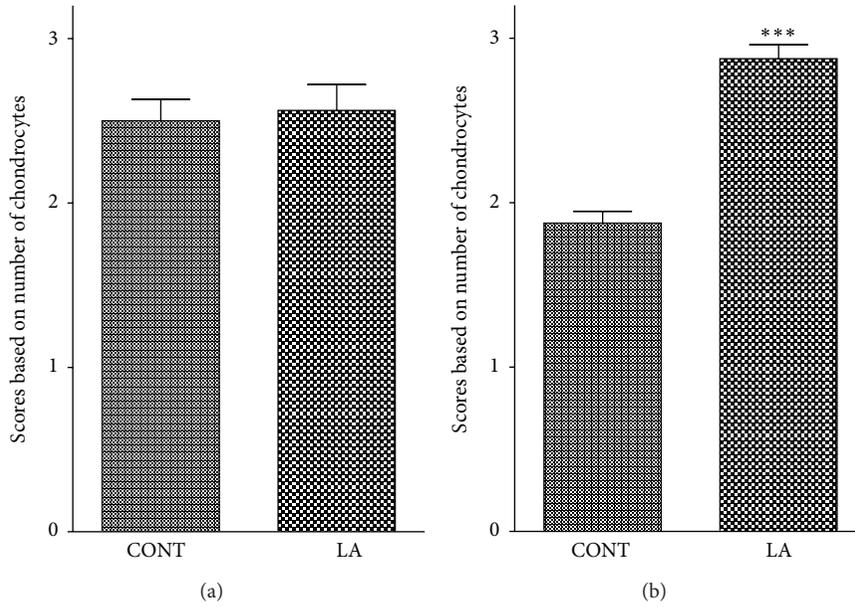


FIGURE 3: Effects of laser acupuncture on cell density in the proliferative and hypertrophic zones of the growth plate. The proliferative (a) and hypertrophic (b) zones of the growth plate were scored in accordance with the number of chondrocytes within the same defined region. CONT, control group; LA, laser acupuncture group. Each value is the mean \pm SEM. *** $P < 0.005$ versus control group.

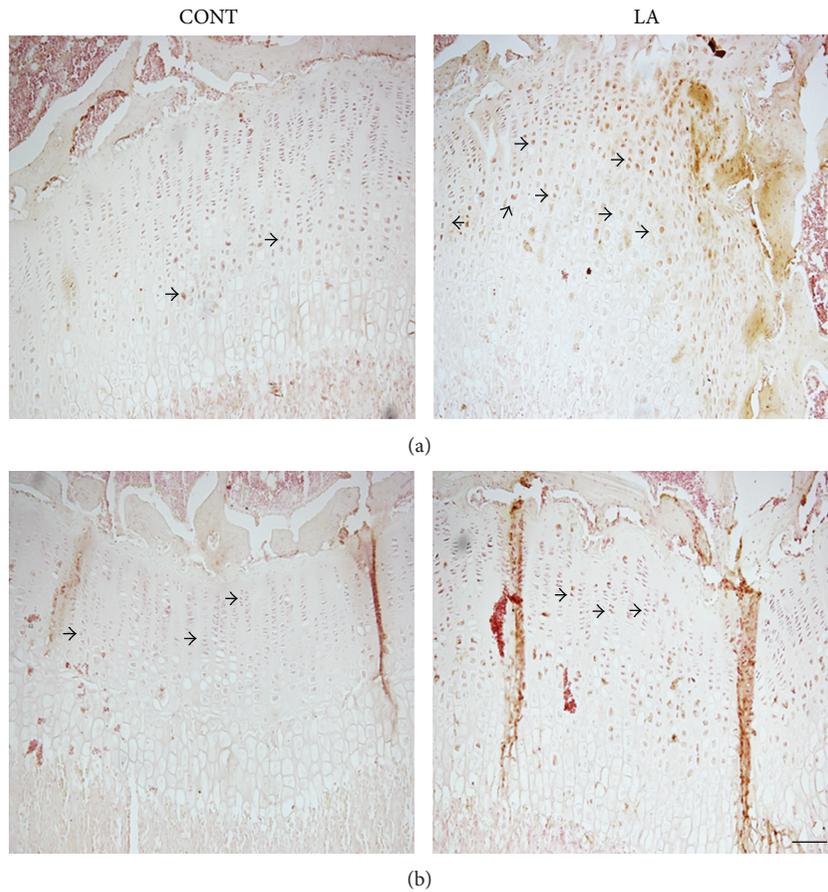


FIGURE 4: Effects of laser acupuncture on BMP-2 and IGF-1 expression in the growth plate. Representative images of immunostaining for BMP-2 (a) and IGF-1 (b) in proximal tibial growth plates of control and laser acupuncture-treated rats are shown. Arrows indicate brown positive staining with BMP-2 or IGF-1. CONT, control group; LA, laser acupuncture group. Scale bar = 100 μm .

important roles in regulating growth plate chondrogenesis and longitudinal bone growth as well as embryonic skeletal development. BMP-2 stimulates chondrocyte proliferation in the proliferative zone of the growth plate and also causes an increase in chondrocyte hypertrophy [24]. BMP-2 immunostaining was intense in hypertrophic zone but weak in the proliferative zone of the growth plate. As expected, BMP-2 expression particularly in hypertrophic chondrocytes of the growth plate was increased in the laser acupuncture-treated group compared with the control group (Figure 4(a), arrow denotes brown staining indicative of BMP-2 expression). IGF-1 is an important factor to augment longitudinal bone growth by stimulating growth plate chondrocyte proliferation [25]. IGF-1 immunostaining was relatively higher in the proliferative zone than the hypertrophic zone. But, as expected, IGF-1 expression was similar between control and laser acupuncture-treated rats (Figure 4(b), arrow denotes brown staining indicative of IGF-1 expression).

Although the mechanism of laser acupuncture is not completely understood, the following effects of LLLT are known: increases of cell growth, cell regeneration, and cellular activity [26]. This may help to explain the positive mechanism of laser acupuncture on longitudinal bone growth. Our data in this study are actually in accordance with the effects of LLLT on cell activity; the stimulation of acupoint with low-level laser increased the height and the chondrocyte numbers in the hypertrophic zone and thus promoted the longitudinal bone growth rate. Also, the regulation by systemic hormones such as GH and IGF-1 on longitudinal growth and final height is well known [27]. Therefore, another possibility to explain the action mechanism of LA on bone growth is that LA may enhance the bone growth in length through the regulation of the hormone system. Further studies are necessary for better understanding of the effects of LA on bone growth.

A few of studies have demonstrated the effects of LLLT on epiphyseal growth, but results are controversial. Some findings support that LLLT has the effects to induce chondrogenesis in vitro [28] and to improve cartilage structure in vivo [29, 30], while others prove that LLLT has no [31] or even negative [19] effect on bone growth. These differences require further study.

In conclusion, laser acupuncture induces the longitudinal bone growth in adolescent rats through the induction of BMP-2 and IGF-1, suggesting that this treatment may have a clinical potential in promoting longitudinal bone growth in children.

Conflict of Interests

No conflict of interests was declared.

Acknowledgments

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Research Article

Electroacupuncture and Rosiglitazone Combined Therapy as a Means of Treating Insulin Resistance and Type 2 Diabetes Mellitus: A Randomized Controlled Trial

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Aims. To evaluate the efficacy of rosiglitazone (TZD) and electroacupuncture (EA) combined therapy as a treatment for type 2 diabetes mellitus (T2DM) patients by randomized single-blind placebo controlled clinical trial. **Methods.** A total of 31 newly diagnostic T2DM patients, who fulfilled the study's eligibility criteria, were recruited. The individuals were randomly assigned into two groups, the control group (TZD, $N = 15$) and the experimental group (TZD + EA, $N = 16$). Changes in their plasma free fatty acid (FFA), glucose, and insulin levels, together with their homeostasis model assessment (HOMA) indices, were statistically compared before and after treatment. Hypoglycemic activity (%) was also compared between these two groups. **Results.** There was no significant difference in hypoglycemic activity between the TZD and TZD + EA group. The effectiveness of the combined therapy seems to derive from an improvement in insulin resistance and a significant lowering of the secreted insulin rather than the effect of TZD alone on T2DM. The combined treatment had no significant adverse effects. A lower plasma FFA concentration is likely to be the mechanism that causes this effect. **Conclusion.** This combined therapy seems to suppress endogenous insulin secretion by improving insulin resistance via a mechanism involving a reduction in plasma FFA. This trial is registered with ClinicalTrials.gov NCT01577095.

1. Introduction

Diabetes mellitus is a syndrome associated with a disordered metabolism and inappropriate hyperglycemia that can be due to either an absolute deficiency in insulin secretion or reduction in the biological effectiveness of insulin. Type 2

diabetes is the predominant form of diabetes worldwide and accounts for 90% of cases globally [1, 2]. Alarming increases in the prevalence of diabetes have occurred in Asia [3]. A number of factors have been shown to play an important role in the development of the disease and these include excessive caloric intake, a sedentary lifestyle, and abdominal

visceral obesity [4]. In addition, many circulating inhibitors, including free fatty acids (FFAs), have also been found to be involved in reducing insulin sensitivity [5]. From a pathology point of view, patients with type 2 diabetes have a number of metabolic abnormalities including (1) resistance to the action of insulin in muscle, fat tissue, and liver; (2) defective insulin secretion, especially under glucose stimulus; and (3) increased glucose production by the liver [6].

Management of type 2 diabetes is a great challenge to physicians both because of the disease's complex pathology and because of the multiple chronic complications associated with the disease. The usual treatment for type 2 diabetes mellitus includes life style modification, exercise, diet therapy, oral antihyperglycemic drugs, and insulin. The thiazolidinediones represent a unique class of drug that may directly decrease insulin resistance by enhancing insulin action in skeletal muscle, liver, and adipose tissue [7]. Two of these compounds, rosiglitazone and pioglitazone, have been approved for clinical use in type 2 diabetic patients. In the UK Prospective Diabetes Study (UKPDS), reduction in the risk of microvascular complications (retinopathy, nephropathy, and neuropathy) was found in the intensive treatment group of patients with new onset type 2 diabetes [8]. However, it should be realized that there are possible unfavorable events associated with achieving lower glycemic targets such as hypoglycemia, bulky combinations of medications, and expense. Although present-day management of type 2 diabetes is more effective than before, as time goes by the effectiveness of drug treatment deteriorates in these patients. Novel therapies and different kinds of treatment for type 2 diabetes need to be developed in the future.

Acupuncture is a part of traditional Chinese medicine. According to traditional Chinese medicine theory, acupuncture regulates "Qi and Blood" and is likely to affect the bioavailability of substances taken internally and in the process may influence the absorption, distribution, metabolism, and/or excretion of substances [9]. In addition, researchers have begun using electroacupuncture (EA) rather than classical acupuncture. This approach combines traditional needle acupuncture with an electrical current passing through the needles into the acupoints. This seems to produce hypoglycemic responses, and, using EA at different frequencies, also causes the release of endogenous opioid peptides that activate specific receptors [10]. In this context, the potential of EA as a treatment for hyperinsulinemia is an important issue because type 2 DM may eventually develop into pancreatic failure [11].

Although the insulin sensitizer rosiglitazone improves insulin sensitivity, there are some adverse effects in terms of liver function and the induction of fluid retention. Since EA has been shown to improve insulin activity in our previous studies [12, 13], a combination of EA's effects in terms of enhancing insulin activity with the use of an insulin sensitizer may potentially be a new modality for the treatment of diabetes mellitus in humans [14]. In addition, this combined therapy may also improve insulin sensitivity and regulate the secretion of insulin, which ought to help to block any worsening of pancreas functioning.

The main purpose of this pilot study was to evaluate a combination of EA and drug therapy, namely, treatment with

the insulin sensitizer, rosiglitazone, and to explore whether this improves insulin activity among type 2 diabetic patients. Therefore, we performed a clinical randomized controlled trial (RCT) on type 2 diabetic patients treated with rosiglitazone alone and compared these with patients treated with rosiglitazone combined with EA. The endpoints assessed were a reduction in blood glucose levels and/or improvements in insulin activity.

2. Materials and Methods

2.1. Study Design and Patient Population. We designed a randomized single-blind placebo controlled clinical trial to compare the reduction in blood glucose level and/or improvements in insulin resistance among type 2 diabetic patients who had been diagnosed within the last 5 years. These individuals were treated with rosiglitazone alone or with rosiglitazone combined with EA using a frequency of 15 Hz at the bilateral Zusanli acupoints. In addition, we studied possible factors associated with improvements in insulin resistance that might be associated with any synergism between EA and rosiglitazone. This was done by assaying plasma FFA, plasma insulin concentration, and calculating the HOMA index [15] for each group of patients. The study protocol was approved by the Institutional Review Board (IRB) of China Medical University Hospital in Taiwan and informed consent was requested and obtained from all study participants. These patients were recruited from the clinical endocrinology and metabolism section of internal medicine and the cooperative center of Chinese and Western Medicine, China Medical University Hospital in Taiwan.

2.2. Inclusion and Exclusion Criteria. We recruited patients who were eligible under the following criteria. The inclusion criteria were firstly that all were native Taiwanese patients aged from 20 to 65 years who had been diagnosed with type 2 diabetes mellitus within the last 5 years and who continued to use same antihyperglycemic agents to control their diabetes during the period of this study as previously. Secondly, their assessment was compatible with the diagnostic criteria of diabetes mellitus according to American Diabetes Association [16] in that (a) the patient presented with any one of the characteristic symptoms of diabetes mellitus such as thirst, polyuria, polyphagia, and weight loss with an any time single blood glucose estimation in excess of 11.1 mmol/L; (b) that the patient presented with a fasting blood glucose level in excess of 6.7 mmol/L after an overnight fast of 8 hours; and (c) that the patient had a 2-hour blood glucose estimation in excess of 11.1 mmol/L after a 75 g glucose load via an oral glucose tolerance test after an overnight fast of 8 hours.

The exclusion criteria were (1) individuals with nephrotic syndrome (urine protein over 3.5 g/day), edema or renal failure (serum creatinine over 115 μ mol/L); (2) individuals who had been diagnosed with heart failure (NYHA Fc III~IV) or who had had a pacemaker implanted; (3) individuals with abnormal liver function (GOT and GPT levels twofold above the normal range) or a diagnosis of liver cirrhosis; (4) individuals with a high HbA1C level (HbA1C above 9%); (5) pregnant women; (6) individuals who were receiving

a thiazolidinedione class drug already; (7) individuals who were receiving insulin therapy already; (8) individuals who received an other therapy during the period of study; (9) individuals who were suffering from a homeostasis disorder or other systemic diseases; and (10) individuals who did not comply with the treatment during the study period.

2.3. Electroacupuncture. The EA treatment used the bilateral Zusanli acupoints, which are located on the anterior tibia muscle near the knees; these were identified based on previous studies [17]. After adjusting the EA apparatus to 15 Hz/10 mA (Han's Healthronics Likon, Taipei, Taiwan), 1.5-unit acupuncture needles (44 mm/32 gauge) were inserted 10–30 mm into the muscle layer at the selected acupoints. After needles were inserted to bilateral Zusanli acupoints, no specific manipulation was carried out; they were only inserted to the specific depth, which gave a sensation of *de-qi* and then electrical stimulation was begun. The positively charged (red pole) clip was connected to the right needle and the negatively charged (black pole) clip was connected to the left needle. In addition, in order to evaluate the effectiveness of the acupoint stimulating therapy and to avoid any variation that might influence the results, only one acupuncture doctor was invited to participate in this study.

2.4. Protocol. The patients enrolled in this clinical single-blind placebo controlled trial were distributed by permuted-block randomization. In each block, a random number for each treatment was generated on a calculator and this was used to decide the treatment group. Each patient was assigned to one of two groups according to this method of randomization by an independent research assistant. All patients were blinded to treatment assignment during the period of study. Body height, body weight, and body mass index were measured before treatment. Biochemical serum markers, namely, blood sugar, liver function, creatinine (Cr), HbA1C, lipid profile, insulin level, and FFA level were measured before treatment for each group. Blood sugar, insulin, and FFA levels were measured after treatment in order to allow an investigation of the efficacies of the protocols.

In the experimental (TZD + EA) group, enrolled patients were treated with 8 mg rosiglitazone 30 min before experiment after overnight fast for 8 hours. The TZD + EA group patients then received EA stimulation at the bilateral Zusanli acupoints with intensity of 15 Hz/10 mA for 30 min. Five mL of blood was drawn from cubital vein before and after EA stimulation for evaluation.

The placebo (TZD) group patients were treated with the same dose of rosiglitazone 30 min before experiment after an overnight fast for 8 hours. During the period of study, only electrode placement took place at the bilateral Zusanli acupoint with no acupuncture manipulation or EA stimulation; this acted as the placebo treatment. Similar to above, five mL of blood was drawn from cubital vein before and after placebo treatment for evaluation.

2.5. Laboratory Measurements. Plasma glucose (mmol/L) concentrations were determined using a spectrophotometric

system (COBAS System, Roche Diagnostics Ltd., Rotkreuz, Switzerland) and commercially available enzymatic kits run in duplicate. The plasma FFA levels (meq/L) were determined spectrophotometrically on the COBAS System (Roche Diagnostics) using a commercially available nonesterified fatty acid kit (Randox Laboratories Ltd, Ardmore, United Kingdom). The plasma insulin levels were measured using an ELISA kit (Linco Research, St. Charles, MO, USA) [18]. In brief, samples were incubated for 2 hours at room temperature in a shaker and exposed to peroxidase conjugate and antibodies bound to a 96-well plate. The conjugate acted on the 3,3',5,5'-tetramethylbenzidine. The reaction was stopped by adding 1 M sulfuric acid 100 μ L and the mixture was shaken to produce a colorimetric endpoint, which was measured spectrophotometrically. The values obtained were in international unit of peptide per liter of plasma.

2.6. Outcome Measures. The primary endpoint was plasma glucose level after treatment in each group patients and a comparison of the hypoglycemic activity between the two independent groups. The secondary endpoints were a comparison of the changes in plasma insulin level, HOMA index, and FFA level after treatment for each group of patients together with a comparison of the FFA level percent change between the two independent groups. All measurements were performed by one independent technician who was blinded to the treatment assignments of the patients throughout the study and the side effects of all the treatments were also recorded.

2.7. Statistical Analysis. The hypoglycemic activity (%) was calculated as follows: $(G_i - G_t) \times (100/G_i)$; where G_i is the initial glucose concentration and G_t is the concentration after treatment. All values were expressed as means \pm SEM in the figures and tables. The Wilcoxon signed-rank test was applied to assess differences by treatment for each group of dependent samples. The Mann-Whitney test was applied to compare the differences between two independent groups. For all comparisons, a P value less than 0.05 (two-sided) was considered statistically significant.

3. Results

3.1. Baseline Data. In this study, 49 patients were recruited and 31 patients completed the clinical protocol with one blood sample being excluded from analysis because of hemolysis. The flowchart of the interventions is shown in Figure 1. All patients were recruited from April, 2006 to May, 2007 at China Medical University Hospital, Taichung, Taiwan and this trial was stopped by a prior setting protocol. A total of 18 patients declined to participate in this study after it was explained to them by the researcher. The male:female ratios were 10:6 for the TZD + EA group and 8:7 for the TZD group. The baseline characteristics of the intension-to-treat patients in the two groups are shown in Table 1. No statistical difference at baseline was observed among these two groups by non-parametric Mann-Whitney test. In addition, cases were followed by telephone to identify any relevant adverse effects after completion of this study. No obvious side effects

TABLE 1: Baseline data obtained from the intention-to-treat patients in the experimental (TZD + EA) and placebo groups (TZD).

Groups	TZD + EA (N = 16)	TZD (N = 15)	P-Value
Age (Years)	48 ± 2	50 ± 2	0.74 (NS)
Body Height (cm)	167 ± 2	165 ± 3	0.27 (NS)
Body Weight (Kg)	73 ± 3	69 ± 3	0.54 (NS)
BMI (Kg/m ²)	26 ± 1	25 ± 1	0.93 (NS)
Glucose (mmol/L)	9.4 ± 0.5	9.1 ± 0.6	0.51 (NS)
HbA1C (%)	8.10 ± 0.45	8.15 ± 0.44	1.00 (NS)
Cr (μmol/L)	71.6 ± 2.7	69.8 ± 5.3	0.20 (NS)
GPT (IU/L)	31 ± 4	28 ± 3	0.71 (NS)
Cholesterol (mmol/L)	5.1 ± 0.3	4.9 ± 0.3	0.44 (NS)
Triglyceride (mmol/L)	1.6 ± 0.3	1.6 ± 0.3	0.81 (NS)
Take OHA (Yes : No)	10 : 6	10 : 5	0.91 (NS)

All values are shown as mean ± SEM except for the use of oral hypoglycemic agents (OHA); TZD + EA = experimental group, who were patients that received 8 mg rosiglitazone and electroacupuncture; TZD = placebo group, who were patients that received 8 mg rosiglitazone only. Independent groups were compared using the nonparametric Mann-Whitney test. NS, means no significant difference, *P* > 0.05.

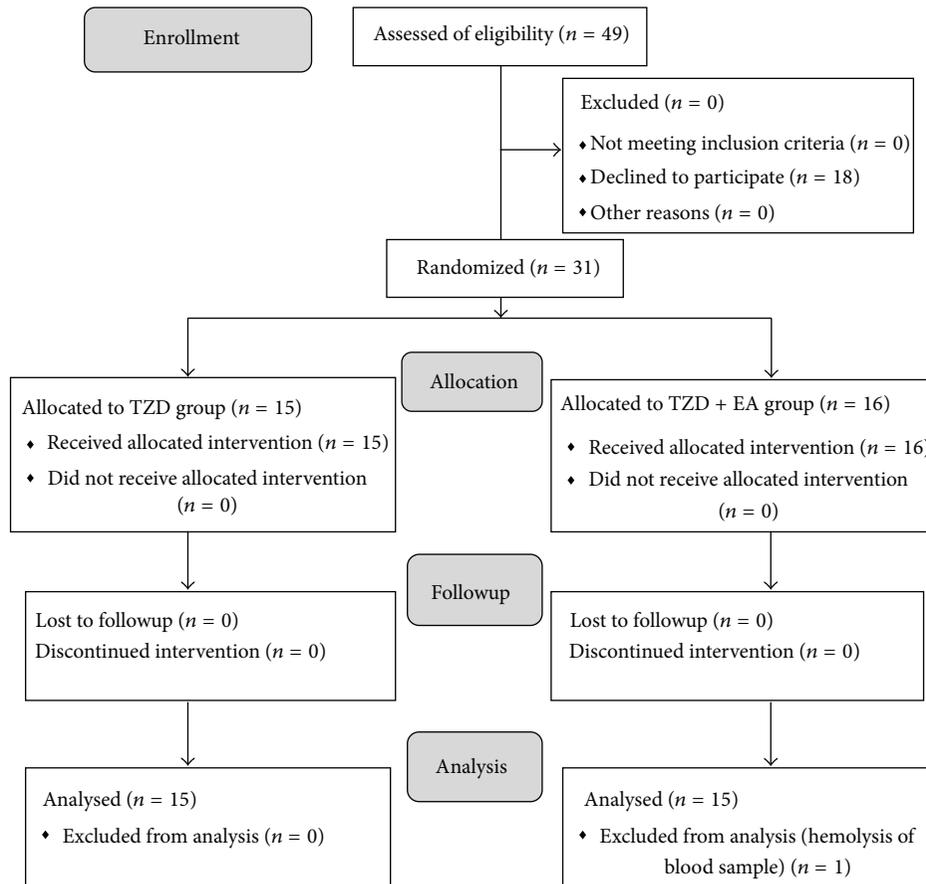


FIGURE 1: The flowchart diagram of progress through the various phases of this two-arm randomized trial according to CONSORT, which stands for consolidated standard for reporting trials.

TABLE 2: Effects of combined and single-dose TZD therapy on plasma glucose.

Group	Before treatment	After treatment	Hypoglycemic activity (%)
TZD + EA ($N = 15$)	8.4 ± 0.4	8.2 ± 0.5	-2 ± 2
TZD ($N = 15$)	7.7 ± 0.6	$7.4 \pm 0.6^*$	-5 ± 2

Plasma glucose concentrations are presented as mean \pm SEM (mmol/L), N = number of patients; the Wilcoxon signed-rank test was used to assess differences in the means of the plasma glucose concentration of each group before and after treatment; $*P < 0.05$. The non-parametric Mann-Whitney test was used to compare the difference in hypoglycemic activity between the two independent groups, $P > 0.05$.

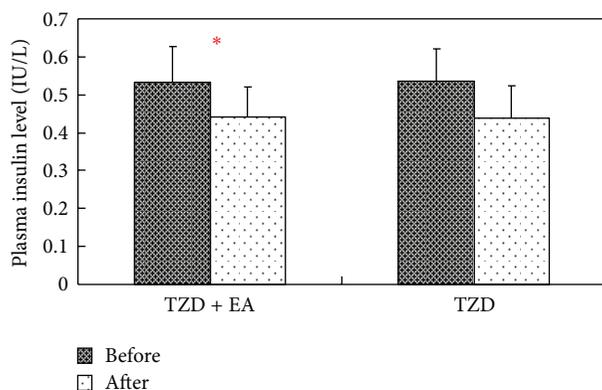


FIGURE 2: Effects of combined therapy or single therapy on plasma insulin concentration among type 2 diabetic patients before and after treatment. TZD + EA = patients receiving 8 mg rosiglitazone stat and electroacupuncture; TZD = patients receiving 8 mg rosiglitazone stat only; Wilcoxon signed-rank test was used to assess differences in the means of the groups, $*P < 0.05$.

were observed during this study except for one case where a localized wheal at an acupoint occurred due to scratching after EA treatment. Antihistamine ointment was prescribed for this and the skin recovered well; the patient was able to tolerate further therapy during the trial after this treatment.

3.2. Effect of Combined Therapy on Plasma Glucose Level. After one treatment of each group, a trend in hypoglycemic response was noted for both groups, but a significant hypoglycemic effect was only noted in the TZD group (7.7 ± 0.6 to 7.4 ± 0.6 mmol/L, $P < 0.05$) by Wilcoxon signed-rank test (Table 2). Furthermore, there was no significant difference in hypoglycemic activity (%) between the combined therapy (EA + TZD, $n = 15$) and monotherapy (TZD, $n = 15$) groups after treatment by Mann-Whitney test (Table 2).

3.3. Effect of Combined Therapy on Plasma Insulin Level and HOMA Index. A significant reduction in plasma insulin level was noted after treatment only in the TZD + EA group ($P < 0.05$) by Wilcoxon signed-rank test (Figure 2). Simultaneously, the HOMA indices before and after treatment were calculated in order to evaluate insulin resistance within the combined therapy and TZD groups. The results showed that the HOMA index after treatment was significantly lower than before treatment for both groups ($P < 0.05$) by Wilcoxon signed-rank test (Figure 3).

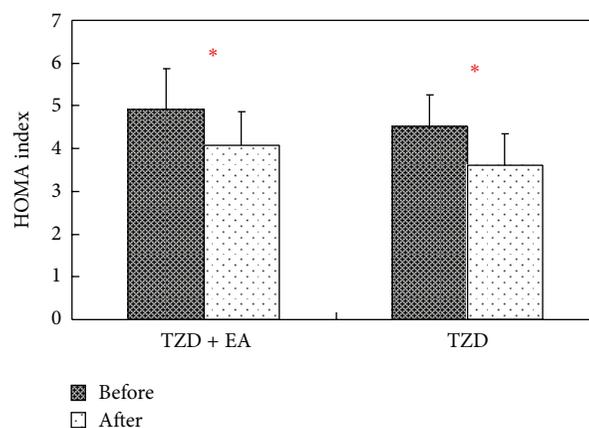


FIGURE 3: Effects of combined therapy or single therapy on insulin sensitivity as evaluated by HOMA index among type 2 diabetic patients before and after treatment. TZD + EA = patients receiving 8 mg rosiglitazone stat and electroacupuncture; TZD = patients receiving 8 mg rosiglitazone stat only; HOMA index = (fasting plasma glucose \times fasting plasma insulin)/22.5; Wilcoxon signed-rank test was used to assess differences in the means of each group, $*P < 0.05$.

3.4. Effect of Combined Therapy on Plasma FFA Level. The plasma FFA levels were significantly decreased for both groups ($P < 0.05$) by Wilcoxon signed-rank test (Figure 4). Nevertheless, no significant difference in plasma FFA lowering activity (%) was observed between the two groups by Mann-Whitney test.

4. Discussion

Although clinical studies have demonstrated that acupuncture is useful because it can lower plasma glucose levels [19], most studies have focused on its effects with respect to diabetic neuropathy [20]. The commonly used acupoints for DM like syndromes include Pishu (BL20), Geshu (BL17), Zhongwan (CV12), Sanyinjiao (SP6), Neiguan (PC6), and Zusanli (ST36) [21]. In this study, we have carried out the first randomized clinical trial that compares the effect of combined therapy (rosiglitazone + EA) on insulin resistance in type 2 diabetic patients with rosiglitazone therapy alone. This design was based on the previous animal studies, which have shown that EA stimulation at the bilateral Zusanli acupoints produced a greater hypoglycemic response than EA stimulation at Zhongwan acupoints, and that 15 Hz EA is able

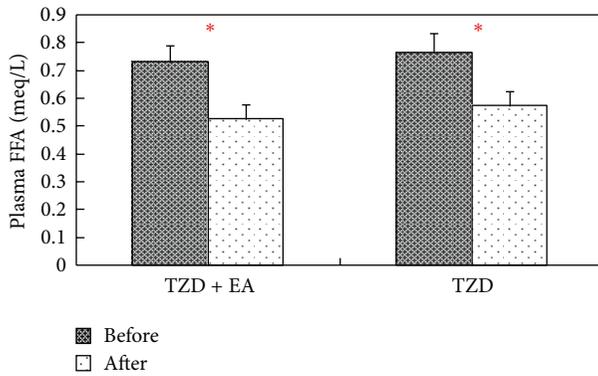


FIGURE 4: Effect of combined therapy or single therapy on plasma FFA concentration among type 2 diabetic patients before and after treatment. TZD + EA = patients receiving 8 mg rosiglitazone stat and electroacupuncture; TZD = patients receiving 8 mg rosiglitazone stat only; Wilcoxon signed-rank test was used to assess differences in the means of each group, * $P < 0.01$.

to produce a better hypoglycemic effect than 2 Hz EA on the same acupoints [17, 22]. On another hand, according to the animal studies, the hypoglycemic activity was obtained after 30-min EA, and rosiglitazone was taken orally 30 min before the patient entered into this experiment to allow absorption and metabolism of drug. This is the reason why the blood was taken 30 min after EA treatment. Since the metabolic abnormalities of type 2 diabetes are complex, more than one medication is often needed for the majority of patients over time to allow maintenance of plasma glucose levels at as near-normal range as possible in order to reduce microvascular complications [23]. At present, the thiazolidinediones are approved for use in combination with metformin, sulfonylureas, glinides, and insulin [24]. Therefore, the combination with EA is possible an alternative.

In the present study, no significant difference in hypoglycemic activity between the patients in the combined therapy group and the patients in the TZD group was identified; nonetheless a trend towards a lowering of plasma glucose levels was found for the patients in both groups. These results are different from our previous animal studies, which showed that the plasma glucose lowering activity of rosiglitazone was increased by EA in both normal and neonatal STZ-induced type 2 diabetic rats [14]. A number of conditions were different between the present study and the earlier animal studies, particularly in which the animal studies were carried out under general anesthesia, while the human subjects were fully conscious during the clinical trial. One possibility is that the plasma glucose lowering effect in patients from the combined treatment group may have been masked by environmental factors, such as pain and stress during EA. Another possibility is that there may have been selection bias because a higher percentage of male patients were excluded because they were already being prescribed thiazolidinedione class drugs.

There were some other limitations that affected this study. We chose an inclusion for subjects with a hemoglobin A1C

below 9%; however, it was not possible to determine whether this was appropriate. The results may have been different if we had recruited patients with HbA1C values between 7% and 10%. Furthermore, although no significant hypoglycemic effect was detected when the combined treatment group was compared with the single drug treatment group, this result may have been related to the relatively short period of treatment. Finally, the sample size in the present study was small and any significant differences between the two groups may have been masked by this. The sample size was also limited by a disappointing recruitment rate, and the protocol was terminated by the researchers based on the available circumstances, and because the prior guarantee period of the protocol from the IRB had ended. Both of these events resulted in the sample size being smaller than that of the prior setting. As a result of these limitations, nonparametric statistical methods were applied to evaluate the differences between the two groups and between before and after treatment.

The normal secretion of insulin is essential to the maintenance of normal glucose tolerance, and an abnormally high secretion of insulin is a consistent finding among type 2 diabetic patients. Usually, insulin resistance and hyperinsulinemia are the main clinical causes of type 2 diabetic among patients, and these indicate the presence of an impaired biological response to either exogenously administered or endogenously secreted insulin [25]. When there is insulin resistance, beta cells compensate for the insulin resistance by increasing insulin secretion even in the presence of normal glucose concentrations. Compensatory hyperinsulinemia due to insulin resistance reflects a combination of increased insulin production and decreased insulin clearance, but most evidence seems to suggest that increased insulin secretion is the predominant factor [26]. In this study, the patients in both the combined treatment group and the single TZD treatment group exhibited a lower HOMA index after treatment. Interestingly, the combined therapy was able to significantly reduce the higher levels of insulin secretion found in these type 2 diabetic patients, which will result in a meaningful relief in terms of pancreas stress that may lead to pancreas failure. That is to say, this combined therapy seems to have the potential benefit of extending the time before pancreatic beta cell failure occurs with type 2 diabetic patients rather than improving insulin resistance. This result is compatible with our previous studies that have shown that insulin sensitivity is enhanced by EA stimulation at the bilateral Zusanli acupoints of normal male Wistar rats and streptozotocin induced diabetic rats when evaluated by the HOMA index [12, 13]. Furthermore, in another study on the effect of EA on insulin sensitivity among steroid background male Wistar rats, the EA group rats were found to also exhibit lower HOMA indices compared to the non-EA group rats [27]. It has been proposed that this effect occurs via the enhancement of endogenous insulin activity by EA. Since abnormal hyperinsulinemia has been found to diminish insulin sensitivity, elevated serum levels of insulin may cause insulin resistance by downregulating insulin receptors and desensitizing postreceptor pathways [28]. Other studies also showed that 24 hours and 72 hours of sustained physiological hyperinsulinemia in a normal individuals are able to

specifically inhibit the ability of insulin to increase nonoxidative glucose disposal and that this is associated with an impaired ability of insulin to stimulate glycogen synthase activity [29]. Our results are similar to previous studies showing that suppression of insulin secretion in insulin resistant individuals results in increased insulin sensitivity [30]. This suggests that a combination therapy consisting of one-dose of rosiglitazone and EA together is able to suppress endogenous insulin secretion, which in turn improves insulin activity as evaluated by the HOMA index. Thus, we conclude that EA stimulation at a specific acupoint may have an important potential role for the treatment of type 2 diabetes because this treatment is able to enhance insulin activity.

Although the combined therapy was able to enhance insulin activity by suppressing endogenous insulin secretion in this study, biochemical evidence for the mechanistic basis of EA activity in this context needs to be explored. Previous studies have shown that 15 Hz EA at the bilateral Zusanli acupoints is able to enhance insulin activity by decreasing plasma FFA concentration and upregulating the expression of insulin signal proteins in steroid background male Wistar rats [27]. Another study has shown that the mechanism by which FFA induces insulin resistance involves the intramyocellular and intrahepatocellular accumulation of triglycerides and diacylglycerol, which then reduce tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and IRS-2 [31]. In addition, studies have shown that EA (15 Hz) at the Zusanli acupoint treatment is able to induce a hypoglycemic response in streptozotocin induced diabetic rats by stimulating the cholinergic nerves, which in turn stimulate the expression of insulin signaling proteins [32]. Additionally, the action of EA has a combined effect that involves both cholinergic nerve stimulation and increased nNOS activity via a lowering of the plasma FFA concentration; the result is enhanced glucose tolerance as described in our recently published reports [13, 27, 33, 34]. Taking the above findings as a whole, we were therefore interested in the role of FFA and how it influences insulin activity when diabetic patients are treated using the combination therapy.

FFA is one of the key factors that influences insulin activity and elevated FFA levels are predictive of the progression from impaired glucose tolerance to diabetes [35]. The majority (>80%) of patients with early T2DM in the USA are overweight [36] and characterized by long-term elevations in their plasma FFA concentration. These are not suppressed as normally they occur following ingestion of a mixed meal [37] or in response to insulin [38]. In addition, insulin is a potent inhibitor of lipolysis and suppresses the release of FFA from the adipocyte by inhibiting the enzyme hormone-sensitive lipase [31]. In our study, a significant plasma FFA lowering effect was noted with both groups of patients. These results are compatible with other studies showing that in early type 2 diabetics, the ability of insulin to inhibit lipolysis and to reduce the plasma FFA concentration is markedly impaired [31]. In addition, it has been found that progressively elevated plasma FFA concentrations seem to induce gluconeogenesis and eventually compensatory hyperinsulinemia; these may then lead to insulin resistance in muscle and liver. This contrasts with a decrease in plasma FFA concentration, which

is able to inhibit glucose-stimulated insulin secretion [32]. Thus, in addition to the documented analgesic effect of EA, we have shown in this study that there is a possible systemic effect of EA whereby there is modulation of FFA levels that can, in turn, influence insulin secretion in patients with type 2 diabetes.

In conclusion, the combined therapy of electroacupuncture and rosiglitazone seems to be able to suppress endogenous insulin secretion, which then improves insulin activity as evaluated by the HOMA index. This seems to occur through a reduction in plasma FFA levels and does not seem to have any major side effects among patients with type 2 DM. Therefore, in the near future, a long-term and large sample size assessment of combined therapy is needed to evaluate the possible benefits that this approach may have in terms of improving the risk profiles of type 2 DM patients.

Abbreviations

EA:	Electroacupuncture
TZD:	Rosiglitazone
T2DM:	Type 2 diabetes mellitus
FFA:	Free fatty acid
HOMA:	Homeostasis model assessment index.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Rong-Tsung Lin and Huei-Chin Pai have been involved in drafting the paper and in the acquisition/analysis of the data. Yu-Chen Lee, Chung-Yuh Tzeng, and Tai-Hao Hsu have assisted for the acquisition of funding and provided the environment for the research. Ying-I Chen has managed the procedures including communication and contact with patient. Chin-Hsien Chang, Pei-Hsiu Hung, Chin-Chun Tsai, and Jaung-Gen Lin have supervised and monitored the procedures carried out during this study. Shih-Liang Chang have made substantial contributions to conception and design, revising the paper, critically analyzing the intellectual content of the paper, and producing the paper for submission to the journal. Dr. Rong-Tsung Lin and Dr. Huei-Chin Pai contributed equally to this study.

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Research Article

Proteomic Analysis of the Effect of Acupuncture on the Suppression of Kainic Acid-Induced Neuronal Destruction in Mouse Hippocampus

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Kainic acid (KA) is a neurotoxin that induces epileptic seizures and excitotoxicity in the hippocampus. Acupuncture is frequently used as an alternative therapy for epilepsy, and it has been known to protect hippocampal neurons against KA toxicity. Using proteomic analysis, we investigated protein expression changes in the hippocampus following acupuncture stimulation at HT8. Eight-week-old male C57BL/6 mice (20–25 g) received acupuncture treatment at HT8 acupoint bilaterally once a day for 3 days and were then administered KA (30 mg/kg) intraperitoneally. Twenty-four hours after KA injection, neuronal survival and astrocyte activation in the hippocampus were measured, and protein expression in the hippocampus was identified by 2-dimensional electrophoresis. Acupuncture stimulation at HT8 suppressed KA-induced neuronal death and astrocyte activation in the hippocampus. We identified the changes in the expression of 11 proteins by KA or acupuncture stimulation at HT8 and found that acupuncture stimulation at HT8 normalized the expression of dihydropyrimidinase-related protein 2 and upregulated the expression of transcriptional activator protein pur-alpha, serine/threonine-protein phosphatase 5, and T-complex protein 1 subunit alpha, which are related to the survival of neurons. These results suggest that acupuncture stimulation at HT8 changes protein expression profiles in the hippocampus in favor of neuronal survival in KA-treated mice.

1. Introduction

Kainic acid (KA) is a potent agonist to the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor. The administration of KA commonly induces epileptic seizures and excitotoxic cell death in the hippocampus, similar to that observed in temporal lobe epilepsy patients [1, 2]. Therefore it has been widely used for seizure animal studies as a trigger to induce temporal lobe epilepsy in animal models [3].

Acupuncture has been used to alleviate various kinds of neurological disorders, including epilepsy and seizure [4],

but the mechanism of acupuncture for epilepsy treatment remains elusive. Among acupoints, HT8 has been used as a therapeutic acupoint for epilepsy. In KA-induced epileptic seizure animal studies, acupuncture stimulation at HT8 reduces the severity of epileptic seizures, regulates GABA-mediated signaling by increasing glutamate decarboxylase [5], and suppresses KA-induced hippocampal cell death by suppressing microglia activation and cytokines [6]. These results indicate that acupuncture stimulation at HT8 protects hippocampal cells from KA toxicity, but it is still unknown which proteins are related to the action of the acupuncture stimulation.

Two-dimensional electrophoresis (2-DE) is a method that separates proteins based on electrical charge and molecular weight from complex protein mixtures extracted from biological samples such as cells and tissues, and this method can detect changes in protein expression levels and isoforms. Using this technique, we investigated the acupuncture-associated proteomic profile changes in the hippocampus in KA-induced mouse model of epilepsy.

2. Methods

2.1. Animals and Grouping. Male C57BL/6 mice (8 weeks old, weighing 20–23 g; Orient Bio, Inc., Republic of Korea) were housed at room temperature ($22 \pm 3^\circ\text{C}$) under a standard 12 h light/dark cycle (lights on at 07:00 h) with unlimited access to food and water. The animals were handled in accordance with the current guidelines established in the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, 1985), and all efforts were made to minimize animal suffering and reduce the number of animals used. The mice were randomly assigned to four groups: the saline group ($n = 9$) was injected with normal saline and did not receive acupuncture stimulation; the KA group ($n = 9$) was injected with KA (Sigma, St. Louis, MO) and did not receive acupuncture stimulation; the KA + HT8 group ($n = 9$) was injected with KA and received acupuncture stimulation bilaterally at acupoint HT8; and the KA + ST36 group ($n = 6$) was injected with KA and received acupuncture stimulation bilaterally at acupoint ST36 as the control group for acupuncture stimulation.

2.2. Acupuncture Stimulation. Between 10:00 and 10:30 a.m., the mice in the KA + HT8 group were lightly immobilized by an assistant who grasped the loose skin behind the ears with thumb and forefinger, and acupuncture needles (0.18×8 mm; Dongbang Acupuncture, Inc., Republic of Korea) were inserted at the bilateral HT8 acupoints. HT8 is located on the palmar surface of the forelimbs, between the fourth and fifth metacarpal bones [7]. The needle was inserted to a depth of 1 mm and was turned at a rate of two spins and counter-spins per second for 30 s, with removal immediately afterward. The entire stimulation lasted 60 s. For the KA + ST36 group, the same procedure was applied to acupoint ST36. ST36 is located at the proximal one-fifth point on the line from the depression lateral to the patella ligament to the anterior side of the ankle crease [7]. The positions of the acupoints in mice correspond anatomically to their locations in humans. The stimulation was repeated three times: once a day for 3 days. The animals in the saline and KA groups were immobilized in a similar fashion for the 60 s.

2.3. Kainic Acid Injection. Thirty minutes after the last acupuncture stimulation, KA (30 mg/kg of free base; Sigma) was injected intraperitoneally with a BD Ultra-fine II insulin syringe (Becton, Dickson, and Company, Franklin Lakes, NJ, USA) into the mice in the KA, KA + HT8, and KA + ST36 groups. The mice in the saline group were injected with normal saline instead of KA.

2.4. Immunohistochemistry. Twenty-four hours after the KA injection, the mice ($n = 6$ from each group) were perfused with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). The brains were removed from the craniums, postfixed for a day, washed in 0.1 M PB, and immersed in 30% sucrose solution for storage at 4°C prior to sectioning. Frozen sections ($40 \mu\text{m}$) were cut using a Leica CM3050S cryostat (Leica microsystems, Wetzlar, Germany).

To identify degenerating neurons in the hippocampus, cresyl violet staining was performed. The sections were mounted on silane-coated slides, air-dried, and incubated for 1 min in a 1% solution of cresyl violet. Next, the sections were washed thoroughly in cold tap water, rinsed briefly in 1% acetic acid solution for 10 s, dehydrated by immersion in ascending grades of alcohol, cleared with xylene, and coverslipped using mounting medium.

To detect the activation of astrocytes in the hippocampus, the sections were incubated with glial-GFAP (Cell Signaling Technology, Beverly, MA, USA) primary antibodies diluted 1:1000 for 24 h at 4°C . After washing in 0.05 M phosphate-buffered saline (PBS), the sections were incubated with biotinylated anti-rat IgG (Vector Laboratories, Inc., Burlingame, CA) for 1 h at room temperature and then incubated with ABC reagent (Vector Laboratories) for 1 h at room temperature. The sections were then washed in PBS, incubated with 0.02% diaminobenzidine and 0.003% hydrogen peroxide in 0.1 M Tris-HCl-buffered saline (pH 7.5) for 5 min, rinsed with PBS, mounted on gelatin-coated slides, air-dried, dehydrated, and coverslipped.

The histological pictures were taken using a bright-field, phase contrast Axio Scope A1 microscope (Carl Zeiss, Oberkochen, Germany), and Axiocam ICc3 camera (Carl Zeiss). The hippocampal cell death and the astrocyte activation were quantitated in terms of the optical density in the CA3 of the hippocampus using Image-pro plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

2.5. Two-Dimensional Gel Electrophoresis. Twenty-four hours after the KA injection, the mice in the saline, KA, and KA + HT8 groups ($n = 3$ from each group) were killed by CO_2 gas; the brain was immediately and rapidly removed from the cranium, and the hippocampus was extracted, weighed and stored at -80°C until use.

The hippocampi were homogenized directly by motor-driven homogenizer (PowerGen125, Fisher Scientific, Pittsburgh, PA, USA) in sample lysis solution composed of 7 M urea, 2 M Thiourea containing 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) dithiothreitol (DTT) and 2% (v/v) pharmalyte and 1 mM benzamidine. Proteins were extracted for one hour at room temperature with vortexing. After centrifugation at $15,000 \times g$ for one hour at 15°C , insoluble material was discarded and the soluble fraction was used for two-dimensional gel electrophoresis. Protein concentration was assayed by the Bradford method [8].

IPG dry strips (4–10 NL IPG, 24 cm, Genomine, Republic of Korea) were equilibrated for 12–16 hours with 7 M urea, 2 M thiourea containing 2% CHAPS, and 1% DTT, 1%

pharmalyte, and respectively, loaded with 200 μg of sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor II electrophoresis unit and EPS 3500 XL power supply (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. For IEF, the voltage was linearly increased from 150 to 3,500 V during 3 hours for sample entry followed by constant 3,500 V, with focusing complete after 96 kVh. Prior to the second dimension, strips were incubated for 10 minutes in equilibration buffer (50 mM Tris-Cl, pH6.8 containing 6 M urea, 2% SDS, and 30% glycerol), first with 1% DTT and second with 2.5% iodoacetamide. Equilibrated strips were inserted onto SDS-PAGE gels (20 \times 24 cm, 10–16%). SDS-PAGE was performed using the Hoefer DALT 2D system (Amersham Biosciences) according to the manufacturer's instructions. 2D gels were run at 20°C for 1,700 Vh, and the 2D gels were then stained with Coomassie G250 as described by Anderson et al. [9].

Quantitative analysis of digitized images was carried out using the PDQuest (version 7.0, Bio-Rad, Hercules, CA, USA) software according to the protocols provided by the manufacturer. The quantity of each spot was normalized to total valid spot intensity. Protein spots that deviated over 1.4-fold in expression level compared with control or normal sample were selected for the significant expression variation.

2.6. Peptide Mass Fingerprinting. For protein identification by peptide mass fingerprinting (PMF), protein spots were excised, digested with trypsin (Promega, Madison, WI, USA), mixed with α -cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid, and subjected to MALDI-TOF analysis (Microflex LRF 20, Bruker Daltonics, Billerica, MA, USA) as described by Fernandez et al. [10]. Spectra were collected from 300 shots per spectrum over m/z range 600–3000 and calibrated by two-point internal calibration using trypsin autodigestion peaks (m/z 842.5099, 2211.1046). The peak list was generated using Flex Analysis 3.0. The threshold used for peak picking was as follows: 500 for minimum resolution of monoisotopic mass, 5 for S/N. The search program MASCOT, developed by Matrix science (<http://www.matrixscience.com/>), was used for protein identification by PMF. The following parameters were used for the database search: trypsin as the cleaving enzyme, a maximum of one missed cleavage, iodoacetamide (Cys) as a complete modification, oxidation (Met) as a partial modification, monoisotopic masses, and a mass tolerance of ± 0.1 Da. The PMF acceptance criterion was probability scoring.

2.7. Western Blotting. The anti-PURA primary antibody was purchased from Cell Signaling Technology (Beverly, MA, USA) and the anti-PP5 antibody was from Abcam (Cambridge, UK). For the Western blot, samples (50 mg protein) were loaded on 10% SDS-PAGE. After separation, the proteins were transferred to NC membrane. The membrane was shaken for 1 h at RT in TBS that contained 0.1% Tween-20, 5% skim milk, and 0.2% BSA. The membrane was incubated for 1 h at RT with primary antibodies (anti-PURA or anti-PP5, 1: 1000) in TBS that contained 0.1% Tween-20. The primary antibodies were detected with an HRP-conjugated

secondary antibody (anti-rabbit, 1: 1000) and then visualized with ECL (Pierce, Rockford, IL, USA). These blots were then reprobated with an anti- β -actin antibody (1: 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The band intensities of the detected proteins were measured by densitometry.

2.8. Statistical Analysis. All data are expressed as the mean \pm S.E.M. and analyzed by one-way ANOVA with the Neuman-Keuls posthoc test. All statistical testing was performed using Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of Acupuncture on KA-Induced Hippocampal Cell Death. We assessed the KA-induced cell death in the CA3 using cresyl violet staining. As a result, the optical densities of the CA3 region in KA (84.40 \pm 5.34%) and KA + ST36 (89.02 \pm 5.18%) groups were significantly reduced ($P < 0.05$ versus saline group), whereas the density in the KA + HT8 group was significantly elevated (105.76 \pm 3.25%) compared to that in the KA group ($P < 0.05$, Figure 1).

3.2. Effect of Acupuncture on GFAP Expression in the Hippocampus. The optical densities in the KA (140.45 \pm 8.70%) and KA + ST36 (136.45 \pm 9.80%) groups were significantly higher than those in the saline group (100.00 \pm 14.54%, $P < 0.05$). However, the density was significantly lower in the KA + HT8 group (110.79 \pm 5.93%) compared with that in the KA and KA + ST36 groups ($P < 0.05$ versus each group, Figure 2).

3.3. Proteins Differentially Expressed in the Hippocampus. To obtain the protein profiles of each group, 2-DE was performed with the protein extracts from the hippocampus. About 600 polypeptide spots could be revealed in the pH 3–10 interval with Coomassie G250 staining (Figures 3(a) and 3(b)). After matching the replicated maps, differential changes in intensities among the mice in the saline, KA and KA + HT8 groups were limited to 11 proteins: valosin containing protein (VCP), ubiquitin-like modifier-activating enzyme 1 isoform 1 (ULMAE-1), ATP synthase subunit d (ATPS), heat shock 70 kDa protein 4L (HSP70), heat shock protein 4 like, isoform CRA.c (HSP4L), dihydropyrimidinase-related protein 2 (CRMP-2), transcriptional activator protein pur-alpha (PURA), pyruvate dehydrogenase protein X component (PDX), serine/threonine-protein phosphatase 5 (PP5), T-complex protein 1 subunit alpha (TCP-1 α), and uncharacterized protein LOC433182 (LOC433182). Compared to the expression of VCP in the saline group, that in the KA group was significantly increased ($P < 0.05$). That in the KA + HT8 group was also increased, but not significantly. The expressions of ULMAE-1, ATPS, HSP70, and HSP4L in the KA and KA + HT8 groups were significantly decreased compared to that in the saline group. CRMP-2 was actually decreased by KA injection ($P < 0.01$), but acupuncture stimulation at HT8 significantly restored it ($P < 0.05$

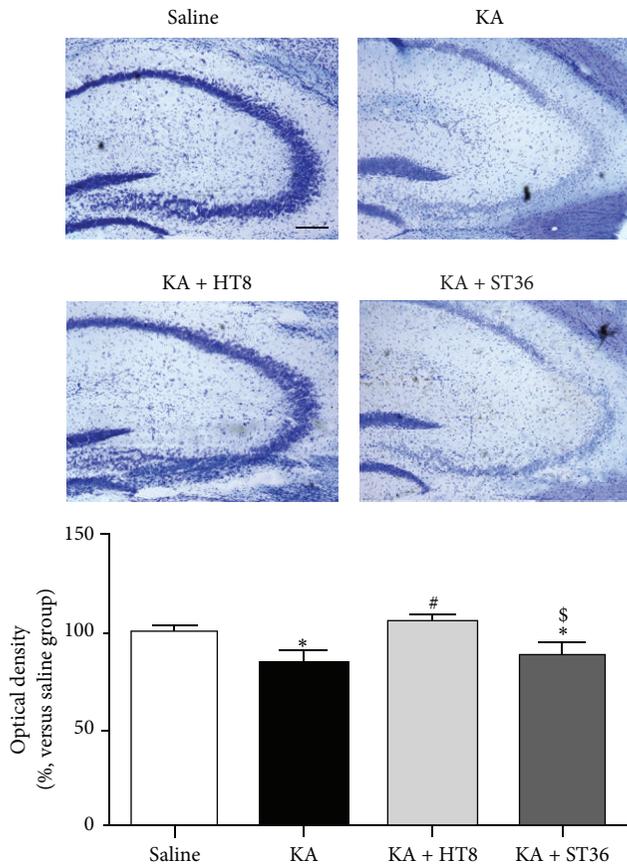


FIGURE 1: Neuroprotective effect of acupuncture stimulation at acupoint HT8 in hippocampal CA3 region after kainic acid (KA) administration. KA destroys neurons in the hippocampus, whereas acupuncture stimulation at HT8 prevents this destruction. Saline: the saline-injected control group; KA, the KA-injected group; KA + HT8: the acupuncture at HT8 with KA injection group; and KA + ST36, the acupuncture at ST36 with KA injection group. Scale bar represents 200 μm . Data are expressed as the mean \pm SEM. * $P < 0.05$ versus the saline group. # $P < 0.05$ versus the KA group. \$ $P < 0.05$ versus the KA + HT8 group.

versus KA group). The levels of PURA, PDX, PP5, TCP- α , and LOC433182 were unchanged by KA injection, but acupuncture stimulation at HT8 significantly increased them (Table 1, Figures 3(c) and 3(d)).

3.4. Confirmation of Altered Proteins by Western Blot Analysis. To verify the reliability of the proteomics analysis, PURA and PP5 were selected as representative proteins and subjected to western blotting. The results of triplicate western blots for the proteins using protein extracts from the hippocampus of mice in the saline, KA and KA + HT8 groups were consistent with those of the 2-DE (Figure 4).

4. Discussion

Our results demonstrate that acupuncture stimulation at HT8 protects against KA-induced neuronal death and astrocyte

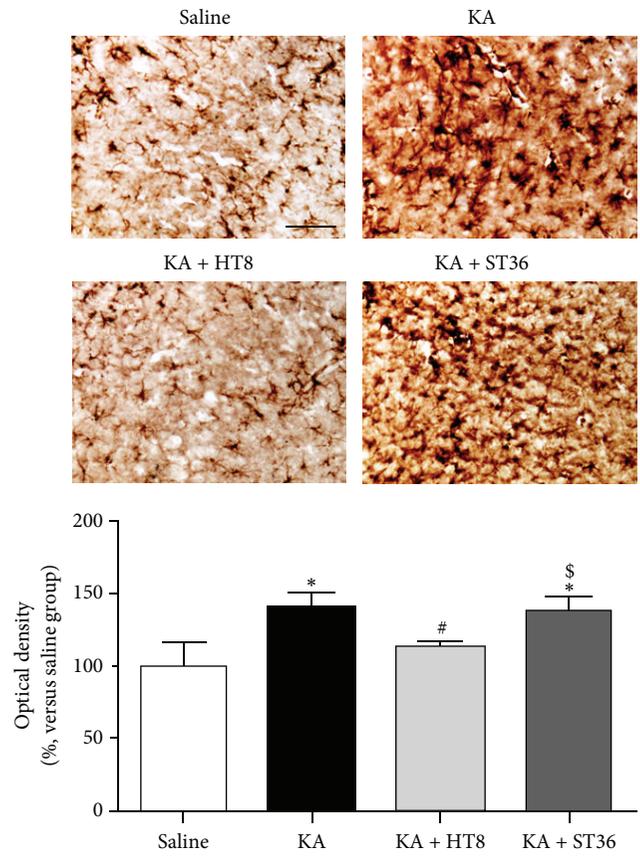


FIGURE 2: The activation of astrocytes in the hippocampus after kainic acid (KA) administration. KA upregulated GFAP activation in the hippocampus, whereas acupuncture stimulation at HT8 prevented this activation. Saline: the saline-injected control group; KA: the KA-injected group; KA + HT8, the acupuncture at HT8 with KA injection group; and KA + ST36: the acupuncture at ST36 with KA injection group. Scale bar represents 50 μm . Data are expressed as the mean \pm SEM. * $P < 0.05$ versus the saline group. # $P < 0.05$ versus the KA group. \$ $P < 0.05$ versus the KA + HT8 group.

activation in the hippocampus. The protein expression profiles in the hippocampus of the saline, KA, KA + HT8 groups were compared using 2-DE, MALDI-TOF analysis, and peptide fingerprinting MS, and eleven proteins were differentially expressed.

During excitotoxin-induced neurodegeneration, microglia and astrocytes are activated, which contributes to hippocampal neuronal death through induction of inflammatory mediators [11]. KA is an excitotoxin, and the administration of KA causes epileptic seizures and activates microglia and astrocytes in the hippocampus. Therefore the deactivation of microglia and astrocytes plays an important role in reducing KA-induced neuronal cell death [12]. In this study, we confirmed that KA induces neuronal death and astrocyte activation in the hippocampus and that acupuncture stimulation at HT8 can suppress them. This result indicates that acupuncture stimulation at HT8 suppresses KA-induced neuronal cell death in the CA3 of the hippocampus, and the

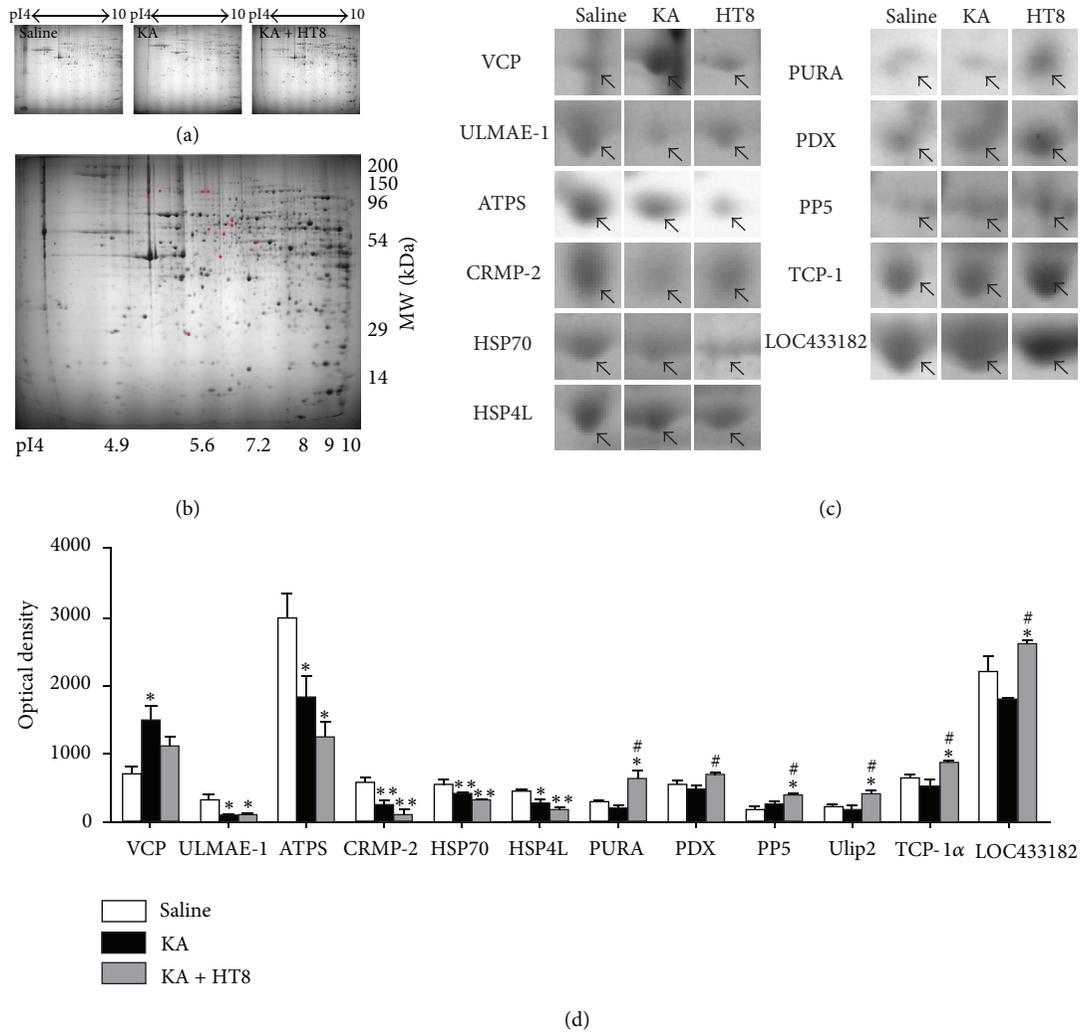


FIGURE 3: Protein profiles with differential expression. (a) Hippocampal tissue protein profiles obtained over different pI ranges. (b) Representation of identified differential protein spots (red) in 2-DE gel templates among the saline, KA and KA + HT8 groups. (c) Differential expression profiles of 11 proteins. Arrowheads on cropped images of 2-DE gels represent protein spots that showed different changes among the saline, KA and KA + HT8 groups. (d) Quantitative analyses of downregulated or upregulated proteins by kainic acid (KA) and acupuncture stimulation at HT8. The spot intensities were derived from Coomassie-stained 2-D gels. VCP: valosin containing protein; ULMAE-1, ubiquitin-like modifier-activating enzyme 1 isoform 1; ATPS: ATP synthase subunit d; HSP70: heat shock 70 kDa protein 4L; HSP4L: heat shock protein 4 like isoform CRA.c; CRMP-2: dihydropyrimidinase-related protein 2; PURA: transcriptional activator protein Pur-alpha; PDX: pyruvate dehydrogenase protein X component; PP5: serine/threonine-protein phosphatase 5; TCP-1α: T-complex protein 1 subunit alpha; LOC433182: uncharacterized protein LOC433182. Data are expressed as the mean ± SEM. **P* < 0.05 and ***P* < 0.01 versus the saline group. #*P* < 0.05 versus the KA group.

deactivation of astrocytes can account for the neuroprotective effect of the acupuncture stimulation in this study.

VCP is an ubiquitin-dependent ATPase involved in protein lysis through the ubiquitin proteasome system associated with vesicle transport and fusion [13], 26S proteasome function, and peroxisome assembly [14]. It plays an important role in the ubiquitin-mediated protein degradation pathways in neurodegenerative disorders [15], and KA administration increases the level of VCP, which causes endoplasmic reticulum (ER) stress [16]. In this study, the administration of KA significantly increased the VCP expression; this increase was suppressed by acupuncture stimulation at HT8. Thus,

acupuncture stimulation at HT8 may reduce KA-induced ER stress by suppressing VCP expression.

CRMP-2, which is in the collapsin response mediator protein family, induces axon guidance and growth and is the most abundant CRMP family member within the brain [17]. Changes in the level of CRMP-2 protein have been related to neurodegenerative disorders [18], and upregulation of CRMP-2 contributes to neuroprotective actions in reactive oxygen species (ROS) stress [19]. KA increases ROS production [20], therefore the upregulation of CRMP-2 may contribute to the suppression of neuronal death from KA toxicity. We found that acupuncture stimulation at HT8 restored

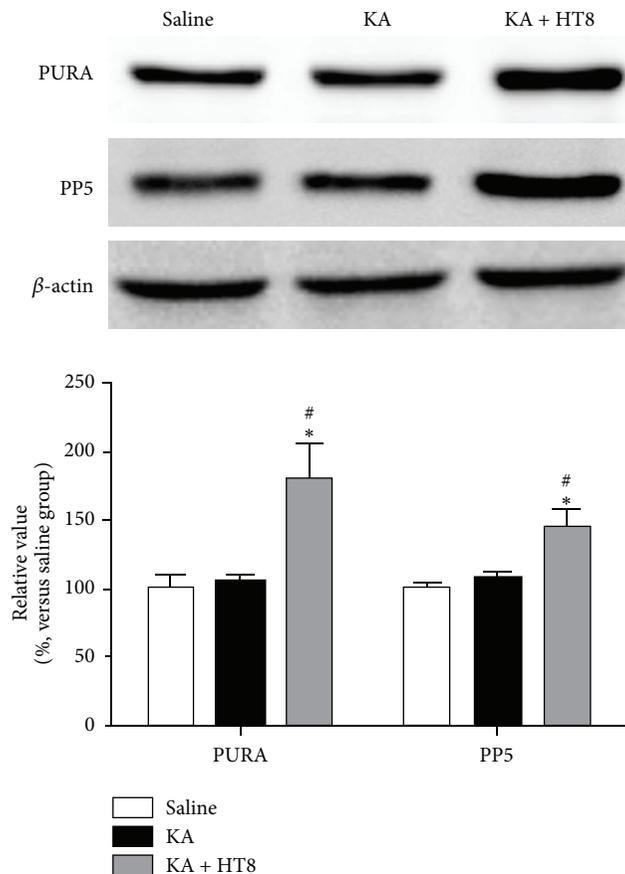


FIGURE 4: Validation of proteomic results using western blot analyses of PURA and PP5 proteins in the hippocampus of mice. The same trends detected in the 2-DE analyses were confirmed for these two proteins. PURA: transcriptional activator protein Pur-alpha. PP5: serine/threonine-protein phosphatase 5. Data are expressed as the mean \pm SEM. * $P < 0.05$ versus the saline group. # $P < 0.05$ versus the KA group.

the CRMP-2 level that was decreased by KA administration, which suggests that the restoration of the CRMP-2 level by the acupuncture stimulation contributed to the neuroprotective effect against KA toxicity.

PURA binds sequence-specific single-stranded deoxyribonucleic acid or ribonucleic acid and affects initiation of DNA replication and gene transcription, especially in the hippocampal CA3 region [21]. The decrease of PURA reduces postsynaptic density protein 95 [22], which contributes to neuronal cell loss after status epilepticus [23] and KA administration [24]. PP5, a serine/threonine phosphatase, is widely expressed in brain including hippocampus [25] and suppresses apoptosis by regulating c-Jun N-terminal kinase phosphorylation [26] and apoptosis signal-regulating kinase 1 activity [27]. T-complex protein 1 (TCP-1), a chaperonin family member, that folds protein properly [28], supports the maintenance of the native forms of cytoskeletal proteins [29], and the overexpression of all TCP-1 subunits suppressed Neuro2a cell death induced by the cytotoxicity of polyglutamine-expansion proteins [30]. Taken together, the increases of PURA, PP5, and TCP-1 have the potential to protect hippocampal neurons against KA toxicity. In this study, acupuncture stimulation at HT8 upregulated the

levels of PURA, PP5, and TCP-1 α , which may contribute to the suppression of hippocampal neuronal death induced by KA.

In conclusion, this study demonstrates that acupuncture stimulation at HT8 protects against KA-induced neuronal damage in the hippocampus, and that the acupuncture-mediated neuroprotection may be due, in part, to the normalization of the altered expression of VCP and CRMP-2, which are implicated in cell death mechanisms following KA administration. Furthermore, we propose that the PURA, PP5 and TCP-1 α upregulation by acupuncture stimulation at HT8 may be a significant event in the neuroprotective effect of acupuncture.

Disclaimer

The funders had no role in the study design, data collection and analysis, and the decision to publish the paper.

Conflict of Interests

The authors declare that no competing financial interests or conflict of interests exist.

TABLE 1: Differentially expressed protein profiles of the protein spots after acupuncture stimulation at HT8.

Name	Theoretical		Gel		MASCOT score	Peptides matched	Sequence coverage (%)	KA/Saline	Average ratio ^a	
	Mr (kD)	pI	Mr (kD)	pI					KA + HT8/KA	KA + HT8/Saline
Valosin containing protein, isoform CRA_a	90.868	5.14	89.7	5.13	276	34	47	2.103*	-1.324	1.588
Ubiquitin-like modifier-activating enzyme 1 isoform 1	118.931	5.43	103.11	5.24	160	24	30	-3.576*	1.259	-2.841*
ATP synthase subunit d, mitochondrial	18.795	5.52	28.02	5.50	133	15	67	-1.631*	-1.471	-2.400*
Heat shock 70 kDa protein 4L	95.178	5.54	101.62	5.69	257	29	39	-1.321**	-1.245	-1.645**
Heat shock protein 4 like, isoform CRA_c	101.159	5.73	101.34	5.84	280	30	35	-1.718*	-1.480	-2.543**
Dihydropyrimidinase-related protein 2	62.638	5.95	56.96	5.87	210	26	53	-1.897**	1.417*	-1.339
Transcriptional activator protein Pur-alpha	34.976	6.07	46.54	6.20	110	11	38	-1.329	2.949*	2.220*
Pyruvate dehydrogenase protein X component, mitochondria	54.250	7.63	53.67	6.29	86	12	23	-1.189	1.523*	1.281
Serine/threonine-protein phosphatase 5	57.437	5.83	60.31	6.50	79	14	27	-1.293	1.688*	2.182*
T-complex protein 1 subunit alpha	60.867	5.82	64.14	6.53	177	21	44	-1.232	1.683*	1.366*
Uncharacterized protein LOC433182	47.453	6.37	50.76	7.20	221	24	55	-1.120	1.458*	1.302*

Saline: saline-injected group; KA: KA-injected group; KA + HT8: KA-injected group with acupuncture stimulation at HT8 acupoint. ^aAverage ratio calculated considering 3 replicate gels. A minus means that the protein expression was decreased. * $P < 0.05$ and ** $P < 0.01$ between the groups.

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Research Article

Research on Effects of the Thermal Stimulation by Moxibustion at Different Temperatures on Cardiac Function in Rats and on Mast Cells in the Local Site of Moxibustion

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Objective. To observe effects of the thermal stimulation by moxibustion at different temperatures on cardiac function in bradycardia rat model and on mast cells in the local site of moxibustion at the Ximen Acupoint and to compare the differences of the effects of moxibustion at different temperatures. **Method.** Establish the bradycardia rat model with propranolol and observe effects of the thermal stimulation by moxibustion at different temperatures (38°C and 46°C). **Results.** The thermal stimulation by moxibustion at 2 temperatures may increase HR, MAP, LVSP, and +dp/dtmax and reduce t-dp/dtmax in bradycardia rats; the 46°C moxibustion group shows greater regulating effects on cardiac function in rats than that in the 38°C moxibustion group ($P < 0.05$). The thermal stimulation by moxibustion at 2 temperatures may promote degranulation of mast cells in the local site of moxibustion at the Ximen Acupoint; the degranulation rate in the 46°C moxibustion group is higher than that in the 38°C moxibustion group ($P < 0.05$). **Conclusion.** There is a certain association between the effect on the target organ and the effect in the local site of moxibustion. The moxibustion effect possibly resulted from local mast cells degranulation and different thermoreceptors activated by the thermal stimulation at different temperatures.

1. Introduction

As an important method in the traditional Chinese medicine, moxibustion is featured mainly with the “warm” advantage, by which it carries out stimulation at the Acupoint or the lesions and works by “stimulation from the outside and regulation from the inside”. The skin is the direct action site of moxibustion stimulation; selection of appropriate warm thermal stimulation to local skin, characteristics of responses to stimulation, and activation of relevant regulating mechanisms are based on the biological characteristics of skin, that is to say, to explore the effect law and action mechanism of moxibustion, attention has to be paid to the action characteristics of moxibustion and the characteristics of the action object of moxibustion. Modern neurobiological studies have demonstrated that [1] the body and skin tissues are distributed with protein receptors (transient receptor potential (TRP)) sensing different temperature ranges, which have their respective thresholds in sensing temperatures and

may be activated by different warm thermal stimulation. The characteristics in the change of local temperatures, caused by moxibustion therapy, are greatly consistent with categories and distribution characteristics of relevant temperature-sensing subtypes of TRP, such as the TRPV1 in the TRP family which may be activated by thermal stimulation ($>43^{\circ}\text{C}$) [2]. Recent studies have found that TRPV1 expression and activation of skin cutin cells may induce inflammatory reaction [3]. Activated TRPV1 can make mast cell degranulation release proinflammatory and itch causing media to participate in the inflammatory reaction [4]. The inflammatory reaction is associated with pathological physiological function in the cardiac arrhythmias [5]. Moreover, TRPV1 has an important regulating effect on the cardiovascular system [6]. Moxibustion can effectively regulate blood, blood flow and microcirculation condition, and diastolic function [7, 8]. Studies have shown effects of acu-moxibustion associated with mast cells in acupoints [9]. From the previously mentioned, attention needs to be paid to what effects may be produced by the

thermal stimulation at two temperatures below and above 43°C on cardiac function in rates, as well as on skin in the local site of moxibustion, whether there is any difference or association of effects, and whether they may provide any basis for optimizing the clinical operating program of moxibustion.

2. Materials and Methods

2.1. Materials

2.1.1. Animals and Grouping. Healthy male SD rats of clean grade, with body weight of 280–380 g, Certificate no. SCXK (Shanghai) 2007-0005, were purchased from Shanghai Slac Laboratory Animal Co., Ltd. and bred in batches. The experiment was carried out in 1 week after the rats were accommodated. The rats were divided into the following groups by the random number table: Model Control Group, Moxibustion Group 1, and Moxibustion Group 2. The number of rats in each group would support to obtain 8 complete data successfully. During the experiment, the animal treatment measures were in strict accordance with the regulations in the *Instructive Opinions on Humanitarian Treatment of Laboratory Animals*, issued in 2006 by the Ministry of Science and Technology of the People's Republic of China and the provisions for care and use of laboratory animals by Nanjing University of Traditional Chinese Medicine.

2.1.2. Main Laboratory Equipment. Moxa strips for animals (in the diameter of 7 mm, Nanyang Chinese Medicine Moxa Company), thermostatic operating table for animals (ST-1 model, Chengdu Instrument Factory), multichannel physiological signal acquisition system (RM 6240 model, Chengdu Instrument Factory), temperature measuring device (HC-04 model, Hangzhou Hongchang Technology Company), microsyringe pump (ALC-IP800, Shanghai Alcott Biotech Co., Ltd.), full automatic tissue processor (TP1020, German LEICA Company), paraffin slicing machine (RM2145, German LEICA Company), optical microscope (BX60, Japanese OLYMPUS Company), and video camera (DP71 Image Acquisition System, Japanese OLYMPUS Company) were used.

2.2. Methods

2.2.1. Electrophysiological Experimental Method. Fast a male SD rat overnight for 12 hours, with free access to water, weigh the rat, carry out anesthesia of the rat with 20% urethane (ethyl carbamate) in the dose of 1 g/kg body weight, fix the rat in the thermostatic operating table for animals, and connect with the electrocardiograph and the temperature transducer (via the anus of the rat); once the electrocardiogram and body temperature of the rat are stable (37°C), carry out intubation in the left ventricle, as well as in the femoral artery and femoral vein of the right lower limb, to measure left ventricular pressure and arterial pressure, and administer the drug intravenously. Model control group 10 minutes after the operation, if the measurement indexes are stable, start

recording data; 5 minutes later, rapid inject 0.4% inderal (4 mg/kg) via the femoral vein; then slowly inject intravenously to maintain inderal at the dose of 0.25 mg/kg/min with the microsyringe pump for 60 min, to establish the bradycardia model; no any other interventions are carried out. Moxibustion group 1: establish the model as described in the model control group. At 20 min during administration of inderal at the maintenance dose, ignite the customized slender moxa strip (20 cm long and in the diameter of 7 mm), carry out moxibustion directly to stimulate at the Ximen(PC4) Acupoint in the left upper limb of the rat for 10 min, place the probe of the temperature controller at the surface of the skin at the acupoint for moxibustion to monitor real-time temperature, and control the temperatures in the local skin for moxibustion at 38°C ± 1°C (Figure 1(a)); the flaming moxa strip and skin are 35 ± 5 mm apart; after moxibustion, continue observation for another 30 min. Moxibustion group 2: establish the model as described in the model control group. At 20 min during administration of inderal in the maintenance dose, carry out moxibustion for thermal stimulation of 10 min at 46°C, carry out the intervention similar to that as described in moxibustion group 1; during thermal stimulation by moxibustion, control the temperature in the local skin for moxibustion at 46°C ± 1°C Figure 1(b), and the flaming moxa strip and skin are 12 ± 2 mm apart; after moxibustion, continue observation for another 30 min. During moxibustion, gently tap any moxa ashes at the tip of the moxa strip to the ashtray, based on the combustion state of the moxa strip, to always maintain cleanliness of the operating interface. The Ximen Acupoint in the left limb is selected; the positioning method of the acupoint is the anthropomorphic comparative method, that is, Ximen: In the inner side at the 1/2 folding point of the forearm, and in the middle of the gap between the radius and the ulna. The reason for the choice of the temperature is that, based on our observations, less than 38°C thermal stimulus, the human body for the warm feeling is not obvious, and above 46°C thermal stimulus, the human body for the hot feeling is very obvious, difficult to continue to endure. During the whole course, using RM6240B multichannel physiology recorder (parameters: acquisition frequency 1 kHz, scan speed 40 ms/div, and filter frequency 30 Hz) to simultaneously monitor and record many indexes for cardiac function and hemodynamics, including heart rate (/min), mean femoral artery pressure (mmHg), left ventricular systolic pressure (mmHg), maximum rate of left ventricular pressure rise (mmHg/s), and time (ms) from the beginning of left ventricular systole to the maximum rate of left ventricular pressure rate, and so forth.

2.2.2. Morphological Experimental Method

(1) Obtain Materials. After completion of the monitoring and recording of the electrophysiology experiment, sacrifice the rats and obtain materials. Rapidly extract skin tissues in the local site at the Ximen Acupoint in the rats of each group with the ophthalmic forceps and the surgical scissors, and place into 10% formaldehyde solution to store and fix for 1 week.

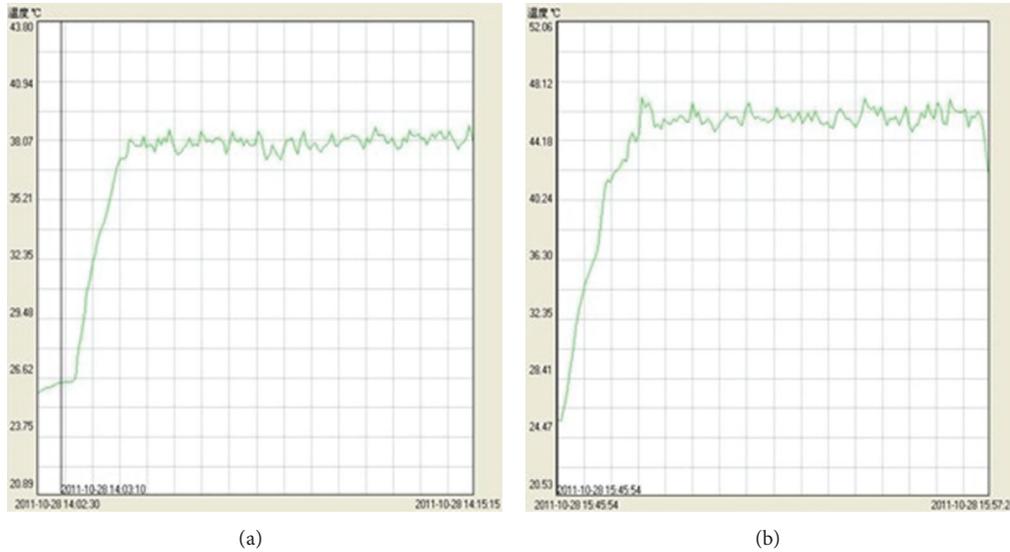


FIGURE 1: Sample curve picture of the point skin temperature during hot stimulation process of moxibustion group, Moxibustion Group 1 (a), Moxibustion Group 2 (b).

(2) *Processing prior to Staining.* (1) *Trimming:* take out the skin tissues that are sheared and then place them in 10% formaldehyde solution and fix them for 1 week, trim the tissues with the surgical knife to a size of approximately 0.7 cm * 0.7 cm * 0.3 cm, and then place them into 70% alcohol to remove any residual formaldehyde solution in the tissues. (2) *Dehydration:* place the trimmed tissues into the full automatic tissue processor for dehydration. (3) *Embedding:* carry out paraffin embedding of the processed tissues. (4) *Slicing:* carry out common slicing by the slicing machine to obtain slices in the thickness of 5–6 μm , and place them into hot water to be flattened. Then slowly take out the slices, rapidly shake off water, and then slowly place them on the well-labeled glass slides, to prevent any air bubbles formed between tissue and slide; in case of any air bubbles, prick off carefully with the acupuncture needle. Bake on a slice-baking plate until the paraffin turns transparent, and place into the slice-baking oven, bake overnight at 60–64°C, and then allow to cool for use.

(3) *Toluidine Blue Staining.* (1) Commonly remove paraffin from slices, and then wash with water; (2) dip-dye with 0.5 toluidine blue solution (to 0.5 g of toluidine blue dye, add water to the volume of 100 mL); (3) wash with water, to remove any excessive staining solution; (4) carry out differentiation with the differentiation solution (mix 0.5 mL of glacial acetic acid and 99.5 mL of distilled water and shake well); (5) wash with water and air-dry; (6) carry out dehydration gradually with alcohol; (7) treat with xylene until transparent; and (8) carry out fixation of slices with neutral balsam.

2.2.3. *Statistical Method.* The data are expressed as mean \pm standard deviation ($\bar{X} \pm S$) and are analyzed and processed by the SPSS17.0 statistical software. The data obtained from each group are subject first to the homogeneity test of variance. In

case of homogeneity of variance, the one-factor analysis of variance is used; if there is difference of population means, the pair-wise comparisons are carried out by the LSD test (in case of heterogeneity of variance, the pair-wise comparisons are carried out by Brown-Forsythe approximate analysis of variance and Games-Howell method); *t* test is used for comparison between two independent samples. It is regarded as significant in case of $P < 0.05$.

3. Results

During the experiment, all deaths due to anesthesia, operative accidents, or drug administration, and so forth, as well as all abnormal responses to intervention of the established model, are not included for observation; 8 valid experimental cases were obtained in each group, so a total of 24 cases were obtained.

3.1. *Results from Electrophysiological Experiment.* Statistical analysis was carried out by the 5 min means for each of the indexes collected, such as HR, MAP, and cardiac function (LVSP, $+dp/dt_{\text{max}}$, and $t-dp/dt_{\text{max}}$). To reduce any statistical errors due to individual differences, the changes before and after intervention in each experimental animal were expressed in percent: Change Rate (%) = (value after intervention – value before intervention)/value before intervention \times 100%. The results were provided as follows.

3.1.1. *Characteristics in the Changes of HR and MAP in the Brachycardia Rat Models Induced by Inderal.* The administration of inderal resulted in rapid decrease of HR and MAP in rats (on average, in 60 minutes, HR was decreased by $34.98\% \pm 5.86\%$, and MAP was decreased by $33.21\% \pm 5.63\%$). In 20 minutes after administration of inderal, the model was stable successfully; during 40-min maintenance

TABLE 1: Mean change percentage of the model group HR, MAP (%).

Model group	N	HR	MAP
60 minutes after administration of inderal	8	34.98 ± 5.86	33.21 ± 5.63
After 20-min administration of inderal	8	-0.16 ± 1.98	0.37 ± 2.09

after 20 min administration of inderal, the mean change rate of HR was $-0.16\% \pm 1.98\%$ and that of MAP was $0.37\% \pm 2.09\%$. The results showed that the method was able to establish brachycardia rat models with stable effects (Table 1).

3.1.2. Waveform Changes of Left Ventricular Pressure and Arterial Pressure before and after Model Establishment and Intervention by Moxibustion. After the model was established successfully with inderal, the waveforms of left ventricular pressure and arterial pressure were obviously decreased than normal; after thermal stimulation by moxibustion at 38°C and 46°C , the waveforms of left ventricular pressure and arterial pressure were recovered to some extents than those in the model (Figure 2).

3.1.3. Intergroup Comparisons of Mean Change Rates of Each of the Indexes, Such As HR, MAP, LVSP, $+dp/dt_{max}$, and $t-dp/dt_{max}$, after Intervention by Moxibustion. Compared with those in the model control group, the thermal stimulation in the Moxibustion group 1 was able to increase HR, MAP, LVSP, and $+dp/dt_{max}$ and reduce $t-dp/dt_{max}$ in rats; however, the effects were not significant ($P > 0.05$). Compared with those in the model control group, the thermal stimulation in the Moxibustion group 2 was able to increase HR, MAP, LVSP, and $+dp/dt_{max}$ and reduce $t-dp/dt_{max}$ in rats, and the effects were significant ($P < 0.05$); moreover, the effects of the thermal stimulation in the moxibustion group 2 were higher than those in the moxibustion group 1 ($P < 0.05$) (Table 2).

3.2. Results from the Morphological Experiment. The morphology of mast cells was observed under the 10×40 optical microscope, and mast cells with degranulation in skin were counted.

3.2.1. Results of Morphological Observations of Mast Cells. Findings of mast cells in slices of skin tissues in the area of the Ximen Acupoint were observed under optical microscope. (1) Model control group: the mast cells were of smooth cell walls and of complete morphology (Figure 3(a)); (2) moxibustion group 1: the mast cells in the local site were of incomplete morphology and were with ruptured cell membranes (Figure 3(b)); (3) moxibustion group 2: the mast cells in the local site were with ruptured cell membranes, and a large amount of granules were scattered around the cell body, and there were significant degranulations (Figure 3(c)).

3.2.2. Degranulation Rate of Mast Cells. The criteria for counting mast cells with degranulation were: count as 1 when the mast cell was with ruptured cell wall, and there were

scattered granules in the matrix. Degranulation rate = count of mast cells with degranulation/total count of mast cells $\times 100\%$. The mean of degranulation rates from 4 slices which were closest to the local site of moxibustion was calculated in each tissue sample, add the mean of degranulation rates of this group all tissue samples together, and divided by the number of samples in each group, then the degranulation rate of mast cells could be calculated.

If no intervention was carried out to the skin at the Ximen Acupoint in rates, the degranulation rate in the local site was as low as 12.9%; after thermal stimulation by moxibustion, the degranulation rate of mast cells was significantly increased ($P < 0.05$) (Table 3).

4. Discussion

From ancient times, moxibustion is one of the important methods in treatment of cardiovascular diseases in clinical practices. It is a crucial problem for moxibustion research how to objectively assess the effects of moxibustion on heart and blood vessels, to explore the possible mechanism for differences of effects, resulted from stimulation by moxibustion at different temperatures as an influencing factor, and to provide a basis for optimizing the clinical operating program of moxibustion. In this experiment, propranolol, a β -receptor blocker, was used to establish the acute brachycardia animal model [10, 11]. It is an easy and simple technique, with good repeatability. The narcotic agent, urethane, is of wide safety margin, with an easily controlled dose, and has small toxicity to heart. The cardiovascular rat model is of good stability, and moxibustion at the Ximen Acupoint has good effects against arrhythmia; they have been verified well in the previous studies of the research group [12, 13]. In this experiment, the indexes including HR, MAP, LVSP, $+dp/dt_{max}$, and $t-dp/dt_{max}$ were analyzed comprehensively; in this way, it may completely reflect the effects of the thermal stimulation by moxibustion on myocardial contractility and peripheral circulation center function. HR may reflect change in the myocardial contractility visually and clearly. After opening of the aortic valve, the left ventricle ejects blood to the artery. The stronger the left ventricle contracts, the more blood it ejects and then the higher the blood pressure is; the afterload resisting ejection of the left ventricle is mainly the peripheral blood pressure (the mean femoral artery pressure was mainly observed in this experiment). The left ventricular systolic function is associated with the afterload of the left ventricle. MAP may reflect the change of the peripheral circulation center function. $+dp/dt_{max}$ is very sensitive to interventions by various variable stresses and, to a certain extent, is influenced by and positively correlated with heart rate and the preload and afterload; the increase of $+dp/dt_{max}$ with concurrent reduction of $t-dp/dt_{max}$ suggests increase of myocardial contractility. The results of this study found that the thermal stimulation by moxibustion at 2 temperatures (38°C and 46°C) may promote increase of HR and MAP in propranolol-induced brachycardia model rats, recovery of LVSP and $+dp/dt_{max}$, and reduction of $t-dp/dt_{max}$, suggesting that the thermal stimulation by moxibustion at 2 temperatures (38°C

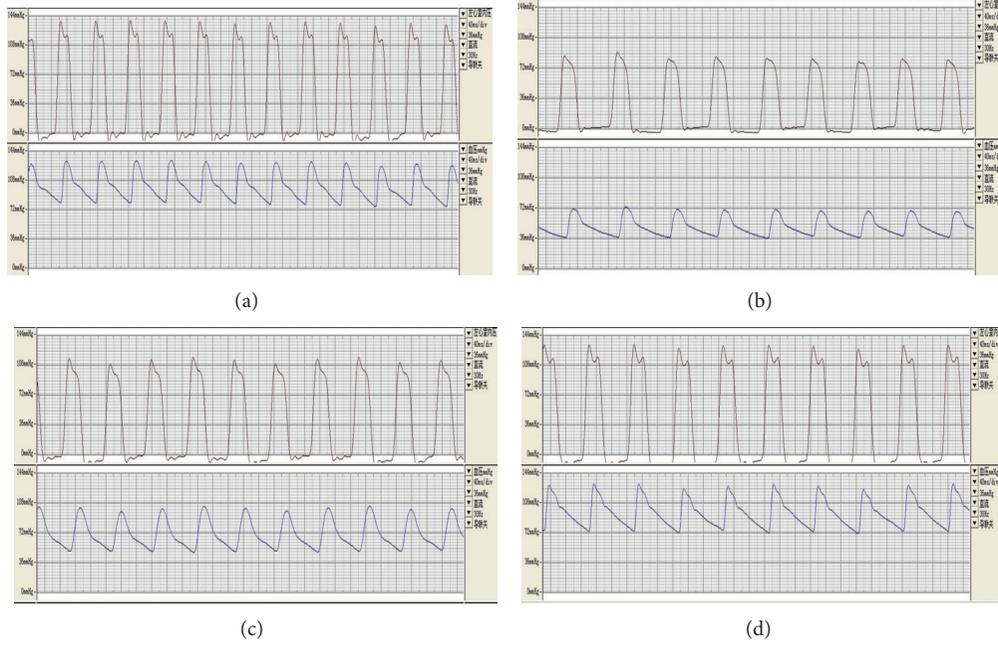


FIGURE 2: Sample waveshape picture of the LVP, AP of each group: normal condition (a), the model was established successfully with inderal (b), after thermal stimulation at 38°C (c), after thermal stimulation at 46°C (d).

TABLE 2: Mean change percentage of the HR, MAP, LVSP, +dp/dtmax, and t-dp/dtmax of different groups after moxibustion treatment.

Group	N	HR (/min)	MAP (mmHg)	LVSP (mmHg)	+dp/dtmax (mmHg/s)	t-dp/dtmax (ms)
Model control group	8	-0.023 ± 0.099	-0.033 ± 0.086	0.045 ± 0.093	0.057 ± 0.14	0.018 ± 0.113
Moxibustion group 1	8	0.044 ± 0.212	0.063 ± 0.078	0.087 ± 0.03	0.14 ± 0.052	-0.026 ± 0.04
Moxibustion group 2	8	0.143 ± 0.074* [▲]	0.425 ± 0.284* [▲]	0.255 ± 0.109* [▲]	0.358 ± 0.143* [▲]	-0.19 ± 0.16* [▲]
Analysis of variance						
F		10.660	14.963	13.875	13.568	7.217
P		0.001	0.000	0.000	0.000	0.004

Note: the values in the table for each index value variation rate.

Compared with model control group * $P < 0.05$.

Compared with moxibustion group 1 [▲] $P < 0.05$.

and 46°C) has the effects of improving cardiac function in bradycardia rats, and the effects of the thermal stimulation by moxibustion at 46°C are obviously stronger than those of the thermal stimulation by moxibustion at 38°C. Our results are in conformity with ancient doctors' point of view that enough strength of thermal stimulation achieve good curative effect.

The ancient doctors emphasis moxibustion heat stimulates the skin local inflammatory response is beneficial to the treatment of diseases. Our study demonstrated that the thermal stimulation by moxibustion may influence the morphology of mast cells in the local site of moxibustion, promote their degranulation, and similarly, the thermal stimulation by moxibustion at 46°C is obviously stronger than that by moxibustion at 38°C. The results suggested that higher or lower mast cell degranulation rate influenced the improvement of cardiac function, so the characteristics of warm thermal stimulation by moxibustion are certainly associated with the effects on the target organ, as well as on the local site of

moxibustion. The local site of moxibustion is the direct object of moxibustion action, and is one of the key links for the starting mechanism of the moxibustion effects. The therapeutic effect of moxibustion is to regulate the body by stimulating the acupoints, while the regulating effects are dependent on receptors in the acupoint area. Recent studies have believed that the skin is an important neuroendocrine organ, which may synthesize and secrete many neurotransmitters and peptide substances [14], while the TRP channel is probably the significant potential molecular target of the warm thermal effects by moxibustion. The TRP channel is widely distributed in mast cells in the body [15], mainly including TRPV1 and TRPV2, which opening effects may directly influence the function and efficacy of mast cells. Especially, TRPV1 calcium ion channels of mast cells may participate in regulating mast cell biological functions and the pathological process [16]. Some scholars proposed the "axonal reflex-linkage hypothesis" [17], in which the synaptic connection between mast cells and nerve fiber acts as a relay baton and the

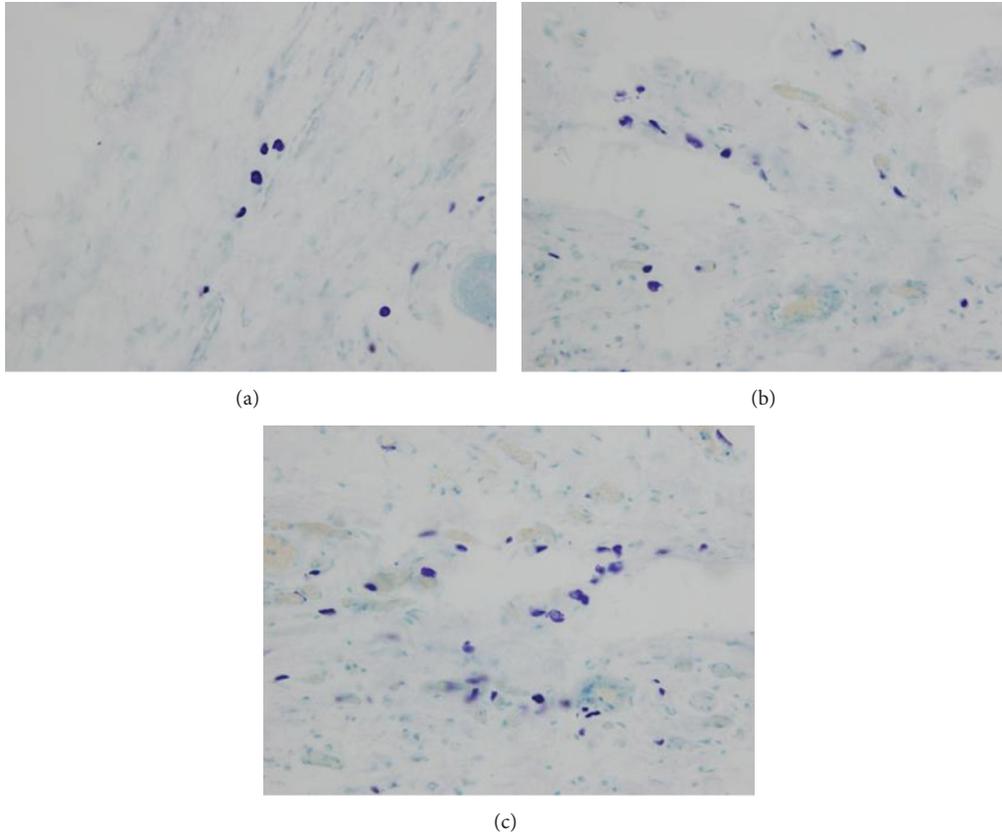


FIGURE 3: Morphologies of Mast cells in the Local Site of the Ximen Acupoint: Model Control Group (a), Moxibustion Group 1 (b), Moxibustion Group 2 (c).

TABLE 3: Degranulation rate of mast cells.

Group	<i>N</i>	Degranulation rate of mast cells %
Model control group	8	12.9 ± 2.8
Moxibustion group 1	8	37.6 ± 5.9*
Moxibustion group 2	8	68.5 ± 6.3* [▲]

Note: compared with model control group * $P < 0.05$.
 compared with moxibustion group 1 [▲] $P < 0.05$.

information is transmitted in the form of stimulating mast cells to degranulate and release active substances and then to activate adjacent nerve terminals. Galli proposed “Mast cells cytokine cascade reaction” [18], based on the characteristics of Mast cells secreting many transmitters and cytokines. Therefore, we deduced that different moxibustion methods may produce different characteristics of temperature changes, activate different TRP subtypes, induce different biological effects, and finally achieve different therapeutic effects.

In this study, the relevant new biological knowledge of the skin was used as the basis for this study, to focus on differences of the effects due to warm thermal stimulation by moxibustion at different temperatures and to explore their association with physiological feedback responses from different thermoreceptors in the body. This was because

that, during the previous research on the action mechanism of moxibustion, we paid more attention to the effects of moxibustion therapy on the diseased target organ and overlooked the unique path and mechanism of starting effects by moxibustion on the target organ. Every effective therapeutic measure may produce effects on the target organ, and the specificity of its action path is the important basis for existence of the method. Based on the stimulation-response process of thermal stimulation by moxibustion → local reception → temperature → chemical conjugation linkage → biological information cascade reaction → effect realization [19], we believed that, due to different temperatures of thermal stimulation, there are differences of excitation receptors; the thermal stimulation at 46°C may activate TRPV1 in the local site of moxibustion and promote degranulation of Mast cells, and the consequent effects may participate in regulation of cardiac function; therefore, the thermal stimulation at 46°C has better improvement effects on cardiac function in bradycardia rats. The thermal stimulation and the resulting inflammatory reaction are the most basic features of moxibustion effect [20], so in our study, attention was paid to the characteristics of the effects in the local site of moxibustion; it may help in further research of the paths of neurology, endocrine, and immune network interaction of moxibustion and also provide a new path to clarify the action mechanism of the effects of moxibustion.

Abbreviations

AP:	Arterial pressure
+dp/dtmax:	Maximum rate of left ventricular pressure rise
HR:	Heart rate
LVSP:	Left ventricular systolic pressure
MAP:	Mean arterial pressure
MCs:	Mast cells
TRP:	Transient receptor potential
TRPV1:	Transient receptor potential vanilloid subfamily member 1
t-dp/dtmax:	Time from the beginning of left ventricular systole to the maximum rate of left ventricular pressure rate.

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Research Article

Reduced Use of Emergency Care and Hospitalization in Patients with Traumatic Brain Injury Receiving Acupuncture Treatment

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Background. Little research exists on acupuncture treatment's effect on patients with traumatic brain injury (TBI). **Methods.** Using Taiwan's National Health Insurance Research Database, we conducted a cohort study to compare the use of emergency care and hospitalization in TBI patients with and without acupuncture treatment in the first year after TBI. The adjusted relative risks (RRs) and 95% confidence intervals (CIs) of high use of emergency care and hospitalization associated with acupuncture treatment were calculated in multivariate Poisson regression models with generalized estimating equation. **Results.** The means of medical visits of emergency care and hospitalization were lower in TBI patients with acupuncture treatment than in those without acupuncture treatment. After adjustment, acupuncture treatment was associated with decreased risk of high emergency care visits (beta = -0.0611 , $P = 0.0452$) and hospitalization (beta = -0.0989 , $P < 0.0001$). The RRs of high medical visits and expenditure for hospitalization associated with acupuncture treatment were 0.62 (95% CI = 0.50–0.76) and 0.66 (95% CI = 0.53–0.83), respectively. **Conclusion.** Patients with TBI who receive acupuncture treatment have reduced the use of emergency care and hospitalization in the first year after injury. The mechanisms of effects of acupuncture on TBI warrant further investigations.

1. Introduction

Traumatic brain injury (TBI), a common injury across every age group and both sexes [1–4], causes disability and death worldwide. It causes 1.1 million emergency visits, 235,000 hospitalizations, and 50,000 deaths in the United States every year [5, 6]. The socioeconomic impacts and burden of diseases for disability following TBI are potentially long term or lifelong [2–5, 7–14]. While the epidemiology, natural history, risk factors, and outcomes of TBI have been established [1–17], TBI treatment and rehabilitation are still crucial problems of global concern.

Despite the remarkable developments in modern Western medicine (a medical system included physicians, surgeons,

and other healthcare professionals (such as nurses, pharmacists, and therapists) to treat symptoms, illness, and diseases using biochemical drugs, radiation, or surgery in the clinics or hospitals) in modern times [18], there is great public interest in complementary and alternative medicine, such as acupuncture [19]. Acupuncture is an important part of traditional Chinese medicine (TCM) widely used in Taiwan [20–25] and other Asian and Western countries [26–29]. Acupuncture's effectiveness has been widely confirmed [30–32], and almost a quarter of Taiwan's people (23%) used acupuncture between 1996 and 2002 [25].

There has been no population-based epidemiological study with comprehensive study design documenting the effectiveness of acupuncture treatment on patients with TBI.

A recent investigation reported that acupuncture had a beneficial effect on cognition and on perception of sleep or sleep quality for patients with TBI [33]. However, that study was limited by small sample size, poor study design, and inadequate adjustment. It was also reported that acupuncture may reduce medical expenditure for patients with chronic neck pain or angina [34, 35]. Based on the findings from previous reports, our study sought to investigate the effectiveness of acupuncture treatment on patients with TBI in a nationwide population-based cohort study with matching procedure by propensity score and multivariate adjustment.

2. Methods

2.1. Study Design and Population. Taiwan's National Health Research Institutes has documented all medical claims for insured beneficiaries since 1996 and provides these as the National Health Insurance Research Database for public access. With patient identification numbers scrambled, data files can be secured to protect patient privacy. Information available for this study included gender, birth date, disease codes, health care rendered, medicines prescribed, diagnoses for admissions and discharges, and medical institutions and physicians providing services. Details were described in our previous studies [1–3, 20]. From a longitudinal cohort population-based database of a randomly selected one million insured subjects, we identified persons aged ≥ 20 years with newly diagnosed TBI who made visits for medical care in 2000–2008 as our eligible study patients (Figure 1). In order to confirm that all patients with TBI in our study were incident cases, only new-onset TBI cases were included in this study; people with previous medical records of TBI within five years before the index date were excluded. The diagnosis of TBI was validated in previous studies [1–3]. Overall, we identified 66,026 new-onset TBI patients aged ≥ 20 years; we excluded mortality cases after TBI within one year. Among new-onset TBI patients, 3495 had used at least two packages (one package included 6 treatments) of acupuncture treatment after injury within the first year. For each patient with TBI who had acupuncture treatment, we randomly selected 4 subjects without acupuncture treatment from patients with TBI as controls matched by age, sex, low income, density of TCM physicians, and coexisting medical conditions (such as hypertension, mental disorder, diabetes mellitus, stroke, ischemic heart disease, hyperlipidemia, migraine, and epilepsy). We conducted our analysis using propensity score-matched pair procedure. Nearest-neighbor algorithm was applied to construct matched pairs, assuming that the proportion of 0.95 to 1.0 is perfect. We followed TBI patients with and without acupuncture treatment for one year and collected their corresponding medical records.

The outcome of this study is the high use (including frequency and medical expenditure) of emergency care and hospitalization for people with a diagnosis of TBI in the first year after this injury. This study used matching procedure with propensity score to minimize the difference of sociodemographic factors and coexisting medical conditions between TBI patients with and without acupuncture treatment. This study's objective is to investigate whether

acupuncture treatment reduces the use frequency and medical expenditure for emergency care and hospitalization for patients with TBI.

2.2. Criteria and Definition. We defined TBI according to the International Code of Diseases, Ninth Edition, Clinical Modification (ICD-9-CM 800–805, 850–854). Coexisting medical conditions included hypertension (ICD-9-CM 401–405), mental disorders (ICD-9-CM 290–319), diabetes (ICD-9-CM 250), stroke (ICD-9-CM 430–438), ischemic heart disease (ICD-9-CM 410–414), hyperlipidemia (ICD-9-CM 272.0–272.4), migraine (ICD-9-CM 346), and epilepsy (ICD-9-CM 345). From individuals' health reimbursement claims, regular renal dialysis (including hemodialysis and/or peritoneal dialysis) was also considered a coexisting medical condition for TBI patients in this study. As Taiwan has 359 townships and city districts, we calculated the population density (persons/km²) for each of these administrative units. Based on population density, these areas were stratified into tertiles of low, moderate, and high urbanization [20–22]. In this study, TCM physicians were defined as physicians licensed by Taiwan's Department of Health who practiced TCM in the legal clinics or hospitals. We calculated the density of TCM physicians (TCM physicians/10,000 persons) using the number of TCM physicians per 10,000 residents for each administrative unit. The first, second, and third tertiles were considered as areas with low, moderate, and high physician density.

We classified the frequency and medical expenditure for emergency care and hospitalization into quintiles [36]. Patients in the highest quintile of medical visits were defined as those having high visits for emergency care or hospitalization. Patients in the highest quintile of medical expenditure (calculated in US dollars) were defined as having high medical expenditure for emergency care or hospitalization.

2.3. Statistical Analysis. The annual prevalence of the utilization of acupuncture treatment by patients with TBI was calculated between 2000 and 2008. The Cochran-Armitage trend tests were analyzed for the prevalence of acupuncture use in 2000–2005 and 2005–2008 (Figure 2). With matching procedure of propensity score, we matched factors including age, sex, low income, density of TCM physicians, diabetes, hypertension, hyperlipidemia, mental disorders, ischemic heart disease, stroke, migraine, and epilepsy between TBI patients with and without acupuncture treatment. The mean and standard deviation of visits and medical expenditure of emergency care and hospitalization were compared with unpaired *t*-tests between patients who had TBI with and without acupuncture treatment. We performed multiple linear regressions to analyze the use of emergency care and hospitalization associated with acupuncture treatment. The adjusted relative risks (RRs) and 95% confidence intervals (CIs) of high medical expenditure of emergency care and hospitalization associated with acupuncture treatment were calculated in the multivariate Poisson regression analyses with generalized estimating equation. All analyses were performed using Statistical Analysis Software version 9.1 (SAS Institute Inc.,

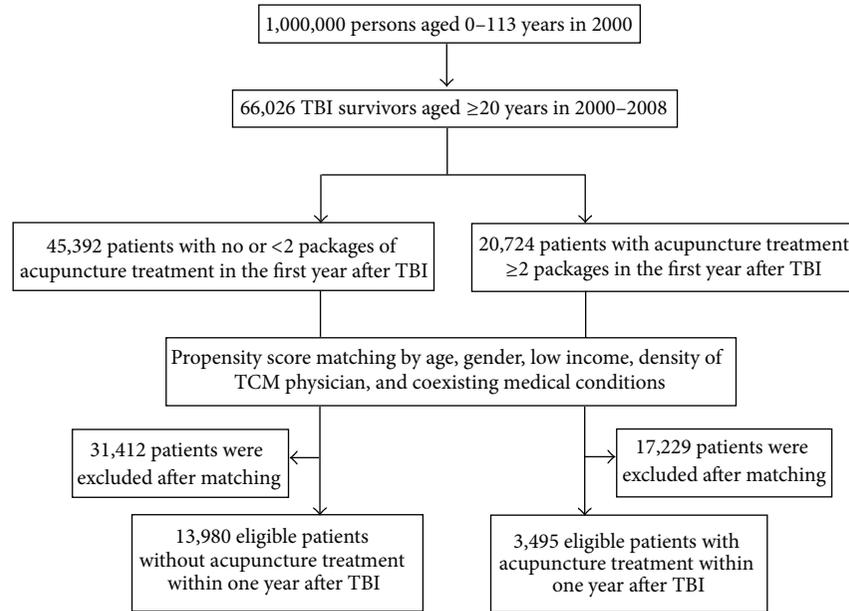


FIGURE 1: Selecting eligible TBI patients with and without acupuncture treatment (TBI: traumatic brain injury; TCM: traditional Chinese medicine).

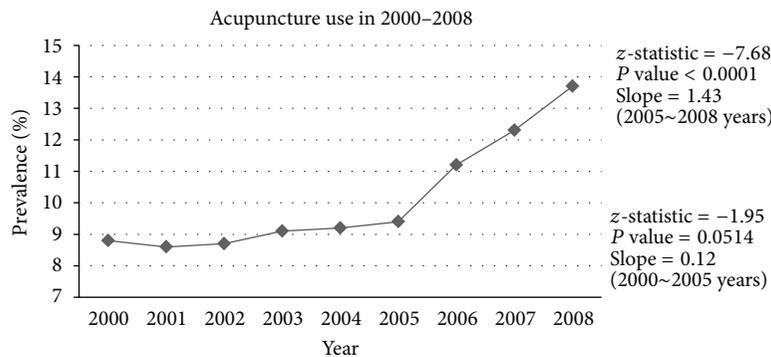


FIGURE 2: Annual prevalence of use of acupuncture treatment by patients with traumatic brain injury (by Cochran-Armitage trend test).

Cary, NC, USA). A two-sided probability value of <0.05 was considered significant.

3. Results

The increased trend of utilization of acupuncture treatment for patients with TBI is shown in Figure 1. The slopes of increased use of acupuncture treatment in 2000-2005 and 2005-2008 were 0.12 ($P = 0.0514$) and 1.43 ($P < 0.0001$), respectively. After matching procedure with propensity score, there was no significant difference in age, sex, low income, density of TCM physician, diabetes, hypertension, hyperlipidemia, mental disorders, ischemic heart disease, stroke, migraine, and epilepsy between TBI patients with and without acupuncture treatment (Table 1).

Compared with TBI patients without acupuncture treatment, patients with TBI who underwent acupuncture treatment had lower medical expenditure for emergency care (44.0 ± 154.2 versus 54.6 ± 520.0 US dollars, $P = 0.04$) and hospitalization (517.2 ± 2588.3 versus 941.0 ± 5099.7 US

dollars, $P < 0.0001$) within the first year after TBI (Table 2). The means of medical visits for emergency care (0.48 ± 1.21 versus 0.56 ± 2.02 visits, $P = 0.006$) and hospitalization (0.29 ± 0.85 versus 0.39 ± 0.98 visits, $P < 0.0001$) were also lower in TBI patients with acupuncture treatment than in TBI patients without acupuncture treatment.

In patients with TBI, acupuncture treatment was associated with reduced visits for emergency care ($\beta = -0.0611$, $P = 0.0452$) and hospitalization ($\beta = -0.0989$, $P < 0.0001$) during the postinjury year (Table 3). The decreased medical expenditure for hospitalization ($\beta = -11772.47$, $P < 0.0001$) for patients with TBI was also associated with acupuncture treatment.

After adjustment for age, sex, low income, TCM physician density, diabetes mellitus, hypertension, hyperlipidemia, mental disorder, migraine, epilepsy, ischemic heart disease, and stroke (Table 4), TBI patients with acupuncture treatment had decreased risk of high medical visits for hospitalization (RR = 0.62, 95% CI = 0.50-0.76) in the first year compared with TBI patients without acupuncture treatment. The

TABLE 1: Matched characteristics and coexisting medical conditions for patients who had traumatic brain injury with and without acupuncture treatment.

	Acupuncture treatment				P value
	No (N = 13980)		Yes (N = 3495)		
	n	(%)	n	(%)	
Age, years					1.00
20–29	3024	(21.6)	756	(21.6)	
30–39	2464	(17.6)	616	(17.6)	
40–49	2596	(18.6)	649	(18.6)	
50–59	2356	(16.9)	589	(16.9)	
60–69	1632	(11.7)	408	(11.7)	
70–79	1448	(10.4)	362	(10.4)	
≥80	460	(3.3)	115	(3.3)	
Mean ± SD	47.34 ± 18.03		47.39 ± 17.80		0.96
Gender					1.00
Female	7980	(57.1)	1995	(57.1)	
Male	6000	(42.9)	1500	(42.9)	
Low income					1.00
No	13944	(99.7)	3486	(99.7)	
Yes	36	(0.3)	9	(0.3)	
Density of TCM physician					1.00
Low	1600	(11.4)	400	(11.4)	
Moderate	5304	(37.9)	1326	(37.9)	
High	7076	(50.6)	1769	(50.6)	
Coexisting medical conditions					
Hypertension	2364	(16.9)	591	(16.9)	1.00
Mental disorder	2128	(15.2)	532	(15.2)	1.00
Diabetes mellitus	1064	(7.6)	266	(7.6)	1.00
Stroke	784	(5.6)	196	(5.6)	1.00
Ischemic heart disease	664	(4.8)	166	(4.8)	1.00
Hyperlipidemia	556	(4.0)	139	(4.0)	1.00
Migraine	200	(1.4)	50	(1.4)	1.00
Epilepsy	88	(0.6)	22	(0.6)	1.00

SD: standard deviation; TCM: traditional Chinese medicine.

TABLE 2: Visits and medical expenditure of emergency care and hospitalization between patients with and without acupuncture treatment in the first year after traumatic brain injury.

	Acupuncture treatment		P value
	No (N = 13980)	Yes (N = 3495)	
Frequency of visits, mean ± SD			
Emergency care	0.54 ± 1.73	0.47 ± 1.16	0.01
Hospitalization	0.38 ± 0.99	0.29 ± 0.89	<0.0001
Medical expenditure, mean ± SD			
Emergency care	46.6 ± 154.9	42.3 ± 145.8	0.12
Hospitalization	872.6 ± 5717.6	480.2 ± 2426.6	<0.0001

SD: standard deviation.

relative risks of high medical expenditure for hospitalization associated with acupuncture treatment in the first year were 0.66 (95% CI = 0.53–0.83).

4. Discussion

Using Taiwan's nationwide insurance database, we reported an increasing trend of acupuncture use for patients with TBI between 2000 and 2008. Using comprehensive study design with the matching procedure of propensity score, our study

discovered decreased use of medical resources for emergency and hospitalization for patients in their first post-TBI years after receiving acupuncture treatment. The present study is the first to report the effects of acupuncture treatment for patient with TBI using population-based investigation from Taiwan.

4.1. Potential Confounding Factors. Age and sex determined the medical conditions and complications for people after their traumatic brain injuries. The utilization of TCM or

TABLE 3: Multiple linear regression analysis of medical visits and expenditure for emergency care and hospitalization for patients having traumatic brain injury with and without acupuncture treatment in the first year after injury.

	Acupuncture treatment*	
	Beta	P value
Medical visits		
Emergency care	-0.06	0.0452
Hospitalization	-0.10	<0.0001
Medical expenditure		
Emergency care	-130.44	0.1260
Hospitalization	-1172.47	<0.0001

*Adjusted for age, gender, low income, TCM physician density, diabetes mellitus, hypertension, hyperlipidemia, mental disorder, migraine, epilepsy, ischemia heart disease, and stroke.

TCM: traditional Chinese medicine.

acupuncture was also associated with age and sex [20–24]. In general, males and older people had worse outcomes after TBI. In contrast, females and young people were more likely to use acupuncture treatment. In addition, low income is a factor associated with TBI risk [1–3]. Density of TCM physicians and low income were related factors for utilization of acupuncture [21]. To investigate the effects of acupuncture treatment on TBI in the current study, we used propensity score to match the effects of age, sex, low income, and density of TCM physicians between TBI patients with and without acupuncture treatment. To accurately estimate post-TBI high medical visits and expenditure for emergency care and hospitalization, residual confounding effects were also controlled in the multivariate linear regressions and Poisson regression models with generalized estimating equation.

Hypertension, mental disorders, diabetes, ischemic heart disease, stroke, and epilepsy were risk factors for TBI [1]. Epilepsy and migraine were identified as common complications after TBI [2]. Compared with non-TBI patients, patients with TBI had increased risk of stroke [4]. To eliminate confounding effects from these TBI-related coexisting medical conditions, we used matching procedure with propensity score and multivariate adjustment to control hypertension, mental disorders, diabetes, stroke, ischemic heart disease, hyperlipidemia, migraine, and epilepsy [1–3].

4.2. Possible Explanations. Insomnia is a frequent problem in patients with TBI. A pilot intervention study found that acupuncture has a beneficial effect on perception of sleep or sleep quality and on cognition in a small sample of TBI patients [33]. Acupuncture treatment was thus suggested as an economical substitute for some medical services and pharmaceuticals; this was a finding of some importance to insurers, healthcare practitioners, and policy makers [27].

In our study, patients with TBI who received acupuncture treatment had reduced use of emergency care and hospitalization in the first year after injury compared with those without acupuncture treatment. We propose two possible explanations. First, the effect of acupuncture treatment on TBI patients was due to biological effects from acupuncture. The

stimulation of acupuncture may lead to activation of frontal-limbic-striatal brain regions, with the pattern of neural activity somewhat different for each acupuncture point [19]. A study from Taiwan also showed the cerebral hemodynamic responses of acupuncture stimulation modes and implied that its mechanism was not only based upon afferent sensory information processing but also had the hemodynamic property altered during external stimulation [37]. Acupuncture intervention involves complex modulations of temporal neural response and involves multiple levels of differential activities of a wide range of brain networks [38]. It can enhance the poststimulation spatial extent of resting brain networks to include antinociceptive memory and affective brain regions. This modulation and sympathovagal response may relate to acupuncture analgesia and other potential therapeutic effects [39]. Second, patients with TBI who choose acupuncture treatment may have better knowledge, attitudes, and practices regarding physical rehabilitation, which we believe could reduce adverse events after TBI.

4.3. Study Limitation. This study has some limitations. First, as we used retrospective medical claims data from health insurance that lacked detailed patient information on lifestyle as well as physical, psychiatric, and laboratory examinations, we were unable to differentiate whether these factors influenced the effects of acupuncture treatment on TBI patients.

Second, as our study used ICD-9-CM codes claimed by physicians for the TBI without clarifying the severity of disease, some of those higher utilization and expenditure might be associated with more severe conditions. Our study would have been improved if we had Glasgow coma score information [7]. Third, this study was limited by the data provided by insurance claims which might be underestimated because very minor TBI might not seek medical treatment. In addition, our study could not show the acupuncture points used in patient care due to the limited information from National Health Insurance Research Database.

5. Conclusion

Using Taiwan's National Health Insurance Research Database, our comprehensive designed retrospective cohort study suggested that patients with TBI who receive acupuncture treatment had reduced use of emergency care and hospitalization in the first year of injury. Our findings implicate that acupuncture treatment may reduce symptoms and illness from major complications for patients with TBI. However, these assumptions need future prospective studies to provide information on the effects of acupuncture treatment on post-TBI mortality and complications, such as stroke, mental disorders, epilepsy, migraine, and fracture. Clinical trials are also needed to clarify the acupuncture points that best address TBI patient needs. The mechanisms of activation and deactivation and their effects on TBI warrant further investigations as well.

Conflict of Interests

The authors declare that they have no conflict of interests.

TABLE 4: Risks of high medical visits and of high emergency care and hospitalization for patients with and without acupuncture treatment in the first year after traumatic brain injury.

	No acupuncture			Acupuncture treatment*		
	Cases	RR	(95% CI)	Cases	RR	(95% CI)
High medical visits						
Emergency care	800	1.00	(Reference)	17	0.88	(0.75–1.02)
Hospitalization	640	1.00	(Reference)	99	0.62	(0.50–0.76)
High medical expenditure						
Emergency care	707	1.00	(Reference)	172	0.97	(0.83–1.14)
Hospitalization	506	1.00	(Reference)	84	0.66	(0.53–0.83)

* Adjusted for age, gender, low income, TCM physician density, diabetes mellitus, hypertension, hyperlipidemia, mental disorder, migraine, epilepsy, ischemia heart disease, and stroke.

CI: confidence interval; RR: relative risk; TCM: traditional Chinese medicine.

Authors' Contribution

Chun-Chuan Shih and Hsun-Hua Lee contributed equally to this work and should be considered cofirst authors. Ta-Liang Chen and Chien-Chang Liao contributed equally to this work and should be considered cocorresponding authors.

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Review Article

On the G-Protein-Coupled Receptor Heteromers and Their Allosteric Receptor-Receptor Interactions in the Central Nervous System: Focus on Their Role in Pain Modulation

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The modulatory role of allosteric receptor-receptor interactions in the pain pathways of the Central Nervous System and the peripheral nociceptors has become of increasing interest. As integrators of nociceptive and antinociceptive wiring and volume transmission signals, with a major role for the opioid receptor heteromers, they likely have an important role in the pain circuits and may be involved in acupuncture. The delta opioid receptor (DOR) exerts an antagonistic allosteric influence on the mu opioid receptor (MOR) function in a MOR-DOR heteromer. This heteromer contributes to morphine-induced tolerance and dependence, since it becomes abundant and develops a reduced G-protein-coupling with reduced signaling mainly operating via β -arrestin2 upon chronic morphine treatment. A DOR antagonist causes a return of the Gi/o binding and coupling to the heteromer and the biological actions of morphine. The gender- and ovarian steroid-dependent recruitment of spinal cord MOR/kappa opioid receptor (KOR) heterodimers enhances antinociceptive functions and if impaired could contribute to chronic pain states in women. MOR1D heterodimerizes with gastrin-releasing peptide receptor (GRPR) in the spinal cord, mediating morphine induced itch. Other mechanism for the antinociceptive actions of acupuncture along meridians may be that it enhances the cross-desensitization of the TRPA1 (chemical nociceptor)-TRPV1 (capsaicin receptor) heteromeric channel complexes within the nociceptor terminals located along these meridians. Selective ionotropic cannabinoids may also produce cross-desensitization of the TRPA1-TRPV1 heteromeric nociceptor channels by being negative allosteric modulators of these channels leading to antinociception and antihyperalgesia.

1. Introduction

The monoamine-peptide interactions have been of great interest [1]. How did they, in fact, interact at the molecular level? One possibility was that the monoamine and peptide signals became integrated through direct neuropeptide-monoamine receptor-receptor interactions in the plasma membrane. We began to test this hypothesis in 1980-1981 in membrane preparations of various Central Nervous System

(CNS) regions and found that neuropeptides could modulate the binding characteristics, especially the affinity, of the monoamine receptors in a receptor subtype specific way [2, 3]. Thus, intramembrane receptor-receptor interactions did exist besides indirect actions via phosphorylation and changes in membrane potential. The results were in line with earlier findings by Limbird et al. in 1975, showing negative cooperativity in β -adrenergic receptors, which may be explained by the existence of receptor homodimers leading

to orthosteric site-site interactions [4]. It was also clear that adapter proteins can be involved in mediating the receptor-receptor interactions in brain membranes [5, 6].

In this paper we give a brief overview of the modulatory role of receptor heteromers in the pain pathways in the CNS and in peripheral nociceptors. They seem to be integrators of nociceptive and antinociceptive wiring (WT) and volume transmission (VT) signals with a major role for the opioid receptor heteromers. Their relevance in the mechanisms for the antinociceptive actions of acupuncture will be discussed.

2. Primary Afferents of the Dorsal Horn Mediating Nociception

We have different types of nociceptors coming into the dorsal horn (primary afferent fibres). One major class includes medium diameter myelinated (A δ) afferents that mediate well-localized fast pain. The second class of nociceptor includes small diameter unmyelinated "C" fibers that mediate poorly localized, slow pain [7]. There exist a very precise laminar organization of the dorsal horn of the spinal cord. The unmyelinated, peptidergic C and myelinated A δ nociceptors terminate most superficially, synapsing upon large projection neurons in lamina I and interneurons located in outer lamina II. Thus, primary afferent nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord (Figure 1, left).

3. Pain Pathways and Their Regulation through WT and VT Signals Integrated through Receptor-Receptor Interactions in Heteromers

A subset of these projection neurons transmits information to the somatosensory cortex via the thalamus, providing information about the location and intensity of the painful stimulus [7]. Other projection neurons reach the cingulate and insular cortices via relay stations in the lower brainstem (parabrachial nucleus) and amygdala, contributing to the emotional component of the pain experience (Figure 1, left). This ascending information also passes on to neurons of the rostral ventral medulla, including the raphe and parapape area and the periaqueductal gray of the midbrain to activate descending feedback systems like distinct bulbo-spinal serotonin (5-HT) and noradrenergic (NA) neurons [8] that regulate the pain transmission output from the spinal cord, especially the lateral paragigantocellular reticular 5-HT neurons may be involved in this process.

This important descending inhibitory control of nociception in the dorsal horn of the spinal cord (Figure 1, left) involves especially the periaqueductal gray-rostral ventromedial medulla, the dorsal reticular nucleus of the medulla, and the ventrolateral medulla [9]. The descending bulbo-spinal NA and 5-HT neuron systems [10–12] play a substantial role [13–15]. Noxious stimulation excites the 5-HT neurons of the lateral paragigantocellular reticular and raphe magnus nuclei [16], building up group B3 of Dahlstroem and Fuxe [10] and these 5-HT neuron systems produce strong analgesia and

the former also cardiovascular activation. Also descending projections to the dorsal horn from DA neurons in the posterior hypothalamus participate in control of pain [17]. The major descending inhibitory bulbo-spinal systems are under the control of high densities of mu opioid receptors [18, 19]. Blockade of the opioid receptors in the medullary reticular nucleus dorsalis prevents analgesia produced by diffuse noxious inhibitory controls [20]. The anti-nociceptive actions of the descending inhibitory pathways in the spinal cord involves the indirect activation of opioid receptors in the dorsal horn, especially in the superficial layers. The major mode of communication in the descending inhibitory monoamine pathways of nociception is extrasynaptic volume transmission involving monoamine diffusion in the um range [21] and activation of their extrasynaptic receptors located on the nociceptors and their target nerve cells mainly found in the superficial layers in the dorsal horn. However, wiring transmission also exists in these descending systems through formation of monoamine synapses. When neuropeptides are released from these descending inhibitory pathways long distance volume transmission can develop in the order of 0.1–1 mm and also involve CSF volume transmission provided the peptides do not undergo rapid degradation [22].

In these pain circuits we will describe how opioid receptor containing heteromers may play a role in the modulation of pain transmission, offering novel targets for antinociceptive drugs. They may be involved in conveying the antinociceptive actions of acupuncture [23] since inter alia electroacupuncture has been shown to increase transcription and translation of enkephalins in the rostral ventrolateral medulla of rats, a region involved in regulation of not only circulation and respiration but also pain [24]. The enkephalin peptides (short distance diffusion) and β -endorphin (long distance diffusion) mainly operate via VT [25–27] and likely modulate the pain circuits via receptor-receptor interactions in receptor heteromers built-up of synaptic protomers and of opioid receptor protomers. In this way synaptic transmission signals and VT signals become integrated giving a balance in nociceptive and antinociceptive signaling in the CNS (Figure 1).

4. Principal Features of the Receptor Heteromers

As mentioned in the introduction, the concept of intramembrane receptor-receptor interactions in receptor heteromers was born in the analysis of neuropeptide-monoamine receptor-receptor interactions in 1980-81. The concept of G protein-coupled receptor (GPCR) heteromers was later confirmed in 1998-1999 by studies reporting that two non-functional GPCR monomers, gamma amino butyric acid (GABA)B1 and GABAB2 receptors, can assemble in a signalling heterodimer, the GABAB receptor at the cell surface [28, 29].

5. The Receptor-Receptor Interaction Toolbox

5.1. Fluorescence and Bioluminescence Resonance Energy Transfer Methods. Different resonance energy transfer methods have been used to study the existence of GPCR

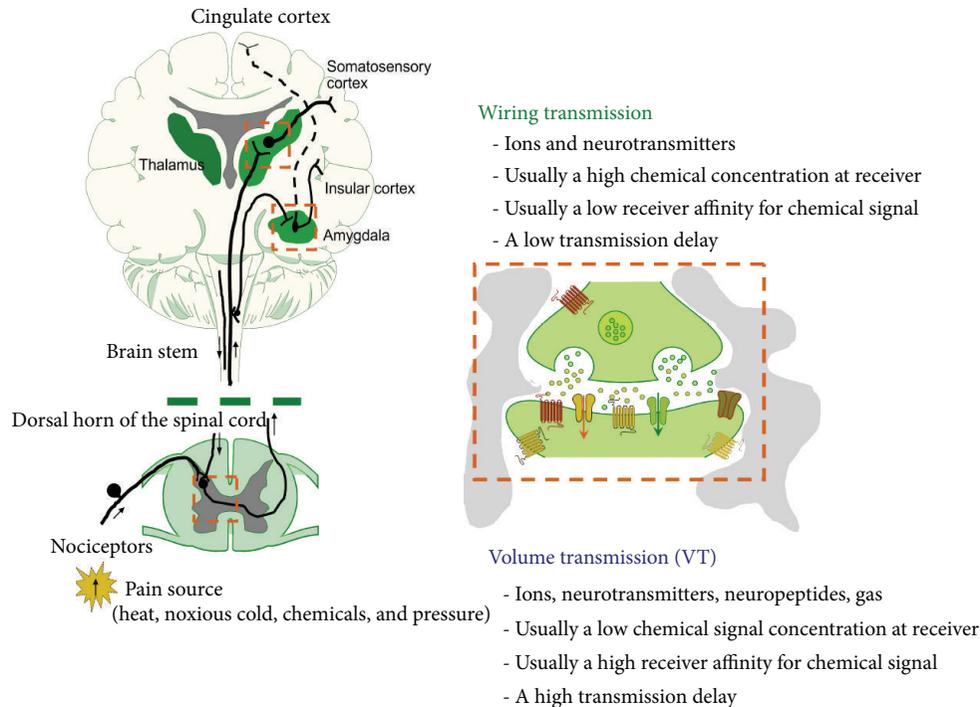


FIGURE 1: Pain pathways and their regulation through WT and VT signals integrated through receptor-receptor interactions in heteromers. (left) A schematic overview of the ascending main circuits mediating pain. When a noxious stimulus is encountered, afferent nociceptors convey noxious information to projection neurons within the dorsal horn of the spinal cord. Neurotransmitters released here bind to and activate postsynaptic receptors on pain transmission neurons. In turn, the axons of pain transmission neurons ascend, predominantly contralaterally, to the brain and carry the information about the noxious stimulus to higher centers (somatosensory cortex via the thalamus with information about location and intensity of the painful stimulus or the insular cortices via connections in the brainstem (parabrachial nucleus) and amygdala within the affective component of the pain experience). The descending inhibitory pathways to the dorsal horn from the brainstem involving inter alia the NA, 5HT, and DA pathways (see text) are also indicated. They exert antinociceptive actions in the pain circuits of the dorsal horn. (right) The diagram shows a few prominent of many possible mediators and cell-cell interactions in the spinal cord dorsal horn, thalamus, or amygdala. In these pain circuits opioid receptor containing heteromers may play a role in the modulation of pain transmission, offering novel targets for antinociceptive drugs. The enkephalin peptides (short distance diffusion) and β -endorphin (long distance diffusion) mainly operate via VT and likely modulate the pain circuits via receptor-receptor interactions in receptor heteromers built-up of synaptic protomers and of opioid receptor protomers. In this way synaptic transmission signals and VT signals become integrated giving a balance in nociceptive and antinociceptive signaling in the CNS. The descending inhibitory pathways to the dorsal horn involving inter alia the monoamine pathways also mainly communicate via VT (see text).

heteromers [30–33]. Fluorescence resonance energy transfer (FRET) method using for example, the CFP-YFP pair in the labeling of two receptors. If the receptors physically interact the distance is short enough (10 nm or less) to allow energy transfer from the donor CFP on one receptor to the acceptor YFP on the other receptor, thus a FRET signal develops from YFP. At the cell surface in living cells, the time-resolved FRET (TR-FRET) method have been developed to study receptor heteromers, by conjugating donor and acceptor fluorophore molecules to antibodies against each protomer in the heteromer of interest. TR-FRET is based on the engagement of a resonance energy transfer process between a lanthanide, such as europium (Eu^{3+}) or terbium (Tb^{3+}) cryptate, as a donor molecule and a compatible acceptor chromophore, such as alexafluor 647 or allophycocyanin [30].

Principle of the detection of GPCR heterodimerization using the bioluminescence resonance energy transfer BRET method is similar to the FRET method except a *Renilla*

luciferase-GFP₂/YFP pair is used in the tagging of the two receptors, in the presence of the substrate h-coelenterazine or coelenterazine-400 on which *Renilla luciferase* acts to produce through oxidation a bioluminescent signal. Then the energy transfer between the generated luminescence and YFP or GFP₂ occurs when the distance between these proteins is less than 10 nm leading to a fluorescence emission from YFP or GFP₂ [21].

On the other hand, the principle of bimolecular fluorescence complementation (BiFC) methods is based on the complementation of the N-terminal and C-terminal fragment of a fluorescent protein (e.g., YFP) [34]. After interaction of the tagged receptors, the protein fragments reconstitute a functional fluorescent protein interpreted as a result of GPCR heteromer formation.

A drawback of these methods is the fact that they involve the ectopic expression and/or overexpression of the fusion receptors, thereby, sometimes promoting the formation of

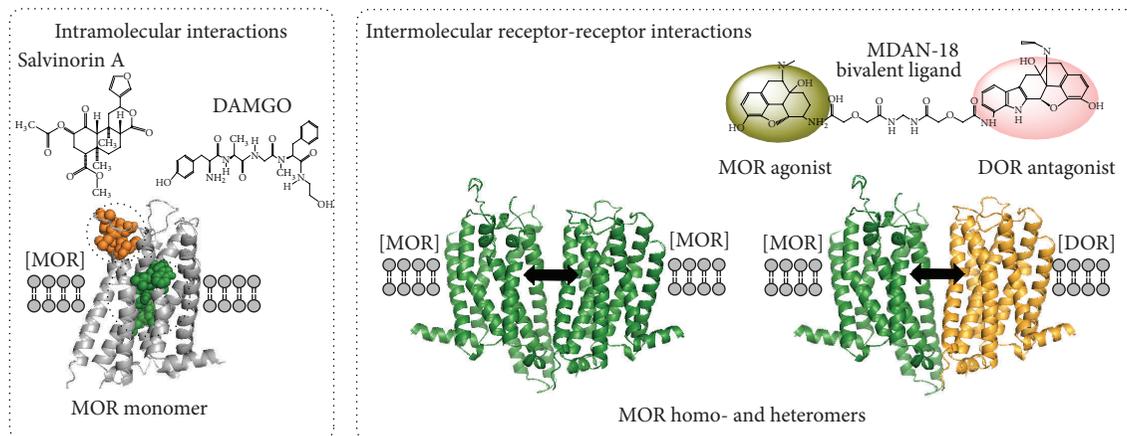


FIGURE 2: Intra- and intermolecular allosteric receptor-receptor interactions. Allosteric mechanisms make possible the integrative activity taking place intramolecularly in monomers (left) or intermolecularly in homo-/heteromers (right). As one example of the intramolecular allosteric mechanisms is the allosteric binding of salvininorin A to the extracellular site of MOR, which partially affects the activity of the orthosteric MOR binding site via a conformational change [105]. Intermolecular allosteric mechanisms take place through the formation of different types of receptor homo-/heteromers and receptor/protein complexes which can change the function of an individual receptor present in a homomer or heteromer. Another example based on the intermolecular heteromer interactions is the use of heterobivalent ligands containing a MOR agonist and an DOR antagonist linked through a spacer of variable size which may function as useful molecular probes for targeting the MOR-DOR heteromer and in this way counteracting the DOR antagonism on MOR function. Such compounds may have a potential use in pharmacotherapy of pain.

artefacts. It is therefore advisable, whenever possible, to consider the physiological expression levels of the receptor pairs under study.

5.2. In Situ Proximity Ligation Assay. However, despite extensive experimental results supporting the formation of GPCR heteromers in heterologous systems (mainly by BRET and FRET methodologies), the existence of such heteromers in the CNS and other tissues remains largely unknown, mostly because of the lack of appropriate methodology. Recently, a well-characterized in situ proximity ligation assay (in situ PLA) has been adapted to confirm the existence of GPCR heteromers in brain slices *ex vivo* [35–39].

In situ PLA is based on a pair of antibodies that can bind to target proteins and to which oligonucleotides have been attached. When the so-called proximity probes recognize a target, for example, the receptor heteromer, the attached oligonucleotides are brought into a sufficiently close spatial proximity to allow them to join followed by ligation of the two linear oligonucleotides into a circular DNA molecule. This newly formed DNA circle strand can serve as a template for rolling circle amplification (RCA), resulting in a long single-stranded rolling circle product (RCP) attached to one of the proximity probes. Since the RCP is linked to the proximity probe, it is attached at the site where the proximity probe bound, which means that it can be used to reveal the location of the receptor complex [40, 41]. The RCPs can then be detected and quantified by hybridizing fluorescent oligonucleotides to the repeated sequences of the RCPs, rendering them visible by fluorescence microscopy. With the in situ PLA method the striatal adenosine 2A receptor (A2AR)-dopamine D2 receptor (D2R) heteromers and D2R-oxycotin

receptor heteromers have for example, been shown [35, 39]. Also the hippocampal and the mesencephalic raphe FGFR1-5-HT1AR heteromers have recently been demonstrated [36, 37]. The in situ PLA procedure represents a high selectivity and sensitivity assay to demonstrate GPCR heteromers in brain [38].

6. Allosteric Receptor-Receptor Interactions

In the beginning, allosteric mechanisms were only discussed in terms of intramolecular interactions within a receptor between orthosteric and allosteric sites. This was the classic pharmacology (Figure 2, left).

Now we have moved into a novel pharmacology, where intermolecular receptor-receptor interactions can occur and results in novel receptor recognition, pharmacology and signaling (Figure 2, right). Intermolecular allosteric mechanisms through the receptor interface produce these changes involving also receptor/protein complexes. An example of the novel pharmacology is the use of heterobivalent ligands [42–44] containing, for example, MOR agonist and DOR antagonist pharmacophores linked through a spacer of variable size which may function as useful molecular probes for targeting the MOR-DOR heteromer and in this way counteracting the DOR antagonism on MOR function [43] (Figure 2, right). Such compounds may have a potential use in pharmacotherapy of pain.

Allosteric mechanism causes a marked rise of the repertoire of GPCR recognition, pharmacology, trafficking and signaling of the participating protomers. This is achieved through changes in recognition, G protein selectivity, and signaling cascades with among others switching from G

proteins to β -arrestin or to calmodulin [33, 45]. Its function may also change by becoming linked to Receptor Tyrosine Kinases (RTKs) or to ion channel receptors [36, 37, 46].

The term moonlighting protein is used to describe multifunctional proteins in which several functions can be found in a single strand of amino acids unrelated to splicing, posttranslational changes, and so forth [47, 48]. In GPCR heteromers moonlighting is brought about by the allosteric receptor-receptor interactions altering the function of the receptor protomers of the heteromer through conformational changes in single strands of amino acids [46].

7. The Receptor Interface

We are interested in the receptor interface since it can be a target for novel drugs by their ability to block or mimic the allosteric receptor-receptor interactions [21, 49–51]. The interface in the A2AR-D2R heteromer can be given as an example [50, 51]. It shows helix-helix interactions in the plasma membrane between A2AR TMIV and D2R-TMV. Intracellular electrostatic interactions between D2R IC3 and A2AR C-terminal tail involve positively charged arginines in the D2R IC3 and negatively charged residues in the A2AR especially phosphorylated serine [50–54]. Electrostatic interactions may represent important hot spots in the receptor heteromer interface. The prototype was the A2AR-D2R heteromer but it exists also in the A2AR-D3R and A2AR-D4R interface [55], giving an amazing stability of the heteromers based on the arginine-phosphate bond [54].

Based on a mathematical approach developed by Dr. Tarakanov, we have deduced, based on 48 pairs of receptors that form or not form heterodimers, a set of triplet amino acid homologies that may be critically involved in receptor-receptor interactions [56]. We call it the triplet puzzle. We showed how such triplets of amino acid residues and their “teams” may be used to construct a kind of code that help determine which receptors should or should not form heterodimers. We propose a “guide-and clasp” manner for receptor-receptor interactions where “adhesive guides” may be the triplet homologies [57–60].

The pro-triplet theory has recently become validated [61, 62] underlining its impact on understanding the receptor interface of the heteromers. On the other hand, the proposed contra-triplets, postulated to block the formation of heteromers, still remain to be documented through experimental work. The lack of studies based on the specificities of the established heteromers hamper a proper prediction of the proposed contra-triplets, that now can be optimized through new experimental data.

8. On the Existence of MOR, DOR, and KOR and Their Participation in Receptor Heteromers

The MOR, DOR, and KOR and their heteromers are of special interest since they play a major role in mediating the antinociceptive transmission of the enkephalin and β -endorphin neurons in case of MOR and DOR and of the dynorphin neurons in case of KOR (Figure 3). It is known from the

fine work of the Watson group that the DOR and especially MOR have a widespread distribution in the brain including the regions of the pain circuits [63]. There exist partial overlaps in the brain and spinal cord of the distribution of the MOR and DOR systems. A widespread distribution is true also for KOR that partially overlaps with the MOR and DOR distribution in the brain and spinal cord. Pain control is all about the balance of activity in the pain and anti-pain systems in the spinal cord, the brainstem, the thalamus, the limbic system and the somato-sensory cortex.

The first opioid receptor heteromer to be discovered was the DOR-KOR heteromer in 1999 by Jordan and Devi [64]. Then in 2000, the MOR-DOR heteromer was demonstrated by George et al. [65] and in 2010 the MOR-KOR heteromer was identified in spinal cord membranes by Chakrabarti et al. [66] and found to be sex specific. They all participate in the modulation of pain.

Together with the existence of opioid heteromers, alternative splicing of the opioid receptor subtypes may help to reconcile the differences between pharmacological subtypes and the results by molecular cloning of only three opioid receptor subtypes. However, also other mechanisms participate [67]. The formation of different types of opioid receptor heteromers through allosteric mechanisms over the receptor interface may contribute to the binding of receptor interacting proteins, producing additional pharmacological subtypes. This can involve, in the latter case, allosteric mechanisms in the receptor-protein interface.

9. MOR-DOR Heteromers and Their Modulation of Pain Circuits

Earlier findings showed that there exist some MOR agonist/DOR antagonist interactions in morphine actions that can be explained by the existence and function of the MOR-DOR heteromer [68], namely, the following.

- (i) Selective DOR blockade with a DOR antagonist reduces the development of morphine tolerance and dependence.
- (ii) Chronic administration of morphine results in an upregulation of DORs in rats.
- (iii) The intensity of the withdrawal syndrome after chronic morphine treatment correlates with the level of DOR binding sites.
- (iv) An antisense oligodeoxynucleotide to DOR was shown to prevent the development of morphine tolerance and dependence after chronic morphine administration [69].
- (v) In DOR knockout mice morphine retained its MOR-mediated analgesic activity without producing tolerance with chronic administration.

All these findings can be explained by DOR exerting an antagonistic allosteric influence on the MOR function in a MOR-DOR heteromer.

In line with the results summarized above, acute *in vitro* experiments on MOR-DOR heteromers in cell lines

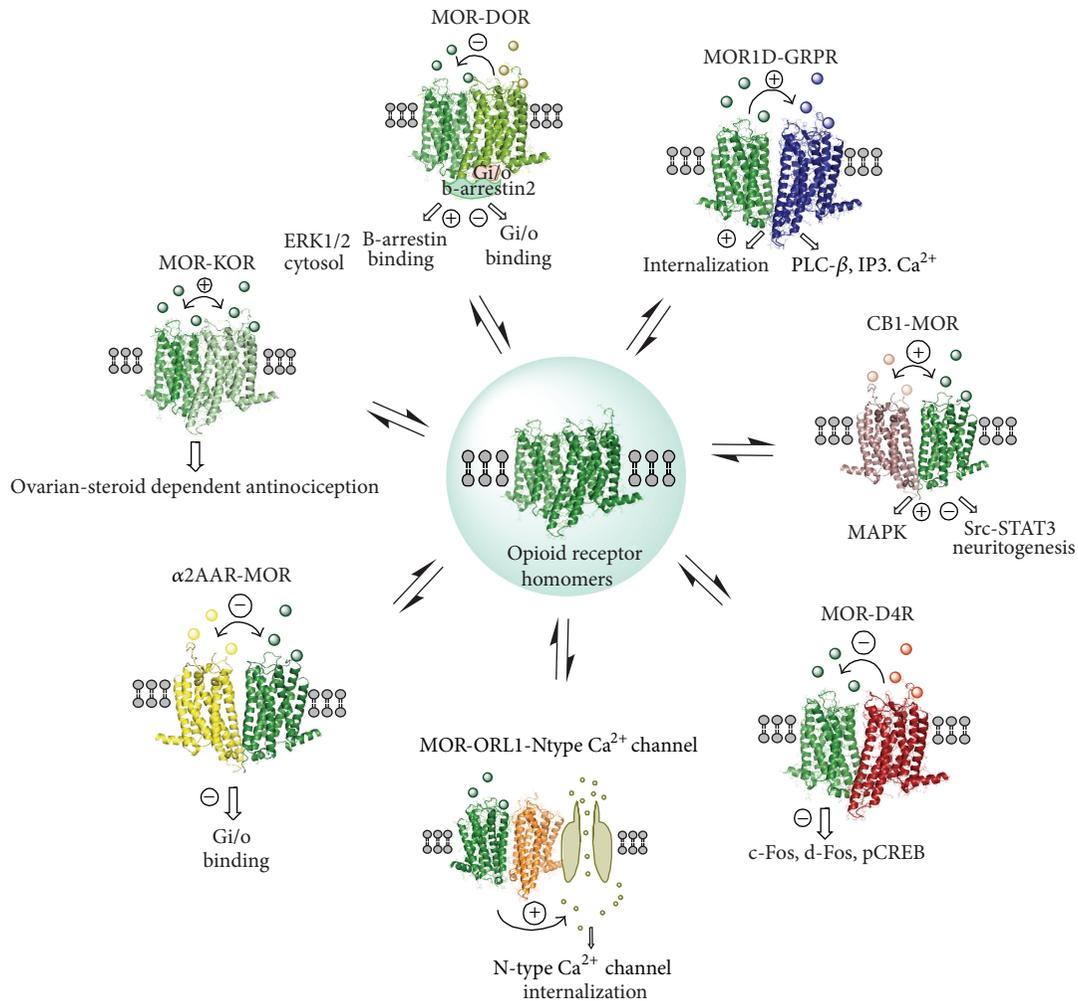


FIGURE 3: Receptor-receptor interactions in different types of opioid receptor heteromers in the CNS and their potential role in pharmacotherapy of pain. The homo- and heterodimers would allow direct physical interactions between the receptors making possible the allosteric receptor receptor interactions between them. The functional balance between these oligomers determines the final functional output and thus the eventual cellular response. The schematic representation depicts some of the principal, nonexclusive, molecular mechanisms by which opioid heteromers produce novel functions.

give evidence that the DOR antagonist enhances MOR recognition, Gi/o coupling and inhibition of cAMP levels. These actions correlated with potentiated morphine analgesia [70].

9.1. Hypothesis on the Role of the MOR-DOR Heteromer in Opioid-Induced Tolerance and Dependence. An interesting concept was introduced by Rozenfeld et al. in 2007 [71] to show the difference in the signaling of MOR homomers versus MOR-DOR heteromers upon repeated morphine treatment. You have a mixture of MOR heteromers and homomers in the plasma membrane and the MOR homodimer activation leads to a rapid G protein-mediated ERK1/2 phosphorylation. pERK1/2 goes to the nucleus, where it activates transcription factors contributing to morphine induced analgesia. Instead in the MOR-DOR heterodimer increasing,

under chronic morphine treatment, the allosteric mechanism is different. It has switched the coupling from G protein to β -arrestin2. You have instead a slow β -arrestin2-mediated ERK1/2 phosphorylation. pERK is retained in the cytoplasm and activates cytoplasmic substrates, such as p-p90srk (Ribosomal protein S6 kinase, 90 kDa) with reductions of changes in gene expression and reduction of morphine action [72].

Also a time course difference in the MOR homodimer versus MOR-DOR heterodimer mediated ERK phosphorylation in primary dorsal root ganglion neurons was measured. Cotreatment of the heterodimer with a combination of a MOR agonist and a DOR antagonist after chronic treatment with morphine leads through altered allosteric receptor-receptor interaction to a dissociation of β -arrestin2 from the heteroreceptor complex and return of the Gi/o binding

and coupling to the heteromer and the biological actions of morphine. It may also be associated with a certain disruption of MOR-DOR heteromers into MOR homomers increasing the MOR homomer/MOR-DOR ratio (Figure 3).

9.2. Experimental Evidence for Targeting the MOR-DOR Heteromer as a Strategy in Antinociceptive Therapy. Based on the hypothesis stated above of an increased formation of MOR-DOR heteromers upon chronic morphine treatment contributing to morphine induced tolerance and dependence, novel bivalent compounds with a MOR agonist pharmacophore and DOR antagonist pharmacophore have been developed [43]. Furthermore, opioid-induced tolerance and dependence in mice is modulated by the distance among pharmacophores in a bivalent ligand series, several being substantially more potent than morphine. It offers a new approach for the development of analgesics devoid of tolerance and dependence. One problem with some bridged bivalent compounds is that they may reduce dissociation of the MOR-DOR heteromer and exert a negative allosteric influence on MOR signaling in spite of the block produced by the delta opioid antagonist at the orthosteric site of the DOR.

9.3. Effects of Chronic Morphine Treatment on the MOR-DOR Heteromer in the CNS. The above hypothesis is now also supported by new evidence. Gupta and colleagues have found increased abundance of MOR-DOR heteromers after chronic morphine administration [73]. Chronic, but not acute, morphine treatment caused an increase in the abundance of MOR-DOR heteromers in key areas of the CNS that are implicated in pain processing. Because of its distinct signaling properties, the MOR-DOR heteromer may, as outlined above, be a therapeutic target in the treatment of chronic pain and addiction [73].

This fine piece of work was possible through a subtractive immunization strategy to generate antibodies that selectively recognize the endogenous MOR-DOR heteromer but does not recognize either MORs or DORs [74]. Such heteromer specific antibodies may also block or activate the heteromer without influencing the homomer adding to their use as tools in the analysis of the function of these heteromers. Increases could be observed in the rostral raphe region of the medulla oblongata, rich in 5-HT neurons projecting to the dorsal horn, as well as in areas of relevance to reward and mood like ventral tegmental area, nucleus accumbens, prefrontal cortex and hippocampus.

All the data support a role of this heteromer in morphine tolerance and dependence, since this heteromer develops a reduced G protein-coupling with signaling mainly operating via β -arrestin2 (see above). Treatment with DOR antagonists reduces the β -arrestin2 coupling in the MOR-DOR heteromer and enhances MOR binding, signaling and morphine-induced antinociception; which may enhance MOR-DOR disruption. It will be of substantial interest to study if acupuncture could favor the formation of MOR homomers versus MOR-DOR heteromers during chronic morphine treatment.

10. MOR-KOR Heteromers and Their Involvement in Gender and Ovarian-Steroid Dependent Antinociceptive Actions

The field of MOR-KOR heteromers is also exciting. Formation of MOR-KOR heterodimer is gender-dependent and mediates female-specific opioid analgesia [66] (Figure 3). Spinal morphine antinociception in females, but not males, required the concomitant activation of spinal MOR and KOR. The evidence shows that spinal cord expression of the MOR-KOR heterodimer is sexually dimorphic and dependent on the stage of the estrous cycle. It is elevated in proestrus with high estrogen receptor (ER) levels as seen from coimmunoprecipitation studies obtained with anti KOR antibodies on the spinal cord [66].

The evidence also shows that the contribution of dynorphin/KOR (part of MOR-KOR heterodimer) to spinal morphine antinociception is dependent on the stage of the ovarian cycle [66, 75]. Spinal morphine antinociception was quantified using the tail-flick test during diestrus and proestrus. The dynorphin antibody and the KOR antagonist counteracted the morphine-induced anti-nociception in proestrus but not in diestrus. Dynorphin was shown to be linked to the KOR protomer of the MOR-KOR heteromer. It represents a molecular switch that shifts the function of KOR and thereby endogenous dynorphin from pronociceptive to antinociceptive actions. Thus, KOR-MOR heteromer could be a novel molecular target for pain control in women.

Further work has indicated that spinal synthesis of estrogen and concomitant signaling by membrane ER regulate spinal MOR-KOR heterodimerization and female-specific spinal morphine antinociception [76, 77]. There exists coexpression of MOR-IR (immunoreactivity) with ER alpha (ER-alpha), GPR30 (a GPCR for estrogen), or KOR in the superficial dorsal horn. Colocalization of MOR-IR and ER-alpha-IR, MOR- and GPR30-IR and MOR-KOR-IR is found in nerve cell bodies and fibers in the superficial dorsal horn. Thus, MOR, KOR, ER-alpha, and GPR30 appear to be coexpressed in neurons of the spinal dorsal horn [76].

Biochemical and behavioral experiments suggest that ERs work in a cooperative manner as part of a macromolecular complex to increase KOR/MOR expression. Estradiol (E2) (spinally synthesized and ovarian derived) triggers the formation of a signaling complex that contains multiple ERs and enhances heterodimerization of KOR and MOR. Transcriptional effects of progesterone (P4) are essential either for the formation of the ERs signaling complex and/or the heterodimerization of KOR with MOR [76, 77].

In our view the formation of receptor mosaics of activated membrane ER complexes and MOR and/or KOR may take place markedly enhancing the formation of MOR-KOR heteromers in the plasma membrane through allosteric changes in MOR and/or KOR. Possibly, progesterone may contribute through transcriptional increases of opioid receptor interacting proteins that may be essential for the MOR-KOR heteromer formation from the ERs-MOR and/or ERs-KOR heteromer complexes.

In summary, the gender- and ovarian steroid-dependent recruitment of spinal cord MOR-KOR heterodimers would provide a way to influence the balance between antinociceptive and pronociceptive functions of the spinal dynorphin/KOR opioid system. Impaired formation of MOR-KOR heteromers could be a biological determinant of various types of chronic pain states that are substantially more common in women than men [66].

11. Opioid Receptor-Like 1 Receptor Heterodimerize with Other Members of the Opioid Receptor Family

The nociceptin receptor or opioid receptor-like receptor 1 (ORL1) belong to the class of Gi/o-linked receptors [78, 79] and is activated by the endogenous 17 amino acid polypeptide ligand orphanin FQ (nociceptin). ORL1 heterodimerize with the other members of the opioid receptor family and can cointernalize each one of them upon agonist exposure.

Upon dimerization with opioid receptors, ORL1 regulation of N-type calcium channels is altered. ORL1 can function as a molecular link that allows MORs to trigger N-type calcium channel internalization [80]. Thus, MOR-ORL1 heterodimers are shown to associate with N-type calcium channels, with activation of MORs triggering N-type channel internalization, but only in the presence of ORL1. Evans et al. [80] found that when coexpressed with the channels alone, ORL1 could trigger internalization of the N-type channels in a nociceptin dose-dependent manner. Without ORL1 expression, activation of MORs by DAMGO did not affect N-type channel surface expression, consistent with a lack of internalization. However, when both MOR and ORL1 were coexpressed, DAMGO application resulted in a dose-dependent loss of N-type channels from the cell surface but not as marked as with ORL1 alone. These findings give the evidence that ORL1 serves as a molecular link allowing MORs to regulate N-type channel surface expression.

These results are of high interest since opioid and opioid-like receptors play a key role in controlling pain signaling in primary afferent terminals in the dorsal horn by two primary mechanisms [81, 82]. These mechanisms are activation of G protein-coupled inwardly rectifying potassium channels, and inhibition of N-type calcium channels in nerve terminals within the dorsal horn of the spinal cord, both reducing neuronal excitability. In conclusion, formation of opioid/ORL1 heterodimers exerts a profound effect on nociceptive processing [80].

Looking at the primary afferents to the dorsal horn clearly activated C and A δ nociceptors release a variety of neurotransmitters, activating output neurons in lamina I of the dorsal horn forming part of the pain pathways from the dorsal horn. One important location of the opioid-ORL1 heteromers may therefore be at the central terminals of these nociceptors to inhibit release of transmitters from them involving increased internalization of the N-type Ca²⁺ channels as well as activation of inwardly rectifying K⁺ channels. Again it would be of high interest to explore how acupuncture would modulate the formation of these receptor heteromeric complexes in the dorsal horn.

12. Do the Alpha-2A Adrenergic Receptor-MOR Heteromers Have a Role in Pain Processing Pathways?

Early work indicates that agonists acting at the alpha-2A adrenergic receptor subtype (alpha-2AAR) and opioid receptors have analgesic properties and act synergistically when co-administered in the spinal cord. The alpha-2AAR subtype is the primary mediator of alpha2 adrenergic spinal analgesia and is necessary for analgesic synergy with opioids and feedback inhibition of capsaicin-induced hyperalgesia [83, 84]. Other findings also demonstrated that alpha-2AAR potentiated morphine analgesia. Thus, a mutual potentiation of anti-nociceptive effects of morphine (opioid agonist) and clonidine (alpha-2AAR agonist) was demonstrated between the antinociceptive effects of intrathecal clonidine and systemic morphine which may be effective in the treatment of chronic pain states [85–87].

The question is if alpha-2AAR-MOR heteromers can participate in these synergistic actions? The major origin of NA innervation of the dorsal horn by the descending bulbo-spinal NA systems [10, 12] is the locus coeruleus (LC) [11]. NA or clonidine significantly reduces the evoked release of glutamate from spinal cord synaptosomes [88] and the release of substance P (SP)-like material and calcitonin gene related peptides (CGRP) from spinal cord slices [89]. Such actions could explain the antinociceptive actions of alpha-2AAR activation.

Immunoreactivity for both alpha-2AAR and MOR is observed in the superficial layers of the dorsal horn of the spinal cord. The primary localization of the alpha-2AAR in the rat spinal cord is on the terminals of capsaicin-sensitive, SP-containing primary afferent fibers (colocation with MOR IR). Thus, alpha-2AAR-MOR heteromers may exist on these terminals.

The role of the receptor-receptor interactions in the alpha-2AAR and MOR heteromers was found to be an unexpected one [90]. There exists a conformational antagonistic crosstalk between alpha-2AAR and MORs in their control of cell signaling upon coactivation (Figure 3). Activation of MOR by morphine modulates alpha-2AAR signaling by a direct strong antagonistic conformational change that propagates from MOR to alpha-2AAR within 0.4 s. The inhibition of Gi activation in the reverse direction also suggests a conformational propagation from alpha-2AAR to MOR. The conformational spread conveyed by the two agonists, noradrenaline and morphine leads to functional inhibition upon agonist coactivation, called cross-inhibition [90]. This is likely a means of rapidly preventing overstimulation of the same signaling pathway as also discussed for alpha-2AAR-Neuropeptide Y receptor (NPY receptor) interactions [91], which results in a cross-inhibition of alpha-2AAR and NPY receptors, both coupled to Gi/o, in biochemical and functional studies on vasodepressor responses. These studies may serve as a model for understanding fast desensitization mechanisms in several signaling pathways. These results suggest that combined agonist activation of alpha-2AAR-MOR heteromers could play a role in counteracting excessive analgesia.

It follows that synergy of alpha-2AAR agonist and morphine in antinociception cannot be explained by receptor-receptor interactions in the alpha-2AAR-MOR heteromers. In this case, interactions at the level of signaling pathways and ion channels controlled by the corresponding homomers may be involved as well as a location in different nerve cells of the neuronal network synergizing in favoring an output pathway leading to antinociceptive effects.

13. On the Localization and Functional Roles of Cannabinoid CB1 Receptors in Pain-Processing Pathways

Synergistic interactions also exist between cannabinoid and opioid analgesia [92]. The cannabinoid CB1 receptors (CB1) are activated by the endocannabinoids, 2-arachidonoylglycerol (2-AG) and anandamide which are recognized for mediating retrograde signaling at glutamate and GABA synapses mediating depression of depolarization induced suppression of excitation and of inhibition, respectively [93]. They likely communicate via an extracellular vesicle mediated form of VT [94, 95]. They can be formed by budding from lipid rafts (shedding vesicles) and may impinge on the plasma membrane of target cells to transfer lipid rafts with associated receptor oligomeric complexes and lipid messengers like the endocannabinoids.

CB1s are present at many locations in the pain networks namely in peripheral terminals of primary sensory neurons, at synapses in the spinal cord, and in pain circuits of the brain. At spinal synapses, CB1 could be on nerve terminals of afferent neurons, on interneurons, and/or on terminals of pathways originating in supraspinal regions [96].

A strong colocalization of CB1 and MOR has been observed in lamina II interneurons [97]. The CB1 action can also involve the inhibition of N-type Ca^{2+} channels and activation of inwardly rectifying K^{+} channels in afferent terminals and dorsal horn neurons as discussed for the alpha-2AAR and MORs.

14. On the Role of CB1-MOR Heteromers for Neuroplasticity in Pain Pathways

Rios et al. in 2006 demonstrated that CB1 forms heteromers with opioid receptors [98]. A BRET signal is formed with MOR, DOR and KOR in cellular models. Thus, coexpression of opioid receptors with CB1, but not with chemokine receptors, leads to a significant increase in the level of BRET signal giving evidence for the existence of CB1-opioid receptor heteromers (Figure 3).

Simultaneous activation of MOR and CB1 leads to a significant attenuation of the increase in MAPK phosphorylation response seen upon activation of the individual protomers. Thus, upon agonist coactivation antagonistic receptor-receptor interactions develop in the CB1-MOR heteromers as observed for the alpha-2AAR-MOR heteromers. However, when the CB1 protomer alone is activated in the CB1-MOR heteromer there is a marked increase in signaling compared with the agonist activation of CB1 monomer/homomer.

Similar results were obtained on neurite outgrowth in Neuro-2A cells expressing MOR and CB1's. Agonist-induced neurite outgrowth in Neuro-2A cells treated with a combination of 100 nM DAMGO or morphine, and 100 nM CB1 agonist HU-210 is markedly reduced upon coactivation while increased with single agonist activation. Upon coactivation a substantial cross-inhibition of the phosphorylation of Src and STAT3 is observed [98].

Antagonistic allosteric interactions in CB1-MOR heteromers may underlie the attenuation of the Src-STAT3 pathway signaling which could be one of the mechanisms leading to reduction of neurite outgrowth. MOR-CB1 interactions will thus upon coactivation lead to cross-inhibition of neurogenesis involving inhibition of the Src-STAT3 pathway. Such a phenomenon may be of substantial importance. Thus, it may lead to counteraction of the plasticity changes seen in discrete pain networks leading to chronic pain [99]. If so, coactivation of MOR and CB1 is the way to go since single activation of the protomers leads to increases in plasticity [98]. It should be considered that RTK can also be involved in these plasticity responses forming a heterotrimer with the CB1-MOR heterodimer making possible integration of transmission and trophic signaling already at the plasma membrane level [8, 36, 37, 100].

15. On the Existence of CB1-D2R and D4R-MOR Heteromers and Their Role in Addiction

There exist indications for the existence also of a CB1-D2R heteromer in which A2AR may participate [101, 102] in the striatopallidal GABA neurons, a key pathway in reward mechanisms. D2R play a major role in cocaine addiction development where the receptor-receptor interactions in CB1-D2R heteromers may exert beneficial actions. During a state of dominance of D2R activation, a negative-feedback regulation of D2R remains through the D2R-mediated release of anandamide inducing an antagonistic CB1-D2R interaction which counteract the exaggerated activation of the D2Rs. In this heteromer, through allosteric receptor-receptor interactions, CB1 may also become coupled to Gs [101, 103] to reduce the downstate induced by the excessive D2R activation, contributing to addiction development.

There may also exist D4R-MOR heteromers in the nucleus accumbens and dorsal striatum of relevance to the treatment of addiction (Figure 3). Thus, D4R activation decreases MOR IR in the striatal islands [104] and D4R can modulate the affinity of MOR in striatum. Furthermore, D4R activation counteracts the morphine induced increases in the striatal expression of the transcription factors c-Fos, deltaFosB and P-CREB [105]. These results can be explained on the existence of antagonistic D4R-MOR interactions in striatal D4R-MOR heteromers [106]. D4R-D2R heteromers [107] and D4R homomers [108] have previously been shown to exist. It will be of interest to evaluate if acupuncture treatment in drug addiction can modulate the striatal CB1-D2R and D4R-MOR heteromers and their antagonistic receptor-receptor interactions.

16. MOR Isoform 1D (MOR1D) Heterodimerizes with Gastrin-Releasing Peptide Receptor (GRPR) in the Spinal Cord: A Key Role in Morphine Induced Itch

In 1983 we discovered neuronal gastrin releasing peptide (GRP) IR in the rat CNS [109]. In the revision we were forced to add also bombesin like IR in the description of the IR since the reviewer claimed it was not possible to fully exclude this possibility. GRP was discovered in porcine nonantral gastric tissue [110]. C-terminal specific antisynthetic porcine GRP sera R-6902 and R-6903 were used showing GRP-like IR in brain tissue extracts. As control was used an antibombesin (BN) serum with the major immunological determinant residing in the 6-7 peptide sequence of BN which is lacking GRP. The results favored the existence of GRP-like IR terminals especially found in the marginal layer and in the substantia gelatinosa of the dorsal horn having a codistribution with SP IR terminals.

Sun and Chen discovered that GRPR mediates the itch sensation in the spinal cord which brought GRP transmission into the spotlight [111]. In 2011 Liu et al.'s group [112] demonstrated coexpression of GRPR and the MOR isoform MOR1D in Lamina I of the spinal cord but not with the MOR1 isoform.

MOR1D was shown to heterodimerize with GRPR in the spinal cord, relaying itch information [112]. Spinal opiates were found to produce itch through MOR1D-GRPR heteromerization leading to cross activation of GRPR signaling (PLC- β /IP3-dependent Ca^{2+} signaling pathway) (Figure 3). They showed that morphine triggers internalization of both GRPR and MOR1D, while GRP specifically triggers GRPR internalization and morphine-independent scratching [112].

The data suggest that opioid-induced itch is an active process concomitant with but independent of opioid analgesia, occurring via the unidirectional cross-activation of GRPR signaling by MOR1D-GRPR heterodimerization. The evidence demonstrates that the C-Terminus of the MOR1D is critical for the MOR1D-GRPR heterodimer formation. The difference between MOR1 and MOR1D isoforms lies in a motif consisting of seven amino acids (RNEEPSS) located in the C-terminus of the MOR1D.

To test the spinal functions of the heteromer, a Tat-fusion peptide (Tat-MOR1D CT) was synthesized. The Tat-motif (YGRKKRRQRRR) belong to a trans-activating domain of a HIV protein that can permeate the cell membrane allowing, after intrathecal injection into the spinal cord, the introduction of the fused MOR1D C-terminal (RNEEPSS motif) into the cells. Introduction of the Tat-MOR1D CT permits its competition with MOR1D for physical contacts with GRPR *in vivo*. It specifically blocked morphine induced scratches while leaving GRP induced scratches intact, morphine induced analgesia unaltered and a reduction in the coimmunoprecipitation of MOR1D-GRPR levels.

New insights into opioid-induced itch prevention was in this way obtained. They also demonstrated that molecular and pharmacologic inhibition of PLC- β 3 and IP3R3, two

downstream effectors of GRPR, specifically blocked morphine induced scratches but not morphine induced analgesia [112]. Based on these observations it would be of high interest to test if acupuncture can counteract the formation of the MOR1D-GRPR heteromers. This would give a biological basis for its use in treatment of itch.

17. Nociceptors in the Peripheral Nervous System and Their TRPA1-TRPV1 Heteromeric Complexes

Of high interest are the peripheral nociceptors and their Transient receptor potential cation channel, subfamily A, member 1 (TRPA1)-Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) heteromeric complexes. TRPV1, one heat nociceptor, is the most famous one, since it represents the capsaicin or vanilloid receptor, activated by ingredients in "hot" chili peppers [7]. TRPA1 is a chemical nociceptor. It is a receptor for pungent ingredients in mustard and garlic plants, isothiocyanates and thiosulfonates. These nociceptor terminals also express a host of sodium channels and potassium channels (such as TRAAK and TREK-1) that modulate nociceptor excitability and/or contribute to action potential propagation [7]. The capsaicin TRPV1 is a nonselective cation channel that is structurally related to members of the TRP family of ion channels [113]. The membrane topology and domain structure of TRPV1 have been predicted. TRPV1 ion channel has high Ca^{2+} permeability and the capsaicin activation of this channel kills the cells.

By means of acceptor bleaching FRET, a direct interaction between TRPA1 and TRPV1 on the plasma membrane was observed [114]. The increase in donor emission between TRPA1 and TRPV1 in the plasma membrane is just as large as between the corresponding homomers TRPA1-TRPA1 and TRPV1-TRPV1 as has been demonstrated by measurement of the FRET signal efficiency.

Mustard oil activates single channel currents in TRPA1 and TRPA1-TRPV1 expressing CHO cells. Vigorous activation of channels in TRPA1 and TRPA1-TRPV1 expressing cells was observed but not in untransfected CHO cells with multiple conductance states, performed in cell-attached configuration in voltage clamp mode [114]. However, differences of the current voltage relationships have been found in the single channel activities of TRPA1 alone and TRPA1-TRPV1 heteromers. The single channel mustard oil induced conductance (IMO) current-voltage I-V relationships for TRPA1-containing cells showed hardly any rectification. In contrast, the TRPA1-TRPV1 channel heteromers resulted in an outward rectification with a high conductance slope, for the outward versus the inward parts of the I-V curve, respectively. Thus, the ion channel function becomes altered through the TRPA1-TRPV1 heteromerization. Also, results on the properties of single channel mustard oil induced conductances (IMO) in TRPA1 in WT and TRPV1 KO sensory neurons validated these findings. IMO exhibited substantially greater activity at positive voltages in WT neurons compared with TRPV1 KO neurons [114].

These results support the hypothesis that TRPV1 and TRPA1 in nociceptors may form a heteromeric receptor ion channel complex and that TRPV1 can influence intrinsic characteristics of the TRPA1 channel also independent of intracellular calcium [114, 115]. Transmission of inflammatory stimuli by nociceptors (namely damage-sensing sensory neurons) is also mutually controlled by TRPA1 and TRPV1 channels. This functional interaction between TRPV1 and TRPA1 could occur indirectly via recruitment of second messengers, such as intracellular Ca^{2+} and/or directly, involving allosteric receptor-receptor interactions between these receptor channels within a heteromeric complex.

The demonstrated pharmacological cross-desensitization between capsaicin and mustard oil responses can involve desensitization of TRPA1 and TRPV1 ion channel activities in their heteromeric complexes which may contribute to inhibition of nociceptor signaling leading to antihyperalgesia and antinociception [114, 115]. One mechanism for the antinociceptive actions of acupuncture along meridians may be that it enhances the cross-desensitization of the TRPA1-TRPV1 heteromeric complexes within the nociceptors located along these meridians. This process may *inter alia* involve changes in the flow of volume transmission signals in channels containing extracellular fluid and nociceptors along the meridians modulating the sensitivity of the nociceptors [22, 82].

18. Iontropic Cannabinoid Receptors in Peripheral Antinociception and Antihyperalgesia

There is also a role of ionotropic cannabinoid receptors (ICR) in peripheral antinociception and antihyperalgesia. The known ICRs are members of the family of transient receptor potential channels (TRP) and remarkably include TRPV1, TRPV2, TRPV4, TRPM8, and TRPA1 (see above). The majority of ICRs are expressed in nociceptive sensory neurons, which can detect and respond to noxious mechanical, thermal and chemical stimuli. Nevertheless, the cannabinoids produce a profound antihyperalgesia and the mechanism has not yet been established [115].

One possible hypothesis addressing this issue is that partial activation of ICRs does not necessarily generate excitation (i.e., action potential) of nociceptors. From this perspective, it is interesting that cannabinoids are not full agonists for TRP channels. Indeed, cannabinoids typically evoke a slow generation of small inward currents and Ca^{2+} accumulation. As a result, cannabinoid-gated responses might not reach the threshold levels required to excite nociceptors. Moreover, slow depolarization of nociceptor membrane potentials might lead to inactivation of voltage gated channels that, in turn, inhibits the generation of action potentials.

To understand how activation of ICRs leads to inhibition of nociceptors, molecular mechanisms of desensitization of TRP channels by ICR-activating cannabinoids have been studied [115]. The results indicate that cross desensitization between the TRPA1 and TRPV1 channels (see also above) in sensory neurons can involve multiple separate mechanisms. Cannabinoids may desensitize TRPV1 channels via activation

of calcineurin and dephosphorylation of the ion channel. Homologous desensitization of TRPV1 can occur by application of TRPV1-selective cannabinoids, and heterologous desensitization of TRPV1 can occur by administration of TRPA1-selective cannabinoids (e.g., WIN55212). Cannabinoids can also desensitize TRPA1 via activation of a calcium-independent pathway.

Based on the existence of TRPA1-TRPV1 heteromeric complexes we may also hypothesize the following mechanism: Ionotropic cannabinoids can activate antagonistic allosteric channel-channel interactions in such types of heteromeric complexes. This allosteric mechanism especially upon coactivation of the two TRP channels by selective ionotropic cannabinoids may produce cross-desensitization of the two nociceptor channels leading to antinociception and antihyperalgesia. The ionotropic cannabinoids may best be regarded as negative allosteric modulators of TRPA1-TRPV1 heteromeric complexes and other types of TRP heteromeric complexes. It is presently unknown to which extent metabotropic CB1 and CB2 may participate in the modulation of the peripheral nociceptors and in the mediation of the actions of the ionotropic cannabinoids. A dynamic interplay between CB1/CB2 and TRP channels as to heteromerization is however, an interesting possibility.

Other mechanisms for acupuncture induced analgesia likely also exist since analgesic effects also develop in distant parts of the body. There exists a mechanism called diffuse noxious inhibitory control (DNIC) which results in reduction of pain from the experimental noxious stimulus when a nociceptive stimulus is applied to a region remote to the test area [20, 116, 117]. DNIC has *inter alia* been shown to be activated in experimental peripheral mononeuropathy [118] where peripheral mechanisms may mainly be involved like sensitization of damaged nerve fibers. However, acupuncture can activate pathways involved in DNIC as found in studies on trigeminal caudalis neurons in rats [119]. However, lower pain intensities are used in human acupuncture which may explain why the analgesic effects of acupuncture in humans are less than a DNIC effect of a painful noninvasive stimulus [116].

It should also be noted that sensory neuropeptides like substance P may be released from nociceptors upon acupuncture and both acupuncture and sensory neuropeptides increase cutaneous blood flow [120]. It is of particular interest that somatostatin peptides may produce systemic analgesic effects [121]. Thus, somatostatin can produce inhibition of the cross-excitation between adjacent primary afferent terminals in rats induced by antidromic stimulation of primary afferents leading to inhibition of peripheral hyperalgesia. Therefore, one additional mechanism for acupuncture analgesia should be considered namely that somatostatin can be released by acupuncture from sensory nerve terminals along the meridians. It may then diffuse and flow along the interstitial fluid channels of the meridians for short (extrasynaptic mode) and/or long distances of volume transmission [122] to activate somatostatin receptors located on the plasma membrane of the nociceptors to reduce their firing and produce reduction of pain. The findings of Guo et al. (2008) [121] indicate that released somatostatin may also use the surrounding vascular beds to reach via the circulation adjacent primary

afferents and also via this mode of communication produce peripheral analgesic effects via activation of somatostatin receptors.

19. Future Directions

The role of the receptor heteromers in pain modulation may be studied along the following lines.

- (i) Understanding the role of MOR-DOR, MOR-KOR, alpha-2AAR-MOR, and CBI-MOR heteromers in key pain circuits in the CNS. Of special interest will be to outline the role of opioid receptor heteromers as a target for the treatment of pain including acupuncture and the role of MORID-GRPR heteromer in itch and as a target for the anti-pruritus actions of acupuncture.
- (ii) Understanding the role of TRPA1-TRPV1 heteromeric ion channel complexes in nociceptor function and their role in the antinociceptive and antihyperalgesic actions of cannabinoids.
- (iii) Characterization of the receptor interfaces in distinct opioid receptor and TRPA1-TRPV1 heteromers and putative ERs-MOR-KOR heteromeric complexes. The receptor interface is a novel target enabling modulation of the allosteric receptor-receptor interactions.
- (iv) Understanding the pharmacology of the above receptor heteromers. A major targets for the therapeutic effects of antinociceptive drugs which may mediate side effects.
- (v) Understanding the link of potential changes in distinct opioid receptor and TRPA1-TRPV1 heteromer structure and function to plasticity changes in the pain pathways in chronic pain syndromes. Discovery of novel key receptor heteromers is still to come.
- (vi) Finally, discrete heteromers may also be targets for chemical ingredients mediating the medicinal properties and the side-effects of plants that is, herbal medicine.

Glossary

The usage of terms in medicine often varies widely. For this reason, it is convenient and helpful to authors and readers if words can be used with an agreement in their technical meaning. The definition provided in this Glossary are intended to be specific and explanatory and to serve as a useful framework, not as a constraint on future development for members who work in the field of pain or are interested in this review article topics (all definitions are taken from Andreas Kopf Guide Pain Management in Low-Resource Settings, International Association for the Study of Pain).

Acupuncture. Acupuncture is a procedure involving the stimulation or inhibition at an anatomical location on or in the skin by a variety of techniques. A number of effects on pain physiology have been identified, the most important

being the activation of the endogenous opioid system and the spinal modulation of pain signaling through activation of touch fibers ($A\beta$ fibers).

Analgesia. Absence of pain in response to stimulation that would normally be painful. The stimulus is defined by its usual subjective effects.

Hyperalgesia. An increased response to a stimulus that in normally painful. Hyperalgesia reflects increased pain on supra-threshold stimulation. For pain evoked by stimuli that usually are not painful, the term allodynia is preferred, while hyperalgesia is more appropriately used for cases with an increased response at a normal threshold, or at an increased threshold, such as in patients with neuropathy. It should also be recognized that with allodynia the stimulus and the response are in different modes, whereas with hyperalgesia they are in the same mode.

Meridian. In the Chinese medicine acupuncture means each of a set of pathways in the body along which vital energy is said to flow. There are twelve such pathways associated with specific organs.

Nociception. Nociception is the sensory component of pain. It encompasses the peripheral and the central neuronal events following the transduction of damaging mechanical, chemical or thermal stimulation of sensory neurons (nociceptors).

Nociceptor. A receptor preferentially sensitive to a noxious stimulus or to a stimulus that would become noxious if prolonged. Often called a pain receptor.

Noxious Stimulus. A noxious stimulus is one that is damaging to normal tissue.

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Review Article

An Exploration of the Needling Depth in Acupuncture: The Safe Needling Depth and the Needling Depth of Clinical Efficacy

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Objective. To explore the existing scientific information regarding safe needling depth of acupuncture points and the needling depth of clinical efficacy. *Methods.* We searched the PubMed, EMBASE, Cochrane, Allied and Complementary Medicine (AMED), The National Center for Complementary and Alternative Medicine (NCCAM), and China National Knowledge Infrastructure (CNKI) databases to identify relevant monographs and related references from 1991 to 2013. Chinese journals and theses/dissertations were hand searched. *Results.* 47 studies were recruited and divided into 6 groups by measuring tools, that is, MRI, in vivo evaluation, CT, ultrasound, dissected specimen of cadavers, and another group with clinical efficacy. Each research was analyzed for study design, definition of safe depth, and factors that would affect the measured depths. Depths of clinical efficacy were discussed from the perspective of de-qi and other clinical observations. *Conclusions.* Great inconsistency in depth of each point measured from different subject groups and tools exists. The definition of safe depth should be established through standardization. There is also lack of researches to compare the clinical efficacy. A well-designed clinical trial selecting proper measuring tools to decide the actual and advisable needling depth for each point, to avoid adverse effects or complications and promote optimal clinical efficacy, is a top priority.

1. Introduction

Acupuncture is an important part of traditional Chinese medicine and has been used for millennia of years to treat various clinical disorders based on ancient Chinese medicine theory. In recent one hundred years, acupuncture has become one of the most popular complementary and alternative therapies in the world. More than 100 million citizens in the European Union make use of complementary and alternative medicine (CAM) today. According to EICCAM files, the most commonly used CAM therapies in Europe are homeopathy, acupuncture, phytotherapy (i.e., herbal medicine), anthroposophic medicine, naturopathy, traditional Chinese herbal medicine, osteopathy, and chiropractic. In 2007, almost 4 out of 10 adults had used CAM therapy in the past 12 months. Results from the 2007 NHIS found that approximately one in nine children (11.8%) used CAM therapy in the past 12 months. Between 2002 and 2007, increased use was seen

among adults for acupuncture, deep breathing exercises, massage therapy, meditation, naturopathy, and yoga in the United States [1]. Given the fact of the rising incidence of chronic disease and stress-related illness in the West, along with an expanding awareness of the unwanted side effects of pharmaceutical treatment, there has been an increased utilization of acupuncture as a contemporary health care option [2]. Acupuncture is also practiced by about 40,000 physicians in Germany [3]. One of the three most commonly used methods of CAM is acupuncture in the United Kingdom [4]. There are 12%~19% of individuals who had received acupuncture treatment in Europe [5]. A practitioner in UK reported that an estimated 10.0% of the UK population had received any CAM therapy (an estimated 6.5% had used one of the five main therapies: acupuncture, homeopathy, chiropractic, osteopathy, or herbal medicine) [6]. Acupuncture points are known as specific locations of the body that are needled during acupuncture treatment. Acupuncture

points are located along meridians that have been defined by ancient writings of Chinese medicine since thousands of years ago. Traditionally, acupuncture points are localized using cun (or Tong Shen Cun) as proportional measurement. Ancient writings of acupuncture guidelines also refer to anatomical landmarks to help localize the needling position. Cuns usually used in the documents are as follows [7].

1.1. Proportional Bone (Skeletal) Cun (B-Cun). This method divides the height of the human body into 75 equal units. Using joints on the surface of the body as the primary landmarks, the length and width of every body part is measured by such proportions. The specific method is as follows: divide the height of the human body into 75 equal units and then estimate the length and width of a certain part of the body according to such units. One unit is equal to one cun.

1.2. Finger Cun (F-Cun). This method is based on the finger cun of the person to be measured for acupuncture point locations.

1.3. Fingerbreadth (F-Breadth). This method utilizes the width of the distal phalanx of the middle finger. This should be distinguished from the middle finger cun.

For example, the individual distance between nipples measures 8 cun, and the individual interscapular distance measures 6 cun. Several research reports have discussed the anatomy and physiology of acupuncture points in order to understand the therapeutic mechanism of acupuncture [39–42]. However, the actual mechanism by which acupuncture works remains controversial. The majority of these studies have been of an experimental nature or in vitro cadaver studies and lack discussions regarding needling depth. Acupuncture is generally considered to be a safe treatment. Most reported adverse events were minor complications such as needling pain, hematoma, nausea, vomiting, and fainting. Ancient Chinese literature and historical texts have also documented the adverse effects of acupuncture. There are two descriptions about the possible critical complications of acupuncture in chapter 60 of *Huangdi Neijing: Spiritual Pivot*. “Unskillful doctors may kill the patients instead of saving their lives.” “Violation of the rules in performing needling therapy will kill the patients instead of saving their lives.” Deep insertion at the acupuncture point Qupen (ST12) may cause dyspnea, cough, and even collapse of the lung. Complications in acupuncture practice may result from violations of sterile procedure and/or negligence of the practitioners. Serious side effects include cardiac tamponade [43, 44], pneumothorax [45, 46], endocarditis [47–49], hepatitis [50–52], chylothorax [53], and spinal cord injury [50, 54], and minor side effects include fainting [55, 56] and skin reactions [57–60]. Pneumothorax is the most common mechanical organ injury associated with acupuncture treatment [50, 61–64], and the related reports are from the United States [65–67], Canada [68], The Netherlands [69, 70], France [71], Norway [63], Portugal [72, 73], Denmark [74], Taiwan [75], Japan [50, 76], China [45, 77], and Hong Kong [78]. Based on the

facts mentioned above, we understand that the safe needling depth for acupuncture therapy is a very important issue for clinical practice. However, there is only meager and confusing information about the safe needling depth in ancient classics of Chinese medicine and modern acupuncture textbooks. As some adverse events are preventable through preventive measures, there stands the need for urgent standardization regarding safe depth of acupuncture. We are also interested to learn if needling depth is correlated with the clinically observed therapeutic effects. In this paper, we provide a critical review of the current researches classified by the measuring tool on safe needling depth of acupuncture points as well as the therapeutic depth with clinical efficacy.

2. Methods

A comprehensive search of the literature that was published from 1991 to 2013 was undertaken using the following key words: acupuncture, acupoint, needling depth, safe depth, dangerous depth, de-qi, therapeutic effect/efficacy, and their synonyms. These terms were used to search the following databases: PubMed, EMBASE, Cochrane, Allied and Complementary Medicine (AME), The National Center for Complementary and Alternative Medicine (NCCAM), and China National Knowledge Infrastructure (CNKI) databases. Additional articles were also identified from the reference list of identified articles. Chinese journals, theses, and dissertations that we thought might be relevant to our study were hand searched. We excluded the animal studies.

3. Results

47 studies from 1991 to 2013 were recruited into the review. As there are not many researches specific for depth of acupuncture points, we tried to include as many articles as possible. Most of the studies were retrospective, nonrandomized clinical trials without control group. The characteristics of subjects, sample size, investigated acupuncture points with the associated body region/meridians, parameters used for comparison, related factors, results and suggestions/conclusions of the researches and related factors and the results and suggestions and conclusions of the researches were summarized in the following tables classified by their measuring tool, that is, magnetic resonance imaging (MRI), in vivo subjects evaluation, computed tomography scan (CT), ultrasound, and dissected specimens of cadavers, in Tables 1, 2, 3, 4, and 5. Table 6 depicts the investigations regarding clinical efficacy.

3.1. The Safe Needling Depth

3.1.1. Researches Using MRI Images for Measurement of the Depths of Acupuncture Points. Magnetic resonance imaging (MRI) is a medical imaging technique used in radiology to visualize internal structures of the body in detail. MRI provides better contrast between the different soft tissues of the body compared with other medical imaging techniques such as CT scans or X-rays which makes it the more

TABLE 1: Summary of researches using MRI images for measurement of the depths of acupuncture points.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Li 2011 [8]	10 male, 10 female	BL18	Gender, angle, side, BL, BW, BMI, and thumb cun	(1) Male subjects had greater safe depth (mean 25.1 mm versus 22.52 mm) (2) Right side points had greater depths (3) The perpendicular depths of right side points correlated with BW, thumb cun; depths of left side points correlated with BL, BW, BMI, and thumb cun
Fu 2011 [9]	10 male, 10 female	BL20	Gender, angle, side, BL, BW, and BMI	(1) No side differences (2) With gender differences except when needling angle is 45 degrees (3) The safe depth did not correlate with BL, BW, and BMI
Chuan 2011 [10]	10 male, 10 female	BL19	Gender, angle, BL, BW, and BMI	(1) The male subject had greater safe depth of perpendicular needling (2) If needled with the angles of 30 and 45 degrees, no gender difference noted (3) The depth may vary profoundly under different angles, for example, 2.02 to 4.0 cm
Yen 2011 [11]	10 male, 10 female	BL17	Gender, angle, side, BL, BW, and BMI	(1) No obvious side difference (2) The various needling angles and safe depth did not correlate with BMI (3) Mean depth of perpendicular depth in male is 4.11 cm and 3.16 cm in females
Han 2010 [12] and Wen et al. 2011 [13]	10 male, 10 female	SP21	Gender, angle, BL, BW, BMI, body cun	(1) The safe needling angle should be about 15 degrees towards the skin surface (2) Difference in safe depth existed in various needling angles (3) The safe depth did not correlate with BL, BW, BMI, thumb cun, and body cun
Yu 2010 [14]	10 male, 10 female	CV14, ST19	Gender, angle, BL, BW, BMI, and body cun	(1) No gender differences in both points (2) the safe depth of CV14 did not correlate with BL, BW, BMI, or body cun (3) the safe depth of ST19 correlated with BW, BMI
Ho 2010 [15]	10 male, 10 female	ST18, GB24	Gender, angle, side, and BMI	(1) No side difference for ST18 (2) Female subjects had greater depths generally for ST18 (3) Safe depths for ST18 and GB24 correlated significantly with BMI
Wu 2010 [16]	10 male, 10 female	LR14	Gender, angle, side, and BMI	(1) Perpendicular needling depth was greater in female subjects (2) No gender difference (3) Dangerous depth of LR14 correlated with BMI significantly
Dong 2010 [17] and Cheng and Dong 2012 [18]	10 male, 10 female	CV15, CV13	Gender, angle, BL, BW, BMI, and body cun	(1) The safe depth for CV15 ranged from 16.99 to 53.47 mm with no gender difference (2) The safe depth for CV13 ranged from 17.25 to 62.74 mm with no gender difference (3) The safe depth of both points did not correlate with BL, BW, and body cun
Wang 2009 [19]	11 male, 9 female	BL10	Gender, side, angle, BL, BW, neck girth, and thumb cun	(1) No side and angle differences noted in both groups (2) Male subjects had greater depth (3) The safe depth did not correlate with any of the parameters

TABLE 1: Continued.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Chang 2009 [20]	11 male, 9 female	GB20	Gender, angle, BL, BW, BMI, neck girth, and head girth	(1) The needling direction towards the nose tip would be the safest way (2) Mean safe depth in males ranged from 35.25 to 42.75 mm while 29.92 to 36.19 in females (3) Safe depth correlated with neck, head girth, BW, and BMI, but needling angle did not
Bai 2009 [21]	11 male, 9 female	GV16	Gender, angle, BL, BW, BMI, neck girth, and thumb cun	(1) Male subjects had greater depth with various needling angles (2) Needling depth correlated positively with BW, BMI, and neck girth (3) Safe depth ranged 27.08–45.55 mm
Wu 2009 [22]	11 male, 9 female	BL1, ST1	Gender	(1) The safe depth was defined as the 75% of dangerous depth (2) Perpendicular needling depth for BL1 was 4.14 mm (19.87 mm if along the axis of eyeball) and 17.85 for ST1 (3) With gender differences
Lu 2009 [23]	11 male, 9 female	GV15	Gender, angle, side, BL, BW, BMI, neck girth, and thumb cun	(1) Perpendicular needling depth was greater in males (2) Oblique needling depth was greater in females at an angle of around 15 degrees (3) Depth in male subjects correlated with BL, BW, and neck girth while depth in female group correlated with none of those parameters

appropriate measuring tool detecting acupuncture points in specific body regions. Unlike CT scans or traditional X-rays, MRI does not use ionizing radiation. Table 1 summarizes 14 studies (composed of 16 papers) that met the search criteria. These studies investigated 17 various acupuncture points in the head, face, chest, abdomen, and back region. Factors that may affect the measured depth including gender, body length (BL), body weight (BW), right or left side points, needling angle, body mass index (BMI), neck girth, and cun (thumb or body cun) were used for comparison. The results do not reach unanimity and contradict each other. For example, male subjects had greater safe depth only in BL18, BL19, and GV16 [8, 10, 16, 19, 21, 23]. The perpendicular depths of right side points correlated with BW, thumb cun while the depths of left side points correlated with BL, BW, BMI, and thumb cun in BL18 [8]. Needling depth correlated positively with BW, BMI, and neck girth in ST18, GB24, LR14, GB20, GV16, and GV15 [15, 16, 20, 21, 23]. On the other hand, the safe depth did not correlate with BL, BW and BMI in BL20, BL17, SP21, CV14, ST19, CV13, and CV15 [9, 11–14, 17, 18].

3.1.2. Researches Using In Vivo Evaluation Methods in Real Subjects. We recruited 8 studies that met the search criteria and are summarized in Table 2. Under this category, two studies were specifically conducted to treat patients with low back pain [24] and intervertebral herniation of cervical spines [28]. We observed a significant correlation between the interscapular distance and the thickness of the soft tissue layer with the BMI at BL25, BL26. As a result, using proportional

methods is relevant for the success of acupuncture therapy [24]. Association between nerve contact and de-qi was also discussed. The rate of median nerve penetrations by the acupuncture needle at P6 was surprisingly high, but these seemed to carry no risk of neurologic sequelae. De-qi at P6 did not depend on median nerve contact, nor did it prevent median nerve penetration which confirms the idea that acupuncture is a safe treatment method [25]. The definition of safe depth should be less than 70% of dangerous depth as suggested in most of the similar investigations [26, 27]. From the needling angle perspective, safe needling angle should be 10 degrees more than dangerous angle at 7 points from bladder meridian (1st side line) [27, 29]. The measured depth of GV14, all back bladder meridian points, and some chest points were greater than documents from ancient writings [28, 29]. The depths of all back bladder meridian points and some chest points highly correlated with body thickness and Tong Shen Cun [29]. Points of female chest had greater depth than male. De-qi depth is related to therapeutic effect; however, no correlations between the de-qi depth and electric resistance of each point in the chest and back regions were revealed [29, 31].

3.1.3. Researches Using CT Scan Images for Measurement of the Depths of Acupuncture Points. One of the mainstreams of measuring acupuncture points is using images from CT scans (13 studies were recruited in this review). One study defined the T/S ratio (therapeutic depth over safety depth). The therapeutic depth was defined as the depth at which

TABLE 2: Summary of researches using in vivo evaluation methods in real subjects.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Groenemeyer et al. 2009 [24]	58 patients with low back pain	BL25, BL26	BMI	(1) An association between de-qi and needle location existed (2) The distance between BL25 and BL6 to the vertebral line was 3.49 ± 0.58 and 3.32 ± 0.53 cm, respectively (3) There was a significant correlation between the interscapular distance and the thickness of the soft tissue layer with the BMI at both acupuncture points
Streitberger et al. 2007 [25]	50 patients receiving acupuncture including PC6 bilaterally (97 wrists)	PC6	Nerve penetrated or contacted	(1) Association between nerve contact and de-qi was discussed. De-qi was elicited in 85 cases. No association between the number of nerve contacts and de-qi was found (2) The mean distance from the needle tip to the nerve was 1.8 mm (standard deviation 2.2; range 0–11.3). Nerve contacts were recorded in 52 cases, in 14 of which the nerve was penetrated by the needle
Dong et al. 2004 [26]	32 adults and 10 cadavers	7 points from bladder meridian (2nd side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth
Li et al. 2004 [27]	32 adults and 10 cadavers	7 points from bladder meridian (1st side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side, and needling angles	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth (4) Safe needling angle should be 10 degrees more than dangerous angle
He et al. 2004 [28]	40 patients of HIVD of C spine	GV14	BL, BW, and AW	(1) Depth ranges 36–75 mm with a mean of 54.6 mm. The safe depth should be within 36 mm (2) Measured depth was greater than documents from ancient writings
Lin 1997 [29]	80 cadavers (including 30 newborns) and 240 adults for safety depth; 300 real subjects for de-qi depth	all back bladder meridian points and chest points	Gender, Tong Shen Cun, BL, BW (normal, over- and underweight) DQ, and AW	(1) Depths were deeper as compared to ancient writings. The depths highly correlated with body thickness and Tong Shen Cun (2) De-qi depth was related to therapeutic effect (3) De-qi depths of chest points were greater in females but not in back points
Lin and Wang 1994 [30]	300 adults	Total of 75 acupoints in head, neck, trunk and lower limb	Gender, BW, and DQ	(1) Discussed de-qi depth but not safe depth (2) Depth of de-qi was greater in males and people with greater body weight (3) Depths in neck region were more superficial in trunk and limbs
Lin 1991 [31]	107 adults	Acupoints in the chest and back of subjects receiving acupuncture therapy	Gender, BW (normal, over- and underweight), BL, and DQ	(1) Overweight group had the greatest de-qi depth (2) Points of female chest had greater depth than male (3) No correlations between the de-qi depth and electric resistance of each point

TABLE 3: Summary of researches using CT scan images for measurement of the depths of acupuncture points.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Chen et al. 2009 [32]	204 pediatric patients aged 7–15	12 abdominal acupuncture points CV-3, CV-4, CV-6, CV-10, CV-12, CV-14, KI-12, ST-24, ST-25, SP-15 LV-13, and LV-4	Gender, age, BW, and waist girth	(1) Using the therapeutic depth over safety depth ratio (T/S ratio) as the indicator of therapeutic depth (2) No significant difference in the T/S ratio between genders (3) The T/S ratio of these 12 acupuncture points ranged from 0.67 to 0.88 and increased significantly with body weight, age, and waist girth (4) The therapeutic depth of abdominal acupoints was closer to the safe depth in overweight and older children aged 7 to 15 (5) No significant difference between genders
Groenemeyer et al. 2009 [24]	58 patients with low back pain	BL25, BL26	BMI	(1) An association between de-qi and needle location existed (2) The distance between BL25 and BL6 to the vertebral line was 3.49 ± 0.58 and 3.32 ± 0.53 cm, respectively (3) There was a significant correlation between the interscapular distance and the thickness of the soft tissue layer with the BMI at both acupuncture points
Yang et al. 2008 [33]	41 adults	GV16	Rohrer index	(1) The safe needling depth should be less than 75% of the dangerous depth (2) The safe depths of GV16 were different for persons of different somatotypes ranging 27.73–33.39 mm
Chen et al. 2008 [34]	219 pediatric patients aged 7–15	12 acupoints along the conception vessel (CV): CV-2 to CV-7 and CV-9 to CV-14	Gender, age, BW, and waist girth	(1) The safe depth of 12 acupoints significantly increased with age, body weight, and waist girth in pediatric patients aged 7–15 (2) There were large variations of the 12 points among different age and body weight groups (3) The safe depths were 1.3–2.1 times deeper in the 12–15-year-old group than in the 7–9-year-old group and 1.7–3 times deeper in overweight children than in underweight children
Chern et al. 2006 [35]	32 adults	BL13	Rohrer index (<1.2, 1.2–1.5, and >1.5), side	(1) Right side points seemed to be deeper, especially in people with Rohrer index <1 (2) Safety depth should be within 75% of the measured distance in each group; that is, 34, 25, and 23 mm
Li et al. 2005 [36]	32 adults	GV14, SI15, GV5, and GV4	Rohrer index: <1.2, 1.2–1.5, and >1.5	The safe depths (75% of dangerous depths) were different for different somatotypes; for example, the needling depth for GV14 was 32.86 ± 3.96 mm for the thin person group and 47.93 ± 5.30 mm for the fat person group
Dong et al. 2004 [26]	32 adults and 10 cadavers	7 points from bladder meridian (2nd side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth

TABLE 3: Continued.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Li et al. 2004 [27]	32 adults and 10 cadavers	7 points from bladder meridian (1st side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side, and needling angles	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth (4) Safe needling angle should be 10 degrees more than dangerous angle
Lin 1997 [29]	80 cadavers (including 30 newborns) and 240 adults for safety depth; 300 real subjects for de-qi depth	All back bladder meridian points and chest points	Gender, Tong Shen Cun, BL, BW (normal, over- and underweight) DQ, and AW	(1) Depths were deeper as compared to ancient writings. The depths highly correlated with body thickness and Tong Shen Cun (2) De-qi depth was related to therapeutic effect (3) De-qi depths of chest points were greater in females but not in back points
Sheu and Lin 1992 [37]	120 adults	28 points in the chest from conception vessel, kidney meridian, stomach meridian, pericardium meridian, lung meridian, spleen meridian, and gallbladder meridian	gender, BW (normal, over- and underweight), and BL	(1) Significant differences in chest points within the same sex existed (2) For different body sizes, statistically significant differences for each point appeared
Lin et al. 1991 [38]	240 adults (120 in each group)	22 points in the back; 28 points in the chest	Gender, BW (normal, over- and underweight), and BL	(1) No gender differences on back loci (2) Significant differences in each point with different body sizes (3) Female chest points had greater depths (4) The results should be more accurate than cadaver study

the needle is in the muscular layer of specific acupuncture point. Chen et al. suggested that the T/S ratios were between 0.67 (SP-15) and 0.88 (CV-6, CV-10). The therapeutic depth of abdominal acupoints was closer to the safe depth in overweight and older children aged 7 to 15 [32]. As for the definition of safe needling depth, it should be less than 75% of the dangerous depth [33, 35, 36], but there were two studies reported that it should be report that to be 70% of the dangerous depth [26, 27]. Depths from in vivo CT images revealed that they were greater than the ones retrieved from cadavers [26, 27]. According to the research of Lin, there were significant differences in chest points within the same sex, however, female chest points had greater depths [37]. In children subjects, the safe depths of studied points (CV-2 to CV-7 and CV-9 to CV-14) were 1.3–2.1 times deeper in the 12–15-year-old group than in the 7–9-year-old group and 1.7–3 times deeper in overweight children than in underweight children. The depths increased significantly with age and body size yet with large variations [34].

3.1.4. Researches Using Ultrasound Images for Measurement of the Depths of Acupuncture Points. We included 2 studies under this category. Lian suggested that needling depths of acupoints BL11 to BL21 ranged from 12–40 mm and not affected by age, body sizes, and disease types. Gender and side differences also existed, and depths measured were shorter

compared to ancient writings [79]. Streitberger et al. found that there was no association between the number of nerve contacts and de-qi when needling at PC6, and the mean distance from the needle tip to the nerve was 1.8 mm (standard deviation 2.2; range 0–11.3). The ultrasound has the advantage of acquiring real-time images; as a result, the authors were able to observe actual nerve contacts by the needle tip, and thus the possible complication of nerve penetration was recorded for analyses (leaving no neurological sequelae) [25].

3.1.5. Researches Using Dissected Specimens of Cadavers for Measurement of the Depths of Acupuncture Points. We included 21 investigations under this category. This is another measuring method used extensively in early investigations which continues to be valuable for certain points. A few studies defined the safe depth and dangerous depth for needling, respectively. For example, five studies suggested that the safe depth should be less than 70% of dangerous depth [26, 27, 86, 89, 95, 96]. Yan et al. suggested that the safe depth of GV15, GV16, GB20, and BL1 should be 80% of the measured depth [97]. Li et al. defined the safe depths to be within 75% of the measured depths because they used in vivo CT images, which should be greater than the ones from cadavers [27]. In short, most of the authors chose 75% or 80% from their clinical experience rather than conclusive anatomical evidence. Consequently, there is no universal

TABLE 4: Summary of researches using ultrasound images for measurement of the depths of acupuncture points.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Lian 1995 [79]	89 adults	From BL11 to BL21 (11 points)	Gender, age, BL, BW, disease type, side, and AW	(1) Depths ranged 12–40 mm (2) Depth was not affected by age or body sizes (3) Points of male subjects and right side of the body had greater depths (4) Disease type did not affect depth (5) Depths measured were shorter compared to ancient writings
Streitberger et al. 2007 [25]	50 patients receiving acupuncture including PC6 bilaterally (97 wrists)	PC6	Nerve penetrated or contacted DQ	(1) Association between nerve contact and de-qi was discussed. De-qi was elicited in 85 cases. No association between the number of nerve contacts and de-qi was found (2) The mean distance from the needle tip to the nerve was 1.8 mm (standard deviation 2.2; range 0–11.3). Nerve contacts were recorded in 52 cases, in 14 of which the nerve was penetrated by the needle

definition of dangerous depth, safe depth, or therapeutic depth. Wang proposed that the needling depth of ST7 and SI18 revealed no side difference, and the mean inserting depths from skin surface to sphenopalatine ganglion are 49.9 and 46.6 mm, respectively, which may change by different puncturing directions [81]. As for gender and side perspective, there was no gender difference in dangerous depth in SI14, ST12, BL1, L12, BL13, GV14, GV15, GV16, and GB20 and acupoints in back and lumbar regions [82, 84, 85, 95]. There was no side difference in dangerous depth in ST7, ST12, SI18, GV15, GV16, and GB20, 7 points from bladder meridian (2nd side line), 7 points from bladder meridian (1st side line), 28 acupoints in back and lumbar regions, and 23 chest points [26, 27, 81, 84, 95–97]. Details of each research are summarized in Table 5.

3.2. The Needling Depth of Clinical Efficacy. This section is composed of 11 researches. Lin had first investigated the needling depths of acupuncture points regarding de-qi in a series of researches since 1991 [29–31]. He proposed that de-qi depth was related to therapeutic effect. Depth of de-qi was greater in males and people with greater body weight except for chest points in females. He also found no correlations between the de-qi depth and electric resistance of the acupuncture points. Chen et al. also used the therapeutic depth over safety depth ratio (T/S ratio) as the indicator of therapeutic depth. There was no significant difference in the T/S ratio between genders, and the T/S ratio of these 12 acupuncture points ranged from 0.67 to 0.88 and increased significantly with body weight, age, and waist girth [32]. Groenemeyer et al. suggested that an association between de-qi and needle location exists [24]. However, Streitberger et al. found no association between the number of nerve contacts and de-qi [25]. The depth of needle penetration counted for the clinical efficacy of relief of muscle pain [102]. Deep

puncturing at ST7 was more effective than routine puncturing, and the total effective rate in deep puncturing group is superior than that in shallow puncturing group [98]. Itoh et al. suggested that immediate pain relief in muscle group (deep insertion for 10 mm) was better than that in skin group (insertion for 3 mm) [102]. Deeper insertion also induced more dull sensations as compared to shallow insertions which induced more sharp sensations. In addition, needle rotation significantly increased the dull sensations [99]. Lu and Tang confirmed the various needling depths ranged from 2–12 mm as documented in Lingshu (Miraculous Pivot) used for treating irritable bowel syndrome of diarrhea [101].

4. Discussion

To our knowledge, this is the most comprehensive research to review all the studies regarding safe needling depth and clinical efficacy of acupuncture points. We tried to include researches as many as possible to provide a solid foundation for evidence based medicine in terms of advisable needling depth when performing acupuncture treatment. Evidence based medicine allows researchers or clinicians nowadays to ask a more extensive spectrum of miscellaneous research questions such as “Is acupuncture more effective than placebo?” or “Is CAM therapy along with wanted care more effective than wanted care alone?” These issues could be dealt with through appropriate study designs.

Studies of acupuncture safety indeed need special attention to needling depth issues. For example, the relationship between the effect of acupuncture analgesia and needling direction, angle, and depth has been optimized to enhance the analgesic effect as suggested by Fan et al. The result shows that all the 3 factors are the key influences. However, studies addressed the more specific correlation between needling depth, and issues aroused from safety or clinical efficacy are still far from enough [103].

TABLE 5: Summary of researches using dissected specimens of cadavers for measurement of the depths of acupuncture points.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Oh et al. 2012 [80]	4 adult cadavers	PC6	—	(1) The acupuncture needle should not be inserted deeply at PC6 in order to minimise the risk of trauma (2) It seemed likely that various kinds of noninvasive stimulation at PC6 may be similarly effective as needling (3) Careful insertion of the needle with respect to patients' sensations and better anatomical knowledge of the forearm can help to prevent unexpected needle penetration of the median nerve or persistent median artery
Wang et al. 2009 [81]	15 adult cadavers	ST7, SI18	Side	(1) No side difference (2) Mean inserting depth of ST7 and SI18 to sphenopalatine ganglion were 49.9 and 46.6 mm
Xie et al. 2007 [82]	46 adult cadavers	SI14 and GV14	Gender	(1) No gender difference (2) The mean dangerous depth for perpendicular insertion was 60.60 mm for SI14 and 55.93 mm for GV14. (3) Suggested depth for perpendicular needling of SI14 and GV14 was within 42 mm in adult
Chen et al. 2007 [83]	46 cadavers	CV22, ST11	—	(1) The needle not only easily injured the upper pleural cavity but also damaged the big blood vessel, the vagus nerve in the mediastinum and the cervical root (2) The safety depth of ST11 ranged 23.7–52.8 mm
Xie et al. 2006 [84]	46 cadavers	ST12	Gender, side	(1) No gender or side difference (2) The mean dangerous depth of male was 34.97 mm and female 31.41 mm (3) The depth for perpendicular needling of SI12 is within 22.50 mm
Xie et al. 2006 [85]	46 cadavers	BL12, BL13	Gender, angles of needle insertion (15, 20, 25, 30, and 40 degrees)	(1) The mean dangerous depth for perpendicular insertion was 49.51 mm of BL12 and 44.88 mm of BL13 (2) It was safe for oblique insertion toward the medial of chest in an angle exceeding 20 degrees (3) No gender difference
Xu et al. 2006 [86]	48 adult cadavers	BL1	—	(1) The mean depth between the skin and the anterior ethmoidal artery was 18.25 ± 4.45 mm, with an angle of 12.5 ± 5.5 degrees (2) The depth from the skin to the optic nerve tunnel frontal point was 43.37 ± 7.84 mm (3) Needling depth should not exceed 30.36 mm (70% of measured depth) to avoid injury of the optic nerve
Lou et al. 2006 [87]	80 limbs (40 cadavers)	ST36	Angle	(1) Average depth was 2.22 cm (2) Maximal depth was 4.42 cm if needled obliquely (3) Safe depth was generally less than 5 cm

TABLE 5: Continued.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Chen et al. 2006 [88]	46 cadavers	CV22, ST11, ST12, GB21, EX-B1, and BL11	—	(1) Risk of pleural injury may exist when inserting needle perpendicularly in these points (2) Divergence existed in observed needling depths such as 22.5–61.3 mm for ST12
Dong et al. 2004 [26]	32 adults and 10 cadavers	7 points from bladder meridian (2nd side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth
Li et al. 2004 [27]	32 adults and 10 cadavers	7 points from bladder meridian (1st side line)	Rohrer index: <1.2, 1.2–1.5, and >1.5, side, and needling angles	(1) No side difference (2) Depths from in vivo CT images were greater than ones from cadavers (3) Safe depth should be less than 70% of dangerous depth (4) Safe needling angle should be 10 degrees more than dangerous angle
Yan et al. 2004 [89]	51 cadavers	74 points from neck, chest, back, and abdomen	Angles of insertion	(1) Safe depth should be less than 70% of dangerous depth (2) Needling angles were suggested such as 65 degrees rather than perpendicular insertion for points in the bladder meridian
Zhang et al. 2001 [90]	51 cadavers	17 acupoints of abdomen	Gender, side	(1) The dangerous depth of most abdominal points were similar and within 11–17 mm (2) KI11 had the greatest depth up to 25 mm (to the urinary bladder) but with divergence in standard error
Zhang et al. 2001 [91]	57 cadavers	ST12	—	(1) The mean dangerous depths for perpendicular insertion downward was 38.34 mm (2) Safety depth was within 26.83 mm (70% of the dangerous depth)
Piao and Zhong 2001 [92]	6 cadavers	B2 (lumbar levels)	different lumbar vertebrae: L1–L5	The depth ranged 4.2–5.75 cm in different lumbar levels with greater depths in lower lumbar levels
Chen et al. 1998 [93]	20 adult cadavers	BL40	Side	(1) Safe depth (from skin to tibial nerve): 15 mm for left side and 16 mm for right side, less than the depth from current used textbook (2) Depth from skin to deep vein: 35 mm
Ge 1998 [94]	16 cadavers	GV15, GV16	Thumb Tong Shen Cun	Safe depth of GV15 (42.46–55.86 mm) and GV16 (43.46–57.42 mm) correlated with thumb Tong Shen Cun
Zhang et al. 1998 [95]	51 cadavers	Total of 28 acupoints in back and lumbar region	Gender, side	(1) No side difference in dangerous depth except BL17, BL18 for male and BL17 for female (2) Points of bladder meridian closer to the spine had greater depths. Divergence existed between points (3) There was no gender/side difference

TABLE 5: Continued.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Zhang et al. 1998 [96]	51 cadavers	23 chest points	Gender, side	(1) The average dangerous depths of 23 chest acupoints were obtained. KI27 had the greatest dangerous depth up to 26 mm. Others ranged from 11.87 to 17.64 mm (2) Divergence existed between points (3) There was no gender/side difference (4) Safe depth should be less than 70% of dangerous depth
Lin 1997 [29]	80 cadavers (including 30 newborns) and 240 adults for safety depth; 300 real subjects for de-qi depth	All back bladder meridian points and chest points	Gender, Tong Shen Cun, BL, BW (normal, over- and underweight) DQ, and AW	(1) Depths were deeper as compared to ancient writings. The depths highly correlated with body thickness and Tong Shen Cun (2) De-qi depth was related to therapeutic effect (3) De-qi depths of chest points were greater in females but not in back points
Yan et al. 1996 [97]	51 cadavers	GV16, GV15, GB20, and BL1	Gender, side	(1) The safe depths (80% of the measured depth) were GV16: 40.08 mm, GV15: 38.10 mm, GB20: 39.77 mm, and BL1: 34.25 mm (2) No gender or side difference

Most of the researches included in this review are retrospective in nature along with small sample size and lack randomization and control group, and most of the researches did not apply the WHO Standard Acupuncture Point Locations, let alone that they employed different measuring tools. Therefore, we observed the great inconsistency of measured needling depth among different subject groups. The inconsistency may also result from the following variables not strictly controlled in most of the researches:

- (1) the difference in ethnicity;
- (2) the age of the subjects;
- (3) the gender difference;
- (4) the subjects were not all divided in to groups by a more specific index for body sizes such as BMI;
- (5) most of the subjects were not grouped by their underlying condition, that is, healthy subjects or with specific medical conditions;
- (6) the definitions of safe needling depth, dangerous needling depth were obscure including the needling angle of specific acupuncture point and which side of the body the points were located.

All these facts lead to discrepancy in the safe depth measurements. For example, the suggested safe depths for GV16 are 27.05–45.55 mm (using MRI images) [21], 27.73–33.39 mm (using CT images) [33], 40.08 mm (using dissected specimens) [97], and 43.46–57.42 mm (using dissected specimens) [94].

In order to lift the level of acupuncture safety, it is necessary to utilize modern imaging technology to explore

the safe depth of each acupuncture point all over the human body. We should take the regional and anatomical properties of different groups of points into consideration when deciding the measuring tools. For example, MRI imaging is very suitable for the acupuncture points in the abdominal and back regions due to the excellent ability to obtain soft tissue details while CT scan images are extremely helpful when detecting chest points. Ultrasound is a convenient tool which can obtain real-time images when trying to observe the clinical efficacy simultaneously, especially applied in vivo subjects. Using direct specimen dissections of cadavers is another measuring method. After frozen, anticorrosive, positioning, and dyeing process, the original elasticity and properties of the corpse tissues have been lost; the concerns which may arise from this kind of study are that specimens are drier and smaller than living human tissues. This may lead to possible inconsistency in measured needling depth. For example, Li et al. [27] and Dong et al. [26] compared the depths of 7 points from bladder meridian and found that depths measured via in vivo CT images were greater than ones from dissections of cadavers.

The measurement of needling depth involves the detection of soft tissue mostly. Different measurement methods often result in possible discrepancies due to the characteristic of each measuring tool. For example, Fiirgaard et al. suggested that MRI is superior in estimating the volume of acoustic neuroma than CT scan and with less inter-examiner difference [104]. MRI was reported to be better in identifying suspected disease of the brain and cervical spinal cord [105]. The sensitivity for detecting bony osteolytic lesions was 51.7% for radiography, 74.7% for computed tomography, and 95.4% for magnetic resonance imaging as reported by Walde et al. [106].

TABLE 6: Summary of researches involving the correlation between therapeutic effect and needling depth.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
He et al. 2012 [98]	63 subjects with trigeminal neuralgia (32 in deep and 31 in shallow puncturing group)	ST7, LI4, LV3, BL2, ST2, and Jiacheng Jiang	Pain index (VAS), traditional Chinese medicine symptoms index, and clinical therapeutic effect	(1) The total effective rate was 93.8% in deep puncturing group, superior to that of 87.1% in shallow puncturing group (2) No adverse reaction was observed in both groups (3) Deep puncturing at ST7 to the depth of sphenopalatine ganglion was more effective than routine puncturing
Park et al. 2011 [99]	5 participants	LI13, LU4 and 2 control points	Needling depth, needle rotation, and oscillation	(1) Pilot study using ultrasound tried to explore the correlation between de-qi sensation and needling depth/needle manipulation (2) Shallower insertion induced more sharp sensations (3) Deeper insertion induced more dull sensations (4) Needle rotation significantly increased the dull sensations
Skjeie et al. 2011 [100]	7 randomized patients (3 in placebo group and 4 in acupuncture treatment group)	ST36	Bilateral insertion at ST36 at the depth of 12 mm, reduction of crying time from baseline	(1) A pilot, open, randomized, and single-blinded controlled trial to assess the feasibility of acupuncture treatment for infantile colic (2) No adverse events were reported (3) Acupuncture group had more reduction of crying time from baseline
Lu and Tang 2011 [101]	21 cases of irritable bowel syndrome of diarrhea	IR13, CV12, ST25, CV4, LR14, LI11, LI4, SP9, ST36, and LR3	The scale for the severity degree of symptom (IBS-SSS)	(1) Various needling depths ranged 2–12 mm as documented in Lingshu (Miraculous Pivot) (2) After treatment, there was significant change in IBS-SSS, and the effective rate may reach 90.5% (3) The longer the session of treatment was, the better the efficacy was obtained (4) Confirmed the needling depth recorded in Lingshu
Itoh et al. 2011 [102]	22 healthy volunteers	Tender point in the extensor digital muscle, in the skin, and in the nonsegmental limb (anterior tibial muscle)	Pressure pain threshold, electrical pain threshold, and needling depth (3 mm to 10 mm)	(1) Randomized controlled trial (2) Immediate pain relief in muscle group (depth of 10 mm insertion into extensor digital muscle) which was better than skin group (depth of 3 mm) (3) Acupuncture stimulation of muscle increases the PPT and EPT of fascia. The depth of needle penetration was important for the relief of muscle pain
Chen et al. 2009 [32]	204 pediatric patients aged 7–15	12 abdominal acupuncture points CV-3, CV-4, CV-6, CV-10, CV-12, CV-14, KI-12, ST-24, ST-25, SP-15 LV-13, and LV-4	Gender, age, BW, and waist girth	(1) Using the therapeutic depth over safety depth ratio (T/S ratio) as the indicator of therapeutic depth (2) No significant difference in the T/S ratio between genders (3) The T/S ratio of these 12 acupuncture points ranged from 0.67 to 0.88 and increased significantly with body weight, age, and waist girth (4) The therapeutic depth of abdominal acupoints was closer to the safe depth in overweight and older children aged 7 to 15 (5) No significant difference between genders

TABLE 6: Continued.

Authors and year	Subjects and sample size	Investigated acupuncture points and their body regions/meridians	Parameters used in comparison and related factors	Results, suggestions, and conclusions
Groenemeyer et al. 2009 [24]	58 patients with low back pain	BL25, BL26	BMI	(1) An association between de-qi and needle location existed (2) The distance between BL25 and BL6 to the vertebral line is 3.49 ± 0.58 and 3.32 ± 0.53 cm, respectively (3) There was a significant correlation between the interscapular distance and the thickness of the soft tissue layer with the BMI at both acupuncture points
Streitberger et al. 2007 [25]	50 patients receiving acupuncture including PC6 bilaterally (97 wrists)	PC6	Nerve penetrated or contacted DQ	(1) Association between nerve contact and de-qi was discussed. De-qi was elicited in 85 cases. No association between the number of nerve contacts and de-qi was found (2) The mean distance from the needle tip to the nerve was 1.8 mm (standard deviation 2.2; range 0–11.3). Nerve contacts were recorded in 52 cases, in 14 of which the nerve was penetrated by the needle
Lin 1997 [29]	80 cadavers (including 30 newborns) and 240 adults for safety depth; 300 real subjects for de-qi depth	All back bladder meridian points and chest points	Gender, Tong Shen Cun, BL, BW (normal, over and under-weight) DQ, and AW	(1) Depths were deeper as compared to ancient writings. The depths highly correlated with body thickness and Tong Shen Cun (2) De-qi depth was related to therapeutic effect (3) De-qi depths of chest points were greater in females but not in back points
Lin and Wang 1994 [30]	300 adults	Total of 75 acupoints in head, neck, trunk, and lower limb	Gender, BW, and DQ	(1) Discussed de-qi depth but not safe depth (2) Depth of de-qi was greater in males and people with greater body weight (3) Depths in neck region were more superficial in trunk and limbs
Lin 1991 [31]	107 adults	Acupoints in the chest and back of subjects receiving acupuncture therapy	Gender, BW (normal, over- and underweight), BL, and DQ	(1) Overweight group had the greatest de-qi depth (2) Points of female chest had greater depth than male (3) No correlations between the de-qi depth and electric resistance of each point

Yet MRI scanogram is slightly less accurate compared with radiographic scanogram in detecting limb length differences [107].

In addition, we also found several studies discussing the divergence among different measuring tools when investigating various body tissues including adipose volume (visceral or subcutaneous), muscle thickness, bone loss, and cartilage thickness. All these body tissues are relevant to the measurement of acupuncture points. However, the results indicated that no single measuring method would be more suitable than the other one (MRI, CT, and ultrasound). There was no significant difference between CT and MRI in the measurement of adipose tissue, glenoid bone loss, hip cartilage thickness, pheochromocytoma and in vivo skeletal muscle. Ultrasound was as good as MRI in the evaluation of supraspinatus and deltoid muscle with high correlation coefficients (0.96 and 0.97, resp.). MRI was also proved to be

as precise as direct cadaver measurement to evaluate adipose tissue.

There was no difference using either ultrasound or CT for the measurement of visceral fat volume. While ultrasound was as good as CT and MRI to assess intra-abdominal adipose tissue [108–117].

Some authors compared the results of MRI/CT imaging with cadavers in measuring adipose tissue [110], skeletal muscle [115], and hyaline cartilage thickness of hips [117] and found that both MRI and CT can serve as a good reference tool in the measurement. But Stevens-Simon et al. reported that the results of their study do not support the validity of ultrasound measurement of visceral adiposity as a measure of central adiposity in postpartum teenagers [118].

Consequently, the results in terms of measuring specific body tissues using different measuring methods are ambiguous and sometimes conflicting with each other. This fact may

TABLE 7: Mean values for needling depths of acupoints in the chest in different-sized male subjects.

Acupoints	Overweight adults	Normal adults	Underweight adults	<i>F</i>
	Mean \pm 95% C.I.	Mean \pm 95% C.I.	Mean \pm 95% C.I.	
Tiantu (CV22)	3.94 \pm 0.19	2.87 \pm 0.26	2.34 \pm 0.65	19.61*
Xuanji (CV21)	0.94 \pm 0.11	0.64 \pm 0.10	2.30 \pm 0.10	38.22*
Huagai (CV20)	0.89 \pm 0.11	0.057 \pm 0.10	0.25 \pm 0.10	39.46*
Zigong (CV19)	0.89 \pm 0.11	0.60 \pm 0.10	0.29 \pm 0.10	35.79*
Yutang (CV18)	0.88 \pm 0.14	0.53 \pm 0.10	0.28 \pm 0.09	31.32*
Danzhong (CV17)	0.86 \pm 0.11	0.52 \pm 0.11	0.25 \pm 0.09	36.96*
Zhongting (CV16)	0.95 \pm 0.14	0.56 \pm 0.13	0.34 \pm 0.11	25.57*
Shufu (KI27)	4.19 \pm 0.46	3.29 \pm 0.48	2.32 \pm 0.49	16.20*
Yuzhong (KI26)	2.98 \pm 0.27	2.20 \pm 0.25	1.50 \pm 0.27	34.18*
Shencang (KI25)	2.59 \pm 0.21	2.00 \pm 0.21	1.26 \pm 0.22	42.93*
Lingxu (KI24)	2.56 \pm 0.17	2.13 \pm 0.23	1.49 \pm 0.19	32.09*
Shenfeng (KI23)	2.47 \pm 0.17	1.96 \pm 0.17	1.44 \pm 0.17	40.78*
Bulang (KI22)	2.33 \pm 0.15	1.95 \pm 0.17	1.46 \pm 0.15	33.64*
Qihu (ST13)	5.24 \pm 0.48	4.15 \pm 0.55	2.88 \pm 0.47	23.71*
Kufang (ST14)	3.82 \pm 0.36	3.10 \pm 0.31	2.02 \pm 0.29	32.21*
Wuyi (ST15)	3.11 \pm 0.27	2.64 \pm 0.52	1.38 \pm 0.17	26.51*
Yingchuang (ST16)	2.78 \pm 0.21	2.35 \pm 0.53	1.33 \pm 0.22	19.52*
Ruzhong (ST17)	2.59 \pm 0.19	2.07 \pm 0.38	1.23 \pm 0.20	27.28*
Rugen (ST18)	2.27 \pm 0.16	1.78 \pm 0.22	1.19 \pm 0.20	33.89*
Tianchi (PC1)	2.64 \pm 0.21	2.25 \pm 0.72	1.18 \pm 0.21	11.97*
Yunmen (LU1)	6.73 \pm 0.55	5.14 \pm 0.57	3.26 \pm 0.79	32.78*
Zhongfu (LU2)	5.05 \pm 0.62	3.69 \pm 0.47	2.20 \pm 0.70	24.06*
Zhourong (SP20)	3.71 \pm 0.50	2.70 \pm 0.35	1.68 \pm 0.51	21.06*
Xiongxiang (SP19)	3.15 \pm 0.37	2.26 \pm 0.23	1.53 \pm 0.43	23.38*
Tianxi (SP18)	2.88 \pm 0.29	2.05 \pm 0.25	1.32 \pm 0.19	40.68*
Shidou (SP17)	2.61 \pm 0.21	1.91 \pm 0.22	1.28 \pm 0.18	45.01*
Zhejin (GB23)	3.48 \pm 0.39	2.43 \pm 0.26	1.73 \pm 0.37	28.00*
Yuanye (GB22)	4.52 \pm 0.41	3.07 \pm 0.34	2.20 \pm 0.33	43.58*

X : mean depth; units are provided in centimeters.

X \pm 1.96 SD: 95% confidence interval.

* *P* < 0.01; *F* is the statistic for one-way ANOVA.

The definitions for “Overweight adults,” “Normal adults,” and “Underweight adults” are following the guidance of the Department of Health, Taiwan: “The suggested ideal body weight of Taiwanese people.” As such, readers from outside Taiwan should bear in mind that ideal body weights differ between countries. The specified needling depths in the table are a suggested guide only.

partially explain the inconsistency of needling depth of each acupuncture point as reviewed in our study.

Some authors discussed the interobserver reliability as well. Botser et al. thought that CT was found to have higher interobserver reliability than MRI when deciding the degree of femoral anteversion [119]. MRI was again found to have less interexaminer difference in calculating the soft tissue volume [104]. Both studies pointed out that some of the measuring errors may come from the man-made technical faults.

As a result, it is very difficult to obtain a general conclusion regarding safe needling depth using these results; future researches are warranted which will be discussed later in the paper.

Some theses about using MRI for safe needling depth detection we included in this study seem to have the same sample population (number, sex ratio, age, and BMI are consistent with each other) which may result in bias in the

interpretation of study results. In addition, the sample size is limited to 20 subjects which lack the power for further analysis of gender, age, and body size differences.

From the review, we learn that many factors may influence the measurement of needling depth. They include gender, age, body size (such as body length, body weight, waist girth, and BMI), which side of the body, angle of needling, and so forth. Chou et al. have reviewed part of the researches previously [120]. Among these factors, body size is always considered the most significant one which complies with our general understanding that subjects with greater body size would have greater measured depth in most of the acupuncture points. However, anatomical and structural difference exists in different human body regions when we take account of the factor like BMI.

The measured depth in body region with frequent accumulation of fat would be highly correlated with BMI. BMI

TABLE 8: Mean values for needling depths of acupoints in the chest in different-sized female subjects.

Acupoints	Overweight adults	Normal adults	Underweight adults	<i>F</i>
	Mean \pm 95% C.I.	Mean \pm 95% C.I.	Mean \pm 95% C.I.	
Tiantu (CV22)	4.46 \pm 0.42	3.69 \pm 0.46	3.01 \pm 0.80	7.39*
Xuanji (CV21)	1.37 \pm 0.27	1.03 \pm 0.25	0.41 \pm 0.33	9.81*
Huagai (CV20)	1.18 \pm 0.29	0.88 \pm 0.23	0.49 \pm 0.38	5.24*
Zigong (CV19)	1.32 \pm 0.24	0.97 \pm 0.19	0.50 \pm 0.40	9.73*
Yutang (CV18)	1.42 \pm 0.25	1.00 \pm 0.20	0.56 \pm 0.46	9.89*
Danzhong (CV17)	1.55 \pm 0.23	1.07 \pm 0.22	0.71 \pm 0.57	8.63*
Zhongting (CV16)	1.75 \pm 0.003	1.41 \pm 0.32	0.90 \pm 0.76	4.17*
Shufu (KI27)	4.31 \pm 0.72	3.36 \pm 0.47	2.23 \pm 0.46	9.90*
Yuzhong (KI26)	2.93 \pm 0.44	2.46 \pm 0.33	1.53 \pm 0.55	9.31*
Shencang (KI25)	2.69 \pm 0.39	2.29 \pm 0.29	1.53 \pm 0.46	8.35*
Lingxu (KI24)	2.70 \pm 0.35	2.40 \pm 0.26	1.69 \pm 0.57	7.19*
Shenfeng (KI23)	2.89 \pm 0.32	2.46 \pm 0.24	1.79 \pm 0.60	9.52*
Bulang (KI22)	2.95 \pm 0.30	2.49 \pm 0.23	1.79 \pm 0.45	11.15*
Qihu (ST13)	4.73 \pm 0.77	3.75 \pm 0.57	2.46 \pm 1.10	8.14*
Kufang (ST14)	3.51 \pm 0.51	3.04 \pm 0.45	2.06 \pm 1.07	5.52*
Wuyi (ST15)	3.05 \pm 0.52	2.72 \pm 0.38	1.76 \pm 0.82	5.61*
Yingchuang (ST16)	2.94 \pm 0.47	2.66 \pm 0.37	1.90 \pm 0.78	4.08*
Ruzhong (ST17)	2.91 \pm 0.42	2.58 \pm 0.33	1.93 \pm 0.62	4.73*
Rugen (ST18)	2.9200 \pm 0.4038	2.444 \pm 0.3052	1.6857 \pm 0.5636	9.4451*
Tianchi (PC1)	3.34 \pm 0.69	2.86 \pm 0.37	1.89 \pm 0.73	5.37*
Yunmen (LU1)	5.84 \pm 0.79	4.42 \pm 0.76	3.51 \pm 1.59	6.52*
Zhongfu (LU2)	4.43 \pm 0.55	3.74 \pm 0.47	2.81 \pm 1.45	5.39*
Zhourong (SP20)	3.82 \pm 0.59	3.49 \pm 0.46	2.37 \pm 1.15	4.72*
Xiongxiang (SP19)	3.70 \pm 0.53	3.31 \pm 0.42	2.23 \pm 0.82	6.40*
Tianxi (SP18)	3.41 \pm 0.53	3.06 \pm 0.44	2.07 \pm 0.79	5.25*
Shidou (SP17)	3.34 \pm 0.53	2.80 \pm 0.40	1.83 \pm 0.72	7.49*
Zhejin (GB23)	3.63 \pm 0.57	3.43 \pm 0.43	2.24 \pm 0.99	5.24*
Yuanye (GB22)	4.06 \pm 0.64	3.72 \pm 0.54	2.39 \pm 1.08	5.48*

X : mean depth; units are provided in centimeters.

X \pm 1.96 SD: 95% confidence interval.

**P* < 0.01; *F* is the statistic for one-way ANOVA.

The definitions for “Overweight adults,” “Normal adults,” and “Underweight adults” are following the guidance of the Department of Health, Taiwan: “The suggested ideal body weight of Taiwanese people.” As such, readers from outside Taiwan should bear in mind that ideal body weights differ between countries. The specified needling depths in the table are a suggested guide only.

is the most widely used method to show the increase in fat amount in the whole body. Nevertheless, BMI does not reliably reflect the body fat composition as only body weight and length are taken into consideration [121]. Fat distribution and sexual dimorphism further explain the likely existing gender difference [122, 123]. Mathematician Nick Trefethen believes that the body mass index formula traditionally used to work out if someone is overweight is flawed, and he has come up with his own formula. And he found short people are actually more overweight than they think they are, while tall people are not as overweight as they are being told (Daily Mail. PUBLISHED: 00:08 GMT, January 21, 2013). He claimed a new method for calculating BMI, but again only body height and weight are considered as reference factors. Probably along with waist and neck girth, simple anthropometric measures could amend the weakness of BMI as a single indicator of body size.

We hereby recommend future research suggestions regarding determining the safe needling depth. Firstly, an international congress should be convened to reach unanimous agreement on the definition of safe needling depth of each acupuncture point and acupuncture point localization method for the future study design. Secondly, factors like gender, age, BMI (or other index to differentiate body sizes), right/left side of the limb, insertion angle, and de-qi should be controlled as much as possible. Subjects (maybe crossing races) should be as many as possible, and the study design should better be randomized control studies. We should try our best to cover every acupuncture point in the whole body. Thirdly, in vivo research is better than retrospective images or specimen dissections. MRI seems to be a better tool to obtain more detailed information of anatomical structures surrounding the acupuncture needle, especially the soft tissue. Fourthly, we also suggest that multicenter

TABLE 9: Mean values for needling depths of acupoints in the back from different-sized male subjects.

Acupoints	Overweight adults	Normal adults	Underweight adults	<i>F</i>
	Mean \pm 95% C.I.	Mean \pm 95% C.I.	Mean \pm 95% C.I.	
Dazhui (GV14)	6.76 \pm 0.41	5.39 \pm 0.40	4.81 \pm 0.54	20.90*
Taodao (GV13)	6.35 \pm 0.40	5.24 \pm 0.40	4.66 \pm 0.47	17.03*
Shenzhu (GV12)	5.39 \pm 0.37	4.79 \pm 0.35	4.10 \pm 0.34	14.13*
Shendao (GV11)	4.86 \pm 0.32	4.30 \pm 0.30	3.65 \pm 0.23	18.95*
Lingtai (GV10)	4.88 \pm 0.32	4.27 \pm 0.30	3.56 \pm 0.21	22.69*
Zhiyang (GV9)	4.86 \pm 0.33	4.20 \pm 0.27	3.47 \pm 0.19	27.32*
Jianzhongshu (SI15)	7.43 \pm 0.50	6.47 \pm 0.59	5.77 \pm 0.70	8.18*
Dazhu (BL11)	6.98 \pm 0.54	6.19 \pm 0.49	5.36 \pm 0.79	7.33*
Fengmen (BL12)	6.21 \pm 0.50	5.53 \pm 0.47	5.08 \pm 0.77	3.95*
Feishu (BL13)	5.70 \pm 0.49	5.15 \pm 0.48	4.67 \pm 0.66	3.73*
Jueyinshu (BL14)	5.37 \pm 0.47	4.76 \pm 0.41	4.39 \pm 0.57	4.42*
Xinshu (BL15)	5.04 \pm 0.67	4.54 \pm 0.43	4.27 \pm 0.50	2.25*
Dushu (BL16)	5.18 \pm 0.47	4.52 \pm 0.48	4.13 \pm 0.45	5.34*
Geshu (BL17)	5.30 \pm 0.47	4.55 \pm 0.46	4.18 \pm 0.47	6.28*
Jianwaishu (SI14)	6.05 \pm 0.39	5.39 \pm 0.43	5.00 \pm 0.63	4.79*
Fufen (BL41)	5.10 \pm 0.45	4.37 \pm 0.37	4.38 \pm 0.59	3.40*
Pohu (BL42)	4.40 \pm 0.37	3.75 \pm 0.35	3.56 \pm 0.50	5.06*
Gaohuang (BL43)	3.98 \pm 0.35	3.34 \pm 0.35	2.98 \pm 0.41	7.99*
Shentang (BL44)	3.75 \pm 0.36	2.98 \pm 0.30	2.57 \pm 0.35	13.39*
Yixi (BL45)	3.70 \pm 0.46	2.76 \pm 0.28	2.28 \pm 0.37	16.03*
Geguan (BL46)	3.66 \pm 0.45	2.63 \pm 0.28	2.33 \pm 0.36	15.08*
Quyuan (SI13)	5.36 \pm 0.32	4.76 \pm 0.35	4.31 \pm 0.42	8.78*

X: mean depth; units are provided in centimeters.

X \pm 1.96 SD: 95% confidence interval.

**P* < 0.01; *F* is the statistic for one-way ANOVA.

The definitions for "Overweight adults," "Normal adults," and "Underweight adults" are following the guidance of the Department of Health, Taiwan: "The suggested ideal body weight of Taiwanese people." As such, readers from outside Taiwan should bear in mind that ideal body weights differ between countries. The specified needling depths in the table are a suggested guide only.

should collaboration be carried out to collect statistically valuable information that can be used to increase the safety of acupuncture. Difference among different measuring methods can be understood better.

When it comes to the discussion of clinical effect of acupuncture, de-qi is frequently mentioned. De-qi means a sensation that is often elicited to enhance the effect of acupuncture treatment [124, 125]. In the ancient acupuncture literature (Huangdi) Neijing chapter Suwen indicated that de-qi may have the root in subcutaneous tissue, connective tissues, and muscles layers according to variable conditions including severity of diseases. Some practitioners of acupuncture refer to de-qi as "needle grasp," a biomechanical phenomenon characterized by an increase in the force necessary to pull the needle out of the tissue. It has been proposed that the sensation of needle grasp is due to the contraction of skeletal muscle [126] or winding of connective tissue around the needle during needle rotation [127]. By using electric impedance, Lin showed that muscle layers were the major site of de-qi in the 22 acupoints in the back [128]. The role of the nervous system in de-qi has also been well described, and some researches in this review shared their opinions as well [24, 129]. The relevant nerves can be found in certain region

surrounding the acupuncture point, and it is likely that nerves are provoked during needle manipulation. As discrepancy in definition, mechanism, and location of de-qi still exists, the depth to elicit de-qi in each point and the correlation with clinical efficacy warrants, further researches.

Only limited researches addressed the needling depth with therapeutic efficacy with good designs, and were not all acupuncture points were investigated thoroughly. Lin conducted a meta-analysis of the published evidence concerning acupoint needling depths, with the aim of providing a uniform guidance. Their group's CT scanning results indicate safe needling depths for acupoints in the back and chest for different-sized people, that is, normal, over- and underweight adults, and for sex differences (Tables 7, 8, 9, and 10) [130].

Some clinicians have shared their clinical experiences about the optimal needling depth of to treat some diseases [131]. As there are still a lot of confusions in needling depth of acupuncture points from the ancient times to the present, which has negatively influenced the standardization and international exchanges of acupuncture science, it remains to be settled as soon as possibly [132]. After convinced that acupuncture is inherently safe, how to avoid the possible associated adverse events and complications is a top priority.

TABLE 10: Mean values for needling depths of acupoints in the back in different-sized female subjects.

Acupoints	Overweight adults	Normal adults	Underweight adults	<i>F</i>
	Mean \pm 95% C.I.	Mean \pm 95% C.I.	Mean \pm 95% C.I.	
Dazhui (GV14)	6.37 \pm 0.71	5.21 \pm 0.70	4.35 \pm 0.19	5.91*
Taodao (GV13)	6.01 \pm 0.45	4.97 \pm 0.58	4.13 \pm 0.88	8.58*
Shenzhu (GV12)	5.36 \pm 0.35	4.40 \pm 0.39	3.60 \pm 0.64	15.31*
Shendao (GV11)	5.01 \pm 0.38	3.97 \pm 0.30	3.40 \pm 0.64	18.12*
Lingtai (GV10)	4.89 \pm 0.35	3.83 \pm 0.28	3.28 \pm 0.63	21.03*
Zhiyang (GV9)	4.90 \pm 0.41	3.86 \pm 0.29	3.37 \pm 0.67	15.06*
Jianzhongshu (SI15)	6.62 \pm 0.48	5.87 \pm 0.62	4.15 \pm 1.36	10.43*
Dazhu (BL11)	6.42 \pm 0.53	5.39 \pm 0.62	4.27 \pm 0.96	8.77*
Fengmen (BL12)	5.83 \pm 0.39	4.78 \pm 0.61	4.13 \pm 1.08	6.97*
Feishu (BL13)	5.32 \pm 0.38	4.43 \pm 0.46	4.03 \pm 0.97	6.66*
Jueyinshu (BL14)	5.07 \pm 0.47	4.25 \pm 0.43	3.73 \pm 0.87	6.40*
Xinshu (BL15)	4.91 \pm 0.51	3.97 \pm 0.32	3.63 \pm 0.88	8.36*
Dushu (BL16)	4.90 \pm 0.49	3.86 \pm 0.30	3.63 \pm 0.80	10.35*
Geshu (BL17)	4.91 \pm 0.47	3.92 \pm 0.37	3.58 \pm 0.92	8.57*
Jianwaishu (SI14)	5.57 \pm 0.41	4.91 \pm 0.48	3.85 \pm 0.81	8.83*
Fufen (BL41)	4.85 \pm 0.31	4.07 \pm 0.42	3.07 \pm 0.85	12.60*
Pohu (BL42)	4.35 \pm 0.28	3.53 \pm 0.31	2.60 \pm 0.74	19.67*
Gaohuang (BL43)	3.96 \pm 0.32	3.15 \pm 0.31	2.30 \pm 0.61	18.00*
Shentang (BL44)	3.67 \pm 0.35	2.75 \pm 0.27	2.02 \pm 0.48	21.33*
Yixi (BL45)	3.59 \pm 0.37	2.56 \pm 0.23	1.88 \pm 0.54	24.99*
Geguan (BL46)	3.59 \pm 0.40	2.53 \pm 0.28	1.78 \pm 0.49	22.77*
Quyuan (SI13)	5.16 \pm 0.37	4.32 \pm 0.39	3.08 \pm 0.58	18.83*

X : mean depth; units are provided in centimeters.

X \pm 1.96 SD: 95% confidence interval.

**P* < 0.01; *F* is the statistic for one-way ANOVA.

The definitions for "Overweight adults," "Normal adults," and "Underweight adults" are following the guidance of the Department of Health, Taiwan: "The suggested ideal body weight of Taiwanese people." As such, readers from outside Taiwan should bear in mind that ideal body weights differ between countries. The specified needling depths in the table are a suggested guide only.

We should put more efforts on how to reduce the risk by starting exploring the safe needling depth as well as the depth of clinical efficacy efficiently and correctly.

5. Conclusion

From the current review we found that there is great inconsistency in terms of safe needling depths measured from different subject groups and via different measuring tools. The depths measured in each research were somehow influenced by the different measuring methods as they all have distinguished advantages/disadvantages as compared with one another. The results of related researches fail to provide the solid support to decide the best measuring tool among conventional cadaver specimens, CT, MRI or ultrasound either. The characteristics of subjects such as ethnicity, gender, age, body size, underlying diseases, and the needling details (such as needling angle, which anatomical region of the body, which side of the body, and if with de-qi) all contributed partially to the measured depths. The definition of safe depth and standard localization method should be established by standardization through international conference. There is also lack of well-designed researches to compare the therapeutic effects thus making the proper

needling depth of clinical efficacy in each acupuncture point remain obscure. A well-designed clinical trial (to control variable strictly, recruit more subjects, etc.) to decide the actual and advisable needling depth for each acupuncture point, to avoid adverse effects or complications and promote optimal clinical efficacy, is a top priority. In vivo MRI imaging may serve as a good study method.

Different levels of treatment assessment of acupuncture then can be suggested by European Information Centre on complementary and Alternative Medicine (EICCAM II), that is, efficacy (is it more effective than placebo or standard?), effectiveness (is it helpful in usual care?) and efficiency (how is the cost benefit relation?).

Conflict of Interests

The authors have indicated no financial conflict of interests.

Authors' Contribution

Jaung-Geng Lin and Pei-Chi Chou contributed equally to this work, and Jaung-Geng Lin, Pei-Chi Chou and Heng-Yi Chu should be considered co-first authors.

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Research Article

“Intensity-Response” Effects of Electroacupuncture on Gastric Motility and Its Underlying Peripheral Neural Mechanism

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The aim of this study was to explore the “intensity-response” relationship between EAS and the effect of gastric motility of rats and its underlying peripheral neural mechanism by employing ASIC3 knockout (ASIC3^{-/-}), TRPV1 knockout (TRPV1^{-/-}), and C57BL/6 mice. For adult male Sprague-Dawley ($n = 18$) rats, the intensities of EAS were 0.5, 1, 3, 5, 7, and 9 mA, respectively. For mice ($n = 8$ in each group), only 1 mA was used, by which C fiber of the mice can be activated. Gastric antrum motility was measured by intrapyloric balloon. Gastric motility was facilitated by EAS at ST36 and inhibited by EAS at CV12. The half maximal facilitation intensity of EAS at ST36 was 2.1–2.3 mA, and the half maximal inhibitory intensity of EAS at CV12 was 2.8 mA. In comparison with C57BL/6 mice, the facilitatory effect of ST36 and inhibitive effect of CV12 in ASIC3^{-/-} mice decreased, but the difference was not statistically significant ($P > 0.05$). However, these effects in TRPV1^{-/-} mice decreased significantly ($P < 0.001$). The results indicated that there existed an “intensity-response” relationship between EAS and the effect of gastric motility. TRPV1 receptor was involved in the regulation of gastric motility of EAS.

1. Introduction

Acupuncture therapy, as a traditional Chinese medicinal treatment, has been widely used in clinical practice in oriental countries. And it has been more accepted by practitioners and patients worldwide after its therapeutic effects for the treatments of postoperative dental pain, nausea, and vomiting have been confirmed by NIH in 1997 [1]. Electroacupuncture (EA) is a modification of conventional manual acupuncture to stimulate acupoints with electrical current. It appears to induce more consistently reproducible effects in both clinical and animal researches than manual acupuncture [2].

During the last decades, a large number of studies have been performed to investigate the effects of acupuncture on gastrointestinal secretion, motility, and gastric myoelectrical activity [3–5]. Some regular responses of gastrointestinal tract induced by acustimulation have been observed in various

studies. In animal models, acupuncture at hindlimb has been reported to accelerate delayed gastric emptying [6], restore impaired gastric accommodation in vagotomized dogs [7], and relax the gastric fundus in rats [8] via the parasympathetic pathway, whereas application of acupuncture at the abdomen was more likely to inhibit gastrointestinal motility [9, 10] via the sympathetic pathway. Most studies have mainly focused on whether acupuncture treatment is effective for restoring gastrointestinal disorders. However, few were performed to explore the “intensity-response” relationship between electroacupuncture stimulation (EAS) and the effect. In the present paper, according to the threshold of activating peripheral III (A δ) and IV (C) primary afferent fibers [11], EAS with different intensities was introduced to reveal the “intensity-response” effects between EAS and the effect of gastric motility.

Previous studies showed that $A\beta$ and $A\delta$ mechanical receptors, as well as C-polymodal receptors, played important roles in the acupuncture stimulation perception [12–14]. But what afferent fibers mediate the regulatory effect of EAS on the internal organs was ignored. Acid sensing ion channel 3 (ASIC3) is a member of the DEG/ENaC family which is known to mediate mechanical responsiveness [15] and located mainly in $A\beta$ primary afferent fibers innervating the skin and muscle [12, 16]. Transient receptor potential vanilloid (TRPV1) belongs to TRPV subfamily, which is expressed in sensory $A\delta$ and C fibers. It can be activated by capsaicin, noxious heat, low PH, and voltage and closely related to noxious physical detection [17–19]. In our previous study, these two knockout mice have been utilized to observe the effect of EAS on mechanical and thermal pain thresholds, which showed that both EA and thermal stimulation of the right ST36 can raise mechanical and thermal pain thresholds in TRPV1 $^{-/-}$ and C57BL/6 mice, but stimulation should be more stronger in TRPV1 $^{-/-}$ mice [20].

In the present study, both ASIC3 knockout (ASIC3 $^{-/-}$) mice and TRPV1 knockout (TRPV1 $^{-/-}$) mice were employed to establish dysfunction of $A\beta$ and $A\delta$ /C afferent fiber mice models, respectively, in order to investigate the roles of $A\beta$ and $A\delta$ /C fibers in the EAS-modulated gastric motility.

2. Materials and Methods

2.1. Animal Preparation. Male Sprague-Dawley (SD) rats ($n = 18$), weighing 250–300 g, were purchased from Institute of Animal, Academy of Chinese Medical Sciences. Male ASIC3 $^{-/-}$ mice ($n = 8$), TRPV1 $^{-/-}$ mice ($n = 8$), and C57BL/6J mice ($n = 8$), weighing 25–30 g, were purchased from Jackson Lab (USA) and bred at the China Academy of Chinese Medical Science Animal Care Facility. The animals were housed under a 12 h light/dark with free access to food and water. All animals were treated according to the Guide for Use and Care of Medical Laboratory Animals from Ministry of Public Health of People's Republic of China.

2.2. Gastric Motility Recording. The animals were fasted overnight with free access to water. For anesthesia, 10% urethane (1.0–1.2 g/kg, via intraperitoneal route) was administered. About 1 h after the urethane administration, the animals were under deep anesthesia, and the trachea was cannulated but not immobilized to keep respiratory tract unobstructed. A catheter was inserted into one of the jugular veins for infusion. A small longitudinal incision was made in the duodenum about 1 cm from the pylorus. A small balloon made of flexible condom rubber was inserted via incision of the duodenum into the pyloric area of rat and kept in position by tying the connecting catheter to the duodenum. And another catheter (inner diameter of 1 mm) was also inserted into the same hole by incision in order to drain digestive juices secreted from stomach. The balloon was filled with about 0.2–0.3 mL warm water to keep pressures at about 100 mmH₂O. For the operation of the mice, a smaller balloon filled with

0.05–0.08 mL warm water was inserted into the pyloric area to keep the pressures at about 100 mmH₂O.

Pressure in the balloon was measured by a transducer through a thin polyethylene tube (1.5 mm in outer diameter) and then input into a polygraph amplifier (NeuroLog, NL900D). The signal was captured online and analyzed off-line using a data acquisition system (Power-Lab/4s, AD Instruments) and Chart 5.2 software. Demifasting gastric motor activity was recorded as a control for at least 30 min before any stimulation. The gastric motility induced by EAS was compared with the background activity in terms of average amplitude (the average difference between the cyclic maxima and minima in the selected cycles), integral (returns the integral of the selection, calculated as the sum of the data points multiplied by the sample interval), and frequency (per minute) of gastric contraction waves. Systemic blood pressure and heart rate were continuously monitored by using of BIOPAC data acquisitionsystem (MP150, USA), and rectal temperature kept constantly around 37°C by a feedback-controlled heating blanket (DC, USA).

Gastric motility during and after EAS was compared with background activity. If the change rates of gastric motility during or after EAS were 15–20% of the basal activity, the response was then considered to have an excitatory or inhibitory effect. The first EA stimulus was applied when gastric motility wave maintained stable, usually at about 30 minutes after the surgical procedure. Different intensities of EAS, including 0.5 mA ($<T_{A\delta}$), 1 mA ($<T_{A\delta}$), 3 mA ($>T_{A\delta}$, $<T_C$), 5 mA ($>T_C$), 7 mA ($>T_C$), and 9 mA ($>T_C$), were applied at ST36 or CV12 in an ascending order. The latter stimulus can only be applied when the gastric motility recovered to control state. The background gastric activity and gastric activity during and after EAS were recorded continuously, 60 s for each session.

2.3. Electroacupuncture Stimulation (EAS) of CV12 and ST36. Rats were randomly divided into ST36 group ($n = 9$) and CV12 group ($n = 9$). A needle (0.3 mm in diameter) was inserted into the skin and its underlying muscles at acupoints Zhongwan (CV12) and Zusanli (ST36) on the body. CV12 was located at center of abdomen, in middle line of the body. ST36 was located bilaterally at the anterior tibia muscles near the knees. EAS was performed at unilateral ST36 or CV12 for 60 s. A pair of noninsulated needle electrodes inserted into the skin of the acupoints with 0.3 cm distance. The needles were connected to an electronic stimulator (SEN-7103, Nihon Kohden) with the parameters as follows: duration: 1 ms, pulse frequency: 15 Hz. For rats, the current intensities were 0.5, 1, 3, 5, 7, and 9 mA, respectively. For mice, only 1 mA EAS was administrated.

2.4. Statistical Analysis. Changes in the average amplitude and integral were calculated according to (the value during EAS-the value pre EAS)/the value pre EAS \times 100%. The data obtained before and after treatment in the same group or different group was compared statistically by a paired t -test or unpaired t -test. $P < 0.05$ was considered as a statistical significance. All data are expressed as mean \pm SE.

The data was fitted with (1), where C is set to be 500, a is set to be 50, and b is set to be 30:

$$Y = \frac{C}{(1 + \exp(a - b) * X)}. \quad (1)$$

3. Results

3.1. Gastric Motility under Resting Condition. The gastric motility of the rats and mice was detected by recording the intragastric pressure. When the intrapyloric balloon pressure was increased to about 80–200 mmH₂O, the rhythmic waves of contractions in pyloric area were observed. With regard to gastric motor characteristics, both the changes of intragastric pressure and rhythmic contraction were noteworthy. Generally, the intragastric pressure represents the index of gastric tone motility, and rhythmic contraction represents gastric peristalsis induced by circular muscle contractions, similar to slow wave of gastric motor activity. The pressure was maintained at about 100 mmH₂O as baseline by expanding the volume of the balloon with warm water, rhythmic contractions occurred at a rate of four to six per minute, and these rhythmically gastric contractions were recorded in both the rats and mice.

3.2. Facilitatory Effect of Gastric Motility Induced by ST36 and Its Intensities Response Effects of the Rats. EAS at ST36 induced facilitatory effects which were related to the intensities. Figure 1(a) showed typical responses of gastric motility following EAS with various intensities for 60 s. Figures 1(b) and 1(c) summarized the responses obtained from all 9 tested rats. It should be noted that when the stimulation was less than 1 mA, there was no significant response of gastric motility (amplitude changes: 0.5 mA: $2.4 \pm 1.1\%$, 1 mA: $4.7 \pm 2.4\%$, $P > 0.05$) (integral changes: 0.5 mA: $7.8 \pm 2.8\%$, 1 mA: $12.7 \pm 5.8\%$, $P > 0.05$). However, 3 mA, 5 mA, 7 mA, and 9 mA EAS at ST36 elicited a significant enhancement on the amplitude and integral of gastric contraction compared with the background activities (amplitude changes: 3 mA: $37.9 \pm 5.8\%$, 5 mA: $43.7 \pm 3.7\%$, 7 mA: $52.3 \pm 4.4\%$, 9 mA: $53.1 \pm 5.4\%$, $P < 0.01$, $P < 0.001$) (integral changes: 3 mA: $47.2 \pm 3.2\%$, 5 mA: $55.2 \pm 5.3\%$, 7 mA: $64.9 \pm 5.6\%$, 9 mA: $64.3 \pm 6.2\%$, $P < 0.001$). The facilitation of EAS at ST36 appeared from a low intensity with an EC₅₀ value of approximately 2.3 mA for amplitude (Figure 1(b)) and 2.1 mA for integral (Figure 1(c)), which means that EAS with 2.1–2.3 mA can obtain 50% of the maximum facilitatory effect. For the intensity of EAS above 5 mA, the response efficiency did not increase correspondingly as intensities increasing, which indicated that the effects may hit a “plateau region” when the stimulating intensity reached to a certain level.

Figure 1(d) illustrated the impact of EAS at ST36 on the frequency of gastric motility. Intensity of EAS lower than 1 mA failed to produce any significant response (frequency changes: 0.5 mA: $0.22 \pm 0.22/\text{min}$, 1 mA: $0.33 \pm 0.16/\text{min}$, $P > 0.05$), while 3 mA, 5 mA, 7 mA, and 9 mA EAS at ST36 induced significant enhancement on the frequencies of gastric motility compared with the background activities (frequency changes: 3 mA: $0.44 \pm 0.17/\text{min}$, 5 mA:

$0.67 \pm 0.16/\text{min}$, 7 mA: $0.64 \pm 0.23/\text{min}$, 9 mA: $0.67 \pm 0.23/\text{min}$, $P < 0.05$, $P < 0.01$). The maximal facilitatory response of the frequency appeared as the intensities reached to 5 mA.

3.3. Inhibitory Effect of Gastric Motility Induced by CV12 and Its Intensities Response Effect of the Rats. EAS at CV12 induced inhibitory effects which were also related to the intensities. Figure 2(a) showed typical responses of gastric motility following EAS with different intensities for 60 s, and Figures 2(b) and 2(c) summarized the responses obtained from all 9 tested rats. EAS with all the intensities at CV12 induced significant inhibition effects on the amplitudes and integrals of gastric contraction (amplitude: 0.5 mA: $-11.1 \pm 2.7\%$, $P < 0.05$; 1 mA: $-18.8 \pm 3.2\%$, 3 mA: $-42.0 \pm 5.5\%$, 5 mA: $-56.7 \pm 10\%$, 7 mA: $-56.3 \pm 10\%$, and 9 mA: $-57.3 \pm 7.2\%$, $P < 0.01$) (integral: 0.5 mA: $-17.0 \pm 3.2\%$, $P < 0.01$; 1 mA: $-34.0 \pm 2.3\%$, 3 mA: $-50.1 \pm 3\%$, 5 mA: $-64.4 \pm 3.2\%$, 7 mA: $-64.0 \pm 3.7\%$, and 9 mA: $-63.4 \pm 2.5\%$, $P < 0.001$). The inhibition of EAS at CV12 appeared from a low intensity (0.5 mA), with IC₅₀ value of approximately 2.8 mA for both amplitude and integral (Figure 2(b)). This means that EAS with 2.8 mA can obtain 50% of the maximum inhibitory effect. When the intensity reached to 5 mA, the response efficiency did not increase correspondingly. The “plateau region” also appeared in the CV12 which induced the inhibitory effects.

Figure 2(d) displayed the impact of EAS on the frequency of gastric motility by CV12. Intensities of EAS lower than 1 mA had no significant influence on the frequencies (frequency changes: 0.5 mA: $-0.22 \pm 0.22/\text{min}$, and 1 mA: $-0.54 \pm 0.24/\text{min}$, $P > 0.05$). But 3 mA, 5 mA, 7 mA, and 9 mA EAS at CV12 induced a significant inhibition on the frequency of gastric motility compared with the background activities (frequency changes: 3 mA: $-2.11 \pm 0.22/\text{min}$, 5 mA: $-3.0 \pm 0.5/\text{min}$, 7 mA: $-3.11 \pm 0.45/\text{min}$, and 9 mA: $-3.22 \pm 0.42/\text{min}$, $P < 0.01$). The inhibitory response of the frequency was prone to be maximal when the intensity reached to 5 mA.

3.4. Facilitatory and Inhibitory Effects of EAS on Gastric Motility Require ASIC3 and TRPV1 Receptors. The previous data showed that there existed a possibility of “intensity-response” relationship between stimulation and effects of gastric motility. We speculated that the EAS with intensities of activation A δ and C fiber played important roles for modulating gastric motility. According to the threshold of C fiber of mice [21], 1 mA was administrated. EAS with 1 mA at ST36 induced facilitatory effects of gastric motility, and the amplitude as well as integral increased by $45.8 \pm 1.7\%$ and $57.2 \pm 3.1\%$, respectively, in C57BL/6 mice. Notably, the facilitatory effects partly diminished in ASIC3 and TRPV1 knockout mice (Figures 3 and 4). The facilitatory effects reduced a little in ASIC3^{-/-} mice but markedly in TRPV1^{-/-} mice (amplitude: $20.6 \pm 2.1\%$; integral: $34.6 \pm 3.2\%$, $P < 0.001$, Figures 3(b) and 3(c)) compared with that in C57BL/6 mice so did the inhibitory effects by CV12 in ASIC3^{-/-} and TRPV1^{-/-} mice ($P < 0.001$, Figures 4(b) and 4(c)). The frequency increased by $17.5 \pm 3.8\%$ in C57BL/6 mice via 1 mA EAS at ST36. The facilitatory effects on frequency slightly reduced in ASIC3^{-/-} mice but significantly in TRPV1^{-/-}

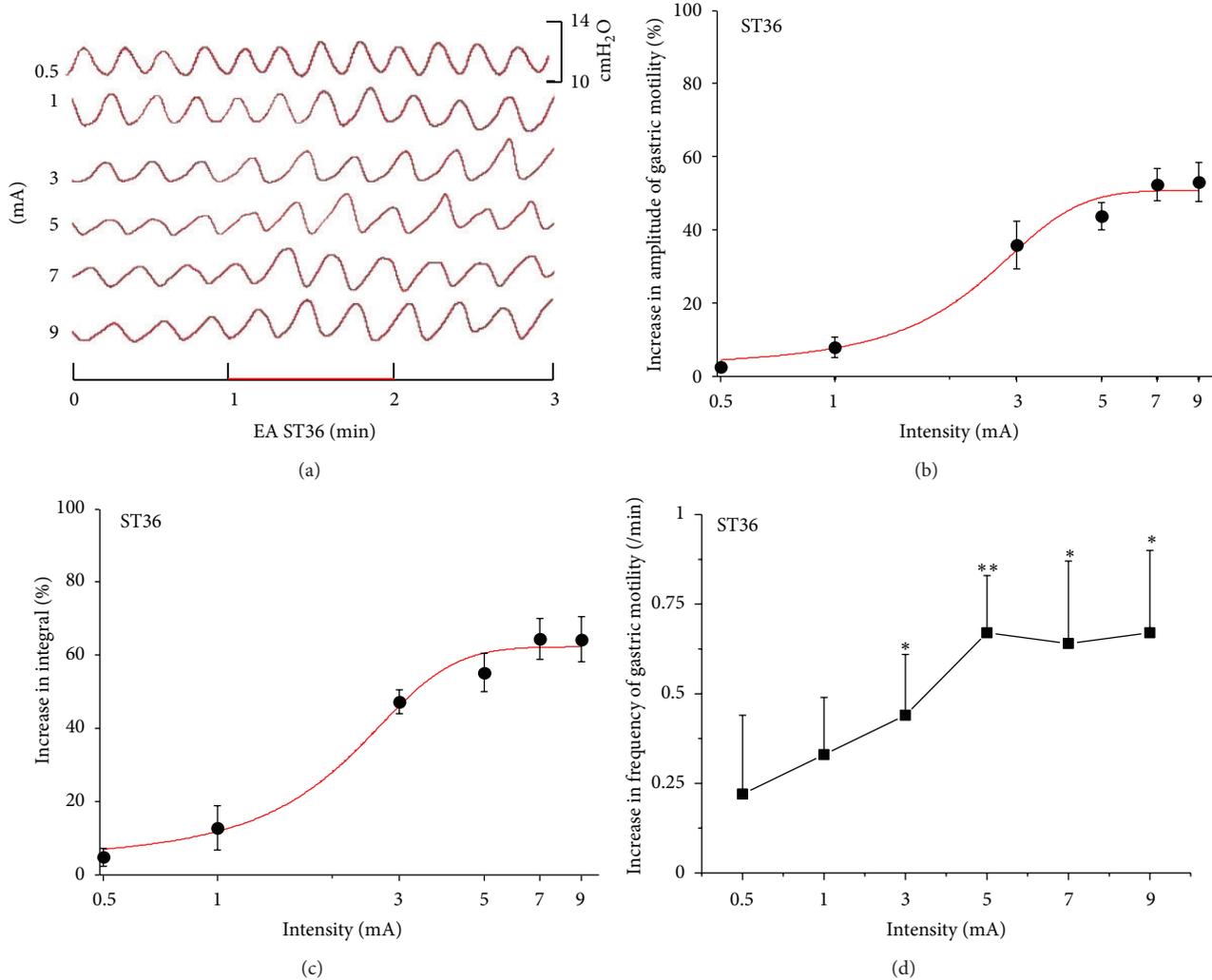


FIGURE 1: Gastric motility in response to EAS at ST36 with different intensities in rats. (a) Representative examples of the alterations of gastric contraction wave induced by different intensities of EAS at ST36. (b), (c), and (d) displayed the facilitatory effects of EAS at ST36 on the amplitude, integral, and frequency of gastric motility, respectively ($n = 9$; * $P < 0.05$, ** $P < 0.01$, versus background activities).

mice (frequency: $5 \pm 2.1\%$, $P < 0.05$, Figure 3(d)) so did the inhibitory effects by CV12 in ASIC3^{-/-} and TRPV1^{-/-} mice ($P < 0.05$, Figure 4(d)). Taken together, these observations provided direct evidence for the role of TRPV1, rather than ASIC3, in EAS-mediated facilitatory and inhibitory effects on gastric motility.

4. Discussion

In the present study, we investigated the “intensity-response” relationship between EAS and the effect of gastric motility in rats. And we firstly observed which afferent fibers were involved in the effect of EAS on gastric motility by using of knockout mice. Our findings strongly indicated the existence of “intensity-response” effects of EAS on gastric motility. EAS at ST36 induced facilitatory effects which were related to the intensities. After data fitting, the EC₅₀ (the half maximal facilitation intensity) of EAS at ST36, was 2.1–2.3 mA, which was near the threshold of A δ fiber. EAS at CV12 displayed

inhibitory effects which were also related to the intensities. The IC₅₀ (the half maximal inhibitory intensity) of EAS at CV12, was about 2.8 mA, which was also near the threshold of A δ fiber. These data suggested that the activation of A δ fiber was important for EAS-modulated gastric motility. Further study in ASIC3 and TRPV1 knockout mice showed that both ASIC3 and TRPV1 receptors were involved in the effects of EAS on gastric motility, but there was a quantity difference in the changes of gastric motility between ASIC3 and TRPV1 knockout mice. TRPV1 played a more important role in the effects of EAS.

Based on another experiment in our research group, 1 mA was strong enough to activate the C primary afferent fiber in mice. The different gastric responses induced by 1 mA EAS between ST36 and CV12 were mainly caused by diverse somatoautonomic reflexes; that is, the facilitatory effect of EA at ST36 was mediated via the parasympathetic pathway, whereas the inhibitory effect of EA at abdomen was reasoned to be attributed to the sympathetic pathway.

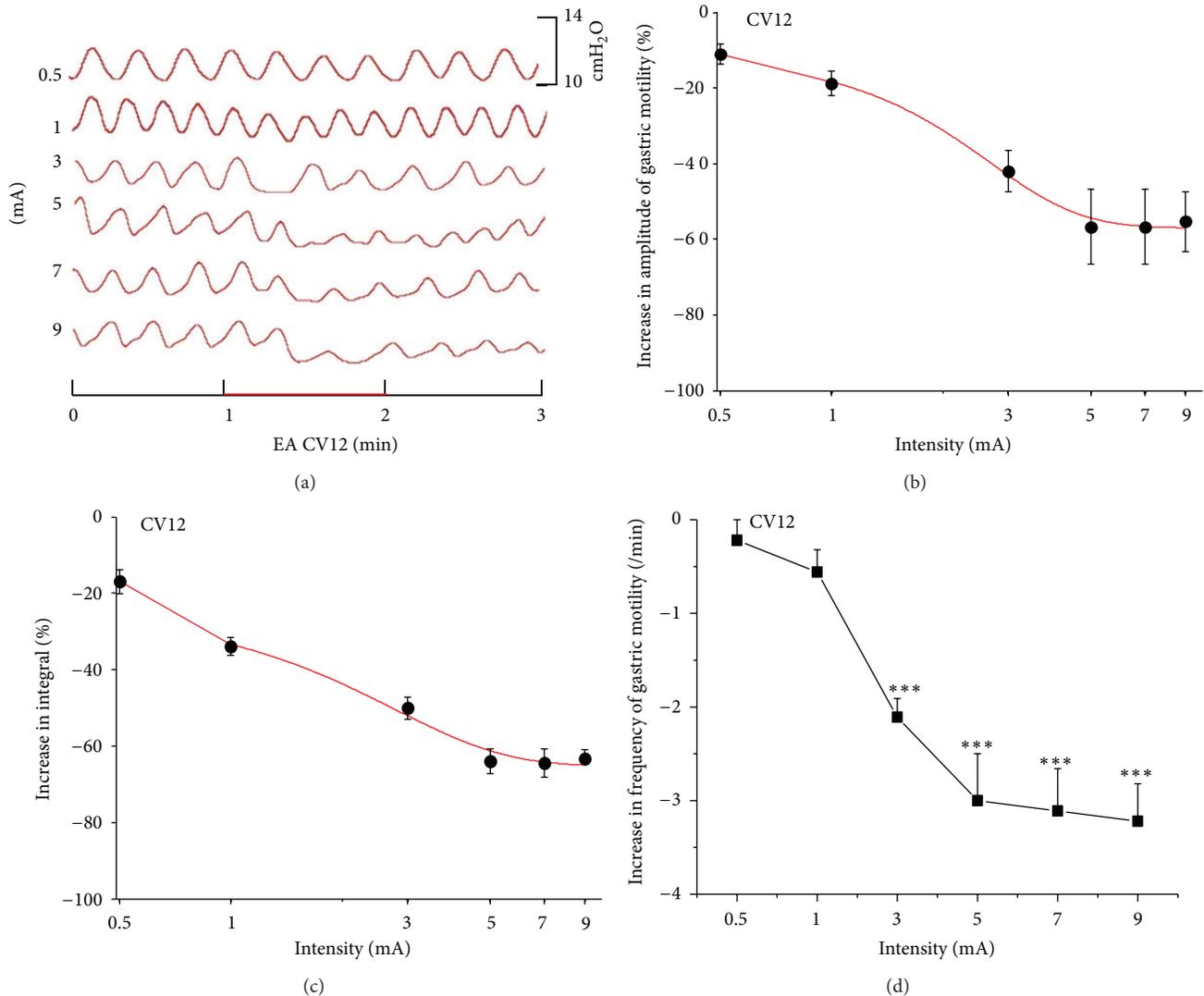


FIGURE 2: Gastric motility in response to EAS at CV12 with different intensities in rats. (a) Representative examples of the alterations of gastric contraction wave induced by different intensities of EAS at CV12. (b), (c), and (d) displayed the inhibitory effects of EAS at CV12 on the amplitude, integral, and frequency of gastric motility, respectively ($n = 9$; *** $P < 0.001$ versus background activities).

The involvement of the opioidergic pathway has also been frequently reported [18, 22]. EA was more likely to activate various afferent fibers of rats including groups II-III [19], groups III-IV [23], or groups II-IV [24]. Recent study showed that the subepidermal nerve fibers showed the colocalization of TRPV1 with peripherine, a marker for the C and A β fibers. Relationship between TRPV1 and effects of acupuncture was further investigated recently. Our previous study suggested an involvement of TRPV1 receptors in acupuncture analgesia [20]. Wang et al. showed that EA at ST36 and ST37 reduces zymosan-induced colorectal hypersensitivity through regulating TRPV1 expression [25]. Moreover, the expression of TRPV1 in subepidermal nerve fibers was significantly increased by EAS at BL40, which indicated that TRPV1 may play a role in local effect of the EA [26]. According to the result of this study, the modulatory effects of EAS at both ST36 and CV12 were barely changed in ASIC3-/-

mice compared with C57BL/6 mice. However, the potency of stimulating these two acupoints decreased significantly in TRPV1-/- mice. These results suggested that A δ and C fiber were more critical than A β fiber in the effects of EA-modulated gastric motility. In another somatovisceral reflex study, Noguchi et al. revealed that to decrease duodenal motilities, EAS to the abdomen needed to be strong enough to excite group IV (C) fibers of intercostal nerves. To increase motilities, EAS to the hindpaw needs to be strong enough to excite the higher threshold group III (A δ) fibers of tibial nerves. Their results also indicated the critical roles of A δ and C primary afferent fibers in effective regulation of EAS on visceral organ, which were quite similar to our results [25].

It is generally believed that acupuncture at different acupoints produces different effects, and the site-specific inhibitory or facilitatory effects of acupuncture on gastric motility had already been proposed [22, 27, 28]. In the present

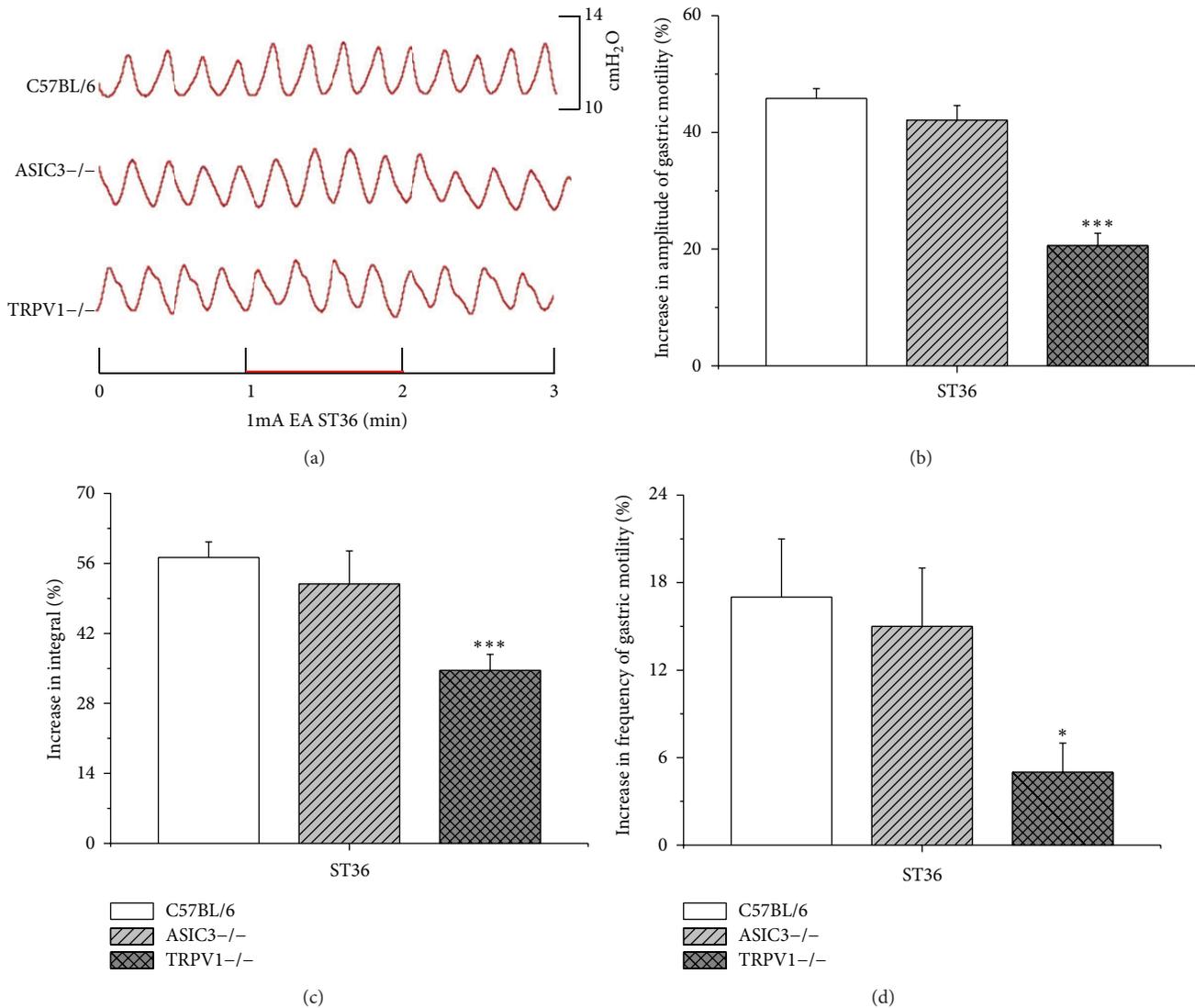


FIGURE 3: Gastric motility in response to 1 mA EAS at ST36 in three groups of mice. (a) Representative examples of the alterations of gastric contraction wave induced by 1 mA EAS at ST36. (b), (c), and (d) displayed the comparison of the facilitatory effects of 1 mA EAS at ST36 on the amplitude, integral, and frequency of gastric motility, respectively, among three groups of mice (C57BL/6, $n = 8$; ASIC3^{-/-}, $n = 8$; TRPV1^{-/-}, $n = 8$; * $P < 0.05$, *** $P < 0.001$ versus C57BL/6).

study, we found that EAS with different intensities at ST36 induced facilitatory responses of gastric motility, whereas EAS at CV12 produced an inhibitory impact on gastric motility. The consistent results have been reported in previous studies [17, 29]. The facilitatory effects of EAS at ST36, as well as inhibition effects of EAS at CV12, ranged from 20% to 60% approximately. The effects reached saturation when the intensity got to a certain level. It was also manifested that EAS had a relative narrow band control for the gastric motility and EAS modulation was a kind of self-limiting and self-regulation to promote the regulation of homeostasis of the body, which demonstrated that EAS modulation is a safe therapy.

5. Conclusion

There existed “intensity-response” relationship between stimulation and effects on gastric motility. TRPV1 receptor was

involved in the regulation process of EAS. It is necessary to activate A δ fiber to get remarkable modulatory effects, and these effects tended to maximization when C fiber was activated.

Authors' Contribution

The experiments were done by Yang-Shuai Su, Wei He, and Chi Wang, and they contributed equally to the work. Hong Shi, Hongyan Shang, and Juanjuan Xin were responsible for the mice identifying. Yufeng Zhao and Ling Hu provided advice on the statistical analyses and data interpretation. Yangshuai Su, Xianghong Jing, and Wei He drafted and finalized the paper. Xianghong Jing and Bing Zhu were responsible for the conception, design, and overseeing the implementation of the study.

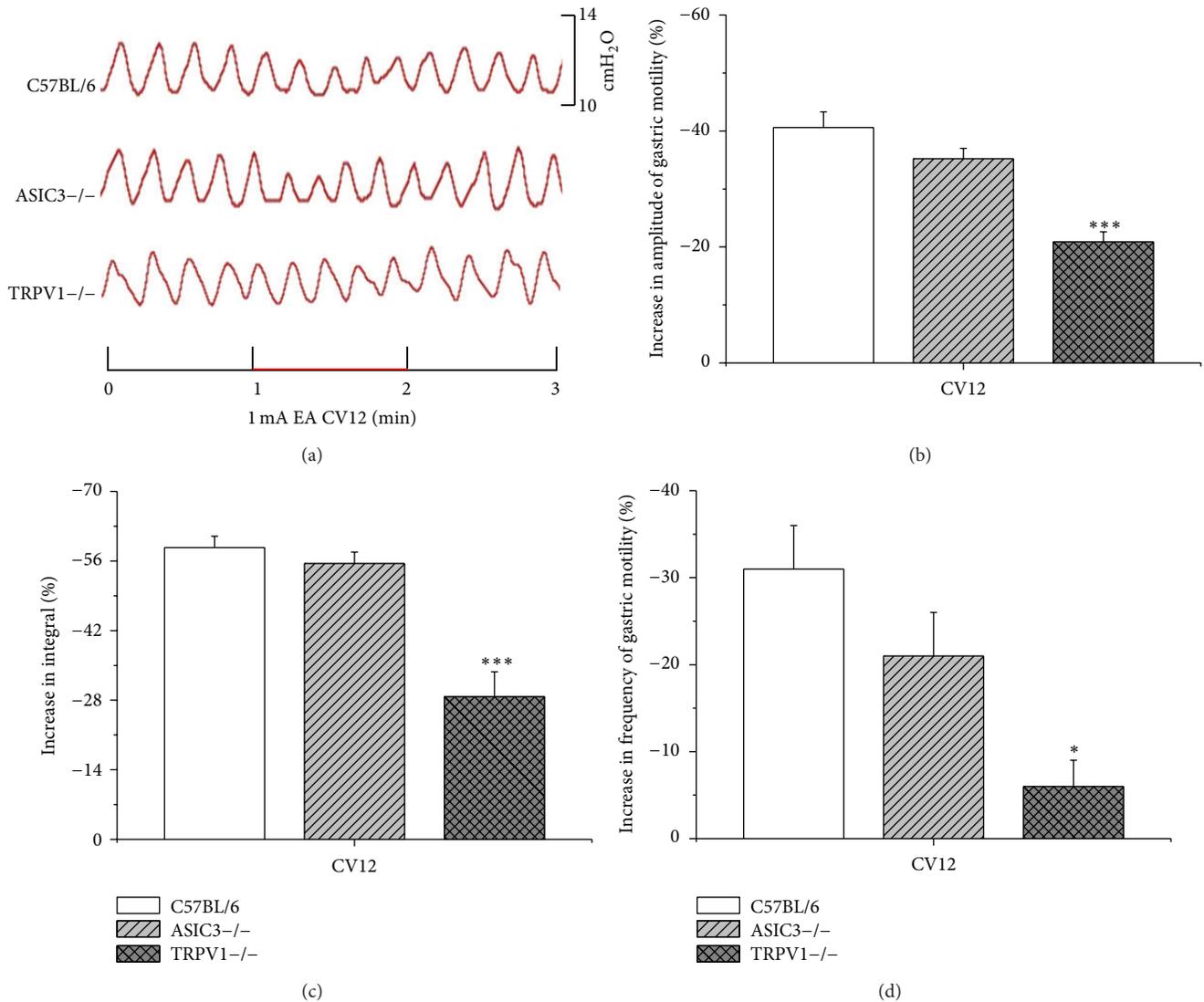


FIGURE 4: Gastric motility in response to 1 mA EAS at CV12 in three groups of mice. (a) showed representative examples of the alterations of gastric contraction wave induced by 1 mA EAS at CV12. (b), (c), and (d) displayed comparison of the inhibitory effects of 1 mA EA at CV12 on the amplitude, integral, and frequency of gastric motility, respectively, among three groups of mice (C57BL/6, $n = 8$; ASIC3^{-/-}, $n = 8$; TRPV1^{-/-}, $n = 8$; * $P < 0.05$, *** $P < 0.001$ versus C57BL/6).

Conflict of Interests

All the authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Effects of Moxibustion Temperature on Blood Cholesterol Level in a Mice Model of Acute Hyperlipidemia: Role of TRPV1

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Objectives. To compare the effects of moxibustion at two different temperatures (38°C and 46°C) on the blood cholesterol level in a mice model of acute hyperlipidemia, to detect the different expression levels of transient receptor potential vanilloid subfamily 1 (TRPV1) in the dorsal root ganglions of the wild mice, and to explore the correlation between TRPV1 and moxibustion's cholesterol-lowering effects. **Method.** Two different mice models were used: C57BL/6J wild type (WT) and TRPV1 gene knockout (TRPV1^{-/-}). Each model was randomly divided into control group and model group with three subgroups after acute hyperlipidemia was established: model control group, 38°C moxibustion group, and 46°C moxibustion group. The mice in 38°C group and 46°C group were subject to moxibustion. After the therapy, the cholesterol concentration in serum was measured, and the expression of TRPV1 was quantified. **Results.** In WT mice, moxibustion caused a decrease in blood cholesterol level and upregulation of TRPV1 at the mRNA level, which was significantly greater in the 46°C group. In contrast, in TRPV1^{-/-} mice, the differences of cholesterol-lowering effects of moxibustion were lost. **Conclusions.** Temperature is one of the important factors affecting the effects of moxibustion, and the cholesterol-lowering effect of moxibustion is related to the activation of TRPV1.

1. Introduction

Moxibustion is a unique therapeutic method in traditional Chinese medicine (TCM), which has long been used to treat human diseases through stimulating certain regions or sites of lesion on the body surface by applying heat with ignited moxa wool. Investigators analyzed the biophysical features of moxibustion and pointed out that the thermal effect or temperature is one of the important determinants of the therapeutic efficacy of moxibustion [1]. Heat produced by moxibustion stimulates not the only epidermis but also the subcutaneous tissue and muscles. Thermal stimulus is an important factor for the therapeutic effects of moxibustion. Thermal stimulation mainly changes the temperature of the skin around the acupuncture points on the body surface to induce effects.

Normal body temperature is the necessary condition for metabolism and vital movement of the body. The body regulates its temperature by using its periphery and central thermoreceptors. In mammals, ion channels are regarded as

the primary thermal signal conductors for thermoreception. Some ion channels can be directly activated by changes of body and skin temperature, as represented by transient receptor potential (TRP), a class of diverse ion channels that play significant roles in regulating a wide spectrum of cellular processes [2]. TRP was firstly described by Cosens and Manning [3] when they recorded transient potential, as opposed to the normal continuous potential, in the related mutants of the drosophila's retina photoreceptor. TRPV family members are nonselective ion channels which are highly relevant to mammals' thermoreception. When TRPV is activated, the inflow of Ca²⁺ is induced. Increased intracellular Ca²⁺ concentration causes changes in physiological and pathophysiological function [4]. TRPV1, TRPV2 (transient receptor potential vanilloid subfamily 2), TRPV3 (transient receptor potential vanilloid subfamily 3), and TRPV4 (transient receptor potential vanilloid subfamily 4), also known as capsaicin receptors and the vanilloid receptors, are major TRPVs that are thermal sensitive. Among these, TRPV1, the most extensively studied TRPV, has been believed to play a key role

in response to thermal fluctuations in mice [5]. It is mainly distributed in the dorsal root ganglion neurons, trigeminal ganglion, nodosal ganglion, and spinal and peripheral nerve endings [6]. TRPV1 is not only sensitive to capsaicin but can also be activated by harmful heat challenge ($>43^{\circ}\text{C}$), acid ($\text{pH} < 5.90$), inflammatory mediators (such as metabolites of arachidonic acid), tissue injuries, changes of extracellular osmolarity, intracellular redox condition, and electrostatic charge, indicating that TRPV1 is a polymodal receptor [7]. TRPV1 $-/-$ mice were found less affected or not affected by heat. Blockers increase body temperature in animals and humans, indicating that TRPV1 is a necessary biomolecule in the heat and pain sensation and body temperature regulation [8–10]. Yet, whether TRPV1 is involved in the thermal effect of moxibustion remained unexplored.

In clinical practice, a variety of moxibustions such as moxibustion with seed-sized moxa cone, moxibustion with moxa cone, and moxibustion with moxa stick all can make patients feel “heat.” The special feeling called “feeling of moxibustion” can be presented as “warm, hot, burnt, and painful.” Different ways of moxibustions can produce different temperature changes and activate different thermoreceptors on the skin to induce different biological effects. But does the thermal stimulation or the pain stimulation play the key role in moxibustion? What is the optimal temperature of moxibustion for patients to tolerate yet with the best therapeutic effects? Can moxibustion at different temperatures cause different therapeutic effects? Is there a correlation between the mechanism of moxibustion and the activation of TRPV1? These questions remained yet to be answered.

This study was designed to investigate the potential role of TRPV1 in regulating the thermal dependence of the therapeutic efficacy of moxibustion. The temperature was set at 38°C which is lower than 43°C and 46°C which is higher than 43°C , respectively, as the interference conditions. TRPV1 WT mice and TRPV1 $-/-$ mice were used to establish a mouse model of acute hyperlipidemia. Influences of moxa roll moxibustion on the blood cholesterol level and the expression of TRPV1 in the dorsal root ganglions of mice under different temperatures were assessed.

2. Materials and Methods

2.1. Experimental Materials

2.1.1. Experimental Animals. Two-month-old C57BL/6J WT mice of 18–22 g were purchased from the Comparative Medicine Centre of Yangzhou University (License code: SCLXX (SU)2007-0001) and were maintained in the Experimental Animals Center of Nanjing University of Chinese Medicine (clean). Two-month-old TRPV1 $-/-$ mice of 18–22 g were purchased from the Model Animal Research Center of Nanjing University (Certification Code: 0006920) and were maintained in the SPF Experimental Animals Center of Nanjing University of Chinese Medicine. Use of animals was in accordance with the *Guidelines to Treat the Experimental Animals* published by the Ministry of Science and Technology of People's Republic of China [11].

Figure 1 proved that TRPV1 $-/-$ mice in this experiment were successful TRPV1 $-/-$ models.

2.1.2. Major Experimental Instruments. The following instruments were utilized in our experiments: moxibustion stick with a diameter of 7 mm (Nanyang Hanyi Moxibustion Technology Development Co., Ltd.), temperature-measuring instrument (HC-04 type, provided by HZNG Zhou Hong Chang Technology Co., Ltd.), analytical balance (Sartorius, Germany, BL310, BL21S), tissue homogenizer (Eppendorf, Haimen, China), whirlpool oscillator (XW-80A, Kylin-Bell, Haimen, China), PCR cyclor (Labnet, America MultiGene Gradient), Fluorescence quantitative PCR cyclor (DA An Gene Co., Ltd. of Sun Yat-Sen University, DA7600), DNA electrophoresis meter (Liuyi Instrument Company, Beijing, DYY-6B), gel imager (BIO-RAD, America, Gel Doc XR), agent knit of composing the first chain of cDNA (Fermentas, Lithuania, PC0002), operative light microscope (SXP-1C Yiguang Instrument Co., Ltd., Shanghai), and ice maker (Aisinuo, China, GM-100K).

2.2. Experimental Methods

2.2.1. Method to Establish Models. The mouse model of acute hyperlipidemia was created according to the procedures described in *Experimental Method in Pharmacology*. Briefly, the egg yolk extracted from fresh eggs was blended with physiological saline to make 75% eggnog. Mice under fasting state for 16 h were weighed and injected with the eggnog at a dose of 0.5 mL per 20 g per mouse. After 20 h, the mice were used for experimental measurements [12].

2.2.2. Subgroups. A total of 32 C57BL/6J WT mice of 2-month age were divided into control ($n = 8$) and model ($n = 24$) groups. The mice of the model group were randomly subdivided into the model control, 38°C moxibustion, and 46°C moxibustion subgroups with 8 mice in each category. Similarly, a total of 24 TRPV1 $-/-$ mice were first divided into control ($n = 6$) and model ($n = 18$) groups. The TRPV1 $-/-$ mice of the model group were further divided into model control, 38°C moxibustion, and 46°C moxibustion subgroups with 6 mice in each category.

2.2.3. Acupuncture Points. To fix the acupuncture points we took human as the model: Shenque acupuncture point was located in the juncture formed by the margo superior of the manubrium sterni along the ventral median line of the mice and the upper 3/4 and lower 1/4 of external genital organs [13]; Zusanli acupuncture point was located in the posterior and lateral part of knee joint 5 mm under the capitulum fibulae [14].

2.2.4. Temperature-Setting Preexperiment. A preexperiment was performed before the formal experiment at under 26°C . Hair located $1\text{ cm} \times 2\text{ cm}$ around the acupuncture points of the normal WT mice was cut off to expose local skin which was then disinfected by alcohol tampon. Mice were fixed in the dorsal decubitus position by self-made fixation clamps.

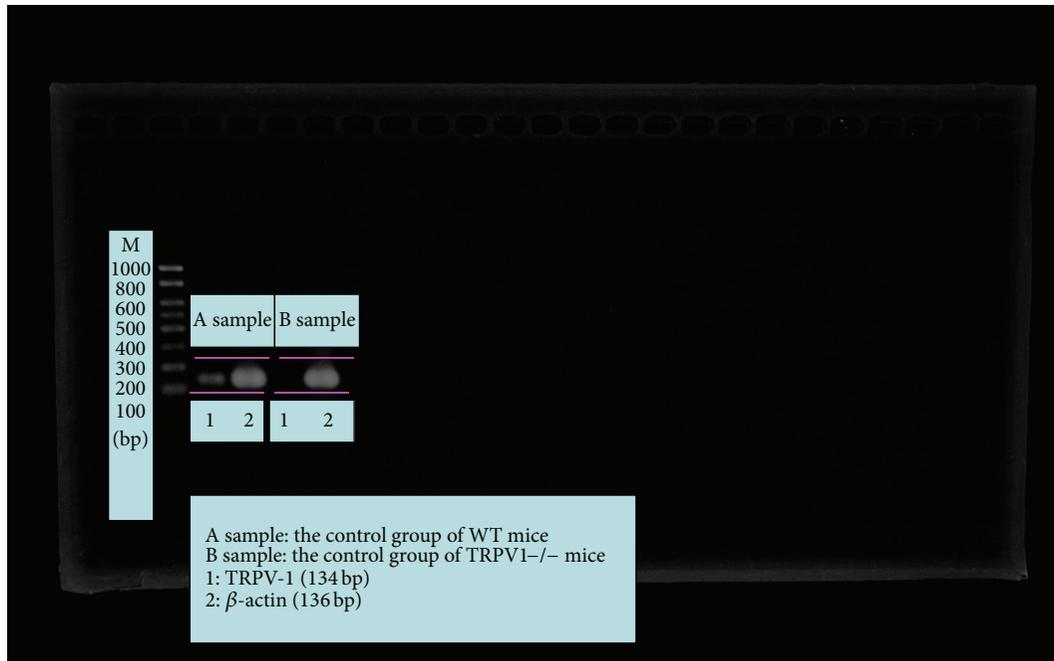


FIGURE 1: Comparison of electrophoretograms in the control WT mice and the control TRPV1^{-/-} mice.

Animal-use moxa sticks with the diameter of 7 mm and length of 20 cm were burned to operate moxibustion directly on the acupuncture points. The probe of temperature-control instrument was put on the skin where acupuncture points were located to detect instantaneous temperature. Temperature was controlled by adjusting the distance between moxa sticks and the skin, blowing the fire of the stick, and adjusting the frequency of flipping the ash of moxa stick. These steps were performed repeatedly until the temperature was stabilized, and then the distance between the moxa stick and the skin where acupuncture points were located was measured. When the distance was 35 ± 5 mm, the temperature was stabilized at $38^\circ\text{C} \pm 1^\circ\text{C}$; when the distance was 10 ± 2 mm the temperature was stabilized at $46^\circ\text{C} \pm 1^\circ\text{C}$. Figures 2 and 3, respectively, show the temperature curves on the local skin during moxibustion at 38°C and 46°C .

2.2.5. Treatments. Treatments were performed at the same day when models were established. Room temperature was fixed at 26°C . Hair located $1\text{ cm} \times 2\text{ cm}$ around the acupuncture points of the mice was cut off to expose the skin which was then disinfected by alcohol tampon. Mice were fixed in the dorsal decubitus position by self-made fixation clamps. After the mice had become quiet without struggling, moxibustion was performed. The method to control the temperature was the same as in the preexperiment. Moxibustion was performed in both Shenque and double Zusanli points for 10 min per point per day for 2 days in total. During the treatment, mice in the control and the model control groups were sham-treated without moxibustion.

Usage of the self-made fixation clamp is shown in Figure 4. Fix the mouse in supine position on the plank, and

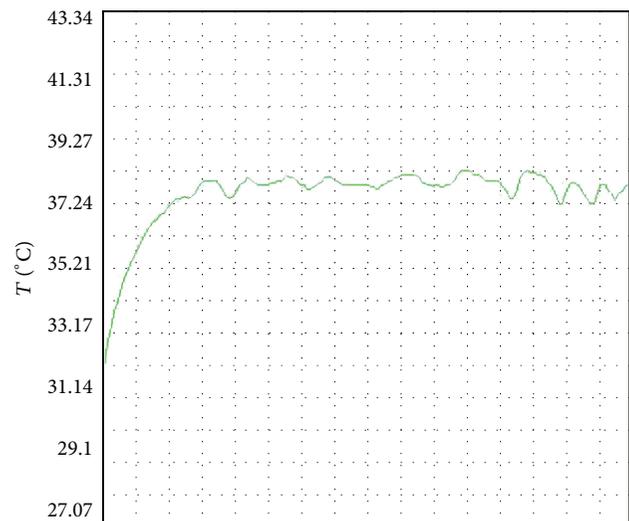


FIGURE 2: Comparison of temperature curves on the local skin during moxibustion at 38°C .

fix the limbs through the rubber ring; strain the four jaws with the rubber band to prevent the mouse from moving; then, fix the rubber band onto the adjacent nail.

2.2.6. Materials. After moxibustion treatment, blood samples were taken by excising the eyeballs. Serum was extracted by centrifuging at 3000 r/min for 15 min. The supernatant was collected into a 0.5 mL Eppendorf tube and then stored at -20°C for later uses.

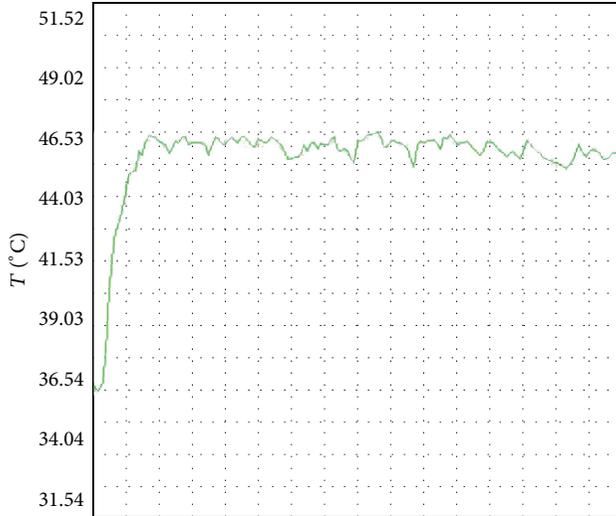


FIGURE 3: Comparison of temperature curves on the local skin during moxibustion at 46°C.

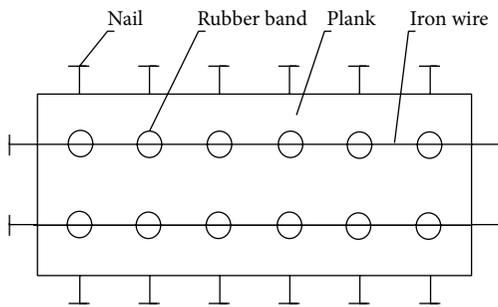


FIGURE 4: A plane view of the self-made fixation clamp.

After sacrificing, the back skin of the mice was cut open along the spine which was separated on ice bath. Canalis spinalis was cut open along the abdominal side of the spine. Nerve fibers were cut and stripped. Under a light microscope, dorsal root ganglions at lumbar segments were dissected from apertura spinalis by microforceps. Residual nerve fibers were ticked out. Dorsal root ganglions were placed into a tube and frozen in liquid nitrogen for later uses.

2.2.7. Serum Testing. Serum samples were sent to Biochemical Room of Clinical Laboratory of Zhongda Hospital Southeast University where the serum cholesterol level was determined by oxidase reaction.

2.2.8. Real-Time PCR. Sequence of the primer and the products of PCR: Mus- β -actin primer (136 bp): Forward primer: 5-GCAGAAGGAGATTACTGCTCT-3 Reverse prim 5-GCTGATCCACATCTGCTGGAA-3 Mus-TRPV-1: primer (134 bp): Forward (oIMR1561) primer: 5-CCTGCT-CAACATGCTCATTG-3 Reverse (oIMR1562) primer: 5-TCCTCATGCACTTCAGAAA-3.

After extracting RNA, the purity and concentration of RNA were measured. The first-strand cDNA was synthesized

TABLE 1: Comparison of serum cholesterol level of C57BL/6J WT mice in each group ($\bar{x} \pm s$).

Groups	Cholesterol (mmol/L)	F	P
Control group	2.17 \pm 0.46	25.31	0.000
Model control group	5.24 \pm 0.76 [#]		
38°C group	4.41 \pm 1.21		
46°C group	2.44 \pm 0.29 ^{▲*}		

Notice. [#] $P < 0.001$ ($P = 0.000$) versus control; [▲] $P < 0.001$ ($P = 0.000$) versus model control; ^{*} $P < 0.01$ ($P = 0.003$) versus moxibustion 38°C.

by reverse transcription. Real-time PCR amplification was performed with each sample in triplicate. The reaction reagents included 2X PCR Master Mix (SYBR Green) 10 μ L, 1 μ L template (cDNA diluted for 10 times), 2 μ L primer MIX (F/R 10 μ M, resp.), and DEPC water 7 μ L, 20 μ L. PCR products were quantified by the 2- $\Delta\Delta$ CT method.

2.2.9. Statistical Analysis. SPSS17.0 statistical software was adopted to analyze the data. All data are presented as $\bar{x} \pm s$ and processed by one-factor analysis of variance. LSD method was used to compare data between groups.

3. Results

3.1. Comparisons of Serum Cholesterol in WT C57BL/6J Mice. Compared with the mice in the control group, the mice in the model control group showed significantly increased serum cholesterol level ($P < 0.01$), indicating that the model of acute hyperlipidemia was successfully established. In contrast to the mice in the model control group, mice subjected to moxibustion at 38°C and at 46°C both demonstrated decreased serum cholesterol levels; only the difference between the model control group and 46°C moxibustion group reached statistical significance ($P < 0.001$). Notably, the blood cholesterol drop was to a significantly greater extent in animals subject to 46°C moxibustion than those with 38°C moxibustion (Table 1).

3.2. Serum Cholesterol Levels in TRPV1-/- Mice. Like the WT animals, the TRPV1-/- mice with acute hyperlipidemia had significantly higher serum cholesterol concentration ($P < 0.01$). The serum cholesterol concentration was not significantly altered by moxibustion at either 38°C or 46°C ($P > 0.05$). There was no significant difference between 38°C moxibustion and 46°C moxibustion (Table 2).

3.3. mRNA Levels of TRPV1 in the Dorsal Root Ganglions of C57BL/6J WT Mice. Figure 5 demonstrated that the relative TRPV-1 mRNA expression in the moxibustion at 46°C group differed significantly from the control group ($P < 0.01$); the relative TRPV-1 mRNA expression in the moxibustion at 46°C group differed significantly from the model control group ($**P < 0.01$). Differences between the moxibustion at 46°C group and the moxibustion at 38°C were significant ($**P < 0.01$), indicating that the temperature of moxibustion

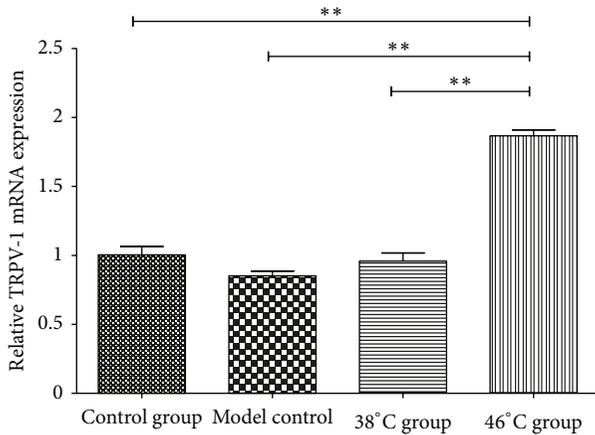


FIGURE 5: Relative TRPV-1 mRNA levels in C57BL/6J WT mice.

TABLE 2: Serum cholesterol level in TRPV1^{-/-} mice ($\bar{x} \pm s$).

Groups	Cholesterol (mmol/L)	F	P
Control group	3.66 ± 0.42		
Model control group	6.00 ± 1.26 [#]	3.30	0.04
38°C group	4.96 ± 0.62		
46°C group	4.58 ± 2.16		

Notice. [#] $P < 0.01$ ($P = 0.006$) versus control.

was an important factor affecting TRPV1 mRNA expression in the dorsal root ganglions of mice.

3.4. PCR Experiment Results of Dorsal Root Ganglions of TRPV1^{-/-} Mice. Figure 6 proved again that TRPV1 in the TRPV1^{-/-} mice has been successfully knocked out.

4. Discussions

Moxibustion can stimulate the human body's regulating system to prevent and treat a disease by producing thermal effects on the specific position to stimulate thermoreceptors on the skin. Moxibustion has had a long history, and its therapeutic mechanisms won wide attentions. Ancient Chinese doctors had already noticed the importance of moxibustion's thermal effects. *Treatise on the Appropriateness of Different Methods according to Locality* concluded that "coldness in organs can cause many diseases and these diseases should be treated with moxibustion." Wang Bing, a renowned ancient Chinese doctor, said "moxibustion meant to burn moxa to treat diseases," indicating that burning was the key element for moxibustion. Recent studies have pointed to thermal stimulation as the major factor of moxibustion, and heat produced by burning was the key in therapy [15, 16].

Lingshu Cijiezhexie concluded "smooth running of fire and Qi will make blood and body fluid flow." *Wai Tai Mi Yao* introduced "if the temperature at which moxibustion is performed to the patient is not high enough, the patient's moxibustion feeling will not be strong enough to cure the disease." Medical scholars in ancient China emphasized "heat" which is also called "moxibustion feeling"

and pointed out that therapeutic effects of moxibustion would only show when the fire and Qi in the body were running smoothly which took heat at the right temperature as a prerequisite [17]. Modern acupuncturists also paid attention to the relationship between the temperature of moxibustion and its therapeutic effects. For example, Ling et al. [18] observed, with moxibustion under different temperatures and on different skin regions, the activation of subnucleus reticularis dorsalis (SRD) at dorsal segment of rats' medulla oblongata. According to their study, moxibustion under 40°C or 42°C could not activate SRD on any area; above 46°C, the area stimulated by moxibustion showed linear correlation with the discharging activity of SRD and under the same temperature, and discharging activity of SRD did not increase with the enlargement of stimulated area. Xinmin et al. [19] observed the relationship between the pyretolysis effects of moxibustion at different temperatures and the activities of temperature-sensing neurons in the thermotaxic center. According to their study, moxibustion under 48°C showed significant rivalry effects against endotoxin (ET) and discharging effects of heat-sensitive neuron (HSN); moxibustion at 40°C showed no obvious effects. Xinmin et al. thought that moxibustion under different temperatures might activate different thermoreceptors on the skin.

The present study revealed the relationship between the temperature of moxibustion and its therapeutic effects. During the repeated preexperiments in early periods, we controlled the moxibustion temperatures at 38°C ± 1°C and 46°C ± 1°C on the targeted skin areas by using HC-04 temperature-measuring instrument. A temperature value of 46°C is considered the thermal threshold that the human body can tolerate or feel the pain of being slightly burned while 38°C is the temperature that is a little higher than the body temperature of humans and makes humans feel comfortable.

In this study, WT mice in the 46°C moxibustion group showed a stronger cholesterol-lowering effect than those in the 38°C moxibustion group, supporting the notion that temperature is an important factor in determining the therapeutic effects of moxibustion. Many clinical reports [20, 21] also put forward that warm feeling on the skin area where moxibustion is performed can never cure diseases; it is widely accepted that burning feeling is necessary to generate beneficial effects with moxibustion; burning feeling produced by thermal stimulation is induced by the activation of skin receptors. This "burning feeling" is regarded as a good sign for the appropriate strength of moxibustion. During moxibustion, simulating certain regions on the body by applying heat with ignited moxa wool is the key procedure. Therefore, in clinical practice temperature setting for moxibustion should always be seriously considered.

We selected two different mice models, because TRPV1^{-/-} mice is a successful model based on C57BL/6J WT mice, the two type of mice were the same species, that is why they can be used to control study of TRPV1. Our results also showed that in WT animals after moxa roll moxibustion at different temperatures, the upregulation of TRPV1 expression in dorsal root ganglions was considerably greater in the 46°C moxibustion group than in the 38°C moxibustion group.

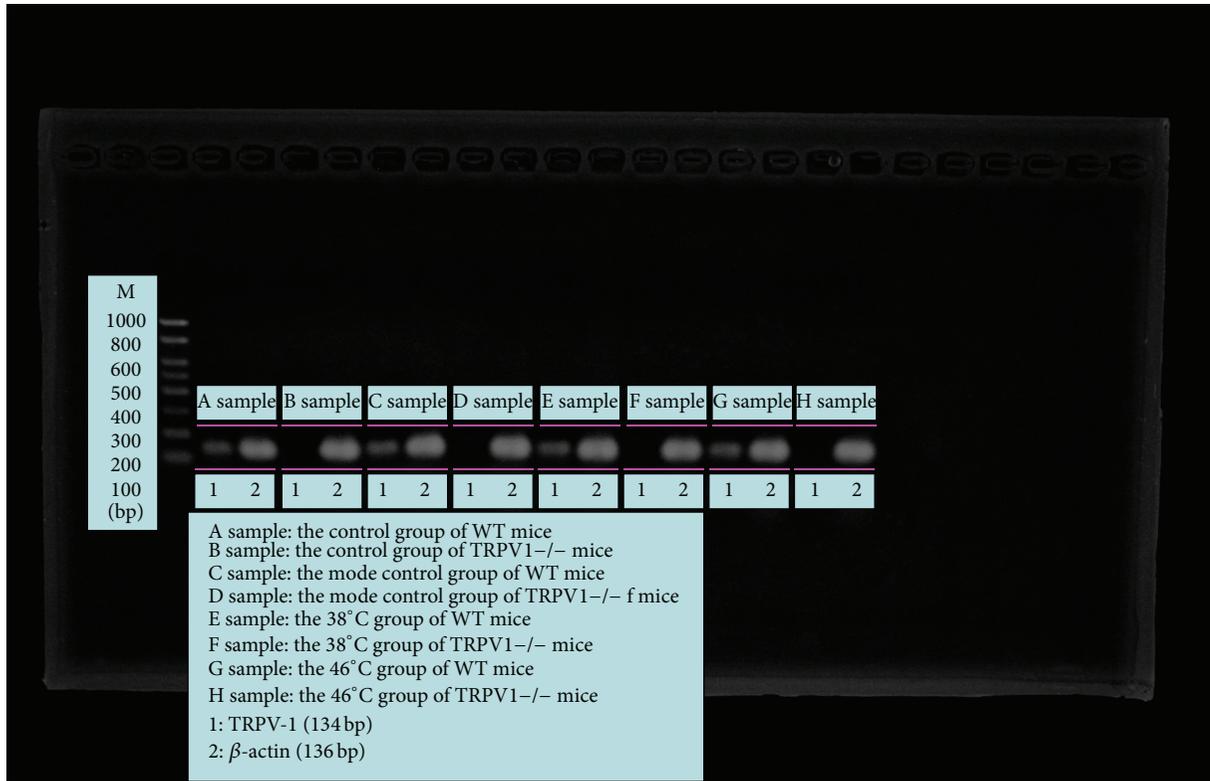


FIGURE 6: Electrophoretograms of four PCR experiments on WT mice and TRPV1^{-/-} mice.

Similarly, the cholesterol-lowering effect in the mice with acute hyperlipidemia was also found to be stronger with 46°C moxibustion than with 38°C moxibustion. In TRPV1^{-/-} mice, however, no significant differences in cholesterol level were observed with moxibustions either at 46°C or 38°C or between these two temperatures. These findings indicate that TRPV1 is an important molecular regulator that confers the temperature dependence of the cholesterol-lowering effects of moxibustion. And there was a relationship between the starting mechanism of moxibustion's cholesterol-regulating mechanism and the activation of TRPV1.

Thermal effects are important for moxibustion's therapeutic effects. The heat can stimulate many kinds of skin receptors and influence the biochemical metabolism of nervous system and histiocytes. Some investigators have recorded the discharging activities of temperature-sensing nerve fibers [22]. They found that sensory neurons express proteins that can transmit temperature and can be activated by specific temperature changes to work as molecular temperature detectors. Thermal stimulation of moxibustion is in fact an energy-transmitting process, and TRP family may be the primary transducer for this process. TRPV1 is a thermoreceptor and can be activated at the temperature above 43°C. Moxibustion at 46°C can activate TRPV1 and induce the inflow of Ca²⁺ which can cause the muscle fibers to act. Production of action potential will be transmitted to the central nervous system to start moxibustion effect.

Thus, manifestation of moxibustion effect is correlated with the activation of TRPV1 channels. The optimal temperature for tolerable moxibustion with the best therapeutic

effects should be a threshold higher than 43°C. considering that the sample size was statistically significant, we chose this quantity of mice in each group. The larger the sample size is, the more convincing the study is. So we will consider it for the experimental design in the future. To understand how the thermal stimulation during moxibustion initiates the signaling cascade and to translate the signal into moxibustion effects and whether other TRP family members are also involved in moxibustion effects, further studies are needed.

Conflict of Interests

The authors declared that there is no conflict of interests to this work.

Acknowledgment

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Research Article

Electroacupuncture Attenuates 5'-Guanidinonaltrindole-Evoked Scratching and Spinal c-Fos Expression in the Mouse

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The present study was undertaken to investigate the influence of electroacupuncture (EA) on compulsive scratching in mice and c-Fos expression elicited by subcutaneous (s.c.) administration of a known pruritogen, 5'-guanidinonaltrindole (GNTI) to the neck. Application of EA to Hegu (LI4) and Quchi (LI11) acupoints at 2 Hz, but not 100 Hz, attenuated GNTI-evoked scratching. In mice pretreated with the μ opioid receptor antagonist naloxone, EA 2 Hz did not attenuate GNTI-evoked scratching, whereas EA at 2 Hz did attenuate GNTI-evoked scratching in mice pretreated with the κ opioid receptor antagonist nor-binaltorphimine. Moreover, intradermal (i.d.) administration of the selective μ opioid receptor agonist [d-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin acetate (DAMGO) attenuated GNTI-evoked scratching behavior, while s.c. administration of DAMGO was ineffective. GNTI provoked c-Fos expression on the lateral side of the superficial layer of the dorsal horn of the cervical spinal cord. Application of 2 Hz EA to LI4 and LI11 decreased the number of c-Fos positive nuclei induced by GNTI. It may be concluded that application of 2 Hz EA to LI4 and LI11 attenuates scratching behavior induced by GNTI in mice and that the peripheral μ opioid system is involved, at least in part, in the anti-pruritic effects of EA.

1. Introduction

Itch is a sensation that provokes the desire or reflex to scratch [1, 2]. It is a leading sign of skin diseases but also occurs in some systemic diseases. Yosipovitch et al. [3] classified itch according to its origin: (1) pruritoceptive (originating in the skin, due to inflammation, dryness, or other skin damage); (2) neuropathic (arising out of disease located at any point along the afferent pathway); (3) neurogenic (central in origin, but without evidence of neuropathology); and (4) psychogenic, as in the delusional state of parasitophobia. Recent advances in pruritus research have partially elucidated the signaling molecules and neuronal pathways involved in itch transmission [4]. Pruritus may cause considerable debilitation, but treatment is often ineffective [5]. Antihistamines may relieve

itching in various allergies, but they do not lessen itch in skin, liver, or kidney diseases.

Acupuncture has been used in traditional Chinese medicine for over 2,500 years [6]. Practice of acupuncture as a treatment modality has steadily increased in Western countries [7] and is now known as "complementary medicine" due to its efficacy in the treatment of certain physical and psychological disorders. In 1997, the US National Institutes of Health issued a report in which acupuncture is listed as an effective treatment modality against many disorders with fewer side effects as compared to medications or surgery [7]. The WHO has published a guide listing 64 disorders/symptoms, including clinical conditions associated with pruritus, where acupuncture treatment can improve or ameliorate the severity of clinical conditions in question [8]. Two different

strategies are employed in acupuncture therapy, that is, manual acupuncture (MA) and electroacupuncture (EA); the latter is a modified form of traditional MA. Most studies use EA because it can be standardized according to frequency, voltage, waveform, length, and so forth.

Several clinical studies have demonstrated the efficacy of acupuncture in alleviating itch. In particular, acupuncture has been shown to relieve histamine-induced itch [9], type I hypersensitivity itch [10], nasal itch [11], refractory uremic pruritus [12], and neurogenic pruritus [13]. In these studies, the Hegu (LI4) and Quchi (LI11) acupoints are most commonly used. The mechanism whereby acupuncture relieves itch has not been studied in detail.

The highly selective κ opioid receptor antagonist 5'-guanidinonaltrindole (GNTI) [14] has been shown to precipitate immediate, excessive scratching in mice [15], using the itch model described by Kuraishi et al. [16]. The scratch behavior induced by subcutaneous (s.c.) injection of GNTI to the neck is abolished by the κ opioid agonist, nalfurafine [15]. Expression of the *c-fos* gene or Fos protein is widely used as a marker in identifying the neural pathways involved in the integration of noxious inputs [17–21]. Nojima et al. [22] reported spontaneous itch-related scratching behavior, which can be correlated with *c-Fos* expression in the superficial dorsal horn of the spinal cord in a rat model of dry skin. Inan et al. [23] reported that GNTI provoked *c-fos* expression in neurons located at the lateral area of the dorsal horn.

In this study, EA experiments were conducted to (1) establish the applicability of EA in alleviating GNTI-induced scratch in a murine model; (2) determine the frequency-dependent anti-scratch activity of EA; (3) correlate the efficacy of EA with expression of Fos protein in the spinal cord; and (4) define the involvement of opioid receptors in EA-mediated anti-scratch activity.

2. Methods

2.1. Animals. Male ICR mice (25–30 g; BioLasco Taiwan Co., Ltd., Taiwan) were housed under a 12:12 light/dark cycle with food and water available *ad libitum* in our animal facility for at least 4 days prior to the experiments, which were conducted between 10:00 and 17:00. The experimental procedures were approved by the China Medical University Institutional Animal Care and Use Committee, in accordance with the care and use of laboratory animal guidebook from the Chinese Taipei Society of Laboratory Animal Sciences. The experiments were designed to keep the number of mice at a minimum and care was taken to minimize suffering.

2.2. Electroacupuncture. Mice were individually acclimated in rectangular observation boxes for 1 hr and then anesthetized with 1.5% isoflurane. Under anesthesia, a pair of stainless steel acupuncture needles (36-gauge) was inserted 2 mm deep into the murine equivalent of the human Hegu (LI4) and Quchi (LI11) acupoints. The murine LI4 is located on the first dorsal interosseus, radial to the midpoint of the second metacarpal bone in the forelimb. The murine LI11 is located in the depression on the lateral end of the cubital crease in the forelimb, when the elbow is fully flexed [24]. A

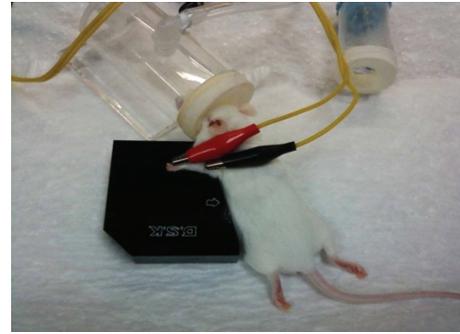


FIGURE 1: Photograph of a mouse under isoflurane anesthesia with acupuncture needles inserted at Hegu (LI4) and Quchi (LI11) acupoints. LI4 is located on the first dorsal interosseus, radial to the midpoint of the second metacarpal bone in the forelimb. LI11 is located in the depression on the lateral end of the cubital crease in the forelimb, when the elbow is fully flexed [24].

pair of acupuncture needles (32-gauge) was also inserted 3–4 mm deep into the bilateral murine equivalent of the human Zusanli (ST36). The ST36 is located on the anterolateral side of the hindlimb near the anterior crest of the tibia, in a depression below the knee on the tibialis anterior muscle [26, 27]. EA stimuli were delivered by an EA Trio 300 stimulator (Ito, Japan) at an intensity of 2 mA for 20 min at either 2 or 100 Hz with a pulse width of 150 μ s [25]. Figure 1 shows an anesthetized mouse receiving EA applied to LI4 and LI11.

2.3. Effect of Electroacupuncture on Pruritogen-Evoked Scratching Behavior. After termination of EA, mice were allowed to recover from anesthesia for 30 min. Saline or GNTI (0.3 or 0.6 mg/kg) were injected s.c. in the midline behind the neck. To minimize the effects of anesthesia, the number of hind leg scratches directed to the back of the neck was counted for up to 40 min [19, 23, 25, 28–30]. All test agents were administered in a dose volume of 0.01 mL/g.

2.4. Opioid Receptor Antagonist Study. The μ opioid receptor antagonist, naloxone (s.c.; 1 mg/kg) [30, 31], and the κ opioid receptor antagonist, nor-binaltorphimine (s.c.; 10 mg/kg) [25], were used to pretreat mice. Naloxone (s.c.; 1 mg/kg) was administered at 70 min before GNTI injection; nor-binaltorphimine (s.c.; 10 mg/kg) was administered at 24 hr before GNTI injection [25]. EA was applied to the LI4 and LI11 acupoints before GNTI administration. After termination of EA, mice were allowed to recover from anesthesia for 30 min. GNTI (0.3 mg/kg) was injected s.c. in the midline behind the neck. The number of scratches was counted for 40 min after pruritogen injection.

2.5. Opioid Receptor Agonist Study. This study used the μ opioid receptor agonist [d-Ala², N-Me-Phe⁴, Gly⁵-ol]-enkephalin acetate (DAMGO) [32]. Mice were individually acclimated in rectangular observation boxes for at least 2 h and then injected intradermally (i.d.) or s.c. with DAMGO (10 nmol/site [33]) at the back of the neck. Ten minutes later, the pruritogen GNTI (0.3 mg/kg) was injected s.c. in the

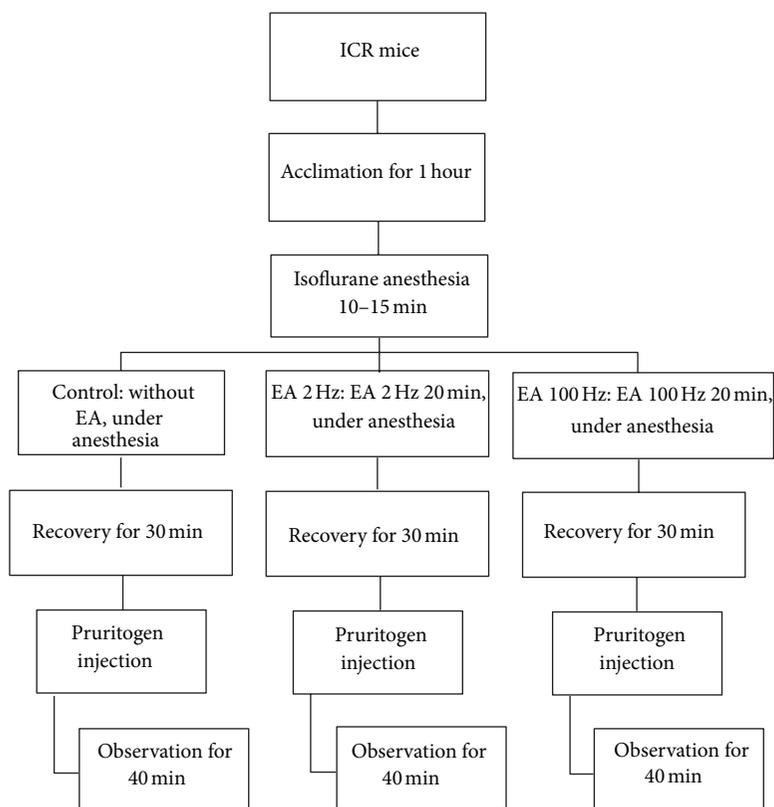


FIGURE 2: Flow chart depicting steps involved in the EA study.

midline behind the neck of mice. The number of hind leg scratches directed to the back of the neck was counted for 30 min [30, 34].

2.6. Fos Immunoreactivity in Cervical Spinal Cords. The immunohistochemistry procedure was similar to that described by Inan et al. [25]. In brief, 2 hr after the injection of saline or GNTI (0.6 mg/kg), animals were deeply anesthetized with urethane (1.2 g/kg; i.p.) and perfused intracardially with ice-cold 0.1 M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M PBS. The cervical spinal cords were removed and postfixed in the same fixative overnight at 4°C. Tissue samples were transferred to 30% sucrose solution for at least 3-4 d before sectioning. Cervical spinal cord (C5-C7) sections (30 μm) were cut at -25°C with a freezing microtome (Leica CM3050 S). Free-floating sections were stored in PBS at 4°C until immunohistochemistry was performed. Fifteen cervical spinal cord sections from each mouse were randomly selected for immunohistochemistry.

Tissues were processed for c-Fos immunoreactivity by the avidin-biotin complex procedure [35]. They were first treated with 3% H₂O₂ to quench endogenous peroxidase activity, washed twice for 10 min with PBS, and blocked with 20% normal goat serum (1:20) at room temperature for 2 hr. The sections were then incubated in a shaker for 2 days at 4°C with rabbit c-Fos antibody (1:1000 dilution) (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After thorough rinsing, sections were incubated in biotinylated anti-rabbit

immunoglobulin G secondary antibody (1:300 dilution; Vector Laboratories, Burlingame, CA, USA) for 2 hr at room temperature. Following two 10 min rinses with PBS, the sections were incubated in an avidin-biotin-peroxidase complex at room temperature for 90 min (1:300 dilution; Vectastain ABC Elite kit, Vector Laboratories). Following three 10 min washes in Tris-buffered saline, sections were reacted with 0.05% diaminobenzidine/0.001% H₂O₂ solution for 4-5 min and washed 3 times for 10 min with Tris-buffered saline. Sections were mounted on slides with 0.25% gel alcohol, air-dried, dehydrated with graded ethanol (50%, 70%, 95%, and 100%, for 6 min each) followed by xylene (twice for 10 min), and coverslipped with Permount. c-Fos positive nuclei were observed under a light microscope and counted at 200x magnification.

2.7. Chemicals. Diaminobenzidine was purchased from Calbiochem (La Jolla, CA, USA); GNTI, DAMGO, naloxone hydrochloride, and nor-binaltorphimine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.8. Data Analysis. Statistical comparisons were performed with Student's *t*-test or one-way ANOVA followed by Tukey's post hoc test. All data are expressed as the mean ± standard error of the mean (S.E.M.). *P* < 0.05 was considered statistically significant.

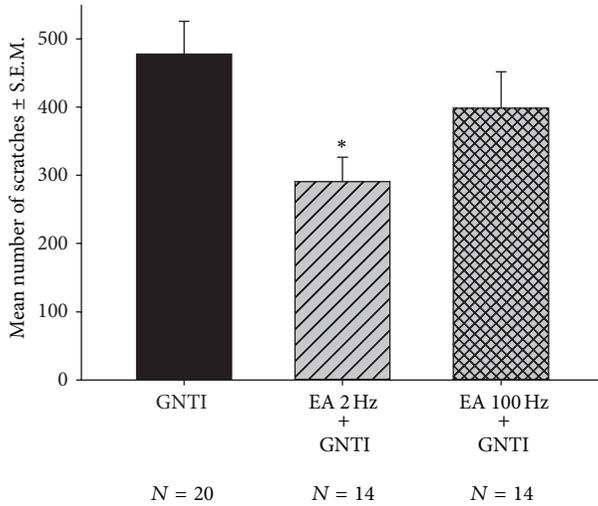


FIGURE 3: Effects of EA at 2 Hz and 100 Hz on GNTI- (0.3 mg/kg) induced scratching. EA was applied to the LI4 and LI11 acupoints before GNTI administration. The number of scratches was counted for 40 min after pruritogen injection. EA at 2 Hz, but not 100 Hz, attenuated GNTI-induced scratching behavior (* $P < 0.05$ compared to controls). Between-group comparisons for each group were performed by ANOVA, followed by Tukey's test.

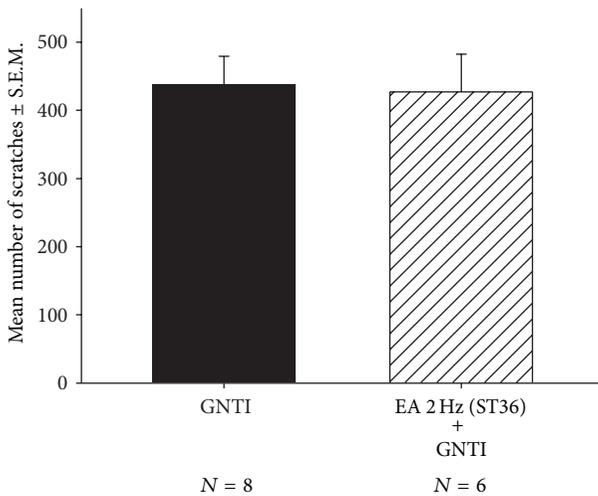


FIGURE 4: Effects of EA at 2 Hz on GNTI- (0.3 mg/kg) induced scratching. EA was applied to the bilateral ST36 acupoints prior to GNTI administration. The number of scratches was counted for 40 min after GNTI injection. EA at 2 Hz had no effect upon GNTI-induced scratching behavior. Between-group comparisons for each group were performed by Student's t -test.

3. Results

3.1. Attenuation of GNTI-Evoked Scratching by EA at 2 Hz but Not 100 Hz. To test the effects of EA at two different frequencies (2 and 100 Hz) applied to LI11 and LI4 on GNTI-induced scratching behavior, mice were divided into 3 groups: (1) control, (2) 2 Hz EA, and (3) 100 Hz EA (Figure 2). In contrast to the EA groups, mice in the control group received isoflurane but did not undergo EA. After anesthesia, mice

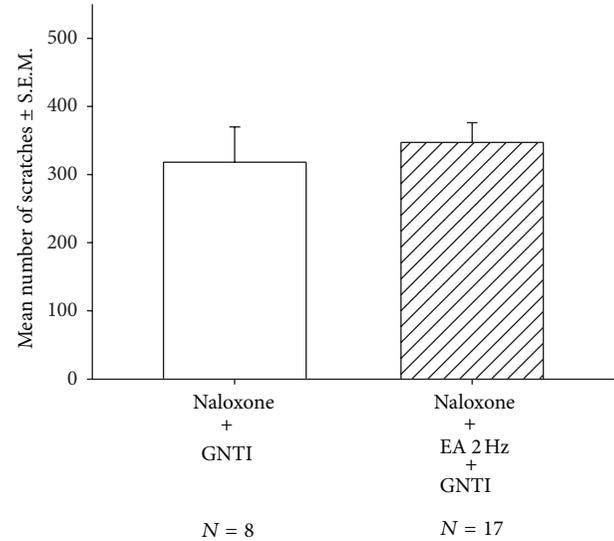


FIGURE 5: Effects of EA at 2 Hz on GNTI- (0.3 mg/kg) induced scratching in naloxone-pretreated mice. Naloxone (s.c.; 1 mg/kg) was administered 70 min before GNTI injection, that is, 5 min before isoflurane anesthesia for EA. EA was applied to the LI4 and LI11 acupoints before GNTI administration. The number of scratches was counted for a period of 40 min after pruritogen injection. EA failed to attenuate GNTI-induced scratching behavior in naloxone-pretreated mice. Between-group comparisons for each group were performed by Student's t -test.

in each group were allowed to recover for 30 min and then injected with GNTI (0.3 mg/kg). Mice started scratching 2-3 min following the GNTI injection. This strong, stereotypic behavior continued throughout the 40 min observation period. At the end of 40 min, the averaged numbers of scratches in the control, 2 Hz EA, and 100 Hz EA groups were 477.7 ± 48 ($n = 20$), 290.6 ± 36.1 ($n = 14$), and 398.3 ± 53.7 ($n = 14$), respectively (Figure 3). Compared with the control group, pretreatment with 2 Hz EA decreased GNTI- (0.3 mg/kg) evoked scratching by 39.2% ($P < 0.05$), whereas 100 Hz EA pretreatment had no such effect ($P > 0.05$).

3.2. EA at 2 Hz Applied to ST36 Did Not Affect GNTI-Induced Scratching. To test the effects of EA at 2 Hz applied to bilateral ST36 acupoints on GNTI-induced scratching behavior, mice were divided into 2 groups: (1) control, (2) 2 Hz EA applied to bilateral ST36 (Figure 4). At the end of a 40 min observation period, the averaged numbers of scratches in the control group and 2 Hz EA were 438.1 ± 41.1 ($n = 8$) and 427.2 ± 55.4 ($n = 6$), respectively (Figure 4). The between-group number of scratches did not differ significantly. It appears that pretreatment with 2 Hz EA to bilateral ST36 did not affect GNTI- (0.3 mg/kg) evoked scratching.

3.3. Naloxone Prevented the Effects of EA (2 Hz) on GNTI-Induced Scratching. To test whether EA attenuates GNTI-induced scratching in mice pretreated with naloxone (1 mg/kg; s.c.), animals were divided into 2 groups: (1) naloxone-pretreated controls and (2) naloxone-pretreated plus EA. The

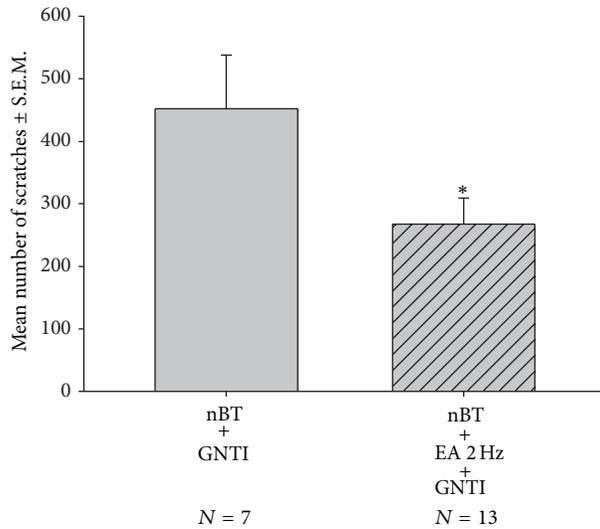


FIGURE 6: Effects of EA at 2 Hz on GNTI- (0.3 mg/kg) induced scratching in nor-binaltorphimine-pretreated mice. Nor-binaltorphimine (10 mg/kg) was subcutaneously administered 24 hr before GNTI injection [25]. EA was applied to the LI4 and LI11 acupoints before GNTI administration. The number of scratches was counted for 40 min after pruritogen injection. EA attenuated GNTI-induced scratching behavior in nor-binaltorphimine-pretreated mice (* $P < 0.05$). Between-group comparisons for each group were performed by Student's t -test.

scratching behavior was observed for 40 min. After 40 min, the averaged numbers of scratches in the naloxone-pretreated controls and naloxone-pretreated plus EA mice were 318.4 ± 51.6 ($n = 8$) and 347.2 ± 28.8 ($n = 17$), respectively (Figure 5). EA did not significantly attenuate GNTI-induced scratching behavior in naloxone-pretreated mice.

3.4. Nor-Binaltorphimine Did Not Alter the Effects of EA (2 Hz) on GNTI-Induced Scratching. To test whether EA attenuates GNTI-induced scratching in mice pretreated with nor-binaltorphimine (10 mg/kg; s.c.), animals were divided into 2 groups: (1) nor-binaltorphimine-pretreated controls and (2) nor-binaltorphimine-pretreated plus EA. The scratching behavior was observed for 40 min. After 40 min, the averaged numbers of scratches in the nor-binaltorphimine-pretreated controls and (2) nor-binaltorphimine plus EA mice were 452.3 ± 85.9 ($n = 7$) and 267.5 ± 41.9 ($n = 13$), respectively (Figure 6). Thus, EA significantly attenuated GNTI-induced scratching behavior by 41% in nor-binaltorphimine-pretreated mice.

3.5. Attenuation of GNTI-Induced Scratching by Intradermal Administration of DAMGO, Not by Subcutaneous Administration of DAMGO. To further clarify the roles of peripheral versus central μ opioid receptors in the anti-pruritic effect of EA, we compared the effects of DAMGO by i.d. and s.c. injection. In the intradermal studies, mice received injections of saline (controls) or DAMGO (10 nmol/site) at the back

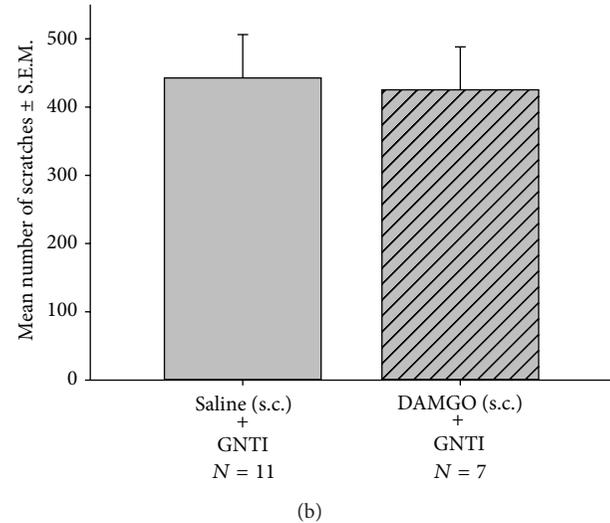
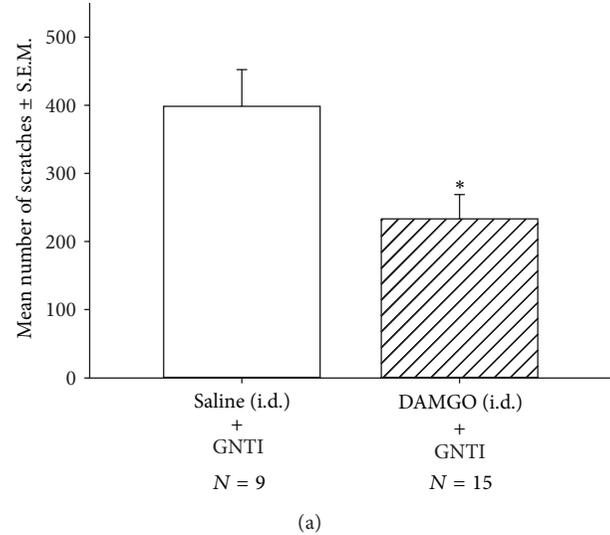


FIGURE 7: Effects of DAMGO by i.d. injection (a) and by s.c. injection (b) on GNTI- (0.3 mg/kg) induced scratching. (a) Mice were administered an i.d. injection of saline or DAMGO (10 nmol) at 15 min before GNTI (0.3 mg/kg). (b) Mice were administered a s.c. injection of saline or DAMGO (10 nmol) at 15 min before GNTI (0.3 mg/kg). The number of scratches was counted for 30 min after GNTI injection. DAMGO (10 nmol/site) attenuated GNTI-induced scratch behavior by i.d. administration but not s.c. administration. (* $P < 0.05$ compared to controls). Between-group comparisons for each group were performed by Student's t -test.

of the neck. After 15 min, animals were injected with GNTI (0.3 mg/kg; behind the neck). After 30 min, the number of scratches was 398.3 ± 53.5 ($n = 9$) for the control group and 233.1 ± 35.6 ($n = 15$) for the DAMGO-treated group (Figure 7(a)). Thus, intradermal pretreatment with DAMGO significantly decreased the number of scratches induced by GNTI (0.3 mg/kg) by 41.5% ($P < 0.05$) as compared to the control group.

In the subcutaneous studies, similar procedures were performed as above, while DAMGO was injected s.c. at the

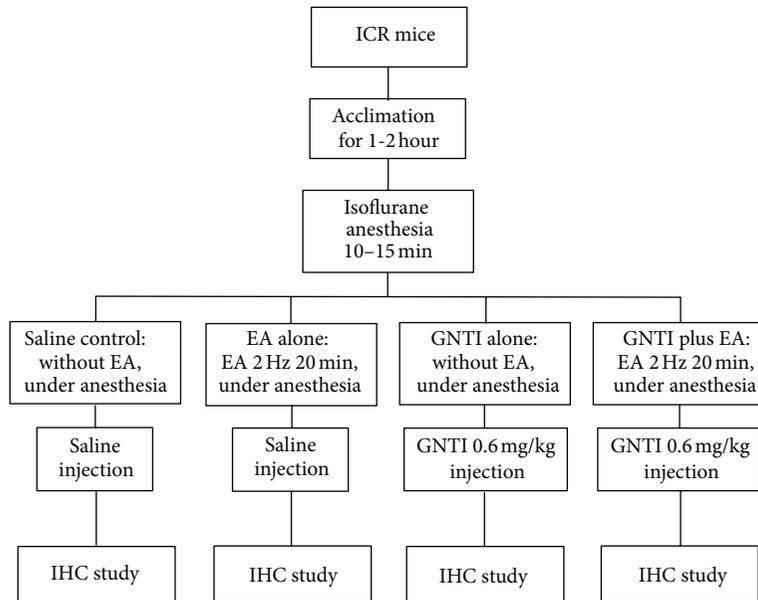


FIGURE 8: Flow chart depicting steps involved in the immunohistochemistry (IHC) study.

back of the neck. After 30 min, the number of scratches for the control group was 442.6 ± 63.4 ($n = 11$) compared with 425.3 ± 62.8 ($n = 7$) for the s.c. DAMGO-treated group (Figure 7(b)). Thus, subcutaneous pretreatment with DAMGO did not affect scratch behavior induced by GNTI (0.3 mg/kg).

3.6. 2 Hz EA Attenuates GNTI-Induced Scratching at a Higher Dose. To evaluate the effects of 2 Hz EA on a higher dose of GNTI-induced scratching behavior, the mice were divided into 2 groups: (1) GNTI 0.6 mg/kg (controls) and (2) GNTI 0.6 mg/kg with 2 Hz EA. After 40 min, the averaged numbers of scratches for the control and 2 Hz EA groups were 618.2 ± 78.7 ($n = 10$) and 383.1 ± 62 ($n = 10$) (Figure 11(a)). Pretreatment with 2 Hz EA significantly decreased GNTI- (0.6 mg/kg) induced scratching by 38% as compared to the control group ($P < 0.05$).

3.7. Attenuation of GNTI-Induced c-Fos Expression by EA. This series of studies was undertaken to test the effects of EA on GNTI- (0.6 mg/kg) induced c-Fos expression in superficial layers of the cervical spinal cord. Mice were divided into 4 groups: saline control; EA alone; GNTI alone; GNTI plus EA (Figure 8).

GNTI significantly increased the number of c-Fos positive nuclei on the lateral side of the superficial layers of the dorsal horn as compared to the saline control group ($P < 0.01$; Figures 9 and 10). While 2 Hz EA alone had no significant effect on c-Fos expression (Figure 9), pretreatment with 2 Hz EA significantly reduced the number of c-Fos positive nuclei induced by GNTI (Figures 10 and 11(b); $P < 0.05$; $n = 8$).

4. Discussion

4.1. EA Inhibits Pruritogen-Induced Scratching. While the influence of acupuncture on pain has been extensively studied, much less is known about the effects of acupuncture on itch. Several clinical studies have demonstrated the efficacy of acupuncture on itch in humans [12, 36–38]. Investigations by Han et al. [25] explored the anti-pruritic effects of acupuncture in a rat model of hindlimb scratching. In their study, itch-associated scratching behavior was induced by i.d. injections of 5-HT into the rostral back, and MA or EA at the frequency of 2 and 120 Hz significantly reduced the number of scratching bouts over a period of 60 min as compared to that of control rats. Further, pretreatment of the rats with nor-binaltorphimine inhibited the anti-pruritic effect of 120 Hz EA. The authors suggest that the anti-pruritic effects of acupuncture using high-frequency EA is due to κ opioid receptor activation.

In the present study, we first established a different animal scratching model, that is, GNTI-induced scratching behaviors [23] suitable for assessing the antipruritic effects of EA. Our results show that application of 2 Hz EA to LI4 and LI11 significantly diminished GNTI-induced scratching behavior, whereas 100 Hz EA had no such effect. EA (2 Hz) applied to ST36 had no significant effect upon GNTI-induced scratching behavior. To the best of our knowledge, this is the first report to demonstrate that EA inhibits scratching in a frequency-dependent manner by a pruritogen other than 5-HT [25]. The findings indicate that different frequencies of EA should be considered in the treatment of pruritus caused by different pruritogens. Further, LI4 and LI11 are preferred over the ST36 for pruritus treatment, in spite of ST36 being recognized as the important acupoint for acupuncture analgesia [39–41].

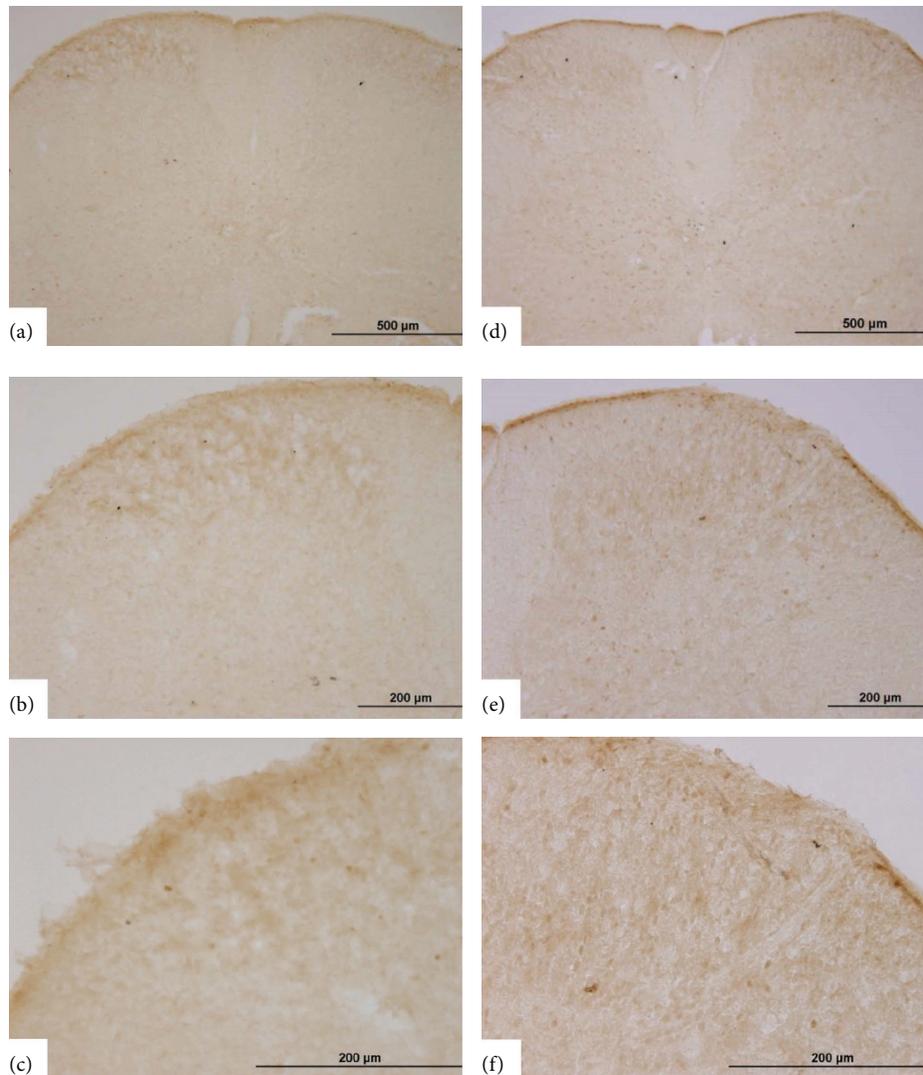


FIGURE 9: Representative photomicrographs of c-Fos expression in the spinal cord. c-Fos was not detected in the superficial layers of the dorsal horn following saline injection (a)–(c) and after EA (2 Hz) application at the LI4 and LI11 acupoints (d)–(f). (a)–(c) represent one spinal section, with increasing magnification, while (d)–(f) are from another spinal section, with increasing magnification.

4.2. Pain and Itch. The sensations of pain and itch share many interactions in acute transmission and sensitization processes, but they have differences as well. Pain sensation evokes withdrawal behavior, while itch evokes scratching as a reflex or conscious mechanical stimulation to relieve the hazardous stimulant and act as a helpful warning of potential hazards [42].

Separate sets of neurons mediate itch and pain inputs from the peripheral nerve [43]. Primary afferents for pain sensation are mechanosensitive and mechanoinsensitive A δ - and polymodal C-fibers [44, 45], whereas primary afferents for itch sensation are histamine-activated mechanoinsensitive C-fibers [46] and cowhage-activated mechano sensitive fibers [47]. Although stimuli follow the spinothalamic pathway, neurons stimulated by histamine project to the posterior part of the ventromedial nucleus of the thalamus [45], whereas neurons stimulated by pain project to the ventroposterior lateral nucleus of the thalamus [48].

Recent research has identified that, rather than there being a clear set of separate specific mediators for pain and itch, most receptor systems may participate in both pain and itch processing in the keratinocyte and nerve ending [42]. Substance P, interleukins, opioids, endothelins, proteases, and others may be involved in itch and pain sensations [42]. However, recent research has proposed that gastrin-releasing peptide, abbreviated as GRP, is a key neurotransmitter in itch sensation in the spinal cord [49, 50].

Itch may be inversely related to pain [51]. Evidence indicates the involvement of the central μ opioid receptor system in itch generation. This phenomenon is particularly relevant to spinally administered μ opioid receptor agonists, which induce segmental analgesia but also induce itch [52]. Intracerebroventricular administration of β -funaltrexamine, a selective μ opioid receptor antagonist, inhibited the scratching behavior induced by intradermal substance P [53]. However, κ opioid agonists reduce experimental itch, instead of

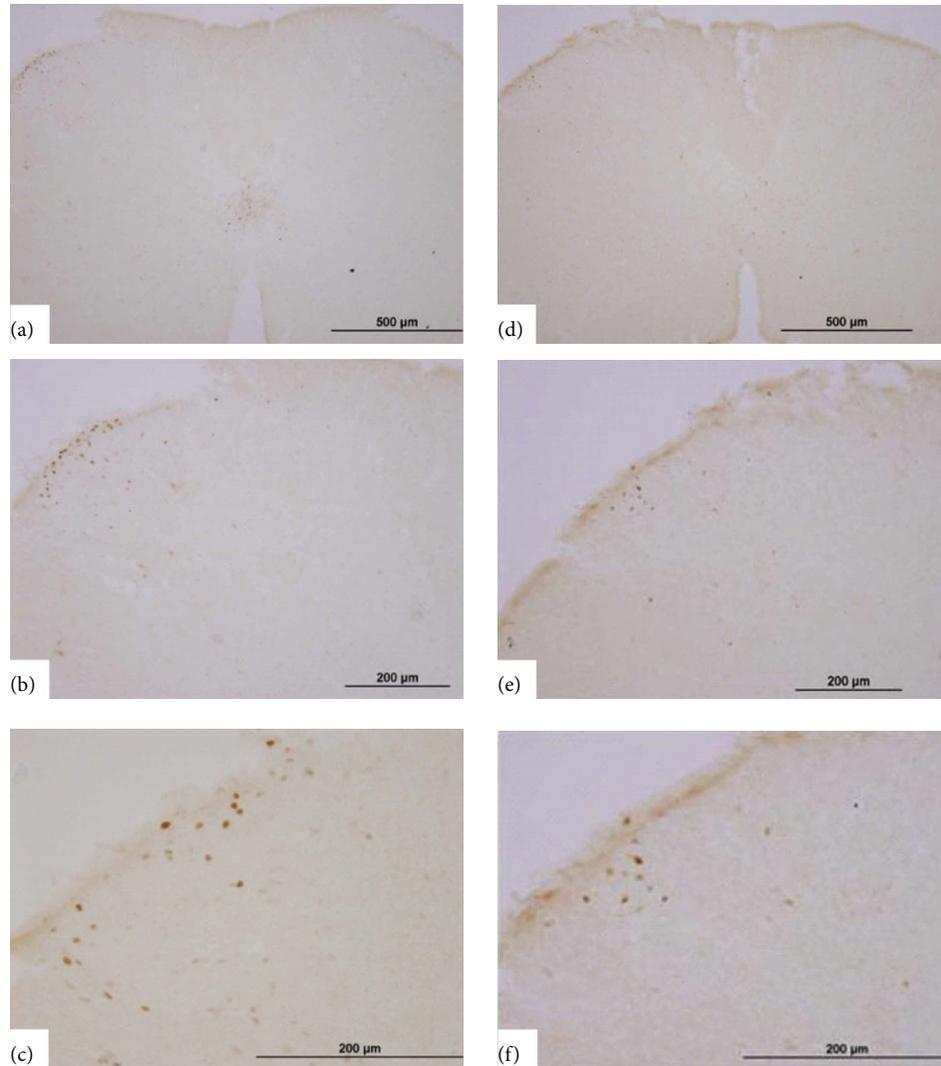


FIGURE 10: Representative photomicrographs of c-Fos expression induced by GNTI in the spinal cord and the effects of EA. GNTI (0.6 mg/kg; s.c.; behind the neck) induced c-Fos expression on the lateral side of the superficial lamina of the dorsal horn of the cervical spinal cord without (a)–(c) and with (d)–(f) EA (2 Hz) pretreatment. (a)–(c) represent one spinal section, with increasing magnification, while (d)–(f) are from another spinal section, with increasing magnification.

inducing itch [54], and κ opioid antagonists induce itch in animal experiments [55]. While the effects of opioids in the central nervous system have been investigated in detail, local production of opioids in the skin is a recent finding. The role of peripheral opioids on itch remains to be explored.

4.3. Peripheral μ Opioid Receptors May Be Involved in the Effects of EA. One of the better characterized mechanisms that may underlie EA relates to a release of endogenous opioids and activation of opioid receptors. Early studies demonstrated the role that endogenous opioids may play in acupuncture-induced analgesia [56]. EA at a lower frequency (2 Hz) stimulates the release of β -endorphin, enkephalin, and endomorphin within the CNS [57–59], whereas EA at a higher frequency (100 Hz) induces the release of dynorphin

[60]. Accordingly, 2 Hz EA analgesia may be mediated by μ and δ opioid receptors, while 100 Hz EA analgesia is mediated by κ opioid receptors.

To clarify the relationship between the anti-pruritic effect induced by EA (2 Hz) and opioid receptors, we tested the effects of naloxone and nor-binaltorphimine. In mice pretreated with naloxone, a μ opioid receptor antagonist, EA 2 Hz did not attenuate GNTI-evoked scratching, whereas when mice were pretreated with nor-binaltorphimine, a κ opioid receptor antagonist, EA 2 Hz did successfully attenuate GNTI-evoked scratching. These results reveal that the antipruritic effects of EA (2 Hz) are prevented by a μ opioid receptor antagonist. The effect of EA on GNTI-induced scratching may be related to μ , but not κ , opioid receptors.

As mentioned above, evidence indicates the central μ opioid receptor may elicit itch. However, the peripheral μ

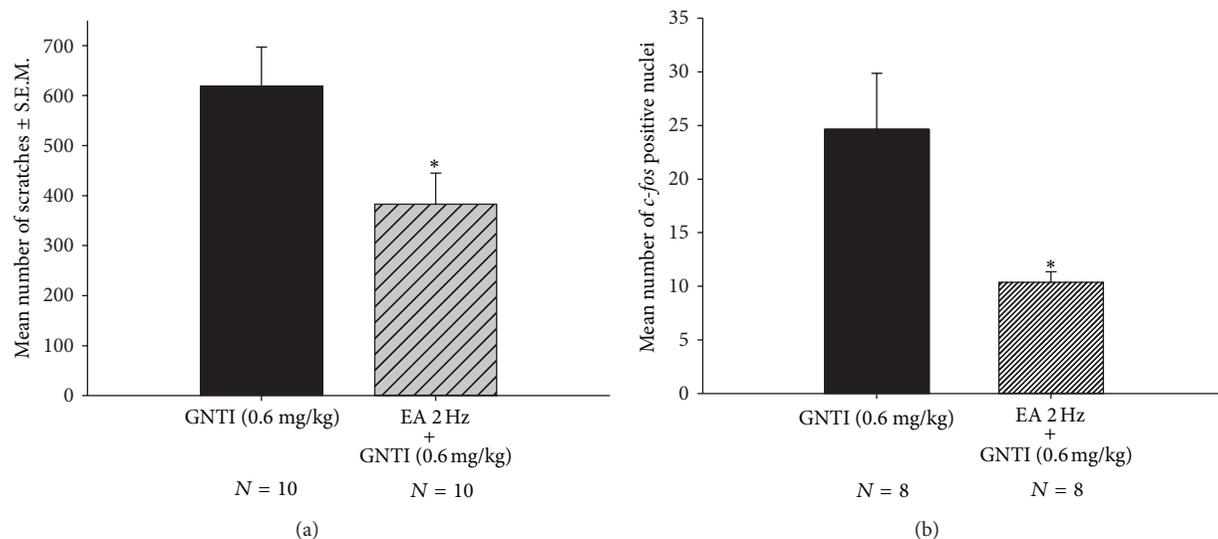


FIGURE 11: Effects of EA on GNTI- (0.6 mg/kg) induced scratching behavior (a) and c-Fos expression (b). (a) Effects of EA 2 Hz (applied to the LI4 and LI11 acupoints) on GNTI- (0.6 mg/kg) induced scratches. The number of scratches was counted for 40 min after injection of GNTI. EA at 2 Hz reduced GNTI- (0.6 mg/kg) induced scratching behavior ($*P < 0.05$ compared to control by Student's *t*-test). (b) The numbers of c-Fos positive nuclei were counted and averaged from 12 randomly chosen cervical sections from each animal in each group. EA at 2 Hz was applied to the LI4 and LI11 acupoints before administration of GNTI (0.6 mg/kg, s.c.). EA (2 Hz) significantly decreased the number of c-Fos positive nuclei ($*P < 0.05$, Student's *t*-test; *N*: the number of animals).

opioid receptor may have a different role in itch sensation [61]. To further clarify the roles of peripheral and central μ opioid receptors in the anti-pruritic effects of EA, we compared the effects of DAMGO by i.d. and s.c. injection. It is thought that intradermal injection of DAMGO can locally activate peripheral μ -opioid receptors, while s.c. injection of DAMGO can activate both peripheral and central μ opioid receptors. We found that DAMGO (10 nmol/site) attenuated GNTI-induced scratch behavior by i.d. administration but not s.c. administration. The results suggest that only peripheral activation of μ -opioid receptors can inhibit GNTI-induced scratching behavior. Similar results were also observed from another study in that i.d. administration of DAMGO reduced endothelin-1 induced-pruritic behavior [61]. It is also noted that EA is able to activate peripheral μ receptors on peripheral nerve terminals [62]. These results suggest that the peripheral μ opioid receptor may be involved in the anti-pruritic effects of low-frequency EA against GNTI-induced scratching.

4.4. EA Decreases GNTI-Induced c-Fos Expression. As mentioned earlier, peripheral receptors uniquely sensitive to pruritogens have yet to be firmly identified. The selectivity of the stimulus appears to be related to a differential connectivity at the spinal level, where a specific class of responsive dorsal horn neurons is located. From there, the stimulus travels via the lateral spinothalamic tract to the thalamus and to the cerebral cortex, where it causes the sensation of itch [46]. The expression of Fos protein or *c-fos* transcript indicates a population of neurons are activated or excited by nociceptive (noxious) inputs [18, 63]. Nojima et al. [22] suggested that spontaneous itch-related scratching behavior

is associated with c-Fos expression in the superficial dorsal horn activated by pruritogens including opiates, 5-HT, and histamine. Viewed in this context, the localization of Fos in the dorsal horn may provide a useful indicator relative to the distribution of individual neurons activated by noxious inputs in awake, behaving animals [17, 18, 20–22]. In regard to the area of c-Fos expression in the spinal cord, it is reported that formalin, the pain inducer, induces an increase in c-Fos expression on the medial side of the superficial and deeper layers of the dorsal horn of the lumbar spinal cord in mice, while the pruritogen GNTI induces an increase in c-Fos expression on the lateral side of the superficial lamina of the dorsal horn of the cervical spinal cord [64].

In this study, immunohistochemistry was employed to address the issue as to whether a reduction of GNTI-induced c-Fos expression is correlated with EA. The results showed that EA alone did not increase c-Fos expression and that GNTI (0.6 mg/kg) induced c-Fos expression on the lateral side of the superficial layer of the dorsal horn in the cervical cord. Administration of 2 Hz EA to the LI4 and LI11 acupoints decreased the number of c-Fos positive nuclei induced by GNTI. These results further support the contention that EA attenuates noxious stimuli imposed by GNTI. This is the first report demonstrating that EA decreases pruritogen-induced c-Fos expression in a mouse model.

Several clinical trials have shown that acupuncture or EA relieves histamine-induced itch [9], type I hypersensitivity itch [10], nasal itch [11], refractory uremic pruritus [12], and neurogenic pruritus [13]. Our findings not only support the suitability of EA in relieving pruritus but also underscore the observation that EA should be evaluated at different frequencies to take into account the multiple signaling molecules and mechanisms that may underlie pruritus.

5. Conclusion

EA diminishes the scratching behavior induced by GNTI in a murine model. The peripheral μ opioid system is involved, at least in part, in the anti-pruritic effects of EA. Collectively, the application of EA to the LI4 (Hegu) and LI11 (Quchi) acupoints holds promise as an effective anti-pruritic treatment modality.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Prevalence and Correlates of Discomfort and Acceptability of Acupuncture among Outpatients in Chinese Acupuncture and Moxibustion Departments: A Cross-Sectional Study

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Objective. This study aims to give a profile of discomfort and acceptability of acupuncture, including the prevalence and association with demographic and acupuncture-related factors. **Methods.** A cross-sectional study was conducted in Beijing, China. Outpatients of acupuncture and moxibustion departments were recruited using purposive sampling. 925 subjects were interviewed with an anonymous questionnaire. Multinomial and binary logistic regression were used to analyze factors affecting discomfort and acceptability of acupuncture. **Results.** The average VAS value of 925 subjects' acupuncture discomfort was 2.66 ± 2.02 , within the range of mild discomfort. Acupuncture was easily accepted by 81.1% of the subjects. Results of logistic regression were as follows: (1) subjects with a better knowledge of acupuncture, or a greater fear of pain or needles, experienced more "moderate to severe discomfort" and showed a decreased acupuncture acceptance ($P < 0.001$ or $P < 0.01$); (2) Acupuncture with less discomfort or implemented by a more qualified doctor was easy to be accepted ($P < 0.001$); (3) subjects aged 20–29 preferred to report "moderate to severe discomfort" while those aged 40–59 preferred to report "slight discomfort" ($P < 0.001$). **Conclusion.** Acupuncture is an acceptable therapy with less discomfort, which can be greatly affected by fear of pain or needles, age, knowledge of acupuncture, and professional title of acupuncturist.

1. Background

Acupuncture, as a main component of traditional Chinese medicine (TCM), has been adopted on diseases prevention and treatment for over 2,000 years in China. It exerts its effects through stimulating of acupoints with acupuncture needles, and thus triggers the body's own ability to prevent diseases [1]. Due to its excellent efficacy, acupuncture has been increasingly accepted and used by practitioners and patients worldwide.

A fact is that acupuncture is a minimally invasive therapy, which may induce anxiety and fear to some patients [2]. When acupuncturists insert needles or perform acupuncture manipulation, patients may produce various sensations, such

as sourness, numbness, distending, or pain [3]. According to TCM theory, sensations induced by acupuncture are closely related to deqi, a traditional acupuncture terminology which describes the connection between acupuncture needles and the energy pathways of the body and is essential for curative effect [4]. In our opinion, acupuncture sensations are in essence negative emotional experience for patient whether belonging to deqi or not. From this perspective, we named them discomfort of acupuncture. This study is designed to answer these two questions. To what degree do patients experience discomfort of acupuncture? What are the influence factors? By finding answers for these two questions, acupuncturists can change the acupuncture regimen accordingly, and thus improve patients' experience of acupuncture.

Though acupuncture is more and more used all over the world, there are few profiles on its discomfort, acceptability and influence factors. Discomfort of acupuncture strongly affects its acceptability. Our study showed that 44.2% (69/156) of the outpatients were reluctant to choose acupuncture because of fear of needles. In addition, acupuncture acceptability could also be affected by patients' characterizations, demographic data, and disease pattern: predominance, with physical symptoms such as diseases of the musculoskeletal system or injury, was the striking characteristic among acupuncture patients [5, 6].

In short, profiles of acupuncture discomfort and acceptability are still uncertain. Moreover, personalized treatment is one of the acupuncture characteristics, and different conditions of patients directly influence the treatment protocol. Therefore, it is useful to understand factors associated with discomfort and acceptability of acupuncture. This pilot survey focuses on the discomfort and acceptability of acupuncture, and the associated demographic and acupuncture-related factors, to provide useful suggestions on relieving discomfort of acupuncture and improving acceptance and personalized acupuncture treatment.

2. Methods

2.1. Study Design. A cross-sectional survey was conducted between May and August 2010 to assess the prevalence of discomfort and acceptability of acupuncture and associated factors. The study was conducted with a purposive sampling in three hospitals in Beijing: Guang'anmen Hospital affiliated to China Academy of Chinese Medical Sciences, Dongzhimen Hospital affiliated to Beijing University of Chinese Medicine, and Dongfang Hospital affiliated to Beijing University of Chinese Medicine, which were selected from a total of six top traditional Chinese medicine hospitals firstly, and then every two acupuncture consulting rooms were randomly selected from each of the three hospitals.

Outpatients from the six consulting rooms at the three selected hospitals, who were willing to be interviewed and were able to complete the questionnaire, participated in the survey. The subjects were asked to do the survey within 10 minutes after completing acupuncture treatment. As we did not collect any personal identifiable information, voluntary provision of information was deemed to be consent. Subjects were invited to complete an anonymous questionnaire (for more details see Supplementary Material available online at <http://dx.doi.org/10.1155/2013/715480>) under the guidance of trained interviewers. A total of 928 subjects were recruited from May 4th to August 31st. Data from 925 subjects were included in the analysis. Three incomplete questionnaires were not included.

2.2. Data Collection

2.2.1. Information Collected. We collected data on subjects' discomfort of acupuncture (one item), acceptability of acupuncture (one item), demographic profile (three items), and acupuncture-related information (five items).

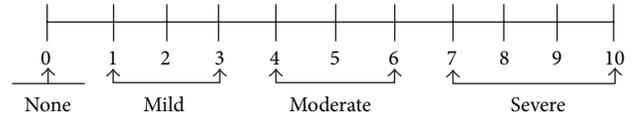


FIGURE 1: Severity of discomfort of acupuncture by VAS.

2.2.2. Discomfort of Acupuncture. Discomfort of acupuncture refers to certain sensations felt by subjects during the treatment, such as aching, soreness, distension, heaviness, numbness, or dull pain that causes negative emotions, nervousness, fears, or hostility toward acupuncture. Discomfort of acupuncture was measured by the question: "How is your feeling of discomfort caused by acupuncture?" Subjects were asked to answer the question within 10 minutes after acupuncture treatment. The severity of discomfort was marked using visual analogue scale (VAS), a numerical scale from 0 to 10 where 0 means no discomfort and 10 means the severest discomfort (see Figure 1). Severity of discomfort was evaluated by values of VAS [7]. A value of 0 on the VAS indicates no discomfort, 1 to 3 indicates mild discomfort, 4 to 6 moderate discomfort, and 7 to 10 indicates severe discomfort.

2.2.3. Acceptability of Acupuncture. Acceptability of acupuncture was assessed by the question: "How is your acceptability of acupuncture?" There were two options for selection, "Difficult to accept" or "Easy to accept."

2.2.4. Demographic Profile. To understand the influence of demographic characteristics toward discomfort and acceptability of acupuncture, the gender, age and education level were taken into consideration. Age was classified into seven segments: ≤ 19 , 20–29, 30–39, 40–49, 50–59, 60–69, and ≥ 70 . Educational level was determined based on the International Standard Classification of Education (ISCED) [8]. We classified educational level into three categories: primary education and below (ISCED level: 0–1), secondary education (ISCED level: 2–4), and tertiary education (ISCED level: 5–8).

2.2.5. Acupuncture-Related Information. To explore acupuncture-related factors affecting discomfort and acceptability of acupuncture, five items were adopted in the questionnaire. These items included whether it was the first acupuncture experience, whether subjects were afraid of acupuncture needles, the extent of fear of pain, subjects' knowledge of acupuncture, and professional title of acupuncturist.

2.2.6. Data Collection. The questionnaire was first given to 5 outpatients to assure clarity of concepts for subjects. The data was collected by two postgraduate students who majored in acupuncture and received formal training on questionnaire interviewing. To ensure research quality, a supervisor performed a spot check on completed questionnaires for completeness and consistency at the time of interview.

3. Statistical Analysis

Statistical analysis was performed using SPSS statistics 18.0. Binary logistic regression with backward stepwise was used to assess the relationship between acceptability of acupuncture and demographic, discomfort, or acupuncture-related variables. Chi-square test was used to test for association between acupuncture discomfort and demographic or acupuncture-related variables; variables that were shown to be significantly associated with discomfort of acupuncture were entered into multinomial logistic regression. $P < 0.05$ was considered statistically significant.

4. Results

4.1. Discomfort of Acupuncture. The average VAS value of 925 subjects' acupuncture discomfort was 2.66 ± 2.02 , within the range of mild discomfort. 146 subjects (15.8%) did not feel any discomfort; the majority of subjects (53.9%) felt slight discomfort; 188 subjects (20.3%) felt moderate discomfort; and 92 (9.9%) felt severe discomfort. "Moderate discomfort" and "severe discomfort" responses were analyzed together (Table 1).

4.2. Acceptability of Acupuncture. Among 925 subjects, 750 subjects (81.1%) reported that acupuncture was easy to be accepted, and 175 (18.9%) reported that acupuncture was difficult to be accepted (Table 1).

4.3. Demographic Profile. Out of 925 subjects (mean age \pm standard deviation: 47.97 ± 2.00 years), 40.2% were male and 59.8% were female. 33.7% had completed tertiary education, 58.6% secondary, and 7.7% primary education (Table 1).

4.4. Acupuncture-Related Factors. There were 17.4% of subjects receiving acupuncture treatment for the first time, and 21.2% of subjects reported fear of needles. When asked how much they were afraid of pain, 32.9% of subjects chose "not at all", 30.9% and 36.2% chose "very much" and "a little," respectively.

Overall, most of the subjects were characterized by the following features: the previous acupuncture experience (82.6%), fear of acupuncture needles (78.8%), fear of pain (67.1%), little knowledge of acupuncture (80.8%), and seeking help from more qualified acupuncturists (82.9%). Details are shown in Table 1.

4.5. Factors Affecting Discomfort of Acupuncture. Results of chi-square tests showed that age ($\chi^2 = 32.83$, $P = 0.001$), fear of needles ($\chi^2 = 34.15$, $P < 0.01$), knowledge of acupuncture ($\chi^2 = 13.17$, $P = 0.01$) and fear of pain ($\chi^2 = 53.75$, $P < 0.01$), were significantly different among varying degrees of acupuncture discomfort. Table 2 showed the multinomial logistic regression predicting the odds of reporting acupuncture discomfort as no discomfort or slight discomfort.

Results showed that subjects reported more "moderate to severe discomfort" than "no discomfort" in several conditions: (1) if they had a fear of needles (OR = 0.26, 95% CI = 0.14–0.49); (2) if they were afraid of pain (a little versus not at all: OR = 0.22, 95% CI = 0.13–0.37; very much versus not at all: OR = 0.24, 95% CI = 0.14–0.41); (3) if they showed a better knowledge of acupuncture (a little versus not at all: OR = 0.43, 95% CI = 0.26–0.71, very well versus not at all: OR = 0.40, 95% CI = 0.21–0.77); and (4) if they were aged 20–29 (20–29 versus ≥ 70 : OR = 0.35, 95% CI = 0.13–0.90).

In the comparisons between "slight discomfort" and "moderate to severe discomfort," fear of needles, fear of pain and age rather than knowledge of acupuncture, showed significant differences. Subjects experienced more "moderate to severe discomfort" than "slight discomfort" if they were afraid of needles (OR = 0.50, 95% CI = 0.36–0.72) and pain (a little versus not at all: OR = 0.47, 95% CI = 0.32–0.69; very much versus not at all: OR = 0.50, 95% CI = 0.33–0.75), while subjects aged 40–49 reported more "slight discomfort" (40–49 versus ≥ 70 : OR = 1.85, 95% CI = 1.04–3.30).

4.6. Factors Affecting Acceptability of Acupuncture. The influence of variables (i.e., discomfort of acupuncture, demographic, and acupuncture-related factors) on acceptability of acupuncture was analyzed by binary logistic regression with backward stepwise. During the analysis procedure, variables of gender, educational level, and age were removed from the equation successively. The residual variables were significantly associated with acceptability of acupuncture, including discomfort of acupuncture, first acupuncture experience, fear of needles, fear of pain, knowledge of acupuncture, and professional title of acupuncturist (Table 3).

Acupuncture discomfort and professional title of acupuncturist were positively associated with acupuncture acceptability. A lower discomfort (no discomfort versus moderate to severe discomfort: OR = 3.11, 95% CI = 1.45–6.72, $P = 0.004$; slight discomfort versus moderate to severe discomfort: OR = 1.90, 95% CI = 1.29–2.82, $P = 0.001$) and a higher professional title of acupuncturist (senior title versus primary title: OR = 3.22, 95% CI = 1.95–5.34, $P < 0.001$; middle title versus primary title: OR = 2.59, 95% CI = 1.58–4.23, $P < 0.001$) were significantly associated with greater willingness to accept acupuncture, while other variables were significantly associated with a decreased willingness to accept acupuncture, referring primarily to first acupuncture experience (OR = 0.62, 95% CI = 0.39–0.99, $P = 0.047$), a greater fear of pain (very much versus not at all: OR = 0.18, 95% CI = 0.10–0.31, $P < 0.001$; a little versus not at all: OR = 0.42, 95% CI = 0.24–0.74, $P < 0.001$), a better knowledge of acupuncture (very well versus not at all: OR = 0.32, 95% CI = 0.16–0.61, $P = 0.001$; a little versus not at all: OR = 0.32, 95% CI = 0.18–0.56, $P < 0.001$), and fear of needles (OR = 0.34, 95% CI = 0.22–0.51, $P < 0.001$).

5. Discussion

In present study, a female predominance was observed (female: male = 1.48:1), and the age distribution displayed

TABLE 1: Characteristics of subjects.

Variables	Number	%
How's your feeling of discomfort caused by acupuncture?		
No discomfort (VAS = 0)	146	15.8
Slight discomfort ($1 \leq \text{VAS} \leq 3$)	499	53.9
Moderate to severe discomfort ($4 \leq \text{VAS} \leq 10$)	280	30.3
How is your acceptability of acupuncture?		
Difficult to accept	175	18.9
Easy to accept	750	81.1
Gender		
Female	553	59.8
Male	372	40.2
Age		
≤ 19	57	6.2
20–29	140	15.1
30–39	112	12.1
40–49	147	15.9
50–59	229	24.8
60–69	130	14.1
≥ 70	110	11.9
Educational level		
Primary education and below (ISCED level: 0-1)	71	7.7
Secondary education (ISCED level: 2-4)	542	58.6
Tertiary education (ISCED level: 5-8)	312	33.7
First acupuncture experience: Is this your first acupuncture experience?		
No	764	82.6
Yes	161	17.4
Fear of needles: Are you afraid of acupuncture needles?		
No	729	78.8
Yes	196	21.2
Fear of pain: How much are you afraid of pain?		
Not at all	304	32.9
A little	335	36.2
Very much	286	30.9
Knowledge of acupuncture: How much do you know about acupuncture?		
Not at all	217	23.5
A little	530	57.3
Very well	178	19.2
Professional title of acupuncturist		
Resident physician	158	17.1
Attending physician	363	39.2
Chief physician	404	43.7

a peak at around fifties. Our findings were consistent with previous report [9]. The educational levels of subjects in our study were mainly secondary education or higher. Majority of subjects believed that acupuncture induced slight or no discomfort (69.7%) and was easy to be accepted (81.1%). There were 11.4% of subjects considering that acupuncture was easy to be accepted, although they chose moderate or severe discomfort. This may be interpreted by subjects' strong expectation for good effectiveness of acupuncture. Overall, acupuncture causes a little discomfort and it can be accepted

easily, which was consistent with the finding that 81% of subjects considered acupuncture process to be comfortable and relaxing [10].

Till now, there were few studies on acupuncture discomfort and acceptability. Fear of pain or needles, as negative emotional experience, could cause greater discomfort. Multinomial regression analysis showed that subjects experienced more discomfort if he or she had a stronger fear toward pain or needles. Compared with subjects aged ≥ 70 , subjects aged 20–29 preferred to report "moderate to severe discomfort"

TABLE 2: Multinomial logistic regression: Odds of subjects reporting less discomfort of acupuncture ($n = 925$).

Characteristic/subcategory	Discomfort of acupuncture: No. (%)			No discomfort ^a		Slight discomfort ^a	
	No	Slight	Moderate to severe	OR (95% CI)	P value	OR (95% CI)	P value
Age							
≥70	20 (18.1)	51 (46.4)	39 (35.5)	1.00		1.00	
≤19	7 (12.3)	35 (61.4)	15 (26.3)	1.12 (0.37–3.41)	0.841	2.02 (0.94–4.32)	0.070
20–29	8 (5.7)	76 (54.3)	56 (40.0)	0.35 (0.13–0.90)	0.030	1.16 (0.66–2.03)	0.605
30–39	11 (9.8)	66 (58.9)	35 (31.3)	0.78 (0.32–1.94)	0.596	1.57 (0.86–2.85)	0.142
40–49	22 (15.0)	88 (59.9)	37 (25.1)	1.37 (0.62–3.02)	0.443	1.85 (1.04–3.30)	0.037
50–59	52 (22.7)	114 (49.8)	63 (27.5)	1.69 (0.84–3.39)	0.138	1.39 (0.82–2.37)	0.226
60–69	26 (20.0)	69 (53.1)	35 (26.9)	1.21 (0.55–2.65)	0.638	1.31 (0.71–2.39)	0.379
Fear of needles: Are you afraid of acupuncture needles?							
No	132 (18.1)	407 (55.8)	190 (26.1)	1.00		1.00	
Yes	14 (7.2)	92 (46.9)	90 (45.9)	0.25 (0.14–0.48)	<0.001	0.50 (0.36–0.72)	<0.001
Fear of pain: How much are you afraid of pain?							
Not at all	79 (26.0)	171 (56.3)	54 (17.8)	1.00		1.00	
A little	36 (10.7)	176 (52.5)	123 (36.7)	0.22 (0.13–0.37)	<0.001	0.47 (0.32–0.69)	<0.001
Very much	31 (10.8)	152 (53.1)	103 (36.0)	0.24 (0.14–0.41)	<0.001	0.50 (0.33–0.75)	0.001
Knowledge of acupuncture: How much do you know about acupuncture?							
Not at all	51 (23.5)	110 (50.7)	56 (25.8)	1.00		1.00	
A little	72 (13.6)	291 (54.9)	167 (31.5)	0.43 (0.26–0.71)	0.001	0.85 (0.58–1.24)	0.394
Very well	23 (12.9)	98 (55.1)	57 (32.0)	0.40 (0.21–0.77)	0.006	0.82 (0.51–1.32)	0.423

OR: odds ratio; CI: confidence interval; ^aThe reference category is “moderate to severe discomfort”.

while those aged 40–59 preferred to report “slight discomfort.” It was unexpected that a better knowledge of acupuncture led to more discomfort. In clinical practice, some patients showed great willingness to acupuncture treatment because of good effectiveness; however, their nervousness did not decrease with treatment sessions. Considering this fact, the result of knowledge of acupuncture may be somewhat understood. Nevertheless, further research is needed to understand the relationship between the knowledge and acupuncture discomfort. In addition, the previous acupuncture experience, educational level, and professional title of acupuncturist showed no significant influence on discomfort. So did the factor of gender.

Acupuncture discomfort, which is a negative emotion like pain, can be predicted to be influenced by gender. The relationship between gender differences and pain has been reported a lot, but the conclusions are different. Most reports showed that males exhibited greater pain tolerance than females [11, 12], but a systematic review failed to show a clear and consistent pattern of gender differences in pain sensitivity [13]. Paradigm to investigate the role of gender in pain perception was mainly based on laboratory-induced thermal, pressure, chemical, or visceral pain, but the application of the paradigms for clinic pain is questionable [13]. In our study, we

did not find a significant gender difference toward discomfort of acupuncture, which is similar to the result of systematic review.

Binary logistic regression analysis of acupuncture acceptability showed that subjects could accept acupuncture more easily if they had less discomfort (no or slight discomfort) and were treated by acupuncturist with a higher professional title. However, they may have great difficulties in accepting acupuncture if they had a fear of pain or needles and a better knowledge of acupuncture. The more serious the negative experiences (e.g., discomfort, fear of pain and acupuncture) are, the more difficult the acupuncture is accepted by subjects. Patient without acupuncture experience showed more difficulty in accepting acupuncture treatment. However, the impact of the first acupuncture experience on acupuncture discomfort was little enough to be disregarded ($P = 0.047$).

Subjects could consider acupuncture easy to be accepted when they were treated by an acupuncturist with a higher professional title, consistent with the previous report [14]. Our results showed that better knowledge of acupuncture may cause more discomfort, and lead to a decreased willingness to acupuncture acceptance. A possible reason was that subjects gained acupuncture knowledge mainly from their own experience, in which negative experience tended to be

TABLE 3: Binary logistic regression: Odds of subjects reporting better acceptance of acupuncture ($n = 925$).

Characteristic/Subcategory	Acceptance of acupuncture		Odds Ratios	
	Difficult to accept	Easy to accept	OR (95% CI)	<i>P</i> value
First acupuncture experience: Is this your first acupuncture experience?				
No	135 (17.7)	629 (82.3)	1.00	
Yes	40 (24.8)	121 (75.2)	0.62 (0.39–0.99)	0.047
Fear of needles: Are you afraid of acupuncture needles?				
No	95 (13.0)	634 (87.0)	1.00	
Yes	80 (40.8)	116 (59.2)	0.34 (0.22–0.51)	<0.001
Fear of pain: How much are you afraid of pain?				
Not at all	21 (12.0)	283 (93.1)	1.00	1.00
A little	63 (18.8)	272 (81.2)	0.42 (0.24–0.74)	0.002
Very much	91 (31.8)	195 (68.2)	0.18 (0.10–0.31)	<0.001
Knowledge of acupuncture: How much do you know about acupuncture?				
Not at all	20 (9.2)	197 (90.8)	1.00	
A little	119 (22.5)	411 (77.5)	0.32 (0.18–0.56)	0.001
Very well	36 (20.2)	142 (79.8)	0.32 (0.16–0.61)	<0.001
Professional title of acupuncturist				
Resident physician	59 (37.3)	99 (62.7)	1.00	
Attending physician	63 (17.4)	300 (82.6)	2.59 (1.58–4.23)	<0.001
Chief physician	53 (13.1)	351 (86.9)	3.22 (1.95–5.34)	<0.001
Discomfort of acupuncture: How is your discomfort feeling caused by acupuncture?				
Moderate to severe discomfort	87 (31.1)	193 (68.9)	1.00	
Slight discomfort	79 (15.8)	420 (84.2)	1.90 (1.29–2.82)	0.001
No discomfort	9 (6.2)	137 (93.8)	3.11 (1.45–6.72)	0.004

OR: odds ratio; CI: confidence interval.

exaggerated. Further research is needed to understand the relationship of the knowledge, discomfort and acceptability of acupuncture.

Overall, fear of needles and pain can cause more discomfort, resulting in decreased acceptance. However, it is worth mentioning that pain endurance can be influenced by pain-related self-efficacy and positive self-instruction [11]. Positive outcome expectancy also indicates a marked improvement in patients' self-reports of anxiety, pain and distress [15, 16]. Therefore, communication and encouragement before acupuncture can hopefully improve patients' fear of pain and acupuncture, thus reducing discomfort and increasing acceptance. Our results showed that a senior professional title of acupuncturist had barely any impact on discomfort, but it can help to increase acupuncture acceptance.

Due to limited staff and resources, our study was completed only in six acupuncture consulting rooms from three top TCM hospitals in Beijing; lower grade hospitals and communities were not included. Besides, purposive sampling instead of random sampling was used in the study. Therefore

our study sample may not be sufficiently representative. Secondly, other factors that may affect acupuncture discomfort and acceptability were not included in this study, such as details of sociodemographic information (income, occupation, marital status, etc.), diagnosis of diseases, needling location selecting, and the needling depth. In addition, only the severities of the sensations induced by acupuncture were recorded; the type of sensation was neglected. Acupuncture sensations were categorized into two types, namely, sensations that cannot hurt as deqi, mainly including aching, soreness, and pressure, followed by tingling, numbness, dull pain, heaviness, warmth, fullness, and coolness, and sensations that can hurt as noxious stimulation, for example, sharp pain [17–19]. A certain type of sensation may contribute to both acupuncture discomfort and deqi. By neglecting the type of discomfort, we cannot differentiate useful discomfort constituting deqi from actual discomfort sensations. Finally, due to the cross sectional nature of this study, our findings should be interpreted as associations rather than implying causality.

6. Conclusions

In conclusion, acupuncture is an acceptable therapy with less discomfort. Discomfort and acceptability of acupuncture can be affected by fear of pain, fear of needles, age, knowledge of acupuncture, and professional title of acupuncturist. Based on our results, methods to relieve discomfort and improve acceptance of acupuncture can be taken accordingly.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Z. Liu and B. Liu designed this study and revised the paper. HF. Xu drafted the paper and performed the statistical analysis. SN. Guo and JN. Wu participated in data acquisition. M. Y. Lim and J. Liu revised the paper. All the authors read and approved the final paper.

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Review Article

Efficacy and Safety of Acupuncture in Preterm and Term Infants

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The aim of the paper was to review the literature about safety and efficiency of acupuncture therapy in term and preterm infants. We searched Medline, EMBASE, and Cochrane Central Register of Controlled Trials using a predefined algorithm, reviewed abstracts from the Pediatric Academic Society annual meetings (2000–2012), and performed a manual search of references in narrative and systematic reviews. A total of 26 studies identified met our search criteria. Only 6 of these studies met our inclusion criteria; however, two studies had to be excluded because the manuscripts were published in Chinese. Hence, only four studies were included in our analysis. Three of the four studies evaluated the effects of acupuncture on infantile colic, and one assessed pain reduction during minor painful procedures in preterm babies. The limited data available suggests that acupuncture could be a safe nonpharmacologic treatment option for pain reduction in term and preterm infants and could also be a non-pharmacologic treatment option to treat infantile colic. Currently acupuncture in infants should be limited to clinical trials and studies evaluating short- and long-term effects and should be performed only by practitioners with adequate training and experience in neonatal/pediatric acupuncture.

1. Introduction

Traditional Chinese Medicine (TCM) has been practiced in China for over 2000 years. TCM remained the main form of medical treatment within China before western medicine was introduced in the past 100 years. Traditional Chinese Medicine includes (i) massage therapy (= *Tuina*), (ii) moxibustion, (iii) and acupuncture. According to the available literature the pillars of pediatric treatment were massages, diets based on the five elements, and medicinal therapy. Observational studies reported that TCM is a popular treatment in children [1, 2]. In addition, there is increasing demand of complementary and alternative medicine (CAM) treatments in the pediatric population [3–7]. This trend towards CAM might be explained by dissatisfaction with conventional medicine as well as positive reports from friends and family [8–10].

There is a lack of data to support acupuncture, TCM, or CAM in children [11–14]. Only a few studies investigated the effect of acupuncture in children demonstrating positive effects on obesity [15], skin irritations, constipation, and pain [16, 17]. In addition, there is emerging evidence in individual cases of acupuncture in neonates [18] and to treat infantile colic [19–21]. Limitations of acupuncture in children are (i) their fear of needles and pain, which makes acupuncture difficult to perform in young children; (ii) infants or toddlers lacking cooperation, which makes the precise use of needle points challenging [22]; (iii) risk of infection caused by needle prick injuries [5, 23]; and (iv) safety of acupuncture being a major concern, particularly during early infancy when responses are difficult to evaluate.

Jindal et al. [24] reviewed the current evidence for acupuncture treatment including nausea and vomiting, asthma and seasonal allergic rhinitis, neurologic and gastrointestinal

disorders, pain, and addiction. Overall, the most evidence for acupuncture comes from studies managing postoperative and chemotherapy-induced nausea/vomiting. Acupuncture seems to be most effective in preventing postoperative induced nausea in children. Acupuncture also appears to be well tolerated in children, has a low incidence of side effects, but fewer needles should be used when treating infants.

In general, acupuncture in children is usually limited to brief light needling using one-way needles, treatment by using acupressure, and giving mild stimulation and laser acupuncture.

The development of laser acupuncture allows new treatment options in children [25]. Laser acupuncture provides a noninvasive therapeutic approach, thus excluding the risk of infection caused by needle prick injuries [13, 24]. But the central and peripheral effects of laser acupuncture in infants have only been sporadically evaluated [26]. In particular the applied doses and the time of stimulation are a matter of ongoing discussions. However, there is increasing evidence from observational studies that acupuncture is a potential nonpharmacologic treatment for infants, term and preterm newborns, during their hospitalization in the intensive care unit [27, 28]. In particular newborn infants are exposed to sedative and analgesic medications, which are often prescribed for a prolonged period of time during their intensive care admission. Hence, the use of alternative or adjunctive comfort measures might decrease neonatal exposure to potentially neurotoxic agents. In addition, a pilot study by Golianu et al. [29] investigated the effect of acupuncture for the management of neonatal opioid and benzodiazepine withdrawal. The results are eagerly awaited. Also a study protocol for a Cochrane Review proposes to evaluate the effect of acupuncture in neonates with hypoxic ischemic encephalopathy compared with standard care but the details have never been published [30].

The aim of the paper was to review the literature about safety and efficiency of acupuncture therapy in term (between 37 and 42 weeks gestation), preterm infants (less 37 weeks gestation), and infants with infantile colic (infants within the 1st year of age).

2. Methods

2.1. Search Strategy. We searched Medline, EMBASE, and Cochrane Central Register of Controlled Trials using a predefined algorithm (the appendix), reviewed abstracts from the Pediatric Academic Societies annual meetings (2000–2012), and performed a manual search of references in narrative and systematic reviews. Discrepancies regarding inclusion were resolved through discussion among the review team.

2.2. Study Selection. Studies meeting the following criteria were included in the review: randomized control trial; comparing acupuncture versus placebo or versus medical treatment in preterm and term infants. The following outcomes were assessed: safety, efficiency, all-cause mortality, and death. Studies describing preterm infants were eligible if infants were born and treated <37 weeks gestation. Term

infants were included if gestation age was between 37 and 42 weeks. In addition, studies investigating acupuncture during the neonatal period (day 1 to 28 after birth) were eligible. For infants with infantile colic studies were eligible for inclusion within the 1st year of age.

2.3. Data Extraction. Data were recorded using a standardized data collection form to record study design and methodological characteristics, patient characteristics, interventions, and outcomes, thereof, relative risk and 95% confidence interval (CI), as well as information regarding randomization mode, allocation concealment, blinding, and intention-to-treat analysis. Data extraction was independently performed by two investigators (GMS, WR) and discrepancies were resolved by consulting a third investigator (BU) through discussion.

2.4. Assessment of Methodological Quality. We assessed the methodological quality of the included trials and the risk of bias conferred by using elements of the Cochrane collaboration tool for assessing risk of bias [31]. The domains used in the present systematic review pertained to randomization and allocation concealment (selection bias), blinding (performance and detection bias), and adherence to the intention-to-treat principle (attrition bias).

2.5. Statistical Analysis. We planned to measure the principal summary as weighted mean difference (WMD) for continuous outcomes, relative risk (RR), and the absolute risk reduction (RD) for dichotomous outcomes. For each trial, we planned to retrieve or calculate the crude RR and RD estimates and corresponding 95% CIs for the assessed outcomes. We planned to explore heterogeneity using a chi-square test and the quantity of heterogeneity using the I^2 [32] statistic [32]. We planned to summarize RR and RD estimates using random-effects models [33]. Analyses were performed in RevMan version 5 (Cochrane Collaboration, 2010). All P values are 2-tailed. We planned to calculate the numbers needed to treat (NNT) for all outcomes where the pooled estimates of RR were statistically significant. The study is reported according to the PRISMA checklist (Figure 1) [34].

3. Results

A total of 26 studies identified met our search criteria (the appendix). However, 20 studies had to be excluded as they were evaluating the effect of acupuncture to resolve (i) breech presentation, (ii) mastitis during lactation, or (iii) pain during labor. Only 6 studies met our inclusion criteria (Figure 2); however, two further studies had to be excluded because the manuscripts were published in Chinese. Hence, only four studies were included in our analysis. Three of four studies evaluated the effects of acupuncture on infantile colic and one assessed pain reduction during minor painful procedures in preterm babies. In addition, we identified 28 abstracts from the Annual Meeting of the Pediatric Academic Societies addressing acupuncture in children. No abstract was identified reporting acupuncture in infants or newborn babies.

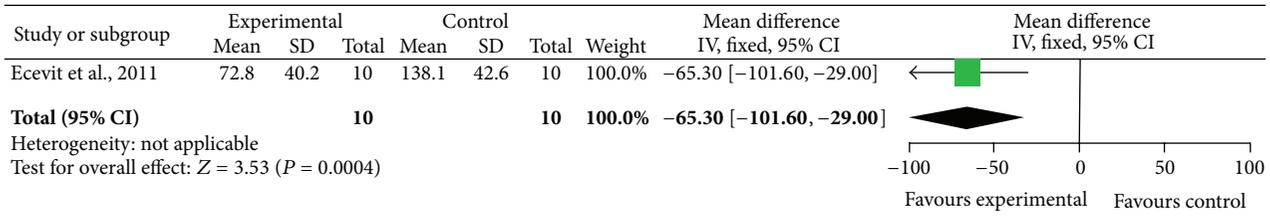


FIGURE 1: Forest plot of crying time for heel prick procedure in preterm infants with and without acupuncture.

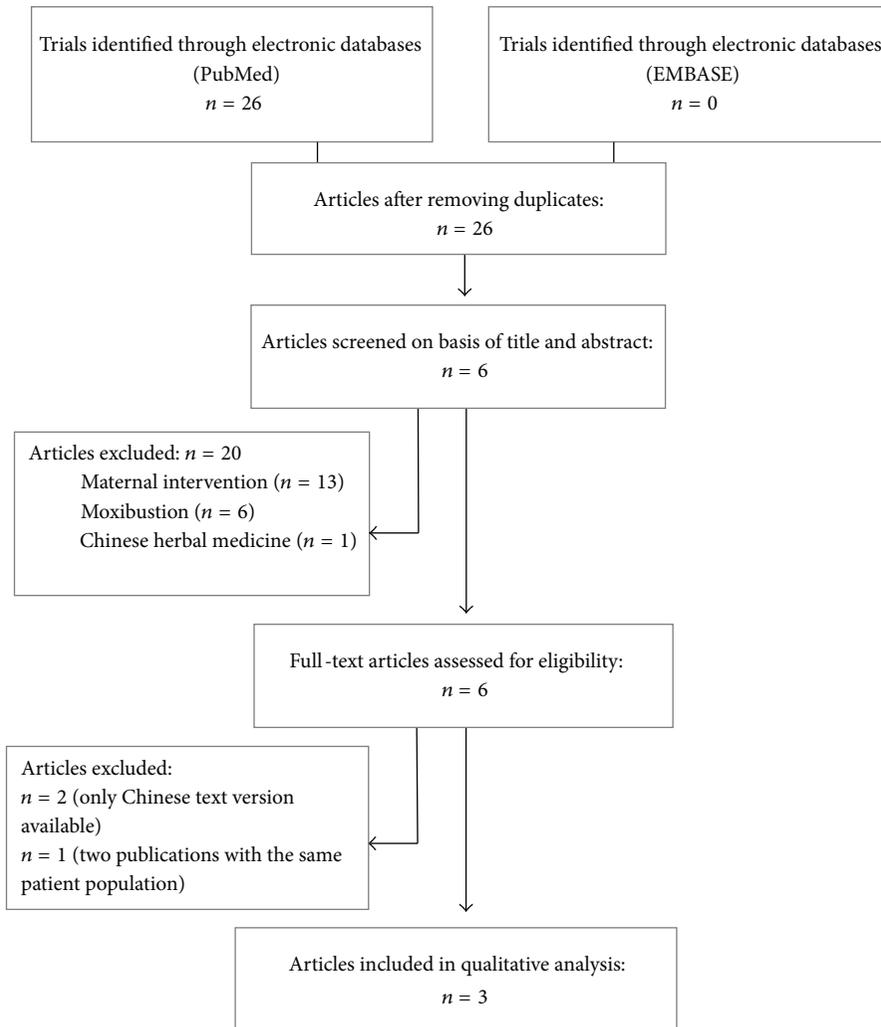


FIGURE 2: PRISMA flow chart.

Risk of bias assessment of included trials is presented in Table 1. We planned extensive statistical analysis; however, the trials identified (3x infantile colic, 1x pain in preterm infants) did not allow any of the planned analysis described in Section 2.5.

3.1. Pain in Preterm Infants. We identified one trial assessing the effect of acupuncture in preterm babies during minor painful procedures [28]. Using cross-over design 10 preterm infants were randomized to receive breast milk only or breast milk and acupuncture for a heel prick for blood gas analysis. Each infant acted as their own control and received either

breast milk only on day one and on the following day breast milk and acupuncture or vice versa. Oxygen saturation, systolic and diastolic blood pressure, respiratory rate, and heart rate were similar before and after heel prick within groups. Crying duration (Figure 1) and neonatal infant pain scale scores (Figure 3) during heel prick were significantly lower in neonates who received acupuncture.

3.2. Infantile Colic. Two studies assessed crying [19, 20] and a third study assessed feeding, stooling, and sleeping patterns [35]. Overall a total of 121 infants were included to assess infantile colic.

TABLE 1: Risk of bias assessment of randomized controlled trials investigating acupuncture in preterm and term infants.

Study	Study population	Comparison	Primary outcome measures	Sequence generation	Allocation concealment	Blinding of participants, personnel, and outcome	Incomplete outcome data	Selective outcome reporting	Funding bias
Ecevit et al. [28]	Preterm infants ($n = 10$)	Breast milk only or breast milk and acupuncture	Crying duration during heel prick for blood gas analysis	Unclear	Unclear	Unclear	Unclear	Unclear	Unclear
Landgren et al. [19, 35]	2–8 weeks old infants ($n = 90$)	Structured program versus structured program and needle acupuncture	Remission of infantile colic	Low	Low	Low	Low	Low	Low
Reinthal et al. [20]	Median 6 weeks old infants ($n = 40$)	Intervention versus control group	Crying intensity, frequency, duration of, crying and pain related behavior	Unclear	Unclear	Unclear	Low	Low	Low

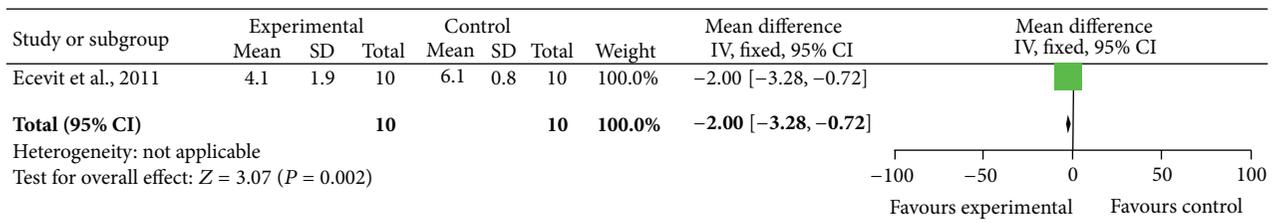


FIGURE 3: Forest plot of the neonatal infant pain scale score with and without acupuncture in preterm infants.

Reinthal et al. quasirandomized 40 infants with excessive crying (median age: six weeks) to conventional or light needling treatment. Parents were blinded to the group assignment. Infants received acupuncture at the LI4 point on both hands for approximately 20 seconds on four occasions compared to conventional group. Parents had to complete pre- and posttreatment questionnaires to assess intensity, frequency, and duration of crying as well as pain-related behavior. Light needling resulted in a significant reduction in the rated crying intensity. Pain-related behavior like facial expression was also significantly less pronounced in the light needling group as compared to the control group. In addition, parents rated light needling as more effective in improving all symptoms than the control group.

Landgren et al. [19] assessed the effect of acupuncture to reduce duration and intensity of crying in infantile colic. Eighty-one of the ninety included infants (2–8 weeks) completed a three weeks structured program consisting of six visits to an acupuncture clinic. Parents were blinded to the allocation of their children. Infants randomized to the treatment group received standardized acupuncture for 2 sec at the LI4 point in addition to standard of care. Infants randomized to acupuncture had a significant lower duration of fussing in the 1st (74 versus 129 min) and 2nd weeks

(71 versus 102 min). In addition, a significant shorter duration of colicky crying in the 2nd intervention week (9 versus 13 min) was observed. In the same patients Landgren et al. [35] also assessed the infants sleep and stooling behavior (frequency and size). In addition, side effects were assessed using a parental questionnaire. Infants randomized to acupuncture had an increased stooling frequency compared to control group. No side effects were recorded. Overall, minimal needling acupuncture had no significant effect on feeding, stooling, or sleep.

4. Discussion

4.1. Efficacy and Safety in Preterm Infants. One trial used breast milk compared to acupuncture and breast milk to assess pain during minor painful procedure in preterm infants [28]. Overall, the acupuncture was well tolerated, and the mean crying time (Figure 1) and the neonatal infant pain scale scores (Figure 3) were significantly lower in preterm infants receiving acupuncture compared to the control group. Although a significant reduction in crying time and pain score was observed in preterm infants receiving acupuncture, these results have to be interpreted with caution. The study by Ecevit et al. [28] had a very small sample size of 10 preterm

infants using cross-over design. Light needling acupuncture was performed using acupuncture point Yintang (EXHN3), which is located midway between the medial ends of the eyebrows. Although a short and restful sleep and a significant decrease in heart rate during the procedure was observed, this might be due to the sedative effects of acupuncture point Yintang (EX-HN3) [28].

Further evidence about efficacy and safety in preterm infants comes from observational studies [23, 27]. Gentry et al. described 10 preterm and term infants receiving acupuncture in a retrospective chart review [27]. They observed a significant decrease in sedative and analgesic use in 5/8 infants treated with acupuncture therapy for agitation, over a time period of 2 weeks to 5 months [27]. Raith et al. described the first case report of laser acupuncture in a preterm infant [23]. After treatment a reduction in heart rate over time was observed [23]. In addition, acupuncture has been described for infantile cerebral palsy, neonatal stress and during hypoxic ischemic encephalopathy, and neonatal abstinence syndrome [18, 30, 36].

No study using needle acupuncture described any skin breakdown, infection, hematoma, or allergic reactions. In addition, no patient distress or discomfort was observed [27, 28]. Raith et al. [26] compared skin temperature before and 5 and 10 min after local laser needle acupuncture. On average an increase in local skin temperature of about 1°C was observed. In one case a maximum temperature of 37.9°C was observed [26]. However, the temperature increase was similar to transcutaneous CO₂ measurements [37]. Furthermore, it remains unknown whether repeated needle stimulation may alter sensory processing and responses to subsequent painful stimuli, similar as heel pricks in infants, skin breakdown, or infection.

In summary current evidence suggests that acupuncture is feasible; however, more evidence is needed to determine efficacy and safety of this treatment in preterm and term infants. Only practitioners with adequate training and experience in neonatal/pediatric acupuncture should perform acupuncture treatments.

4.2. Efficacy and Safety in Infants with Infantile Colic. Infantile colic is a common painful clinical condition associated with signs of distended intestines and an increase in colon peristalsis. We identified three studies evaluating acupuncture for the management of infantile colic [19, 20, 35]. All studies used the LI 4 (Hegu) point, which is considered to be one of the most effective acupuncture points for general pain control. In addition, it has been reported that LI4 interacts with serotonin and melatonin release and thereby with the circadian rhythm [38]. LI 4 is an acupuncture point in the large intestine meridian located on the radial side in the middle of the 2nd metacarpal bone. LI 4 is easily accessible and therefore easy to use in particular in young infants. The studies by Landgren et al. [19, 35] used short needling intervals of two seconds alternating between the right and left hands. The study by Reinthal et al. used “light needling” for 20 seconds bilaterally as minimal acupuncture technique [20]. Both trials reported a reduction crying frequency and intensity in the acupuncture group compared to controls. Limitations

of Reinthal’s study are (i) infants were older, which could have contributed to the remission rates, (ii) parents were blinded, but the same nurse who met the parents performed the acupuncture [20]. The main limitation from Landgren et al. [19] study is the increased crying incidence in the acupuncture group. Parents could have interpreted the crying as being in the treatment group, therefore providing a more positive feedback in the questionnaire. Overall, randomized trials reported that acupuncture reduced crying behavior of infants suffering from colic. Further evidence for efficacy and safety of acupuncture comes from observational studies [38, 39]. Reinthal et al. evaluated changes in gastrointestinal function after minimal acupuncture in 913 term infants at a mean age of 1.6 weeks [39]. Bilaterally light needling stimulation of LI4 was performed for 10–20 seconds daily [39]. Overall, frequency of regurgitation, belching, drooling, inflated stomach, and frequency of defecation decreased after treatment. In summary, acupuncture was well tolerated, safe, and with no serious side effects reported. Crying as a response to pain was the main side effect in the reported trials. Thirty-two of the 256 infants in the acupuncture group cried for more than 10 sec during the interventions compared to 14 infants in the control group. In addition, 37/256 infants cried >1 minute during acupuncture. Landgren et al. reported slight bleeding after needling in 1/256 acupuncture treatments in 81 randomized patients [19].

In summary current evidence suggests that acupuncture is safe, effective, and a cheap method to treat infantile colic [20].

4.3. Gaps of Knowledge to Treat Term and Preterm Infants. Currently acupuncture for term and preterm infants should be limited to clinical trials. Laser or needle acupuncture has been described [19, 23, 26–28, 35]; however, it remains unclear which treatment option is superior to treat preterm or term infants. Randomized trials should compare laser and needle acupuncture for the treatment of newborn infants. All included studies used a “light needling” technique. However, it remains unclear if a deeper needling technique would have yielded different results. The duration of acupuncture in the reported studies was very short, and a comparison of different acupuncture treatment duration is lacking. Acupuncture treatment is associated with a significant nocturnal increase in endogenous melatonin secretion and significant improvements in sleep onset latency, arousal index, total sleep time, and sleep efficiency [38]. Further studies are needed to clarify this relationship. In addition, clinical trials should focus on advantage, safety, and efficacy of acupuncture in the neonatal population.

5. Conclusion

Acupuncture has the potential to decrease neonatal exposure to potentially neurotoxic analgesic and sedative agents during their early life. The limited data available suggests that acupuncture is a safe nonpharmacologic treatment option for pain reduction in term and preterm infants. However, no study has evaluated long-term effects of acupuncture. Currently acupuncture should be limited to clinical trials and

studies evaluating short- and long-term effects are urgently needed.

Appendix

Search Strategies for PubMed (Last Search: February 14, 2013).
Limits activated: Humans, Randomized Controlled Trial, Clinical Trial (Phase I–IV), Child: birth to 18 years, Infant: birth to 23 months, Infant: 1–23 months, Newborn: birth to 1 month

- (1) MeSH descriptor *Infant*, explore all trees (Result: 40689)
- (2) MeSH descriptor *Newborn*, explore all trees (Result: 19274)
- (3) MeSH descriptor *Acupuncture*, explore all trees (Result: 714)
- (4) ((1) AND 2) AND 3 (Result: 26).

Search Strategies for EMBASE (Last Search: February 14, 2013).
Limits activated: Humans, 1980 to current, Randomized Controlled Trial, Clinical Trial or controlled clinical trial, and (infant <to one year> or child <unspecified age

- (1) MeSH descriptor *Infant*, explore all trees (Result: 13023)
- (2) MeSH descriptor *Newborn*, explore all trees (Result: 4645)
- (3) MeSH descriptor *Acupuncture*, explore all trees (Result: 240)
- (4) ((1) AND 2) AND 3 (Result: 0).

Abbreviations

TCM:	Traditional chinese medicine
CAM:	Complementary and alternative medicine
RCT:	Randomized control trial
CI:	Confidence interval
RR:	Relative risk
RD:	Absolute risk reduction
WMD:	Weighted mean difference
NNT:	Number needed to treat
HIE:	Hypoxic ischemic encephalopathy
LI:	Large intestine
LI4:	Large intestine 4
GI:	Gastrointestinal.

Disclosure

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Authors' Contribution

Conception and design was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger. The literature

review was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger. Analysis and interpretation of the data was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger. Drafting of the paper was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger. Critical revision of the paper for important intellectual content was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger. Final approval of the paper was done by Georg M. Schmölzer, Wolfgang Raith, and Berndt Urlesberger.

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Research Article

ATP Release from Mast Cells by Physical Stimulation: A Putative Early Step in Activation of Acupuncture Points

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In Chinese medicine acupuncture points are treated by physical stimuli to counteract various diseases. These stimuli include mechanical stress as applied during the needle manipulation or tuina, high temperatures as applied during moxibustion, and red laser light applied during laser acupuncture. This study aimed to investigate cellular responses to stimuli that might occur in the tissue of acupuncture points. Since they have a characteristically high density of mast cells that degranulate in response to acupuncture, we asked whether these processes lead to ATP release. We tested in *in vitro* experiments on mast cells of the human mast-cell line HMC-1 the effects of the physical stimuli; mechanical stress was applied by superfusion of the cells with hypotonic solution, heat was applied by incubation of the cells at 52°C, and red laser light of 657 nm was used for irradiation. We demonstrate that all the stimuli induce ATP release from model human mast HMC-1 cells, and this release is associated with an intracellular free Ca²⁺ rise. We hypothesize that ATP released from mast cells supplements the already known release of ATP from keratinocytes and, by acting on P2X receptors, it may serve as initial mediator of acupuncture-induced analgesia.

1. Introduction

The treatment of specific points on the body surface by physical stimuli has been shown to affect various body functions including pain sensation [1, 2] and the cardiovascular system (see, e.g., [3]). Physical stimuli are also applied in traditional Chinese medicine (TCM) [4–6]: in needling acupuncture mechanical stress occurs during manipulation of lifting, thrusting, and twisting [5], and in moxibustion high temperatures exceeding 50°C are applied to the skin. More recently acupoints were treated with blue and red low-level laser light [7], termed laser acupuncture.

Mast cells (MCs) play a significant role in the pathophysiology of many diseases including asthma and allergies,

pulmonary fibrosis, and rheumatoid arthritis [8]. In addition to these deleterious activities, MCs are involved in protection from inflammation and help to maintain tissue homeostasis [9]. MCs are ubiquitous in the body, especially in interface, connective tissue, and mucous membranes. Skin is the main location of MCs [9], which makes the MCs easily accessible to the external physical stimuli. Recently, MCs were shown to participate in the mechanism of analgesia induced by needling acupuncture [5], moxibustion [10], and laser acupuncture [11]. In cellular biological studies, MCs can be activated by stretch and swelling, by application of heat or red laser irradiation that can be monitored morphologically and electrophysiologically [6, 12, 13]. Activation of transient receptor potential channel TRPV2 in the mast-cell membrane

was suggested to be involved [6]. Once MCs are activated, their released mediators can be expected to activate sensory nerve fibers [14], and adenosine has been suggested as mediator of acupuncture-induced analgesia [15].

Extracellular nucleotides are important autocrine/paracrine mediators in various tissues. Increasing evidence suggests that extracellular ATP functions as a stress-responsive molecule, and mechanically induced ATP release is a cell-regulated process that could be observed in the absence of cell lysis [16]. In particular, mechanical stresses, such as stretch, shear, medium change, or osmotic stress, have been shown to evoke ATP release from various cell types [17]. Hypotonic shock represents experimentally convenient and frequently used surrogate of mechanical stress, with which it shares many common characteristics, including transient cytoskeleton reorganization, elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), and stimulation of other signaling pathways [18]. In some cell types, ATP release induced by mechanical stimulation correlates tightly with $[\text{Ca}^{2+}]_i$ elevation, suggesting the involvement of Ca^{2+} -dependent exocytosis [19].

ATP release in response to mechanical stimulation from keratinocytes [15, 16] has been demonstrated, and activation of P2X2 and P2X2/3 receptors located on sensory nerve endings [15, 17] can be expected. Activation of purinergic signaling cascade in response to acupuncture has been suggested [20], and involvement of adenosine receptors in mediating local antinociceptive effects could be demonstrated in mice [15].

Based on our previous studies on mechanical stress-, heat- and red laser light- induced degranulation and activation of TRPV2 channels of MCs [6, 12, 13], we investigated whether these physical stimuli can liberate ATP from MCs. The human leukemia mast-cell line HMC-1 was used as a model system. The purpose of the study was to find a cellular basis for acupuncture effects; we suggest that ATP release from MCs might contribute to stimulation of P2X receptors [20–23] as an early step.

2. Methods

2.1. Cell Culture. The HMC-1 was kindly provided by Dr. J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). The cells were cultured as described previously [6]. In brief, cells were incubated in IMDM (Gibco, Invitrogen, Grand Island, NY, USA), supplemented with 2 mM L-glutamine, 25 mM HEPES, 10% (v/v) fetal bovine serum (Gibco, Invitrogen, Australia), and 1% penicillin and streptomycin (Gibco, Invitrogen, Grand Island, NY, USA), in a 95% humidity controlled incubator with 5% CO_2 at 37°C.

2.2. Solutions and Reagents. Physiological solution (PS) contained (mM): 140 NaCl, 5 KCl, 1 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 10 HEPES, pH 7.4 (adjusted with NaOH). 50% hypotonic solution (HS) was prepared by adding equivalent distilled water to PS. Osmolarity of the solutions was checked with a freezing point osmometer (Micro Osmometer 3300, Advanced instruments Inc., Norwood, MA, USA). Ca^{2+} -free

solution was prepared by omitting CaCl_2 and supplementing with 0.1 mM or 5 mM ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) to chelate trace Ca^{2+} .

100 mM N-Ethylmaleimide (NEM) (Sigma) was prepared in 100% ethanol and diluted into 200 μM with IMDM medium before experiments. To apply NEM to cells, at the beginning of an experiment 50 μL of upper supernatant in each sample was removed and replaced with 50 μL IMDM containing 200 μM NEM to obtain the final concentration of 100 μM . Probenecid (Sigma) 250 mM stock solution was prepared in 1 M NaOH. Calcium Green-1 AM (Invitrogen) and Fura-2 AM (Invitrogen) were dissolved in 20% (w/v) Pluronic F-127 (Invitrogen). 25 mM 1, 2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA-AM) (Sigma) was prepared with DMSO. All stock solutions were stored at -20°C and diluted into bath solution to working concentrations when used. DMSO and Pluronic F-127 were kept at less than 1% in all test solutions.

2.3. ATP Measurements

2.3.1. Changes in ATP Release in Response to Mechanical Stimulation. In *in vitro* experiments, mechanical stimulation can be applied to cells in suspensions in different ways, for example, by hypotonic swelling, shear stress, or pressure stretch [24]. Here we exposed the HMC-1 cells to hypo-osmotic solution. ATP release in response to osmotic stress was measured with high temporal resolution using a flow-through filter chamber and open circuit perfusion system. Briefly, 1–2 mL of HMC-1 cell suspension ($0.5\text{--}1.5 \times 10^6$ cells/mL) was gently introduced, by gravity flow, into the polycarbonate filter chamber. It consisted of 25 mm or 13 mm diameter polycarbonate filter membrane with 1 μm average pore size, which was mounted in appropriate polypropylene Swin-Lok Filter holder (Nucleopore, Whatman, Florham Park, NJ, USA). The chambers had internal volume of 700 μL or 300 μL , respectively. Cells were superfused with warm PS solution (37°C, in-line SF-28 heater, Warner Instrument Co., Hamden, CT, USA) at 1.3 mL/min. After an equilibration period in PS for 30–40 min, 50% hypotonic shock was applied by HS perfusion of the chamber ($t = 0$), and the perfusate was collected at 30 s or 60 s intervals with fraction collector Frac-100 (Pharmacia). ATP in the samples was quantified by luciferase-luciferin luminescence assay (ATP Assay Mix and ATP Assay Mix Dilution Buffer, Sigma-Aldrich Canada, Ltd). Luminescence was measured by Turner TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

2.3.2. ATP Release in Response to Laser Irradiation. For low-level laser stimulation a CW laser of 656.7 nm (SB2007047, Shanghai University of TCM, China) was used with an output power of 35 mW. The diameter of the light spot was 0.4 cm.

HMC-1 cells were cultured in phenol-red-free IMDM medium. Cell density was adjusted to approximately 3.5×10^4 cells/mL. Aliquots of 100 μL cell suspensions were transferred into 1.5 mL Eppendorf tubes and placed in an incubator to equilibrate for 3 h. Following equilibration, some

samples were exposed to red laser irradiation for 5 min as treated group and the remaining samples were the control group. 10 μL rLuciferase-Luciferin (rL/L) reagents (Promega company, USA) were added into each sample and the luminescence was measured by the Luminometer (Promega company, USA).

2.3.3. ATP Release in Response to Heat. Samples were prepared as for the irradiation experiments. To determine temperature dependence of ATP release, the samples were kept at room temperature or placed into prewarmed water bath for 3 min at 42°C and 52°C, respectively. Before the measurement of the luminescence the heated samples were cooled down to room temperature for 1 min.

ATP release was presented as changes of ATP concentration in the perfusate aliquots collected at different time points and expressed in $\text{nM}/10^6$ cells. Calibration of luciferase-luciferin luminescence *versus* ATP standards was always performed with corresponding solutions used in the experiment. Moreover, all test compounds that were added to the extracellular solution during the ATP efflux experiments were also examined for their ability to directly interfere with luciferase bioluminescence.

2.4. $[\text{Ca}^{2+}]_i$ Measurements. For $[\text{Ca}^{2+}]_i$ measurements in the experiments with mechanical stimulation cells were loaded (1 h, room temperature) with 10 μM Fura-2-AM in physiological solution containing 0.02% Pluronic F127 and 2.5 mM Probenecid, followed by 30 min deesterification in PS-containing Probenecid. For fluorescence imaging, a Fura-2-loaded cells were introduced into an imaging/perfusion chamber (RC-20, volume 48 μL) attached to a heated platform (P-5, Warner Instruments Co.) on the stage of an inverted microscope (Nikon TE300). A thin vacuum grease barrier was made at one end of the chamber, close to the perfusion outlet, to trap cells in the chamber and prevent their wash out during perfusion. The imaging chamber was perfused continuously with a warm solution (37°C) via an in-line heater (SF-28, Warner Instruments Co.) at ~ 0.5 mL/min. The cells were illuminated for 100 ms with alternate light wavelengths of 340 and 380 nm, using a high-pressure mercury lamp (100 W) via interference filters (Chroma Technology Corp., Brattleboro, VT, USA) mounted on a filter wheel (Sutter Lambda 10-C, Sutter Instrument Co., Novato, CA, USA) and a dichroic mirror (510/540 nm, Chroma Technology Corp.). Fluorescence images were recorded at 15 s intervals with a digital camera and stored for later analysis. Fura-2 measurements are presented as the fluorescence F_{340}/F_{380} ratio. To chelate intracellular Ca^{2+} , cells were preloaded with 25 μM BAPTA-AM for 30 min at room temperature in PS.

For $[\text{Ca}^{2+}]_i$ measurements in the experiments with laser irradiation and heat application, $[\text{Ca}^{2+}]_i$ measurements were performed as described previously [12]. In brief, HMC-1 cells grown on glass cover slips coated with poly-L-lysine (Sigma Chemical) were loaded with 4 μM Calcium Green-1 AM in IMDM loading buffer for 1 h at room temperature. The loaded cells were superfused with PS. All solutions used in the fluorescence experiments contained 2.5 mM Probenecid. Irradiation experiments were performed at room

temperature. In heating tests, 42°C and 52°C were controlled by a Temperature Control Device (PTC-20, NPI, Tamm, Germany). Photos were taken every minute. Images were digitized and averaged (five frames), background corrected, and analyzed by an image-processing system (Wasabi, Hamamatsu, Japan). Fluorescence intensities of individual cells in the field of view were determined by averaging the image intensities collected from regions of interest within each cell.

In some experiments, Ca^{2+} influx from extracellular space was abolished by using nominally Ca^{2+} -free extracellular medium containing 0.1 mM EGTA to chelate any trace of Ca^{2+} .

2.5. Data Analysis. For data presentation and analysis, ORIGIN software (OriginLab, Northampton, MA, USA) was used. Data are expressed as mean \pm SEM. The n values give the number of measurements obtained from different samples of cells; the N values the number of single cells analyzed in fluorescence measurements. Differences between sample means were evaluated by Student's t -test or Kruskal-Wallis test, and a P value <0.05 was considered to represent significant difference.

3. Results

3.1. Stimulation by Mechanical Stress. For mechanical stimulation of HMC-1 cells the perfusion solution in the flow-through filter chamber was changed at $t = 0$ to 50% hypotonic solution. Figure 1(a) shows that ATP release transiently increased peaking after about 2 min and was followed by a decay that lasted approximately 10 min to a level that was even lower than that before stimulation. Short-term removal of extracellular Ca^{2+} , that is, 3–5 min had no significant effect on the increase of ATP release by hypotonic shock ($P = 0.4795 > 0.05$, at 2 min). Fura-2 fluorescence measurements of $[\text{Ca}^{2+}]_i$ response showed a time course similar to the ATP release, that is, a peak of $[\text{Ca}^{2+}]_i$ at 1.5–2 min after stress application followed by a decay phase (Figure 1(b)). Similar to the ATP release, the $[\text{Ca}^{2+}]_i$ response was not significantly affected by removal of extracellular Ca^{2+} ($P = 0.42503$, at 2 min), suggesting that Ca^{2+} mobilization from intracellular stores plays a dominant role in this response.

To test the role of intracellular Ca^{2+} in hypotonic stress-induced ATP release, cells were pretreated with a Ca^{2+} chelator 25 μM BAPTA-AM before they were subjected to hypotonic shock. Figure 2(a) shows that in 50% hypotonic, Ca^{2+} -free solution ATP release was significantly diminished in BAPTA-loaded cells compared to controls ($P = 0.04953$, at 2 min). Figure 2(b) shows that also $[\text{Ca}^{2+}]_i$ response was almost completely abolished in these cells demonstrating a tight correlation between ATP release and $[\text{Ca}^{2+}]_i$ elevations induced by the hypotonic stress ($P = 0.04953$, at 2 min).

3.2. Stimulation by Red Laser Light. To investigate the effect of red laser light on ATP release, HMC-1 cells were exposed to laser light of 656.7 nm at 35 mW. For cells that had been exposed for 5 min to laser light, the amount of released ATP was higher than in the controls by $10.7 \pm 4.0\%$

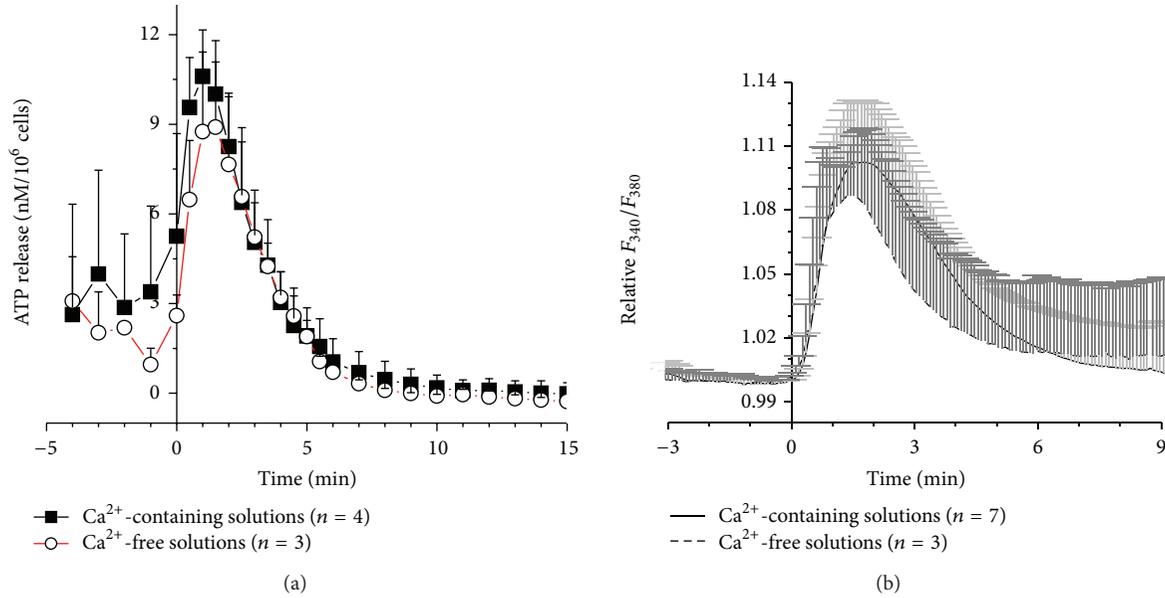


FIGURE 1: Time course of ATP release (a) and intracellular Ca²⁺ responses (b) induced by 50% hypotonic shock of HMC-1 cells in Ca²⁺-containing (filled squares ($n = 4$) or solid line ($n = 7$), resp.) and in Ca²⁺-free solutions (open circles ($n = 3$) or broken line ($n = 3$), resp.). Data represent averages of n measurements \pm SEM. The respective curves in (a) and (b) are not significantly different on the basis of $P > 0.05$.

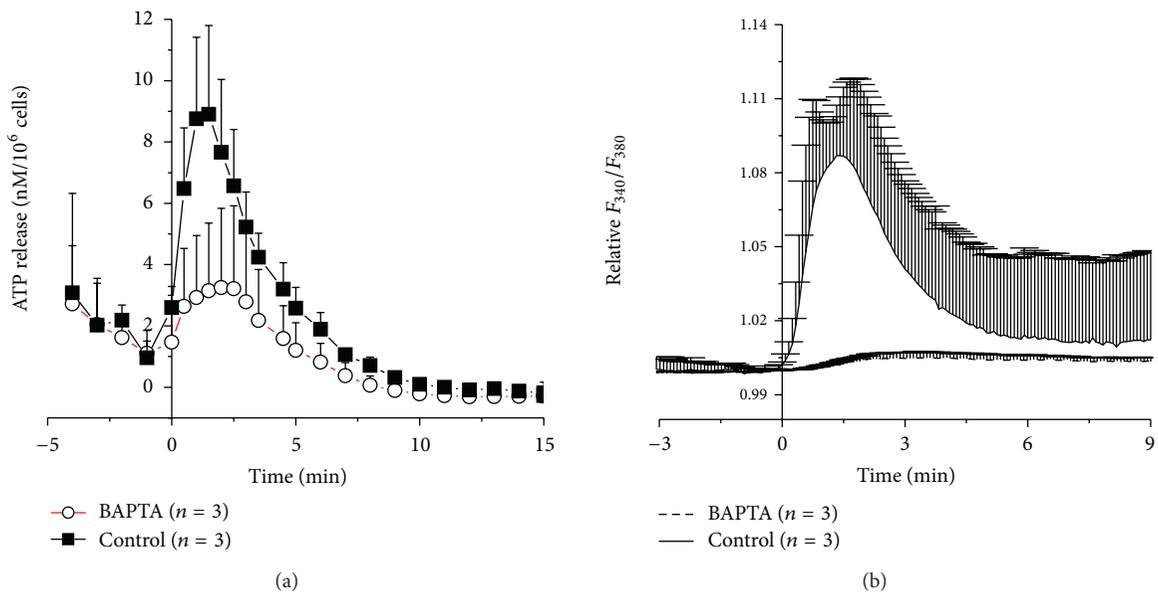


FIGURE 2: Time course of ATP release (a) and intracellular Ca²⁺ responses (b) induced by 50% hypotonic shock of HMC-1 cells before (filled square or solid line, resp.) and after treatment with BAPTA (open circles or broken line, resp.) in Ca²⁺-free solution. Data represent averages of 3 measurements each \pm SEM. The respective curves in (a) and (b) are significantly different on the basis of $P < 0.05$.

($P = 0.0299$, $n = 8$). The concentration of released ATP for untreated control cells varied between different experiments from 4.5 nM to 8.7 nM. Figure 3(a) shows averaged data for the light-induced ATP release normalized to the ATP level in control cells.

Figure 3(b) illustrates that also [Ca²⁺]_i mobilization was induced by red laser irradiation. An increase of [Ca²⁺]_i by $3.5 \pm 0.6\%$ ($P = 4.6 \times 10^{-6}$, $N = 17$) and $21.5 \pm 1.1\%$ ($P = 1.5 \times 10^{-11}$, $N = 15$) appeared when HMC-1 cells were

exposed to irradiation for 1 min and 5 min, respectively. The presence of 5 mM EGTA partially prevented [Ca²⁺]_i elevation induced by 1 min irradiation.

3.3. Stimulation by High Temperature. Figure 4(a) shows that incubation at 42°C increased the released ATP content from 8.0 ± 1.3 nM to 17.4 ± 1.0 nM ($P = 0.0253$), and an even more significant increase to 49.5 ± 5.0 nM ($P = 1.8 \times 10^{-4}$) was found at 52°C. In order to exclude that this response

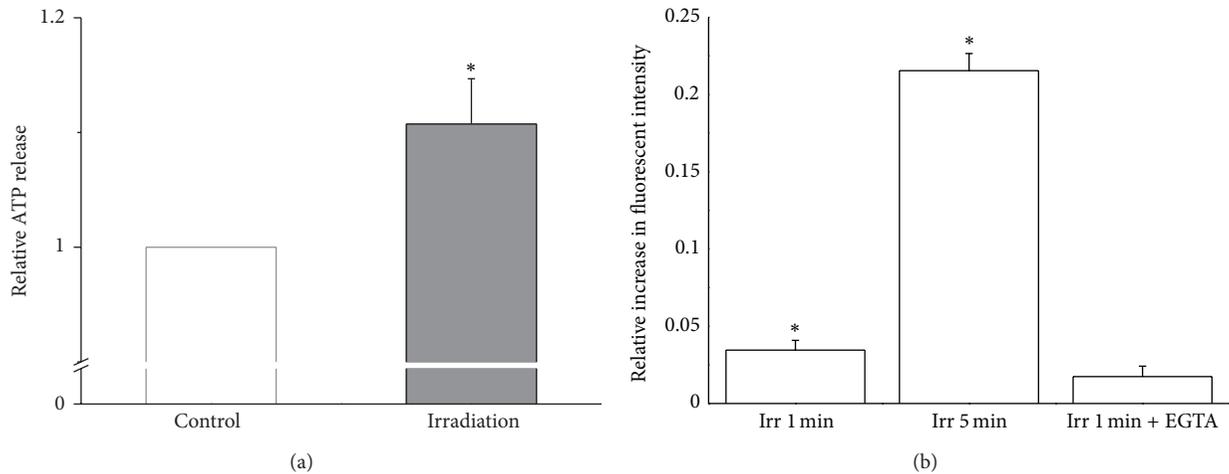


FIGURE 3: (a) Normalized ATP content in supernatant from untreated cells (Control) and of cells after having been treated for 5 min with red laser light (Irradiation). Data were normalized to the controls of the respective batch of cells and represent averages of 8 determinations (\pm SEM). One corresponds to $6.0 \text{ nM}/10^6$ cells. (b) Relative increase in intracellular Ca^{2+} in response to 1 and 5 min of red laser light compared to control cells; measurements were performed with cells untreated (Irradiation) and cell treated for one min with EGTA (+EGTA). The data represent averages \pm SEM ($N = 15-17$). *Significant difference compared to control.

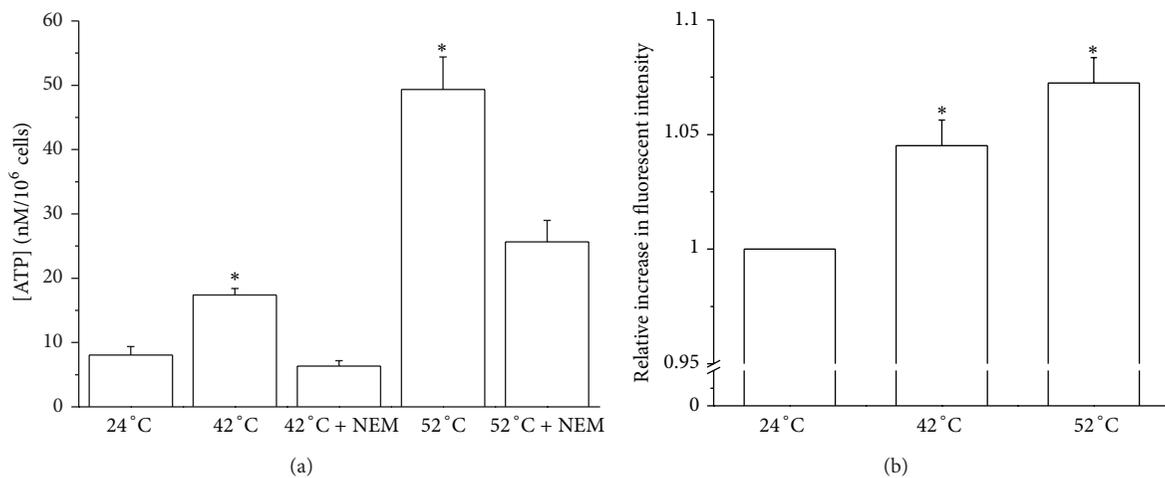


FIGURE 4: (a) ATP content in cell suspension after 3 min of incubation at different temperatures. Data represent averages \pm SEM ($n = 4-16$). (b) Relative increase in fluorescent intensity of HMC-1 cells in response to higher temperatures. The data represent averages \pm SEM ($N = 13$). *Significant difference compared to 24°C .

was only due to cell lysis, experiments were performed in solution containing $100 \mu\text{M}$ NEM, an inhibitor of exocytosis [25]. In the presence of NEM the concentration of released ATP was reduced to $6.4 \pm 0.8 \text{ nM}$ at 42°C , which was close to the control value. At 52°C the ATP content dropped to $25.6 \pm 3.3 \text{ nM}$. The result indicates that at least a large fraction of ATP release is mediated by exocytosis.

Similar to the mechanical and laser light stimuli, also heat could enhance $[\text{Ca}^{2+}]_i$ in HMC-1 cells. The relative fluorescent intensity increased by $4.5 \pm 1.1\%$ ($P = 0.00112$, $N = 13$) and $7.3 \pm 1.1\%$ ($P = 9.0 \times 10^{-6}$, $N = 13$) at 42°C and 52°C , respectively.

4. Discussion

Recently we have shown that physical stimulation of MCs results in mast-cell degranulation [6, 12, 13], which forms

an early step in acupuncture effects [5]. Here we have demonstrated that physical stimuli applied to MCs led to the release of ATP to the extracellular medium. These stimuli include hypo-osmotic stress with a transient stimulation reaching a maximum at about 2 min after the stimulation was initiated. This transient signal was paralleled by an increase in intracellular Ca^{2+} with nearly identical time course. While the intracellular $[\text{Ca}^{2+}]_i$ returned to the level before stimulation, the amount of ATP release dropped to even lower concentrations than before stimulation indicating partial depletion of ATP stores during the period of elevated release. Neither the release of ATP nor the increase in $[\text{Ca}^{2+}]_i$ was significantly affected if the experiments were performed in Ca^{2+} -free medium; this observation suggests that the release of Ca^{2+} from intracellular stores might be involved in the process of ATP release. The hypothesis is supported by

the finding that loading of the cells with the Ca^{2+} chelator BAPTA completely blocked the Ca^{2+} signal and strongly inhibited the ATP release. Similar observations were reported for hypotonic stress-induced ATP release from A549 cells, a model of human type 2 alveolar cells [18].

Irradiation with red laser light also stimulated ATP release. This increase in ATP release was also accompanied by an increase in $[\text{Ca}^{2+}]_i$. In contrast to hypo-osmotic stress, the 1 min irradiation-induced increase in $[\text{Ca}^{2+}]_i$ was partially reduced when the cells were kept for 1 min in Ca^{2+} -free medium indicating that also extracellular Ca^{2+} contributes to this process. This agrees with our previous work, which demonstrated that red laser irradiation activated TRPV2 channels allowing Ca^{2+} uptake from extracellular space [6]. Basal ATP release from HMC-1 cells was observed when cells were kept at room temperature; however, its rate was significantly increased at elevated temperatures. The effect was more pronounced at 52°C than at 42°C . The temperature-dependent increase in ATP release cannot be attributed to cell lysis but rather to the combined effects of elevated temperature on exocytosis and Ca^{2+} homeostasis. Involvement of regulated exocytosis in the observed osmotic stress-induced and red laser light-induced ATP release is indicated by the accompanied increase of $[\text{Ca}^{2+}]_i$ and strong inhibition of the release by NEM.

The physical stimuli applied in this investigation to the MCs are used in Chinese medicine to stimulate acupuncture points: mechanical stimulation during the needling, heat during moxibustion, and red light in laser acupuncture. Acupuncture and moxibustion have been demonstrated to be effective in analgesia [26]. Recently, pain relief by laser acupuncture has attracted attentions [11, 27, 28]. Purinergic signaling is known to participate in the mechanisms of pain sensation [29], and ATP is one of the main purinergic agonists in the purinergic system. Release of ATP from keratinocytes has also been suggested to be involved in acupuncture-dependent analgesia [20]. P2X3 homomeric and P2X2/3 heteromeric receptors are found predominantly in sensory nerve endings [30] and hence likely receptors for ATP released by physical stimulation of MCs and keratinocytes. Our results suggest that ATP release within the acupuncture point may be an initial step that may lead to stimulation of P2X3 and P2X2/3 receptors in peripheral nerve endings, which may account for acupuncture-induced analgesia [29]. For further elucidation of MC-neuron, interaction in response to the physical stimuli is under investigation. Since our data are obtained from *in vitro* experiments, further animal tests are needed for support.

Abbreviations

BAPTA-AM:	1,2-Bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
EGTA:	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

HS:	Hypotonic solution
MCs:	Mast cells
NEM:	N-Ethylmaleimide
PS:	Physiological solution
TRPV:	Transient receptor potential channel (valinoidsensitive).

Conflict of Interests

The authors have no financial relationships with the corporations and software providers mentioned in the paper.

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Research Article

Effects of Moxa (*Folium Artemisiae argyi*) Smoke Exposure on Heart Rate and Heart Rate Variability in Healthy Young Adults: A Randomized, Controlled Human Study

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Objective. To determine the effects of the moxa smoke on human heart rate (HR) and heart rate variability (HRV). **Methods.** Fifty-five healthy young adults were randomly divided into experimental ($n = 28$) and control ($n = 27$) groups. Experimental subjects were exposed to moxa smoke ($2.5 \pm 0.5 \text{ mg/m}^3$) twice for 25 minutes in one week. ECG monitoring was performed before, during, and after exposure. Control subjects were exposed to normal indoor air in a similar environment and similarly monitored. Followup was performed the following week. Short-term (5 min) HRV parameters were analyzed with HRV analysis software. SPSS software was used for statistical analysis. **Results.** During and after the first exposure, comparison of percentage changes or changes in all parameters between groups showed no significant differences. During the second exposure, percentage decrease in HR, percentage increases in lnTP, lnHF, lnLF, and RMSSD, and increase in PNN50 were significantly greater in the experimental group than in control. **Conclusion.** No significant adverse HRV effects were associated with this clinically routine 25-minute exposure to moxa smoke, and the data suggests that short-term exposure to moxa smoke might have positive regulating effects on human autonomic function. Further studies are warranted to confirm these findings.

1. Introduction

Moxibustion, one of the classical therapies of Traditional Chinese Medicine (TCM), uses the heat generated by burning moxa floss (usually made by *Folium Artemisiae argyi*) to stimulate acupuncture points [1]. It is used widely in acupuncture clinics in China and other Asian countries to treat various diseases, especially chronic conditions such as osteoarthritis, asthma, gastrointestinal disorders, and insomnia [2–5]. Smoke is an unavoidable aspect of the therapy. The aim of this study was to evaluate the effects of moxa smoke exposure on human heart rate variability (HRV) parameters.

A moxibustion session typically lasts 20–30 minutes, and patients are often treated several times a week for several

weeks. Patients are exposed to the smoke during treatment, while acupuncturists are commonly exposed for prolonged periods during clinical practice. Because of recent concerns as to the safety of the therapy, specifically the potential toxicity of the smoke, many clinics no longer use moxibustion, thus depriving patients of the benefits of this unique treatment. Evaluation of the safety and the effects of moxa smoke is imperative.

Concerns about moxa smoke are similar to those regarding tobacco smoke and air pollutants. Many studies show that exposure to tobacco smoke and air pollutants is positively associated with adverse effects in the respiratory, immune, nervous, and cardiovascular systems [6–11]. Active and passive exposures to tobacco smoke have been found

to increase sympathetic nervous system activity and reduce parasympathetic nervous system modulation and HRV [12–14]. Particulate air pollutants affect heart rate (HR), blood pressure, vascular tone, blood coagulation, the progression of atherosclerosis [15], and HRV [16–19]. To our knowledge, the influence of moxa smoke on HRV in the human body has not been sufficiently investigated.

HRV refers to the time variation coefficient between successive heart beat cycles. It is one of the most promising quantitative markers of autonomic nerve system activity [20]. There is a growing recognition of the role of HRV abnormalities in cardiovascular disease [21–23], and HRV decrease is a strong predictor of mortality [24]. HRV measurement in time and frequency domains is a convenient, noninvasive tool for autonomic nervous physiology evaluation, and short-term (5-minute) recording gives reliable measurements [25]. The purpose of this study was to determine whether exposure to moxa smoke influences HR and HRV in healthy subjects.

2. Methods

2.1. Subjects. Participants, most of them students of Beijing University of Chinese Medicine or other nearby universities plus some residents of the area around the University, were recruited between March 2012 and July 2012. The study protocol was approved by the Human Medical Ethics Committee of Beijing University of Chinese Medicine and was registered in the Chinese Clinical Trial Registry (ChiCTR-TRC-12002445). Written informed consent was secured from all participants.

Inclusion criteria required that subjects be normal and healthy according to the American Society of Anesthesiologists Physical Status Classification System, that is, that they have no organic, physiologic, biochemical, or psychiatric disorders, smoke <5 cigarettes per day [26], and are between the ages of 18 and 50.

Individuals were excluded if they (1) had a history of addiction to alcohol or drugs, (2) had had contact with moxa smoke within one month of the test, (3) had used medications within two weeks of the test, (4) had had a cold or other illnesses within one week of the test, (5) had ingested food or drink containing caffeine or alcohol, smoked, or done strenuous exercise within four hours of the test, and (6) were pregnant or lactating.

Participants were instructed to refrain from tobacco, alcohol, medications, and strenuous exercise and to avoid contacting moxa smoke or any other abnormal gas during the two-week test.

2.2. Study Protocol

2.2.1. Equipment and Setting. The trial was performed at the Beijing University of Chinese Medicine in two adjacent, bright, quiet, and similarly laid-out rooms equipped with beds. Ambient temperature and humidity were kept between 24°C~26°C and 40%~50% and monitored by a meteorological parameter recorder (Kestrel NK3000, USA).

Room 1 had normal indoor air. In Room 2, moxa smoke was generated by burning moxa sticks (three-year-old pure moxa, 1.8 cm × 20 cm, Nanyang Hanyi Moxa Co., Ltd.,

China). A digital dust indicator (P5L2C, Binta Green Technology Co., Ltd., Beijing, China) that detects particulate matter <10 μm in diameter (PM₁₀) levels and a volatile organic compound (VOC) detector (model no. PGM-7320, kit MiniRAE3000, Rae Systems, Inc., USA) were set beside the participant to monitor the air. In the moxa room, PM₁₀ and total VOC levels were kept between 2.5 ± 0.5 mg/m³ and 4.2 ± 1.3 mg/m³, respectively, which accord with average moxa smoke levels in acupuncture clinics [27]. In Room 1, PM₁₀ and total VOC levels detected in this trial were lower than 0.01 and 0.2 mg/m³, respectively.

This was a two-arm, open, and randomized study (*N* = 55). After reading and signing the consent form, the participant was assigned to the experiment or control group by computer-generated random allocation. Group assignments were performed by a statistician blinded to the study design. Each assignment was sealed in an opaque envelope that was opened for the respective participant by the investigator prior to treatment.

2.2.2. Experimental Group. Testing consisted of three phases, one immediately after the other. In phase 1, subjects entered Room 1 and were encouraged to relax in a supine position. After 5–10 minutes of rest, ECG monitoring was performed for 5 minutes. In phase 2, they entered Room 2. After a 5-minute rest in a supine position, ECG monitoring was performed for 20 minutes. In phase 3, they returned to Room 1 for another 5-minute ECG recording (see Figure 1). After each ECG, subjects recorded their subjective sensations and emotions on a questionnaire. These questions were about whether they have experienced drowsiness, shortness of breath, cough, choking, irritation in nose, pharynx, and eyes, body temperature changes, or any other discomfort that might be associated with moxa smoking exposure.

2.2.3. Control Group. Control subjects were similarly monitored but remained in Room 1 during phase 2 (see Figure 1).

The test was performed on each subject twice in a single week to accord with routine clinic practice. One week later, subjects in both groups returned for another 5-minute ECG (see Figure 2).

2.3. ECG Monitoring and Short-Term (5 min) HRV Data Analysis. With the subjects supine, three ECG electrodes were placed on their right subclavian and double costal arch regions. A data acquisition instrument (DATAQ Instrument Inc., MODEL:DI-720-USB, USA) was connected to the electrodes and a computer. To allow the heart beat to become steady, ECG recording was started 5–10 minutes after they lay down.

ECGs were analyzed by a specialist blinded to group assignment. After removal of extraneous noise, normal-to-normal beat intervals were analyzed for time- and frequency-domain parameters in 5-minute epochs using standard algorithms and HRV analysis software (Catholic University of Leuven). Time-domain analysis estimates the variation of differences between successive RR intervals through statistically developed indices. Frequency-domain

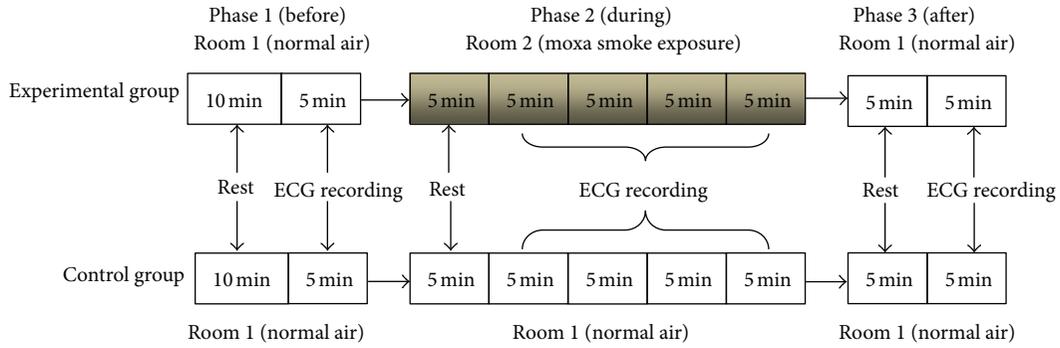


FIGURE 1: Experimental procedures for tests 1 and 2.

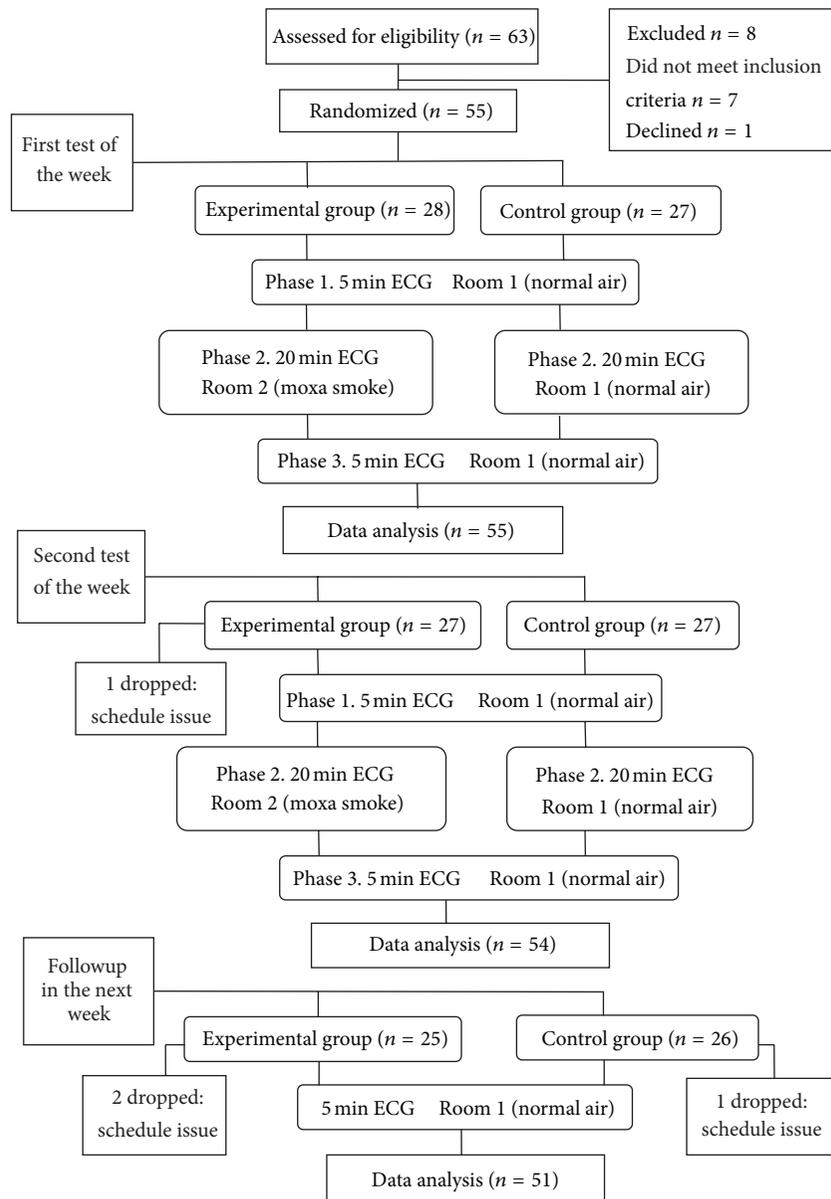


FIGURE 2: Flow of participants through the whole trial.

TABLE 1: HRV parameters used in this trial.

Variable	Units	Description
Time-domain parameters		
SDNN	msec	the standard deviation of all NN intervals, an estimate of overall variability
rMSSD	msec	the square root of the mean of the squared differences between adjacent NN intervals, an estimate of the short-term components of variability
pNN50	%	the proportion derived by dividing NN50 (the number of interval differences of successive normal-to-normal intervals greater than 50 ms) count by the total number of normal-to-normal intervals
Frequency-domain parameters		
TP	msec ²	total power, frequency range <0.4 Hz
HF	msec ²	power in the high-frequency range (0.15–0.4 Hz), considered to be mediated mainly by vagal activity
LF	msec ²	power in the low-frequency range (0.04–0.15 Hz), suggested to be mediated by both sympathetic and parasympathetic activities
LF/HF	Ratio	an indicator of the balance of the sympathetic and parasympathetic systems

TABLE 2: Baseline characteristics of study subjects.

Variables		Experimental group ($N = 28$)	Control group ($N = 27$)
Gender ^a	Male/female	12/16	12/15
Age ^b	Years	24.5 ± 2.5	25 ± 2.3
BMI ^b	kg/m ²	21.2 ± 2.9	21.1 ± 2.3
Ethnic group ^a	Han/others	27/1	26/1
Nationality ^a	China/Singapore	26/2	26/1
Smoking history ^a	Yes/no	0/28	0/27
Regular exercise ^a	Yes/no	11/17	7/20
Emotional condition ^a	Good/ok/bad	11/17/0	13/14/0
Mean HR ^b	bpm	67.57 ± 8.95	70.02 ± 7.31
SDNN ^c	ms	40.43 (12.89)	43.02 (26.26)
RMSSD ^c	ms	35.08 (24.80)	34.02 (13.8)
PNN50 ^c	%	14.93 (32.12)	14.02 (17.21)
lnTP ^c		7.17 (0.77)	7.17 (1.18)
lnHF ^c		6.31 (1.21)	6.02 (1.36)
lnLF ^c		5.77 (0.8)	5.99 (0.96)
LF/HF ^c		0.76 (0.82)	0.83 (0.72)

^b Mean ± standard deviation; ^c median (interquartile range). No significant differences were found between the groups based on ^aChi-square test, ^bindependent two-sample *t*-test, or ^cMann-Whitney *U* Test.

analysis estimates respiratory-dependent, high- and low-frequency power through spectral analysis. Widely used HRV parameters [25, 28, 29] were employed in this study (Table 1).

2.4. Statistical Analysis. SPSS17.0 statistical software was used for data analysis. The paired *t*-test was used to compare HR data of different phases in the same group; the independent sample *t*-test was used to compare data from different groups. A related sample nonparametric test (Wilcoxon) was used to compare all HRV parameter data of different phases in the same group, and an independent sample nonparametric test (Mann-Whitney *U* Test) was used to compare data from different groups.

TP, HF, and LF data were transformed into natural logarithms (ln) for better analysis. The four-segment data of phase 2 were calculated into one mean value for comparison with data from the other two phases. Percentage changes ((mean value in phase 2/value in phase 3 – value in phase 1)/value in phase 1 × 100%) or changes (mean value in phase 2/value in phase 3 – value in phase 1) of all data were used for comparisons between the groups.

3. Results

3.1. Baseline Characteristic of Study Subjects. There were no statistically significant baseline differences between the groups (Table 2).

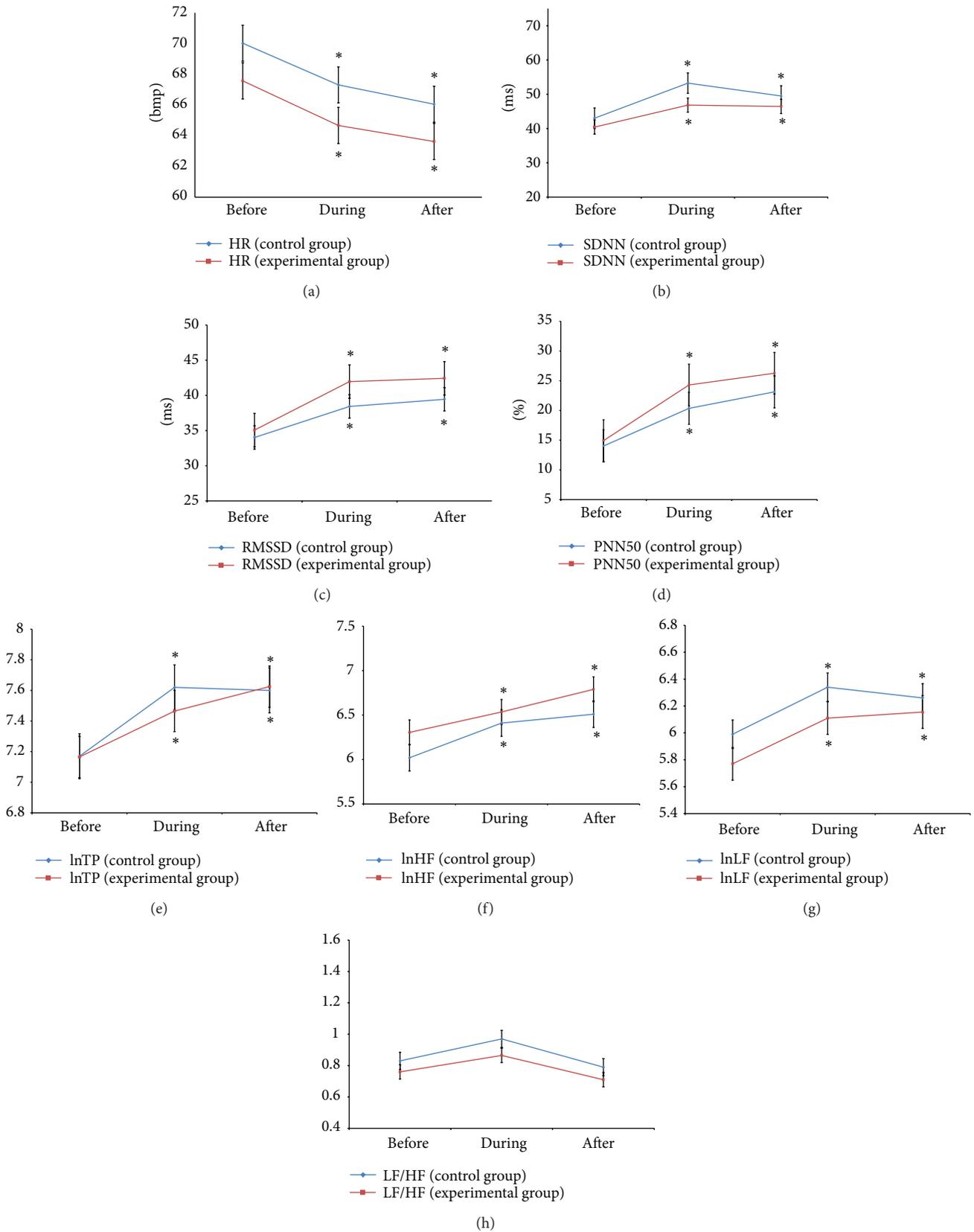


FIGURE 3: Changes in all indicators in the first test of the week. Values are expressed as (a) mean and (b)–(h) median. * indicates a significant difference ($P < 0.05$) between during/after periods and before periods in the same group using (a) paired t -test and (b)–(h) Wilcoxon test.

TABLE 3: Comparison of the percentage changes/changes in HR and HRV parameters between the groups in the first test of the week.

	Experimental group	Control group	P value
HR ^{1,a}			
Before-during	-0.04 ± 0.03	-0.04 ± 0.04	0.75
Before-after	-0.06 ± 0.05	-0.06 ± 0.04	0.92
SDNN ^{1,b}			
Before-during	0.19 (0.35)	0.20 (0.29)	0.74
Before-after	0.22 (0.42)	0.14 (0.33)	0.62
RMSSD ^{1,b}			
Before-during	0.18 (0.38)	0.09 (0.35)	0.78
Before-after	0.18 (0.36)	0.15 (0.42)	0.89
PNN50 ^{2,b}			
Before-during	4.03 (10.34)	4.88 (11.44)	0.56
Before-after	4.32 (8.46)	5.26 (15.3)	0.72
lnTP ^{1,b}			
Before-during	0.03 (0.09)	0.04 (0.09)	0.58
Before-after	0.05 (0.08)	0.04 (0.07)	0.60
lnHF ^{1,b}			
Before-during	0.05 (0.1)	0.04 (0.11)	0.46
Before-after	0.05 (0.09)	0.04 (0.11)	0.45
lnLF ^{1,b}			
Before-during	0.03 (0.15)	0.04 (0.09)	0.57
Before-after	0.03 (0.17)	0.04 (0.18)	0.82
LF/HF ^{2,b}			
Before-during	0.11 (0.51)	0.09 (0.34)	0.89
Before-after	-0.03 (0.64)	-0.15 (0.85)	0.79

Number 1 indicates percentage changes; number 2 indicates data changes. Values are expressed as ^amean ± standard deviation and ^bmedian (interquartile range). No significant differences were found between the groups using ^aindependent two-sample *t*-test and ^bMann-Whitney *U* test.

3.2. Comparisons: First Test. In phases 2 and 3, during and after exposure, HR ($P < 0.05$) was significantly reduced, and SDNN ($P < 0.05$), RMSSD ($P < 0.05$), PNN50 ($P < 0.05$), lnTP ($P < 0.05$), lnLF ($P < 0.05$), and lnHF ($P < 0.05$) were significantly increased in both groups (Figures 3(a)–3(g)). No significant change was found in LF/HF in either group (Figure 3(h)).

Comparison of the percentage changes/changes in HR and HRV parameters showed no significant differences between groups (Table 3).

3.3. Comparisons: Second Test. In phases 2 and 3, during and after exposure, each group had significant reductions in HR ($P < 0.05$) and significant increases in all HRV parameters ($P < 0.05$; Figures 4(a)–4(h)) except for LF/HF ratio in phase 3 of the experimental group (Figure 4(h)).

In phase 2, the experimental group's percentage decrease in HR ($P < 0.001$), increases in RMSSD ($P < 0.001$), lnTP ($P < 0.001$), lnHF ($P < 0.001$), and lnLF ($P < 0.001$), and increase in PNN50 ($P = 0.02$) were significantly greater than those in control. In phase 3, LF/HF ratio increase was significantly lower in the experimental group than in control ($P = 0.005$; see Table 4).

3.4. Comparisons: Follow-Up Test. Mean HR ($P = 0.039$) in the experimental group was significantly lower than that in

control; other indicators were not significantly different in the two groups (Table 5).

3.5. Participants' Sensations. During moxa smoke exposure, seventeen experimental group subjects felt sleepy and relaxed. One felt refreshed; stomach and bowel movement improved in another. Ten complained of choking and irritation in nose, pharynx, and eyes. One had difficulty in breathing. Eight had no unusual sensations.

In the control group, two subjects felt sleepy; one had neck discomfort; one had numbness in the hand. Twenty-three felt nothing unusual.

4. Discussion

No harmful HR and HRV effects were observed during exposure to clinical levels of moxa smoke. Evidence for this is that there were no differences in HR and HRV, either immediately (after 10 minutes) or at followup a week after exposure, between the experimental group exposed to moxa smoke and the control group without such exposure. These results might explain why reports of adverse reactions associated with smoke produced in this ancient therapy are so rare.

In contrast to retrospective studies based on clinical observation, our present study was a well-controlled, randomized, and prospective study to examine possible adverse

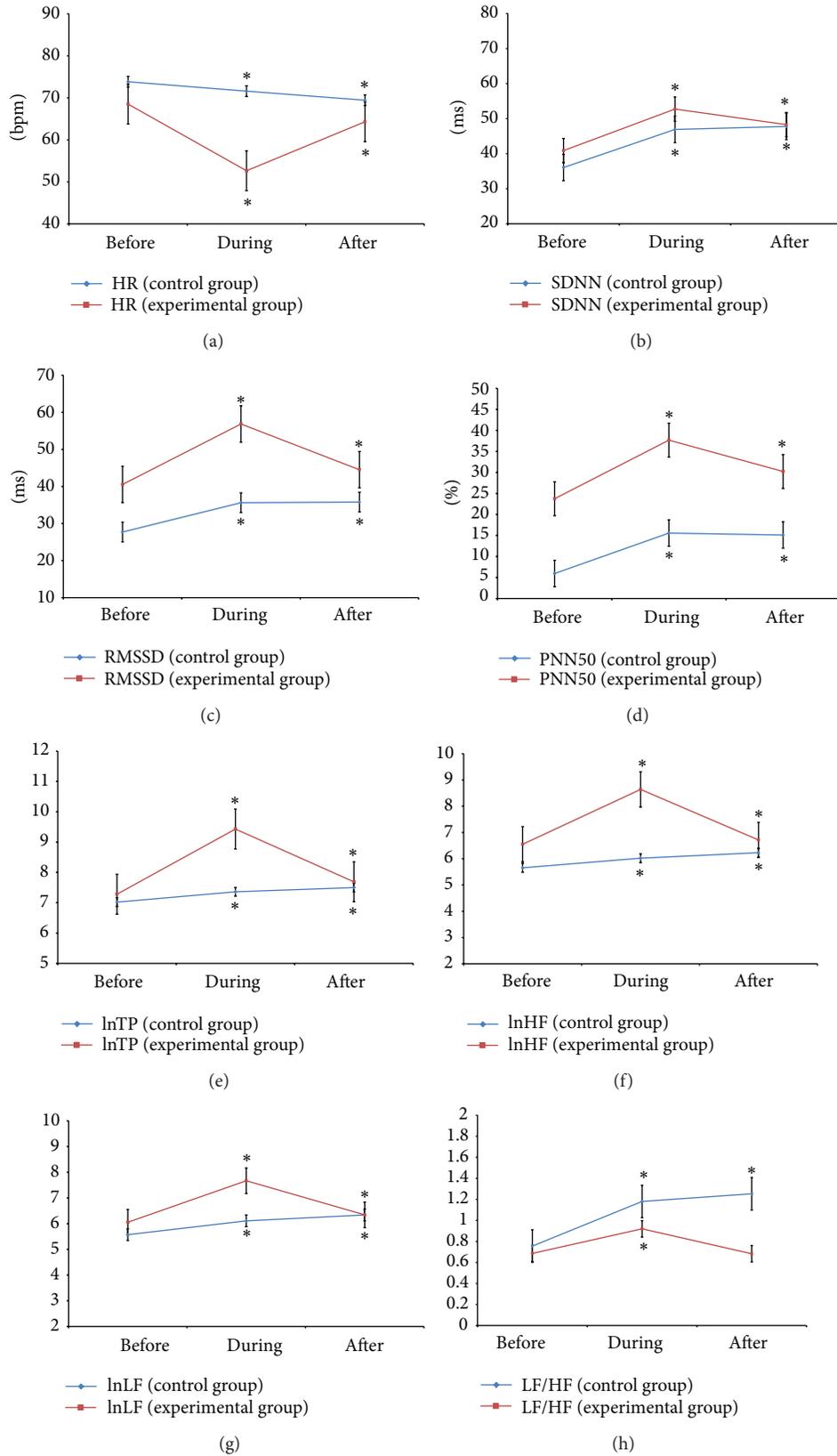


FIGURE 4: Changes in all indicators in the second test of the week. Values are expressed as (a) mean and (b)–(h) median. * indicates a significant difference ($P < 0.05$) between the during/after phases and phase 1 in each group using (a) paired t -test and (b)–(h) Wilcoxon test.

TABLE 4: Test 2: percentage changes/changes in HR and HRV parameters.

	Experimental group	Control group	P value
HR ^{1,a}			
Before-during	-0.23 ± 0.02*	-0.03 ± 0.09	<0.001
Before-after	-0.06 ± 0.04	-0.06 ± 0.09	0.63
SDNN ^{1,b}			
Before-during	0.21 (0.32)	0.15 (0.41)	0.76
Before-after	0.16 (0.36)	0.28 (0.57)	0.56
RMSSD ^{1,b}			
Before-during	0.42 (0.29)*	0.19 (0.30)	<0.001
Before-after	0.12 (0.37)	0.24 (0.57)	0.47
PNN50 ^{2,b}			
Before-during	9.78 (17.32) [△]	5.02 (7.81)	0.02
Before-after	4.26 (13.59)	10.59 (17.26)	0.34
lnTP ^{1,b}			
Before-during	0.3 (0.09)*	0.05 (0.08)	<0.001
Before-after	0.04 (0.09)	0.09 (0.13)	0.18
lnHF ^{1,b}			
Before-during	0.29 (0.07)*	0.03 (0.09)	<0.001
Before-after	0.06 (0.1)	0.06 (0.19)	0.71
lnLF ^{1,b}			
Before-during	0.31 (0.21)*	0.07 (0.15)	<0.001
Before-after	0.10 (0.19)	0.15 (0.23)	0.15
LF/HF ^{2,b}			
Before-during	0.18 (0.34)	0.32 (0.84)	0.30
Before-after	0.13 (0.69) [△]	0.44 (1.19)	0.005

Number 1 indicates percentage changes; number 2 indicates data changes. Values are expressed as ^amean ± standard deviation and ^bmedian (interquartile range). The symbols * and [△] indicate significant differences ($P < 0.001$ and $P < 0.05$, resp.) between the groups using ^aindependent two-sample t -Test and ^bMann-Whitney U Test.

TABLE 5: HR and HRV parameters, follow-up test.

	HR ^a	SDNN ^b	RMSSD ^b	PNN50 ^b	lnTP ^b	lnHF ^b	lnLF ^b	LF/HF ^b
Control group	72.92 ± 7.83	44.07 (22.26)	34.01 (21.12)	12.37 (21.9)	7.22 (1.04)	5.94 (1.62)	5.83 (1.04)	0.93 (1.17)
Experimental group	67.68 ± 9.74*	52.72 (26.91)	46.16 (38.15)	29.75 (45.16)	7.56 (1.07)	6.73 (1.54)	6.19 (1.35)	1.01 (1.45)

Values are expressed as ^amean ± standard deviation and ^bmedian (interquartile range). The symbol * indicates a significant difference ($P < 0.05$) between the two groups according to the independent two-sample t -test.

effects of moxa smoke. The study is unique in moxa smoke concentration, length of exposure to the smoke, and its carefully controlled and monitored experimental environment, all of which mimic actual clinical moxibustion practice. The sample size is comparable to those reported in similar studies on exposure to other types of potentially hazardous smoke [12–14, 30].

The HRV effects that we observed in this moxa smoke study contrast with findings of air pollution and tobacco smoke studies, which show harmful effects on human health [12–19]. This difference might be the result of the unique constituents of moxa smoke. Moxa floss (burning material of moxibustion) is made from the mugwort leaf (*Folium Artemisiae argyi*), and its smoke contains multiple essential oils, suspended particulate matters, and products of chemical oxidation [31]. Wheeler et al. [32] tested the chemical products of moxibustion in clinically common dosages and

found that neither carbon monoxide nor volatile compounds that present safety hazards are produced under clinical conditions. Air pollution studies show that the suspended particles in polluted air can reduce HRV by affecting the neurological system and consequently affecting the cardiovascular system by increasing HR and blood coagulation and decreasing hemoglobin to cause oxidative stress [33–35]. The respirable particles in moxa smoke mainly consist of unknown, ultrafine particles [27], which might be one reason why we observed no adverse HRV effects from clinical levels of the smoke. However, further investigation is needed to confirm and refine our finding.

Interestingly, in the second test we observed positive HR and HRV parameter changes in the experimental group compared to control. These include decrease in mean HR and increases in both time-domain analysis HRV (RMSSD, PNN50) and frequency-domain (TP, HF, and LF) during the

25-minute moxa smoke exposure (Table 4). HRV has been widely applied as a marker of autonomic nervous activity. Tension of the autonomic nervous system is maintained by opposing actions of the sympathetic and parasympathetic systems. RMSSD, PNN50, and HF are primarily thought to reflect parasympathetic influences. LF has been shown to reflect both sympathetic and parasympathetic influences. The LF/HF ratio is widely used as a relative marker of sympathetic nervous activities or sympathovagal balance [25, 36]. The HRV changes found in this study appear to be linked to the restorative functions of the autonomic system. These include an increase in total variability shown by increased TP and an increase in parasympathetic nervous activity shown by increased RMSSD, PNN50, and HF. LF/HF increase after moxa smoke exposure was significantly lower in experimental subjects than in control, which may indicate that moxa smoke drives autonomic nervous activity toward a balanced state. These findings are consistent with those of our previous pilot study in which 24 healthy volunteers exposed to moxa smoke had significant reduction in HR and increase in total HRV during and after 20 minutes of exposure to moxa smoke [37]. This suggests that moxa smoke has a regulating effect on human autonomic system function and that moxa smoke inhalation might have short-term stress-alleviating effects.

Moxa smoke effects and mechanisms have not been well investigated. We speculate that the effects are similar to those of aromatherapy, as a number of studies [38–42] show that inhalation of certain aromas can induce HRV increase and HR reduction, indicating beneficial autonomic nervous system regulation. Mechanisms of these effects might be pharmacological and/or psychological [43]. The pharmacological hypothesis is that the odor directly interacts with and affects the autonomic nervous system/central nervous system and/or endocrine systems. On the one hand, the pharmacological compound might enter the bloodstream by way of nasal or lung mucosa; on the other, the odor might stimulate the olfactory nerves and the limbic system of the brain. In the clinic, moxibustion is often used to treat insomnia, anxiety, and depression [4, 5]. However, it is unclear whether the treatment effects are induced by heat at the acupuncture point or by the moxa smoke. The present study provides some information for distinguishing the respective roles of heat and smoke. Further studies to elucidate the mechanisms of moxibustion are warranted.

We are aware of the limitations of the present study. Our data only show HRV effects from short-term exposure to moxa smoke; in normal acupuncture practice, patients usually receive multiple moxibustion treatments, and practitioners are usually exposed to the smoke for years. These factors warrant a long-term observational study. Furthermore, because the participants in our study were not blinded, we cannot rule out the possibility of placebo effect. Additionally, our subjects were young and healthy; these results might not reflect how moxa smoke affects the elderly or chronically ill.

Nevertheless, this study is an important step toward understanding the effects of moxa smoke. Our results provide useful information on the feasibility of a future, larger trial and will make it possible to calculate adequate sample sizes for such research.

In conclusion, our data show that short-term moxa smoke exposure at clinical concentrations poses no hazards to patients' HR and HRV and suggest that moxa smoke has a positive regulating effect on human autonomic function. Future studies are needed to further investigate the effects and the safety of moxa smoke.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgments

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Research Article

Electroacupuncture-Induced Dynamic Processes of Gene Expression Levels of Endogenous Opioid Peptide Precursors and Opioid Receptors in the CNS of Goats

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In order to investigate the dynamic processes of mRNA levels of proenkephalin, proopioidmelanocortin, prodynorphin, and opioid receptors (δ -, μ -, and κ -receptor) induced by electroacupuncture (EA) in the central nerve system, goats were stimulated by EA of 60 Hz for 0.5 h at a set of Baihui, Santai, Ergen, and Sanyangluo points. The pain threshold was measured using the method of potassium iontophoresis. The mRNA levels of the three opioid peptide precursors and three opioid receptors were determined with quantitative real-time PCR and the levels of Met-enkephalin with SABC immunohistochemistry at 0.5 h before and at 0, 2, 4, 6, 8, 12, and 24 h after EA. The results showed that the pain threshold correlated ($P < 0.01$) with Met-enkephalin immunoactivities in the measured nuclei and areas of goats. The analgesic aftereffect lasted for 12 h at least. The mRNA levels of the three opioid peptide precursors and three opioid receptors began to increase at 0 h, reached the peak during the time from 4 h to 6 h or at 12 h, and remained higher at 24 h after EA was discontinued. These results suggested that the initiation of gene expression of opioid peptides and the three receptors may be associated with EA-induced analgesic aftereffect.

1. Introduction

Electroacupuncture (EA) is a modern version of acupuncture and extensively used in the clinic practice because it has better analgesic effect and its stimulation can be objectively quantified and controlled [1]. EA has been used not only for effective treatment of painful diseases, but also for successful relief of pain in various operations, such as cesarean section, gastrectomy, enterectomy, and castration in human or animals [2–4]. Since the 1960s, many scientists have investigated the mechanism by which electroacupuncture induces analgesic effect. Early studies showed that analgesia induced by EA was involved in modulations of neurotransmitters (serotonin, acetylcholine, catecholamine, etc.) in the central nerve system (CNS) [5]. Latter, studies verified that neuromodulators, especially some endogenous opioid peptides (enkephalin, β -endorphin, and dynorphin), played a more important role in EA-induced analgesia. EA can promote the release of different endogenous opioid peptides (EOPs) which act on

their corresponding receptors (δ -, μ -, or κ -receptor) to exert analgesic effect [6–9].

Previous studies indicated that EA not only induces an “immediate analgesia,” but also causes an analgesic aftereffect (analgesia lasts for a while after EA is discontinued). This aftereffect plays an important role in the treatment of painful diseases and is conducive to the recovery from the surgery. So far the mechanisms by which acupuncture induces analgesic aftereffect have not been fully studied. Some reports showed that EA induced the gene expression of opioid peptide precursors, such as proopioidmelanocortin (POMC, precursor of endorphin), proenkephalin (PENK, precursor of enkephalin), and prodynorphin (PDYN, precursor of dynorphin) in the CNS of rats [10–12]. The initiation of EOP gene expressions is inferred to replenish the consumed opioid peptides because opioid peptides are immediately decomposed by some specific enzymes after they are induced to release and act on their corresponding receptors. However, the roles of the gene expressions of endogenous opioid

peptides and their receptors in EA-induced aftereffect are not completely confirmed.

Analgesia induced by EA has been proven to vary in animal species [1]. In order to quantitatively estimate the degree of acupuncture-induced analgesia, some researchers compared the dosage of some anesthetic in the anesthetic group with its dosage in the EA plus anesthetic group with the same complete analgesia. They found that EA resulted in the reduction of the dosage of anesthetics in the EA plus anesthetic group in human, rat, and goat by 45%–55%, 50%–60%, and over 75%, respectively [13, 14]. It is clear that the analgesic effect induced by EA in goats (a ruminant) is superior to that in rats or human. Ruminants should be optimal model animals for researches on the mechanisms of EA-induced analgesia. In the present study, goats were stimulated with EA for 30 min to determine the relationships of the gene expression of Met-enkephalin (M-ENK), beta-endorphin (β -EP), and dynorphin (DYN-A) and their receptors (δ -, μ -, and κ -receptor) with pain threshold and levels of enkephalin (a representative of opioid peptides) in order to probe into the mechanisms of EA-induced aftereffect.

2. Materials and Methods

2.1. Animal Preparation. One hundred and eight healthy 1- to 2-year-old hybrid male goats, weighing 23–28 kg, purchased from the goat farm of Hubei Agricultural Academy of Science, were used in this experiment (54 goats for the measurement of gene expression levels of endogenous opioid peptides and opioid receptors, another 54 goats for the measurement of M-ENK levels). All goats drunk freely and were maintained on a dry grass diet which was supplemented with a cereal-based concentrate. They were dewormed and accustomed to being approached. Feed was withheld for 24 h before the start of the experiments. The experiments were performed in a quiet environment, and the ambient temperature fluctuated between 23°C and 24°C. The experimental protocol was approved by the Animal Care Center, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, China.

2.2. Electroacupuncture. A set of Baihui (hundred meetings), Santai (three platforms), Ergen (ear base), and Sanyangluo (three Yang communication) points was selected for EA. The anatomic location of these points has been described in detail in veterinary medicine [13, 15]. The Baihui and Santai points on the dorsal midline and the Ergen and Sanyangluo points on the left side of the body were chosen in this study. Needle insertion and EA were conducted with the method reported by Liu et al. [13]. Experimental animals were restrained in right recumbency and stimulated with EA at 60 Hz for 0.5 h via WQ-6F Electronic Acupunctoscope (Beijing Xindonghua Electronic Instrument Co., Ltd., Beijing, China). The sham control goats which were only dealt with needles left in the acupoints for 0.5 h without electricity were restrained as the experimental goats.

2.3. Determination of Pain Threshold. At 0.5 h before and at 0, 2, 4, 6, 8, 12, and 24 h after EA, the pain threshold was

measured on the center of the left flank using the method of potassium iontophoresis as described in detail by Cheng et al. [16]. The pain threshold in the sham control was measured at 0.5 h before needle insertion and at 0 h after needle withdrawal.

2.4. Measurement of Gene Expression Levels of Endogenous Opioid Peptides and Opioid Receptors. Six goats were taken from the experimental goats at 0.5 h before and at 0, 2, 4, 6, 8, 12, and 24 h after EA, respectively, anesthetized with intravenous administration of xylydinothiazoline at 3 mg/kg, and slaughtered for the measurement of gene expression levels of endogenous opioid peptides and opioid receptors. According to the results of repeated pretest, gene expression of endogenous opioid peptides and opioid receptors in most nuclei and areas in the CNS reached a higher level at about 4 h after EA. Therefore, six sham control goats in the present study were euthanized for brain sampling at 4 h after needle withdrawal. The goat's brain, hypophysis, and a part of the adjacent spinal cord were immediately taken out of the skull and cervical vertebral canal. The brain was transected into 17 sections quickly with the method described by Cheng et al. [16]. The nuclei and areas were identified according to the photographic atlas of the goat brain and the morphological characteristics of the neurons [17–19]. The analgesia-related nuclei and areas were obtained with 4–8 mm diameter plastic tubes dealt with 1% DEPC solution and then put into RNastore solution (Beijing Tiangen Biological Technology Ltd., Beijing, China) to prevent RNA degradation. The mRNA levels of EOP precursors and the three opioid receptors were examined in the nuclei or areas in bilateral brain regions of goats.

The gene expression levels of PENK, POMC, PDYN, and opioid receptors (δ -, μ -, and κ -receptor) were measured through the method of quantitative real-time PCR using ABI Prism 7500 real-time PCR instrument (ABI Co., USA). The PCR primers were designed according to the sequences of β -actin-mRNA (GeneBank accession no. AF481159), PENK-mRNA (GeneBank accession no. NM174141), POMC-mRNA (GeneBank accession no. NM001009266), PDYN-mRNA (GeneBank accession no. NM174139), δ -receptor-mRNA (GeneBank accession no. NM001191148), μ -receptor-mRNA (GeneBank accession no. AF266480), and κ -receptor-mRNA (GeneBank accession no. DQ065757). The PCR products were tested through the method of normal PCR. The homology between the referenced sequences and the products' sequences was greater than or equal to 95%. The sequences of PCR products had been submitted to GeneBank (accession nos. GU169095 for PENK, GU167924 for POMC, GU169905 for PDYN, JQ756319 for δ -receptor, JQ241177 for μ -receptor, and JQ241178 for κ -receptor). Their upstream and downstream primers were presented in Table 1. All experimental data were analyzed using the method of $2^{-\Delta\Delta Ct}$ through SDSShell software (ABI Co., USA).

2.5. Measurement of M-ENK Level. The level of M-ENK was measured through the method of SABC immunohistochemistry. The experimental goats were taken and anesthetized as

TABLE 1: The quantitative real-time PCR primers of different genes.

Gene	Upstream primer	Downstream primer	Length of PCR product	Referenced GenBank no.
β -Actin	5'-ACACGGTGCCCATCTACGA-3'	5'-CCTTGATGTACGGACGATTT-3'	159 bp	AF481159
PENK	5'-GGCGACCGTGAGGGCAGAATG-3'	5'-GCAGGTTTCCAGGTCCTTGAG-3'	139 bp	NM174141
POMC	5'-GGCGGCCGAGAAGAAGGAC-3'	5'-CTTGATGATGGCGTTTTTGA-3'	148 bp	NM001009266
PDYN	5'-CTTTCCTCACTCCCTTCACCG-3'	5'-TCTCCACCAGGCTGCTACTCA-3'	186 bp	NM174139
δ -receptor	5'-TGCTCTCCATTGACTACTACA-3'	5'-AAGAGGAACACGGCAGATTTTG-3'	289 bp	NM001191148
μ -receptor	5'-AGTGTGTTATGGGCTGATGA-3'	5'-GCGTCCAGCAGACAATGAACACAG-3'	137 bp	AF266480
κ -receptor	5'-CGTCTTCGTCTGCTGCTGG-3'	5'-CAAGGAAGGCGTAGAGAATGG-3'	158 bp	DQ065757

above. According to the previous research results that opioid peptides in the CNS were released to the higher level at the end of EA [16], six sham control goats in this study were anesthetized and perfused immediately after needle withdrawal. The goats were fixed with the infusion of 4% paraformaldehyde through bilateral carotid arteries. The goat's brain was taken out and transected into 17 sections with the method described by Cheng et al. [16]. Each of the subsections was embedded in a paraffin block, sectioned at 5 μ m, mounted on polylysine-coated slides, deparaffined, and rehydrated sequentially. Four serial slides were chosen from near the middle of each section for immunohistochemical staining. Of these four slides, the three were incubated with rabbit-anti-M-ENK IgG (1:100) (Wuhan Boster Biological Technology Ltd., Wuhan, China), while the rest was incubated with PBS instead of the corresponding antibody as negative control. Experimental procedures of SABC immunohistochemistry followed the instructions provided by the reagent company (Wuhan Boster Biological Technology Ltd., Wuhan, China). The cytoplasm of positive cells was stained as brown yellow. Optical density of the stained nuclei or areas in the CNS was obtained with a light microscope connected to a video-based and computer-linked system (high-resolution pathological image analysis system-1000, Wuhan Qianping Ltd., Wuhan, China). This system was programmed to calculate the mean optical density (MOD) for three fields of each slide examined under 400x magnification. The level of M-ENK in each nucleus or area was represented with the mean value % of the mean optical density from the three slides.

2.6. Statistical Analysis. Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, USA). All the data presented as mean \pm SD. Pain threshold, mRNA levels of EOP precursors, and opioid receptors were used for ANOVA followed by the Bonferroni post hoc test. The correlation coefficient (Pearson's) was used to examine the relations between pain threshold and levels of M-ENK. Statistical significance was evaluated by determining if the *P* value was equal to or less than 0.05.

3. Results

3.1. Pain Threshold Changes Induced by Electroacupuncture. The analgesic effects of experimental goats were expressed as the pain threshold (Figure 1). The pain threshold of

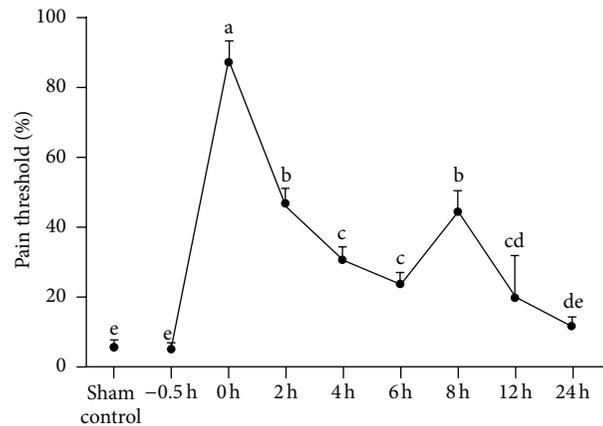


FIGURE 1: Pain threshold of goats stimulated by EA (mean \pm SD, %, *n* = 6). The same letter indicated that no significant difference between pain thresholds at two different time points (*P* > 0.05), and different letters indicated significant difference (*P* < 0.05).

experimental goats at 0.5 h before EA was not different (*P* = 1.00) from that of sham control goats. After EA stimulated the goats, the pain threshold increased and reached the peak at 0 h. Then the pain threshold gradually decreased, began to rebound at 6 h, came to the second peak at 8 h, and then fell gradually again. At 0, 2, 4, 6, 8, and 12 h after EA, the pain threshold increased by 88%, 47%, 32%, 24%, 46%, and 21%, respectively. The pain threshold during the time from 0 to 12 h after EA was higher (*P* < 0.05) than that at 0.5 h before EA. The pain threshold value at 8 h was higher (*P* < 0.01) than that at 4 h, 6 h, 12 h, or 24 h although it was lower (*P* < 0.01) than that at 0 h after EA. There was no difference (*P* = 1.00) between pain thresholds at 2 h and 8 h.

3.2. mRNA Levels of Endogenous Opioid Peptide Precursors in the CNS of Goats. PENK, POMC, and PDYN are the precursors of enkephalin, endorphin, and dynorphin, respectively. The mRNA levels of three endogenous opioid peptide precursors were measured in the analgesia- and distribution-related nuclei or areas. These nuclei or areas mainly included nucleus accumbens (ACB), caudate nucleus (CAU), amygdala (AMY), supraoptic nucleus (SON), paraventricular nucleus of hypothalamus (PVH), ventromedial nucleus of hypothalamus (VMH), arcuate nucleus (ARC), paraventricular nucleus of thalamus (PVT), periaqueductal gray

TABLE 2: Levels of PENK gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
ACB	1.06 \pm 0.05 ^d	1.00 \pm 0.00 ^d	1.15 \pm 0.12 ^{cd}	1.29 \pm 0.18 ^{cd}	1.68 \pm 0.21 ^b	2.01 \pm 0.24 ^a	1.43 \pm 0.17 ^{bc}	1.17 \pm 0.16 ^{cd}	1.23 \pm 0.14 ^{cd}
CAU	1.03 \pm 0.02 ^d	1.00 \pm 0.00 ^d	1.11 \pm 0.10 ^{cd}	1.26 \pm 0.26 ^{bcd}	1.75 \pm 0.21 ^{ab}	2.04 \pm 0.19 ^a	1.26 \pm 0.31 ^{bcd}	1.49 \pm 0.35 ^{bcd}	1.54 \pm 0.48 ^{abc}
AMY	1.17 \pm 0.08 ^e	1.00 \pm 0.00 ^e	1.35 \pm 0.19 ^{cd}	1.41 \pm 0.11 ^{cd}	1.57 \pm 0.13 ^{bc}	1.31 \pm 0.12 ^d	1.74 \pm 0.06 ^b	2.10 \pm 0.10 ^a	1.51 \pm 0.13 ^{cd}
PVH	1.06 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.31 \pm 0.09 ^d	1.38 \pm 0.11 ^{cd}	1.89 \pm 0.12 ^b	2.34 \pm 0.10 ^a	1.57 \pm 0.13 ^c	2.06 \pm 0.19 ^b	1.53 \pm 0.15 ^{cd}
VMH	1.24 \pm 0.16 ^e	1.00 \pm 0.00 ^e	1.29 \pm 0.06 ^{de}	1.47 \pm 0.10 ^{cd}	1.69 \pm 0.17 ^{bc}	2.42 \pm 0.18 ^a	1.44 \pm 0.21 ^{cd}	1.63 \pm 0.15 ^{bc}	1.87 \pm 0.20 ^b
PAG	1.22 \pm 0.21 ^f	1.00 \pm 0.00 ^f	1.64 \pm 0.15 ^{ef}	2.46 \pm 0.37 ^{cd}	2.86 \pm 0.37 ^{ab}	3.37 \pm 0.41 ^a	2.68 \pm 0.43 ^{bc}	2.02 \pm 0.36 ^{de}	2.22 \pm 0.33 ^{de}
DR	1.17 \pm 0.08 ^d	1.00 \pm 0.00 ^d	1.37 \pm 0.09 ^{cd}	1.55 \pm 0.05 ^{bcd}	1.98 \pm 0.22 ^{ab}	2.42 \pm 0.42 ^a	1.93 \pm 0.43 ^{ab}	1.61 \pm 0.37 ^{bc}	1.75 \pm 0.31 ^{bc}
HB	1.05 \pm 0.04 ^f	1.00 \pm 0.00 ^f	1.22 \pm 0.07 ^{def}	1.29 \pm 0.17 ^{cde}	1.54 \pm 0.21 ^{bc}	1.16 \pm 0.07 ^{ef}	1.48 \pm 0.16 ^{bcd}	1.72 \pm 0.14 ^{ab}	1.93 \pm 0.18 ^a
PBN	1.16 \pm 0.07 ^e	1.00 \pm 0.00 ^e	1.37 \pm 0.10 ^{de}	1.50 \pm 0.23 ^{cd}	1.77 \pm 0.31 ^{bc}	2.21 \pm 0.25 ^a	1.44 \pm 0.15 ^{cd}	1.67 \pm 0.21 ^{bcd}	1.89 \pm 0.19 ^{ab}
GI	1.05 \pm 0.09 ^e	1.00 \pm 0.00 ^e	1.27 \pm 0.06 ^d	1.39 \pm 0.17 ^{cd}	1.61 \pm 0.12 ^c	1.93 \pm 0.18 ^b	2.39 \pm 0.20 ^a	1.59 \pm 0.11 ^c	1.33 \pm 0.11 ^d
SOL	1.08 \pm 0.12 ^e	1.00 \pm 0.00 ^e	1.33 \pm 0.11 ^d	1.49 \pm 0.21 ^{bcd}	1.71 \pm 0.21 ^b	2.12 \pm 0.16 ^a	1.36 \pm 0.13 ^{cd}	1.52 \pm 0.17 ^{bcd}	1.65 \pm 0.22 ^{bc}
NH	1.16 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.54 \pm 0.13 ^d	2.17 \pm 0.22 ^c	3.22 \pm 0.13 ^b	4.27 \pm 0.43 ^a	2.19 \pm 0.37 ^c	1.59 \pm 0.24 ^d	2.17 \pm 0.23 ^c

There was difference ($P < 0.05$) between the values with different letters, and no difference ($P > 0.05$) with the same letters in a line. The letters in the following tables have the same meanings.

TABLE 3: Levels of POMC gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
CAU	1.03 \pm 0.02 ^c	1.00 \pm 0.00 ^c	1.25 \pm 0.09 ^{bc}	1.38 \pm 0.16 ^b	2.03 \pm 0.23 ^a	1.37 \pm 0.14 ^b	1.81 \pm 0.16 ^a	1.27 \pm 0.05 ^b	1.46 \pm 0.13 ^b
AMY	1.09 \pm 0.05 ^d	1.00 \pm 0.00 ^d	1.28 \pm 0.08 ^{cd}	1.34 \pm 0.08 ^{bc}	1.64 \pm 0.23 ^{ab}	1.39 \pm 0.22 ^{abc}	1.69 \pm 0.30 ^a	1.25 \pm 0.06 ^{cd}	1.39 \pm 0.10 ^{abc}
PVH	1.13 \pm 0.03 ^e	1.00 \pm 0.00 ^e	1.54 \pm 0.12 ^{cd}	1.96 \pm 0.20 ^{ab}	2.31 \pm 0.17 ^a	1.39 \pm 0.25 ^d	1.84 \pm 0.14 ^{bc}	1.51 \pm 0.12 ^{cd}	1.49 \pm 0.36 ^{cd}
VMH	1.10 \pm 0.06 ^f	1.00 \pm 0.00 ^f	1.17 \pm 0.07 ^{ef}	1.31 \pm 0.13 ^e	2.36 \pm 0.10 ^a	1.72 \pm 0.16 ^{bc}	1.86 \pm 0.15 ^b	1.35 \pm 0.11 ^{de}	1.57 \pm 0.13 ^{cd}
ARC	1.13 \pm 0.03 ^e	1.00 \pm 0.00 ^e	1.36 \pm 0.13 ^d	2.17 \pm 0.12 ^b	1.54 \pm 0.14 ^{cd}	2.24 \pm 0.16 ^b	2.76 \pm 0.21 ^a	2.08 \pm 0.13 ^b	1.78 \pm 0.14 ^c
PAG	1.06 \pm 0.03 ^d	1.00 \pm 0.00 ^d	1.41 \pm 0.10 ^c	1.66 \pm 0.21 ^{bc}	2.56 \pm 0.16 ^a	1.84 \pm 0.10 ^b	1.66 \pm 0.17 ^{bc}	1.43 \pm 0.20 ^c	1.64 \pm 0.22 ^{bc}
NRM	1.06 \pm 0.04 ^d	1.00 \pm 0.00 ^d	1.42 \pm 0.11 ^c	1.92 \pm 0.14 ^b	2.54 \pm 0.18 ^a	1.61 \pm 0.25 ^{bc}	1.44 \pm 0.27 ^c	2.32 \pm 0.20 ^a	1.47 \pm 0.14 ^c
GI	1.13 \pm 0.03 ^e	1.00 \pm 0.00 ^e	1.57 \pm 0.09 ^d	2.61 \pm 0.19 ^a	1.93 \pm 0.17 ^{bc}	2.14 \pm 0.27 ^b	2.65 \pm 0.19 ^a	1.81 \pm 0.15 ^{bcd}	1.67 \pm 0.20 ^{cd}
SOL	1.05 \pm 0.07 ^c	1.00 \pm 0.00 ^c	1.47 \pm 0.09 ^b	2.12 \pm 0.17 ^a	1.79 \pm 0.18 ^{ab}	1.61 \pm 0.19 ^b	1.93 \pm 0.36 ^{ab}	1.70 \pm 0.48 ^{ab}	1.83 \pm 0.13 ^{ab}
NH	1.07 \pm 0.03 ^e	1.00 \pm 0.00 ^e	1.33 \pm 0.12 ^d	1.42 \pm 0.18 ^d	1.96 \pm 0.14 ^c	2.81 \pm 0.08 ^a	1.75 \pm 0.08 ^c	1.43 \pm 0.06 ^d	2.24 \pm 0.19 ^b

(PAG), dorsal raphe nucleus (DR), habenular nucleus (HB), parabrachial nucleus (PBN), nucleus raphe magnus (NRM), gigantocellular reticular nucleus (GI), solitary nucleus (SOL), neurohypophysis (NH), and spinal cord dorsal horn (SCD).

The mRNA levels of PENK changed in the measured nuclei or areas in a similar pattern after EA stimulated the animals; they increased ($P < 0.01$) with the peak at 6 h in ACB, PAG, DR, NH, CAU, VMH, PBN, SOL, and PVH, at 8 h in GI, at 12 h in AMY, or at 24 h in HB (Table 2). PENK mRNAs remained higher levels ($P < 0.05$) at 24 h after EA than at 0.5 h before EA in the measured nuclei or areas except ACB. The nucleus or area sequence of the amplitude by which PENK mRNAs increased were NH > PAG > DR = VMH > GI > PVH > PBN > SOL > AMY > CAU > ACB > HB.

The mRNA levels of POMC increased ($P < 0.01$) with one or two peaks in the measured nuclei or areas after EA (Table 3). The peak of POMC mRNA levels occurred at 2 h in SOL, at 4 h in CAU, PVH, VMH, and PAG, or at 6 h in NH. There were two peaks of POMC mRNA levels which appeared

at 2 h and 8 h in ARC and GI, or at 4 h and 12 h in NRM, or at 4 h and 8 h in AMY, respectively. POMC mRNA levels remained higher ($P < 0.05$) at 24 h after EA than at 0.5 h before EA in the measured nuclei or areas.

The mRNA levels of PDYN began to increase ($P < 0.05$) in most of the measured nuclei or areas at 2 h or 4 h (Table 4). They reached the first peaks at 4 h in AMY, PVT, PAG, PBN, SCD, and CAU or at 6 h in PVH, VMH, SON, and SOL. The second peaks of PDYN mRNA levels, occurred at 12 h, were higher ($P < 0.05$) than the first peaks in all the measured nuclei or areas. PDYN mRNA levels at 12 h increased by times from 0.96 to 2.21, compared with those at 0.5 h before EA stimulated the animals. PDYN mRNAs in AMY, PAG, SCD, and SON remained higher levels ($P < 0.05$) at 24 h while mRNA levels of PDYN in PVT, PBN, PVH, VMH, SOL, and CAU returned to the preacupuncture level ($P > 0.05$).

There were no differences ($P > 0.05$) in mRNA levels of PENK, POMC, or PDYN between sham control goats and

TABLE 4: Levels of PDYN gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
CAU	1.08 \pm 0.03 ^{de}	1.00 \pm 0.00 ^{de}	1.05 \pm 0.09 ^{de}	0.87 \pm 0.03 ^e	1.68 \pm 0.09 ^b	1.36 \pm 0.07 ^c	1.07 \pm 0.02 ^d	1.96 \pm 0.08 ^a	1.12 \pm 0.07 ^d
AMY	1.17 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.30 \pm 0.08 ^d	1.53 \pm 0.06 ^c	1.87 \pm 0.09 ^b	1.30 \pm 0.03 ^d	1.40 \pm 0.05 ^{cd}	2.75 \pm 0.12 ^a	1.40 \pm 0.08 ^{cd}
PVH	1.06 \pm 0.02 ^c	1.00 \pm 0.00 ^c	1.23 \pm 0.12 ^c	1.29 \pm 0.09 ^c	1.46 \pm 0.11 ^c	1.82 \pm 0.09 ^b	1.25 \pm 0.13 ^c	2.86 \pm 0.24 ^a	1.16 \pm 0.04 ^c
VMH	1.09 \pm 0.04 ^d	1.00 \pm 0.00 ^d	1.10 \pm 0.07 ^d	1.17 \pm 0.11 ^{cd}	1.45 \pm 0.13 ^c	1.88 \pm 0.11 ^b	1.10 \pm 0.07 ^d	2.57 \pm 0.16 ^a	1.18 \pm 0.08 ^{cd}
SON	1.10 \pm 0.03 ^e	1.00 \pm 0.00 ^e	1.15 \pm 0.13 ^{de}	1.22 \pm 0.09 ^{de}	1.61 \pm 0.07 ^{bc}	1.84 \pm 0.07 ^b	1.35 \pm 0.12 ^{cd}	2.53 \pm 0.14 ^a	1.37 \pm 0.11 ^{cd}
PVT	1.20 \pm 0.08 ^b	1.00 \pm 0.00 ^b	1.22 \pm 0.10 ^b	1.27 \pm 0.15 ^b	2.11 \pm 0.18 ^a	1.41 \pm 0.16 ^b	1.23 \pm 0.14 ^b	2.40 \pm 0.25 ^a	1.21 \pm 0.11 ^b
PAG	1.15 \pm 0.05 ^e	1.00 \pm 0.00 ^e	1.21 \pm 0.11 ^{de}	1.59 \pm 0.11 ^c	1.89 \pm 0.07 ^b	1.51 \pm 0.13 ^{cd}	1.25 \pm 0.11 ^{de}	3.11 \pm 0.09 ^a	1.36 \pm 0.09 ^{cd}
PBN	1.07 \pm 0.03 ^d	1.00 \pm 0.00 ^d	1.27 \pm 0.09 ^{cd}	1.31 \pm 0.14 ^c	1.66 \pm 0.09 ^b	1.26 \pm 0.07 ^{cd}	1.08 \pm 0.04 ^{cd}	2.78 \pm 0.17 ^a	1.23 \pm 0.11 ^{cd}
SOL	1.11 \pm 0.04 ^c	1.00 \pm 0.00 ^c	1.37 \pm 0.09 ^{bc}	1.43 \pm 0.09 ^{bc}	1.48 \pm 0.20 ^b	1.49 \pm 0.16 ^b	1.15 \pm 0.11 ^{bc}	2.44 \pm 0.23 ^a	1.27 \pm 0.15 ^{bc}
SCD	1.07 \pm 0.02 ^e	1.00 \pm 0.00 ^e	1.33 \pm 0.12 ^{cd}	1.30 \pm 0.06 ^{cd}	1.63 \pm 0.10 ^b	1.39 \pm 0.03 ^c	1.14 \pm 0.04 ^{de}	3.21 \pm 0.15 ^a	1.52 \pm 0.09 ^{bc}

TABLE 5: Levels of δ -receptor gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
ACB	1.05 \pm 0.03 ^d	1.00 \pm 0.00 ^d	1.05 \pm 0.06 ^{cd}	1.19 \pm 0.07 ^c	1.39 \pm 0.11 ^b	1.79 \pm 0.09 ^a	1.52 \pm 0.10 ^b	1.15 \pm 0.13 ^{cd}	1.10 \pm 0.08 ^{cd}
CAU	1.08 \pm 0.04 ^{de}	1.00 \pm 0.00 ^{de}	0.99 \pm 0.05 ^e	1.10 \pm 0.05 ^{de}	1.45 \pm 0.09 ^b	1.71 \pm 0.12 ^a	1.16 \pm 0.12 ^{cd}	1.32 \pm 0.10 ^{bc}	1.29 \pm 0.10 ^{bc}
AMY	1.05 \pm 0.04 ^f	1.00 \pm 0.00 ^f	1.23 \pm 0.09 ^e	1.43 \pm 0.09 ^{cd}	1.59 \pm 0.10 ^{bc}	1.32 \pm 0.09 ^{de}	1.76 \pm 0.09 ^b	2.10 \pm 0.13 ^a	1.48 \pm 0.12 ^{cd}
PVH	1.08 \pm 0.09 ^f	1.00 \pm 0.00 ^f	1.25 \pm 0.08 ^e	1.36 \pm 0.10 ^{de}	1.83 \pm 0.10 ^b	2.22 \pm 0.12 ^a	1.56 \pm 0.10 ^c	2.06 \pm 0.17 ^a	1.49 \pm 0.09 ^{cd}
VMH	1.12 \pm 0.05 ^f	1.00 \pm 0.00 ^f	1.30 \pm 0.07 ^e	1.44 \pm 0.10 ^{de}	1.69 \pm 0.13 ^{bc}	2.24 \pm 0.11 ^a	1.44 \pm 0.12 ^{de}	1.63 \pm 0.09 ^{cd}	1.87 \pm 0.17 ^b
PAG	1.23 \pm 0.16 ^f	1.00 \pm 0.00 ^f	1.64 \pm 0.09 ^e	2.41 \pm 0.19 ^{cd}	2.79 \pm 0.15 ^b	3.30 \pm 0.21 ^a	2.49 \pm 0.17 ^{bc}	2.11 \pm 0.21 ^d	2.16 \pm 0.19 ^d
DR	1.13 \pm 0.07 ^f	1.00 \pm 0.00 ^f	1.29 \pm 0.08 ^{ef}	1.51 \pm 0.08 ^{de}	1.89 \pm 0.16 ^{bc}	2.44 \pm 0.31 ^a	1.96 \pm 0.15 ^b	1.63 \pm 0.10 ^{cd}	1.69 \pm 0.15 ^{bcd}
HB	0.99 \pm 0.06 ^f	1.00 \pm 0.00 ^f	1.19 \pm 0.08 ^e	1.29 \pm 0.11 ^{de}	1.55 \pm 0.11 ^{bc}	1.18 \pm 0.08 ^{ef}	1.48 \pm 0.09 ^{cd}	1.71 \pm 0.10 ^b	1.96 \pm 0.15 ^a
PBN	1.07 \pm 0.04 ^f	1.00 \pm 0.00 ^f	1.37 \pm 0.11 ^e	1.42 \pm 0.13 ^e	1.75 \pm 0.13 ^{bc}	2.20 \pm 0.10 ^a	1.49 \pm 0.08 ^{de}	1.65 \pm 0.12 ^{cd}	1.85 \pm 0.07 ^b
GI	1.04 \pm 0.05 ^e	1.00 \pm 0.00 ^e	1.25 \pm 0.08 ^d	1.35 \pm 0.14 ^d	1.61 \pm 0.08 ^c	1.90 \pm 0.15 ^b	2.36 \pm 0.12 ^a	1.59 \pm 0.08 ^c	1.30 \pm 0.07 ^d
SOL	1.07 \pm 0.03 ^f	1.00 \pm 0.00 ^f	1.33 \pm 0.08 ^e	1.49 \pm 0.10 ^{de}	1.74 \pm 0.09 ^b	2.10 \pm 0.10 ^a	1.37 \pm 0.12 ^e	1.55 \pm 0.10 ^{cd}	1.69 \pm 0.09 ^{bc}
NH	1.10 \pm 0.05 ^e	1.00 \pm 0.00 ^e	1.57 \pm 0.09 ^d	2.24 \pm 0.10 ^c	3.24 \pm 0.13 ^b	4.24 \pm 0.14 ^a	2.34 \pm 0.11 ^c	1.61 \pm 0.12 ^d	2.17 \pm 0.14 ^c

experimental goats at 0.5 h before EA in the measured nuclei and areas.

3.3. mRNA Levels of Endogenous Opioid Receptors in the CNS of Goats. The mRNA levels of δ -, μ -, or κ -receptor were measured in the corresponding nuclei or areas where their ligands are distributed (Tables 5, 6, and 7). The mRNA levels of δ -receptor increased ($P < 0.01$) with the peak at 6 h in ACB, PAG, DR, NH, CAU, PVH, VMH, PBN, and SOL, at 8 h in GI, or at 12 h in AMY. δ -receptor mRNA remained higher levels in the measured nuclei or areas except ACB at 24 h and kept uptrend in NH, VMH, PBN, SOL, and HB. The δ -receptor mRNA levels at the peak in NH, PAG, DR, GI, VMH, PVH, PBN, SOL, AMY, HB, ACB, and CAU increased by 3.24, 2.30, 1.44, 1.36, 1.24, 1.22, 1.20, 1.10, 1.10, 0.96, 0.71, and 0.71 times, respectively.

The mRNA levels of μ -receptor increased ($P < 0.05$) at 0 h after EA and then fluctuated with one or two apparent peaks in the measured nuclei or areas. There was a single peak of μ -receptor mRNA levels which occurred at 4 h in VMH and PAG or at 6 h in NH. There were two peaks of μ -receptor

mRNA levels which appeared at 2 h and 8 h in ARC, GI, and SOL, at 4 h and 8 h in PVH, CAU, and AMY, or at 4 h and 12 h in NRM, respectively. μ -receptor mRNA kept higher levels ($P < 0.05$) at 24 h after EA than at 0.5 h before EA in all the measured nuclei or areas.

The mRNA levels of κ -receptor increased ($P < 0.05$) at 0 h, slightly decreased at 8 h, then increased quickly, and reached the peak at 12 h after EA was terminated. Thereafter, κ -receptor mRNA levels declined again, but remained higher levels ($P < 0.05$) at 24 h, compared with those at 0.5 h before EA. The κ -receptor mRNA levels at 12 h in SCD, PAG, PBN, AMY, PVH, VMH, SON, SOL, PVT, and CAU increased by 1.99, 1.92, 1.67, 1.59, 1.51, 1.43, 1.42, 1.40, 1.31, and 0.81 times, respectively.

There were no differences ($P > 0.05$) in mRNA levels of δ -, μ -, or κ -receptor between sham control goats and experimental goats at 0.5 h before EA in the measured nuclei and areas.

3.4. Levels of Met-Enkephalin in the CNS of Goats. Electroacupuncture induced M-ENK immunoactivities to increase

TABLE 6: Levels of μ -receptor gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
CAU	1.05 \pm 0.03 ^d	1.00 \pm 0.00 ^d	1.26 \pm 0.11 ^c	1.38 \pm 0.15 ^{bc}	1.99 \pm 0.10 ^a	1.35 \pm 0.14 ^{bc}	1.81 \pm 0.13 ^a	1.29 \pm 0.10 ^{bc}	1.48 \pm 0.12 ^b
AMY	1.04 \pm 0.04 ^c	1.00 \pm 0.00 ^c	1.26 \pm 0.11 ^b	1.39 \pm 0.10 ^b	1.53 \pm 0.14 ^a	1.38 \pm 0.08 ^b	1.64 \pm 0.15 ^a	1.26 \pm 0.08 ^b	1.38 \pm 0.09 ^b
PVH	1.11 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.48 \pm 0.10 ^d	2.01 \pm 0.19 ^b	2.26 \pm 0.13 ^a	1.36 \pm 0.08 ^d	1.74 \pm 0.13 ^c	1.47 \pm 0.11 ^d	1.49 \pm 0.10 ^d
VMH	1.12 \pm 0.06 ^e	1.00 \pm 0.00 ^e	1.16 \pm 0.11 ^{de}	1.38 \pm 0.10 ^{cd}	2.24 \pm 0.12 ^a	1.75 \pm 0.11 ^b	1.79 \pm 0.13 ^b	1.39 \pm 0.15 ^c	1.46 \pm 0.12 ^c
ARC	1.08 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.36 \pm 0.16 ^d	2.08 \pm 0.15 ^b	1.50 \pm 0.12 ^{cd}	2.20 \pm 0.12 ^b	2.82 \pm 0.18 ^a	2.10 \pm 0.13 ^b	1.71 \pm 0.10 ^c
PAG	1.18 \pm 0.11 ^e	1.00 \pm 0.00 ^e	1.44 \pm 0.12 ^d	1.71 \pm 0.14 ^{bc}	2.41 \pm 0.21 ^a	1.72 \pm 0.11 ^b	1.67 \pm 0.11 ^{bcd}	1.45 \pm 0.16 ^{cd}	1.54 \pm 0.16 ^{bcd}
NRM	1.09 \pm 0.08 ^d	1.00 \pm 0.00 ^d	1.44 \pm 0.14 ^c	1.99 \pm 0.16 ^b	2.41 \pm 0.15 ^a	1.58 \pm 0.15 ^c	1.45 \pm 0.13 ^c	2.09 \pm 0.08 ^b	1.51 \pm 0.11 ^c
GI	1.21 \pm 0.15 ^e	1.00 \pm 0.00 ^e	1.51 \pm 0.08 ^d	2.54 \pm 0.15 ^a	1.88 \pm 0.18 ^{bc}	2.12 \pm 0.19 ^b	2.47 \pm 0.15 ^a	1.78 \pm 0.14 ^{cd}	1.74 \pm 0.16 ^{cd}
SOL	1.08 \pm 0.07 ^e	1.00 \pm 0.00 ^e	1.44 \pm 0.14 ^d	2.09 \pm 0.13 ^a	1.77 \pm 0.10 ^{bc}	1.63 \pm 0.12 ^{cd}	2.01 \pm 0.28 ^{ab}	1.68 \pm 0.21 ^{cd}	1.72 \pm 0.16 ^{bc}
NH	1.08 \pm 0.06 ^e	1.00 \pm 0.00 ^e	1.33 \pm 0.15 ^d	1.38 \pm 0.15 ^{cd}	2.02 \pm 0.15 ^b	2.53 \pm 0.13 ^a	1.59 \pm 0.13 ^c	1.47 \pm 0.10 ^{cd}	2.23 \pm 0.12 ^b

TABLE 7: Levels of κ -receptor gene expression in the CNS of goats stimulated by EA ($2^{-\Delta\Delta Ct}$, mean \pm SD, $n = 6$).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h
CAU	0.98 \pm 0.06 ^c	1.00 \pm 0.00 ^c	1.11 \pm 0.06 ^c	0.99 \pm 0.09 ^c	1.55 \pm 0.12 ^b	1.41 \pm 0.08 ^b	1.10 \pm 0.09 ^c	1.81 \pm 0.10 ^a	1.10 \pm 0.10 ^c
AMY	1.17 \pm 0.13 ^d	1.00 \pm 0.00 ^d	1.33 \pm 0.10 ^c	1.37 \pm 0.13 ^c	1.65 \pm 0.07 ^b	1.33 \pm 0.14 ^c	1.15 \pm 0.09 ^{cd}	2.59 \pm 0.18 ^a	1.23 \pm 0.15 ^{cd}
PVH	1.10 \pm 0.04 ^d	1.00 \pm 0.00 ^d	1.28 \pm 0.12 ^c	1.32 \pm 0.09 ^c	1.47 \pm 0.13 ^c	1.80 \pm 0.14 ^b	1.24 \pm 0.09 ^c	2.51 \pm 0.21 ^a	1.24 \pm 0.14 ^{cd}
VMH	1.09 \pm 0.05 ^e	1.00 \pm 0.00 ^e	1.15 \pm 0.11 ^{de}	1.20 \pm 0.10 ^d	1.44 \pm 0.13 ^c	1.89 \pm 0.10 ^b	1.15 \pm 0.08 ^{de}	2.43 \pm 0.15 ^a	1.18 \pm 0.09 ^{de}
SON	1.16 \pm 0.14 ^e	1.00 \pm 0.00 ^e	1.22 \pm 0.11 ^{de}	1.29 \pm 0.11 ^d	1.58 \pm 0.11 ^{bc}	1.72 \pm 0.10 ^b	1.45 \pm 0.13 ^{cd}	2.42 \pm 0.22 ^a	1.41 \pm 0.15 ^{cd}
PVT	1.06 \pm 0.03 ^d	1.00 \pm 0.00 ^d	1.26 \pm 0.08 ^c	1.36 \pm 0.08 ^c	2.01 \pm 0.10 ^b	1.42 \pm 0.11 ^c	1.25 \pm 0.13 ^c	2.31 \pm 0.14 ^a	1.29 \pm 0.10 ^c
PAG	1.09 \pm 0.13 ^e	1.00 \pm 0.00 ^e	1.25 \pm 0.08 ^d	1.56 \pm 0.10 ^c	1.93 \pm 0.10 ^b	1.58 \pm 0.08 ^c	1.29 \pm 0.08 ^d	2.92 \pm 0.16 ^a	1.41 \pm 0.14 ^{cd}
PBN	1.07 \pm 0.06 ^e	1.00 \pm 0.00 ^e	1.31 \pm 0.12 ^{cd}	1.35 \pm 0.12 ^c	1.67 \pm 0.09 ^b	1.27 \pm 0.06 ^{cd}	1.13 \pm 0.11 ^{de}	2.67 \pm 0.15 ^a	1.37 \pm 0.16 ^c
SOL	1.11 \pm 0.15 ^d	1.00 \pm 0.00 ^d	1.37 \pm 0.12 ^{bc}	1.45 \pm 0.11 ^b	1.51 \pm 0.13 ^b	1.53 \pm 0.11 ^b	1.16 \pm 0.11 ^{cd}	2.40 \pm 0.14 ^a	1.31 \pm 0.13 ^{bc}
SCD	1.06 \pm 0.04 ^e	1.00 \pm 0.00 ^e	1.38 \pm 0.10 ^{cd}	1.35 \pm 0.10 ^{cd}	1.59 \pm 0.11 ^b	1.42 \pm 0.07 ^{bcd}	1.23 \pm 0.11 ^d	2.99 \pm 0.18 ^a	1.55 \pm 0.12 ^{bc}

($P < 0.05$), to reach the peak at 0 h, and then to fall down gradually to the lowest levels at 4–6 h (but still higher than those at 0.5 h before EA) in most measured nuclei or areas. M-ENK immunoactivities rebounded at 4–6 h and came to the second peak at 6–8 h in SOL, VMH, CAU, PBN, AMY, and PVH. At the end of the experiment, M-ENK immunoactivities remained higher levels in the measured nuclei or areas. M-ENK immunoactivities positively correlated ($P < 0.01$) with the pain threshold in the measured nuclei and areas (Table 8). There were no differences ($P > 0.05$) in M-ENK immunoactivities between sham control goats and experimental goats at 0.5 h before EA in the measured nuclei and areas.

4. Discussion

4.1. The Aftereffect Phenomenon of Analgesia Induced by EA. In the 1970s, researchers used potassium iontophoresis method to quantitatively assess acupuncture-induced change in pain threshold and found that acupuncture caused a gradual increase and slow return in pain threshold [20, 21]. The analgesia during acupuncture is usually believed to be “immediate analgesia” whereas the analgesia which lasts after

acupuncture discontinuation is called “analgesic aftereffect of acupuncture.” Some researchers have paid more attention to this aftereffect because they realize it is an important basis for the treatment of pain diseases. Reports showed that hand acupuncture at 5 Hz at human “Hegu” point produced an increase in pain threshold with a peak occurring 20 to 40 min after the needle insertion, and that the threshold returned to the preacupuncture level about 45 min after the needle was removed [22]. Liang et al. [23] alternatively used 20 Hz and 100 Hz of electroacupuncture to stimulate “Kunlun” acupoint of rats with artificial acute arthritis and found the analgesic effect in the inflammatory region lasted for over 60 min after EA was terminated. Zhao et al. [24] applied alternatively 15 Hz and 100 Hz of EA to “Kunlun” and “Xuanzhong” acupoints of rats with adjuvant arthritis and showed that the analgesic aftereffect lasted for about 12 h. These discrepancies in the duration of acupuncture analgesic aftereffect might be caused by different acupuncture methods (electroacupuncture or manual acupuncture), subjects (human or animal), acupoints, acupuncture parameters (frequencies), and so forth.

Studies show that the analgesic effect induced by EA in goats (a ruminant) is superior to that in rats or human [13, 14]. Therefore, ruminants should be optimal model animals

TABLE 8: M-ENK immunoactivities in the CNS of goats stimulated by EA (mean ± SD, n = 6).

Nuclei and areas	Sham control	-0.5 h	0 h	2 h	4 h	6 h	8 h	12 h	24 h	Correlation with pain threshold (r)
ACB	15.96 ± 1.53 ^c	16.17 ± 2.03 ^c	35.76 ± 4.50 ^a	27.78 ± 2.15 ^b	23.92 ± 2.03 ^b	18.47 ± 2.96 ^c	17.55 ± 0.89 ^c	17.27 ± 1.91 ^c	15.37 ± 1.72 ^c	0.874 ^{**}
CAU	14.19 ± 1.21 ^d	13.67 ± 2.88 ^d	33.06 ± 3.62 ^a	28.69 ± 2.91 ^{ab}	23.48 ± 3.94 ^{bc}	17.72 ± 3.65 ^{cd}	23.02 ± 3.12 ^{bc}	16.21 ± 2.26 ^d	14.80 ± 2.02 ^d	0.819 ^{**}
AMY	10.74 ± 1.55 ^g	10.62 ± 2.07 ^g	47.74 ± 5.94 ^a	37.40 ± 3.14 ^b	28.69 ± 2.91 ^{cd}	34.41 ± 3.69 ^{bc}	25.13 ± 2.12 ^{de}	18.42 ± 1.05 ^f	21.16 ± 1.95 ^{ef}	0.808 ^{**}
PVH	17.11 ± 2.28 ^c	17.68 ± 4.59 ^c	43.99 ± 5.78 ^a	37.71 ± 2.98 ^a	29.18 ± 5.47 ^b	21.78 ± 2.14 ^{bc}	28.14 ± 1.40 ^b	23.95 ± 4.93 ^{bc}	28.41 ± 1.82 ^b	0.729 ^{**}
VMH	12.14 ± 1.81 ^e	11.66 ± 3.21 ^e	41.24 ± 2.53 ^a	31.65 ± 3.07 ^b	29.94 ± 2.72 ^b	19.48 ± 1.57 ^d	27.05 ± 2.55 ^{bc}	24.85 ± 2.30 ^c	18.79 ± 1.29 ^d	0.848 ^{**}
PAG	8.04 ± 0.84 ^e	7.91 ± 1.18 ^e	32.13 ± 2.13 ^a	26.58 ± 1.00 ^b	21.04 ± 3.76 ^c	15.73 ± 1.69 ^d	18.57 ± 1.32 ^{cd}	21.28 ± 1.67 ^c	21.70 ± 1.53 ^c	0.748 ^{**}
DR	11.48 ± 1.64 ^e	10.92 ± 1.73 ^e	28.41 ± 1.82 ^a	22.96 ± 1.94 ^b	19.32 ± 1.64 ^{bc}	14.48 ± 1.06 ^{de}	16.94 ± 0.79 ^{cd}	20.66 ± 1.46 ^{bc}	18.31 ± 0.72 ^{cd}	0.759 ^{**}
HB	16.51 ± 1.44 ^{cd}	15.76 ± 1.46 ^{cd}	26.19 ± 1.21 ^a	24.17 ± 1.04 ^a	19.49 ± 1.75 ^b	19.06 ± 1.87 ^b	17.87 ± 1.04 ^{bc}	16.75 ± 2.35 ^{bcd}	15.52 ± 1.27 ^d	0.837 ^{**}
PBN	7.79 ± 1.10 ^e	6.75 ± 1.24 ^e	26.80 ± 3.72 ^a	23.44 ± 1.47 ^a	19.23 ± 1.62 ^b	14.02 ± 1.88 ^{cd}	16.80 ± 1.87 ^{bc}	12.70 ± 1.12 ^d	11.16 ± 1.53 ^d	0.857 ^{**}
GI	11.56 ± 1.27 ^d	10.51 ± 1.42 ^d	19.13 ± 1.33 ^a	18.14 ± 1.09 ^a	15.03 ± 0.97 ^{bc}	14.60 ± 1.14 ^{bc}	11.70 ± 1.03 ^d	12.73 ± 1.47 ^{cd}	15.70 ± 1.27 ^b	0.605 ^{**}
SOL	14.03 ± 1.77 ^g	14.44 ± 0.93 ^g	49.83 ± 1.15 ^a	40.99 ± 2.05 ^b	30.82 ± 1.40 ^c	24.32 ± 1.69 ^c	27.67 ± 1.34 ^d	26.48 ± 1.25 ^{de}	19.49 ± 1.38 ^f	0.911 ^{**}

** Means the levels of M-ENK correlate with the pain threshold at the 0.01 level.

for researches on the mechanisms of EA-induced analgesia (including analgesic aftereffect). Analgesia induced by EA varies in frequency. Our previous study showed that 60 Hz is suitable for the induction of analgesia in goats. In addition, proper prescription of specific acupoints also influences EA-induced analgesic effect. Numerous studies proved that EA at a set of Baihui, Santai, Ergen, and Sanyangluo acupoints elicited an effective analgesia in cattle and goats [13, 16, 25]. In the present study, we used EA of 60 Hz to stimulate goats at a set of acupoints above and found the analgesic effect lasted for 12 h at least, which is in accordance with Zhao's [24] results in rats. However, we also found the pain threshold did not continue to fall back toward the preacupuncture level, but gradually rose to form an apparent peak once again during the time from 6 to 8 h after EA was terminated. This interesting phenomenon has not been reported in human and small experimental animals. The possible explanation for it is species variation.

4.2. Effects of the Gene Expression of EOPs on the EA-Induced Analgesic Aftereffect. Acupuncture practitioners always try to pursue a lasting and effective analgesic aftereffect for the treatment of painful diseases because it is a major foundation for optimizing times, interval and session of acupuncture. However, the mechanisms of analgesic aftereffect underlying EA have not been completely elucidated yet. According to the phenomenon that the pain threshold gradually returns to the normal level after acupuncture discontinuation, the analgesic aftereffect should be associated with the change in some physiological substances. Numerous studies confirmed that EOPs in the CNS, mainly M-ENK, β -EP, and DYN, played an important role in EA; released EOPs induced by EA acted on their corresponding receptors and exerted immediate analgesic effect [6–9]. Our experiment showed the immunoactivities of M-ENK, a typical representative of EOPs, correlated with the pain threshold after EA was terminated, which indicated that EOPs did participate in the EA-induced analgesic aftereffect. Studies showed that EOPs caused the posttetanic potentiation on the presynaptic nerve terminals of neurons (at least in arcuate nucleus), which led to the lasting synaptic transmission between afferent and efferent pathways [26]. However, these evidences above cannot give a complete explanation for the lasting aftereffect (in some cases even over 24 h) because the released EOPs are enzymolyzed quickly after they combine with their receptors. Therefore, whether EOP gene expression is initiated to replenish the consuming EOPs in EA-induced analgesic aftereffect or not is worthy to be investigated.

Some studies were involved in gene expressions of EOP precursors after EA treatment. Cui [10] found EA-induced PENK mRNA to express increasingly in the spinal cord and medulla oblongata of rats. The study of Chen et al. [27] indicated that EA elicited POMC mRNA to increase in hypothalamus of rats. Guo et al. [12] determined the levels of PENK and PDYN mRNA at 24 h after EA in the nuclei of hypothalamus and medulla oblongata of rats and found that EA caused PENK mRNA levels to increase in SON, ARC, PVH, VMH, NRM, GI, and PDYN mRNA levels to increase in SON, PVH, VMH, and PBN. These researches

above determined EOP precursor mRNAs in a few local districts (nuclei) or at single time point, but did not exhibit their dynamic processes in all analgesia-related nuclei and areas of the CNS.

In this study, dynamic processes of mRNA levels of three EOP precursors (PENK, POMC, and PDYN) were determined in nuclei and areas which were related to analgesic regulation or EOP distribution in the CNS. The results indicated that mRNA levels of the EOP precursors gradually increased, reached the peak during the time from 4 h to 6 h, and remained higher ($P < 0.05$) at 24 h after EA was discontinued in most measured nuclei or areas. In addition, PDYN mRNA levels formed the second peak at about 12 h. Because EOP precursors are translated from their mRNA and then slipped into corresponding EOPs by specific enzymes, mRNA levels of EOP precursors do not completely represent the levels of EOPs. In our previous study, EA at its suitable frequency induced the simultaneous release of the three opioid peptides in extensive analgesia-related nuclei and areas in the CNS of goats [16]. In this study, M-ENK was chosen as a representative of EOPs to investigate the relationships between levels of EOPs and mRNA levels of EOP precursors or pain threshold in the whole experiment. M-ENK levels in the measured nuclei or areas positively correlated ($P < 0.01$) with the pain threshold. The peak of M-ENK levels (at 8 h) lagged behind that of PENK mRNA (at 6 h). The peak (at 8 h) of the pain threshold also lagged behind that (at 4–6 h) of each EOP mRNAs. These results suggested that the initiation of gene expression of the three precursors contributed to the change in pain threshold after acupuncture termination, that is, the analgesic aftereffect.

The role of DYN-A in EA-induced analgesia in the brain is controversial. Han and Xie [28] found that DYN-A did not produce EA-induced analgesia when it was microinjected into the cerebral ventricle of rats. But Zhang et al. [29] made an opposite conclusion with DYN-A microinjection in rats. Our previous study showed that EA induced DYN-A to increase in most analgesia-related nuclei in the CNS of goats [16]. In this study, there were two peaks of PDYN mRNA levels, one occurred at 4 to 6 h and another at 12 h with the former being lower than the latter. Considering the controversy about the role of DYN in the brain, the gene expression of PENK or POMC may play greater role than that of PDYN in maintaining the analgesic aftereffect. The meanings of the reincrease in PDYN mRNA after 8 h in the analgesic aftereffect need to be investigated.

Guo et al. [12] used in situ hybridization method to determine the level of POMC mRNA in ARC at 24 h after rats were stimulated by EA of 2 Hz or 100 Hz at “Zusanli” (St.36) and “Sanyinjiao” (SP.6) and found no change in POMC mRNA. However, our results showed that 60 Hz of EA induced POMC mRNA to increase significantly in goats' ARC at 24 h after EA was terminated. This difference may be caused by different frequencies, acupoints, or species. In addition, research methods may be also a contributor. Quantitative real-time PCR used in this study is believed to be more sensitive and reliable than the semiquantitative mRNA methods such as in situ hybridization, dot blot, and northern blot.

4.3. *Effects of the Gene Expression of Opioid Receptors on the EA-Induced Analgesic Aftereffect.* δ -receptor, μ -receptor, and κ -receptors are three important opioid receptors in the CNS. The affinity of ENK and β -EP with δ -receptor is almost equal to that of them with μ -receptor [30]. DYN has higher affinity with κ -receptor than with δ - or μ -receptor. There are a few reports about the regulation of opioid receptors in EA-induced analgesia. Sun and Han [9] found that EA-induced immediate analgesia is mediated by M-ENK via δ -receptor, β -EP via δ - and μ -receptor, and DYN via κ -receptor. Some studies showed that the mRNA levels of the three opioid receptors increased at the end of EA [31–33]. However, the dynamic process of opioid receptor mRNAs in EA-induced analgesic aftereffect is not clear yet. In the present study, mRNA levels of the three opioid receptors changed in a similar pattern as those of their ligands; they began to increase at 0 h, reached the peak at 4–6 h or 12 h, and remained higher at 24 h after EA was terminated. The results suggested that EA-induced upregulation of opioid receptor genes may play a role in the analgesic aftereffect. More work on this respect is worthy to be done.

5. Conclusion

The pain threshold during the time from 0 to 12 h after EA discontinuation was significantly higher than that before acupuncture, which showed that the EA-induced analgesic aftereffect lasted for at least 12 h in goats. The mRNA levels of three EOP precursors (PENK, POMC, and PDYN) and three opioid receptors (δ -, μ -, and κ -receptor) in most analgesia-related nuclei and areas began to increase at 0 h, reached peaks during the time from 4 h to 6 h or at 12 h, and remained higher at 24 h after EA was discontinued in goats. These results, along with the relationships between the dynamic processes of pain threshold and M-ENK (a representative of EOPs) level or EOP precursor mRNA levels, suggested that the initiation of gene expression of the endogenous opioid peptides and their receptors may contribute to the regulation of EA-induced analgesic aftereffect.

Conflict of Interests

None of the authors has conflict of interests with this submission. None of the authors has conflict of interests with the corporations and the software mentioned in this paper.

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Research Article

Comparison of Electroacupuncture in Restrained and Unrestrained Rat Models

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Acupuncture and electroacupuncture (EA) are widely used to treat a variety of diseases including pain. In preclinical research, EA is usually applied by inserting acupuncture needles into the hindlimbs of rats restrained in small tubes or bags. This restrained model of EA not only causes stress-like behaviors but also is limited in stimulating locations and intensities. In 2004, a novel, unrestrained model of EA was introduced. However, these two EA methods have never been directly compared regarding their analgesic effects and other features such as stress. In the present study, we reported similar analgesic effects between restrained and unrestrained EA in rats of acute inflammatory pain induced by intraplantar injection of CFA. In addition, rats receiving unrestrained EA showed less significant stress-like behaviors and tolerated higher current intensity. These advantages suggest that this unrestrained EA method can replace the traditional restrained procedure with similar analgesic effects and allow for more choices of stimulating intensities and locations.

1. Introduction

Acupuncture and electroacupuncture (EA) can effectively treat a variety of diseases such as pain and nausea. Animals, especially rodents, are now widely used in preclinical research on the neural mechanisms of EA. In the traditional EA model, conscious rodents are restrained in small tubes or bags, with acupuncture needles inserted into their hindlimbs [1, 2]. This restrained EA method had been suggested to cause stress [1], and its analgesic effects might hardly be differentiated from stress-induced analgesia (SIA) [3]. In addition, accumulating evidence suggests that different stimulating parameters, such as current intensity, frequency, and location, all significantly affect the analgesic effects of EA [2, 4–9]. The restrained EA model is limited in stimulating locations and intensities, that only acupoints on hindlimbs can be stimulated with relatively low intensities to avoid stress-like responses such

as vocalizations and muscle twitches. Finally, *in vivo* electrophysiological techniques in consciously behaving rodents are more and more widely used in neuroscience research, including pain studies [10, 11]. The presence of restraining tubes or bags restricts their application in EA research. In 2004, Lao et al. [12] introduced a novel, unrestrained method of EA stimulation. This model does not require restraining tubes or bags and allows for more choices of acupoints, for example, those on the back. However, the analgesic effects, intensity tolerance, and stress-like behaviors have never been directly compared between these two EA methods. The present study was designed to answer these questions.

2. Material and Methods

2.1. Animals. Male Sprague-Dawley rats were provided by the Department of Experimental Animal Sciences, Peking

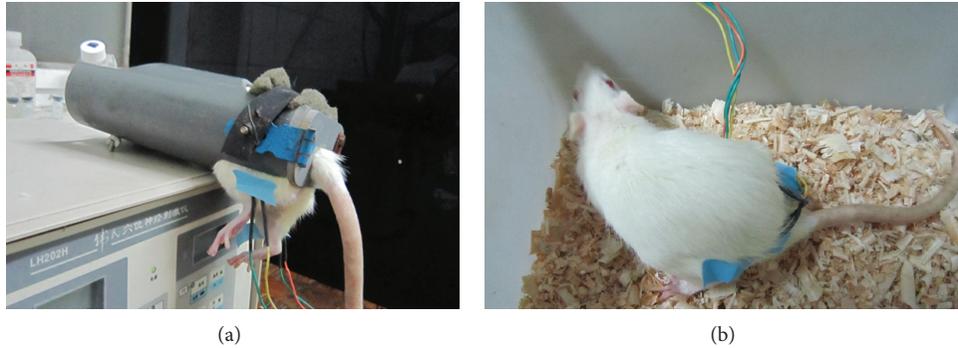


FIGURE 1: Photographs showing the restrained EA (a) and the unrestrained EA (b) procedures.

University Health Science Center. They were housed 4–6 per cage with the temperature maintained at $22 \pm 1^\circ\text{C}$ and kept under a natural light/dark cycle. Food and water were available ad libitum. Rats were handled daily for minimally three days before any experiments. During this period and regardless of their grouping, rats were also habituated to EA tubes, by 10 min daily restraint (Figure 1(a)) but without acupuncture needles), and to EA wires, by 10 min daily free exploration in small cages (Figure 1(b)) with acupuncture needles taped but not inserted onto their bodies). All animal experimental procedures were conducted in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Peking University.

2.2. The CFA Model of Inflammatory Pain, EA, and Thermal Hyperalgesia Measurement. The rat model of acute inflammatory pain was established by intraplantar injection of 0.1 mL Complete Freund's Adjuvant (CFA, Sigma, suspended in an 1:1 oil/saline emulsion, 0.1 mL, and $50 \mu\text{g}$ Mycobacterium tuberculosis) into the left paw [12]. These inflamed rats were then randomly divided into three groups. Rats in the restrained EA group ($n = 12$) and in the unrestrained EA group ($n = 12$) received EA treatment. For restrained EA, rats were restrained in plastic tubes with hindlimbs extending out through two holes (Figure 1(a)). Bilateral GB30 (Huantiao, located at the junction of the lateral 1/3 and medial 2/3 of the distance between the greater trochanter and the hiatus of the sacrum) [12] were stimulated with square waves of 0.2 ms in pulse width and 100 Hz in frequency from an Han's Acupoint Nerve Stimulator (HANS, LH series, manufactured in Peking University). Their intensities were increased in a stepwise manner at 1.0-1.5-2.0 mA, each lasting for 10 min [2]. For unrestrained EA, one investigator gently held the animal, while another swiftly inserted acupuncture needles into bilateral GB30. The needles were stabilized with adhesive tape. The procedure typically lasted less than 20 seconds and caused little distress. These rats were then released in a small cage to receive EA stimulation of the same intensities (Figure 1(b)). If the needles dropped during EA, they were inserted again, and the procedure took a few seconds. To obtain strong analgesic effects, each rat received two EA sessions, one immediately after CFA injection and one 2

hours after injection as previously described [12]. Needles were pulled out, and rats were released into their home cages between these two sessions. Rats in the control group ($n = 10$) were either restrained in tubes as in the restrained EA group ($n = 5$) or freed in a cage as in the unrestrained EA group ($n = 5$) but did not receive EA treatment. No differences of paw withdrawal latency (PWL) changes were detected between these two manipulations at any time points so they were pooled into the control group (restrained controls versus unrestrained controls: 2.5 h: -16.4 ± 3.3 versus -16.9 ± 3.7 ; 5 h: -17.2 ± 2.3 versus -17.8 ± 2.8 ; 24 h: -17.3 ± 2.7 versus -19.9 ± 4.1 , seconds, $P > 0.05$ for all time points).

The PWL was measured in a blind manner before CFA injection and 2.5 hours, 5 hours, and 24 hours after injection. The rat was placed under a clear plastic chamber on a glass surface. A high-intensity beam (IITC, Woodland Hills, CA; setting = 20%, $\approx 45\text{ W}$) was applied onto the plantar surface of the left hind paw from underneath the glass floor. The PWL was measured to the nearest 0.1 s when the rat withdrew its hind paw from the radiant heat stimulus and mean PWL was calculated by averaging the latencies of three tests with 5 min intervals between each test. With this intensity, naive rats showed an average PWL of approximately 20 seconds. Thirty seconds was used as the cutoff time to avoid plantar injuries. The PWL before CFA injection was taken as the baseline. Changes of PWL after CFA injection were calculated by subtracting the baseline PWL from the measured PWL.

2.3. Current Intensity Tolerance and Stress-Like Behaviors. We next tested the highest current intensity that could be tolerated by rats with restrained and unrestrained EA. Naive rats received stimulation in bilateral GB30 as above. The current intensity was increased from 1.0 mA at 0.5 mA steps (60 seconds per step) to the level that caused hindlimb flinches or audible vocalizations and maintained at this level. This procedure lasted 5 minutes so the maximal current intensity was 3.5 mA. The highest current intensity that did not cause any hindlimb flinches or vocalizations was defined as the current intensity tolerance. Five other parameters were measured during this 5 min period: number of hindlimb flinches, number of urination, number of audible vocalizations, duration of audible vocalizations, and number of fecal boli.

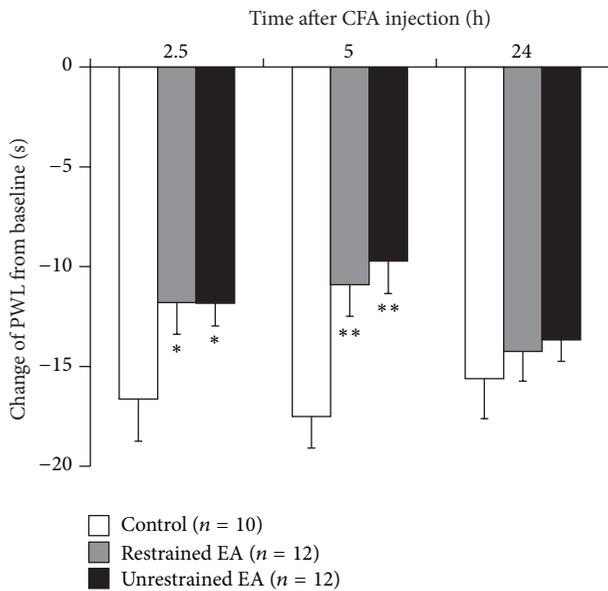


FIGURE 2: Restrained and unrestrained EA produced significant and similar analgesic effects 2.5 and 5 but not 24 hours after intraplantar CFA injection, indicated by decreased changes of PWL from preinjection baseline. * $P < 0.05$, ** $P < 0.01$ compared with the control group, ANOVA with Dunnett's post hoc tests.

3. Results

After subcutaneous CFA injection into the plantar surface of left hind paws, rats developed thermal hyperalgesia, indicated by a sharp decrease of PWL from thermal stimulation which lasted for over 24 hours (Figure 2). Restrained and unrestrained EA produced significant and similar analgesic effects 2.5 and 5 but not 24 hours after injection, indicated by smaller PWL changes from preinjection baselines (2.5 h: $F = 4.15$, both $P < 0.05$; 5 h: $F = 5.06$, both $P < 0.01$; 24 h: $F = 0.36$, both $P > 0.05$ compared with the control group, ANOVA with Dunnett's post hoc test. Figure 2). However, there were no differences between the two EA methods ($P > 0.05$, ANOVA with Dunnett's post hoc test. Figure 2).

In both human volunteers [5, 6] and anaesthetized rodents [9], there is evidence that EA of different intensities exerts analgesic effects through distinct mechanisms. However, it is hard to perform such experiments in awake animals with the restrained EA method, since rats start to show stress-like behaviors, indicated by strong hindlimb flinches, vocalization, and fecal boli, when the current intensity is increased to higher than 2 mA (Figure 3). Using the unrestrained methods, we noticed that the intensity tolerance was significantly higher ($P < 0.01$, Students t -test). Indeed, all rats receiving unrestrained EA could tolerate up to 3.5 mA currents with little indication of stress (Figure 3(a)). The number of hindlimb flinches ($P < 0.01$, Students t -test), the number of urination ($P < 0.01$, Students t -test), the number of audible vocalizations ($P < 0.01$, Students t -test), the duration of audible vocalizations ($P < 0.01$, Students t -test), and the number of fecal boli ($P < 0.05$, Students t test) were all lower in the unrestrained EA group than in the restrained EA group (Figures 3(b)–3(f)).

4. Discussion

The neurobiological mechanisms of EA analgesia are intensively studied worldwide. It is generally accepted that EA promotes the release of analgesic substances such as opioids [4]. However, more recent work indicated distinct mechanisms of EA analgesia between different stimulating frequencies [4, 7], durations [7, 8], intensities [5, 6, 9], locations [2], and subject conditions (healthy versus pathological states) [13]. Thus, various experimental designs and techniques should be applied in combination towards a comprehensive elucidation of EA mechanisms. Rodents are traditionally placed into special tubes or bags for EA stimulation [1]. This method has obvious limitations. Firstly, only a limited numbers of acupoints, mainly those in the hindlimb, could be stimulated. Acupoint specificity has raised lots of attention, not only that different acupoints show distinct analgesic effects under pathological conditions but also that the underlying mechanisms vary as well [2]. The presence of restraining bags or tubes prevents adequate animal experiments on this issue. It also restricts the application of *in vivo* electrophysiological recording in consciously behaving rodents [10, 11] in EA research, since the animal's head is usually inaccessible under such conditions. Finally and most importantly, animals sometimes show stress-like behaviors such as vocalization and hindlimb flinches when restrained in small containers. Such behaviors cause intolerance to high current intensities and confuse mechanisms of EA analgesia with those of stress-induced analgesia (SIA) [3].

In the present study, we tested a new unrestrained EA method first described by Lao et al. [12]. We found that without restraint, rats tolerated a much higher current intensity with few signs of stress-like behaviors. Our experiment in CFA-induced inflammatory pain revealed similar analgesic effects between the restrained and the unrestrained EA methods. These data suggest that the unrestrained EA method could fully replace the restrained model and allow the application of a broader range of stimulating intensities and locations as well as *in vivo* recording in conscious rodents. The presence of stress-like behaviors during restrained EA requested differentiation between EA analgesia and SIA. In clinical situations, acupuncture or EA may cause deqi sensations characterized by aching, pressure, heaviness, and numbness but not negative emotions such as sharp pain [14]. Emotional stress could be easily avoided in humans with sufficient verbal communications and habituation. In rodents, however, stress-like responses frequently occur during EA stimulation under restraint. In the present study, for example, despite the intensive handling and habituation, rats still showed stress-like behaviors such as hindlimb flinches and vocalization in the restraint tube when the current was increased to ~ 2 mA. In an early study, Wan et al. [1] showed that restraining mice in clothing holders alone was sufficient to produce mild anti-nociception, but EA stimulation showed an additional analgesic effect. Thus, restraint becomes a confounding factor in evaluating the real analgesic effects of EA and explaining its mechanisms. In our CFA experiment, we tried to eliminate stress by intensive handling and habituation

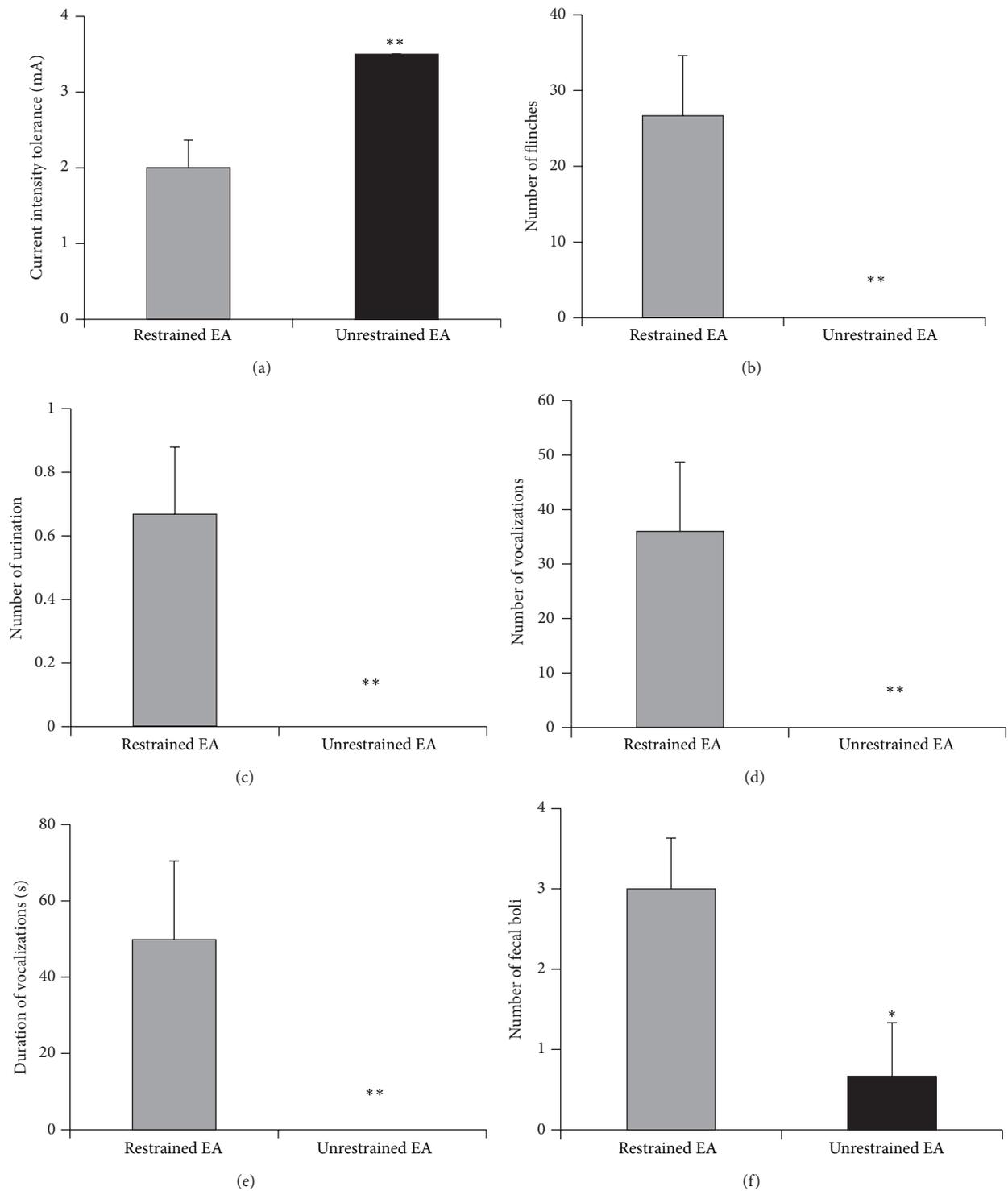


FIGURE 3: Current intensity tolerance and stress-like behaviors during restrained and unrestrained EA. (a) Rats with unrestrained EA could tolerate significantly higher current intensity than rats with restrained EA. Rats with unrestrained EA showed significantly fewer hindlimb flinches (b), number of urination (c), number of audible vocalization (d), duration of audible vocalization (e), and number of fecal boli (f) during EA procedures. * $P < 0.05$, ** $P < 0.01$, and Students t -test.

as well as by using relatively low current intensity (1-1.5-2 mA). Under this condition, little evidence of stress was observed during the procedure. Despite these manipulations, the analgesia effects in the restrained EA model may still be partially caused by SIA. A more direct way to reflect stress level is to measure blood biochemical indexes. Our previous study [13] showed that unrestrained EA increased blood corticosterone levels in rats with intraplantar CFA injection but not in naïve rats without inflammatory pain. Adrenalectomy blocked EA-produced anti-edema, but not EA anti-hyperalgesia [13]. These data suggest distinct working mechanisms of EA in healthy subjects and in those with pathologies, and that changes of hormone levels could be independent from pain behaviors. In the present preliminary study, we observed stress-like behavioral changes associated with restrained naive animals. To better elucidate how blood biochemical indexes change during EA and more importantly, how they correlate with EA induced antinociception, a more carefully designed and controlled future study is required.

5. Conclusions

Overall, rats experienced less stress during the unrestrained EA procedure and tolerated higher current intensity. The absence of restraining bags or tubes also allows for more choices of acupoints and application of electrophysiological techniques that require access to the head of conscious rats. These advantages, combined with similar analgesic effects compared with the traditional restrained EA, suggest that the unrestrained EA method can replace the restrained EA procedure in future research.

Authors' Contribution

H. Zhang and X. Chen contributed equally to this work.

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Research Article

Acupressure Improves the Weaning Indices of Tidal Volumes and Rapid Shallow Breathing Index in Stable Coma Patients Receiving Mechanical Ventilation: Randomized Controlled Trial

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Background. Acupressure has been shown to improve respiratory parameters. We investigated the effects of acupressure on weaning indices in stable coma patients receiving mechanical ventilation. **Methods.** Patients were randomly allocated to one of three treatments: standard care with adjunctive acupressure on one ($n = 32$) or two days ($n = 31$) and standard care ($n = 31$). Acupressure in the form of 10 minutes of bilateral stimulation at five acupoints was administered per treatment session. Weaning indices were collected on two days before, right after, and at 0.5 hrs, 1 hr, 1.5 hrs, 2 hrs, 2.5 hrs, 3 hrs, 3.5 hrs, and 4 hrs after the start of treatment. **Results.** There were statistically significant improvements in tidal volumes and index of rapid shallow breathing in the one-day and two-day adjunctive acupressure study arms compared to the standard care arm immediately after acupressure and persisting until 0.5, 1 hr, and 2 hrs after adjustment for covariates. **Conclusions.** In the stable ventilated coma patient, adjunctive acupressure contributes to improvements in tidal volumes and the index of rapid shallow breathing, the two indices most critical for weaning patients from mechanical ventilation. These effects tend to be immediate and likely to be sustained for 1 to 2 hours.

1. Introduction

One out of three patients in critical care units requires mechanical ventilation [1, 2]. Invasive mechanical ventilation is potentially harmful due to physical (e.g., gastrointestinal

complications [3], pain, and difficult breathing [4]) and psychological factors (e.g., fearfulness and anxiety [4]). Further, 27% of mechanically ventilated patients develop ventilator-associated pneumonia [5] resulting in excess morbidity and mortality. Every effort should be made to wean patients safely

from mechanical ventilation within as short a period of time as possible [2].

Acupressure is the massage of acupuncture points [6] performed with the fingertip or knuckle. In noncontrolled studies of patients with respiratory disease, acupressure has been associated with significant subjective improvements in cough [7] and congestion or dyspnea [8, 9] as well as reductions in depression [10] and improvements in quality of life [11], at least for short periods of time. Systematic reviews have argued that more empirical evidence about acupressure is required [12, 13], including evidence of the value of acupressure in the treatment of respiratory disease.

Since acupressure has potential benefits on respiratory symptoms, it may contribute to the success of weaning critically ill patients from mechanical ventilation and hence reduce weaning failure. The purpose of our study was to examine the potential benefits of adjunctive acupressure on objective parameters (weaning indices) of intubated critically ill patients. We hypothesized that stable coma patients with mechanical ventilation randomized to treatment arms receiving standard care and adjunctive acupressure on either one or two days would show improved weaning indices compared to patients receiving standard care only.

2. Methods

2.1. Design. This study was a prospective randomized clinical trial using a parallel-group design. A sample of medically stable, ventilated coma patients was randomly assigned on a 1:1:1 basis to one of three treatment groups: two days of standard care with adjunctive acupressure on the first day, two days of standard care with adjunctive acupressure on both days, and standard care on both days. This design enabled us to evaluate the immediacy versus delay of any treatment effects, as well as the duration of these effects above and beyond standard care. This was deemed important because a standardized regimen for delivering acupressure has not been proposed to date. Outcomes were measured before, right after, and at half hour intervals up to 4 hours following acupressure for a total of 10 daily measurements and a trial total of 20 measurements. In the standard care only group, measurements were made at the same time points with a pause inserted where acupressure was administered in the one- and two-day adjuvant treatment arms.

2.2. Participant Selection and Assignment. Participants were recruited from three medical intensive care units (ICU) and one respiratory care center (RCC) unit in a 3715-bed tertiary teaching facility in Taoyuan, Taiwan. Eligibility criteria included being 19 years of age or older, diagnosed with coma and having a Glasgow Coma Scale (GCS) score of less than 7 in the absence of sedation, and having been on mechanical ventilation at 6 to 8 cm H₂O levels of pressure support preferably but not necessarily between 7 and 21 days. We conservatively selected low levels of ventilator support because, to our knowledge, this was the first study examining the impact of acupressure on weaning indices of intubated patients in the acute stage of mechanical ventilation. Coma

patients were chosen because this permitted controlling for nonspecific effects such as positive or negative patient expectations, anxiety about pain, variability in care intensity by the nurse administering acupressure, healing rituals that reference East Asian culture, and interpersonal dynamics in the nurse-patient relationship [14].

Exclusion criteria were as follows: neurologic injury or pathology (e.g., myasthenia gravis and hemiplegia); infection (e.g., open tuberculosis, vancomycin-resistant enterococcus, or pandrug-resistant *Acinetobacter baumannii*); acute cardiac vascular dysfunction; other systemic diseases (e.g., diabetes mellitus, systemic lupus erythematosus, or AIDS); receiving sedation or neuromuscular blockade (e.g., midazolam, barbiturates, opiates, propofol, or pancuronium).

The minimum sample size was determined using the software program Power Analysis and Sample Size (PASS 11.0). In our pilot study of 15 patients, we recorded at baseline and right after acupressure treatment mean tidal volumes of 351.00 ± 104.99 and 407.60 ± 110.92 , respectively, and mean indices of rapid shallow breathing of 88.19 ± 44.01 and 70.79 ± 35.56 , respectively. Using these estimates, a sample size of 30 patients in each group would be required to detect an effect size of 0.5 with power of 0.80 at $\alpha = 0.05$. Allowing for a 10% loss rate, a total of 99 recruits (33 per group) would be needed.

One-hundred-forty patients met study criteria over a 15-month recruitment period. The next of kin of 110 subjects provided written informed consent (Figure 1). Subjects were randomized to one of the three study arms. The random allocation sequence was concealed until interventions were assigned. Ninety-four patients completed the study protocol. Sixteen patients were withdrawn from the study: consent was revoked for two patients, eight patients were weaned between randomization and protocol completion, and six patients returned to their previous ventilation mode, that is, no longer tolerating pressure support.

2.3. Rationale for the Selection of Acupoints. Acupuncture is based on the concept that an energy flow (Qi) essential for good health flows throughout the body along 12 main pathways known as meridians [15]. The patterns of this Qi are related to the organs and the tendon-muscular system. The causes of most disorders in the human body are believed to be due to an imbalance in the Qi [16]. Acupuncture and associated methods such as acupressure may correct the imbalance in the Qi at the related acupuncture points located close to the skin along the meridians. The underlying mechanism of most respiratory disorders is believed to be the result of a defective interaction between lungs and spleen and is explained in great detail (and beyond the scope of this paper) elsewhere [17].

The acupuncture points referred to in the present study (Figure 2) were taken from Zhen Jiu Da Cheng (Compendium of Acupuncture and Moxibustion, A.D. 1601), a collection of acupuncture and moxibustion papers from the Han to the Ming dynasties, and edited in 1601 by Yang, a Ming Dynasty acupuncturist [18]. The acupoints selected in this study have been reported to provide substantial relief to

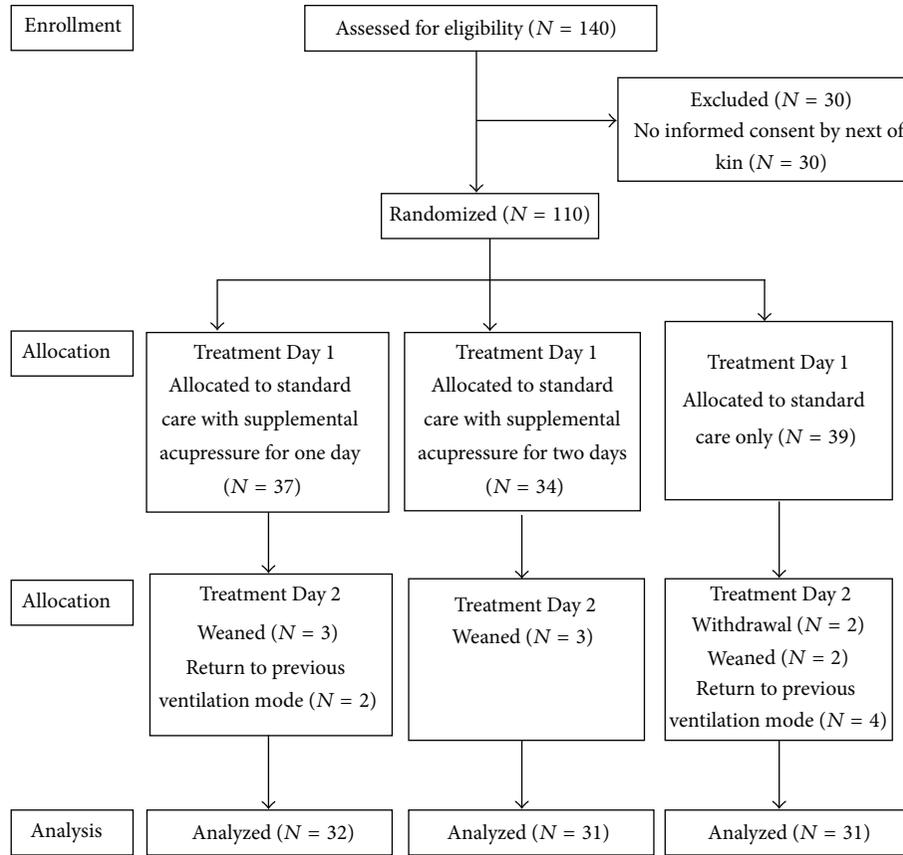


FIGURE 1: Summary data for study recruitment and completion at each time point: baseline, Day 1 and Day 2.

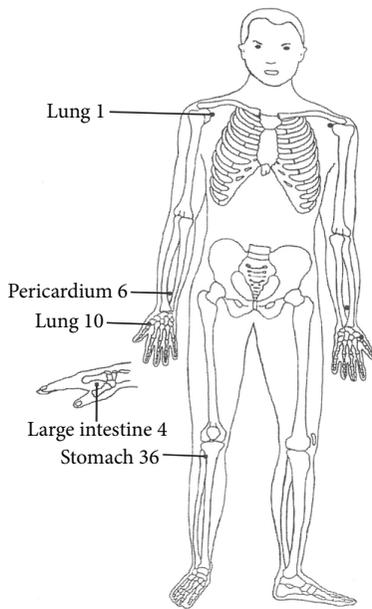


FIGURE 2: Location of acupressure points. Acupressure points Zhongfu (lung 1, LU1), Yuji (lung 10, LU10), Hegu (large intestine 4, LI4), Neiguan (pericardium 6, PC6), and Zusanli (stomach 36, ST36). Each point is located bilaterally.

patients suffering from dyspnea and to enhance the function of the immune system [16]. The principles of treatment of this study are to relieve any disharmonies of the lung involving the Zhongfu (LU1), Yuji (LU10), Hegu (LI4), and the Neiguan (PC6) and to strengthen the spleen (Neiguan (PC6) and the Zusanli (ST36)).

According to Stux and Pomeranz [16], the Zhongfu (LU1) is the trigger point (front Mu point) of the lungs as well as the meeting point (Hui Shu point) of lungs and spleen. Manipulating this point oxygenates the lungs, provides relief of symptoms due to asthma, improves the function of the spleen, and relieves both shoulder and back pain. The second point, the Yuji (LU10), is the slow-flowing Ying point and tends to accelerate the flow along the lung meridian. Manipulation of this point relieves pain, cough, and symptoms of asthma. The third point, the Hegu (LI4), is the source point (Yuan point) and has the energy of the combined channel, since the transverse Luo vessel from the Luo point of the combined channel ends at this point. When this point is manipulated, it relieves headache, dizziness, fever, chills, and coughing. The fourth point, the Neiguan (PC6), is the connecting (Luo) point of the pericardium channel to the Yangchi (TE4). It is the point of confluence of the additional channel Yinwei. By manipulating this point the lungs are warmed, sputum is reduced, and the function of the spleen is improved.

And finally, the uniting point (He point) is located at the Zusanli (ST36), providing an internal equilibrium for the stomach meridian. The manipulation of this point improves the functioning of the spleen and warms the spleen's yang because it is so closely related to the stomach [16].

2.4. Treatment Protocols

2.4.1. Standard Care. Throughout the trial all subjects received their prescribed medication and chest physiotherapy, including chest percussion, positioning, and suction.

2.4.2. Adjunctive Acupressure. Acupressure was administered at the five acupoints referenced above. To make sure that appropriate and standard methods were used, acupressure was administered by one of the coinvestigators (C.H.W.), a nurse who had completed three courses of Traditional Chinese Nursing recommended by the Committee of Chinese Medicine and Pharmacy, Department of Health, Executive Yuan (Acupuncture and Moxibustion in Nursing, Traditional Chinese Traumatology in Nursing, and Traditional Chinese Nursing). She also trained clinically in acupressure technique (including intensity, duration, location, and stimulation of the acupuncture points) in the Department of Acupuncture and Moxibustion, Chang Gung Memorial Hospital.

We identified the acupoints by bone standard, which is the length of equally divided portions of a certain long bone or the distance between two anatomical landmarks, taken as one cun, as the unit of measurement for locating acupoints. The stimulation of the five acupoints was done once daily, first to one side of the body and then on the other side, in the order preferred by the patient, by using the fingers to apply gentle but firm pressure [19]. The amount of pressure applied was dependent upon the acupoints location, the skin thickness, and the adipose tissue in that location. The finger was moved in small circles, or in a back and forth motion, but remained located over the same point on the skin, for up to two minutes. Thus acupressure was performed at five acupuncture points for a period of up to 10 minutes per treatment session [7]. Acupressure was performed at the same time, either morning or afternoon, for each participant. If there were two participants recruited at one day, we arranged them to receive acupressure at different times. We did not recruit more than two participants in any one day.

2.5. Outcome Measures. Eight outcome measurements (heart rate, respiratory rate, mean arterial pressure, peripheral oxygen saturation [SpO_2], tidal volumes [V_T], minute ventilation [V_E], dynamic lung compliance [C_{dyn}], and rapid shallow breathing index the ratio of the frequency of breathing (f) divided by the tidal volume (V_T)) were recorded before, right after, and at 0.5 hrs, 1 hr, 1.5 hrs, 2 hrs, 2.5 hrs, 3 hrs, 3.5 hrs, and 4 hrs after the start of treatment, for a total of 10 recordings per day. The rationale for ten daily measurements was that acupressure may be short-acting, hence the frequency of measures at relatively short intervals, and the goal to capture the immediate, lasting, and delayed effects, if any, of acupressure [20].

Heart and respiratory rate, mean arterial pressure, and arterial oxygen saturation scores were obtained from an adequately calibrated and checked electrocardiographic monitor (Hewlett-Packard M1165A Model 56S, Andover, MA, USA). Tidal volume, minute ventilation, and dynamic lung compliance scores were obtained from the median of the five scores from the display on the ventilator, which also was adequately calibrated and checked.

2.6. Statistical Analysis. Multivariate analyses were performed through the use of generalized estimating equations (GEE) of multiple linear regressions with autoregressive I(AR(1)) correlation [21]. This method takes into account the correlated nature of the data due to repeated measurements after adjusting for the effects of time-dependent and/or time-independent covariates. On the first day a marginal model was used for each dependent variable to establish the population average values of that outcome variable across the ten daily time points after adjusting for the effects of treatment, time, setting, body mass index (BMI), GCS, and baseline values. A similar model was fitted for each dependent variable on the second day. The final model included six covariates: treatment (one-day adjunctive acupressure, two-day adjunctive acupressure, and standard care), time point over two days, corresponding pretreatment (baseline) measurement, setting where patients were treated, BMI, and GCS.

Data were analyzed with SAS v 9.3 (SAS Institute Inc., Cary, NC, USA). The post hoc power analysis was done by using the software program G*Power 3.1.3 [22, 23]. Statistical significance was set at 0.05.

There were no statistically significant differences in baseline characteristics, clinical status variables, and outcome measures between those patients who were withdrawn from the study and those who completed the study protocol. Therefore only the data for those subjects completing the study were analyzed.

2.7. Ethical Considerations. The study was approved by the Institutional Ethics Committee of Chang Gung Memorial Hospital. The study was conducted in accordance with the Declaration of Helsinki as subsequently amended. Subjects were enrolled after written informed consent was provided by next of kin.

3. Results

3.1. Participant Characteristics and Baseline Values. The baseline characteristics of the 94 participants who completed the protocol are shown in Table 1. Mean \pm SD age was 73.3 ± 14.3 years. Forty-eight patients were male compared to 46 who were female. Thirty patients were smokers. There were no statistically significant differences between the three groups except that there were, proportionately, more heavy smokers in the one-day acupressure arm and more female subjects in the two-day acupressure arm. Hence we added smoking and gender as confounding variables in the original GEE model. As Table 2 shows, there were no statistically significant

TABLE 1: Baseline characteristics of participants.

Characteristic	One-day acupuncture (<i>n</i> = 32)	Two-day acupuncture (<i>n</i> = 31)	Standard care (<i>n</i> = 31)	<i>P</i>
	M ± SD	M ± SD	M ± SD	
BMI	22.31 ± 4.78	23.95 ± 5.08	22.83 ± 5.11	0.56*
GCS	4.59 ± 1.50	4.23 ± 1.82	4.81 ± 1.80	0.46*
APACHE II	23.68 ± 8.7 [‡]	25.29 ± 7.58 [§]	25.42 ± 8.83	0.73*
	<i>n</i> (%)	<i>n</i> (%)	<i>n</i> (%)	
Cigarette pack years				0.38 [†]
0	19 (59.4)	24 (77.4)	21 (67.7)	
1–19	2 (6.3)	1 (3.2)	4 (12.9)	
20 or over	11 (34.4)	6 (19.4)	6 (19.4)	
Length of ventilation prior to enrollment, <i>d</i>				0.19 [†]
≤7	8 (25.0)	12 (38.7)	6 (19.4)	
8 to 13	11 (34.4)	13 (41.9)	10 (32.3)	
14 to 20	8 (25.0)	5 (16.1)	7 (22.6)	
≥21	5 (15.6)	1 (3.2)	8 (25.8)	
Sex, male	20 (62.5)	10 (32.3)	18 (58.1)	0.04 [†]
Age, yr, ≥65	28 (87.5)	26 (83.9)	21 (67.7)	0.13 [†]
Hypertension history, present	21 (65.6)	17 (54.8)	18 (58.1)	0.68 [†]
CVA history, present	14 (43.8)	8 (25.8)	6 (19.4)	0.09 [†]
Setting				0.16 [†]
MICU-1	3 (9.4)	5 (16.1)	8 (25.8)	
MICU-3	6 (18.8)	11 (35.5)	7 (22.6)	
MICU-5	20 (62.5)	14 (45.2)	11 (35.5)	
RCC	3 (9.4)	1 (3.2)	5 (16.1)	
Intubation				0.27 [†]
Endotracheal tube	27 (84.4)	21 (67.7)	25 (80.6)	
Tracheostomy	5 (15.6)	10 (32.3)	6 (19.4)	

Data are presented as mean ± SD or number (%) unless otherwise indicated; *Kruskal Wallis tests; [†] χ^2 and Fisher's exact test; BMI: body mass index; GCS: Glasgow Coma Scale; APACHE: acute physiology and chronic health evaluation. [‡]*n* = 25; [§]*n* = 21; ^{||}*n* = 24.

TABLE 2: Comparison of baseline measurements among treatment arms.

Variable	One-day acupuncture (<i>n</i> = 32)	Two-day acupuncture (<i>n</i> = 31)	Standard care (<i>n</i> = 31)	<i>P</i> *
HR, min	91.72 ± 18.10	93.67 ± 18.49	91.58 ± 6.50	0.79
RR, min	21.97 ± 5.39	23.32 ± 7.10	20.74 ± 5.41	0.54
MBP, mm Hg	85.03 ± 15.34	81.81 ± 13.67	83.94 ± 15.44	0.76
SpO ₂ , %	96.53 ± 2.21	96.81 ± 2.57	96.45 ± 2.41	0.60
V _T , mL	390.00 ± 125.70	381.26 ± 114.17	400.10 ± 105.86	0.83
V _E , L/min	8.06 ± 2.34	8.57 ± 3.34	7.83 ± 2.35	0.69
Cdyn, mL/cm H ₂ O	34.14 ± 15.88	32.93 ± 13.70	37.81 ± 13.56	0.26
<i>f</i> /V _T , breaths/min/L	64.74 ± 32.43	69.54 ± 38.10	55.90 ± 23.51	0.40

Data are presented as mean ± SD. *Kruskal Wallis tests; HR: heart rate; RR: respiratory rate; MBP: mean arterial blood pressure; SpO₂: peripheral oxygen saturation; V_T: tidal volume; V_E: minute ventilation; Cdyn: dynamic lung compliance; *f*/V_T: rapid shallow breathing index.

differences between the three treatment arms on the outcome variables of interest at baseline.

3.2. Outcomes

3.2.1. Tidal Volumes. Tidal volumes (Mean ± SD) observed in the three treatment arms across the 20 time points are

presented in Table 3. The 95% confidence intervals (95% CIs) around the means are depicted in Figure 3. The overall mean tidal volumes from baseline to each time point improved after acupuncture treatment in both the one-day and two-day acupuncture groups on the first day, only in the two-day acupuncture group on second day, but not in standard

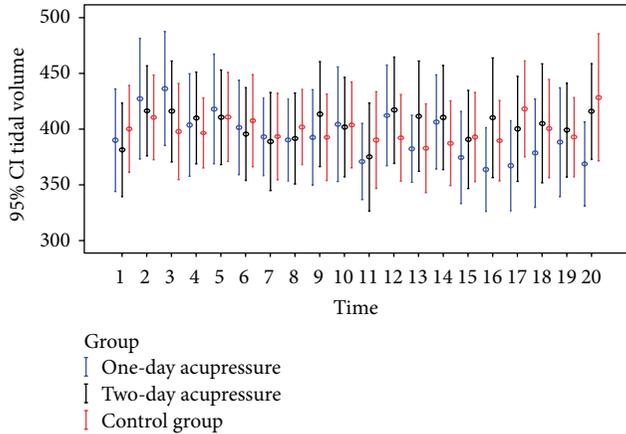


FIGURE 3: Mean and 95% CI of tidal volume measurement for each treatment arm across the twenty time points.

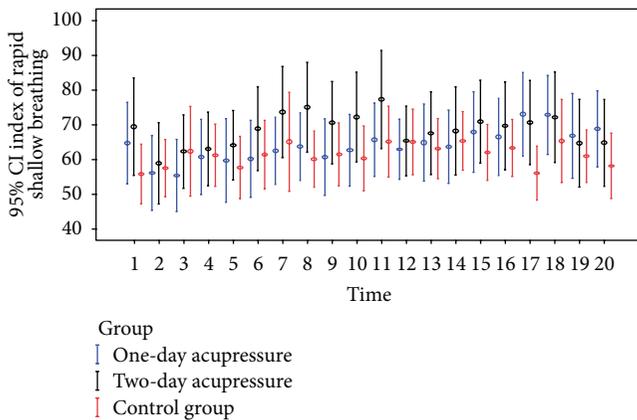


FIGURE 4: Mean and 95% CI of rapid shallow breathing index for each treatment arm across the twenty time points.

care group. The improvement in tidal volumes following the administration of acupressure was maintained for up to four hours in both acupressure arms on the first day and in the two-day acupressure arm on the second day. No relevant changes were observed in the means of the standard care group.

Statistically significant multivariate analysis results obtained using GEE with AR(1) correlation and controlling for effects of treatment, time, setting, BMI, GCS, and baseline values are reported in Table 5, including estimates of linear regression coefficients, standard errors (SE), and z test values and their associated significance levels P . On the first day, compared to subjects in the standard care group, change in tidal volumes from baseline was higher by 48.63 mL ($P = 0.036$) at 0.5 hrs for participants receiving one-day adjunctive acupressure. For those in the two-day adjunctive acupressure arm, changes were higher by 32.32 mL ($P = 0.028$) at 1 hr and 39.71 mL ($P = 0.041$) at 3.5 hrs. On the second day, for those in the two-day acupressure arm, changes were higher by 40.10 mL ($P = 0.008$) right after treatment, by 43.35 mL ($P = 0.014$) at 0.5 hrs, and by

TABLE 3: Mean \pm SD of tidal volume (mL) measurement.

Test time	One-day acupressure (N = 32)	Two-day acupressure (N = 31)	Standard care (N = 31)
0.0 hrs ^a	390.00 \pm 125.70	381.25 \pm 114.17	400.10 \pm 105.86
0.17 hrs ^b	427.21 \pm 148.91	416.29 \pm 109.62	410.39 \pm 103.41
0.5 hrs	436.38 \pm 141.72	416.00 \pm 122.83	397.84 \pm 117.49
1.0 hr	403.34 \pm 127.57	409.97 \pm 112.23	396.48 \pm 85.42
1.5 hrs	417.97 \pm 135.84	410.77 \pm 115.16	410.87 \pm 108.78
2.0 hrs	401.34 \pm 116.92	395.61 \pm 113.33	407.58 \pm 112.27
2.5 hrs	393.06 \pm 96.50	389.06 \pm 119.77	393.38 \pm 105.59
3.0 hrs	390.31 \pm 101.53	391.65 \pm 110.66	401.74 \pm 91.48
3.5 hrs	392.53 \pm 118.56	413.48 \pm 128.07	392.61 \pm 105.75
4.0 hrs	404.28 \pm 142.79	401.90 \pm 121.61	403.81 \pm 104.96
24.0 hrs ^c	370.97 \pm 94.65	375.45 \pm 131.71	390.10 \pm 118.32
24.17 hrs ^d	412.00 \pm 124.19	417.03 \pm 129.46	391.90 \pm 105.09
24.5 hrs	382.50 \pm 82.74	411.26 \pm 134.14	382.87 \pm 108.64
25.0 hrs	406.34 \pm 117.17	410.55 \pm 127.23	387.26 \pm 103.14
25.5 hrs	374.50 \pm 114.10	389.68 \pm 120.77	393.00 \pm 108.86
26.0 hrs	363.90 \pm 104.72	410.32 \pm 146.53	389.65 \pm 98.29
26.5 hrs	367.16 \pm 11.75	400.00 \pm 128.55	418.23 \pm 116.85
27.0 hrs	378.47 \pm 134.52	404.81 \pm 145.53	400.55 \pm 119.9
27.5 hrs	388.25 \pm 134.68	399.13 \pm 114.63	392.90 \pm 96.96
28.0 hrs	369.03 \pm 104.09	415.45 \pm 116.38	428.48 \pm 155.43

^aStart of treatment on Day 1 (study baseline); ^bend of treatment on Day 1; ^cstart of treatment on Day 2, if any; also, elapsed time to first Day 2 measurement since study baseline; ^dend of treatment on Day 2, if any.

38.26 mL ($P = 0.048$) at 1 hr after treatment. There were no statistically significant differences in changes in tidal volumes between the two acupressure groups on either day and across time points (all $P = ns$).

3.2.2. Index of Rapid Shallow Breathing. Table 4 lists the mean \pm SD values on rapid shallow breathing index across treatment arms and time points, and Figure 4 presents the associated 95% CIs. On the first day, mean scores on rapid shallow breathing index scores decreased after adjunctive acupressure treatment for up to 4 hours in the one-day and for up to 2 hours in the two-day acupressure arms. On the second day, the mean declined for up to four hours in the two-day acupressure group only. No relevant changes were observed in the means of the standard care group.

Multivariate analysis examined changes from baseline in rapid shallow breathing index in subjects in the two adjunctive acupressure arms compared to those in the standard care group after adjustment for covariates (Table 5). On the first day, change from baseline to right after treatment was lower by -10.28 breaths/min/L ($P = 0.001$) in the one-day acupressure arm and by -12.28 breaths/min/L ($P = 0.003$) in the two-day acupressure arm. At 0.5 hrs decreases in the index were -15.77 ($P = 0.009$) and -13.72 breaths/min/L ($P = 0.029$) in the one-day and two-day acupressure groups, respectively. At 1 hr, values were lower by -9.41 ($P = 0.030$)

TABLE 4: Mean \pm SD of rapid shallow breathing index (breaths/min/L) measurement.

Test time	One-day acupressure (N = 32)	Two-day acupressure (N = 31)	Standard care (N = 31)
0.0 hrs ^a	64.74 \pm 32.43	69.54 \pm 38.10	55.90 \pm 23.51
0.17 hrs ^b	56.19 \pm 29.77	58.98 \pm 31.84	57.61 \pm 22.47
0.5 hrs	55.51 \pm 28.59	62.35 \pm 28.72	62.43 \pm 35.07
1.0 hr	60.75 \pm 30.11	63.11 \pm 28.82	61.32 \pm 24.44
1.5 hrs	59.82 \pm 33.34	64.15 \pm 27.23	57.73 \pm 24.28
2.0 hrs	60.28 \pm 30.53	68.91 \pm 32.85	61.48 \pm 26.76
2.5 hrs	62.56 \pm 26.85	73.68 \pm 35.67	65.17 \pm 38.65
3.0 hrs	63.74 \pm 26.86	75.06 \pm 35.17	60.22 \pm 22.05
3.5 hrs	60.80 \pm 30.34	70.65 \pm 32.24	61.52 \pm 24.80
4.0 hrs	62.78 \pm 28.24	72.28 \pm 35.09	60.41 \pm 25.41
24.0 hrs ^c	65.74 \pm 29.37	77.26 \pm 38.38	65.14 \pm 27.81
24.17 hrs ^d	63.02 \pm 23.86	65.32 \pm 27.14	65.13 \pm 25.70
24.5 hrs	64.96 \pm 30.71	67.61 \pm 32.36	63.20 \pm 23.50
25.0 hrs	63.69 \pm 29.14	68.21 \pm 34.50	65.39 \pm 22.76
25.5 hrs	68.03 \pm 32.10	70.93 \pm 32.45	62.05 \pm 21.60
26.0 hrs	66.50 \pm 30.77	69.71 \pm 34.35	63.31 \pm 22.26
26.5 hrs	70.96 \pm 27.61	70.72 \pm 32.95	56.16 \pm 21.00
27.0 hrs	72.88 \pm 31.30	72.23 \pm 35.58	65.35 \pm 32.64
27.5 hrs	66.80 \pm 33.66	64.73 \pm 34.51	60.98 \pm 20.75
28.0 hrs	68.87 \pm 30.21	64.84 \pm 33.93	58.24 \pm 25.60

^aStart of treatment on Day 1 (study baseline); ^bend of treatment on Day 1; ^cstart of treatment on Day 2, if any; also, elapsed time to first Day 2 measurement since study baseline; ^dend of treatment on Day 1, if any.

and -11.85 breaths/min/L ($P = 0.020$) in the two respective acupressure groups. The one-day acupressure group also showed a decline by -10.03 breaths/min/L ($P = 0.039$) at 2 hrs. On the second day, subjects in the two-day acupressure arm showed a change of -11.92 breaths/min/L ($P = 0.001$) right after treatment when compared to the standard care group. There were no statistically significant differences in changes in rapid shallow breathing index between the two acupressure groups on either day and across time points.

3.2.3. Other Measures. Scores on the other outcome measures did not show statistically significant changes across the three treatment groups and across time points (data not reported).

3.2.4. Post Hoc Power Analysis. Using the mean \pm SD values for tidal volumes (Table 3) and indices of rapid shallow breathing (Table 4), we calculated the corresponding power to ascertain that we had sufficient statistical power. The power estimates at $\alpha = 0.05$ were 0.86 and 0.97, respectively, for the two parameters of interest.

4. Discussion

The principal findings of this prospective randomized control trial of the effects of acupressure on weaning indices

are two-fold. First, adjunctive acupressure was observed to significantly improve the weaning indices of tidal volumes and rapid shallow breathing index above and beyond the effects of standard care. Second, these effects tended to be immediate and without delayed effect, likely to be sustained for 1 to 2 hours with nominal differentials beyond, and attributable to the acupressure stimulation itself rather than the one- or two-day regimen. Together, these findings suggest that adjunctive acupressure improves the critical weaning indices of tidal volumes and rapid shallow breathing index for a significant but bounded time after treatment. Future randomized controlled trials need to examine whether bilateral stimulation of the Zhongfu (LU1), Yuji (LU10), Hegu (LI4), Neiguan (PC6), and Zusanli (ST36) acupoints, administered for 10 minutes every 1 to 2 hours over sustained periods of time, independently or integrated into weaning protocols, results in accelerated weaning from and decreased rates of relapse to mechanical ventilation.

The differential effect of acupressure on tidal volumes and rapid shallow breathing index but not on six other outcome measures may be attributed to the fact that subjects were clinically stable patients in whom variation in these additional outcome measures is less likely to occur. Further, tidal volumes and rapid shallow breathing index are widely quoted as critical physiological weaning indices [24, 25]. The increased tidal volumes generate adequate transpulmonary pressure gradients to overcome alveolar atelectasis [26, 27]. Decreasing patients' rapid shallow breathing index improves gas exchange and therefore blood gases and blood pH [24].

Although a statistically significant change in tidal volume and rapid shallow breathing index scores was detected in patients receiving acupressure, only those improvements immediately after acupressure exposure as well as 0.5 hr, 1 hr, and 2 hrs after the start of acupressure treatment should be considered clinically significant differences. Additional studies are needed to test the efficacy of different acupressure dosing and timing regimens and to examine the inclusion or exclusion of other acupoints to so optimize acupressure protocols to assist in weaning from mechanical ventilation.

A mechanism of action for the effect of acupressure on tidal volumes and rapid shallow breathing index cannot be proposed at this time. However, a role of hypothalamic and pituitary activation can be hypothesized. Acupuncture (and acupressure by extension) activates myelinated neural fibers that stimulate, among others, the hypothalamus and pituitary gland [28]. This activation releases β -endorphins from the hypothalamus into the spinal fluid and the brain and from the pituitary into the blood stream. First, the analgesic effect of β -endorphins in general may in itself facilitate respiratory function in patients, improve the effectiveness of breathing movements, and translate into greater tidal volumes. Second and more specifically, the tissue- and muscle-relaxant effect of β -endorphins may reduce shallow breathing, enable deeper breathing movements, and thus result in greater tidal volumes. Both may explain why most respiratory patients feel calm, warm, and relaxed during and after acupressure treatments [7, 11], and why most patients with COPD report relief from dyspnea following acupressure

TABLE 5: Response to treatment over time: covariates effect estimates on tidal volumes and rapid shallow breathing index and standard errors obtained from GEE.

	Estimate	Standard error	z value	P value
Tidal volumes (mL) at Day 1				
One-day acupressure × 0.5 hrs	48.63	23.20	2.10	0.036*
Two-day acupressure × 1.0 hrs	32.32	14.68	2.20	0.028*
Two-day acupressure × 3.5 hrs	39.71	19.45	2.04	0.041*
Tidal volumes (mL) at Day 2				
Two-day acupressure × 24.17 hrs	40.10	15.16	2.64	0.008*
Two-day acupressure × 24.5 hrs	43.35	17.63	2.46	0.014*
Two-day acupressure × 25.0 hrs	38.26	19.31	1.98	0.048*
The rapid shallow breathing index (breaths/min/L) at Day 1				
One-day acupressure × right after	-10.28	3.20	-3.21	0.001*
Two-day acupressure × right after	-12.28	4.07	-3.01	0.003*
One-day acupressure × 0.5 hrs	-15.77	6.07	-2.60	0.009*
Two-day acupressure × 0.5 hrs	-13.72	6.27	-2.19	0.029*
One-day acupressure × 1.0 hrs	-9.41	4.33	-2.18	0.030*
Two-day acupressure × 1.0 hrs	-11.85	5.09	-2.33	0.020*
One-day acupressure × 2.0 hrs	-10.03	4.86	-2.06	0.039*
The rapid shallow breathing index (breaths/min/L) at Day 2				
Two-day acupressure × 24.17 hrs	-11.92	3.66	-3.26	0.001*

For treatment, the category “control group” was taken as the reference, and either one-day acupressure or two-day acupressure was used as a representative variable for each of the two groups; for time, the category “baseline” at Day 1 was taken as the reference, right after, 0.5 hrs, 1 hr, 1.5 hrs, 2 hrs, 2.5 hrs, 3 hrs, 3.5 hrs, and 4 hrs were representative variables, the category “24 hrs” at Day 2 was taken as the reference, and 24.17 hrs, 24.5 hrs, 25 hrs, 25.5 hrs, 26 hrs, 26.5 hrs, 27 hrs, 27.5 hrs, and 28 hrs were representative variables. For setting, the category “RCC” was taken as the reference. In addition to treatment, time, setting, BMI, GCS, smoking amount, gender, and baseline values were forced into the model to perform GEE. * $P < 0.05$ compared to controls for the treatment × time interaction.

[8–10]. Third, deeper breathing movements are associated with increases in plasma β -endorphins though it remains unclear whether deeper breathing stimulates β -endorphin release or vice versa. In sum, the effect of acupuncture and acupressure on respiratory function may be directly due to the moderating role of these neuropeptides on respiratory function. The effect may also be indirect due to the analgesic and sedative effect of β -endorphins, which may facilitate patient breathing.

The use of a randomized controlled design strengthened the internal validity of the study’s findings. Being limited to the three intensive care units and one respiratory care center unit of one large medical center limits the external validity and future studies should be multicenter to diffuse any potential class effects for site. Future studies should also examine the effect of acupressure on successful weaning (and relapse as applicable), length of time on mechanical ventilation, length of stay in the critical care unit, and length of hospitalization. Such analyses should be done in general and as a function of the covariates considered in this present study as well as markers of respiratory function, complications of mechanical ventilation, severity of illness, and relevant comorbidities. In addition, about 20–30% of patients do not respond to acupressure regardless of disease condition [29]. Therefore, in order

to optimize the efficacy signal, future studies may want to screen patients for prior exposure to acupressure and whether they failed to respond [29]. As noted, optimal acupressure dosing for weaning from mechanical ventilation needs to be established more firmly along with further specification of protocols in terms of clinical administration, intervals between treatments, and overall length of treatment. According to Chinese tradition acupressure should be given repeatedly for sustained improvements [30] but specific recommendations are lacking at this time. Future studies should also broaden the number of respiratory and weaning indices measured and extend beyond stable coma patients. Lastly, future studies should consider including a control group receiving an adjunctive sham treatment.

5. Conclusion

To the best of our knowledge, this trial was the first to investigate the potential benefits of adjunctive acupressure on weaning indices in a group of stable, mechanically ventilated coma patients. The study provides evidence that adjunctive acupressure may improve tidal volume and rapid shallow breathing index scores—the two indices considered most critical to weaning patients from mechanical ventilation.

Conflict of Interests

No conflicts are declared, including for software and other products mentioned in this paper.

Authors' Contribution

S.-H. Maa and K.-H. Hsu made the study design, S.-H. Maa, C.-H. Wang, K.-H. Hsu, H.-C. Lin, B. Yee, K. MacDonald, and I. Abraham performed the data collection and analysis, and S.-H. Maa, C.-H. Wang, K.-H. Hsu, H.-C. Lin, B. Yee, K. MacDonald, and I. Abraham prepared the paper.

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Research Article

Electroacupuncture Reduces Cocaine-Induced Seizures and Mortality in Mice

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The aims of this study were to characterize the protective profile of electroacupuncture (EA) on cocaine-induced seizures and mortality in mice. Mice were treated with EA (2 Hz, 50 Hz, and 100 Hz), or they underwent needle insertion without anesthesia at the Dazhui (GV14) and Baihui (GV20) acupoints before cocaine administration. EA at 50 Hz applied to GV14 and GV20 significantly reduced the seizure severity induced by a single dose of cocaine (75 mg/kg; i.p.). Furthermore, needle insertion into GV14 and GV20 and EA at 2 Hz and 50 Hz at both acupoints significantly reduced the mortality rate induced by a single lethal dose of cocaine (125 mg/kg; i.p.). In the sham control group, EA at 50 Hz applied to bilateral Tianzong (SI11) acupoints had no protective effects against cocaine. In addition, EA at 50 Hz applied to GV14 and GV20 failed to reduce the incidence of seizures and mortality induced by the local anesthetic procaine. In an immunohistochemistry study, EA (50 Hz) pretreatment at GV14 and GV20 decreased cocaine (75 mg/kg; i.p.)-induced c-Fos expression in the paraventricular thalamus. While the dopamine D₃ receptor antagonist, SB-277011-A (30 mg/kg; s.c.), did not by itself affect cocaine-induced seizure severity, it prevented the effects of EA on cocaine-induced seizures. These results suggest that EA alleviates cocaine-induced seizures and mortality and that the dopamine D₃ receptor is involved, at least in part, in the anticonvulsant effects of EA in mice.

1. Introduction

1.1. Cocaine-Induced Seizures and Death. Cocaine is a widely abused psychomotor stimulant [1, 2]. In addition to its addiction liability, the abuse of cocaine is associated with an array of medical complications [3]. Generalized tonic-clonic seizures and status epilepticus are well-documented neurologic sequelae of cocaine abuse [3, 4]. Seizures may be induced by cocaine after an accidental overdose or after recreational use of relatively low doses of cocaine [5, 6]. The incidence of seizures in cocaine users has been reported to be 2–10% [7]. High doses of cocaine have been associated with a disturbingly high number of sudden deaths in adults; a recent study documented that 3.1% of all sudden deaths in Southwest Spain were related to the use of cocaine [8].

Seizures are considered to be a major determinant of cocaine-related lethality in humans [4] and animals [9]. Sudden death in cocaine abuse may also be attributed to cardiac arrhythmia and intracerebral hemorrhage [10, 11]. Currently, no specific treatment modalities offer protection against cocaine-induced seizures and death in humans.

1.2. Acupuncture. Acupuncture has been used in traditional Chinese medicine for over 2,500 years [12]. The practice has recently gained popularity and is becoming an alternative and/or complementary treatment modality for a variety of disorders worldwide [13]. In 1997, the US National Institutes of Health stated that acupuncture is a useful method for treating many conditions and has fewer side effects as

compared with other contemporary medical treatments, such as surgery or drugs [13]. Two different strategies are used in acupuncture therapy: manual acupuncture (MA) and electroacupuncture (EA). EA is a modified form of traditional MA. The advantage of EA is in its combined therapeutic effects of transcutaneous electric nerve stimulation (TENS) and MA. Most studies use EA because the latter can be standardized by frequency, voltage, waveform, length, and so forth [14]. Studies on animals and humans have demonstrated that acupuncture results in multiple biological responses [15, 16]. The best characterized mechanism is via the endogenous opioid peptides and their receptors [17].

1.3. Treating Epilepsy with Acupuncture. Acupuncture has been used to treat epilepsy in China for thousands of years. Many reports have suggested that acupuncture, TENS and other alternative therapies may produce positive effects in epilepsy [19], although some studies disagree [20]. The major advantage of acupuncture is the absence of side effects. In the clinic, acupuncture at specific acupoints such as the Zusanli (ST36), Dazhui (GV14), and Baihui (GV20) has significantly ameliorated symptoms of epilepsy [21–23]. Although some evidence supports this contention [24–26], the precise mechanism remains unknown. It is noted that acupuncture to GV20 point has been used to treat loss of consciousness and tinnitus, in addition to relief of mental abstraction, sluggish speech, and hysteria [27]. Recent studies have shown that EA inhibits seizures in experimental rat models. These protective effects may be related to increased concentrations of inhibitory amino acids [28], decreased levels of nitric oxide in the central nervous system (CNS) [26, 29], or increased cellular glutamic acid decarboxylase-67 mRNA expression, thereby increasing the production of γ -aminobutyric acid (GABA) [30]. The possibility that EA protects against cocaine-induced seizures and death has not yet been explored.

1.4. Acupuncture and Abused Drugs. Acupuncture has been widely used throughout Asian countries for treating many functional disorders, such as substance abuse and mental illness [31]. Some preclinical data have shown that acupuncture can modify the morphine withdrawal syndrome and suppress alcohol drinking behavior in rats [32, 33]. Further evidence suggests that acupuncture at a specific acupoint (Shenmen; HT7) attenuates the ethanol-induced dopamine release in the nucleus accumbens through the GABA_B receptor [34] and suppresses *c-Fos* expression in the nucleus accumbens and ventromedial striatum following a nicotine challenge in rats sensitized to nicotine [35].

1.5. *c-Fos* Expression. Expression of *c-fos* gene or Fos protein is widely used as a marker to identify neuronal pathways involved in the integration of noxious inputs [36–40]. High doses of cocaine have been reported to induce *c-fos* mRNA expression in many brain regions [41].

In this study, EA experiments were conducted to (1) establish the applicability of EA to cocaine-induced seizure and

mortality in a mouse model, (2) determine the frequency-dependent antiseizure activity of EA, and (3) correlate the efficacy of EA with the expression of Fos protein in the brain.

2. Methods

2.1. Laboratory Animals. Male ICR mice (28–35 g; BioLasco Taiwan Co., Ltd., Taiwan) were housed under a 12:12 h light/dark cycle with food and water available *ad libitum* in our animal facility for at least 4 days prior to the experiments, which were conducted between 10:00 and 17:00 h. The experimental procedures were approved by the China Medical University Institutional Animal Care and Use Committee, in accordance with the Chinese Taipei Society of Laboratory Animal Sciences guidance on care and use of laboratory animals. Experiments were designed to keep the number of mice at a minimum and care was taken to minimize suffering.

2.2. Electroacupuncture. For the EA treatment, animals were covered by paraffin film and restrained by tapes without anesthesia. A pair of stainless steel acupuncture needles (Tianjin HaingLimSou Won Medical CO., Ltd.; Gauge 40) were inserted 3–4 mm deep into the murine equivalent of the human GV14 and GV20, that is, (1) the skin at the location between the last cervical and the first thoracic vertebral spinous processes at the midline of the back, which is equivalent to the human GV14 and (2) at the vertex of the parietal bone, that is, the midpoint of the connecting line between the auricular apices, which is equivalent to the human GV20 acupoints [42, 43]. EA stimuli were delivered by an EA Trio 300 stimulator (Ito, Japan) at 1 mA intensities for a 15 min duration at a frequency of 2, 50, or 100 Hz, with a pulse width of 150 μ s. The two electrodes were connected to the needles, which were inserted into GV20 and GV14. In the control group, animals were also covered by paraffin film and restrained by tape for 15 min [44]. A sham EA was performed by bilateral insertion of a pair of stainless steel acupuncture needles approximately 3–4 mm deep into the middle of each scapula, which is equivalent to the human Tianzong (SI11).

2.3. Behavioral Study. Following the injection of cocaine or saline, animals were placed in a plastic observation box and seizure scores were assessed by the Itzhak scale, which categorizes five stages according to their severity over a 30 min period [18]. The Itzhak stages of seizure are as follows.

Stage 1. Normal behavior (moving about the cage, sniffing, and rearing).

Stage 2. Hyperactivity (running movement characterized by rapid changes in position).

Stage 3. Animal remains in the same place for several seconds with fast repetitive movements of the head, face, mouth, or forelimb, as well as head nodding.

Stage 4. Forelimb clonus and rearing.

Stage 5. Full motor seizures, characterized by clonus of forelimbs and hindlimbs, flexion of head or entire body, and complete loss of postural control.

2.4. Immunohistochemistry Procedure. The immunohistochemistry procedure was similar to that described by Inan et al. [45]. Two hours after the injection of saline or cocaine, animals were deeply anesthetized with urethane (1.2 g/kg; i.p.) and perfused intracardially with ice-cold 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1M PBS. The brains were removed, postfixed for 2 h, and kept in 30% sucrose solution overnight. Brain sections of 50 μ m thickness were cut with a cryostat (LEICA CM 3050). Free-floating sections were stored in PBS at 4°C until immunohistochemistry was performed. Three brain sections, from -2.255 mm to -1.856 mm caudal to bregma, were randomly selected for immunohistochemistry procedures. Tissues were processed for c-Fos immunoreactivity by the avidin-biotin complex procedure [46]. Tissues were initially treated with 3% H₂O₂ to reduce endogenous peroxidase activity, then washed twice for 10 min with PBS and blocked with 20% normal goat serum (1:20) for 2 h at room temperature. The sections were then incubated on a shaker for 2 d at 4°C with a rabbit c-Fos antibody (1:1000 dilution) (sc-52; Santa Cruz, USA). After thorough rinsing, sections were incubated in biotinylated anti-rabbit immunoglobulin G secondary antibody (1:300 dilution; Vector Laboratories, USA) for 2 h at room temperature. Following two 10 min PBS rinses, the sections were incubated in a complex of avidin-biotin-peroxidase in 1:300 solution at room temperature for 90 min (Vectastain ABC Elite kit, Vector Laboratories). Following three 10 min washes in Tris-buffered saline, the sections were placed in a 0.05% diaminobenzidine (Sigma)/0.001% H₂O₂ solution for 4-5 min and washed again with Tris-buffered saline 3 times for 10 min each. Sections were mounted on slides with 0.25% gel alcohol, air-dried, and dehydrated with graded alcohol (50%, 70%, 95%, and 100%, for 6 min each) followed by xylene (3 times for 10 min each), and the coverslip was placed using Permount. c-Fos positive nuclei were observed under a light microscope and counted at 200x magnification. c-Fos immunoreactive nuclei were counted on the captured images using MetaMorph (Universal Imaging Corp.) software. Bilateral calculations were performed on the paraventricular thalamus, amygdala area, and caudoputamen. The number of immunoreactive nuclei was averaged for each mouse.

2.5. Drugs and Chemicals. Cocaine-HCl was purchased from the National Bureau of Controlled Drugs, Department of Health, Taipei, Taiwan. Freshly dissolved in 0.9% NaCl, all test agents were administered in a dose/volume of 10 mg/mL. Cocaine at the dose of 75 mg/kg causes clonic seizures in mice [48]. Cocaine at 125 mg/kg is considered to be a lethal dose [49]. In this study, cocaine at 75 mg/kg and 125 mg/kg was injected intraperitoneally to induce seizures and death. Procaine HCl was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Procaine (250 mg/kg and 400 mg/kg; i.p.) was used to induce seizures and death in mice, respectively

[50]. SB-277011-A (30 mg/kg; s.c.) was purchased from Tocris Bioscience (Ellisville, MO, USA) and was used to block the dopamine D₃ receptor [51].

2.6. Statistical Analysis. Data were expressed as means and the standard error of the mean (SEM). Group comparisons for seizure scores and the number of cocaine-induced c-Fos positive nuclei were evaluated by one-way ANOVA, followed by Tukey's post-hoc test. Group comparisons for mortality rates and seizure incidence were evaluated by chi-square test or Fisher's exact test. Significance was considered at $P < 0.05$ for all tests. Statistical analysis was performed by SPSS version 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Positioning of Acupuncture Needles. We used X-ray images to ensure accurate positioning of acupuncture needles. As shown in Figure 1(a), a mouse was anesthetized with urethane and two acupuncture needles were inserted into the GV14 and GV20 acupoints. Figure 1(b) shows the position of the acupuncture needles, as revealed by X-ray. Figure 1(c) shows the positions of GV14, GV20, and SIII in mice.

3.2. Effects of EA on Cocaine-Induced Seizures. Firstly, the effects of EA were examined on seizures induced by a single dose of cocaine (75 mg/kg) in six groups of mice (a control group, three EA treatment groups, a sham EA group, and a needle insertion group). In the control group, animals were restrained with the same procedure as that used with the EA group but without EA. In the EA treatment groups, EA (2 Hz, 50 Hz, and 100 Hz) was applied for 15 min to GV14 and GV20 acupoints prior to cocaine injection. In the sham EA group, mice received EA (50 Hz) applied to bilateral SIII. In the needle insertion group, needles were inserted into GV14 and GV20 acupoints, but without electrical stimulation. After undergoing control, EA, sham, or needle insertion treatment, all animals received an intraperitoneal injection of cocaine (75 mg/kg). Seizure severity was measured by the 5-stage cocaine seizure scale published by Itzhak [18].

As shown in Figure 2, the average seizure score was significantly lower in animals treated with EA (50 Hz) compared with those in the control and sham EA groups. No such effects were seen with EA 2 Hz, EA 100 Hz, or needle insertion. It appears that EA 50 Hz at the GV14 and GV20 acupoints significantly reduced seizure scores induced by a single cocaine administration (75 mg/kg), whereas EA at 2 Hz and 100 Hz had no such effect. Furthermore, cocaine-induced seizures were not significantly affected by either 15 min of needle insertion into GV14 and GV20 or by EA at SIII.

Table 1 depicts the incidence of seizures (higher or equal to stage 3) induced by cocaine (75 mg/kg; i.p.). Pretreatment of animals with EA (50 Hz) significantly reduced seizure incidence.

3.3. Effects of EA on Cocaine-Induced Death. The effects of EA on death induced by single administrations of a high dose of cocaine (125 mg/kg) were investigated in a series

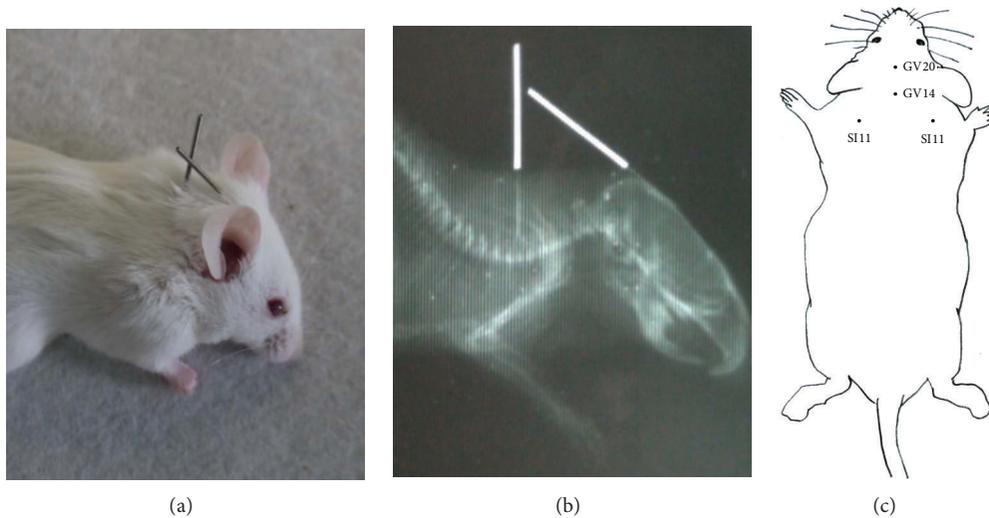


FIGURE 1: Photograph (a) and X-ray (b) of a mouse under anesthesia with acupuncture needles inserted into GV14 and GV20 acupoints. (c) Mouse schematic showing the location of the acupoints used in the study.

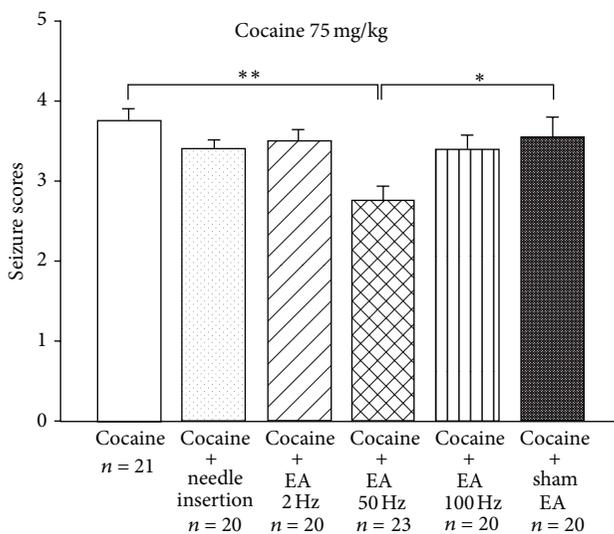


FIGURE 2: Effects of EA at different frequencies (2 Hz, 50 Hz, and 100 Hz), needle insertion exerted at GV14 and GV20 acupoints, and sham EA (50 Hz; at bilateral SI11) on cocaine-induced seizures. Mice were administered with cocaine (75 mg/kg; i.p.) at 1 min after EA or needle insertion. Seizure severity was measured by the Itzhak five-stage scale [18]. Between-group comparisons for each group were performed by one-way ANOVA followed by Tukey's test (* $P < 0.05$; ** $P < 0.01$; n : the number of animals).

of six groups, that is, control group, three EA treatment groups, a sham EA group, and a needle insertion group. After undergoing treatment, all animals received an intraperitoneal injection of cocaine (125 mg/kg).

As shown in Figure 3, mortality rates were significantly lower in the needle insertion and EA (2, 50 Hz) groups as compared to mortality in the control group. The EA (2 Hz) group mortality rate was also lower than that of the sham EA group. Needle insertion into GV14 and GV20, as well as EA

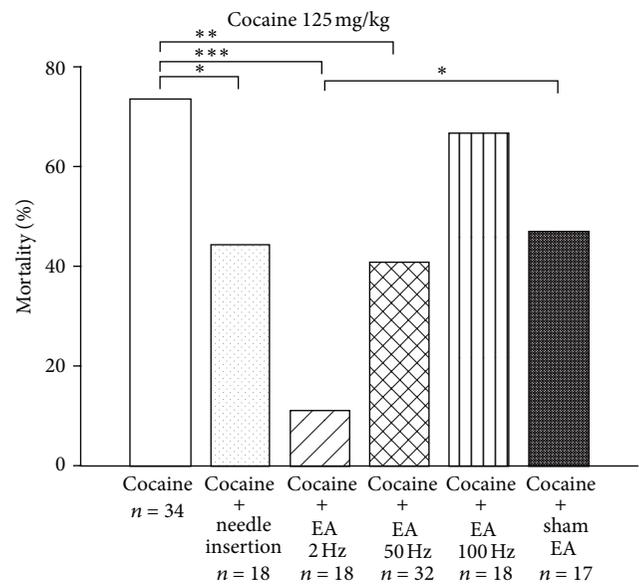


FIGURE 3: Effects of EA at different frequencies (2 Hz, 50 Hz, and 100 Hz), needle insertion exerted at GV14 and GV20 acupoints, and sham EA (at bilateral SI11; 50 Hz) on cocaine-induced mortality rates. Mice were administered with cocaine (125 mg/kg; i.p.) at 1 min after EA or needle insertion and mortality was monitored for 1 hour. Between-group comparisons for each group were performed by chi-square test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n : the number of animals).

at 2 Hz and 50 Hz applied to GV14 and GV20, significantly reduced the mortality rate induced by a single dose of cocaine (125 mg/kg). However, EA at 100 Hz had no such effect. EA at SI11 as a sham control reduced the mortality rate induced by cocaine but did not reach a statistically significant level.

3.4. *Effects of EA on Procaine-Induced Seizure and Death.* In addition to inhibiting dopamine uptake, cocaine acts as

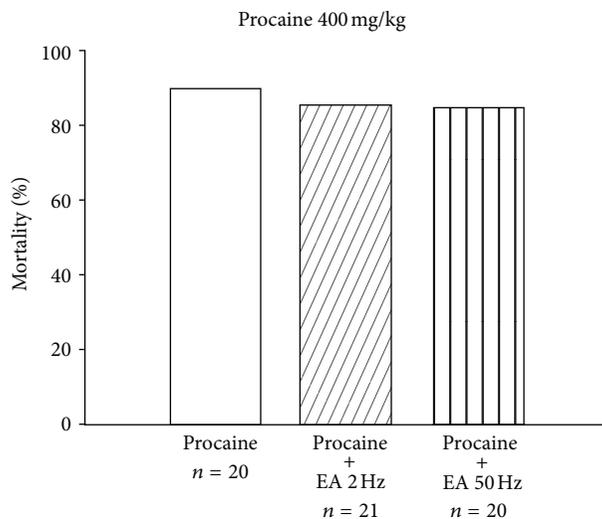


FIGURE 4: Effects of EA at 2 Hz and 50 Hz exerted at GV14 and GV20 acupoints on procaine-induced (400 mg/kg, i.p.) mortality rates. At 1 min after EA, procaine was injected and the mortality was monitored for 1 hour. Between-group comparisons for each group were performed by chi-square test or Fisher's exact test. No significant between-group differences were observed (*n*: the number of animals).

TABLE 1: EA (50 Hz) applied to GV14 and GV20 reduced the incidence of cocaine-induced seizures but did not alter the incidence of procaine-induced seizures.

	Control	EA 50 Hz
Cocaine (75 mg/kg; i.p.)	20/21	14/21*
Procaine (250 mg/kg; i.p.)	8/9	8/9

The proportion of mice exhibiting seizures is shown here. The effects of cocaine, alone and in combination with EA, were compared by Fisher's exact test (**P* < 0.05). Similarly, the effects of procaine, alone and in combination with EA, were compared.

a local anesthetic by blocking voltage-dependent Na⁺ channels [52, 53]. Local anesthetics, such as procaine, can cause CNS and cardiovascular toxicity when plasma concentrations are increased by accidental intravenous injection of a lethal dose of procaine [54]. However, procaine has no CNS stimulant effects compared with those of cocaine. Therefore, the effects of EA on procaine-induced seizures and death were examined.

The seizure incidence of procaine-treated mice (250 mg/kg; i.p.) is shown in Table 1. When animals were pretreated with EA (50 Hz) at acupoints GV14 and GV20, the incidence was not affected. Furthermore, as shown in Figure 4, mortality rates did not differ significantly between the control group and EA (2 and 50 Hz) groups. It appears that EA at 2 Hz and 50 Hz, when applied to GV14 and GV20, fails to affect the mortality rate associated with procaine (400 mg/kg; i.p.) in mice.

3.5. EA Attenuation of Cocaine-Induced c-Fos Expression. This series of studies was undertaken to test the effects of

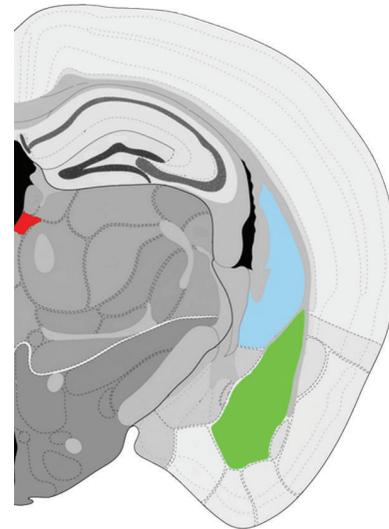


FIGURE 5: Schematic representation of the brain regions in which the c-Fos immunoreactive neurons were counted. The paraventricular thalamus (in red), the caudoputamen (blue), and the amygdala area (including lateral amygdalar nucleus, basolateral amygdalar nucleus, and basomedial amygdalar nucleus; green) are indicated. The drawing has been modified from the Allen Brain Atlas online database (<http://www.brain-map.org/>; [47]).

EA on cocaine-induced (75 mg/kg) c-Fos expression in the mouse brain. Mice were divided into 4 groups: saline control, EA alone, cocaine, and cocaine plus EA.

All mice in the saline control group and cocaine group were restrained without EA. Mice in the EA alone and cocaine plus EA groups received EA (50 Hz) at GV14 and GV20 acupoints. Following the restraining procedure or EA, the mice were allowed to recover for 1 min, before being injected with saline (saline control and EA-alone groups) or cocaine (cocaine group and cocaine plus EA groups).

Figure 5 shows the brain regions in which the c-Fos immunoreactive neurons were counted. Results are shown in Figures 6, 7, and 8. c-Fos expression was examined in the three brain areas, including paraventricular thalamus (Figure 6), amygdala area (Figure 7), and caudoputamen (Figure 8). Few c-Fos positive nuclei were found in these areas in the saline control group. In the EA-alone group, c-Fos positive nuclei were found only in the paraventricular thalamus (Figure 6). It appears that EA 50 Hz alone had no significant effect on c-Fos expression, except in the paraventricular thalamus. In the cocaine group, c-Fos positive nuclei were found in all three areas and at significantly higher levels than those seen in the saline control group. In the cocaine plus EA group, although c-Fos positive nuclei were noted in all three brain areas, c-Fos-positive nuclei were significantly decreased in the paraventricular thalamus (Figure 6) as compared to those in the cocaine group. It appears that pretreatment with EA 50 Hz significantly reduced the number of c-Fos positive cells induced by cocaine in the paraventricular thalamus, but not in the amygdala area (Figure 7) and caudoputamen (Figure 8).

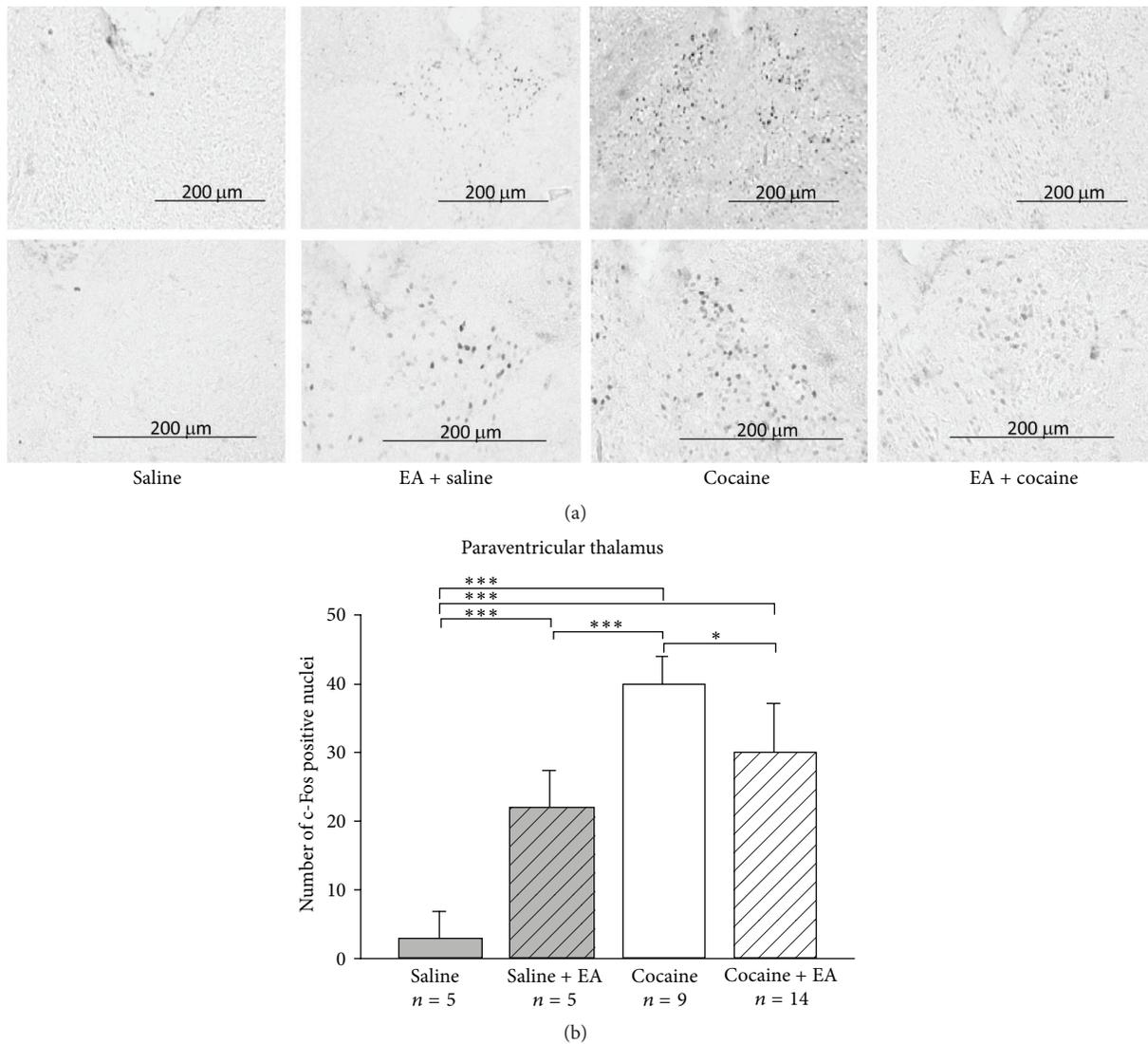


FIGURE 6: (a) Representative photomicrographs of c-Fos expression induced by cocaine in the paraventricular thalamus and the effects of EA. c-Fos expression was not observed among saline controls, whereas c-Fos expression was evident in the paraventricular thalamus among animals in the EA-alone, cocaine-alone (75 mg/kg; i.p.), and cocaine plus EA groups. (b) Effects of EA on controls and cocaine-induced c-Fos expression in the paraventricular thalamus. The numbers of c-Fos positive nuclei were counted and averaged from three randomly chosen sections from each animal in each group. EA at 50 Hz was applied to the GV14 and GV20 acupoints. EA increased c-Fos expression in the paraventricular thalamus, while EA decreased the number of c-Fos positive nuclei induced by cocaine. Between-group comparisons for each group were performed by ANOVA, followed by Tukey's test (* $P < 0.05$; *** $P < 0.001$; n : the number of animals).

3.6. EA Effects on Cocaine-Induced Seizures Blocked by the Dopamine D_3 Receptor Antagonist SB-277011-A. Cocaine enhances monoamine system activity through the blockade of dopamine and serotonin reuptake. However, the mechanism of seizures induced by cocaine is complex and involves interaction of the drug with several neurotransmitter systems as well as with voltage-dependent sodium channels [55]. It is believed that dopamine receptors play an important role in cocaine-induced seizures and death. It is known that the paraventricular thalamus expresses dopamine D_3 mRNA

[56] and we found that EA induced c-Fos expression in the paraventricular thalamus. We therefore tested the possibility that dopamine D_3 receptors may mediate the effects of EA on cocaine-induced seizures.

Four groups were included: a control group, an SB-277011-A group, an EA group, and an SB-277011-A plus EA group. In the SB-277011-A group and EA group plus SB-277011-A, the compound SB-277011-A (30 mg/kg) was administered subcutaneously for 30 min prior to the restraining procedure or EA treatment. After the restraining procedure

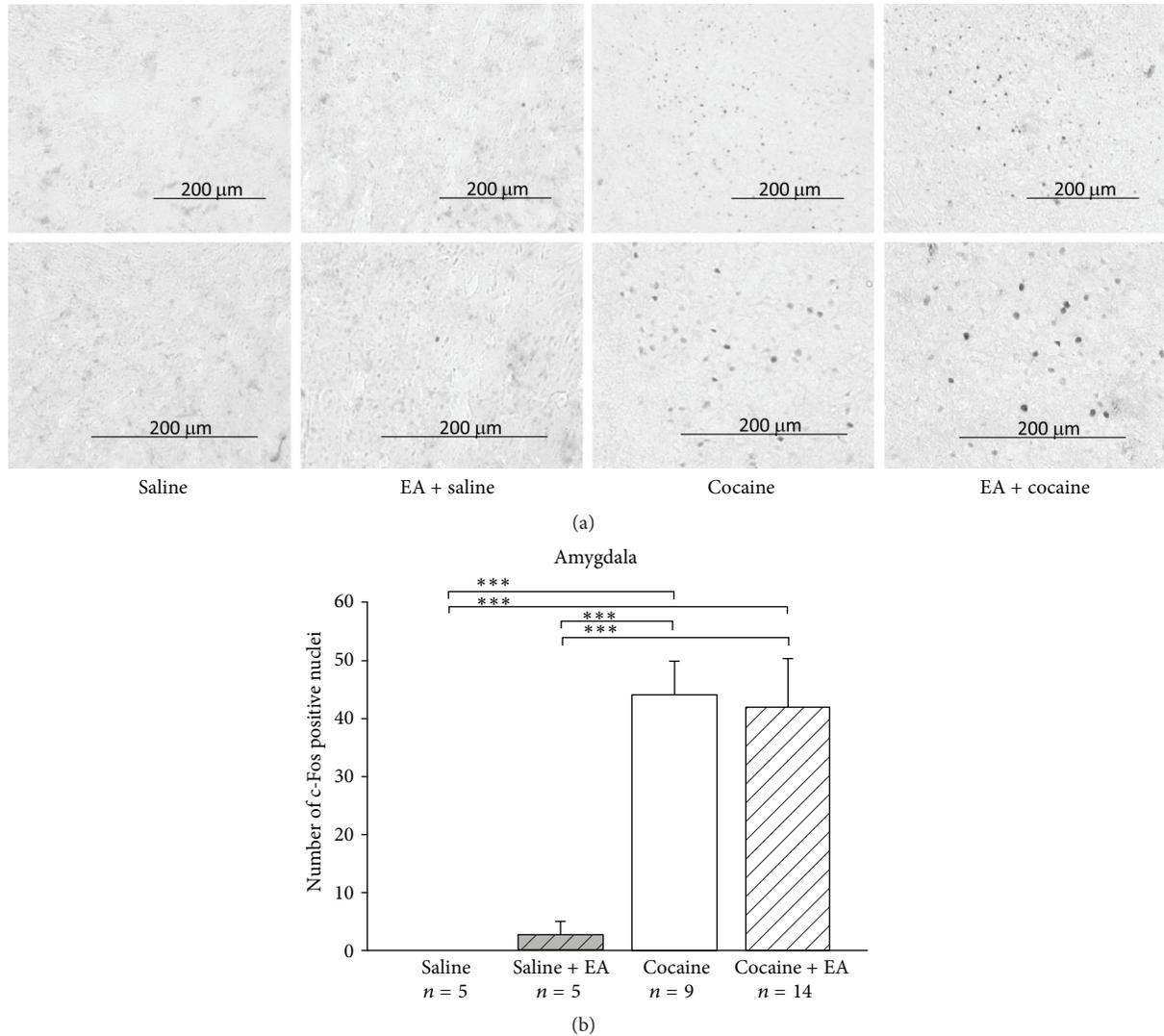


FIGURE 7: (a) Representative photomicrographs of c-Fos expression induced by cocaine in the amygdala and the effects of EA. Whereas c-Fos expression was absent among animals in the saline control and EA plus saline groups, c-Fos expression in the amygdala was observed in animals treated with cocaine (75 mg/kg; i.p.) and cocaine plus EA. (b) Effects of EA on controls and cocaine-induced c-Fos expression in the amygdala. The numbers of c-Fos positive nuclei were counted and averaged from three randomly chosen sections from each animal in each group. EA at 50 Hz was applied to the GV14 and GV20 acupoints. Cocaine administration induced a marked level of c-Fos expression, while EA did not significantly decrease the number of c-Fos positive nuclei induced by cocaine. Between-group comparisons for each group were performed by ANOVA, followed by Tukey's test ($***P < 0.001$; n : the number of animals).

or EA treatment, all animals received an intraperitoneal injection of cocaine (75 mg/kg). Seizure severity was measured by the Itzhak five-stage cocaine seizure scale [18].

As shown in Figure 9, the EA (50 Hz) group had significantly lower average seizure scores compared with those of the remaining three groups. Notably, seizure scores in the SB-277011-A group and SB-277011-A plus EA group did not differ significantly from the control group but were significantly higher than those in the EA group. It appears that while the dopamine D_3 receptor antagonist, SB-277011-A (30 mg/kg; s.c.), did not affect cocaine-induced seizure severity, it did prevent the effects of EA on cocaine-induced seizures.

4. Discussion

4.1. EA Decreases Cocaine-Induced Effects. The psychostimulant and euphoric effects of cocaine are considered to be associated with the blockade of dopamine uptake in the CNS. Acupuncture has been widely used throughout Asian countries for treating various functional disorders, including substance abuse [31]. Immunohistochemical investigations by Lee et al. (2009) [57] reported that acupuncture can reduce repeated cocaine-induced locomotor activity in rats and the expression of tyrosine hydroxylase (TH) in the rat brain, which suggests that acupuncture may effectively inhibit

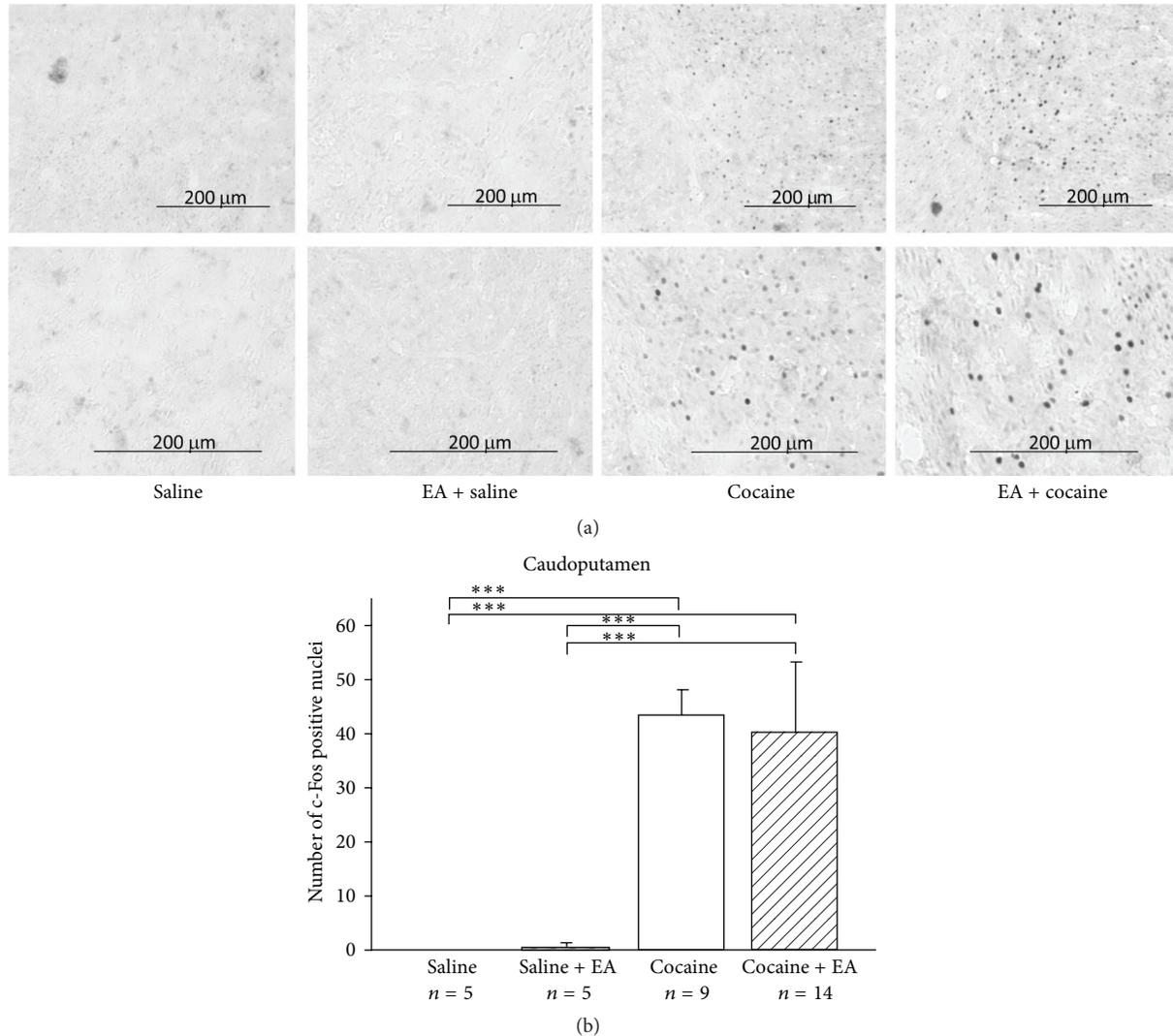


FIGURE 8: (a) Representative photomicrographs of c-Fos expression induced by cocaine in the caudoputamen and the effects of EA. Whereas c-Fos expression was not observed in the saline control and EA plus saline groups, c-Fos expression in the caudoputamen was observed among animals treated with cocaine (75 mg/kg; i.p.), and cocaine plus EA. (b) Effects of EA on controls and cocaine-induced c-Fos expression in the caudoputamen. The numbers of c-Fos positive nuclei were counted and averaged from three randomly chosen sections from each animal in each group. EA at 50 Hz was applied to the GV14 and GV20 acupoints. Cocaine administration induced a marked level of c-Fos expression, while EA did not significantly decrease the number of c-Fos positive nuclei induced by cocaine. Between-group comparisons for each group were performed by ANOVA, followed by Tukey's test ($***P < 0.001$; n : the number of animals).

the behavioral effects of cocaine by modulating the central dopaminergic system.

As mentioned earlier, cocaine abuse is associated with a risk of various medical complications, including seizures and death [3]. It has previously been reported that cocaine at doses ranging from 2.5 mg/kg to 20 mg/kg (i.p.) causes conditioned place preference, representing the rewarding effects of cocaine [58]. At a dose as high as 40 mg/kg (i.p.), cocaine caused seizures in mice [59]. The calculated ED_{50} value for cocaine-induced seizures is 58.84 mg/kg [59]. Cocaine at 75 mg/kg (i.p.) produces seizures in more than 90% of mice [48]. Cocaine at 125 mg/kg is considered to be a lethal dose [49]. In the present study, we found that EA at 50 Hz exerted at GV14 and GV20 acupoints significantly

reduced the seizure severity induced by a single cocaine (75 mg/kg; i.p.) administration. Moreover, needle insertion into GV14 and GV20 as well as EA at 2 Hz and 50 Hz exerted at GV14 and GV20 acupoints significantly reduced the mortality rate induced by a single cocaine (125 mg/kg) administration. Conversely, EA at 50 Hz applied to bilateral SIII acupoints had no such effects. In addition, EA failed to protect against procaine-induced seizure incidence and lethality in mice. We report here for the first time that EA reduced seizures and mortality induced by a high dose of cocaine.

4.2. Cocaine-Induced c-Fos Expression and Effects of EA. Expression of the *c-fos* gene or Fos protein is commonly

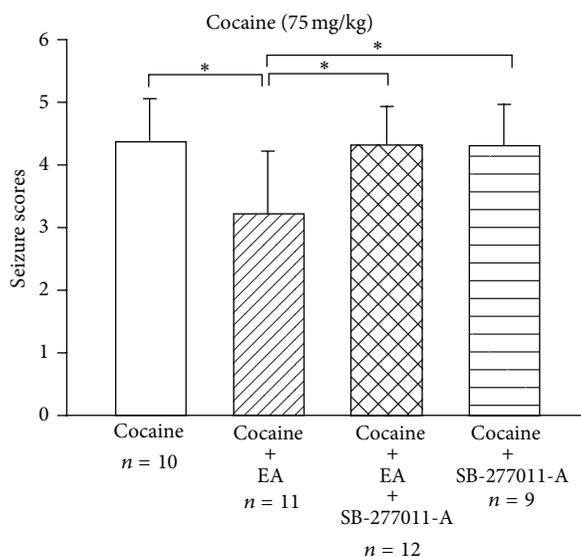


FIGURE 9: Effects of the dopamine D_3 receptor antagonist, SB-277011-A, on the anticonvulsant effects of EA. SB-277011-A (30 mg/kg) was administered subcutaneously 30 min prior to the control restraining procedure or EA treatment. EA (50 Hz) was applied for 15 min to the GV14 and GV20 acupoints prior to cocaine injection. After the restraining procedure or EA treatment, all animals received an intraperitoneal injection of cocaine (75 mg/kg). Seizure severity was measured by the Itzhak five-stage scale [18]. Between-group comparisons for each group were performed by one-way ANOVA, followed by Tukey's test ($*P < 0.05$; n : the number of animals).

used as a marker for neuronal activation, seizure pathways, and sites of action of neuroactive drugs [60–64]. It has been reported that an acute injection of cocaine (65 mg/kg) in rats increased expression of *c-fos* mRNA in the dentate gyrus of the hippocampus and olfactory bulb and limbic cortical regions as well as the striatum and ventromedial hypothalamic nucleus [41].

In this study, we focused on three brain areas: the paraventricular thalamus, amygdala, and caudoputamen. We found that cocaine at a dose of 75 mg/kg (i.p.) induced marked *c-Fos* expression in all areas. Pretreatment with 50 Hz EA significantly reduced the number of *c-Fos* positive cells induced by cocaine in the paraventricular thalamus, but not in the amygdala or caudoputamen.

The thalamus is considered to be an important interface between the ventral pallidum and the dorsal medial prefrontal cortex which may therefore contribute to the development of compulsive drug-seeking behavior [65]. One thalamic nucleus that is of particular interest is the paraventricular thalamus, a component of the dorsal midline thalamic group [66]. The thalamic paraventricular nucleus projects to the nucleus accumbens and other limbic sites, including the prefrontal cortex, amygdala, and hippocampus; these projections are predominantly glutamatergic [67]. The paraventricular thalamus also receives a dopaminergic innervation, in part derived from the ventral tegmental area, and paraventricular thalamus neurons expressing dopamine D_3 mRNA [56]. The

paraventricular thalamus has been implicated in stress reactivity [68], reward-seeking behavior [69], and general arousal activity [70]. Furthermore, inactivation of the paraventricular thalamus prevents context-induced reinstatement of alcohol-seeking [71] and cocaine-primed reinstatement in rats [72]. Paraventricular thalamus lesions block cocaine sensitization [67].

The paraventricular thalamus is related to seizures activity. Mraovitch and Calando [73] used immunocytochemistry to determine the regional and temporal distribution of Fos protein expression in awake and unrestrained rats after a unilateral stereotaxic microinjection of the cholinergic agonist carbachol in the thalamic ventroposterolateral and reticular nuclei, previously shown to cause limbic and generalized seizures [73]. They found that paraventricular thalamus activation occurred after 15 min after administration of epileptic agents.

The amygdala is involved in both temporal lobe epilepsy and in cocaine mechanisms in the brain. In particular, the central nucleus of the amygdala is a highly epileptogenic brain area and, of the amygdaloid nuclei, responds most rapidly to a kindling stimulus [74]. The caudoputamen is a part of the striatum area and plays an important role in control of movement in animals [75–77].

As shown by our results and the published literature [41], acute cocaine administration increases *c-Fos* expression in many brain areas. Among these areas, the paraventricular thalamus and amygdala are structures associated with seizures, while the caudoputamen is associated with movement control. We therefore analyzed *c-Fos* expression in these three specific regions. Our results implicate involvement of the paraventricular thalamus in the effects of EA on cocaine-induced seizures.

4.3. EA Induces *c-Fos* Expression in the Paraventricular Thalamus and Dopamine D_3 Receptors Are Involved in the Effects of EA. Medeiros et al. (2003) reported higher levels of *c-Fos* expression induced by EA at the ST36 point in animals, that is, in the dorsal raphe nucleus, locus coeruleus, posterior hypothalamus, and central medial nucleus of the thalamus [78]. In the present study, we found that EA alone at GV14 and GV20 acupoints induced significant *c-Fos* expression in the paraventricular thalamus.

Recent research indicates that dopamine D_3 receptors may play an important role in cocaine-induced seizures [51]. The D_3 antagonist SB-277011-A has been used to block dopamine D_3 receptors, at doses ranging from 3 mg/kg to 30 mg/kg (s.c.) [18, 79]. SB-277011-A prevented the anticonvulsant effects of the D_3/D_2 receptor agonist (+)-PD-128,907 on cocaine-induced seizures. Notably, the protection afforded by (+)-PD-128,907 was eliminated in D_3 receptor-deficient mice, whereas the anticonvulsant effects of (+)-PD-128,907 were preserved in D_2 receptor knockout mice.

As mentioned above, the paraventricular thalamus expresses dopamine D_3 mRNA, while EA alone induces *c-Fos* expression in the paraventricular thalamus. We therefore sought to determine whether dopamine D_3 receptors mediate the effects of EA on cocaine-induced seizures. Our results

revealed that while the D₃ receptor antagonist SB-277011-A did not affect cocaine-induced seizure severity, it prevented the effects of EA on cocaine-induced seizures. This finding suggests that the D₃ receptor is involved, at least in part, in the anticonvulsant effects of EA.

Interesting topics that remain for future studies include an investigation into whether intraparaventricular thalamus injection of a D₃ receptor antagonist prevents EA-induced anticonvulsive effects and whether a D₃ receptor antagonist antagonizes EA-induced changes to c-Fos expression in the paraventricular thalamus or has any effect on cocaine-induced mortality.

4.4. A Complex Mechanism Underlies Cocaine-Induced Death.

It is noted that EA at 50 Hz only, when exerted at GV14 and GV20 acupoints, significantly reduced seizure severity induced by a single cocaine (75 mg/kg; i.p.) administration. However, needle insertion into GV14 and GV20 acupoints as well as EA at 2 Hz and 50 Hz effectively reduced the mortality rate induced by a single cocaine (125 mg/kg) administration. As mentioned earlier, seizures are considered to be a major determinant of cocaine-related lethality in both humans [4] and animals [9]. However, the mechanism of sudden death in cocaine abuse also includes cardiac arrhythmia and intracerebral hemorrhage [10, 11]. Cocaine produces euphoria, elation, mood elevation, alertness, attention focusing, and fatigue reduction through interactions with monoamine transporters [80]. In addition to the central effects, cocaine also blocks norepinephrine uptake and increases sympathetic activity in the periphery [81]. Overstimulation of sympathetic activity may cause cardiac arrhythmia and intracerebral hemorrhage and hence may contribute to cocaine-induced fatality [10, 11]. EA frequency-specific differences observed in protection against seizures and death may be because the mechanism that induces death is more complicated than that involved in seizures. It is recognized that acupuncture can account for different effects in the autonomic nervous system [82, 83]. For example, the GV14 acupoint is capable of increasing parasympathetic activities while simultaneously suppressing sympathetic activities [82]. This may explain why EA at 2 Hz is particularly effective against cocaine-induced death.

4.5. EA Frequency. The best-known mechanism of EA is via the endogenous opiates and their receptors. Different kinds of endogenous opioid peptides, such as β -endorphin, enkephalin, endomorphin, and dynorphin, reportedly act in a frequency-dependent manner in EA. At a low frequency (2 Hz), EA accelerated the release of β -endorphin and enkephalin in the CNS, whereas high-frequency EA (100 Hz) accelerated the release of dynorphin [17, 84–86]. In the present study, we found that EA applied to GV14 and GV20 acupoints at the frequency of 50 Hz is most effective in reducing seizure severity induced by a single cocaine administration. There were few reports in the literature as to the effects of EA 50 Hz as compared to those of EA 2 and 100 Hz. However, it has been recently reported that EA 60 Hz increases the pain threshold by a greater extent than any

other frequency [87]. EA at a frequency of 60 Hz induced the simultaneous release of met-enkephalin, β -endorphin, and dynorphin-A in extensive analgesia-related nuclei and areas of the CNS, such as the periaqueductal gray, the paraventricular nucleus of the hypothalamus, the ventromedial nucleus of the hypothalamus, the dorsal raphe nucleus, and the nucleus raphe magnus, amongst others. These results suggest that EA at 60 Hz may contribute to optimal analgesic effects. The effects of EA at 50 or 60 Hz deserve to be studied further [87].

No effective treatment currently exists for cocaine-induced seizures and death. Our results suggest that EA reduces seizure severity and death caused by cocaine in an animal model of cocaine abuse. We found evidence for involvement of the dopamine D₃ receptor, at least in part, in the anticonvulsant effects of EA.

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Research Article

Auricular Acupressure Combined with an Internet-Based Intervention or Alone for Primary Dysmenorrhea: A Control Study

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Background. Primary dysmenorrhea is prevalent in adolescents and young women. Menstrual pain and distress causes poor school performance and physiological damage. Auricular acupressure can be used to treat these symptoms, and Internet-based systems are a flexible way of communicating and delivering the relevant information. **Objective.** This study investigates the effects of auricular acupressure (AA) alone and combined with an interactive Internet-based (II) intervention for the management of menstrual pain and self-care of adolescents with primary dysmenorrhea. **Design.** This study adopts a pretest/posttest control research design with a convenience sample of 107 participants. **Results.** The outcomes were measured using the short-form McGill pain questionnaire (SF-MPQ), visual analogue scale (VAS), menstrual distress questionnaire (MDQ), and adolescent dysmenorrheic self-care scale (ADSCS). Significant differences were found in ADSCS scores between the groups, and in SF-MPQ, VAS, MDQ, and ADSCS scores for each group. **Conclusion.** Auricular acupressure alone and a combination of auricular acupressure and interactive Internet both reduced menstrual pain and distress for primary dysmenorrhea. Auricular acupressure combined with interactive Internet instruction is better than auricular acupuncture alone in improving self-care behaviors.

1. Introduction

The rate of primary dysmenorrhea in adolescents and youth worldwide is relatively high: 83.2% in Singapore [1], 82% in Korea [2], 73.3% in Taiwan [3], 72.7% in Turkey [4], 71.1% in Australia [5], 65% in the US [6], 64% in Mexico [7], 68.7% in Hong Kong [8], and 60% in Canada [9]. Primary dysmenorrhea refers to painful menstrual cramps in the lower abdomen without evident pelvic pathologic lesions. This condition occurs just before or at the onset of menstrual flow. Females with primary dysmenorrhea produce excessive amounts of prostaglandins and leukotrienes in the uterus, and these substances cause inflammation, myometrial hypercontractility, and vasoconstriction [10, 11]. The uterus then becomes ischemic and hyperalgesic, resulting in cramps and

systemic symptoms such as nausea, vomiting, and headache. Dysmenorrhea can affect mental concentration during class, restrict social activities, reduce academic achievement [12], increase absenteeism [1, 7, 13], and reduce quality of life [4]. Previous studies have demonstrated that primary dysmenorrhea changes central sensitization to pain perception and alters brain metabolism, particularly in gray matter [14]. These findings indicate that the adolescent brain is sensitive and susceptible to menstrual pain [15].

Nonsteroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives (OCs) are the most commonly used and effective pharmacologic treatments for pain relief from primary dysmenorrhea [16, 17]. In this study, 12–28% of adolescents sought medical care and 60.9–66.9% of adolescents self-medicated themselves with over-the-counter drugs

[7, 18, 19]. Although NSAIDs provide temporary relief from menstrual pain, they may have side effects such as gastrointestinal upset, indigestion, headaches, and drowsiness [20]. Moreover, OC usage is significantly related to higher incidences of irregular uterine bleeding and nausea. Adverse events are more common during the early stage of use [21], and endometriosis is more frequent with long-term use [22]. In traditional Chinese medicine, the acupoint stimuli transmitted to the brain and specific organs in the rest of the body can modulate physiological reactions [23]. This causes the release of various neurotransmitters, which interrupt afferent signals in the central nervous system [24]. Auricular acupressure is a simple noninvasive method of acupoint stimulation. Auricular acupressure can be used to treat the symptoms associated with primary dysmenorrhea, inhibit excessive production of prostaglandins, reduce excitability of the cerebral cortex, and regulate endocrine hormone secretion [25]. Many studies have demonstrated the beneficial effects of auricular stimulation on menstrual pain and distress [26–30].

Most adolescents lack appropriate and sufficient information about menstruation and must be empowered to take charge and manage their own care. Adolescents with dysmenorrhea often receive information about dysmenorrhea from school [3], their mothers, siblings and friends, physicians and nurses [19], and others [8]. Of these sources, health care professionals are the most flexible and adaptable in their approach to providing patients with information [31]. Internet-based systems are flexible communication methods that deliver the latest, most up-to-date information [32], and their use for pain management has become popular [33]. Previous studies using Internet-based learning programs have reported reduced pain [34–36], facilitated knowledge acquisition [37], facilitated self-care education [38], and improved health status [39]. However, the effects of auricular acupressure combined with Internet-based programs on menstrual pain and self-care for dysmenorrheic adolescents may be more comprehensive.

2. Purpose Statements

This study investigates the effects of auricular acupressure (AA) alone or when combined with interactive Internet-based (II) interventions on menstrual pain and self-care in adolescents with primary dysmenorrhea. Significant differences in scores on the short-form McGill pain questionnaire (SF-MPQ), visual analogue scale (VAS) for pain, menstrual distress questionnaire (MDQ), and adolescent dysmenorrhea self-care scale (ADSCS) were hypothesized between and within the groups.

3. Methods

3.1. Research Design and Participants. This study adopts a pretest/posttest control design involving 107 participants with primary dysmenorrhea from a senior high school. We divided participants into two groups. One group received auricular acupressure (AA) alone, whereas the other received

AA combined with an interactive Internet-based (AII) intervention. Figure 1 shows a flowchart of the participants of this study. Inclusion criteria were (1) two or more incidents of menstrual pain experienced in the past six months; (2) VAS > 5; and (3) no swelling, infections, or ulcers in the bilateral ears. Exclusion criteria were (1) known diagnosis of pelvic inflammatory disease, endometriosis, or gynecological surgery, and (2) taking analgesic drugs or herbal medicine for dysmenorrhea. We used G Power version 3 to calculate the sample size and found that each group should contain 45 participants to achieve a statistical power of 0.80 with a statistical significance of 0.05. We calculated the effects of auricular acupressure on pain relief in adolescents with dysmenorrhea based on the study of Yeh et al. (in press). Pain relief (mean \pm standard deviation) was 5.14 ± 2.32 in the experimental group and 3.64 ± 2.49 in the control group. We collected data before and after the interventions and compared the effects of interventions between and within the groups.

3.2. Intervention. Six auricular acupoints were used for relieving dysmenorrhea: *shenmen*, *kidney*, *liver*, *internal genitals*, *central rim*, and *endocrine*. The effects of stimulation depended on the following specific auricular acupoints: *internal genitals* and *endocrine* were for harmonizing and improving endocrine and uterine function [40], *kidney* and *liver* were for normalizing qi and blood and restoring organ function [41], *shenmen* was for alleviating pain and for sedation [29], and *central rim* was for dredging the meridian and normalizing circulation [42]. We adopted a seed-embedding method with cowherb seeds to stimulate the auricular acupoint. Two experts licensed in traditional Chinese medicine confirmed the accuracy and precision of seed positioning and pressing techniques. We placed adhesive plasters containing seeds on the auricular acupoint at the start of menstrual bleeding and removed after pain relief 48 hours later. All participants were instructed to press each acupoint for at least one minute, four times per day until experiencing pain relief.

We created an interactive website to promote the health of those with dysmenorrhea. This website provided clients with nursing care instruction and counseling and served as an interactive communication medium for increasing knowledge about dysmenorrhea and self-care practices related to dysmenorrhea. Two experts in the discipline of obstetrics and gynecology examined the content validity of the program and evaluated the correlation between objectives and content. These experts graded each of the 20 items on a four-point scale for item-objective congruence and relevance. The overall content validity index was 0.95. We divided the contents of dysmenorrhea into nine units as follows.

Unit 1: Hot News. To disseminate information and build consensus, this unit provided a preview of activities and reported the prevalence of dysmenorrhea, legal rights in the workforce relating to dysmenorrhea according to gender equality law, and the health-care concerns of adolescents. *Unit 2: Red Magic Book.* This included an online survey and menstrual diary. Participants were asked to fill out relevant questionnaires before and after self-care learning, and record

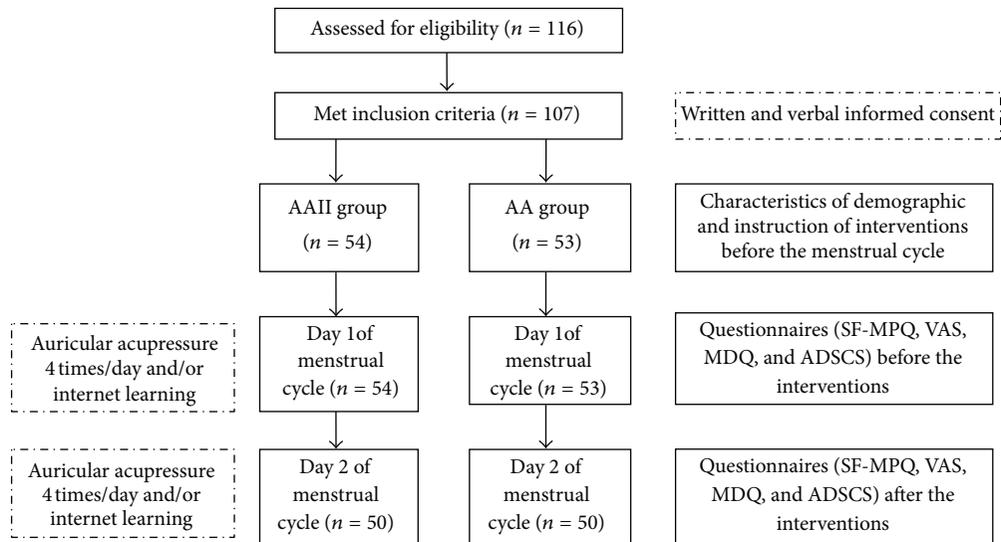


FIGURE 1: The Flow Chart of Research Design and Participants.

the necessary information in their menstrual cycle diaries. This data was charted automatically to create a menstrual cycle chart. *Unit 3: Understanding of Dysmenorrhea.* We used computer-animated videos to describe the menstrual cycle and hormone fluctuation during ovulation and presented the definitions and differences between primary and secondary dysmenorrhea in table format. We also presented the physical and psychological symptoms of dysmenorrhea in interactive format and discussed the effects of dysmenorrhea on daily life. *Unit 4: Caring.* Menstrual care included menstrual care based on the viewpoint of Chinese medicine, self-care approaches such as hot pack, daily menstrual care, and menstrual hygiene. *Unit 5: Auricular Acupressure.* We used pictures with word descriptions to introduce acupoint techniques and the theory, rationale, efficacy, advantages, and precautions associated with these techniques. The photographs explained and illustrated the six auricular acupoint procedures for pain relief. *Unit 6: Professional Counseling.* Professional health-care providers responded to posted questions through this webpage or email. We prioritized questions and answers from participants to present their main concerns and issues. *Unit 7: Diet and Food Properties.* This part of the website presented content relating to daily diet requirements to preserve health, food properties based on the rationale of Chinese medicine, and general information on food properties and herbal cuisine dietetics. *Unit 8: Chat Room.* Using this part of the website, participants could post messages regarding dysmenorrhea or the menstrual cycle to interact with each other and provide support. *Unit 9: ext-linked Websites.* We also provided hyperlinks to representative and authoritative websites on dysmenorrhea to extend the learning experience.

3.3. Measures. We used the short-form McGill pain questionnaire (SF-MPQ) developed by Melzack [43] to assess the quality and intensity of pain. The SF-MPQ includes an 11-item

sensory subscale and four-item affective subscale rated from 0 to 3 (none, mild, moderate, and severe). The Cronbach's alpha for this measure was 0.84 in this study. We also assessed pain intensity on a 0–100 mm visual analog scale (VAS), with the left end labeled as no pain and the right end labeled as unbearable pain. All participants rated their present pain intensity. The test-retest reliability of the VAS for pain was 0.97 [44].

We used the modified 16-item menstrual distress questionnaire (MDQ) developed by Wang [45] to assess the severity of physiological symptoms (pain, water retention, and autonomic reactions) during the premenstrual and menstrual periods. Participants graded each item on a four-point scale from one (no symptoms) to four (severe symptoms). Cronbach's alpha for this measure was 0.83 in this study. Additionally, we used the adolescent dysmenorrheic self-care scale (ADSCS) with seven subscales (including search for knowledge, expression of emotions, seeking assistance, control over external factors, resource usage, self-control status, and Internet information) to evaluate self-care behaviors [46]. Participants graded each item on a six-point Likert scale. We added six items to the 40 original items to confirm the interactive content. Cronbach's alpha for this measure was 0.90 in this study.

3.4. Data Analysis. We analyzed data using IBM SPSS 20.0, and used descriptive statistics to describe demographic characteristics. We also used the chi-square test, paired *t*-test and one-way ANCOVA to analyze the effects of interventions on these characteristics between or within groups. We considered a *P* value of less than 0.05 to be statistically significant.

4. Results

The sample in this study initially consisted of 107 participants. Of these, seven participants withdrew from the study for

TABLE 1: Comparisons of demographic characteristics between the groups.

Variables	AAII (<i>n</i> = 50)	AA (<i>n</i> = 50)	<i>t</i>
	Mean (SD)	Mean (SD)	
Age (years)	16.94 (1.02)	17.94 (0.84)	0.75**
Age at menarche (years)	11.96 (1.21)	12.18 (1.56)	2.46
Menstrual cycle (days)	28.82 (3.74)	29.70 (4.02)	0.29
Menses duration (days)	5.86 (1.16)	5.24 (1.12)	0.002*
	<i>n</i> (%)	<i>n</i> (%)	χ^2
Menstrual regularity			0.00
Yes	30 (60.0)	30 (60.0)	
No	20 (40.0)	20 (40.0)	
Initial onset of menstrual pain			0.35
Menarche	13 (26.0)	11 (22.0)	
<1 year after menarche	15 (30.0)	15 (30.0)	
1-2 years after menarche	10 (20.0)	12 (24.0)	
Others	12 (24.0)	12 (24.0)	
Time of dysmenorrhea			0.25
Day before menses	11 (22.0)	9 (18.0)	
First 2 days in menses	39 (78.0)	41 (82.0)	

AAII: auricular acupressure combined with interactive internet; AA: auricular acupressure.

* $P < 0.05$.

** $P < 0.001$.

personal reasons ($n = 5$) or because they had difficulty using computers ($n = 2$). Finally, each group consisted of 50 participants, with an attrition rate of 6.5%. The average ages of the AAII and AA groups, respectively, were 16.94 ± 1.02 and 17.94 ± 0.84 years at the beginning of the study and 11.96 ± 1.21 and 12.18 ± 1.56 years at menarche. The mean length of the menstrual cycle was 28.82 ± 3.74 and 29.70 ± 4.02 days. Table 1 presents a summary of the demographic characteristics of groups at baseline and shows no significant between-group differences, except in age ($P < 0.001$) and menses duration ($P = 0.008$).

Table 2 shows the improvement in pain management after the AAII and AA interventions. The between-group difference was not significant in pre-to-posttest change in SF-MPQ ($P = 0.81$), VAS ($P = 0.75$), and MDQ ($P = 0.28$) scores. The within-group difference in pre-to-posttest change in SF-MPQ, pain VAS, and MDQ scores was significant for both the AAII ($P < 0.001$) and AA ($P < 0.001$) groups. Table 3 presents a summary of the results of ADSC scale for dysmenorrhea. We found a significant difference in pre-to-posttest change in the total scores between the groups ($P < .001$) and in each group (AAII: $P < .001$; AA: $P = 0.04$). The between-group differences in pre-to-posttest change in scores for all subscales ($P < 0.05$) and the within-group difference in pre-to-posttest change for all subscales in the AAII group ($P < 0.001$) were significant. However, these differences were significant only in the searching for knowledge ($P < 0.001$) and self-control status ($P = 0.02$) subscales in the AA group.

5. Discussion

Most of the participants in this study started with menstrual pain within two years after their menarche and experienced dysmenorrhea within the first two days of menstruation. Other studies have reported similar findings [3, 4, 7, 8, 20, 47, 48]. In this study, the average age at menarche was approximately 12 years, which is slightly lower than that in Turkey (13.38; [4]), Iran (13.3 years; [49]), Korea (13 years; [50]), Nigeria (12.7 years; [13]), Japan (12.5 years; [21]), and Hong Kong, South Africa, and Mexico (12.3 years; [8, 18, 47]). However, early menarche is defined as menarche beginning before age 11-12 [51-53].

The results of this study show that auricular acupressure alone or combined with interactive Internet instruction can reduce menstrual pain and distress. This observation is consistent with previous studies [26, 28, 30]. Auricular acupressure combined with interactive Internet intervention was more effective than auricular acupressure alone in improving dysmenorrhea self-care. Auricular acupressure alone, compared to analgesics, achieved greater improvement in menstrual pain and associated syndromes [29, 54-56]. Auricular stimulation of local pressure receptors results in nerve impulse transmission, and pain decreases or disappears when the intensity of the stimulus exceeds a threshold. Based on this mechanism, auricular acupressure can relieve menstrual pain and distress in adolescents with primary dysmenorrhea.

The interactive Internet-based intervention proposed in this study provided knowledge and information about dysmenorrhea self-care, auricular acupressure techniques, professional counseling, and peer support. Our results are consistent with previous findings showing the efficacy of Internet-based interventions in reducing pain [35, 36, 57, 58] but are not in agreement with the findings of Trautmann and Kröner-Herwig [59]. A systematic review shows that Internet-based programs appear to relieve pain [33]. Autonomic reactions refer to pain, stress, and anxiety [60]. A program of interactive online learning may reduce autonomic reactions by increasing knowledge about self-care techniques, thereby reducing pain and anxiety [34, 57] and stress [57, 61]. Future research should measure autonomic reaction indicators, such as heart rate variability, to clarify the mechanisms involved in menstrual distress and autonomic nervous system activity.

This study also shows that auricular acupressure combined with interactive Internet instruction is better than auricular acupuncture alone in improving self-care for primary dysmenorrhea. Other studies have also shown that Internet- or computer-based interventions enhance knowledge and technical abilities or promote self-care ability in patients dealing with idiopathic carpal tunnel syndrome [62], adolescents preparing for out-patient tonsillectomy procedures [37], and patients attempting to self-manage chronic low back pain [63]. A systematic review of relevant literature shows that Internet-based interventions used to disseminate information on treatment are an effective complementary tool for changing lifestyle habits, diminishing symptom severity, and improving decision-making skills [64]. Thus,

TABLE 2: Outcomes on auricular acupressure combined with interactive internet or alone.

Variables	AAII (<i>n</i> = 50)			AA (<i>n</i> = 50)			ANCOVA <i>F</i>
	Pre-test Mean (SD)	Post-test Mean (SD)	Improvement Mean (SD)	Pre-test Mean (SD)	Post-test Mean (SD)	Improvement Mean (SD)	
SF-MPQ	16.65 (8.88)	4.58 (3.73)	11.98 (8.46) ^{***}	18.80 (9.26)	5.36 (6.76)	13.44 (8.62) ^{***}	0.06
VAS	6.56 (1.36)	1.97 (1.87)	4.59 (1.93) ^{***}	7.17 (1.46)	2.03 (2.02)	5.14 (2.32) ^{***}	0.11
MDQ	29.30 (7.12)	21.88 (7.06)	7.42 (9.28) ^{***}	31.88 (7.60)	21.16 (4.67)	10.72 (6.85) ^{***}	1.18

SF-MPQ: short-form McGill pain questionnaire; VAS: visual analog scale; MDQ: menstrual distress questionnaire.

^aWithin-group differences.

** *P* < 0.001.

TABLE 3: Results of adolescent dysmenorrhea self-care scale.

Variables	AAII (<i>n</i> = 50)			AA (<i>n</i> = 50)			ANCOVA <i>F</i>
	Pre-test Mean (SD)	Post-test Mean (SD)	Improvement Mean (SD)	Pre-test Mean (SD)	Post-test Mean (SD)	Improvement Mean (SD)	
Searching for knowledge	11.54 (4.63)	18.54 (2.94)	7.00 (5.13) ^{***}	13.54 (4.68)	15.46 (4.89)	1.92 (3.06) ^{***}	32.67**
Expression of emotions	24.66 (6.53)	28.50 (4.93)	3.84 (5.09) ^{***}	29.58 (5.57)	29.02 (6.37)	-0.56 (4.59) ^a	7.49*
Seeking assistance	16.68 (4.21)	19.08 (3.34)	2.40 (3.30) ^{***}	18.88 (4.40)	18.66 (4.15)	-0.22 (3.31) ^a	8.77*
Control over external factors	20.52 (5.65)	26.06 (5.71)	5.54 (5.90) ^{***}	23.68 (7.48)	24.62 (7.03)	0.94 (3.96) ^a	14.52**
Resource utilization	47.64 (11.29)	62.12 (16.11)	14.48 (15.63) ^{***}	57.20 (10.69)	58.00 (10.87)	0.80 (6.52) ^a	18.02**
Self-control being	14.86 (5.07)	19.32 (5.01)	4.46 (4.76) ^{***}	16.12 (4.88)	17.50 (3.86)	1.38 (4.18) ^{a*}	10.55*
Internet information	14.34 (5.65)	27.16 (5.47)	12.82 (7.16) ^{***}	19.64 (7.44)	21.14 (5.50)	1.50 (6.20) ^a	50.67**
Total score	150.24 (30.94)	200.78 (32.25)	50.54 (30.16) ^{***}	178.64 (28.60)	184.40 (28.27)	5.76 (18.80) ^{a*}	46.92**

^aWithin-group differences

* *P* < 0.05.

** *P* < 0.001.

Internet-based interventions could be integrated into programs that enhance the knowledge and self-care behaviors of adolescents with primary dysmenorrhea. Overall, the proposed treatment improves self-care behaviors in both the AAI and AA groups, and especially the AAI group. Interactive Internet education programs not only provide knowledge of self-care activities, but also lead to increased self-efficacy [39] and confidence in the use of self-care techniques [38]. Therefore, Internet interventions can increase the effectiveness of auricular acupressure.

5.1. Limitations. This study has some limitations. First, non-randomized clinical research involves inherent limitations. Second, this study does not include a control or placebo group; therefore, the results of a placebo effect are unknown. Third, the sample was only taken from one senior high school, which may limit the generalizability of the results. Fourth, this study only demonstrates the short-term effects of the proposed interventions; the long-term effects remain unknown. Fifth, we collected menstrual pain and distress data through self-reported questionnaires and did not measure physiological indicators. Randomized controlled, longitudinal studies that measure physiological indicators in various geographic locations are recommended. In addition, future research should address the current limitation of accessing interactive Internet-based interventions in the AA group.

6. Conclusion

This study contributes information regarding the effectiveness of employing auricular acupressure combined with interactive Internet-based instruction and auricular acupressure alone in adolescents with primary dysmenorrhea. Our results show that auricular acupressure improves menstrual distress and self-care behavior. The interactive Internet-based intervention in this study, which is flexible and available to adolescents seeking information to manage health-related issues, generated even more efficient self-care behaviors. However, the long-term effects of auricular acupressure combined with interactive Internet-based intervention and auricular acupuncture alone in adolescents with primary dysmenorrhea remain unclear. Future studies should integrate Internet-based interventions with other interventions to improve the self-care of menstrual pain and distress in adolescents. In addition, objective measures of the autonomic nervous system activity (physiological indicators) are needed to enhance the value and reliability of this type of intervention.

Conflict of Interests

None of the authors has any potential conflict of interests in this study.

Authors' Contribution

Y.-J. Wang and J.-G. Lin equally contributed to this work.

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Research Article

Development and Validation of Acupuncture Fear Scale

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Objectives. Strong aversions to acupuncture have been an obstacle to understanding its intrinsic action of acupuncture. Thus, it is necessary to evaluate the nature and extent of fear of acupuncture treatment. Our study aims to develop and validate an instrument that evaluates a patient's fear of acupuncture treatment. **Methods.** We have developed an acupuncture fear scale, a 16-item instrument which assesses the acupuncture fear score and uses it to survey 275 participants in South Korea, thus testing the reliability and validity of the instrument. **Results.** Internal consistency was high (Cronbach's alpha = 0.935). Test-retest reliability (Spearman's rank correlation coefficient) among 33 participants out of 275 ranged from 0.565 to 0.797 ($P < 0.001$). Principal component analysis revealed two factors accounting for 68% of the variance, which are painful sensation and possible adverse events, respectively. The acupuncture fear scale was positively correlated with the total of fear of pain questionnaire-III ($r = 0.423$, $P < 0.001$). **Conclusions.** The acupuncture fear scale can be a valid and reliable instrument that can measure fear of acupuncture treatment. These results strongly suggest that it would be a clinically useful tool to assess fear of acupuncture in the acupuncture clinic setting and an important instrument to understand the complex social-behavioral component of acupuncture modality.

1. Introduction

Fear is an important factor for understanding the characteristics of pain. Pain-related fear influences the perception of the intensity of pain, and it has been reported that fear can be more disabling than pain itself by motivating patients to avoid treatment [1–3]. Fear of injections, for example, is a common concern among patients in healthcare setting. At least 10% of population in a medical setting reports an excessive fear of needles that causes significant avoidance, distress, and/or impairment [4]. Since a person with needle phobia will typically avoid medical care, this condition has been considered a significant impediment in the health care system. In order to minimize needle phobia-related problems and encourage patients to seek treatment, the evaluation of the specific component of fear is very important and has to be considered for any kind of treatment of pain.

Acupuncture is widely used for the treatment of pain, and many clinics, hospitals, and doctors apply it for therapeutic

purposes of pain relief. However, there are still a great number of patients hesitant to receive acupuncture treatment, even though it is well known that acupuncture is a safe therapy in clinical practice when general precautions are followed [5]. This fear of receiving acupuncture treatment could be due to the fear of acupuncture needle penetration—a form of instinctive needle phobia—as acupuncture is generally defined as the procedure of inserting and manipulating needles to various specified points on the body surface [6, 7]. Or it could be simply due to the fear of pain, since fine needles penetrating the skin can also evoke a certain degree of pain during the treatment [8]. Another possibility could be also the concern about adverse effects. It is reported that the most commonly feared adverse effects are needle pain (1% to 45%) from treatments, tiredness (2% to 41%), and bleeding (0.03% to 38%) [9]. Regardless of which component is contributing more significantly, evaluating the patients' fear of acupuncture is necessary in order to provide better treatments.

Several questionnaires have been developed in order to evaluate the specific component of fear. This includes the fear of pain questionnaire-III (FPQ-III), consisting of fear of “severe,” “minor,” and “medical” pain, which has been developed to assess an individual’s fear of a variety of different stimuli that may induce pain [10]. “Medical” pain, one of the subscales of FPQ-III, also includes a number of items that represent painful stimuli in a medical setting. The fear of dental pain questionnaire has been developed to understand the role of fear and pain in the context of dental treatment and a patient’s well-being [11]. However, to our knowledge, an instrument or questionnaire which assesses fear of acupuncture treatment has not been developed yet.

The development of Acupuncture Fear Scale would be useful to deal with the fear of acupuncture-induced pain and to minimize fear-induced adverse events in a clinical setting. The purpose of this study was to develop an instrument which assessed the acupuncture fear and then conduct a survey to test the reliability and validity of the instrument. We obtained reliable information regarding the structure of the response patterns of the Acupuncture Fear Scale. We also tested construct validity by correlating the score of Acupuncture Fear Scale to the ratings of FPQ-III.

2. Methods

2.1. Development of the Acupuncture Fear Scale Questionnaire.

We used a structured interview in which responses are opened to a set of structured questions. This format was useful in reducing the interviewer’s bias and obtaining expansive information from the interviewees. The interviewees ($N = 50$) were asked to describe what they felt afraid of when treated with acupuncture. The answers were roughly categorized into two issues: sensation of pain and possible adverse events. Based on this tendency and qualitative descriptions, four experts—two in the field of acupuncture and one for each Korean language and cognitive psychology—were asked to create the items which describe acupuncture treatment-related situations that may induce fear. The contents of all items were judged for clarity, difficulty, and relevance among them, resulting in a list of 16 items (Table 1). All the experts reviewed the expressions of the 16 items from differing points of view. Five-point Likert-type scales from 1 (not at all) to 5 (extremely) were used. The Korean version of the acupuncture fear scale was translated into English using standardized forward and backward translation procedures [12].

2.2. Participants. A total of 275 participants (male = 74, female = 201) were recruited, and all subjects were between 14 and 66 years old (mean age = 35.15, SD = 12.87). Exclusion criteria were any kind of cognitive problems. People who were working or majoring in the medical field were excluded as well. Thirty-three test-retest participants were randomly chosen out of these 275 questionnaire participants. They received a detailed explanation of the questionnaire, and written consent was obtained. Basic demographic data such as age and gender were acquired from all participants by the questionnaire. This investigation was conducted in

TABLE 1: The 16 items of the acupuncture fear scale.

Acupuncture fear scale items
AFS1. Seeing an acupuncture needle
AFS2. Seeing an acupuncture needle puncturing my skin
AFS3. Seeing an acupuncture needle puncturing another person’s skin
AFS4. The stinging sensation of an acupuncture needle puncturing my skin
AFS5. An acupuncture needle entering my body’s flesh
AFS6. Acupuncture treatment on my face
AFS7. Acupuncture treatment on my hands and/or feet
AFS8. Acupuncture treatment on my torso
AFS9. The possibility of having acupuncture needles in the wrong areas
AFS10. The possible occurrence of unpleasant sensation due to acupuncture treatment
AFS11. The possible occurrence of nerve damage due to acupuncture treatment
AFS12. Persistent pain possibly occurring after acupuncture treatment
AFS13. The possible occurrence of bleeding due to acupuncture treatment
AFS14. The possibility of bruising due to acupuncture treatment
AFS15. The possibility of an infection occurring due to acupuncture treatment
AFS16. The possibility of damage to other parts of my body (parts of the body not exposed to acupuncture treatment) due to acupuncture treatment

accordance with the guidelines of the human subjects committee of Kyung Hee University, Seoul, Republic of Korea.

2.3. Procedures. All participants were asked to fill out the Acupuncture Fear Scale questionnaire on their own. All participants completed the questionnaire in less than 5 minutes. The test-retest data of 33 random subjects were obtained in the same manner five weeks later.

In order to test the validity of the Acupuncture Fear Scale, all participants also filled out the questions on the FPQ-III which is a 30-item self-report questionnaire assessing an individual’s fear of a variety of stimuli that may produce pain. The FPQ-III includes three subscales, severe pain, minor pain, and medical pain, and it has been reported to provide evidence for the validity and utility of the FPQ-III [10].

2.4. Statistical Analysis. We evaluated the internal consistency of the Acupuncture Fear Scale by computing item-total correlations and Cronbach’s α . Spearman’s rank correlation coefficients were calculated to determine test-retest reliability. The paired sample t -test was also implemented to complement the correlation coefficient.

To test construct validity, we performed a principal component analysis to explore the potential number and characteristics of the domains. Varimax rotation was performed to more specific response patterns present, with an eigen

TABLE 2: Demographics of the study's participants ($N = 275$).

	Number (%)
Gender	
Women	201 (73.09)
Men	74 (26.91)
Prior acupuncture experience (within the last 5 years)	
Never	103 (37.45)
1–3 times	79 (28.73)
4–6 times	26 (9.45)
7–9 times	9 (3.27)
≥ 10 times	45 (16.36)
Pain-related conditions	
Yes	68 (24.73)
No	194 (70.55)

*Total sample 275, within cells totals may not sum to 275 because of missing data.

value greater than one as the criterion. We also evaluated the construct validity by correlating scores on the Acupuncture Fear Scale and FPQ-III. Data analyses were performed using the Statistical Package for Social Sciences for Windows 18.0 (SPSS, Chicago, IL, USA). Statistical significance level was set for $P < 0.05$.

3. Results

3.1. Sample Characteristics. A total of 275 subjects (mean age: 35.15 ± 12.87) participated in this survey (Table 2) where seventy-three percent were females. One hundred and three participants ($N = 103$, 37.5%) had no previous experience of acupuncture treatment, and one hundred and seventy-two participants ($N = 172$, 62.5%) had previous experience of acupuncture treatments within the past five years. Sixty-eight participants ($N = 68$, 24.7%) had pain-related conditions.

3.2. Properties of the Scale. The response patterns to the 16-item Acupuncture Fear Scale of all 275 participants are shown in Table 3. The score of the final instrument ranged from 16 to 68 of a possibility from 16 to 80. The mean was 36.63, with a standard deviation of 11.57, and a median of 36.

3.3. Internal Consistency. Cronbach's α was 0.935 for the mean of the item total. It did not become greater when any of the 16 items were removed. Each item had an item-total correlation value greater than 0.5, and five items (AFS4, 5, 7, 8, and 10) had correlation values greater than 0.7 (Table 4).

3.4. Test-Retest Reliability. There was no significant difference in the first test and the second test of any item in the Acupuncture Fear Scale. The retest was conducted 5 weeks after the first test. All items were not significantly different at $P < 0.05$ (Table 5). All items exhibited high Spearman's rank correlation coefficients ranged from 0.565 to 0.797.

3.5. Factor Analysis. The 16 items were subjected to factor analysis to examine the factorial validity of the Acupuncture Fear Scale using principal component extraction and varimax rotation with an Eigen value over 1.0 as the criterion. The two-factor solution explained an acceptable amount of variance (67.741%) in the response (Table 6). Factor 1 (entitled as "sensation of pain") was made up of 8 items with factor loadings of 0.675 to 0.873, which accounted for 51.296% of variance. Factor 2 (entitled as "possible adverse events") consisted of the remaining 8 items with factor loadings of 0.652 to 0.846, which accounted for 16.445% of the variance. When we evaluated the internal consistency of the two factors, Cronbach's α was 0.939 and 0.915 for the two factor scores, respectively.

3.6. Validity. To test construct validity, a correlation analysis was conducted between the Acupuncture Fear Scale and FPQ-III questionnaire (Table 7). The total of Acupuncture Fear Scale was positively correlated with the total of FPQ-III ($r = 0.423$, $P < 0.001$). Factor 1 of the Acupuncture Fear Scale ($r = 0.413$, $P < 0.001$), that is, sensation of pain, exhibited greater correlations with the total of FPQ-III than factor 2 of the Acupuncture Fear Scale ($r = 0.326$, $P < 0.001$), that is, adverse events. In addition, the Acupuncture Fear Scale showed highest correlations with the subscale "fear of Medical pain" among the three subscales of the FPQ-III ($r = 0.498$, $P < 0.001$).

3.7. Subgroup Analysis. We also looked at the influence of factors such as gender, acupuncture experience, and diseases with pain symptom and compared answers to the Acupuncture Fear Scale based on those factors. Female participants scored higher on the Acupuncture Fear Scale compared to male participants (38.14 ± 11.69 versus 32.51 ± 10.23 , $t = 3.656$, $P < 0.001$). Participants without acupuncture experience also showed higher scores on the Acupuncture Fear Scale compared to those with acupuncture experience (40.60 ± 11.15 versus 34.53 ± 10.79 , $t = 5.262$, $P < 0.001$). Younger participants (age < 40) exhibited higher scores on the Acupuncture Fear Scale compared to older participants (age > 40) (37.81 ± 11.87 versus 34.52 ± 10.40 , $t = 2.292$, $P < 0.001$). There were no significant differences between normal participants and patients with pain (35.52 ± 11.42 versus 35.53 ± 10.47 , $t = 0.626$, $P > 0.532$).

4. Discussion

Fear can be a major impediment to apply necessary health care measures, and it is important that reliable and valid assessment tools are available to measure the cause and extent of a fear [2]. Several questionnaires have been developed in the past, such as the fear of dental pain questionnaire [11] or the fear of pain questionnaire [10]. However, there has not been an instrument which assessed the fear of acupuncture treatment, even though acupuncture became increasingly important and popular for the treatment of pain in the past years [5]. The present study was designed to systematically develop an instrument which assesses fears of acupuncture and also test the reliability and validity of this instrument.

TABLE 3: The response patterns to the acupuncture fear scale ($N = 275$).

Item	Not at all (1)	A little (2)	Fair amount (3)	Very much (4)	Extremely (5)
AFS1	82 (29.8)	111 (40.4)	58 (21.1)	20 (7.3)	3 (1.1)
AFS2	73 (26.5)	105 (38.2)	57 (20.7)	31 (11.3)	7 (2.5)
AFS3	115 (41.8)	85 (30.9)	50 (18.2)	22 (8.0)	3 (1.1)
AFS4	67 (24.4)	116 (42.2)	51 (18.5)	38 (13.8)	3 (1.1)
AFS5	71 (25.8)	113 (41.1)	50 (18.2)	36 (13.1)	5 (1.8)
AFS6	14 (5.1)	63 (22.9)	70 (25.5)	87 (31.6)	41 (14.9)
AFS7	82 (29.8)	106 (38.5)	60 (21.8)	23 (8.4)	4 (1.5)
AFS8	75 (27.3)	106 (38.5)	69 (25.1)	24 (8.7)	1 (0.4)
AFS9	26 (9.5)	88 (32.0)	87 (31.6)	62 (22.5)	12 (4.4)
AFS10	67 (24.4)	102 (37.1)	69 (25.1)	31 (11.3)	5 (1.8)
AFS11	47 (17.1)	97 (35.3)	67 (24.4)	47 (17.1)	17 (6.2)
AFS12	78 (28.4)	104 (37.8)	61 (22.2)	26 (9.5)	5 (1.8)
AFS13	102 (37.1)	105 (38.2)	46 (16.7)	21 (7.6)	0 (0)
AFS14	108 (39.3)	117 (42.5)	34 (12.4)	14 (5.1)	2 (0.7)
AFS15	63 (22.9)	92 (33.5)	67 (24.4)	42 (15.3)	11 (4.0)
AFS16	73 (26.5)	108 (39.3)	65 (23.6)	26 (9.5)	3 (1.1)

AFS: acupuncture fear scale, number (percentage).

TABLE 4: Internal consistency of the acupuncture fear scale ($N = 275$).

Item	Score (mean \pm SD)	Item-total correlation
AFS1	2.09 \pm 0.95	0.686
AFS2	2.25 \pm 1.05	0.673
AFS3	1.96 \pm 1.01	0.625
AFS4	2.25 \pm 1.01	0.715
AFS5	2.24 \pm 1.04	0.753
AFS6	3.28 \pm 1.13	0.628
AFS7	2.13 \pm 0.98	0.742
AFS8	2.16 \pm 0.94	0.737
AFS9	2.80 \pm 1.03	0.660
AFS10	2.29 \pm 1.02	0.735
AFS11	2.60 \pm 1.14	0.659
AFS12	2.18 \pm 1.01	0.692
AFS13	1.95 \pm 0.92	0.637
AFS14	1.85 \pm 0.88	0.641
AFS15	2.44 \pm 1.12	0.515
AFS16	2.19 \pm 0.97	0.560

AFS: acupuncture fear scale, *Cronbach's α coefficient of the total is 0.935; each item scored by the patient is on a 5-point Likert scale, from 1 (not at all) to 5 (extremely).

To test its reliability, we evaluated internal consistency and test-retest reliability in this study. Cronbach's α is believed to indirectly indicate the degree to which a set of items or variables measure a single unidimensional latent construct. It generally requires a reliability of 0.70 or higher. In the present study, Cronbach's α coefficient for the Acupuncture Fear Scale reveals strong internal consistency, with a value of 0.935, indicating a reliable measurement of Acupuncture Fear Scale. Furthermore, Spearman's rank correlation coefficient ranged from 0.565 to 0.797 ($P < 0.001$), suggesting that all

items of the Acupuncture Fear Scale have a good test-retest reliability. As all the items of the Acupuncture Fear Scale revealed good internal consistency and test-retest reliability, we can conclude that it is a reliable instrument with a good repeatability and reproducibility.

For the validity of the proposed Acupuncture Fear Scale, we evaluated a correlation analysis between the Acupuncture Fear Scale and the FPQ-III questionnaire. In the current study, we demonstrated that the total of the Acupuncture Fear Scale revealed a positive correlation with the total of the FPQ-III ($r = 0.423$) and the highest correlation with the subscale fear of "medical" pain among three subscales of the FPQ-III ($r = 0.498$). Our results were consistent with the previous finding that fear of dental pain questionnaire exhibited the highest correlation with the subscale fear of "medical" pain of the FPQ-III [11]. It is speculated that items of the Acupuncture Fear Scale contain fear of pain associated with the general medical settings. We also found that the subscale of acupuncture fear, the sensation of pain subscale ($r = 0.413$), exhibited greater correlations with the total of the FPQ-III than the other subscale of acupuncture fear, adverse events subscale ($r = 0.326$). It might be deduced that the sensation of pain subscale could more directly represent the degree of fear of pain than the possible adverse event subscale.

When we asked participants to describe what they were afraid of regarding acupuncture treatment in our preliminary study, most of the answers were narrowed down to two categories: sensation of pain and possible adverse events. In order to examine the factorial validity of the acupuncture fear scale, we conducted a principal component analysis, which revealed two factors accounting for 67.7% of the variance. Factor 1 is made up of 8 items related with sensation of pain, which explained 51.3% of variance. Factor 2 consists of 8 items associated with possible adverse events, which explained 16.4% of variance. These results indicate that most of the

TABLE 5: Test-retest reliability of the acupuncture fear scale ($N = 33$).

Item	Score in the 1st test (mean \pm SD)	Score in the 2nd test (mean \pm SD)	P value*	Spearman's rank coefficient (95% CI)
AFS1	2.12 \pm 0.99	2.12 \pm 1.08	1.000	0.597 (0.331, 0.688)**
AFS2	2.48 \pm 1.09	2.33 \pm 1.16	0.344	0.680 (0.471, 0.829)**
AFS3	2.00 \pm 1.03	2.18 \pm 1.07	0.245	0.649 (0.416, 0.774)**
AFS4	2.42 \pm 1.12	2.24 \pm 0.90	0.280	0.576 (0.299, 0.656)**
AFS5	2.39 \pm 1.05	2.27 \pm 0.91	0.441	0.598 (0.332, 0.690)**
AFS6	3.64 \pm 1.14	3.42 \pm 1.09	0.109	0.781 (0.690, 1.048)**
AFS7	2.33 \pm 1.11	2.33 \pm 1.11	1.000	0.797 (0.732, 1.090)**
AFS8	2.12 \pm 0.93	2.27 \pm 0.98	0.169	0.790 (0.714, 1.071)**
AFS9	2.94 \pm 1.09	3.03 \pm 1.07	0.500	0.750 (0.615, 0.973)**
AFS10	2.24 \pm 0.97	2.45 \pm 1.12	0.214	0.586 (0.314, 0.672)**
AFS11	2.70 \pm 1.26	2.76 \pm 1.23	0.690	0.759 (0.636, 0.994)**
AFS12	2.21 \pm 1.22	2.30 \pm 1.10	0.598	0.648 (0.414, 0.772)**
AFS13	1.97 \pm 1.02	1.97 \pm 0.92	1.000	0.569 (0.288, 0.646)**
AFS14	2.06 \pm 1.06	2.12 \pm 0.93	0.712	0.565 (0.282, 0.640)**
AFS15	2.33 \pm 1.22	2.36 \pm 1.22	0.851	0.716 (0.542, 0.899)**
AFS16	2.27 \pm 1.15	2.18 \pm 1.04	0.521	0.736 (0.584, 0.942)**

AFS: acupuncture fear scale; *paired sample t -test was used; **test-retest reliability is reported with the Spearman's rank correlation coefficient; all items were highly significant at $P < 0.001$.

TABLE 6: Factor variance explained and item factor loadings for the final solution.

Item	Factor loadings	Variance explained (%)
Factor 1: sensation of pain		51.296
AFS1	0.856	
AFS2	0.873	
AFS3	0.764	
AFS4	0.849	
AFS5	0.840	
AFS6	0.675	
AFS7	0.828	
AFS8	0.784	
Factor 2: possible adverse events		16.445
AFS9	0.686	
AFS10	0.710	
AFS11	0.810	
AFS12	0.841	
AFS13	0.735	
AFS14	0.652	
AFS15	0.773	
AFS16	0.846	

AFS: acupuncture fear scale.

participants were afraid of being treated with acupuncture because of the inevitable sensation of pain and possible adverse events. This has been also taken into account by the two subscales of the acupuncture fear scale in the present study, including these two factors: sensation of pain and possible adverse events.

TABLE 7: Correlation coefficients between the acupuncture fear scale and the fear of pain questionnaire ($N = 275$).

	FPQ-III total	FPQ-III severe	FPQ-III minor	FPQ-III medical
AFS total	0.423	0.279	0.346	0.498
AFS factor 1	0.413	0.265	0.311	0.519
AFS factor 2	0.326	0.223	0.294	0.349

AFS: acupuncture fear scale; all correlations were significant at $P < 0.001$.

The results of this study lead to further implications. As acupuncture stimulation can give rise to feelings of faintness upon exposure to feared stimuli, for example, vasovagal syncope in needle-phobic patients, fear of acupuncture pain should be carefully managed in an acupuncture clinic [13]. It is reported that an acupuncture-induced sensation of pain and physiological responses were altered by cognitive manipulation regarding acupuncture modalities [7]. Thus, fear of acupuncture-evoked pain might be able to be alleviated through cognitive therapy and other methods [14]. Regarding the fear of possible adverse events, it might be necessary to provide people with exact health information of adverse events regarding acupuncture treatment, in order to correct exaggerated aversion to acupuncture. There are studies showing that acupuncture is well known to be a safe therapy with a low risk of adverse events in clinical practice [5]. Understanding the patients' fear of acupuncture treatment in a clinical setting would be also useful to minimize fear-induced adverse events and to build a more desirable doctor-patient relationship.

There are a few limitations which need to be considered in this study. As the Acupuncture Fear Scale was originally developed in Korean and translated into English, the English version of this instrument needs to be further tested in

more diverse populations. In addition, because of the higher cultural familiarity and acceptance of acupuncture in East Asian countries, the results might differ in western countries, and therefore, the Acupuncture Fear Scale should be also validated in western countries. As the high value of Cronbach's alpha (>0.90) may suggest unnecessary duplication of content across items [15], it is necessary to develop a short version of the Acupuncture Fear Scale in the future. Since the present study included varying population samples, these various characteristics of populations could be possible confounding factors. In the subgroup analysis, we found that the Acupuncture Fear Scale varied significantly by their gender, age, and experience of acupuncture treatment. In order to quantify the prevalence of a patient's fear of acupuncture score, it is required to compare different populations and subpopulations or to track population shifts over time with a larger number of populations in the future study. It is recommended that the Acupuncture Fear Scale is used to screen all the acupuncture treatment patients for fear of acupuncture to enable a more tailored and effective acupuncture treatment experience.

In sum, all the items of the Acupuncture Fear Scale revealed good internal consistency and test-retest reliability. Principal component analysis revealed two factors, such as sensation of pain and possible adverse events. The Acupuncture Fear Scale has proven to be well validated. Therefore, we can conclude that the Acupuncture Fear Scale we have developed in the current study is a valid and reliable instrument to assess fear of acupuncture treatment. Moreover, we suggest that this questionnaire would be a clinically useful tool to prevent possible adverse events of acupuncture and an important instrument to understand the complex social-behavioral components of the acupuncture modality.

Appendix

Acupuncture Fear Scale

Name:

Date:

Instructions. The following items describe possible reasons that would make you fear receiving acupuncture treatment. Please look at each item and think about how fearful you are with such reasons. Circle one rating per item to rate your fear of acupuncture treatment in relation to each reason.

(1) Seeing an acupuncture needle.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(2) Seeing an acupuncture needle puncturing my skin.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.

(4) Very much.

(5) Extreme.

(3) Seeing an acupuncture needle puncturing another person's skin.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(4) The stinging sensation of an acupuncture needle puncturing my skin.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(5) An acupuncture needle entering my body's flesh.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(6) Acupuncture treatment on my face.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(7) Acupuncture treatment on my hands and/or feet.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(8) Acupuncture treatment on my torso.

- (1) Not at all.
- (2) A little.
- (3) Fair amount.
- (4) Very much.
- (5) Extreme.

(9) The possibility of having acupuncture needles in the wrong areas.

- (1) Not at all.
- (2) A little.

- (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (10) The possible occurrence of unpleasant sensation due to acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (11) The possible occurrence of nerve damage due to acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (12) Persistent pain possibly occurring after acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (13) The possible occurrence of bleeding due to acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (14) The possibility of bruising due to acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.
- (15) The possibility of an infection occurring due to acupuncture treatment.
- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.

- (16) The possibility of damage to other parts of my body (parts of the body not exposed to acupuncture treatment) due to acupuncture treatment.

- (1) Not at all.
 (2) A little.
 (3) Fair amount.
 (4) Very much.
 (5) Extreme.

Authors' Contribution

H.-S. Kim and Y.-J. Kim equally contributed to this study as cofirst authors.

Disclosure

The authors declare that no competing financial interests or conflict of interests exist. The funders had no role in the study design, data collection and analysis, and the decision to publish the paper.

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Research Article

Modifying Bodily Self-Awareness during Acupuncture Needle Stimulation Using the Rubber Hand Illusion

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Background. The rubber hand illusion (RHI) is an experimental paradigm that manipulates important aspects of body self-awareness. **Objectives.** We were interested in whether modifying bodily self-awareness by manipulation of body ownership and visual expectations using the RHI would change the subjective perception of pain as well as the autonomic response to acupuncture needle stimulation. **Methods.** Acupuncture needle stimulation was applied to the real hand during the RHI with (experiment 1) or without (experiment 2) visual expectation while measuring concurrent autonomic changes such as the skin conductance response (SCR). Subjective responses such as perception of the RHI and perceived pain were measured by questionnaires. **Results.** In experiment 1, the amplitude of the increase in SCR was visibly higher during the synchronous session compared with that of the asynchronous session. In experiment 2, the amplitude of the increase of SCR was lower for the synchronous session compared with that for the asynchronous session. Comparing these two experiments, the visual expectation of needle stimulation produced a greater autonomic response to acupuncture stimulation. **Conclusions.** Our findings suggest that the sympathetic response to acupuncture needle stimulation is primarily influenced by visual expectation rather than by modifications of body ownership.

1. Introduction

What is the role of body self-awareness or self-consciousness in a clinical context? Imagine yourself receiving acupuncture needle treatment. Would you expect a difference in your perception depending on whether you looked at the site of needle penetration? Next, imagine a patient with asomatognosia or somatoparaphrenia (a patient who cannot feel his/her body or does not recognize parts of the body as his/her own) receiving moxibustion on a part of the body that is not perceived as self. Would you expect the treatment to have the same effects as with a normal patient who could feel all of the sensations of heat, touch, and pain on the skin?

These questions clearly demonstrate the importance of body awareness or bodily self-consciousness in clinical treatments. Body awareness and self-consciousness are thought

to include both an attentional focus (e.g., visual attention) and awareness of bodily sensations (e.g., proprioception and interoception) [1–4]. Although it is difficult to find unquestionably clear definitions, currently, aspects of bodily self-awareness or self-consciousness are typically investigated by modifying participants' senses of body ownership and embodiment [1, 5, 6]. Body ownership is defined as the sense that the body or body part belongs to oneself (i.e., self-attribution) [7, 8], whereas a sense of embodiment is defined as the experience of being within the borders of one's body (i.e., self-localization) [5, 6, 8, 9]. These senses can be experimentally modified using different perceptual illusions such as the out-of-body illusion, the body-swapping illusion, or most commonly, the rubber hand illusion (RHI) [10, 11].

The RHI is evoked when a subject watches a rubber hand being stroked or touched while their own hand is

stroked or touched simultaneously, leading to the experience of additional body ownership such that the rubber hand also “feels like one’s own hand” [12]. This illusion is thought to arise due to the multimodal integration of the senses of vision, somatosensation (e.g., touch), and proprioception (e.g., position) in the brain [13]. The visual stimulation of the rubber hand matching the tactile stimulation of the actual hand leads to a stable and strong experience of illusory body ownership (embodiment) of the rubber hand, accompanied by a sense of body disownership (disembodiment) of the actual hand. Evidence for illusory body ownership has been found in functional magnetic resonance imaging and skin conductance response (SCR) studies, where threatening the rubber hand during the illusion elicits a measurable cortical anxiety response, indicating that the brain has indeed accepted the rubber hand as part of its own body [14, 15]. Thus, the RHI has been repeatedly shown to be an effective experimental tool for manipulating the sense of body ownership and thereby modifying aspects of bodily self-awareness [16, 17].

Acupuncture, an ancient East Asian therapeutic technique, uses needles to penetrate the skin and needle manipulation to stimulate the body [18]. The specific perception of acupuncture treatment is traditionally termed the *deqi* sensation and includes a sense of soreness and aching that can be experienced as dull pain [19, 20]. Sympathetic responses following acupuncture needle stimulation indicate enhanced SCR in response to both real and sham acupuncture [21, 22]. The clinical effects of acupuncture treatment (e.g., analgesic effects) have been investigated and confirmed in a number of studies in animals and in clinical populations, and many researchers have contributed to identifying the brain processes activated by acupuncture [23]. Nevertheless, the underlying mechanisms of acupuncture and its mode of action are unclear, and psychosocial and contextual factors such as expectation, attention, and body schema may play important roles in the clinical effects of acupuncture [24]. However, no studies are available in which different aspects of bodily self-awareness have been actively manipulated and investigated.

In the current study, we were interested in actively manipulating some aspects of bodily self-awareness through modification of body ownership and visual expectations using the RHI. How would physiological and subjective responses to acupuncture needle stimulation differ during embodied compared with disembodied body conditions? How would these responses differ based on visual feedback during acupuncture stimulation? The aim of our study was to investigate whether these modifications of bodily self-awareness result in different psychophysiological responses when participants are treated with acupuncture needle stimulation.

2. Methods and Materials

2.1. Participants. Thirty-one participants (age, 19–29 years; 16 males and 15 females) recruited by advertisement from the general population of students, staff, and visitors to

Kyung Hee University, Seoul, Republic of Korea, participated in the experiments. Nineteen participants (nine males and 10 females) took part in experiment 1, and 12 participants (seven males and five females) took part in experiment 2. The participants received 10,000 Korean Won (approximately 10 USD) for reimbursement. All participants received a detailed explanation of the study, and written informed consent was obtained. This investigation was conducted in accordance with the guidelines of the human subjects committee of Kyung Hee University.

2.2. Experimental Design. A rubber hand was placed in front of each participant while his/her left hand was hidden from sight. The setup followed standard procedure and was designed almost identically to a previous study [25] (Figure 1). Both experiments followed a within-participants repeated-measures design, and the independent variable was a synchronous versus an asynchronous brush touch on the hand. In both experiments, participants received acupuncture needle stimulation on the hidden (real) left hand immediately after commencing the RHI in the synchronous condition and the control trial in the asynchronous condition. Experiments 1 and 2 were designed almost identically except that in experiment 1, the participants saw the acupuncture needle stimulation applied to the rubber hand in a synchronized and colocalized manner as it was applied to the real hand (visual expectation condition), whereas in experiment 2, they received no visual feedback at all (no visual expectation), meaning that they did not know when or where the acupuncture needle would penetrate their real hand. Thus, a between-subjects design was additionally tested with visual cue as the independent variable (presence or absence of acupuncture needle penetration of the rubber hand at the time of acupuncture needle penetration of the real hand).

2.3. Procedures

2.3.1. Rubber Hand Illusion Induction. The participants were told to fixate on the rubber hand (Korean Prosthetic Limbs Research Institute, Seoul, Korea) and not to look elsewhere. They were also not allowed to move any of their fingers. A small tube was preinstalled on the rubber hand and the real hand in experiment 1, and the acupuncture needle stimulation was followed by tapping the inserted needle on the tube. Two small paintbrushes stroked the rubber hand and the participant’s hidden real left hand as synchronously as possible under one condition (synchronous condition) and asynchronously under the other (asynchronous condition). After 300 seconds of brush stroking, an acupuncture needle was applied to the real hand by inserting the needle into the skin using the small tube. The same procedure was repeated twice, once with synchronous brush stroking and once with asynchronous brush stroking. The order was randomized, so participants were randomly assigned to one of two groups; that is, they received either the synchronous or the asynchronous session first. The participants had to wait 10–15 minutes between the two sessions.

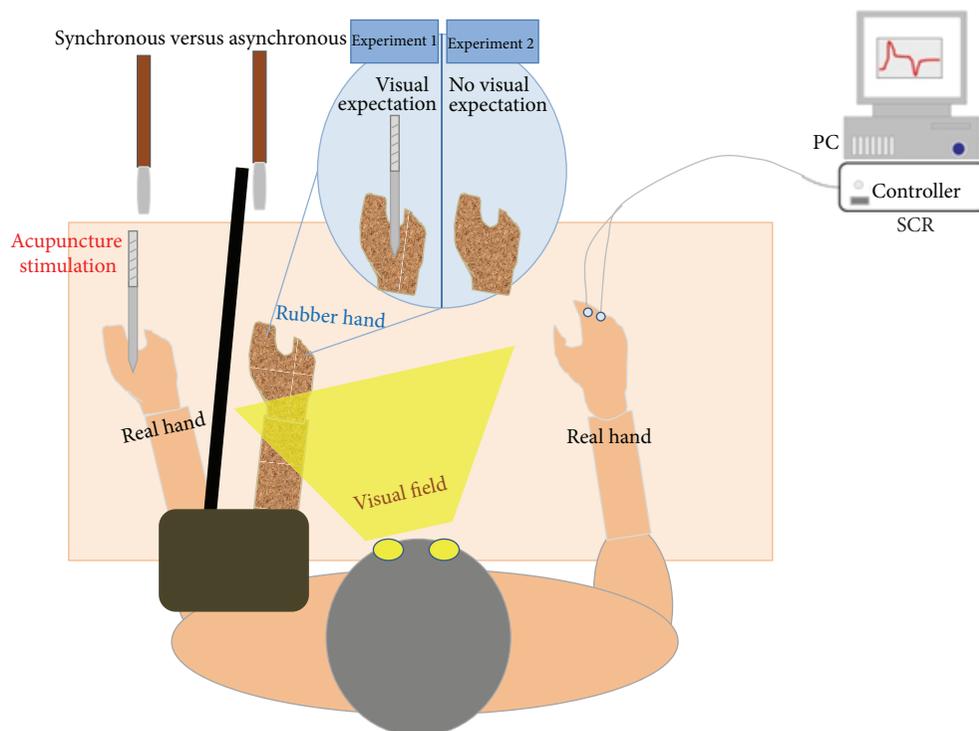


FIGURE 1: Schematic drawing of the experimental setup illustrating the rubber hand illusion with (experiment 1) and without (experiment 2) the visual expectation when participants received acupuncture stimulation on their real hand. Two small paintbrushes stroked the rubber hand and the participant's hidden real left hand as synchronously as possible under one condition (synchronous condition) and asynchronously under the other (asynchronous condition).

2.3.2. Acupuncture Stimulation. Participants were first informed about the experiment and told that physiological data including skin conductance measurements would be taken while they received acupuncture treatment. The participants were told that they would randomly receive either real acupuncture treatment (with needle penetration of the skin) or sham acupuncture treatment (without needle penetration) for each trial to produce uncertainty as to whether they would receive real acupuncture needle stimulation. The Park sham needle was explained and demonstrated in front of all participants. All participants were told that they would participate twice in the same procedure. Then, the participants were led into the experimental room and seated with their left and right arms on a table. They all received acupuncture stimulation with the real needle at acupoint LI4, on the dorsum of the left hand, radial to the midpoint of the second metacarpal bone, in the left hand in front of a curtain. The needles (Dongbang Acupuncture, Inc., Gyeonggi-do, Korea) were 0.25 mm in diameter and 40 mm long. The needles for the demonstration of the sham procedure (Park sham needle; Dongbang Acupuncture) were identical in size and appearance.

2.3.3. Skin Conductance Response Measurement. Two electrodes were placed on the left hand to measure skin

conductance. Skin conductance was recorded from the medial phalanges of the second and third digits of the left hand, with 0.05 M NaCl paste as the electrolyte. Skin conductance was digitized and recorded with a galvanic skin response amplifier (GSR Amp ML116; ADInstruments, Bella Vista, Australia) and a high-performance data acquisition PowerLab 8/30 system (ML870; ADInstruments). Each half-second was deviated from a 1 s baseline prior to inserting the acupuncture needle (cue onset) and averaged across the 15 s of each acupuncture session to assess responses during and after acupuncture treatment, resulting in change scores that reflected increases or decreases from baseline.

2.3.4. Rubber Hand Illusion Questionnaire. After finishing each session (synchronous and asynchronous), the participants reported their perception of the RHI using the Rubber Hand Illusion Perception Scale, which includes nine questions [12]. The participants were also required to give detailed answers to an open-end questionnaire asking about their experience and changes in their perception during the experiment.

2.3.5. Self-Reported Pain Rating. Subjective pain ratings were obtained immediately after each acupuncture stimulation session. The participants evaluated acupuncture-induced pain using a 100 mm visual analogue scale.

2.4. Data Analysis. All values are expressed as mean \pm standard error. The RHI ratings during the synchronous brush stroking (induction of illusory body ownership) and the asynchronous brush stroking session (control) were compared using a paired *t*-test. The subjective pain ratings and the SCR responses were analyzed by mixed between-group analyses of variance (ANOVAs) with one (time) repeated-measure factor. The level of significance was set at 0.05 for all analyses. Statistical analyses were performed using the Statistical Package for Social Sciences for Windows 17.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Self-Assessments of the Rubber Hand Illusion. A significant difference was observed in the self-report RHI questionnaire between the synchronous and asynchronous brush stroking sessions in both experiment 1 (visual expectation condition) (1.8 ± 0.2 versus -0.3 ± 0.3 , $t = 5.883$, $P < 0.001$, Figure 2(a)) and experiment 2 (no visual expectation condition) (1.8 ± 0.3 versus -0.4 ± 0.3 , $t = 5.933$, $P < 0.001$, Figure 2(b)).

3.2. Self-Assessments of Pain. No significant differences were observed between the synchronous and asynchronous brush stroking sessions in self-reported pain in experiment 1 (visual expectation condition) (4.1 ± 0.6 versus 3.5 ± 0.6) or experiment 2 (no visual expectation condition) (2.8 ± 0.6 versus 2.5 ± 0.6) ($F_{[3,46]} = 1.215$, $P > 0.315$).

3.3. Skin Conductance Response. The SCR recordings are presented as the mean SCR change over time (Figure 3). A 4×30 repeated-measures ANOVA was conducted for each SCR recording during acupuncture stimulation, with condition ((synchronous or asynchronous session) \times (with or without visual expectation condition)) as the between-subjects factor and time (measured every 15 s) as the within-subjects factor. The repeated-measures ANOVA showed a significant effect of time ($F_{[3,116]} = 3.406$, $P < 0.001$) and a condition \times time interaction effect ($F_{[3,116]} = 1.610$, $P < 0.001$).

4. Discussion

We modified different aspects of bodily self-awareness using the RHI and observed psychophysiological responses to acupuncture needle stimulation. Bodily self-awareness was manipulated by modifying body ownership (synchronous and asynchronous brush stroking sessions) and visual expectations (with and without visual cues in experiments 1 and 2, resp.). No significant differences in subjective pain ratings were found for the modification of body ownership, but the visual expectation of needle stimulation seemed to determine the patterns of autonomic responses. The disrupted sense of body ownership appeared to notably ameliorate the increase in SCR in response to acupuncture stimulation.

4.1. Bodily Self-Awareness during Rubber Hand Illusion. This is one of the first studies applying the RHI experimental

paradigm (Figure 1) to modify aspects of bodily self-awareness to examining differential responses to acupuncture needle stimulation. Participants' answers to the RHI questionnaire confirmed the stable and successful evocation of the RHI in both experiment 1 (visual expectation condition) and experiment 2 (no visual expectation condition), as the significant differences between the synchronous and asynchronous brush stroking sessions showed (Figure 2). This result confirms that synchronous brushing of the visible rubber hand and of the participant's own hand successfully produced the sense of body ownership and embodiment for the rubber hand while inducing a disruption of body ownership and disembodiment for the real hand. These results are comparable to those of the initial study describing the RHI [12].

4.2. Pain Perception during Rubber Hand Illusion. Acupuncture needles were always applied to the real hand, implying a disruption of body ownership (disembodiment) during the synchronous sessions when the RHI was successfully evoked and a normal sense of body ownership (embodiment) during the asynchronous sessions. We wanted to observe differences in the responses to acupuncture stimulation based on the embodied and disembodied conditions. The question of what happens with the actual hand during the illusion has been a point of recent discussion [17, 26, 27]. Does inducing illusory body ownership result in disembodiment or actual disownership of the real hand? Recent evidence points toward a displacement of sensations from the actual hand, demonstrating that disembodiment induces cooling of the disembodied body part as well as desensitization to tactile stimuli [17, 27]. Furthermore, increased histamine reactivity has been reported during the RHI in the real arm, indicating a rejection of the "replaced" hand by the innate immune system, similar to autoimmune disorders [28]. Previous studies using other body illusions have also reported modulation of perceived pain when body ownership was manipulated [29, 30]. Therefore, we initially expected a difference in subjective pain perception in the disembodied hand during acupuncture stimulation. However, no significant differences were found in the subjective pain ratings across all sessions. Even more surprising was the finding that the subjective pain ratings did not significantly differ between experiments 1 and 2. Previous reports have emphasized the role of vision in bodily self-awareness and body ownership [31, 32]. For example, seeing one's own body while receiving nociceptive stimuli induces an analgesic effect, implicating the involvement of bodily self-awareness and body ownership because seeing another person's body has no effect [33]. Nonetheless, the results of a very recently published study are consistent with our results, as they also reported no differences in subjective pain perception during the RHI [26].

4.3. Autonomic Responses during Rubber Hand Illusion. Although subjective pain ratings did not differ, autonomic responses showed visible differences for the different conditions of modified bodily self-awareness. This result was also consistent with one of our previous studies in which

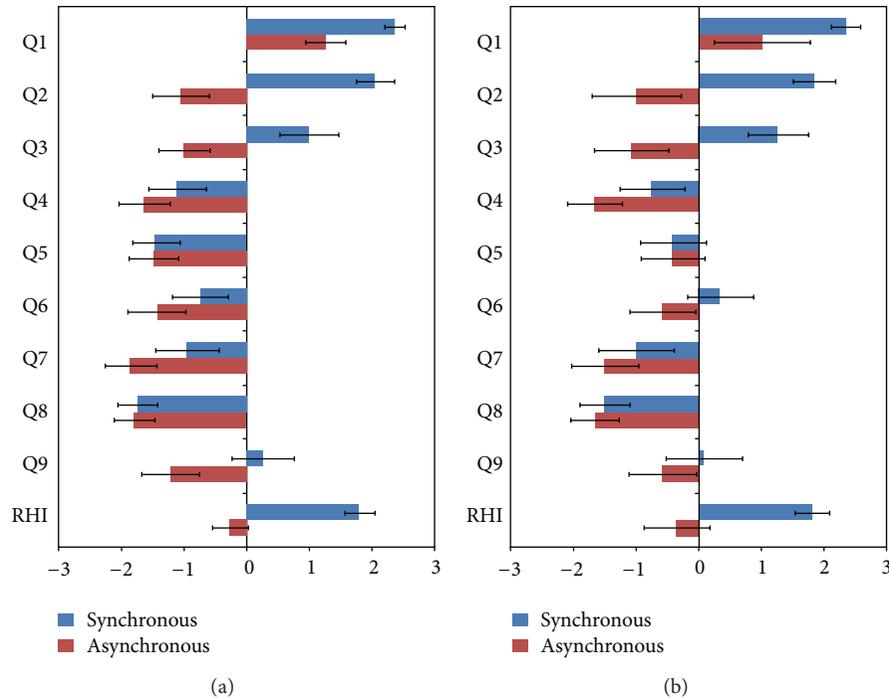


FIGURE 2: The Rubber Hand Illusion Perception Scale. Q1: It seemed as if I were feeling the touch of the paintbrush in the location where I saw the rubber hand touched. Q2: It seemed as though the touch I felt was caused by the paintbrush touching the rubber hand. Q3: I felt as if the rubber hand was my hand. Q4: I felt as if my (real) hand were drifting toward the right (toward the rubber hand). Q5: It seemed as if I had more than one left hand or arm. Q6: It seemed as if the touch I was feeling came from somewhere between my own hand and the rubber hand. Q7: It felt as if my (real) hand were turning “rubbery.” Q8: It appeared (visually) as if the rubber hand were drifting towards the left (towards my hand). Q9: The rubber hand began to resemble my own (real) hand, in terms of shape, skin tone, freckles, or some other visual feature. The first three questions (Q1–Q3) were designed to correspond to the rubber hand illusion. Mean responses to the rubber hand illusion questionnaire statements on a 7-point Likert scale ranging from “strongly disagree (–3)” to “strongly agree (+3),” with standard errors. A significant difference was observed between the synchronous and asynchronous brush stroking sessions under the visual expectation (1.8 ± 0.2 versus -0.3 ± 0.3 , $t = 5.883$, $P < 0.001$, (a)) and no visual expectation conditions (1.8 ± 0.3 versus -0.4 ± 0.3 , $t = 5.933$, $P < 0.001$, (b)). Values are mean \pm standard error.

subjective experiences and physiological responses to real and sham acupuncture stimulation differed from each other [21]. The autonomic responses depended primarily on visual expectation, but they were also influenced by body ownership (Figure 3). The increase in SCR to acupuncture stimulation was significantly higher under the visual expectation condition (experiment 1) compared with the no visual expectation condition (experiment 2). This could be due to the already reported role of vision in bodily self-awareness and body ownership [31–33]. When no vision of the acupuncture needle stimulation was provided (experiment 2), the amplitude of the increase of SCR was lower for the synchronous session compared with that for the asynchronous session (Figure 3), suggesting that sympathetic activation in response to acupuncture needle stimulation decreased in the disembodied condition. Consistent with previous studies where disruption of body ownership reduced temperature and tactile sensitivity in the disembodied hand [17], this could mean that the psychologically induced limb-specific disruption reduced the physiological autonomic response to acupuncture stimulation. When a visual expectation of the acupuncture needle stimulation existed (experiment 1),

the amplitude of the increase in SCR was visibly higher during the synchronous session compared with that of the asynchronous session (Figure 3). In this case, the participants seemed to have already allowed the incorporation of the artificial body part into their “self-representation,” as indicated by the RHI questionnaire responses, and experienced an additional “visual capture” of the acupuncture needling, therefore exhibiting overall higher sympathetic activation to acupuncture stimulation. However, viewing a needle pricking a hand strengthens the perception of pain as well as the anticipation of forthcoming pain [34]. Acupuncture needles can intensify the fear of pain. Therefore, it is also possible that the autonomic response was not specific to acupuncture needling but a more common reaction to potentially pain-related cues.

4.4. Clinical Implications of Bodily Awareness. Why would modifications to bodily self-awareness have important clinical implications? Recent research in the fields of neuroscience and neurology has shown that the conscious sense of one’s physical self is closely linked to the physiological

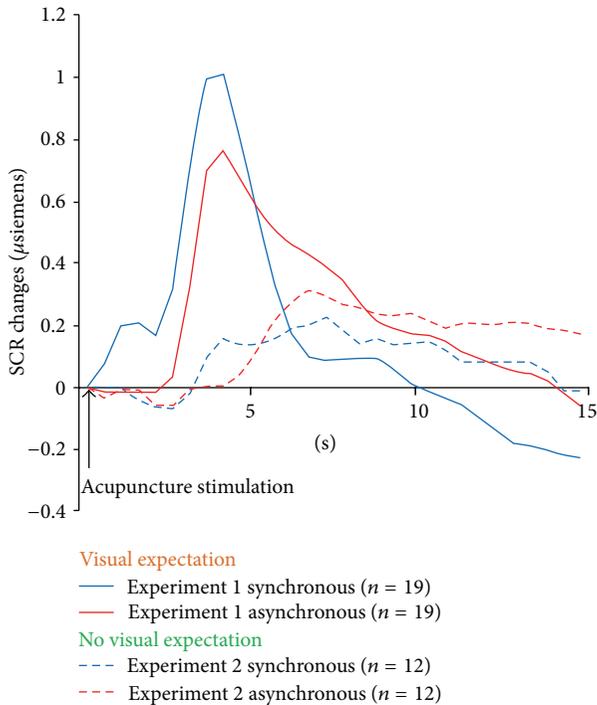


FIGURE 3: Skin conductance response (SCR) recordings are presented as the mean change in SCR over time. A significant condition ((synchronous or asynchronous session) \times (with or without visual expectation condition)) \times time effect was observed ($F_{[3,116]} = 1.610$, $P < 0.001$).

regulation of one's physical self [17, 27]. Moreover, aspects of "body awareness" have increasingly attracted the interest of researchers across many disciplines [3]. Disturbances in body awareness are thought to be related to a variety of diseases such as depression or schizophrenia, as well as to somatoform disorders and eating disorders [2]. Furthermore, individual differences in body awareness, which are closely related to symptom awareness, could possibly be one of the main factors contributing to different patient reactions to the same clinical treatment [4, 35], which has important consequences for the future of personalized medicine.

Acupuncture is not just a treatment consisting of "simple needling." Rather, it should be seen as a complex treatment comprising multimodal sensory stimulation interacting with various psychosocial factors [36]. According to this view, if acupuncture stimulation is applied, there is no single acupuncture effect but rather a total effect of acupuncture comprising different subsets of stimuli. These could include (a) bodily sensations including vision and somatosensation, as well as tactile sensation and the sensation of pain; (b) cognitive factors including attention, expectation, placebo effects, bodily self-awareness, and self-consciousness; and (c) sociocontextual factors such as perception of the clinical environment and the doctor-patient relationship. We suggest that distinguishing between the components of acupuncture effects in terms of bodily self-awareness could be a useful approach to understand the mechanisms of acupuncture treatment.

5. Conclusion

Our focus in this study was to determine how responses to acupuncture needle stimulation would be specifically influenced by modifications of bodily self-awareness. Experiments involving illusions of body ownership have made it possible to manipulate different aspects of bodily self-awareness, revealing much about the physiological and neuroanatomical underpinnings of these illusions and explaining the multisensory mechanisms behind them [5, 6, 27, 37]. Our study is the first attempt to implement these experimental procedures to modify bodily self-awareness and to test psychophysiological responses to acupuncture needle stimulation. Relating and translating the knowledge of this field to acupuncture research could be valuable for a further understanding of underlying common mechanisms. Our findings suggest a new approach to scientific investigation of the effects and mechanisms of acupuncture.

Conflict of Interests

The authors report no conflict of interests.

Acknowledgments

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Review Article

How Does Moxibustion Possibly Work?

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“Acupmoxa” is a hybrid word of “acupuncture” and “moxibustion” that more closely resembles the Chinese ideograph for this treatment. People in Western countries are more familiar with acupuncture, while moxibustion is less popular, partially due to the paucity of scientific studies. Although the evidence-based efficacy of moxibustion needs to be further clarified, the mechanisms by which moxibustion may work include temperature-related and nontemperature-related ones. Local somatothermal stimulation (LSTS), one type of moxibustion, is achieved by application of a heat source to and above the acupoint. Such mild heat stimulation of the acupoint induces little skin damage, in contrast to the burning effect of moxibustion, but does provoke mild oxidative stress in the viscera. Thus, preconditioned LSTS at the peripheral acupoints LR 14 and PC 6 of animals is able to induce visceral HSP70 expression and to protect the liver and the heart against ischemia-reperfusion injury. Nontemperature-related mechanisms include smoke, herbs, and biophysical (far infrared) stimulation. We conclude that LSTS, a remote preconditioning method, has potential clinical usefulness. However, evidence-based efficacy and safety studies involving large-scaled clinical trials are needed in order that this approach will pass muster with Western scientists.

1. Introduction

“Acupmoxa” is a hybrid word of “acupuncture” and “moxibustion” that more closely resembles the Chinese ideograph for this treatment. Acupuncture describes a procedure involving penetration of skin areas (acupoints) by thin metallic needles, which is followed by manipulating the needles manually. Moxibustion describes a technique that applies heat to acupoints by burning compressed powdered herbal material at the acupoints to be stimulated. Acupuncture or moxibustion, either alone or in combination, can be applied when treating patients with a wide range of diseases [1]. In 1980, the World Health Organization (WHO) recommended acupuncture as an effective treatment for forty-three health problems, including respiratory tract disorders, gastrointestinal disorders, eye disorders, and neuromuscular disorders [2]. People in the Western countries are more familiar with acupuncture; in contrast, moxibustion has remained less popular, perhaps partially due to the paucity of relevant scientific studies.

In contrast to the development of Western medicine, which can be traced back to Hippocrates via a clear and

distinct route, Chinese acupmoxa theory was already fully developed by the end of the 2nd century BCE. In 1972, documents written on silk scrolls in a Ma-Wang-Dui tomb sealed in 198 BCE were discovered in China. This discovery included documents that only relate to moxibustion and do not include any references to acupuncture or acupoints. The documents refer to eleven lines of channel (meridians), which suggests that the origins of moxibustion and of meridians are earlier than those of acupuncture and acupoints [3].

2. The Classification and the Efficacy of Moxibustion

Classically, moxibustion is applied to patients with the use of nonmoxa or moxa sticks, and the latter can be applied either directly or indirectly [4]. Direct moxibustion is defined as application of moxa sticks onto or above the destined acupoints on the body surface, while indirect moxibustion is the application of herbs (mugwort, ginger, etc.) between moxa sticks and the acupoints. Sometimes, moxibustion

can be applied over acupuncture needles [5], either with or without scarring, in order to improve efficacy [4, 6].

Based on descriptions in the ancient Chinese literature, the therapeutic effects of moxibustion are associated with treating chronic symptoms related to “deficiencies” and to the prevention of human disorders. Previous studies have demonstrated that moxibustion is effective when used to treat cervical vertigo [7], dysmenorrhea [8], chemotherapy-induced leucopenia [9], and various emergency conditions [10]. Much effort has been devoted to the studies of moxibustion using experimental tumor models, including with or without smoke [11], using different modes [12] and in combination with radiotherapy or taxol treatment [13, 14].

Since the late 20th century, it has been suggested that moxibustion increases fetal activity during the treatment period, cephalic presentation after the treatment period, and cephalic presentation at delivery [6, 15–17]. However, meta-analysis of a large number of investigations over the past two decades has failed to demonstrate that moxibustion effectively produces cephalic inversion during breech presentation [18–20] or is a useful treatment for stroke [21], hypertension [22], rheumatic conditions [23], ulcerative colitis [24], constipation [25], pain relief [26], and cancer support [27]. There is consensus that well-designed randomized controlled trials are needed in order to evaluate the safety and efficacy of moxibustion.

3. Possible Mechanisms of Action of Moxibustion

3.1. The Temperature-Related Mechanisms of Action of Moxibustion. As moxibustion is defined as a technique that applies heat to acupoints by burning herbal materials on the body surface, factors such as temperature, smoke, odor, and herbs are likely to be involved in the possible mechanisms by which moxibustion may work.

3.1.1. Local Somatothermal Stimulation (LSTS). Conventional application of moxibustion evokes multiple sensory stimulations, including temperature, pressure, pain, touch, and smoke stimuli. To avoid difficulties with respect to data interpretation when there are moxibustion-induced multi-sensory stimulations, Chiu et al. used temperature as the only stimulator in their series of studies (Table 1). In brief, local somatothermal stimulation (LSTS), which was compared with whole-body hyperthermia, was achieved by the application of a heat generator to and above (0.5 cm) the acupoint without any contact with the skin surface; furthermore, a fluctuating skin temperature was obtained by intermittently turning on and off the heat generator (4 minutes on and 5 minutes off for three cycles). Usually, it took 27 minutes to complete one LSTS treatment [28]. Usually, LSTS was repeatedly applied at 12-hour intervals. The fluctuation in temperature brought about by the LSTS was designed to make a temperature increase and decrease in relation to the critical point of 42°C, so that the heat-sensitive neural transmission would not be tolerated. It is important to notice that, when

there is such mild heat stimulation, no skin damage such as burning injury or nerve damage can be observed.

3.1.2. LSTS at Acupoints Relaxes the Sphincter of Oddi and the Anal Sphincter via the Neural Release of Nitric Oxide (NO). Several lines of evidence support the idea that NO plays an important role in the gastrointestinal system and acts as a neurotransmitter in nonadrenergic, noncholinergic, or “nitroergic” neurons of the peripheral nervous system [36, 37]. When LSTS was applied onto and 0.5 cm above acupoint GB 24, manometry of sphincter of Oddi (SO) showed that the tonic pressure and phasic contraction pressure of this sphincter were decreased. The LSTS-induced relaxation of the SO could not be blocked by pretreatment with atropine, phentolamine, or propranolol but could be blocked by L-NAME; furthermore the blockage could be reversed by L-arginine and not by D-arginine. These findings suggest that LSTS relaxed the SO via activation of neural L-arginine/NO pathway. The effect of LSTS on SO relaxation could be observed not only in carnivorous species (the cat) and in herbivorous species (the rabbit), but also in humans [28]. In addition, LSTS at designated acupoints (BL 36 and BL 40) was also shown to relax hypertonic anal sphincters in humans [29], possibly via the nitroergic neural release of nitric oxide [30]. The responses of the SO and anal sphincters to LSTS were found to be temperature-specific (42°C) and acupoint-specific; furthermore, the neurotransmitter was nitric oxide.

3.1.3. LSTS at Peripheral Acupoints Induces the Expression of Heat Shock Protein 70 (HSP70) in Corresponding Organs. It is noteworthy that the critical temperature for evoking NO-related sphincteric responses by LSTS is around 42°C, which is similar to the temperature used to induce heat shock protein (HSP) expression in many studies [38, 39]. To test the hypothesis that LSTS at peripheral acupoint without contact with the skin surface is able to induce HSP70 expression in the corresponding visceral organ, LSTS was applied onto and above acupoint LR 14 or acupoint PC 6, and the HSP70 gene expression in the liver and heart, respectively, was analyzed by Western blotting and RT-PCR. Acupoint LR 14 is innervated by the seventh intercostal nerve, and PC 6 is innervated by the median nerve; these have been used in traditional Chinese medicine for the treatment of hepatobiliary and heart disease, respectively. Lin et al. demonstrated that LSTS at the LR 14 acupoint induced HSP70 expression in the liver, but not in the heart. When analyzed by Western blotting and RT-PCR, the LSTS-induced HSP70 expression was determined to be *de novo* synthesis in the liver [31]. On the other hand, LSTS at the PC 6 acupoint induced *de novo* HSP70 expression in the heart, but not in the liver [32]. Taken together, these novel findings suggest that the LSTS-induced visceral HSP70 expression occurs in a meridian-specific manner.

3.1.4. Preconditioning by LSTS Protects Organs against Ischemia-Reperfusion (I/R) Injury. Since HSP70 has been reported to enhance myocardial tolerance against I/R injury [40–42], it was reasonable to postulate that preconditioning by LSTS at peripheral acupoints ought to induce visceral HSP70 expression and protect the relevant organs from subsequent I/R

TABLE 1: Effects of LSTS on peripheral acupoints on visceral functions of the corresponding organs.

Acupoints	Visceral functions	Mechanisms		References
		Regulatory molecules	Serum	
GB 24	Motility of SO ↓	NO ↑		Chiu et al., 1998 [28]
BL 40 and BL 36	Motility of anal sphincter ↓	NO ↑		Jiang et al., 1999 [29] Jiang et al., 2000 [30]
LR 14	Protects the liver from subsequent I/R injury	HSP70 ↑	I/R + LSTS versus I/R: ALT ↓ LSTS versus normal: ALT ↑ I/R + LSTS versus I/R: AST ↓ LSTS versus normal: AST ↑	Lin et al., 2001 [31]
PC 6	Protects the heart from subsequent I/R injury	HSP70 ↑	I/R + LSTS versus I/R: CPK ↓ I/R + LSTS versus I/R: CK-MB ↓	Chiu et al., 2003 [32] Tsou et al., 2004 [33]
BL 37	Protects the muscles from tourniquet-induced neuromuscular injury	ROS ↑ HSP70 ↑	I/R + LSTS versus I/R: CK-MM ↓ LSTS versus normal: CK-MM ↑	Pan et al., 2008 [34] Pan et al., 2012 [35]

SO: sphincter of Oddi; NO: nitric oxide; HSP70: heat shock protein 70; LSTS: local somatothermal stimulation; I/R: ischemia-reperfusion; ROS: reactive oxygen species; ALT: alanine aminotransferase; AST: aspartate aminotransferase; CPK: creatine phosphokinase; CK-MB: creatinine kinase-MB isoenzyme; CK-MM: creatine kinase-MM isoenzyme. Reference number is between square brackets.

injury. When animals were preconditioned with three doses of LSTS at the left PC 6 acupoint (median nerve territory) and this was followed by subsequent I/R injury to the heart, there was a significant decrease in the creatine kinase level of the heart, a significant decrease in the duration of arrhythmia, and a significant decrease in the mortality rate as well as improved mitochondrial respiratory functioning when compared to animals without prior LSTS preconditioning [31]. Furthermore, when animals were preconditioned with one dose of LSTS at the right LR 14 acupoint (7th intercostal nerve territory), followed by subsequent I/R injury to the liver, there were a significant decrease in liver enzymes (ALT/AST) and a significant decrease in malonyldialdehyde (MDA) formation when compared to animals without prior LSTS treatment or to animals with three doses of LSTS treatment [39]. In addition to the above, LSTS has been used in combination with the oral administration of geranylgeranylacetone in order to bring about tolerance of I/R injury to rat livers [43].

Recently, Pan et al. used the rubber band wrapping model to induce I/R injury to the calf muscle induced via rubber band encasement; the animals underwent injury with or without preconditioning by LSTS. No significant change in neuromuscular function was found between the LSTS (-) and LSTS (+) groups on the first day after I/R injury. However, gait stride length, compound motor action potential, and the level of serum creatine phosphokinase MM isoenzyme were found to be significantly improved on the eighth day when there had been one or two doses of LSTS preconditioning compared to the situation without LSTS preconditioning. The results suggest that LSTS preconditioning protects the animals with respect to neuromuscular plasticity when there is tourniquet-induced neuromuscular injury [34].

3.1.5. Effects of LSTS Occur via Somatovisceral Regulation. It is well known that viscerovisceral reflex regulation is a normal physiological response. For example, relaxation of the internal sphincter of the anus (the rectoanal reflex) is observed when rectal pressure is increased. A growing body of evidence suggests acupuncture may adjust visceral function and modulate immune response via a "Somatovisceral" mechanism [44–51]. The fact that acupuncture and related alternatives increase the concentrations of opioids and monoamines in cerebral spinal fluid and the levels of vasoactive intestinal peptide (VIP) and cholecystokinin (CCK) in serum suggests that Somatovisceral regulation occurs via neuroneural or neurohumoral pathways. It is generally accepted that local anesthetics depress completely the transmission of pain and thermal sensations, which are carried by A δ or C-fibers [52]. LSTS at a peripheral acupoint induces nitrenergic neural release of NO in Oddi's and anal sphincters, and this is able to be completely blocked by local infiltration of an anesthetic agent at the LSTS site. Due to how embryonic development takes place, visceral pain is perceived as originating from a somatic area, a phenomenon known as "referred pain." Previously, a role for polymodal receptors (PMRs) in this phenomenon has been suggested based on the fact that PMRs are responsive to both acupuncture and moxibustion stimuli and that thermal sensitivity is essential to moxibustion therapy [53]. These findings are in agreement with the fact that the effects of LSTS are mediated by Somatovisceral regulation via heat-sensitive sensory afferent and NANC motor neurons.

3.1.6. Differences between Acupuncture and LSTS. Previous investigations have demonstrated that I/R injury of the heart can be attenuated by application of either LSTS or electroacupuncture (EA) at the PC 6 acupoint [32, 33]. To

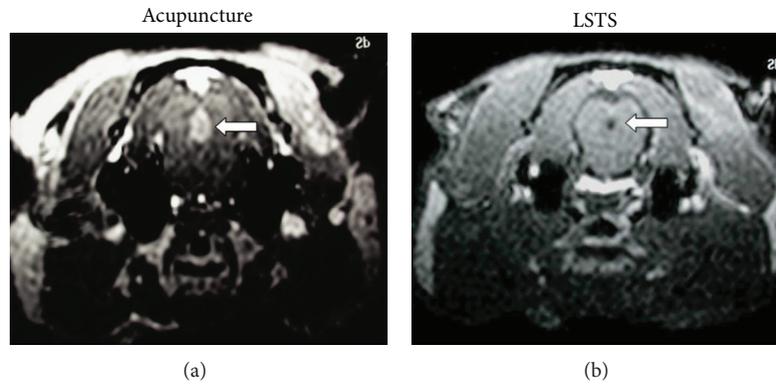


FIGURE 1: Different central manifestations between electrical acupuncture and local somatothermal stimulation at peripheral acupoints. Manganese-enhanced functional magnetic resonance imaging was performed in Sprague-Dawley rats after EA (a) at acupoint LI 4 and LSTS (b) at acupoint GB 24. The results showed that EA induced activation of pain-modulation nuclei such as the periaqueductal grey (PAG); however, in contrast, LSTS did not induce such activation.

investigate the differences in myocardial protein expression between PC 6 stimulation by EA and by LSTS, animals were treated with either LSTS or EA stimulation at acupoint PC 6, and this was followed by harvesting of the heart at different time points for proteomic analysis. The results showed that either PC 6 stimulation by EA or PC 6 stimulation by LSTS had a cardioprotective effect against I/R injury. However, proteins related to energy production and inflammation, such as glycogen synthase kinase-3 α , interleukin-1 β converting enzyme (ICE), natural killer cell protease, and tumor necrosis factor receptors, were found to change in expression to a greater degree in response to EA treatment than to LSTS treatment. In contrast, LSTS increased to a greater extent than EA for various protective proteins, including creatine kinase and HSP70 [33].

There is consensus that acupuncture evokes complex somatosensory sensations and in this way may modulate the cognitive/affective perception of pain; this suggests that many effects are supported by the brain and various other central nervous system (CNS) networks. Modern neuroimaging techniques, such as functional magnetic resonance imaging (fMRI), have provided a means whereby brain activity in humans can be safely monitored. This type of approach is useful when mapping the neurophysiological correlates of acupuncture [54]. A meta-analysis of fMRI acupuncture studies has suggested that acupuncture is able to modulate activity within specific brain areas; however, more high quality studies with more transparent methodologies are needed to improve the consistency across the various different studies [55]. Nevertheless, using manganese-enhanced fMRI in animals, EA has been found to induce activation of pain-modulation nuclei such as the periaqueductal grey (PAG); however, in contrast, LSTS did not induce such activation (Figure 1). This supports the clinical observation that LSTS is not a modality for pain relief [56].

3.1.7. ROS Plays an Important Role in LSTS-Induced Physiological Responses beneath the Acupoint. In order to elucidate the exact mechanism by which LSTS acts beneath the acupoint,

LSTS was applied to the acupoint of animals, and the underlying muscles were then collected at various time intervals after LSTS, namely, at baseline and at 5 min, 15 min, 30 min, and 60 min after baseline. The time-dependent profiles for free radical production and enzymatic scavenging activity were measured. The concentrations of reactive oxygen species, NO metabolites, and malondialdehyde were found to have increased significantly at 5 min after LSTS, whereas scavenging activity was reduced to its lowest level at 5 min (dismutase) and at 15 min (catalase and glutathione) after LSTS. Expression of HSP70 was significantly lower after LSTS when the animals were treated with an NO synthase inhibitor than in the control group without inhibitor. These results suggest that LSTS induces oxidative stress and a scavenging response in the underlying skeletal muscle and that this plays an initial role in the LSTS-induced Somatovisceral regulation mediated by the heat-sensory afferent loop [35].

3.1.8. Limitations of LSTS. It should be noted that LSTS alone at the LR 14 and PC 6 acupoints induces an elevation in serum ALT/AST [31] and cardiac troponin T levels [33], respectively. In addition, LSTS alone on the calf muscle induces a mild elevation in serum creatine kinase MM isoform (CK-MM) levels [34], which supports the hypothesis that preconditioning by LSTS at peripheral acupoints acts as a stress and may cause cellular damage in the corresponding organs. Such sublethal damage is similar to that observed when ischemia preconditioning is carried out, and this seems to protect subsequent I/R injury [57, 58]. Minor injury may be able to initiate complex biochemical cascades that are able to protect against subsequent overwhelming I/R injury. The mechanisms of preconditioning are complicated. Various mediators, including nitric oxide (NO) and adenosine in the first few minutes to hours, inducible NO synthase (iNOS) and antioxidants enzymes in the first to fourth days, and *de novo* synthesized proteins such as HSP in the few days after preconditioning, have all been postulated to protect against I/R injury [59–62]. Thus, LSTS should be cautiously applied to or may be contraindicated for those patients with chronic liver or heart diseases.

3.2. The Nontemperature-Related Mechanisms of Action of Moxibustion

3.2.1. The Effects of Herbs. When the traditional moxibustion technique is carried out, many herbs, including *Artemisia argyi* leaf and ginger, are widely used between the moxa sticks and the skin surface. Using gas chromatography-mass spectrometry (GC-MS) with solid-phase microextraction (SPME), a total of fifty-three compounds, including cyclofenchene, alpha-pinene, alpha-myrcene, D-limonene, caryophyllene, and germacrene D, were identified as well as two volatile components (borneol and borneol acetate) from *Artemisia argyi* flowers [63]. In addition, various nonvolatile substances, such as juniper camphor, caryophyllene oxide, and caryophyllene, have been found in a high proportion of moxa wools [64]. Furthermore, there is evidence supporting that the hypothesis that the increase in temperature induced by moxibustion increases the permeability of the skin to high molecular compounds [65], as well as acting as an aid to the entry of any topical application of salicylate [66].

3.2.2. The Smoke Effects of Moxibustion. Previously, the anti-inflammatory effects of moxa smoke on NO production were demonstrated by Matsumoto H et al. using mouse macrophage-like Raw 264.7 cells. This study showed that the 50% inhibitory concentration (IC₅₀) of lipopolysaccharide-induced NO production by moxa smoke (0.16%) was one order of magnitude lower than the 50% cytotoxic concentration (CC₅₀) (4.67%). The inhibition of NO production by moxa smoke is probably due to both an inhibition of iNOS expression and an inhibition of radical scavenging activity [67]. Moreover, moxa smoke dose-dependently induces internucleosomal DNA fragmentation, activates caspases 3, 8, and 9, and modifies to some extent the expression of various apoptosis-related proteins (Bcl-2, Bad, and Bax) in HL-60 cells. These findings suggest that moxa smoke has potential as an antitumor agent [68]. It should be noted that there has been the advent of strict antismoking legislation in many countries, and as a result there are concerns about the potential effectiveness and toxicity of the volatiles produced by moxibustion. Up to now, no immediate concerns have been raised about the continued use of moxibustion as a therapeutic modality in traditional Chinese medicine [69].

3.2.3. The Far Infrared (FIR) Effects of Moxibustion. It is reasonable to speculate that direct moxibustion with a traditional moxa stick may produce its therapeutic effects via thermal action, while traditional indirect moxibustion may act by producing both modest thermal activation and a sympathetic vibration at the skin surface [70]. Shen et al. demonstrated that intensity of infrared radiation produced by a traditional moxa stick was 43300.41 mV with a peak in the infrared spectrum at 3.5 μm , while the respective radiation intensities of two control experiments using a smokeless moxa stick and a 555 cigarette were 31.15 mV and 37.03 mV with peaks of 7 μm and 3.5 μm , respectively. The infrared radiation intensities of the three traditional media used for indirect moxibustion, monkshood cake, ginger slices, and garlic slices were found to be 520.27 mV, 594.79 mV, and 681.87 mV, respectively, all with

peaks around 7.5 μm . These materials all produced similar spectra, which were quite different from those detected when slices of various control materials (cucumber and carrot) were used [70]. In addition, when moxibustion stimulation at the ST 25 acupoint was carried to treat animal ulcerative colitis, Wang et al. demonstrated that the infrared radiation intensity at fourteen wavelengths at the ST 25 acupoint were significantly different between the normal and model control groups. These findings suggest that biophysical mechanisms may be involved in the moxibustion treatment [71].

4. Adverse Events due to Moxibustion

Although traditional moxibustion has potential as a treatment, it is not entirely risk free, and several kinds of adverse events have been reported, including trauma [72], allergy, burns [73, 74], and infection. There is consensus that both the evidence-based efficacy of moxibustion and the level of patient safety of moxibustion need to be explored in more detail in order to clarify the usefulness and applicability of this ancient technique [75, 76].

5. Clinical Implication of LSTS

There is consensus that the expression of HSPs by prior sublethal hyperthermic preconditioning is able to attenuate the heat-induced cellular responses to a subsequent severe heat challenge [77]. The development of thermotolerance can be initiated by preconditioning animals, not only with repeated hyperthermia, but also with ischemia-reperfusion challenge or low doses of various chemical stressors [78–80]. In addition, accumulating evidence indicates that remote ischemia preconditioning attenuates I/R injury of the heart [81, 82]. In contrast to whole-body hyperthermia, which is likely to induce HSP expression in many organs, LSTS is a local heat stress and induces HSPs in specific corresponding visceral organs. LSTS at the PC 6 acupoint induces myocardial but not hepatic HSP70 expression, while LSTS at the LR 14 acupoint induces hepatic but not myocardial HSP70 expression. The fact that preconditioning LSTS at these two acupoints protect the heart and the liver against subsequent I/R injury, respectively, supports the concept that preconditioning LSTS at peripheral acupoints is a remote preconditioning technique.

6. Conclusion

Moxibustion is an ancient Chinese medical technique. The possible mechanisms by which moxibustion may work include temperature-related factors and nontemperature-related factors; the latter include smoke effects, herbal effects and biophysical effects (far infrared). Compared with whole-body hyperthermia or brief ischemia preconditioning, LSTS (an alternative to moxibustion that avoids skin damage) is an easily applicable preconditioning method for the prevention or treatment of overwhelming subsequent I/R injury. However, evidence-based studies of the efficacy of LSTS as well as safety studies are needed using large-scaled clinical trials

in order that this ancient Chinese technique can pass muster with Western scientists.

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Research Article

The Stimulation Effect of Auricular Magnetic Press Pellets on Older Female Adults with Sleep Disturbance Undergoing Polysomnographic Evaluation

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Study Objectives. To examine the stimulation effect of auricular magnetic press pellet therapy on older female adults with sleep disturbance as determined by polysomnography (PSG). *Design.* Randomized, single-blind, experimental-controlled, parallel-group. *Setting.* Community. *Participants.* Twenty-seven older female adults with sleep disturbance according to the Pittsburgh Sleep Quality Index (PSQI) >5 for at least 3 months were recruited. Participants were screened by both the Hospital Anxiety and Depression Scale (HADS) and the Mini-Mental State Examination (MMSE), as well as polysomnography prior to randomization. *Interventions.* All eligible participants were randomly allocated into the experimental or control group. Both groups were taped with magnetic press pellet on auricular points for 3 weeks. The experimental group was treated by applying pressure on the magnetic press pellets 3 times per day while no stimulation was applied on the control group. *Measurements and Results.* Both groups were measured by PSG and PSQI at the beginning of the study and 3 weeks after the study. Both groups showed improvements on PSQI scores compared to the baseline. One-way analysis of covariance adjusted for baseline scores showed that significant improvements of PSG-derived sleep parameters, such as sleep efficiency, were found in the experimental group. However, no significant differences between groups were observed in the proportion of sleep stages with the exception of Stage 2. *Conclusions.* Auricular therapy using magnetic pellets and stimulation by pressing was more effective in improving the sleep quality compared to auricular therapy without any stimulation.

1. Introduction

Sleep is a periodic state of rest for the body and mind. In humans, normal sleep has five stages that cyclically repeat themselves during an episode of sleep. The first four stages comprise nonrapid eye movement (NREM) sleep and the last stage constitutes rapid eye movement (REM) sleep. Stage 1 is characterized by drowsiness, Stage 2 by light sleep, and Stages 3 and 4 by deep sleep or slow wave sleep (SWS). The NREM sleep does not occur with rapid eye movement and is associated with tissue repair. During REM sleep there are

rapid periodic twitching movements of the eye muscles and accelerated respiration and heart rate, in which dreams occur and memory is thought to be organized. Hence sleep is a vital function to restore energy and maintain homeostasis of our body. However, complaints of sleep difficulties are increasing in modern society. The overall prevalence of symptoms of insomnia in adults ranged from 13.5% to 40%, with higher percentages occurring in the elderly [1–6]. The National Institute on Aging held a survey regarding sleep quality in the elderly population and found that up to 43% of respondents reported difficulty in falling asleep or maintaining sleep [7].

The older adults usually complain more about the sleep dissatisfaction than that in the general population [8]. Changes in sleep patterns with advancing age characterized by decreased total nocturnal sleep time and frequent nocturnal awakening often result in the older adults experiencing a lighter and more interrupted sleep [9]. Their total sleep time and sleep efficiency also decline with age [10]. Furthermore, polysomnographic studies have shown that sleep architecture which depicts the structure and pattern of sleep alters with age [9]. Compared to young adults, polysomnography- (PSG-) derived sleep architecture in older adults presents with increased NREM Stage 1 sleep and reduced amounts or absence of NREM Stage 3 and 4 sleep, indicating less deep sleep (or slow wave sleep) but more light sleep in those subjects [11, 12]. The proportion of REM sleep stays the same but the latency of REM sleep may shorten, resulting in advanced sleep phase and increase in early morning awakening [13]. Consequently, sleep disturbance is highly associated with fatigue, daytime sleepiness, psychological distress, and physical discomfort and, thereby impairing daytime functioning, quality of life as well as physical and mental health [1, 2, 14, 15].

Currently, hypnotic medication is the most popular treatment for insomnia or sleep disturbance, but some people, especially elder people, are susceptible to their adverse effects, such as daytime drowsiness, falls, drug dependence, and rebound insomnia owing to the drug's withdrawal [16]. Therefore, it is essential to explore a safe way or an alternative approach to improve the sleep quality. Evidence has shown that auricular therapies hold great potential on having positive effects on the improvement of insomnia [17–20]. Auricular therapy combines both channel theory and knowledge of nerve distributions in the auriculae to effect the autonomic nervous system and, thereby, promoting sleep quality [21, 22]. However, the paucity of rigorously designed trials was confined to reveal the real effect of auricular therapy for the treatment of insomnia [17–20]. Furthermore, most appraisals of efficacy usually use subjective experience stated as outcome measurements [23–26]. The results may fail to accurately represent the effectiveness of auricular therapy due to lack of robust objective tool measurements. Only a few studies which met the standards of clinical trial used actigraphy in the appraisal of the effectiveness of auricular therapy, but there is still lack of polysomnographic measurement, which is viewed as a golden standard for sleep assessment [27]. Also, magnetic press pellets were only placed on auricular acupoints and no stimulation was given. Nonetheless, based on the experience of traditional Chinese medicine practitioners, the magnetic press pellets placed on the acupoints must be stimulated to exert its effect. The main purpose of this study is to further examine the stimulation effect of auricular press pellet therapy on older adult women with sleep disturbance by using the polysomnography (PSG) measurements.

2. Methods

2.1. Design. This was a randomized, single-blind, and experimental-controlled parallel-group study design to compare the effects of auricular press pellet therapy with or without stimulation. Major assessments were conducted at baseline

and 3 weeks after intervention. This study was approved by the Institutional Review Board of China Medical University Hospital (DMR97-IRB-107).

2.2. Participants. Potential participants over 50 years of age complaining of sleep disturbance were recruited from the local community through advertisements. The inclusion criteria for selecting eligible participants were (1) 50 years of age and above; (2) complaining of sleep disturbance (difficulty in initiating sleep over 30 minutes, maintaining sleep, or awakening prematurely) 3 or more nights per week for at least 3 months; (3) having a global Pittsburgh Sleep Quality Index (PSQI) score greater than 5. In order to avoid the masking effect of hypnotics on sleep, all participants were asked not to take or to be willing to discontinue their use of any hypnotics for at least 1 week prior to the beginning of the study and during the study period.

Potential participants were excluded if they had the following factors involved: (1) sleep disturbance was relevant to obvious environmental factors; (2) a history of alcohol or substance use; (3) their sleep difficulties resulted from medical conditions, including an acute illness, unstable health conditions associated with physical discomfort or pain, malignancy ≤ 5 years prior to study, unstable or uncontrolled cardiac disease, diabetes, hepatic or renal disease, active endocrine disorders, nocturia (>3 times/night) and; (4) serious neurological disorders, such as stroke and Parkinson, or conditions related to impaired cognitive function, such as dementia; (5) the presence of psychiatric disorders; (6) the presence of other sleep disorders, and Apnea-Hypopnea Index (AHI) >10 or periodic limb movements (PLM); (7) persons with shift work. In addition, the Hospital Anxiety and Depression Scale (HADS) and the Mini-Mental State Examination (MMSE) were also screened to confirm participants' anxiety and depression as well as the absence of impaired cognitive function. These potential participants were also excluded if they had an HADS score >11 on either anxiety or depression subscale.

The process of selecting research participants and randomized assignments of groups were summarized in Figure 1. One hundred and eighty-eight potential participants responded and were preliminarily screened for the inclusion criteria by telephone. A total of 89 persons who met the preliminary screen were further confirmed by face-to-face interview and informed of the research process. Forty-two persons agreed to participate in this study and were scheduled for the initial PSG studies. Participants were excluded from this study if they presented with severe sleep apnea ($n = 8$), PLM ($n = 3$), were unable to adapt to the Sleep Center's environment during the first night, or refused to participate ($n = 3$). Because only one male subject remained and gender is a significant factor for sleep [28], data of only female participants were analyzed in this study. Twenty-seven female participants completed this study.

2.3. Procedure. After the prescreening interview, eligible participants were informed about the study and were asked to sign the consent form. They entered a sleep laboratory and

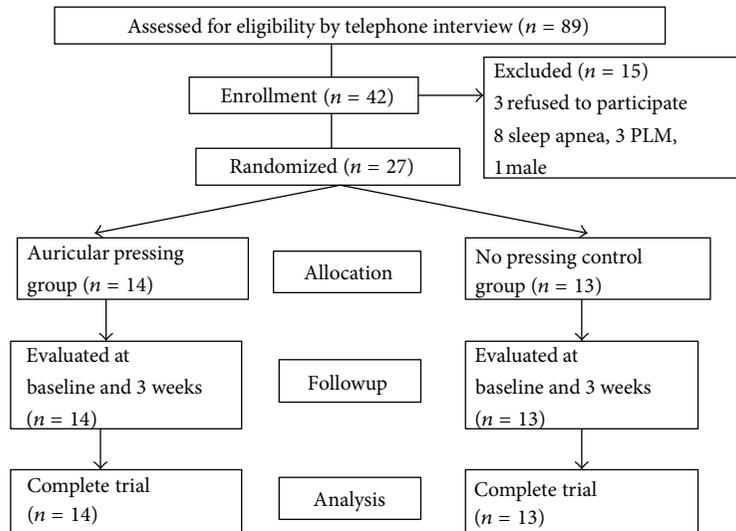


FIGURE 1: Flow of participants through the trial.

underwent PSG screening to further confirm the absence of any sleep disorders, such as sleep apnea and PLM. Following the stratification by sex, qualified participants were randomly assigned to the control or experimental groups using a random digital table. Participants were not aware whether they would be placed into either the experimental group or the control group. Both groups were measured by PSG and PSQI before and after intervention.

2.3.1. Auricular Therapy. Both groups were taped with a round 1.6 mm diameter magnetic press pellets similar in size to that of Semen Vaccariae on the ear acupoints. The magnetic press pellets were purchased from commercial product. Each magnetic press pellet contained an average of 100 gauss or above magnetic flux density examined by an FW Bell 4048 Gauss/Tesla Meter (made in USA). Before taping, the most sensitive auricular points were probed by forceps to further confirm the location. The participant's auricles were sterilized with 75% alcohol pads, and a magnetic press pellet was then taped to the reactive point. The magnetic press pellets and adhesive tape were replaced every 3-4 day to avoid the possibility of local irritation or ulceration on the auricular point. Both ears were taped alternately.

2.3.2. Control Group. Participants assigned to the control group were treated with no additional force applied to the magnetic pellets.

2.3.3. Experimental Group. Participants assigned to the experimental group were given an audiotape and instructions guiding them on how to stimulate the acupoints. They were asked to apply pressure on the magnetic press pellets 3 times a day (morning, afternoon, and 30 minutes before bedtime). Each point was pressed 7 seconds and relaxed 1 second for 100 times with the exception of the point Shenmen that

was pressed 120 times. Each round lasted for about 12 to 15 minutes. The pressure was exerted to the extent of obvious and tolerable feeling of pain, thereby having a swelling pain and warmth feeling on the acupoints. The trial was conducted over a 3-week period.

2.3.4. Selection of Auricular Acupoints. According to Traditional Chinese Medicine (TCM), insomnia is mainly due to a dysfunction between the viscera bowels, imbalance between Yin and Yang, and hampered circulation of Qi and blood in the channels and network vessels [20]. Seven acupoints for sleep improvement were chosen based on the principles of Traditional Chinese Medicine (TCM) and a standardized protocol published by Suen et al. [29]. They are Shenmen, Heart, Kidney, Spleen, Liver, Subcortex, and Occiput. The "Heart" point can quiet the mind. The "Kidney" point supplements essence. The "Spleen" point can dispel dampness and improve digestive function. The "Liver" point can regulate the flow of Qi, especially when insomnia is caused by stagnation of liver Qi. The points "Shenmen" and "Occiput" can calm the mind. The "Subcortex" point can harmonize the excitation and inhibition of the cortex [21]. The precise auricular points are located in Figure 2 and follow the International Standard of Auricular Points [21].

2.4. Measurements

2.4.1. Subjective Sleep Measures: Pittsburgh Sleep Quality Index. The Pittsburgh Sleep Quality Index [30] was used to evaluate self-rating sleep disturbance and quality at the beginning and the end of the study. Nineteen individual items composed the 7 components, including subjective sleep quality, sleep latency, sleep duration, sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction, to evaluate participants' sleep over a 1-month time

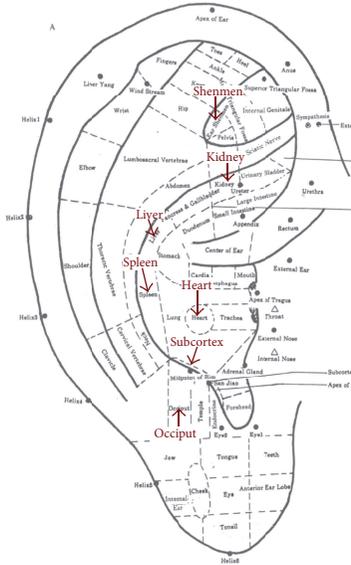


FIGURE 2: The location of selected auricular acupoints—frontal surface.

interval. According to the Likert scale, each component was scored 0–3 points, and the sum of these component scores generated a global PSQI score ranging from 0 to 21. The overall Cronbach alpha of the Chinese version of the Pittsburgh Sleep Quality Index was 0.82 [31]. The higher scores indicated poor sleep quality. A score of 5 or greater was used to differentiate between poor and good sleep. Participants had to score >5 to be eligible for this study.

2.4.2. Objective Sleep Measures: Polysomnography (PSG). Polysomnography is considered a gold standard in assessing sleep status. It can record electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and electrocardiography (ECG) as well as breathing patterns at the same time. Data was obtained using the Alice IV sleep data acquisition system (Respironics, Murrysville, PA, USA). Two consecutive overnight PSG were performed in the sleep center at the beginning and the end of the 3-week period of auricular therapy. PSG results obtained on the first night during the before and after study were not included in the analysis in order to avoid first night effect. Sleep stage scoring of the entire PSG recordings were visually analyzed by a well-trained sleep technician according to the Rechtschaffen and Kales standard. Sleep parameters included sleep latency (SL, lights off to the first epoch of Stage 1 sleep), total sleep time (TST, the sum of sleep Stages 1–4 and REM), sleep period time (SPT, time from the beginning of NREM Stage 1 until final awakening), sleep efficiency (SE, ratio of TST/TIB), waking after sleep onset (WASO), and arousal index. Sleep architecture was also obtained, including Stage 1 to 4 of nonrapid eye movement (NREM) sleep, slow wave sleep (SWS, indicating the deep sleep), and rapid eye movement (REM) sleep [32].

2.4.3. Sleep Apnea and Periodic Limb Movements (PLM). Screening for evidence of sleep apnea and periodic limb

movements (PLM) was done during the first night in the sleep center. The evaluation criteria were based on the Report of the American Academy of Sleep Medicine Task Force [33]. The Apnea-Hypopnea Index (AHI) <10 was used as the screening criterion for this study.

2.5. Statistical Analysis. Descriptive statistics were used to summarize the participants' demographic characteristics. Demographic differences between the experimental and control groups were examined using either *t*-test or χ^2 test. Mean values of PSG sleep parameters (TST, SL, SE, WASO, and sleep stage percents and minutes) and perceived sleep quality (PSQI) were calculated. A one-way analysis of covariance (ANCOVA) with baseline measure as covariate was used to compare changes in PSG and perceived sleep parameters between the experimental and control groups after intervention. Each analysis was performed at the significance level of $P < 0.05$. All statistical data was carried out by using SPSS 17.0 software for windows.

3. Results

3.1. Demographic Data. The demographic information of participants was summarized in Table 1. Their age ranged from 50 to 68 years, with an average of 57 and 56.8 years, respectively, for the experimental group and control group. Average of BMIs of both groups fell within the normal range. There was no difference between two groups in various screening indicators of all participants, such as the degree of cognitive and the level of anxiety and depression. Most participants were married and lived with their spouses or children. In both groups approximately 30% to 40% of the participants were retired or worked as a volunteer with an education level of middle school or below. The duration of insomnia was 92 months in the experimental group and up to 105 months in the control group without significant differences between groups. There were no significant differences in the variance of demographic variables between groups, indicating homogeneous and comparable groups.

3.2. Changes in Subjective Sleep Quality (PSQI) between Pre- and Posttests. Table 2 illustrated the results of the comparative analysis of both groups' PSQI scores using analysis of covariance (ANCOVA). There are no differences between the groups in the global PSQI score as well as the 7 components observed at baseline. After intervention, the mean global score of PSQI reached significant difference ($F_{(1,23)} = 9.10$, $P < 0.01$) between groups, indicating that sleep quality of the experimental group improved more than that of the control group. Significant differences ($P < 0.05$) also existed between two groups within the detailed components of PSQI in the posttest, including sleep quality, sleep duration, sleep efficiency, sleep disturbances, and daytime dysfunction.

3.3. Changes in Polysomnography between Pre- and Posttests. Sleep parameters measured by PSG were summarized in Table 3. The changes of sleep latency and sleep efficiency detected by PSG between the experimental group and the

TABLE 1: Demographic data of participants in the experimental and control group.

Variables	Experiment (<i>n</i> = 14)	Control (<i>n</i> = 13)
	Mean ± SD	Mean ± SD
Age, years	57.0 ± 4.6	56.8 ± 4.9
Insomnia duration, month	92.4 ± 84.39	105.5 ± 102.83
BMI [#] , %	22.59 ± 2.74	22.56 ± 2.98
MMSE [#]	29.2 ± 0.9	28.9 ± 1.3
HADS [#] _anxiety	3.6 ± 3.3	3.4 ± 2.5
HADS [#] _depression	2.9 ± 2.7	2.9 ± 2.4
	<i>n</i> /(%)	<i>n</i> /(%)
Retired	4 (28.6%)	5 (38.5%)
Education level		
Middle school or below	4 (28.6%)	5 (38.5%)
High school	6 (42.8%)	6 (46.2%)
College or above	4 (28.6%)	2 (15.4%)
Marriage: married	11 (78.6%)	10 (76.9%)
Religion		
None or other	2 (14.2%)	2 (15.4%)
Buddhist	6 (42.9%)	5 (38.5%)
Folk religion	6 (42.9%)	6 (46.2%)
Living status		
With spouse	4 (28.6%)	1 (7.7%)
With children	3 (21.4%)	7 (53.8%)
With spouse and children	5 (35.7%)	4 (30.8%)
Other	2 (14.3%)	1 (7.7%)
Other family members suffering from insomnia	6 (42.9%)	7 (53.8%)

[#]BMI: body mass index; MMSE: Mini-Mental State Examination; HADS: Hospital Anxiety and Depression Scale.

control group were also illustrated in Figure 3. The results of the comparative analysis in both groups' sleep parameters were analyzed by analysis of covariance (ANCOVA). Prior to auricular therapy, older adults in both groups had sleep efficiency less than 80%, more than 15 minutes of sleep latency, and arousal index up to 15 times per hour, indicating poor sleep quality in both groups. There were no significant differences in all PSG sleep parameters between groups at the pretest. After intervention, sleep efficiency was increased sleep latency, WASO, and arousal index were decreased significantly ($P < 0.05$) in the experimental group.

Comparing the sleep architecture, no significant differences existed in the parameters of each sleep stages between the two groups at the pretest. After intervention, minutes and percentages of Stage 1 sleep were decreased in the experimental group while increased in the control group. There was no statistical difference in the proportion and duration of other sleep stages between groups with the exception of Stage 2.

4. Discussion

Previous studies have shown that auricular therapy with magnetic press pellets improved subjective sleep quality in patients of all ages [25, 34, 35]. Our study further showed that auricular therapy using magnetic press pellets effectively improved the sleep quality evaluated by both subjective sleep and polysomnography in older community dwelling women with sleep disturbance. Therefore, auricular therapy can be an effective way to improve sleep quality in women with sleep disturbance.

Compared to auricular therapy of using magnetic press pellets without stimulation by pressing, total sleep time and sleep efficiency are increased as well as sleep latency, wake minutes, and Stage 1 sleep are decreased in those of using magnetic press pellets with stimulation. The effects of the auricular magnetic press pellets with stimulation on both subjective and objective sleep qualities are better than that without auricular stimulation. Previous studies have shown that the effect of using magnetic press pellets without stimulation in auricular therapy is better than that of using Junci Medulla or Semen Vaccariae [27]. It suggested that the effectiveness of auricular therapy on sleep improvement is due to the magnetic effect. However, our study further showed that even magnetic press pellets used in auricular therapy are more efficacious when the magnetic press pellets are stimulated against the acupoints. Stimulation is also essential in auricular therapy. Magnetic press pellets with stimulation evoke better sleep improvement.

Most of the previous studies used the subjective feeling of patients to appraise the effectiveness of improvement in sleeping. In our study we emphasized using the PSG instrument in order to objectively evaluate the outcome and effect of auricular therapy, since PSG is viewed as the gold standard to assess sleep and can obtain more comprehensive and objective information. Interestingly, it was found that the level of improvement of sleep quality appraised by PSG was far less than that by subjective feeling. Therefore, the limitation of Hawthorne effect and social desirability bias effect cannot be ignored in subjective appraisal. By using both subjective and objective sleep, assessment is necessary in evaluating the real effect of sleep interventions.

The findings of this study are consistent with the fact that the elderly easily experienced lighter and more fragmented sleep [9, 10]. The results showed that older women with sleep disturbance had difficulties in falling asleep and needed greater than 15 minutes to enter Stage 1 before the auricular therapy. The patients' sleep pattern fluctuated back and forth repeatedly between Stage 1 and Stage 2. This was also accompanied by significantly increased arousal event of up to 15–17 times per hour in the entire night. This led them to remain in a state of light sleep which augments the disruption of sleep. As a result, older women with sleep disturbance could not experience better quality of sleep.

After auricular stimulation, sleep latency became less than 15 minutes in the experimental group, which was obviously shorter than that of the nonstimulation control group. In the findings of the PSG appraisal, the improvement of sleep efficiency of the experimental group increased to 84.04%, but

TABLE 2: Comparisons of seven components and global score of PSQI between the experimental group and the control group by analysis of covariance (ANCOVA).

Items of PSQI	Pretest		Posttest		ANCOVA	
	Experiment (<i>n</i> = 14) Mean ± SD	Control (<i>n</i> = 13) Mean ± SD	Experiment (<i>n</i> = 14) Mean ± SD	Control (<i>n</i> = 13) Mean ± SD	<i>F</i>	<i>P</i> value
C1. Sleep quality	2.47 ± 0.52	2.46 ± 0.52	0.80 ± 0.56	1.31 ± 0.75	4.73	0.04*
C2. Sleep latency	2.87 ± 0.52	2.69 ± 0.63	1.33 ± 0.82	1.77 ± 0.73	2.48	0.13
C3. Sleep duration	2.53 ± 0.64	2.54 ± 0.66	1.07 ± 0.70	1.92 ± 0.95	5.17	0.03*
C4. Sleep efficiency	2.33 ± 0.82	2.62 ± 0.77	0.80 ± 1.15	1.85 ± 1.14	5.41	0.02*
C5. Sleep disturbances	1.00 ± 0.38	1.08 ± 0.28	0.73 ± 0.46	1.15 ± 0.38	7.06	0.01*
C6. Sleeping medication	1.07 ± 1.28	0.77 ± 1.24	0.00 ± 0.00	0.15 ± 0.38	0.84	0.37
C7. Daytime dysfunction	1.20 ± 1.08	1.54 ± 0.97	0.33 ± 0.62	1.08 ± 0.95	4.47	0.04*
PSQI global score	13.40 ± 2.90	13.69 ± 1.97	5.07 ± 2.63	9.23 ± 3.63	9.10	0.00**

P* < 0.05; *P* < 0.01.

TABLE 3: Comparisons of the sleep parameters detected by PSG between the experimental group and the control group by analysis of covariance (ANCOVA).

Sleep parameters	Pretest		Posttest		ANCOVA	
	Experiment (<i>n</i> = 14) Mean ± SD	Control (<i>n</i> = 13) Mean ± SD	Experiment (<i>n</i> = 14) Mean ± SD	Control (<i>n</i> = 13) Mean ± SD	<i>F</i>	<i>P</i> value
Sleep latency (to Stage 1) (min)	17.57 ± 11.27	20.58 ± 13.13	12.04 ± 6.74	24.27 ± 15.48	6.78	0.02*
Total sleep time (min)	356.29 ± 51.61	335.65 ± 54.97	362.14 ± 31.46	314.31 ± 66.96	5.87	0.02*
Sleep period time (min)	423.75 ± 45.67	416.00 ± 49.21	409.18 ± 35.34	401.62 ± 41.89	0.18	0.67
SE [#] (TST/TIB) (%)	79.58 ± 7.54	76.99 ± 12.62	84.04 ± 4.62	72.15 ± 12.78	10.85	0.00**
Sleep stages (min in sleep period time)						
Wake (WASO [#])	61.75 ± 38.72	80.35 ± 48.76	47.04 ± 24.07	87.31 ± 50.42	6.90	0.02*
Stage 1	54.82 ± 42.87	50.85 ± 29.74	42.93 ± 15.60	60.62 ± 50.58	2.22	0.15
Stage 2	208.43 ± 56.22	187.23 ± 41.12	208.46 ± 47.28	158.15 ± 54.67	6.29	0.02*
Stage 3	10.21 ± 15.31	14.62 ± 20.65	11.64 ± 15.45	15.92 ± 21.01	0.17	0.69
Stage 4	0.93 ± 3.06	4.08 ± 12.51	0.64 ± 1.99	2.81 ± 8.50	0.48	0.50
REM	87.61 ± 27.93	78.88 ± 23.55	98.46 ± 34.01	76.81 ± 26.24	2.08	0.16
NREM	274.39 ± 30.12	256.77 ± 42.75	263.68 ± 42.71	237.50 ± 52.26	0.56	0.46
SWS	11.14 ± 18.11	18.69 ± 31.40	12.29 ± 16.64	18.73 ± 27.83	0.02	0.89
Sleep stages (% in sleep period time)						
Wake (WASO [#])	14.36 ± 8.03	19.17 ± 11.28	11.34 ± 5.36	21.96 ± 13.20	6.74	0.02*
Stage 1	13.03 ± 9.89	11.96 ± 6.34	10.43 ± 3.52	15.15 ± 13.16	1.98	0.17
Stage 2	49.50 ± 13.27	45.80 ± 11.54	50.90 ± 10.27	39.18 ± 12.24	4.75	0.04*
Stage 3	2.35 ± 3.52	3.03 ± 4.39	2.86 ± 3.73	3.90 ± 5.00	0.03	0.87
Stage 4	0.21 ± 0.72	0.79 ± 2.34	0.14 ± 0.45	0.68 ± 2.03	0.03	0.87
REM	21.21 ± 4.79	19.04 ± 5.48	24.31 ± 8.61	19.12 ± 6.35	2.73	0.11
Arousal index	14.57 ± 5.01	15.27 ± 2.65	12.99 ± 5.00	16.78 ± 3.97	4.81	0.04*

[#]SE: sleep efficiency; WASO: wake after sleep onset.**P* < 0.05; ***P* < 0.01.

sleep efficiency of the control group did not improve distinctly. Besides, in the aspect of sleep disturbance, indicator of wakefulness (e.g., WASO, arousal index) of the experimental group showed a statistically significant improvement compared to the control group. This showed that with progression of insomnia, intervention of the experimental group can better improve sleep quality. Fascinatingly, in the aspect of sleep architecture, though the two groups show no observable difference in stages of deep sleep (Stages 3 and 4), Stage 1 sleep of the experimental group decreased and transferred to Stage

2 sleep and REM sleep. Auricular stimulation may urge sleep to develop from shallow sleep to deeper intermediate sleep while the number of arousal event significantly decreased, thereby improving sleep quality.

Though the effectiveness of how auricular stimulation can improve sleep quality was not as much as we expected, this procedure is relatively simple and easy to perform. Therefore, it can be considered to be a complementary alternative therapy. The probable mechanism for the improvement of sleep by stimulating the auricle in this study could be explained

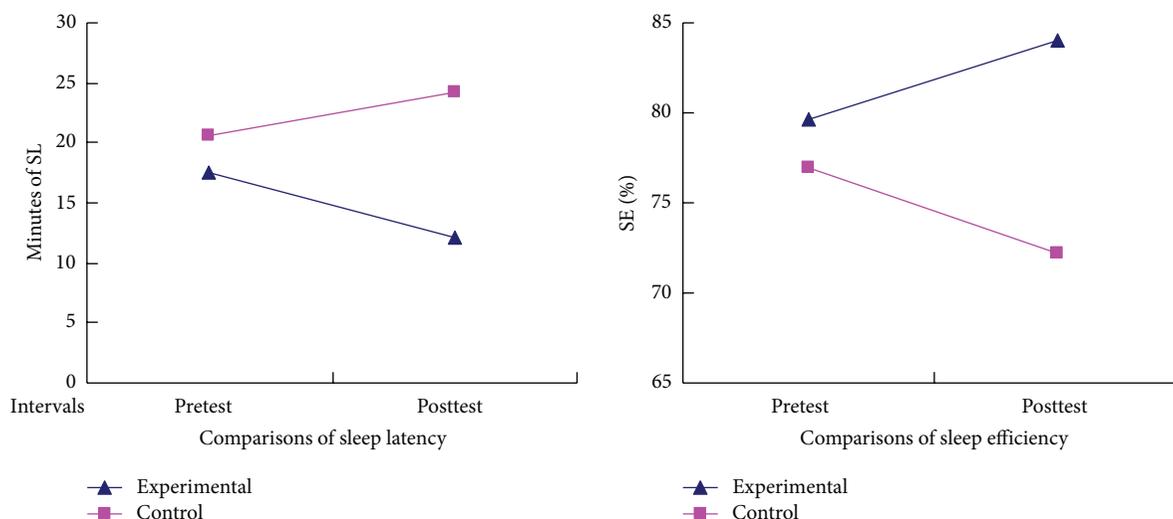


FIGURE 3: The changes of sleep latency and sleep efficiency detected by PSG between the experimental group and the control group.

based on several physiological functions. There are many nerve innervations in the auricle, such as the great auricular nerve, and lesser occipital nerve from the cervical plexus of the spinal ganglia; branches of the ear temporal nerve, facial, glossopharyngeal nerve and vagus nerve from the cranial nerves; and sympathetic nerves which follow the external carotid artery [21]. Therefore, through the regulatory function of the central nervous system, the stimulation of the auricular points plays a regulatory role in the treatment of insomnia. However, stimulation of the auricle is a simple complementary alternative therapy, yet the compliance of the participants towards self-stimulation should be considered. Otherwise, the extent of stimulation could affect the effect of sleep improvement. Although this study used randomized controlled trial and a single-blind design, there were still some limitations in this study. One of the limitations is the small number of participants studied; therefore future studies will require a larger number of participants to substantiate our findings. Another limitation is that the auricular stimulation and outcome measurement were performed by the same investigator, and, thus, the interaction between the investigator and participants may cause subjective measurement bias. The social desirability and Hawthorne effect were difficult to be avoided. Further double-blinded studies are required to determine whether subjective effects existed in the current single-blind study. Moreover, a long-term follow-up study with an increased range of ages and male participants will be necessary to ascertain the effectiveness of auricular stimulation.

Disclosure

This was not an industry-supported study. All authors have indicated no financial conflict of interests.

Authors' Contribution

C. Lo and W.-C. Liao have contributed equally to this paper.

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Review Article

Adverse Events of Acupuncture: A Systematic Review of Case Reports

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Acupuncture, moxibustion, and cupping, important in traditional Eastern medicine, are increasingly used in the West. Their widening acceptance demands continual safety assessment. This review, a sequel to one our team published 10 years ago, is an evaluation of the frequency and severity of adverse events (AEs) reported for acupuncture, moxibustion, and cupping between 2000 and 2011. Relevant English-language reports in six databases were identified and assessed by two reviewers. During this 12-year period, 117 reports of 308 AEs from 25 countries and regions were associated with acupuncture (294 cases), moxibustion (4 cases), or cupping (10 cases). Country of occurrence, patient's sex and age, and outcome were extracted. Infections, mycobacterial, staphylococcal, and others, were the main complication of acupuncture. In the previous review, we found the main source of infection to be hepatitis, caused by reusable needles. In this review, we found the majority of infections to be bacterial, caused by skin contact at acupoint sites; we found no cases of hepatitis. Although the route of infection had changed, infections were still the major complication of acupuncture. Clearly, guidelines such as Clean Needle Technique must be followed in order to minimize acupuncture AEs.

1. Introduction

Traditional acupuncture, which is defined as needling insertion, moxibustion thermal stimulation, and cupping techniques at acupuncture points [1], has become popular in the United States and the rest of the world in recent decades. Data released by the National Institutes of Health (NIH) in 2008 reported that 3.1 million American adults and 150,000 children used acupuncture in 2007. Adult use of acupuncture increased by approximately a million people in the five years from 2002 to 2007 [2]. This increased use brings attention to the safety and quality of the modality.

A number of large surveys on the safety of acupuncture have been conducted, mainly in Europe. Most reported incidents have been fairly minor, and incidence rates were low.

For example, in a prospective survey of 34,000 treatments by traditional acupuncturists, MacPherson et al. [3] found no serious adverse events (AEs) and 43 minor ones, a rate of 1.3 per 1000 treatments. In another prospective survey, Melchart et al. [4] found 7.1% minor AEs and 5 serious ones among 97,733 acupuncture patients. The authors of these studies concluded that serious AEs seem to be rare and that acupuncture is generally a safe intervention.

More than a decade since our last review [5], we have conducted this systematic follow-up review of case reports published between 2000 and 2011 on AEs and complications associated with acupuncture. Our purpose is to (1) estimate the trend of occurrences of the AEs associated with acupuncture over the past 11 years, (2) identify risk factors in acupuncture practice in order to minimize such events, and

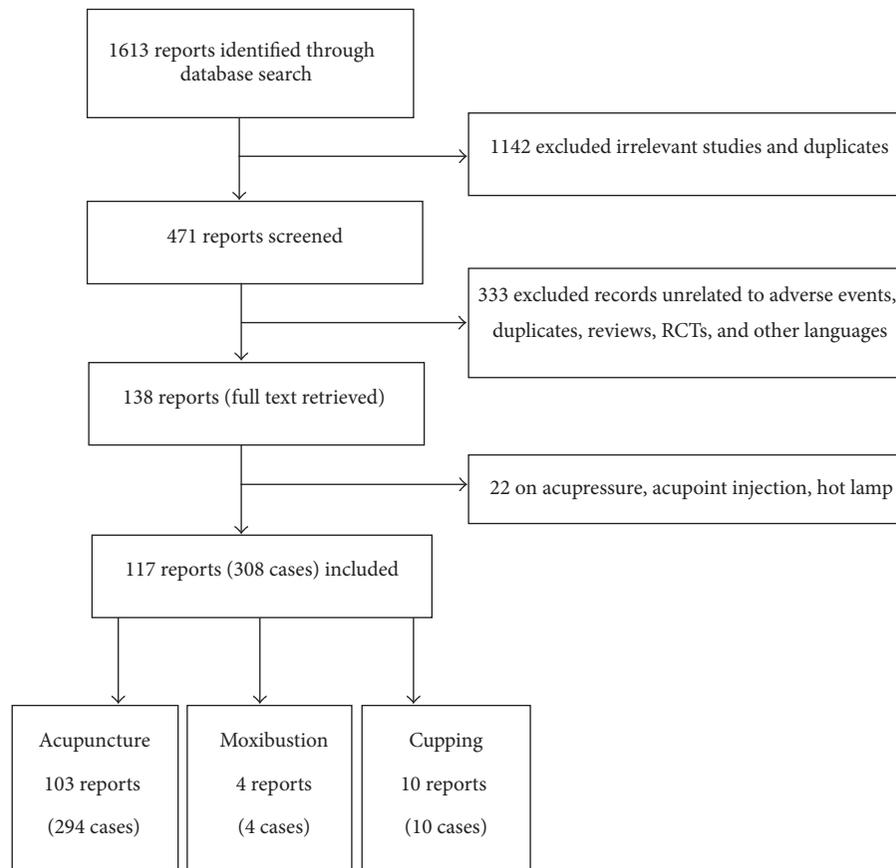


FIGURE 1: Flow chart of the screening process.

(3) recommend safe acupuncture practices based on these reported incidents in order to enhance professional standards of practice.

2. Materials and Methods

2.1. Search Strategy. We searched six databases in an attempt to locate any and all existing English-language case reports on acupuncture AEs published between 2000 and 2011 in electronic form. PubMed, Medline, the Central Information System of Complementary Medicine (CISCOM), Excerpta Medica (EMBASE), Citations in Nursing and Allied Health Literature (CINAHL), and the Complementary and Alternative Medicine for Pain (CAMPAIN) were searched. Search terms were “acupuncture, acupuncture anesthesia, acupuncture analgesia, electroacupuncture, acupuncture points, auricular acupuncture, moxibustion, needling, and cupping.” These terms were combined with “safe, safety, adverse event, adverse reaction, side effects, complications, and risk.”

2.2. Inclusion and Exclusion Criteria. Only original case reports of complications or AEs of acupuncture, moxibustion, and cupping published from 2000 to 2011 were included in this review. Two authors independently screened the titles and abstracts of all papers found from the initial

search. Disagreements between the two authors were resolved through discussion.

We excluded multiple inclusions and analyses of the same AE as well as irrelevant studies. An irrelevant study was defined as a non-case report, such as a review, commentary, or clinical trial.

AEs reporting infection, internal organ or tissue injury, and other severe consequences are categorized as “complications,” defined as an added difficulty; a complex state; a disease or accident superimposed upon another without being specifically related. Peripheral or secondary effects such as syncope, nausea, or immune reactions are classified as “adverse reactions” [5].

2.3. Data Extraction. A total of 1613 papers were found; 117 were relevant (Figure 1). When provided, we extracted author, year of publication, country of occurrence, number of patients affected, disease originally treated, preexisting conditions that might have contributed to the AE, the needling site, the reported AE and its outcome, the practitioner’s training, and the patient’s status at followup. The majority of the reports did not give the date of the AEs. The data were extracted by two independent coauthors, double checked to ensure matching, and organized by whether the AEs were (1) complications or (2) adverse reactions.

TABLE 1: Adverse events associated with acupuncture, moxibustion, and cupping (2000–2011).

Adverse events	Number of cases
<i>Acupuncture</i>	
Complications	284
Infections	239
Isolated incidents	48
Outbreaks	191
Internal organ or tissue injury	38
Pneumothorax	13
Central nerve system	9
Peripheral nerves	4
Heart	5
Other injuries	7
Other complications	7
Adverse reactions	10
<i>Moxibustion</i>	4
<i>Cupping</i>	10
Total	308

3. Results

For the years 2000–2011, a total of 117 reports containing 308 AEs associated with acupuncture (294 cases), moxibustion (4 cases), and cupping (10 cases) were identified from 25 countries and regions (Table 1).

3.1. Acupuncture Complications: Infections. A total of 239 reported cases were infections associated with acupuncture. These include 48 individual isolated cases reported in 45 papers (Table 2) and 191 cases reported in five outbreaks (Table 3). Incidents were reported in 17 countries and regions. Korea reported 162 cases, Canada 33, Hong Kong 7, Australia 8, Japan 5, Taiwan 5, UK 4, USA 6, Spain 1, Ireland 1, France 1, Malaysia 1, Croatia 1, Scotland 1, Venezuela 1, Brazil 1, and Thailand 1. Most of the papers did not report the practitioner's training, but 4 cases were treated by individuals with no medical training or license [6, 7]. One patient with a knee infection died due to renal failure [8]. All other cases recovered after the infection was treated.

3.2. Mycobacterium Infection. Of the 239 cases of infection, 193 (80.75%; 153 from Korea, 32 from Canada, 5 from Hong Kong, 1 from Venezuela, 1 from Brazil, and 1 from Spain) were associated with mycobacterium.

In 2006, Song et al. reported an outbreak of 40 cases of infection in an Oriental medicine clinic in Republic of Korea. Although disposable acupuncture needles were used, the patients developed skin lesions at two or more sites on the body; infections were confirmed by laboratory culture, clinical signs, and histopathology. All patients recovered after active treatment with antibiotics. Reportedly, these patients received hot-pack therapy and gel massage after acupuncture treatment. No further cases were found in that clinic after

equipment sterilization, and regular towel changes were instituted. The authors of the report concluded that the outbreak of infection was due to improper sterilization of equipment applied to the skin after withdrawal of acupuncture needles [52].

In 2006, Tang et al. reported an outbreak of acupuncture-associated bacterial infection in Canada. Between April and December 2002, thirty-two patients developed cutaneous mycobacteriosis after visiting an acupuncture practice in Toronto. Interviews with the patients and acupuncturist revealed that needles were reused and kept in a container of glutaraldehyde disinfectant prior to insertion. The solution was no longer available at the time of the investigation but was probably improperly diluted with tap water [51].

In 2009, Koh et al. reported an outbreak of 109 cases of skin and soft tissue infection in an acupuncture clinic in Republic of Korea. Most patients had at least one skin lesion. Investigators determined that disposable acupuncture needles were used and were unlikely to be the source of infection. Infected patients were all treated by a physical therapy called “interferential current therapy” or “low-frequency therapy.” The authors found that the diluted disinfectant used to sanitize the therapeutic equipment had been prepared several months earlier and was contaminated with *Mycobacterium abscessus*, the likely source of the outbreak [54].

Woo et al. reported four cases of infection by alcohol-resistant mycobacterium, discovered over a two-year period, in patients with skin lesions who were receiving acupuncture treatment in Hong Kong (Table 3). The patients had clinical and/or radiological lesions at acupuncture points. The acupuncturists' training and whether disposable acupuncture needles were used were not reported. The authors recommended that proper infection control guidelines for acupuncture should be mandatory and strictly implemented [50].

3.3. Staphylococcus Infection. Nineteen cases from 14 case reports concern staphylococcus infections associated with acupuncture [14, 15, 17, 21, 25, 27, 28, 30, 33, 38, 39, 46, 47, 53]. Of these, nine patients were infected by methicillin-resistant *Staphylococcus aureus* (MRSA): six from Australia [53], one from Korea [33], one from Taiwan [47], and one from Hong Kong [38].

In the Australian case, Murray et al. reported a 2008 outbreak of eight cases of invasive MRSA, six of them associated with acupuncture (Table 3). After extensive investigation, the authors concluded that the outbreak most likely resulted from a breakdown in sterile technique during the acupuncture procedure and that the MRSA was probably transmitted from the medical practitioner to the patients. At two time points fifteen months apart, that practitioner had been positively colonized with the MRSA strain that caused the infection [53].

3.4. Other Infections. Other infections (31 cases) include septic arthritis [10, 23, 31, 39], necrotizing fasciitis [26, 45, 49], pneumoretroperitoneum [34, 36], facial erysipelas [20],

TABLE 2: Infections associated with acupuncture (48 cases).

First author/year (references)	Country	Cases age/sex	Disease treated	Punctured site	Diagnosis	Practitioner	Followup time
Origuchi 2000 [9]	Japan	67/M	Not stated	Not stated	Infectious aortic aneurysm	Not specified	Recovered (≥ 8 d)
Ishibe 2001 [10]	Japan	13/M	LBP #	Not stated	Septic arthritis	Acupuncturist	Recovered (1 wk)
Woo 2001 [11]	HK	79/F	Knee OA	GB38* (leg)	<i>Mycobacterium chelonae</i>	Not specified	Recovered (3 wk)
Nambiar 2001 [12]	UK	42/F	LBP #	Not stated	Endocarditis	Not specified	Recovered (?)
Shah 2002 [13]	UK	37/M	Tendonitis	BL57* (leg)	Streptococcus	Not specified	Recovered (?)
Leavy 2002 [14]	USA	33/M	Hip pain	Low limb	<i>Staphylococcus aureus</i>	Not specified	Recovered (6 wk)
Laing 2002 [15]	Ireland	45/F	Postoperative recovery	Around tibia	<i>Staphylococcus aureus</i> strain sensitive to methicillin (in knee joint)	Practitioner	Recovered (6 wk)
Uchino 2002 [16]	Japan	47/F	Weight loss	Earlobes	Infected left atrial myxoma (Gram-positive)	Not specified	Recovered (after surgery)
Woo 2003 [17]	HK	73/M	LBP	Back	Staphylococcus	Not specified	Recovered (5 wk)
Ara 2003 [18]	Spain	58/F	Obesity	Abdomen	<i>Mycobacterium chelonae</i>	Not specified	Recovered (3 mo)
Cho 2003 [19]	Korea	56/M	Right flank discomfort	Not stated	<i>Klebsiella pneumoniae</i>	Not specified	Recovered (?)
Kettaneh 2003 [20]	France	70/F	Not stated	Face	Facial erysipelas	Physiotherapist	Recovered (4 wk)
Wiwanitkit 2003 [6]	Thailand	60/F	muscle pain	Not stated	HIV	Non-MD	Not stated
Ha 2003 [21]	Korea	68/F	LBP	Back	Staphylococcus	Not specified	Recovered (4 mo)
Lin 2003 [22]	Australia	44/F	Not stated	Thigh	Tissue abscess and osteomyelitis	Not specified	Recovered (?)
Daivajna 2004 [23]	UK	48/F	LBP	Low back	Septic arthritis	Not specified	Recovered (3 wk)
Studd 2004 [24]	Australia	64/F	Epigastric pain	Abdomen (embedded needles)	Intra-abdominal abscess	Not specified	Recovered (3 wk)
Kim 2004 [25]	Canada	50/M	LBP	Lower back	Discitis from staphylococcus	Acupuncturist	Recovered (?)
Saw 2004 [26]	Malaysia	55/F	Knee OA	Knee	Necrotizing fasciitis	Not specified	Recovered (?)
Chen 2004 [27]	Taiwan	44/M	Nuchal and subscapular pain	Cervical paraspinal and medial scapular region	<i>Staphylococcus aureus</i>	Not specified	Recovered (5 mo)
Vucicevic 2005 [28]	Croatia	53/F	Shoulder stiffness	Shoulder and arm	Staphylococcus pleural empyema	Not specified	Recovered (6 wk)
Bang 2005 [29]	Korea	64/M	LBP	Lumbar paraspinal muscles	<i>Escherichia coli</i>	Not specified	Paraplegic
Seeley 2006 [30]	USA	31/M	Hip pain	Bip, thigh	Staphylococcus bacteraemia	TCM doctor	Recovered (4 wk)
Simmons 2006 [8]	Scotland	69/M	Knee pain	SPI0* (knee)	Cellulitis, septicemia, and pneumonia	Not stated	Death due to renal failure
Tien 2008 [31]	Taiwan	78/M	Knee RA	Knee	<i>Listeria monocytogenes</i> Septic arthritis	Acupuncturist	Recovered (3 wk)
Morgan 2008 [32]	USA	16/F	Weight loss	Auricular	<i>Pseudomonas aeruginosa</i>	Acupuncture parlor	Recovered (21 d)
Lee 2008 [33]	Korea	79/M	LBP	Back	<i>Escherichia coli</i> and MRSA	Not specified	Recovered (76 d)
Hwang 2008 [34]	Korea	25/F	LBP	Back	Pneumoretroperitoneum	OMD	Recovered (1 wk)
Jeong 2009 [35]	Korea	24/F	Weight loss	Both arms	Facial panniculitis	Not specified	Recovered (?)
Hwang 2008 [36]	Korea	22/F	Weight loss	Abdomen	Facial panniculitis	Not specified	Recovered (?)
Hwang 2008 [36]	Korea	25/F	LBP	Not stated	Pneumoretroperitoneum	Licensed OMD	Recovered (7 d)
Wu 2009 [37]	Taiwan	12/M	Neurologic sequelae of encephalitis	Head	Pott's puffy tumor from pseudomonas	Not specified	Recovered (8 wk)

TABLE 2: Continued.

First author/year (references)	Country	Cases age/sex	Disease treated	Punctured site	Diagnosis	Practitioner	Followup time
Woo 2009 [38]	HK	43/F	Knee pain	Knee	MRSA	Not specified	Recovered (3 mo)
Ogasawara 2009 [39]	Japan	50/F	LBP	Lower back	Septic arthritis (MRSA)	Not specified	Recovered (70 d)
Guevara-Patiño 2010 [40]	Venezuela	23/F	Not stated	Not stated	NTM	Not specified	Recovered (6 mo)
Nakajima 2010 [41]	Japan	60/F	Knee pain	Needles embedded at knee	<i>Enterococcus faecalis</i> knee infection	Not specified	Recovered (1y)
Winter 2010 [42]	USA	21/F	Obesity	Auricular	Auricular cellulitis	Acupuncturist	Recovered (2 d)
Kim 2010 [43]	Korea	30/M	Obesity	Auricular	Auricular cellulitis	Acupuncturist	Recovered (1 wk)
Cho 2010 [44]	Korea	53/F	LBP	Lower back	Psoas abscess	Not specified	Recovered (2 wk)
	Korea	59/F	Not stated	Abdomen, thigh	Mycobacterium skin infection	Not specified	Recovered (3 mo)
		77/M	Not stated	Back and abdomen	Cutaneous tuberculosis infection	Illegal treatment	Recovered (1y)
Kim 2010 [7]	Korea	72/F	Not stated	Back, shoulder, and right thigh	Cutaneous tuberculosis infection	Illegal treatment	Recovered (9 mo)
		75/F	Not stated	Back and thigh	Cutaneous tuberculosis infection	Illegal treatment	Recovered (9 mo)
Macuha 2010 [45]	USA	84/M	Osteoarthritis	Left groin	Necrotizing fasciitis	Not specified	Recovered (2 mo)
Buckley 2011 [46]	UK	15/M	Eczema	Around the knee	<i>Staphylococcus aureus</i> endocarditis	Not specified	Recovered (3 mo)
Kuo 2011 [47]	Taiwan	57/M	LBP	Bilateral paraspinal muscles	MRSA	Not specified	Recovered (2 mo)
Castro-Silva 2011 [48]	Brazil	59/M	Ankle pain	Limb	<i>Mycobacterium haemophilum</i> infection	Not specified	Recovered (4 mo)
Hsieh 2011 [49]	Taiwan	44/F	Calf pain	Calf	Necrotizing fasciitis	TCM doctor	Recovered (21 d)

MRSA: methicillin-resistant *Staphylococcus aureus* infection.

NTM: nontuberculous mycobacterial skin infection.

* Acupuncture points.

TABLE 3: Infectious outbreaks associated with acupuncture (191 cases).

First author/year (references)	Country	Cases	Diagnosis	Practitioner	Followup time
Woo 2002 [50]	HK	4	Alcohol-resistant mycobacteria	Not specified	Recovered
Tang 2006 [51]	Canada	32	Mycobacteriosis	Acupuncturist	Recovered
Song 2006 [52]	Korea	40	Mycobacteriosis	Oriental medical clinic	Recovered
Murray 2008 [53]	Australia	6	MRSA	Acupuncturist	Recovered
Koh 2010 [54]	Korea	109	Mycobacteriosis	Acupuncturist	Recovered

TABLE 4: Pneumothoraxes associated with acupuncture (13 cases).

First author /year (reference)	Country	Cases age/sex	Disease treated	Punctured site	Practitioner	Followup
Kao [58]	Taiwan	28/F	Back pain	Thoracic spine bilaterally	Not specified	Recovered (2 d)
Leung 2002 [59]	HK	70/F	Asthma	Thoracic spine bilaterally	Acupuncturist	Not stated
Iwadata 2003 [60]	Japan	72/F	Stiff neck	Thoracic cavity	Acupuncture clinic	Death
Peuker 2004 [61]	Germany	38/F	Breathing problem	Points at chest and upper back (LU1 and BL13)	Medical acupuncturist	Recovered (1 wk)
Saifeldeen 2004 [62]	UK	31/M	Shoulder pain	Right scapular region	Not specified	Recovered (1 wk)
Lee 2005 [63]	HK	36/F	Back pain	Upper back	Registered TCM practitioner	Recovered (5 d)
Chauffe 2006 [64]	USA	27/M	Upper back pain	Upper back (T2-8 levels)	Not specified	Recovered (2 d)
Su 2007 [65]	Singapore	52/F	Chronic bronchitis	Upper back (T3)	Not specified	Recovered (2 d)
Von Riedenauer 2007 [66]	USA	25/M	Shoulder pain	Migration of embedded needles	Not specified	Recovered (1 wk)
Juss 2008 [67]	UK	50/F	Neck and back pain	Acupoints at upper back (BL13, BL14, BL15, and BL16)	Physiotherapist	Recovered (2 d)
Richter 2008 [68]	New Zealand	35/F	Back pain	Back region	Physiotherapist	Recovered (10 d)
Kennedy 2010 [69]	USA	54/F	Musculoskeletal pain	Left side chest	Not specified	Recovered (?)
Inayama 2011 [70]	Japan	37/F	Not stated	Neck and upper back	Acupuncturist	Recovered (12 d)

HIV [6], *Listeria monocytogenes*-caused arthritis [31], and infections by *Enterococcus faecalis* [41] and *Pseudomonas* [32, 37]. Although most of the reports did not state possible cause of the infections, reusable needles were used in a few cases.

3.5. Acupuncture Complications: Organ and Tissue Injuries.

Of 38 cases of organ or tissue injuries, 13 were pneumothoraxes (Table 4); 9 were central nerve system injuries (Table 5); 4 were peripheral nerve injuries (Table 6); 5 were heart injuries (Table 7); 7 were other organ and tissue injuries (Table 8). The cases were distributed among ten countries: 10 from South Korea, 6 from the USA, 6 from Taiwan, 5 from Japan, 3 from the UK, 2 from Germany, 2 from Hong Kong, 1 from Austria, 1 from Iran, 1 from Singapore, and 1 from New Zealand. Although most papers did not report the training background of the practitioner, 3 cases were reportedly treated by individuals with no medical training or license [55–57].

3.6. *Pneumothorax* (Table 4). Of 13 cases of pneumothorax [58–70] associated with acupuncture, the USA reported 3,

the UK 2, Hong Kong 2, Japan 2, Singapore 1, Germany 1, Taiwan 1, and New Zealand 1. Most of these were reported by emergency room physicians. The major patient complaints were dyspnea and chest pain; pneumothorax was confirmed by X-ray. All but one of the 13 patients recovered. A 72-year-old woman died 90 minutes after an acupuncture treatment; autopsy confirmed that the cause was needle penetration of the thoracic cavity [60].

3.7. *Central Nervous System Injury* (Table 5). There were nine cases of central nervous system injury, including five spinal cord injuries [55, 73–75, 77] and four of brain injury [56, 71, 72, 76].

Two of the spinal injuries were caused by migrating broken needles [55, 75]; the others were probably the result of needling too deeply. All patients recovered after treatment.

The brain injuries were an acute intracranial hemorrhage [71], an injury to the medulla oblongata [72], a subarachnoid hemorrhage [76], and an intracranial hemorrhage with cerebellar infarction [56]. Three were due to needle insertion; the medulla injury was caused by a broken needle. Three

TABLE 5: Central nervous system injuries associated with acupuncture (9 cases).

First author /year (reference)	Country	Cases age/sex	Disease treated	Punctured site	Complication	Onset after acupuncture	Practitioner	Followup
Choo 2000 [71]	USA	44/M	Neck pain	GV16 (neck)	Acute intracranial hemorrhage	Immediately	Not specified	Recovered (10 d)
Hama 2004 [72]	Japan	70/M	Not stated	Not stated (broken needle)	Medulla oblongata injury, left facial paresthesia	3 wk	Not specified	Recovered (1 y)
Eftekhari 2005 [73]	Iran	74/M	LBP	Lumbar region	Epidural hematoma	Shortly	Not specified	Recovered (after surgery)
Chen 2006 [74]	Taiwan	30/M	Back pain	Upper back	Epidural haematoma	1 h	Acupuncturist	Recovered (after surgery)
Ulloth 2007 [75]	USA	52/M	LBP	L1, L2, and L3 Vertebrae (embedded needles)	Cerebrospinal fluid fistula	14 mo	Acupuncturist	Recovered (after surgery)
Liou 2007 [55]	Taiwan	29/M	Stiffness of neck	Epidural space at C2 level (a broken needle)	Spinal Cord Injury	3 y	“Nonmedical practitioner”	Recovered (after surgery)
Tsukazaki 2008 [76]	Japan	32/F	Not stated	GV16 (neck)	Subarachnoid hemorrhage	1 d	Oriental medicine clinic	Not stated
Lee 2011 [77]	Korea	58/F	Quadri-paresis neck pain	Neck	Cervical epidural hematoma	1 h	Family physician	Recovered (8 wk)
Heo 2011 [56]	Korea	65/M	Not stated	Posterior neck	Intracranial hemorrhage and cerebellar infarction	3 d	Unauthorized acupuncturist	Recovered (1 mo)

TABLE 6: Peripheral nerve injuries associated with acupuncture (4 cases).

First author /year (reference)	Country	Cases age/sex	Disease treated	Punctured site	Complication	Onset after acupuncture	Practitioner	Followup
Sato 2003 [78]	Japan	62/F	Sciatica	Anterior of the leg	Peroneal nerve palsy	1 d	Not specified	Recovered (4 mo)
Patrick 2005 [79]	USA	63/F	LBP	Low back	Injury of the L5 nerve root	28 y	Not specified	Recovered (after surgery)
Rosted 2007 [80]	UK	47/M	TMD	ST6, ST7 (face)	Bell's Palsy	1 d	Not specified	Recovered (2 wk)
Lee 2008 [81]	Korea	47/M	Abdominal discomfort	PC5 & PC6 (forearm)	Median nerve neuropathy	Shortly	Oriental medicine practitioner	Recovered (1 y)

TABLE 7: Heart injuries associated with acupuncture (5 cases).

First author /year (reference)	Country	Cases age/sex	Disease treated	Punctured site	Complication	Onset after acupuncture	Practitioner	Followup
Kirchgatterer 2000 [82]	Austria	83/F	Not stated	Sternum	Cardiac tamponade	20 min	Experienced acupuncturist	Recovered (2 wk)
Park 2004 [83]	Korea	49/F	Shoulder pain	Shoulders and upper back	Cardiac tamponade	2 h	Not specified	Recovered (after surgery)
Kim 2006 [84]	Korea	70/M	Chronic lung disease	Neck, chest, and abdomen (embed needles)	Right ventricular embolism	1 y	Not specified	Not stated
Song 2010 [85]	Korea	69/F	Pain	Shoulders and neck (implanted needles)	Myocardium injury	10 y	Traditional medicine practitioner	Unknown
Kim 2011 [57]	Korea	54/f	Myalgia and dyspepsia	Chest, abdomen	Hemopericardium	30 min	Unauthorized acupuncturist	Recovered (6 d)

patients recovered after treatment; outcome was not given for the fourth (Table 5).

3.8. Peripheral Nerve Injury (Table 6). Four reported cases of peripheral nerve injury were associated with acupuncture treatment [78–81], one each in Japan, Korea, the USA, and the UK. The injured nerves were the peroneal nerve via acupuncture point GB34 the median nerve via PC5 and PC6, the facial nerve via ST7 and ST8, and the L5 nerve root via a broken needle in the lumbar region. All patients recovered.

3.9. Heart Injury (Table 7). Five cases of heart injury include two of cardiac tamponade [82, 83], one of the hemopericardium [57], one ventricular embolism [84], and one myocardial injury [85]. Of these, two were due to the migration of embedded needles [83, 84] and two were due to needle insertion [57, 82]. Two were caused by an acupuncturist or TCM practitioner, and one by an “unauthorized acupuncturist” [57]. The status of two practitioners was unreported. Three patients recovered; outcome was not reported in the other two cases.

3.10. Other Organ and Tissue Injuries (Table 8). Seven cases of other organ and tissue injuries were found: a pseudoaneurysm of the abdominal aorta [86], a pseudoaneurysm of the popliteal artery [87], acute traumatic pancreatitis [88], an aortoduodenal fistula causing direct communication between the aorta and the GI tract [89], a rectus sheath hematoma [90], ear hematomas [91], and a popliteal arteriovenous fistula [92]. The patient with acute traumatic pancreatitis had been treated with 13 cm needles placed at three sites on the anterior abdominal wall. Abdominal computed tomography revealed small multiple gold acupuncture needles on the anterior abdominal wall and back muscles. The patient’s condition quickly improved with fasting and intravenous fluids [88]. One patient died [89].

3.11. Other Complications of Acupuncture. Seven other complications associated with acupuncture were reported (Table 9): bilateral hand edema [93], epithelioid granuloma at needling sites [94], pseudolymphoma [95], localized argyria [96], pustules [97], pancytopenia [98], and scars at needling sites [99]. The localized argyria and pancytopenia were caused by needles embedded 20 and 17 years earlier, respectively [96, 98], in a type of Japanese acupuncture reported in our previous review [5]. The epithelioid granulomas were caused by silicone coating on the needles [94]. The scars were due to a hot needle technique in which the needles were heated in fire before insertion [99].

3.12. Adverse Reactions Associated with Acupuncture. Ten cases of adverse reactions from acupuncture were found (Table 10): three of syncope from two reports [100, 101]; two of galactorrhoea (spontaneous milk flow) [102, 103]; one of bilateral nystagmus [104]; one of pyoderma gangrenosum due to immune reaction, in which the tissue became necrotic and deep ulcers formed [105]; one of hepatotoxicity [106]; one

of eruptive lichen planus [107]; one of spontaneous needle migration [108]. These unusual cases are uncommonly seen in regular acupuncture practice. The case report authors postulated that these AEs were likely caused by a rare physiological reaction to the acupuncture needle. For example, the case report of spontaneous needle movement involved the acupuncture needles having “spontaneously moved deeper as far as the hilt, travelling an extra depth of 5–10 mm,” which was observed repeatedly on the same patient. Although there was no resulting complicating in this case, the authors cautioned that this could have caused serious complications if the needles had been placed near a vital organ [108].

The syncope cases occurred immediately or several minutes after a first acupuncture treatment; the patients were sitting or semirecumbent during treatment [100, 101].

3.13. Complications Associated with Moxibustion. Four AEs associated with moxibustion were found (Table 11): bruising [109], burns and cellulitis [110], spinal epidural abscess [111], and large superficial basal cell carcinoma [112]. Of these, two were self-administered [111, 112]. An “untrained individual” performed the third [110]; there was no information on the fourth [109].

3.14. AEs Associated with Cupping. Ten AEs associated with cupping were found (Table 12): four from Turkey, three from Korea, two from Taiwan, and one from the UK. Most were minor: keloid scarring [113], burns [114, 115], and bullae [116, 117]. Several were serious: acquired hemophilia A [118], stroke 14 hours after cupping on the back and neck [119], factitious panniculitis [120], reversible cardiac hypertrophy [121], and iron deficiency anemia [122]. These last two cases involved cupping with bleeding [121, 122]. In six cases, there was no information on practitioner training; in the other four, treatment was self-administered.

4. Discussion

Our primary objective in reviewing case reports of AEs associated with acupuncture has been to identify individual cases and outbreaks of AEs and to analyze their possible causes, in order to minimize future acupuncture AEs and enhance safe practice within the profession. How do the objectives and results of this review fit in the context of other available literatures on the safety of acupuncture? Incidence rates for major AEs of acupuncture are best estimated from large prospective surveys of practitioners. Four recent surveys of acupuncture safety among regulated, qualified practitioners, two conducted in Germany [4, 123] and two in the United Kingdom [3, 124], confirm that serious adverse events after acupuncture are uncommon. Indeed, of these surveys, covering more than 3 million acupuncture treatments all together, there were no deaths or permanent disabilities, and all those with AEs fully recovered [125]. Thus, it can be concluded that acupuncture has a very low rate of AEs, when conducted among licensed, qualified practitioners in the West. Recent systematic reviews of RCTs of acupuncture [126–128], in which the acupuncture procedure is also

TABLE 8: Other organ or tissue injuries associated with acupuncture (7 cases).

First author /year (reference)	Country	Cases age/sex	Disease treated	Punctured site	Complication	Onset after acupuncture	Practitioner	Followup
Kim 2002 [86]	Korea	54/M	Abdominal pain	Back	Pseudoaneurysm of abdominal aorta	Immediately	OMD	Recovered (8 d)
Kao 2002 [87]	Taiwan	61/F	Osteoarthritis	Knee	Pseudoaneurysm of the popliteal artery	6 mo	Not specified	Recovered (in 1 y)
Uhm 2005 [88]	Korea	42/F	Dyspepsia	Abdomen	Acute traumatic pancreatitis	5 h	Acupuncture clinic	Recovered (4 d)
Chang 2005 [89]	Korea	68/F	LBP	Abdomen	Aortoduodenal fistula	2 wk	Not specified	Dead
Cheng 2005 [90]	Taiwan	37/F	Weight loss	Abdomen	Rectus sheath hematoma	4 h	Not specified	Recovered (1 mo)
Usichenko 2006 [91]	Germany	78/M	Postoperative pain	Ear lobe (embedded needles)	Ear hematomas	4 d	Not specified	Recovered with discoloration
Kuo 2010 [92]	Taiwan	39/F	Knee soreness	Popliteal fossa	Popliteal arteriovenous fistula	Several years	Not specified	Discharged

TABLE 9: Other complications associated with acupuncture (7 cases).

First author /year (reference)	Country	Case age/sex	Disease treated	Puncture site	Complication	Followup time	Remarks
McCartney 2000 [93]	UK	52/M	LPB	LI4 (Hand)	Bilateral hand edema	Recovered (in 8 wk)	No lab evidence of inflammation
Yanagihara 2000 [94]	Japan	55/F	Shoulder pain and lumbago	Back, hip, neck, legs and arms	Epithelioid granuloma at needling sites	Improved	Caused by silicone coating on needles
Kim 2002 [95]	Korea	37/F	Abdominal discomfort	Not state	Pseudolymphoma	Improved	CD-30 positive
Takeishi 2002 [96]	Japan	66/F	Arthralgia	Extremities	Localized argyria	Not stated	Embedded silver needles 20 y earlier
Murray 2002 [97]	UK	35/M	Tennis elbow	Arm	Pustules	Not stated	Pt has Behcet disease
Vassiou 2003 [98]	Greece	67/F	LBP	Chest & abdomen	Pancytopenia	Not stated	Embedded needles 17 y earlier
Pigatto 2004 [99]	Italy	36/F	Hyperthyroidism	St10 (neck)	Scars at needling site	No improvement	"Hot needle" used

conducted under well-controlled conditions, also found no serious AEs associated with acupuncture [128], although one of these systematic reviews of RCTs separately examined case reports of AEs associated with acupuncture and had findings comparable to ours. However, any medical intervention has the potential to cause damage, particularly when administered by an untrained or unqualified practitioner, or in an unregulated setting. Our objective was thus to identify signals that might suggest the potential for AEs of acupuncture, when administered in specific settings, or when using specific acupuncture styles, and also to compare the patterns of AEs in the past 12 years with the patterns identified in the 35-year period covered by our first review. Comparing the new data with that of the previous review shows the emergence

of some important new patterns, which may be relevant for future regulation and policy making.

Although the majority of the AEs are still infections, the routes of infection have changed. Our present findings include 239 AEs from infection; 191 occurred in five outbreaks of bacterial infection caused by skin contact with unsterilized equipment and dirty towels, in unhygienic clinical settings. In our previous findings, hepatitis cross-infections from patient to patient due to reused needles (94 cases reported in four outbreaks) were the most frequent source of infection. Since the introduction of disposable needles, hepatitis infections have rarely been reported, which is an important achievement that has resulted from the greater regulation of acupuncture practice, particularly the requirement for disposable needle

TABLE 10: Adverse reactions associated with acupuncture (10 cases).

First author /year (reference)	Country	Case age/sex	Disease treated	Puncture sites	Adverse reactions	Remarks
Castro-Durán 2000 [105]	Spain	48/F	Arthralgia	Not stated	Pyoderma gangrenosum	Immune response
Jenner 2002 [102]	UK	41/F	Cancer pain	Points at upper back	Galactorrhoea	Breast cancer
Cole 2002 [100]	USA	25/M	Healthy volunteer for a clinical study	ST36 (bilateral)	Convulsive syncope	Pt was sitting
Campbell 2005 [103]	UK	32/F	Foot pain	Local points at foot	Galactorrhoea (left side)	Pt had no lactation prior to the tx
Kung 2005 [101]	Taiwan	72/M	Arm pain	LIII, TB5 (arm)	Syncope	Pt was sitting
Bradbury 2006 [104]	UK	63/F	Ankle pain	GB34, B40 (leg & ankle)	Syncope	Pt was sitting
Smyth 2007 [108]	Scotland	50/F	Shoulder pain	Points around shoulder	Nystagmus	Semirecumbent position
Hong 2008 [106]	China	55/M	Back pain	Back	Spontaneous needle movement	No complication
Fleming 2011 [107]	UK	52/F	Leg weakness	ST36 (leg)	Hepatotoxicity	Pt was in menopause
		41/F	Back pain	Lower back	Eruptive lichen planus	Immune response

TABLE 11: Adverse events associated with moxibustion (4 cases).

First author /year (reference)	Country	Case age/sex	Disease treated	Moxibustion site	Adverse events	Practitioner	Remarks
Fisman 2002 [109]	Canada	38/M	Not stated	Abdomen	Ecchymoses	Not specified	Pt had a hx of liver disease
Chau 2006 [110]	USA	53/F	Headache	Leg and feet	Cellulitis	Untrained individual	Recovered
Lee 2008 [111]	Korea	78/F	Pain	Fingers	Infection caused spinal epidural abscess	Self	Pt had diabetes
Yun 2009 [112]	Korea	58/M	Abdominal pain	Abdomen	Basal cell carcinoma	Self	Pt. self-treated for 10 y

use. However, in recent years, bacterial infections, including MRSA and mycobacterium, have become pervasive in health-care settings in general [129]. Such infections, a pressing concern for all medical practitioners, including acupuncturists, result from poor hygiene. Hygienic clinical settings, sterilized equipment, and clean supplies are critical for preventing future such infections.

Pneumothorax is still the most common organ and tissue injury. There were also cases of spinal cord injuries due to short, small needles embedded laterally along the spine in the Japanese practice known as *okibari*. The putative mechanism responsible for this AE is that the imbedded needles used in the Japanese *okibari* acupuncture technique could spontaneously migrate within the tissue, with some of them migrating to the spinal cord to cause spinal cord injury [130]. However, this AE has significantly decreased since our previous review, in which 11 cases due to this practice were found. In the present review, we found organ injuries

mainly to be associated with faulty needle insertion. Heart injuries can be fatal, although no death was reported in the five cases we found. Acupuncture training programs must enhance student knowledge of anatomy at each acupuncture point. Supervised clinical internships must provide rigorous training in needle direction, depth of insertion with attention to the size of the patient, and methods of manipulation.

Three cases reported deaths attributed to acupuncture [8, 60, 89]. Two were due to organ injuries [60, 89], and one was due to infection [8]. Of the organ injury deaths, one case from Japan [60] reported that a 72-year-old woman died after bilateral tension pneumothorax following acupuncture. The finding of the autopsy also suggested the patient that may have been injured by the insertion of the needles into the lungs during the previous acupuncture treatments. The second organ injury death, from Korea, reported that a 68-year-old woman died of massive hematemesis resulting from aortoduodenal fistula. The autopsy showed an injury to the

TABLE 12: Adverse events associated with cupping (10 cases).

First author /year (reference)	Country	Case age/sex	Disease treated	Cupping site	Adverse events	Practitioner	Remarks
Birol 2005 [113]	Turkey	36/F	Cough	Back	Keloid scar	Not specified	Recovered (several days)
Kose 2006 [114]	Turkey	30/M	Back pain	Back	10% burns at shoulder and back	Self	Recovered (11 d)
Tuncez 2006 [116]	Turkey	57/F	LBP	Low Back	Suction bullae	Not stated	Diabetic; cupping lasted 40 min
Weng 2008 [118]	Taiwan	58/F	Not stated	Thigh	Acquired hemophilia A	Not stated	Improved (1 wk)
Sohn 2008 [121]	Korea	66/F	Pain	Not specified	Reversible cardiac hypertrophy	Self	Bloodletting with cupping >10 y, recovered (3 mo)
Lee 2008 [122]	Korea	39/M	Musculoskeletal pain	Back	Iron deficiency anemia	Not stated	Bloodletting with cupping Pt. fully recovered
Lin 2009 [117]	Taiwan	55/M	Not stated	Back	Bullae	Not stated	Recovered (several wk)
Blunt 2010 [119]	UK	55/M	Not stated	Back and neck	Hemorrhagic stroke (14 h later)	Not stated	May be due to stimulation of baroreceptor, neck area
Kulahci 2011 [115]	Turkey	32/M	Back pain	Back	Burns on back and shoulder	Mother	Recovered
Moon 2011 [120]	Korea	56/F		Neck and shoulder	Factitious panniculitis	Self	Recovered (3 mo)

abdominal aorta, caused by a deep insertion with a 15 cm long acupuncture needle into the abdomen [89]. The third case was reported from Scotland in which a 69-year-old man died from an infection after acupuncture treatment at the thigh [8]. The patient was later found to have a preexisting pancytopenia (i.e., low white blood cell count), resulting in an increased susceptibility to infection. The case report author, who is also the practitioner, admitted that the patient's skin at the acupuncture point was not cleaned prior to the needle insertion and later found local muscle infection which led to septicaemia. The patient died a few weeks later from a multiorgan failure. These three unfortunate death cases suggest that biomedical knowledge such as anatomy and microbiology is needed in order avoid organ injury and infection. Skin cleansing should also be required, particularly for those patients with immune compromised condition.

There were only a handful of cases reported by practitioners who performed the acupuncture [8, 100, 101, 103, 104, 108] including a death report [8]. The rest of the cases were reported by investigators who were not the acupuncturists who performed the treatment. Most cases of AEs did not report the qualification of the practitioner. We would suggest that future report on AEs of acupuncture should include the information on the training qualification of the practitioners and the procedure used for the treatment, such as whether or not clean needle techniques were used.

Acupuncture safety practice guidance or guidelines such as Clean Needle Technique (CNT) appear to have played a critical role in minimizing the number of AEs associated with acupuncture practice [129]. In the United States, CNT was first addressed by the National Certification Commission for Acupuncture and Oriental Medicine in 1984. This course is designed to train professional acupuncturists on safe practice procedures. Course content includes training on microbiology, infection control, skills of adequately setting up a sterile practice area (e.g., adequate use of disinfectant and sterile equipment), adequate needle insertion, and adequate handling of AEs associated with acupuncture [130]. CNT courses are now offered by the US Council of Colleges of Acupuncture and Oriental Medicine and required by the acupuncture licensing boards of each state; as a result, reported acupuncture AE incidents have significantly decreased in the United States. In our previous review, about half of the 202 cases of AE that we identified were from the USA. However, as our present review shows, AE cases reported from the USA are now rare. Of the 308 cases we found, only 13 were from the United States, and out of 239 cases of infection, only 5 are from the United States. It should be noted that there were very few case reports of AEs from China included in this review, although acupuncture is widely practiced in China. We are aware that cases of AEs associated with acupuncture performed in China are likely to be reported in Chinese

language case reports, which are not reflected in the present review due to language limitation. We are currently preparing a separate review on AEs reported in China.

In conclusion, although serious AEs associated with acupuncture are rare, acupuncture practice is not risk-free. Adequate regulation can even further minimize any risk. We recommend that not only adequate training in biomedical knowledge, such as anatomy and microbiology, but also safe and clean practice guidelines are necessary requirements and should continue to be enforced in countries such as the United States where they exist, and that countries without such guidelines should consider developing them in order to minimize acupuncture AEs.

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Research Article

Electroacupuncture Reduces Carrageenan- and CFA-Induced Inflammatory Pain Accompanied by Changing the Expression of Nav1.7 and Nav1.8, rather than Nav1.9, in Mice Dorsal Root Ganglia

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Several voltage-gated sodium channels (Navs) from nociceptive nerve fibers have been identified as important effectors in pain signaling. The objective of this study is to investigate the electroacupuncture (EA) analgesia mechanism by changing the expression of Navs in mice dorsal root ganglia (DRG). We injected carrageenan and complete Freund's adjuvant (CFA) into the mice plantar surface of the hind paw to induce inflammation and examined the antinociception effect of EA at the Zusanli (ST36) acupoint at 2 Hz low frequency. Mechanical hyperalgesia was evaluated by using electronic von Frey filaments, and thermal hyperalgesia was assessed using Hargreaves' test. Furthermore, we observed the expression and quality of Navs in DRG neurons. Our results showed that EA reduced mechanical and thermal pain in inflammatory animal model. The expression of Nav1.7 and Nav1.8 was increased after 4 days of carrageenan- and CFA-elicited inflammatory pain and further attenuated by 2 Hz EA stimulation. The attenuation cannot be observed in Nav1.9 sodium channels. We demonstrated that EA at Zusanli (ST36) acupoint at 2 Hz low-frequency stimulation attenuated inflammatory pain accompanied by decreasing the expression of Nav1.7 and 1.8, rather than Nav1.9, sodium channels in peripheral DRG neurons.

1. Introduction

Recently, several studies have implied that voltage-gated sodium channels (Navs) might be involved in the development of hyperalgesia produced by inflammation [1–3]. Sensory neurons innervating the muscles are considered to sense muscle pain, and Navs are reported to participate in the process of inflammatory pain. Intraplantar injection of carrageenan and CFA is well documented to produce edema, as well as mechanical and thermal hyperalgesia, and

has often been used as an inflammatory pain model [1–4]. Sodium channel-induced currents have been identified in CNS neurons [3] and in DRG (dorsal root ganglia) neurons [2] which significantly influence the threshold for action potential firing.

Several voltage-gated sodium channels, Nav1.1, Nav1.3, Nav1.6, Nav1.7, Nav1.8, and Nav1.9, have been reported to express in DRG. Of these Navs, Nav1.8 and Nav1.9 have been reported to demonstrate resistance to TTX (tetrodotoxin), regarded as TTX-R (TTX-resistant) [2–5]. Nav1.7, Nav1.8,

and Nav1.9 are usually reported to be participating in inflammation pain and regulating neuron excitability [4]. Notably, previous animal studies have indicated that Nav1.3, Nav1.7, and Nav1.8 play prominent roles in inflammatory pain and can be potentiated by microinjection of carrageenan and CFA into intraplantar [1]. Studies on humans have shown that Nav1.7 is crucial for physiological pain sensations, and mutational alterations to Nav1.7 can result in severe chronic pain sensations [6].

Acupuncture has been widely used for over 3000 years and has been based on the principles of traditional Chinese medicine. Acupuncture is known to stimulate the A δ -fibers [7] and modulate pain sensation by activating C-fibers through the meridian [8]. Acupuncture can be used therapeutically to treat diseases systematically [9]. The analgesic effect of acupuncture is already widely accepted. Several studies have suggested that acupuncture increases the release of endogenous opiates [10], serotonin [11], and adenosine to reduce pain [12]. Low-frequency electroacupuncture at 2 Hz induces enkephalins release to activate μ -receptor. In contrast, high-frequency stimulation releases dynorphins to activate κ -receptors [10].

Navs blockers are usually used for anesthesia and analgesia. To seek for more specific inhibitor with low side effects is possible. The rationale of this study is that Nav1.7, Nav1.8, and Nav1.9 are well known in inflammatory pain. The current study examines the crucial role of Navs and the effect of 2 Hz EA in mechanical and thermal hyperalgesia induced by carrageenan and CFA. We hypothesized that EA could alter expressions of Navs in both carrageenan- and CFA-induced inflammatory pain.

2. Methods

2.1. Animals and EA Pretreatment. Adult ICR female mice aged 8 to 12 weeks were used in the experiment. The usage of these animals was approved by the *Institute of Animal Care and Use Committee of China Medical University* (Permit no. 101-116-N), Taiwan, following the *Guide for the use of Laboratory Animals* (National Academy Press). EA treatment was applied using stainless steel needles (12 mm, 32 G, Yu Kuang, Taiwan) which were inserted into the muscle layer to a depth of 2-3 mm at ST36 acupoint, which is therapeutic in both animal models and clinical study [11]. EA was administered immediately after the injection of carrageenan or CFA and performed every day at the same time (12:00–14:00). A Trio-300 (Japan) stimulator delivered electrical square pulses for 20 min with a 100 μ s duration and a 2 Hz frequency. The stimulation amplitude was 1 mA. The same treatment was given to nonacupoint (the upper lateral gluteal muscle but not GB30 acupoint) to be set as the sham control group entitled S-GM [13]. Another sham control group, entitled S-Acu, was induced by needling into ST36 acupoint without manipulation [14, 15].

2.2. Inflammatory Pain Models. Mice were anesthetized with 1-2% isoflurane and administered a single injection of 20 μ L saline (pH 7.4, buffered with 20 mM HEPES), CFA

(0.5 mg/mL heat-killed *M. tuberculosis* Sigma, St. Louis, MO, MSA), or 3% carrageenan (lambda carrageenan and CFA, type IV; Sigma) in the plantar surface of the hind paw to induce intraplantar inflammation. Behavior tests were conducted at day 4 after induction of inflammation, and DRGs were harvested after behavior tests.

2.3. Animal Behavior of Mechanical and Thermal Hyperalgesia. Mechanical sensitivities were tested at 4 days after intraplantar injections. All experiments were performed at 30 min after EA (room temperature was approximately 25°C). Mechanical sensitivity was measured by testing the force of responses to stimulation with five applications of electronic von Frey filaments (North Coast Medical, Gilroy, CA, USA). Thermal pain was measured with five applications using Hargreaves' test IITC analgesimeter (IITC Life Sciences, Woodland Hills, CA, USA). Both hot-induced pain and cold-induced pain were measured using a hot/cold plate (IITC Life Sciences, Woodland Hills, CA, USA). Total of eight mice were used in each animal's behavior per group.

2.4. Immunohistochemistry. Total of 6 mice were anesthetized with an overdose of choral hydrate and intracardially perfused with saline followed by 4% paraformaldehyde. L3–L5 DRGs were immediately dissected and postfixed with 4% paraformaldehyde. Similar protocols were used as previously described [16]. DRGs were incubated with primary antibodies prepared in blocking solution at 4°C overnight against Nav1.7 (1:1000, Alomone), Nav1.8 (1:1000, Alomone), and Nav1.9 (1:1000, Alomone). The secondary antibodies were goat anti-rabbit (Molecular Probes, Carlsbad, CA, USA). Slides were visualized by use of fluorescence-conjugated secondary antibodies and mounted on cover slips.

2.5. Immunoblotting Assay. L3–L5 DRGs from 6 mice were immediately excised to extract proteins. Total proteins were prepared by homogenized DRG as previously described [13]. Peroxidase-conjugated anti-rabbit antibody (1:5000) was used as a secondary antibody. The bands were visualized by an enhanced chemiluminescent substrate kit (PIERCE) with LAS-3000 Fujifilm (Fuji Photo Film Co. Ltd). Where applicable, the image intensities of specific bands were quantified with NIH ImageJ software (Bethesda, MD, USA).

2.6. Electrophysiology. L3–L5 DRGs were isolated from mice treated with intraplantar saline, CFA, CFA with EA for 4 days. DRG culture and settings for whole-cell patch recording were as previously described [16]. The internal solution contained (in mM) 10 NaCl, 110 CsCl, 20 tetraethylammonium-Cl, 2.5 MgCl₂, 5 EGTA, 3 Mg²⁺-ATP, and 5 HEPES, adjusted to pH 7.2 with CsOH. The external solution contained (in mM) 100 NaCl, 5 CsCl, 30 tetraethylammonium-Cl, 1.8 CaCl₂, 1 MgCl₂, 0.1 CdCl₂, 25 glucose, 5 4-aminopyridine, and 5 HEPES, adjusted to pH 7.4 with HCl. Osmolarity was adjusted to 300 mosm. Recordings were performed in external solution with 500 nM TTX (Tocris, Avonmouth, UK). TTX-R currents were evoked by a 50 ms test pulse between -70 and 50 mV in 10-mV steps from a holding

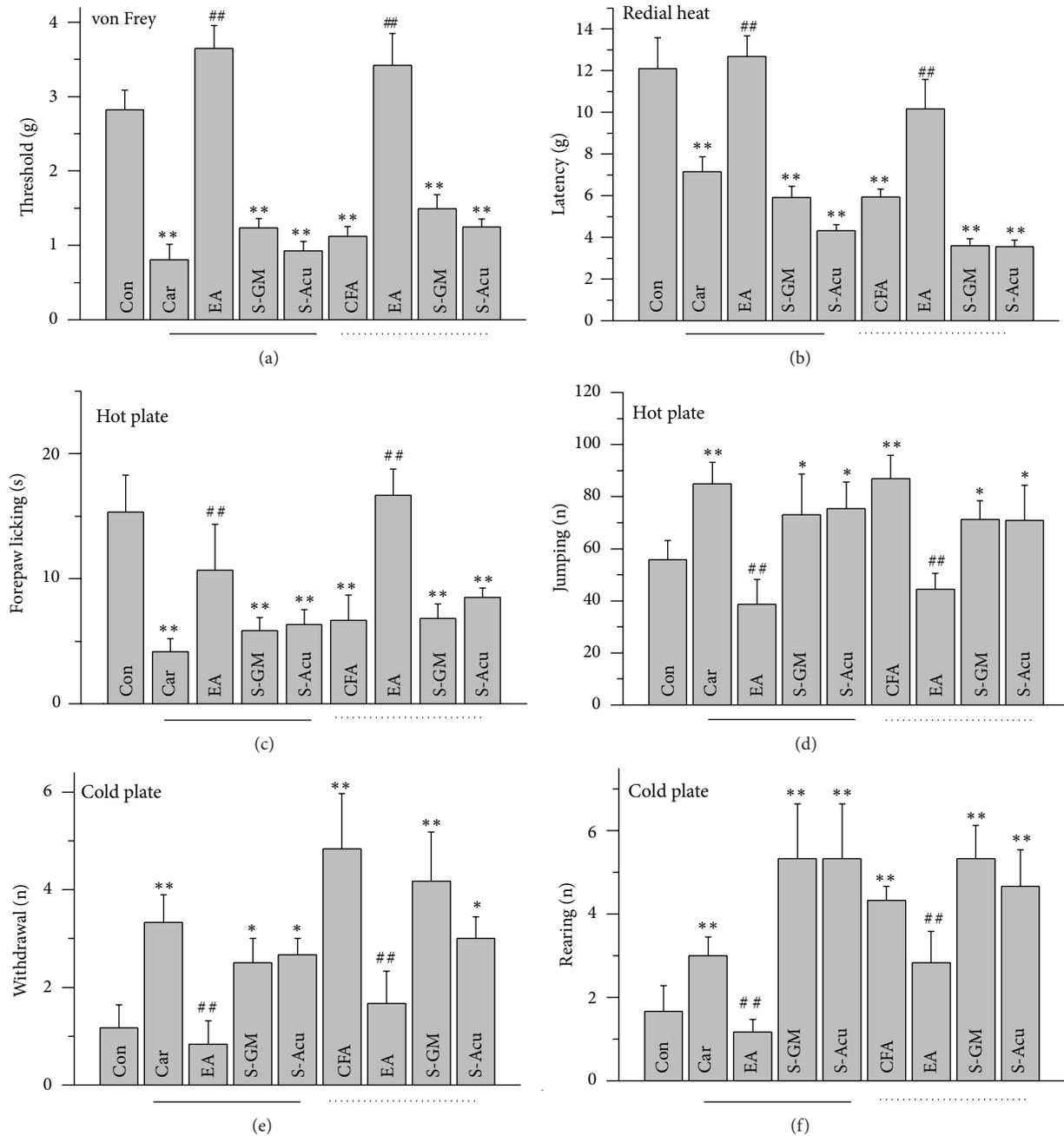


FIGURE 1: Primary inflammation-induced mechanical and thermal hyperalgesia through carrageenan or CFA injection. (a) Electronic von Frey filament test; (b) radial heat assay; (c) and (d) the latency time for forepaw licking and jumping responses after exposure to a hot plate maintained at 50°C. (c) Latency to first forepaw licking, (d) escape times (jumping) within 5 minutes; (e) and (f) the hind paw withdrawal and rearing on exposure to a cold plate kept at 4°C; (e) withdrawal times; (f) rearing times within 5 minutes. Con: saline, Car: carrageenan and CFA: intraplantar injection into hind paw; Car-EA and CFA-EA: after carrageenan or CFA injection, needles inserted at the ST36 acupoint with electrical stimulation at 2 Hz. * $P < 0.05$, as compared to that of the baseline. ** $P < 0.01$, as compared to that of the baseline. # $P < 0.05$; comparison between inflammation and EA-ST36 groups. ## $P < 0.01$; comparison between inflammation and EA-ST36 groups. CFA: complete Freund's adjuvant. Solid lines mean carrageenan-injected group. Dot lines mean CFA-injected group.

potential of -70 mV. All recordings were obtained at room temperature (25°C) and completed within 24 h after plating.

2.7. Statistical Analysis. All statistic data are presented as the mean \pm standard error. Statistical significance between control, inflammation, and EA group was tested using the

ANOVA test, followed by a post hoc Tukey's test ($P < 0.05$ was considered statistically significant).

3. Results

3.1. Inflammatory Pain Models and Behavior. We first showed that intraplantar injection of normal saline did not induce

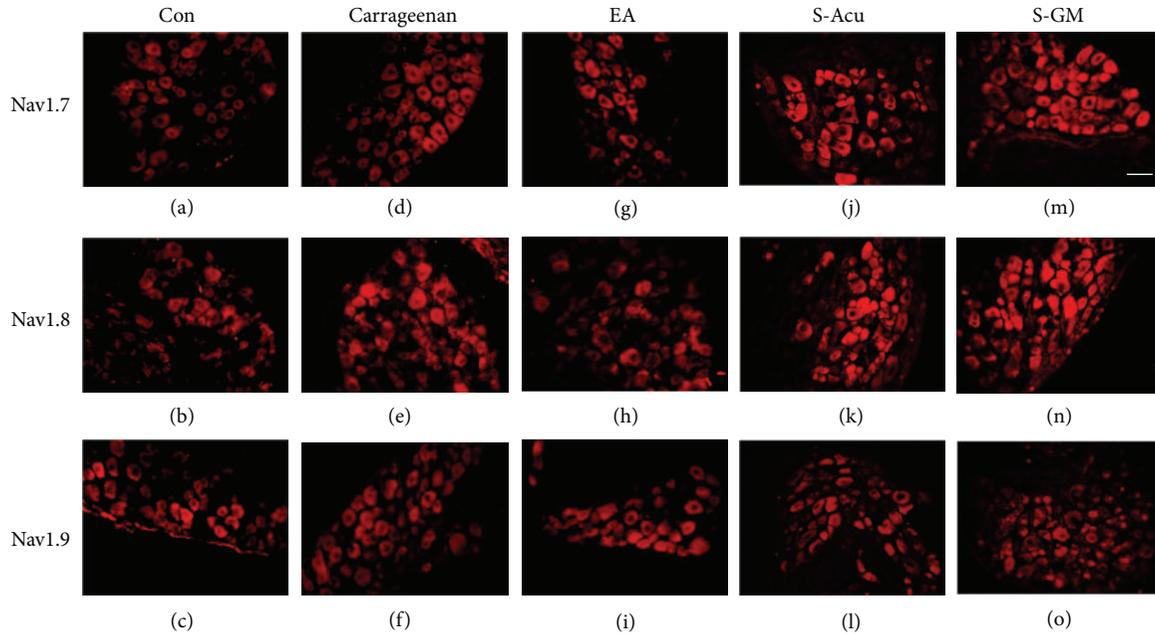


FIGURE 2: Nav1.7 and Nav1.8 expressions were increased in ipsilateral DRGs after intraplantar carrageenan injection and further attenuated by EA at the ST36 acupoint in mice, though Nav1.9 was not different. (a)–(c) Nav1.7, Nav1.8, and Nav1.9 immunoreactive neurons were found in lumbar DRGs at the ipsilateral site of the saline-injected group. (d)–(e) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the carrageenan-injected group, but (f) Nav1.9 immunoreactive neurons were not increased. (g)–(h) Carrageenan-induced increases of Nav1.7 and Nav1.8 were attenuated by EA, as compared to those of the carrageenan-induced group. (i) Nav1.9 immunoreactive neurons were not altered by EA at the ipsilateral site of inflammation. (j)–(k) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the S-Acu group. (l) Nav1.9 immunoreactive neurons were not altered in the S-Acu group. (m)–(n) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the S-GM group. (o) Nav1.9 immunoreactive neurons were not altered in the S-GM group. Scale bar = 50 μ m.

mechanical hyperalgesia to be set as a control group (Figure 1(a), 2.82 ± 0.26 , $n = 8$). Intraplantar injection of carrageenan or CFA successfully produced mechanical hyperalgesia (Figure 1(a), 0.81 ± 0.21 and 1.12 ± 0.13 of carrageenan and CFA, $n = 8$, $P < 0.01$). Low-frequency 2-Hz EA at ST36 reliably attenuated carrageenan- and CFA-induced hyperalgesia (Figure 1(a), 3.64 ± 0.31 and 3.42 ± 0.43 of carrageenan and CFA, $n = 8$, $P < 0.01$). The phenomenon was not observed neither in S-GM (Figure 1(a), 1.24 ± 0.12 and 1.50 ± 0.18 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(a), 0.92 ± 0.13 and 1.25 ± 0.10 of carrageenan and CFA, $n = 8$, $P > 0.05$). We further showed that thermal hyperalgesia was observed in carrageenan-induced inflammatory mice (Figure 1(b), 7.14 ± 0.72 s and 12.1 ± 1.49 s of carrageenan and control, $n = 8$, $P < 0.01$). The same phenomenon was also evoked in CFA-induced inflammatory mice (Figure 1(b), 5.94 ± 0.38 , $n = 8$, $P < 0.01$). Both mechanical and thermal hyperalgesia can be reduced by EA at ST36 (Figure 1(b), 12.69 ± 0.97 and 10.17 ± 1.42 of carrageenan and CFA, $n = 8$, $P < 0.01$). The therapeutic effect was not obtained neither in S-GM (Figure 1(b), 5.91 ± 0.54 and 3.59 ± 0.36 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(b), 4.31 ± 0.31 and 3.56 ± 0.31 of carrageenan and CFA, $n = 8$, $P > 0.05$).

3.2. Thermal Hyperalgesia on the Hot and Cold Plate. Our results displayed that noxious heat can induce thermal pain

with a decreased duration of forepaw licking (Figure 1(c), 4.17 ± 1.05 and 6.67 ± 2.01 of carrageenan and CFA, $n = 8$, $P < 0.01$). The phenotype can be attenuated by EA at ST36 (Figure 1(c), 10.67 ± 3.69 and 16.67 ± 2.11 of carrageenan and CFA, $n = 8$, $P < 0.01$). Similar results can also be obtained from criteria regarding jumping analysis. The phenotype was not observed neither in S-GM (Figure 1(c), 5.83 ± 1.07 and 6.83 ± 1.17 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(c), 5.60 ± 1.17 and 8.40 ± 1.03 of carrageenan and CFA, $n = 8$, $P > 0.05$). The number of jumping instances increased after inflammation treatment (Figure 1(d), 84.83 ± 8.37 and 86.83 ± 9.09 of carrageenan and CFA, $n = 8$, $P < 0.01$). Both carrageenan- and CFA-induced thermal pain can be further ameliorated by EA stimulation (Figure 1(d), 38.67 ± 9.49 and 44.5 ± 6.09 of carrageenan and CFA, $n = 8$, $P < 0.01$). The effect was not obtained neither in S-GM (Figure 1(d), 66.50 ± 5.6 and 69.60 ± 18.74 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(d), 68.75 ± 5.43 and 68.40 ± 12.91 of carrageenan and CFA, $n = 8$, $P > 0.05$). Our results also show that cold hyperalgesia was induced by carrageenan, and CFA intraplantar injection was analyzed with hind paw withdrawal number (Figure 1(e), 3.33 ± 0.56 and 4.83 ± 1.14 of carrageenan and CFA, $n = 8$, $P < 0.01$). Accordingly, similar curative effects of EA were observed in both carrageenan- and CFA-induced inflammatory mice (Figure 1(e), 0.83 ± 0.48 and 1.67 ± 0.67 of carrageenan and CFA, $n = 8$, $P < 0.01$). The effect was not obtained neither in S-GM (Figure 1(e), $2.50 \pm$

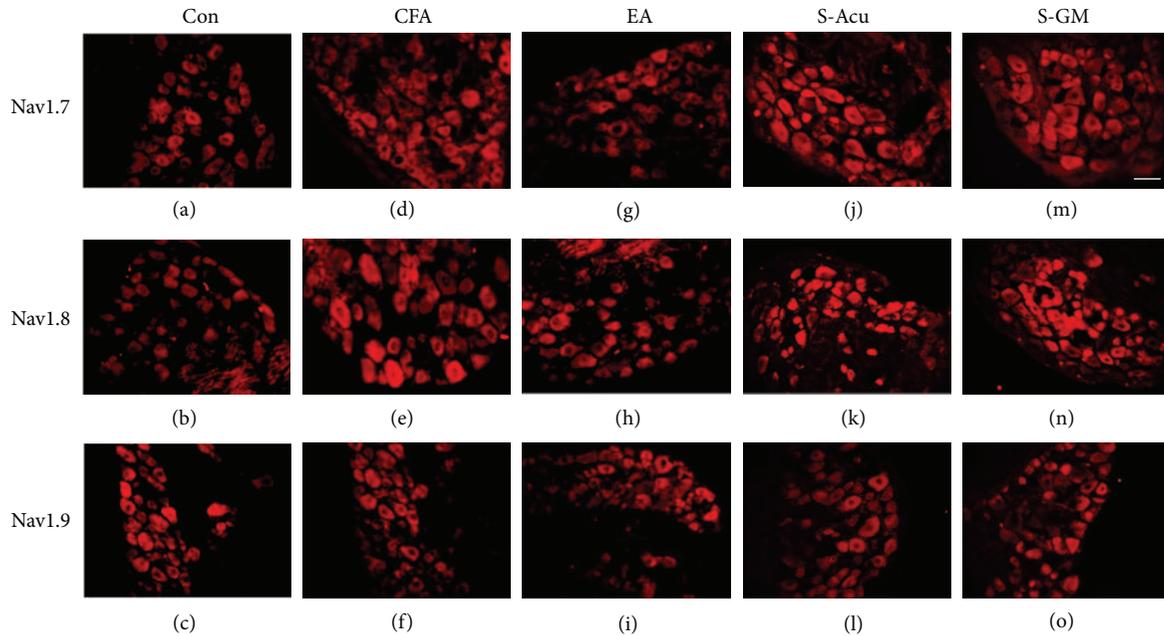


FIGURE 3: Nav1.7 and Nav1.8 expressions were increased in ipsilateral DRGs after intraplantar CFA injection and further attenuated by EA at the ST36 acupoint in mice, though Nav1.9 was not different. (a)–(c) Nav1.7, Nav1.8, and Nav1.9 immunoreactive neurons were found in lumbar DRGs at the ipsilateral site of the saline-injected group. (d)–(e) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the CFA-injected group, but (f) Nav1.9 immunoreactive neurons were not increased. (g)–(h) CFA-induced increases of Nav1.7 and Nav1.8 were attenuated by EA, as compared to those of the CFA-induced group. (i) Nav1.9 immunoreactive neurons were not altered by EA at the ipsilateral site of inflammation. (j)–(k) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the S-Acu group. (l) Nav1.9 immunoreactive neurons were not altered in the S-Acu group. (m)–(n) Nav1.7 and Nav1.8 immunoreactive neurons were increased in the S-GM group. (o) Nav1.9 immunoreactive neurons were not altered in the S-GM group. Scale bar = 50 μm .

0.50 and 4.20 ± 1.24 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(e), 2.67 ± 0.33 and 3.0 ± 0.45 of carrageenan and CFA, $n = 8$, $P > 0.05$). Cold hyperalgesia was induced by carrageenan, and CFA intraplantar injection was analyzed with rearing number (Figure 1(f), 3.0 ± 0.45 and 4.33 ± 0.33 of carrageenan and CFA, $n = 8$, $P < 0.01$). Accordingly, similar curative effects of EA were observed in both carrageenan- and CFA-induced inflammatory mice (Figure 1(e), 1.17 ± 0.31 and 2.83 ± 0.75 of carrageenan and CFA, $n = 8$, $P < 0.01$). The effect was not obtained neither in S-GM (Figure 1(f), 4.60 ± 1.29 and 5.0 ± 0.80 of carrageenan and CFA, $n = 8$, $P > 0.05$) nor in S-Acu group (Figure 1(f), 5.33 ± 1.31 and 4.67 ± 0.88 of carrageenan and CFA, $n = 8$, $P > 0.05$).

3.3. Immunohistochemistry Expression of Navs in DRG Neurons. Our data showed that Nav1.7 sodium channels were distributed in L3–L5 DRG neurons (Figures 2(a) and 3(a)). Intraplantar injection of carrageenan or CFA reliably increased the expression of Nav1.7 sodium channels in L3–L5 DRG neurons (Figures 2(d) and 3(d)). Dramatically, Nav1.7 channels were negatively regulated to a normal level by applying 2 Hz EA treatment at ST36 acupoint (Figures 2(g) and 3(g)). EA-elicited downregulation of Nav 1.7 was not observed neither in sham-Acu (Figures 2(j) and 3(j)) nor in sham-GM groups (Figures 2(m) and 3(m)). Our results also show that Nav1.8 channels were expressed in DRG neurons

in saline-injected neurons (Figures 2(b) and 3(b)). With the injection of carrageenan or CFA, Nav1.8 channels were greatly increased in DRG neurons (Figures 2(e) and 3(e)). The phenomenon was similar to previous results [2]. Importantly, 2 Hz EA at ST36 significantly reverses the overexpression of Nav1.8 channels in DRG neurons (Figures 2(h) and 3(h)). The effects were not obtained from sham-Acu (Figures 2(k) and 3(k)) and sham-GM groups (Figures 2(n) and 3(n)). The expression on Nav1.9 channels was observed in control group (Figures 2(c) and 3(c)). In carrageenan- and CFA-induced inflammation group, the expression of Nav1.9 was similar to that of the control one suggesting absence of the role of Nav1.9 in this model (Figures 2(f) and 3(f)). Similarly, the expression of Nav1.9 channels was not significantly different in the EA-treated group (Figures 2(i) and 3(i)), sham-Acu (Figures 2(l) and 3(l)), and sham-GM groups (Figures 2(o) and 3(o)).

3.4. Immunoblotting Quality of Navs in DRG Neurons. We further showed that Nav1.7 and Nav1.8 channels were increased during carrageenan- and CFA-induced inflammatory pain in mice L3–L5 DRGs by using western blot technique (Figures 4(a) and 4(b)). In contrast, the expression of Nav1.9 sodium channels was not changed in this condition (Figure 4(c)). Our results suggested that Nav1.7 channels were attenuated by 2 Hz EA at ST36 acupoint in carrageenan- and CFA-induced inflammation pain (Figure 4(a) displayed

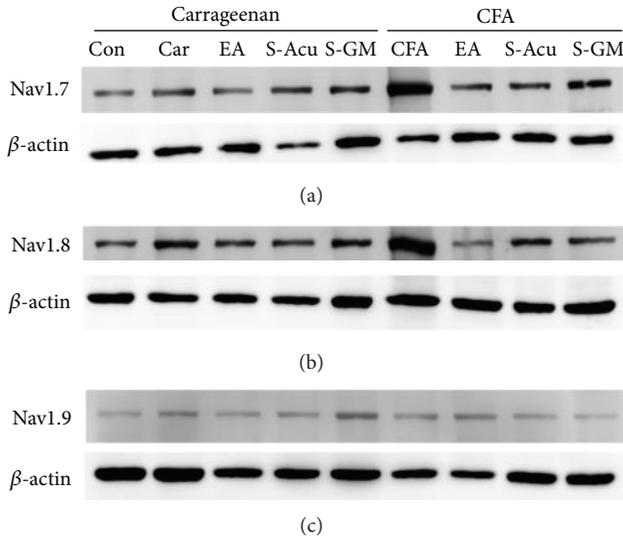


FIGURE 4: Nav1.7 and Nav1.8 protein levels were increased in lumbar DRGs in both intraplantar carrageenan- and CFA-induced inflammation and further attenuated by EA at the ST36 acupoint in mice, but Nav1.9 proteins were not altered. (a) DRGs lysates were immunoreactive with specific antibodies to Nav1.7 and a substantially increased signal at the ipsilateral site, as compared to that of the saline-injected group. Nav1.7 protein levels were attenuated by EA at the ST36 acupoint, as compared to that of the carrageenan- and CFA-induced groups. (b) Nav1.8 displayed similar results to Nav1.7. The protein levels of S-Acu and S-GM were similar to inflamed but not EA group. (c) Nav1.9 protein levels were not changed in both the carrageenan- and CFA-injected sites. Nav1.9 protein levels were not attenuated by EA at the ST36 acupoint, as compared to those of the carrageenan- and CFA-induced groups, either. Nav1.9 proteins were not altered at the ipsilateral site of inflammation and EA stimulation.

a 46.2% decrease in the signal, as compared with the carrageenan group, $n = 6$, $P < 0.05$; a 78.8% decrease in the signal, as compared with the CFA group, $n = 6$, $P < 0.05$). Similar results were observed in Nav1.8 (Figure 4(b)) displayed a 24.8% decrease in the signal, compared with the carrageenan group, $n = 6$, $P < 0.05$; a 30.7% decrease in the signal, compared with the CFA group, $n = 6$, $P < 0.05$). The protein levels of S-Acu and S-GM were similar to inflamed but not EA group suggesting acupoint specificity. Nav1.9 displayed no significant difference per group (Figure 4(c)). Accordingly, our results suggest that 2-Hz EA at the ST36 acupoint has the ability to ameliorate carrageenan- and CFA-induced overexpression of Nav1.7 and Nav1.8, rather than Nav1.9 sodium channels. All data were analyzed and presented in Figure 5.

3.5. Functional Analysis of TTX-R Currents Using Whole-Cell Recording. To determine whether EA attenuates the neuronal excitability after CFA-induced inflammation pain model, we used whole-cell recording to record the TTX-R sodium currents in small-to-medium-size ($<34 \mu\text{m}$) DRG neurons. In control group, TTX-R currents were obtained with membrane potential depolarized to -40 mV . However, intraplantar

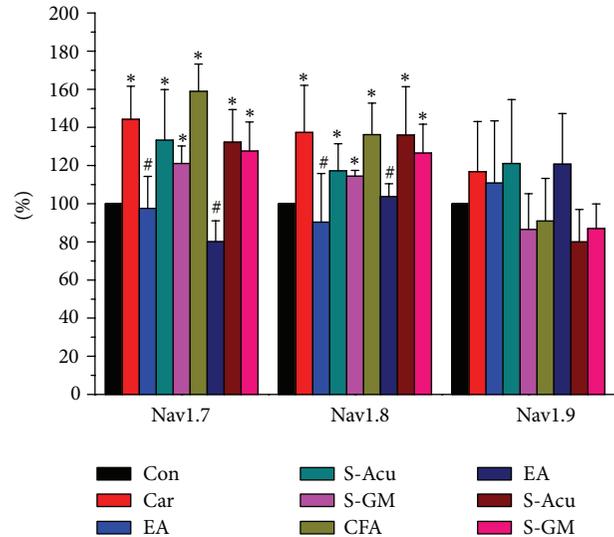


FIGURE 5: Protein levels of Nav1.7, Nav1.8, and Nav1.9 in the L3–L5 DRGs in mice in control, Car, EA, S-Acu, S-GM, CFA, EA, S-Acu, S-GM groups. The percentage of Nav protein levels from lumbar DRGs was presented in each group. * $P < 0.05$, as compared to control group. # $P < 0.05$; comparison between inflammation and EA groups.

inflammation by CFA injection potentiated the amplitudes of TTX-R currents in DRG neurons. The potentiation of TTX-R currents was decreased in DRG neurons obtained from EA-treated group (Figure 6(a)). The relationship between membrane potential and Nav currents was plotted in Figure 6(b).

4. Discussion

In this study, we first established animal models of inflammatory pain by injection of carrageenan or CFA into hind paw. Animals with inflammatory pain showed mechanical and thermal hyperalgesia using a von Frey filament test, Hargreaves' test, and hot/cold plate tests. EA stimulation at the ST36 acupoint reduced inflammatory hyperalgesia in both carrageenan and CFA groups. Our results indicated that Nav1.7 and 1.8, but not Nav1.9, were upregulated in both carrageenan and CFA-induced hyperalgesia, which suggested the important role of Nav1.7 and 1.8 in inflammatory pain. We showed that EA at Zusanli (ST36) acupoint at 2 Hz low-frequency stimulation reduced pain thresholds accompanied by decreasing the expression of Nav1.7 and 1.8, rather than Nav1.9, sodium channels in DRG neurons.

Zhang et al. reported that EA at 10 Hz frequency significantly reduced CFA-induced hind paw edema. Moreover, EA attenuates inflammatory response through the hypothalamus-pituitary-adrenal axis (HPA) and the nervous system [14]. Recently, EA also suppresses the expression of neurokinin-1 in spinal cord dorsal horn induced by inflammation in rats [15]. These phenomena were not observed in sham control groups suggesting the acupoint-specific effect [14, 15]. Our results were consistent with these studies that

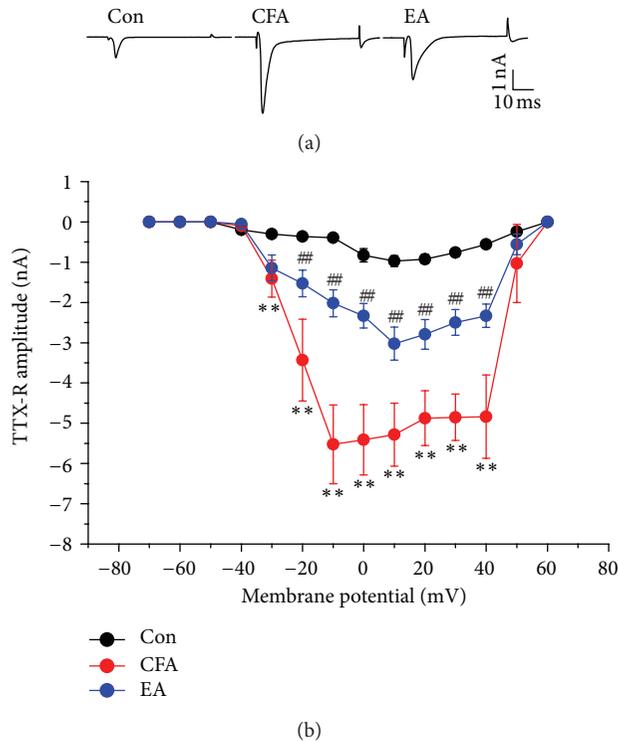


FIGURE 6: Tetrodotoxin-resistant (TTX-R) sodium currents in L3–L5 DRG neurons. (a) Representative TTX-R current traces in Con, CFA, and EA groups. The TTX-R currents were induced by membrane potential depolarized to -40 mV. (b) Mean peak amplitudes of TTX-R currents in each group. ** $P < 0.01$ compared to control group. ## $P < 0.01$ compared to CFA group.

the antinociceptive effect was only observed in EA but not in sham-Acu and sham-GM groups.

Numerous studies have investigated the role of different Navs in pain, neuron excitability, and action potential firing [4, 17]. The most emphatic evidence implicating a specific ion channel participating in pain comes from studies on complete insensitivity to pain using gene knock mice [18, 19]. Nav1.7 was greatly expressed in C-fiber free nerve endings, playing a crucial role in nociceptive information [20]. Recent studies have strongly supported Navs as potential analgesic drugs, according to antisense and knockout mice [6, 21]. Derivatives from benzazepine and imidazopyridine were also developed to block Nav1.7 channels for pain treatment [22]. Our results clearly indicate that EA reliably attenuated carrageenan- and CFA-induced inflammation pain by ameliorating Nav1.7 overexpression. This is the first paper regarding the functional role of acupuncture in pain manipulation and its novel findings pertaining to Nav1.7 channel alteration.

Chronic intrathecal Nav1.8 antisense injection successfully attenuated the Nav1.8-induced current and decreased mechanical allodynia after intraplantar CFA injection [23]. Developing a specific Navs channel blocker is possible for inflammatory pain. A-803467 is a novel specific blocker for the Nav1.8 channel and can ameliorate inflammatory pain in rats [24]. Nav1.9 is a TTX-R sodium channel greatly expressed

in small diameter C-fibers and contributes to membrane properties, particularly in nociceptive neurons [25]. Nav1.9 is also suggested to regulate inflammatory pain thresholds [26]. Animal behavior studies have also demonstrated that deletion of Nav1.9 channel expression prevents inflammatory mediator-induced hyperalgesia [27, 28]. Inflammatory mediators, such as PGE_2 , can reliably increase the Nav1.9 channel current in mice DRG neurons with G-protein activation [29]. Our data provide highly valuable results from investigating inflammation pain regarding ancient acupuncture mechanisms that can be further applied to clinical medicine.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgments

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Research Article

Objectifying Acupuncture Effects by Lung Function and Numeric Rating Scale in Patients Undergoing Heart Surgery

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Rationale. Poststernotomy pain and impaired breathing are common clinical problems in early postoperative care following heart surgery. Insufficiently treated pain increases the risk of pulmonary complications. High-dose opioids are used for pain management, but they may cause side effects such as respiratory depression. **Study Design.** We performed a prospective, randomized, controlled, observer-blinded, three-armed clinical trial with 100 patients. Group 1 ($n = 33$) and Group 2 ($n = 34$) received one 20 min session of standardized acupuncture treatment with two different sets of acupoints. Group 3 ($n = 33$) served as standard analgesia control without additional intervention. Results. Primary endpoint analysis revealed a statistically significant analgesic effect for both acupuncture treatments. Group 1 showed a mean percentile pain reduction (PPR) of 18% (SD 19, $P < 0.001$). Group 2 yielded a mean PPR of 71% (SD 13, $P < 0.001$). In Group 1, acupuncture resulted in a mean forced vital capacity (FVC) increase of 30 cm³ (SD 73) without statistical significance ($P = 0.303$). In Group 2, posttreatment FVC showed a significant increase of 306 cm³ (SD 215, $P < 0.001$). **Conclusion.** Acupuncture revealed specific analgesic effects after sternotomy. Objective measurement of poststernotomy pain via lung function test was possible.

1. Introduction

Median sternotomy represents a surgical technique, in which the sternum is longitudinally incised and opened to get access to the organs of the mediastinum, for example, for heart transplantation, to set bypasses or valves on aortocoronary arteries, or to remove thymus tumors. Poststernotomy pain and impaired spontaneous breathing are common clinical problems in early postoperative care following heart surgery [1, 2]. Insufficiently treated pain impedes coughing and

physiological rib cage expansion and, thus, increases the risk of pulmonary complications such as atelectasis and pneumonia [2].

High-dose application of opioids represents the standard therapy after sternotomy. It is controversially discussed whether opioids after median sternotomy lead to side effects such as respiratory depression, vomiting, nausea, and sedation [3, 4]. Therefore, combination therapy regimens have been applied containing opioids and traditional nonsteroidal anti-inflammatory drugs (tNSAIDs), cy-

cloxygenase-2 (COX-2) inhibitors, alpha2-inhibitors, or other nonopioid drugs such as paracetamol. The effectiveness of these combinations in pain reduction is, however, critically discussed [5–7]. Therefore, there is an urgent need for the improvement of pain therapy after median sternotomy. It has been suggested that electroacupuncture might be a valuable adjunct in managing poststernotomy pain [8, 9]. Whether needle acupuncture is a considerable asset in pain treatment after sternotomy is unknown as yet.

The aim of the present investigation was, therefore, to investigate the effect of needle acupuncture on pain-induced reduction of lung function after median sternotomy. To this end, a prospective, randomized, controlled, patient- and observer-blinded clinical trial was performed with patients undergoing median sternotomy.

2. Methods

The study was designed as a prospective, randomized, controlled, patient- and observer-blinded, three-armed clinical trial. Between September 2005 and August 2008, 100 thoracotomized patients were recruited from the intermediate intensive care unit (IMC) in the Department of Heart Surgery at Heidelberg University Hospital. Informed consent was obtained before subject enrollment according to a clinical trial protocol approved by the local Ethical Committee.

2.1. Inclusion and Exclusion Criteria. Patients were included in the study if they had undergone conventional on-bypass surgery via median sternotomy and reported pain during deep inspiration with an intensity of at least 3 on a 1–10 numeric rating scale under standard analgesia with tNSARs and high-dose opioids. Patients had to be extubated and lucid. Patients meeting these criteria were excluded if they had a medical history of severe obstructive or restrictive pulmonary disease. Due to safety reasons, patients with a blood clotting time exceeding 4 min and/or a platelet count below 50,000 nL under anticoagulative therapy were also not accepted to the study. Additional ineligibility factors included acute general infection (fever, highly elevated leucocytes, or CRP) and/or local infection at the sites of potential needle insertion. Measures specifically influencing pain perception and/or lung function (i.e., inadvertent or accidental application of analgetics, physical exercise, physiotherapy treatment, and breathing exercises) were prohibited during the active 40 min study period (10 min screening and randomization, 5 min baseline assessment, 20 min acupuncture/observation, and 5 min control assessment) and would result in an exclusion from the study. Furthermore, intolerance to acupuncture treatment or sudden worsening of the physical condition would lead to a discontinuation of the study.

2.2. Randomization to Study Groups. Subjects were randomized to 3 study groups: Group 1 and 2 received one single 20 min session of standardized verum acupuncture treatment in addition to standard analgesia. Acupuncture was performed on 12 acupoints using sterile 0.25 × 40 mm surgical stainless steel needles. Needles were stimulated

by rotation for approximately 5 sec during insertion, after 10 min, and before removal after 20 min. Additionally, in both groups, six of the selected 12 acupoints were treated with “blood-letting” or “leopard spotting technique” using sterile 033 mm 29-gauge needles. To rule out systematic acupuncturist-dependent fluctuations in overall treatment effects, all acupuncture treatments were carried out by the same physician, who had advanced training in traditional Chinese medicine (TCM).

The only systematic difference between acupuncture in Group 1 and Group 2 was the acupoint selection. In Group 2, the acupoint selection was based upon common classical Chinese diagnostic features shown by an a priori investigated representative patient collective (10 patients with varied sex, age, and cultural background with postoperative chest pain following sternotomy). A short summary of the acupoint selection is given in the following paragraph. Specific diagnostic technical terms are quoted and described with an auxiliary Western medical explanation in brackets.

According to a well-known part of classical Chinese medical theory—the Shang Han Lun—which is systematized in the “Algor Laedens Theory” of the “Heidelberg model of TCM”, severe postoperative pain arises from “xue stasis” (relative hypoxia of a tissue arising from a disrupted microcirculation) due to “post-traumatic algor” (reduced microcirculation of a tissue following a trauma due to vasospastic reflexes and immunological reactions) [10]. SP 10 was included as a classical acupoint to counteract xue stasis [11]. The common “orb pattern” (specific neuroaffective pattern with defined physical signs) presented by the patient collective indicated “a syndrome of the closing principle”—the simultaneous occurrence of a “splendor yang state” (neuroimmunological reaction with signs of the “stomach” and the “large intestine” orb pattern) and a “yin flectens state” (neuroimmunological reaction with signs of the “pericardium” and “liver” orb pattern) [10]. Respectively, ST 34, ST 44, PC 6, and LIV 2 were chosen as potent acupoints on the aforementioned orb meridians [11]. As all patients showed signs of a distinct “yin deficiency” (a labile neurovegetative regulation due to an overall deficiency of functional tissue), K 3 was added [11].

Acupoint selection in Group 1 served as a verum acupuncture control as it presented a form of inferior acupuncture—acupoints were exclusively selected according to their ascribed therapeutical actions and medical indications propagated by the acupuncture literature. LI 4 was included as a well-known, analgesic acupoint [11]. GV 20 and Ex 1 are generally ascribed psychovegetatively relaxing effects, possibly enhancing overall analgesia [11]. BL 60 is indicated to relief sharp chest pain and to loosen tension in the back muscles, GB 8 is recommended to treat feeling of pressure in the epigastric and thoracic regions, SI 6 is commonly used to uncramp the entire musculoskeletal system, and ST 8 is used to treat dyspnea [11].

A detailed list of the selected acupoints is illustrated in Tables 1 and 2. Group 3 served as a standard treatment control without additional acupuncture intervention. After baseline assessment, patients assigned to Group 3 were advised to avoid physical or mental stress and rest tranquilly in bed for 20 min under the supervision of the study investigator. In

TABLE 1: Acupoint selection in Group 1 (control acupuncture).

Engl. abbr.	Latin abbr.	Latin notation	Chinese notation	Needling technique	Localisation
LI 4	IC 4	Valles coniunctae	Hegu	blood-letting	bilateral
SI 6	IT 6	Senectus felix	Yanglao	blood-letting	bilateral
BL 60	V 60	Olympus	Kunlun	blood-letting	bilateral
Ex 1	Ex 1	Atrium impressionis	Yintang	conventional needle insertion	median
GV 20	Rg 20	Conventus omnium	Baihui	conventional needle insertion	median
GB 8	F 8	Apex auriculi	Erdian/Shuanijue	conventional needle insertion	bilateral
ST 8	S 8	Retinens capitis	Touwei	conventional needle insertion	bilateral

TABLE 2: Acupoint selection in Group 2 (classical Chinese acupuncture).

Engl. abbr.	Latin abbr.	Latin notation	Chinese notation	Needling technique	Localisation
PC 6	PC 6	Clusa interna	Neiguan	blood-letting	bilateral
ST 34	S 34	Monticuli septi	Liangqiu	blood-letting	bilateral
SP 10	L 10	Mare xue	Xuehai	conventional needle insertion	bilateral
ST 44	S 44	Vestibulum internum	Neiting	blood-letting	bilateral
K 3	R 3	Rivulus major	Taixi	conventional needle insertion	bilateral
LIV 2	H 2	Interstitium ambulatorium	Xingjian	conventional needle insertion	bilateral

this way, Group 3 displayed the physiological fluctuations of pain perception and FVC over a 20 min interval without any medical intervention (observation).

Randomization was stratified using consecutively numbered envelopes, containing the details of allocation. The envelopes were filled and sealed prior to study enrolment by an independent assistant according to a password-protected, computer-generated randomisation list. The envelopes were opened in a row by the acupuncturist at the bedside after recruitment. The designated observer and those patients allocated to an intervention arm (Group 1 or 2) were blind to treatment. Patients were informed that the study aimed to compare two different acupuncture treatments with respect to their analgesic effect after sternotomy.

2.3. Efficacy Parameters. Baseline and control efficacy assessment was carried out by a blinded observer and comprised two elements.

- (1) Dynamic pain assessment via numeric rating scale to record changes in subjective pain perception was carried out dynamically—during deep inspiration. Two different numeric rating scales were chosen; to semiquantify baseline chest pain, a conventional 1–10 NRS was used. To record the residual chest pain after treatment/observation in relation to the initial pain intensity, a 0%–100% NRS (subdivided into 10% sections) was implemented. The baseline pain intensity served as the individual “100%—reference pain” for this 0%–100% NRS.
- (2) Measurement of forced vital capacity (FVC) served as a physical and, thus, objective parameter to indicate analgesia-related functional improvements in rib cage expansion.

Percentile pain reduction (PPR) after treatment served as primary efficacy parameter, posttreatment change in FVC

compared to baseline FVC was chosen as secondary efficacy parameter. For primary and secondary endpoint analyses, the Kruskal-Wallis test was performed as a 3-group global test, followed by the Wilcoxon test for post hoc analysis in case of a significant outcome. Correlation and subgroup analysis was performed to further investigate the a priori assumed pathophysiological connection between chest pain intensity and FVC measurement.

3. Results

Out of 367 screened patients, 100 patients met the inclusion criteria and were randomized to one out of three possible study groups. Participant flow through the trial is shown in Figure 1. Randomization resulted in an equal distribution of sociodemographic and clinical characteristics (see Table 3).

Primary endpoint analysis revealed a statistically significant analgesic effect for both acupuncture treatments—acupuncture in Group 1 resulted in a mean PPR of 18% (SD 19, $P < 0.001$), and acupuncture treatment in Group 2 yielded a mean PPR of 71% (SD 13, $P < 0.001$). Figure 2 displays the distribution of PPR outcomes.

In Group 1, acupuncture resulted in a mean FVC increase of 30 cm³ (SD 73), which failed to attain statistical significance (1 versus 3: P -value = 0.303). In Group 2, post-treatment FVC showed a statistically significant, average increase of 306 cm³ (SD 215, 2 versus 3: P -value < 0.001), with a strong correlation to PPR outcomes (spearman's rank correlation coefficient = 0.74). Pain reduction and FVC changes after acupuncture in Group 1 did not correlate in a statistically significant manner ($P = 0.503$). Figure 3 illustrates the distribution of FVC-changes, and Figure 4 displays the correlation of PPR and FVC-changes in Group 2.

Subgroup analysis revealed that meaningful FVC increases (≥ 300 cm³) exclusively occurred in patients with a PPR of 60%, or greater (see Figure 5). Below a PPR of 60%,

TABLE 3: Patients' characteristics at baseline.

	Group 1	Group 2	Group 3 (control)	Total	<i>P</i> value
<i>N</i>	33	34	33	100	
Male gender*	24 (73%)	22 (65%)	26 (79%)	72 (72%)	0.436 [∞]
Age (years)					0.295 [#]
Mean (SD)	65 (10)	68 (11)	66 (10)	66 (10)	
BMI (kg/m ²)					0.515 [#]
Mean (SD)	28 (4)	29 (5)	29 (4)	28 (4)	
Operation type					
On-bypass*	33 (100%)	34 (100%)	33 (100%)	100 (100%)	
Coronary revascularisation*	17 (51%)	20 (58%)	21 (63%)	58 (58%)	
Valve reconstruction or replacement*	15 (45%)	14 (41%)	15 (45%)	44 (44%)	
Aortic reconstruction after dissection*	1 (3%)	0 (0%)	0 (0%)	1 (1%)	
Chest pain baseline (0–10 NRS)					0.653 [#]
Median (IQR)	6 (2)	5 (2)	5 (2)	5 (2)	
Mean (SD)	5.5 (1.2)	5.2 (1.4)	5.3 (1.3)	5.3 (1.3)	
FVC baseline (cm ³)					0.082 [#]
Median (IQR)	1200 (600)	1000 (550)	1200 (400)	1150 (600)	
Mean (SD)	1294 (426)	1147 (405)	1275 (325)	1238 (390)	

*The numbers in parentheses display the percentage for dichotomous variables.

[#]*P* values were calculated using the Kruskal-Wallis test.

[∞]*P* values were calculated using the χ^2 -test.

Abbreviations—SD: standard deviation; IQR: interquartile range.

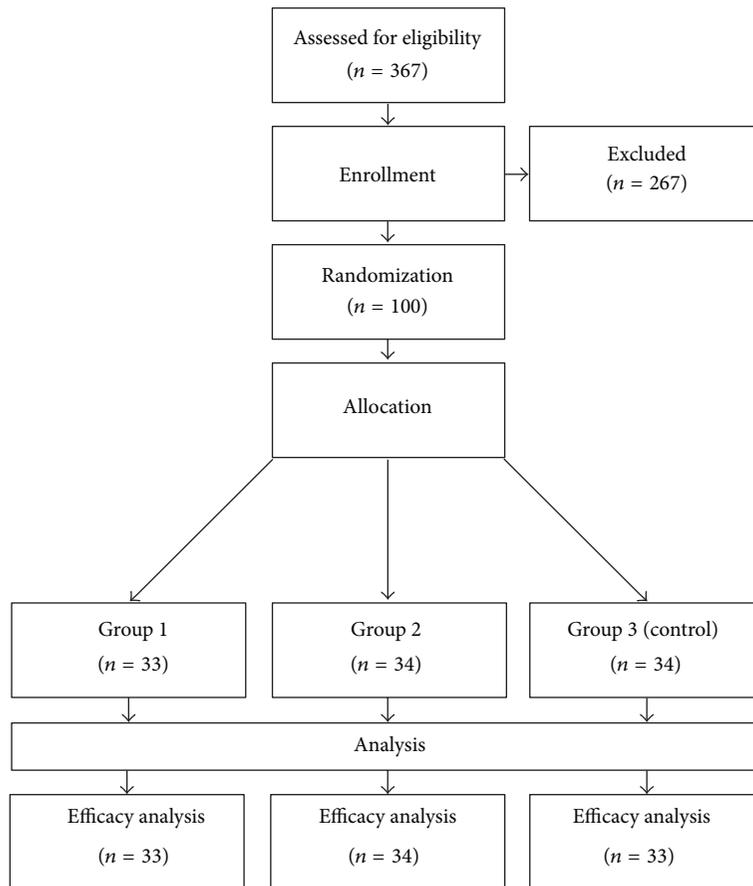


FIGURE 1: Participant flow.

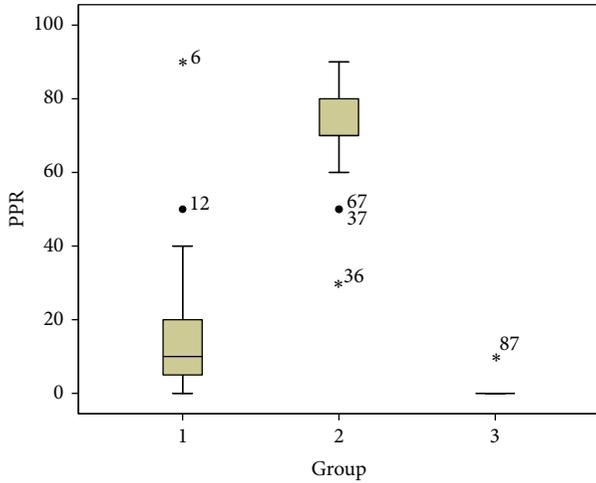


FIGURE 2: Distribution of PPR. Box-and-whisker plots represent lower quartile, median, upper quartile, maximum, minimum, outliers, and extreme values.

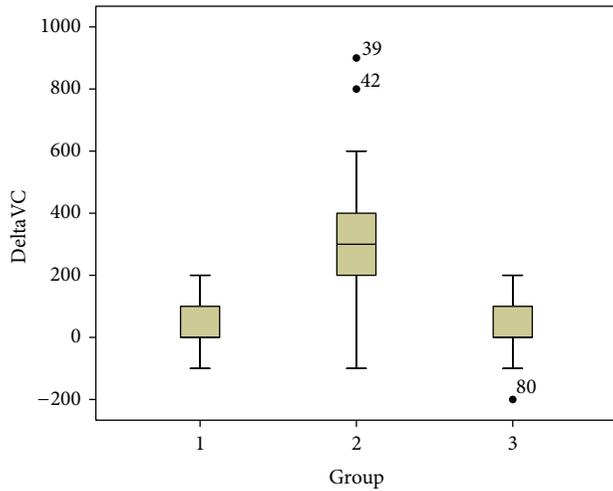


FIGURE 3: Distribution of FVC changes from baseline FVC. Box-and-whisker plots represent lower quartile, median, upper quartile, maximum, minimum, outliers, and extreme values.

the observed changes in FVC remained within the range of normal, acupuncture-independent fluctuations as shown by Group 3 patients.

4. Discussion

In the present investigation, we demonstrated statistically significant differences in immediate analgesic and functional effects between two types of acupuncture treatments compared to a control group in poststernotomy patients. The clinical trial showed that a lung function test can be used as an objective measurement of poststernotomy pain. Interestingly, only clinically significant analgesic effects (PPR $\geq 60\%$) led to functional improvements in lung function. This result further emphasizes the importance of maximal efficacy of postoperative analgesia to prevent pulmonary complications

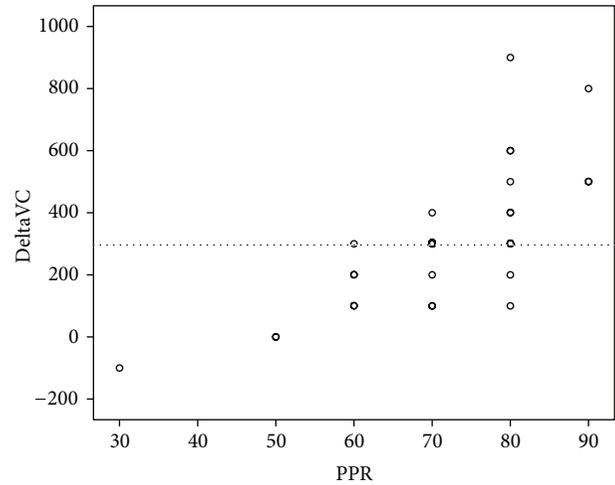


FIGURE 4: Correlation between PPR and posttreatment FVC changes in Group 2. Circles that are more intensely marked represent coordinates that occurred several times. The dotted line represents the lower limit for meaningful FVC changes ($\geq 300 \text{ cm}^3$).

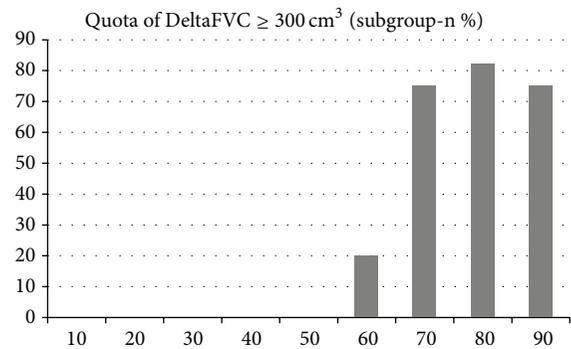


FIGURE 5: Subgroup analysis. Quota of clinically significant FVC increases ($\geq 300 \text{ cm}^3$) in the individual PPR subgroups of Group 2.

[2]. Some limitations of the present study may be discussed. The study period was short with no repeated acupuncture treatments, and the results only mirror immediate analgesic effects. Further studies may be performed in the future to address these issues. A Blinding of acupuncturists did not take place, because this is merely achievable. Furthermore, no placebo control was implemented in the study, since from an ethical point of view, a nontreatment group is not justifiable in patients suffering from severe pain.

It is a long-lasting discussion in the scientific literature that strong postoperative pain and a considerable incidence of chronic pain after cardiac surgery and median sternotomy necessitate effective pain management [7]. A number of opioid-based treatment strategies have been described, including thoracic epidural anesthesia, spinal and intrathecal anesthesia, intercostal and paraventral blocks, or patient-controlled intravenous analgesia (PCS) [7]. In addition to

combining opioids with tNSAIDs, COX-2 inhibitors, alpha2-inhibitors, or other drugs, several nonpharmacological strategies have been reported. Preoperative pain education of patients about postoperative pain resulted in less concerns about pain management [12]. Interestingly, electrical skin stimulation reduces pain perception of the organism. This technique was termed transcutaneous electrical nerve stimulation (TENS), which was successfully applied for poststernotomy pain management in several randomized clinical trials [13–15]. The stimulation of skin for pain reduction indicates that acupuncture as a technique applied since ages to treat pain associated with many diseases and symptoms may also be helpful for poststernotomy pain management. Indeed, electroacupuncture has been reported to reduce poststernotomy pain and to improve pulmonary function [8, 9]. Electroacupuncture is based on the insertion of needles at specific acupoints together with electric current.

In the present investigation, we clearly demonstrated that classical needle acupuncture without electrostimulation also led to significant pain reduction and lung function improvement. This may have impact on future concepts of pain management after median sternotomy in heart surgery. The fact that the acupuncture treatment of Groups 1 and 2 differ in their analgesic effects in the present study indicates that it considerably matters which acupoints are used and contradicts the view that acupuncture might only mediate nonspecific skin stimulation, which may or may not exceed placebo effects [16]. As imaging studies have delivered evidence of acupoint-specific functional magnetic resonance imaging patterns [17, 18] and the selection of acupoints presented the only systematic difference between acupuncture treatment in Groups 1 and 2, the observable difference in analgesic effects can be attributed to the summation effect of all twelve individual acupoint-specific reactions. Furthermore, it can be argued that the diagnosis-dependent selection of acupoints has significantly contributed to the analgesic superiority observed in Group 2. If acupoints are understood as reflex points that elicit specific neurovegetative alterations, it can be speculated that they might only yield satisfactory treatment results if they match the current vegetative status of a patient (which is expressed in the TCM diagnosis) [10]. However, it is also imaginable that psychological confounders may have distorted post-treatment pain assessment; minimal to moderate pain reductions—as frequently observed in Group 1—are possibly underrated if patients were disappointed by the acupuncture treatment effect, while patients, who were positively surprised by the analgesic effect of acupuncture treatment, might tend to overrate its effect.

The exact mechanisms for acupuncture-mediated pain reduction in general and after median sternotomy are not well understood as yet. Different modes of action may account for pain reduction such as the release of endogenous opioids [7], adenosine A1 receptor-mediated antinociceptive effects [19], mast cell degranulation, and the release of substance P, amongst others [20–26].

In conclusion, the present clinical trial demonstrated that acupuncture revealed specific effects in pain management after median sternotomy. Objective measurement of poststernotomy pain by a lung function test was possible

and enabled to distinguish between functionally insignificant pain reduction (resulting in no improvement of breathing) and clinically significant analgesia effects of acupuncture.

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Research Article

The Influence of Skin Microcirculation Blood Perfusion at Zusanli Acupoint by Stimulating with Lift-Thrust Reinforcing and Reducing Acupuncture Manipulation Methods on Healthy Adults

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Background. In traditional Chinese medicine acupuncture manipulation is one of the key factors that affect the curative results of acupuncture and more and more researches focus on how the different acupuncture manipulation techniques influence microcirculation nowadays. In this paper we demonstrate the different influences of lift-thrust reinforcing and reducing on blood perfusion. *Method.* The acupuncture manipulations of lift-thrust reinforcing and reducing were, respectively, applied to the 15 healthy subjects at the Zusanli acupoint and the changes of blood perfusion were monitored by Pericam Perfusion Speckle Imager (PSI). *Conclusion.* Both of the manipulations of lift-thrust reinforcing and reducing increase blood perfusion at Zusanli acupoint while the increasing amount of blood perfusion in the reinforcing group is significantly higher than in the reducing group.

1. Introduction

In recent years, more and more patients in western countries turn to complementary and alternative medicine therapies [1]. Acupuncture especially is one of the most frequently requested of the complementary therapies [2]. According to a national cross-sectional survey, acupuncture provides a substantial contribution to the healthcare [3]. Acupuncture is increasingly used in managing chronic pain and other conditions, such as chronic knee pain, tension-type headache, low back pain, and so on [4–6]. Though it has clinical efficacy and cost-effectiveness, western medical experts have been skeptical of acupuncture's therapeutic value. Acupuncture rational basis underlying its use remains unclear [7]. In traditional Chinese medicine (TCM), acupuncture manipulation is one of the key factors that affect the curative results of

acupuncture [8]. In Qing Dynasty, Li Shouxian said “It's not difficult to understand acupuncture point, but to master the manipulation; it's hard for us to master the manipulation more than to understand acupuncture point; if only you merely comprehend acupuncture point, you couldn't be a distinguished doctor.” He stressed the importance of acupuncture manipulation. In order to apply appropriate acupuncture therapeutic effectiveness, different acupuncture manipulations are required after needle insertion in clinical practice [9]. These manipulations are performed very widely ranging from reinforcing and reducing the needle to twirling the needle and varying the insertion angle, and so forth. With the continuous development and progress of science and technology recently, the research approach and methods of acupuncture manipulation continue to broaden and update. Many scholars adopt a multidisciplinary,

multichannel method to carry out acupuncture reinforcing and reducing manipulations to obtain some meaningful results [10]. Applying different acupuncture manipulations, the degree of nervous excitement, the local oxygen tension, concentration of chemical substances, and the degree of temperature could be changed at point's area [10–13]. Li et al. reported that reinforcing and reducing methods can produce different effects on skin temperature [11]. Some scientists collected different acupuncture manipulation parameters and stabilization of mathematical model [14, 15]. In our lab in 2011, using the parameter tester ATP-II of acupuncture manipulation produced by Shanghai University of TCM we collect the experts acupuncture operation parameters (cycle, frequency, rising time of waveform, falling time of waveform, platform of rising wave, platform of falling wave, rising slope of waveform, falling slope of waveform, increased amplitude of waveform, and decreased amplitude of waveform) of reinforcing lifting and thrusting, reducing lifting and thrusting, establish databases, and build the corresponding mathematical model with the Fourier transform and kernel regression mathematical method. The microcirculation blood perfusion as an important indicator of energy metabolism has attracted the attention of researchers [16–21]. Skin microcirculation is a good indicator of presenting the effect of acupuncture. Different methods of acupuncture on acupoint can cause change in skin blood perfusion [22]. As for the study, we also take the effect of different acupuncture manipulations (e.g., reinforcing lifting and thrusting, reducing lifting and thrusting, even reinforcing-reducing method, and acupuncture group without manipulations) on the blood perfusion of offside Zusanli (ST36) acupoint as the basis of this experiment, in order to observe the influence of different acupuncture manipulations.

2. Methods

2.1. Ethics Statement. This study was reviewed and approved by the Institutional Review Board at the Institute of Acupuncture and Moxibustion, Tianjin University of TCM. Each participant read and signed an informed consent form.

2.2. Subjects. The study was conducted at Experimental Acupuncture Research Centre of Tianjin University of TCM between May 2012 and August 2012. Fifteen healthy volunteer students (7 men and 8 women) aged 25.4 ± 0.99 years (mean \pm SD; range, 24–27 years) were recruited in this study, who had no history of diseases and had not taken any medicine 1 month before the experiment. Each subject had an adequate understanding of the procedure and purpose of this study. Throughout the experiment, the subjects were neither told nor able to see or hear any indication of which needle manipulation type was being performed. There are five groups as follows: control group, acupuncture group without manipulations (no manipulation), reinforcing lifting and thrusting group (reinforcing), reducing lifting and thrusting group (reducing), and even reinforcing-reducing

method group (even). Each group has 15 persons with self-control method, and everyone accepted the above 5 kinds of manipulation methods.

2.3. Procedures

2.3.1. Protocol for Experimental Conditions. The room temperature during the experiment was controlled at about $26 \pm 1^\circ\text{C}$, and relative humidity was maintained 50%–60%, and there was no direct sunlight indoor. The PSI parameter was set as follows: image acquisition rate, 50 Hz; normal resolution, 0.5 mm; 1 frame per second; the working distance is 18 ± 1 cm; the monitor area is 10 (highness) \times 8 (width) cm^2 ; the definition of the Region of Interest (ROI) is in 0.5 cm around the Zusanli acupoint. PSI System is using the PIMSoft software for recording, saving, and analysis. PSI can dynamically and instantly monitor the change of blood flow perfusion in the body and display the image and blood flow curve at the same time. It also has the functions of video broadcast and output. Perfusion Unit (PU) is the unit used for blood perfusion. The higher the PU is, the greater the blood perfusion is. Right side of ST36 (Zusanli acupoint) is based on the national standard name and location (GB/T12346-2006). The sterile disposable needle used in acupuncture is 40 mm in length and 0.3 mm in diameter (Han Yi, Tian Jin). Figure 1 shows Pericam Perfusion Speckle Imager (PSI) and the blood perfusion image of the monitor area.

2.3.2. Operation Rules. Reinforcing group: insert the needle at offside ST36 and do the thrust heavily and lift lightly for 2 minutes after Deqi. Reducing group: insert the needle at offside ST36 and do the thrust lightly and lift heavily for 2 minutes after Deqi. Even group: insert the needle at offside ST36 and do even lifting, thrusting, and rotating for 2 minutes after Deqi. No manipulation group: insert the needle at offside ST36 without any manipulation. Control group: all subjects maintained still, without any intervention. Deqi is believed to be essential for efficacy of acupuncture according to TCM [23, 24].

To make sure of the stability of the acupuncture manipulation, before the experiment begin, the operator has had an operation skill training on the ATP-II acupuncture manipulation parameter tester (which was manufactured by Shanghai University of Traditional Chinese medicine ShangXin medical technology company). We compared the curve charts the operator made with the standard curve charts. The operator did not start the experiment until he reached the standard level. All the acupuncture manipulations during the whole experiment were made by the same person. This ensured consistent experimental conditions and eliminated many potential sources of investigator bias. The preliminary experiment showed that the blood perfusion would return normal 24 hours after needling on right side of ST36. To make sure that the effect of acupuncture disappeared, the operator did each manipulation at least two-day interval, at most three-day interval, and the experiment was done at the same time in different days. Figure 2 shows the acupuncture technique parameter tester and the output curve. Figure 3

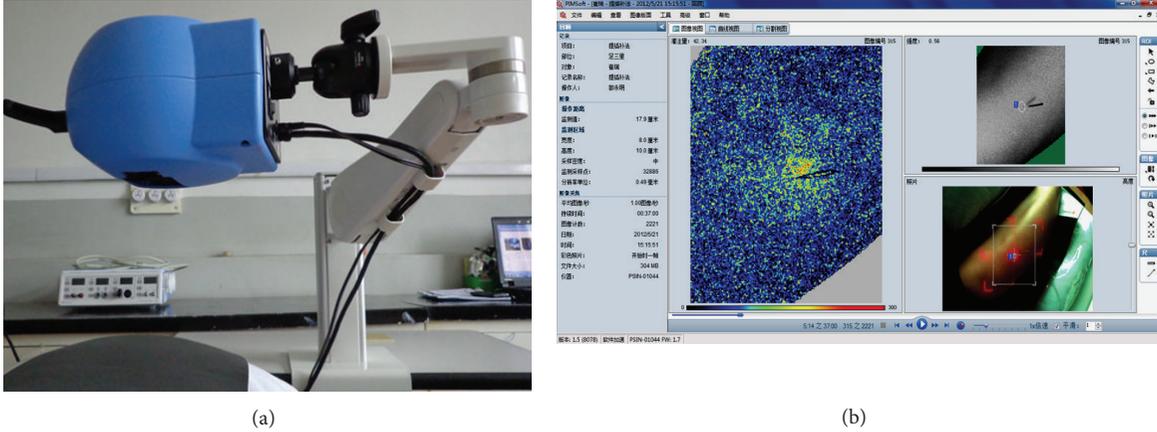


FIGURE 1: (a) Pericam Perfusion Speckle Imager (PSI). (b) The blood perfusion image of the monitor area. The brighter area is the definition of the Region of Interest (0.5 cm around the Zusanli acupoint).

shows the curves of the reinforcing manipulation and the reducing manipulation made by the operator.

2.3.3. Experiment Flow. Every subject lies still for about 20 minutes for acclimatization. Then, skin at Zusanli acupoint was disinfected with alcohol. The control group blood perfusion was monitored about 35 minutes; data of the no manipulation group was recorded for 5 minutes before needling, and 30 minutes after needling; data of acupuncture groups with manipulations (including reinforcing manipulation by lifting and thrusting group, reducing manipulation by lifting and thrusting group, and even reinforcing-reducing manipulation group) were recorded for 5 minutes before needling, and 30 minutes after doing manipulations. The manipulation groups flow diagram is illustrated in Figure 4.

2.3.4. Statistical Analysis. Statistical analyses were all performed in R statistical software. Repeated measurement ANOVA was used to assess the differences in mean blood perfusion between different groups and within single group. The trend over time was shown by the mean and standard deviation. At the same point-in-time, t test is used to find the significant difference.

3. Results

3.1. Study Participants. 15 volunteers were enrolled in the study. All the participants completed the testing protocol.

3.2. Images. Figure 5 is composed of the blood perfusion images. It shows the changes of blood perfusion unit in different groups; the brighter areas indicate higher blood perfusion.

3.3. The Overall Trend of the Blood Perfusion Ratio in Different Groups. One data was recorded at every second. Every subject from each group was recorded for 35 minutes to get 2100 data as x_{ijk} ($i = 1, \dots, 15$ as subjects, $j = 1, \dots, 5$ as groups, $k = 1, \dots, 2100$). To every subject from each group:

Step 1, average data for the first 5 minutes (before needle in acupuncture groups including manipulation groups and no manipulation group) as X_{ij0} , $X_{ij0} = \sum_{k=1}^{300} x_{ijk}$. Step 2, average data in every minute after the first 5 minutes as X_{ijl} , $X_{ijl} = \sum_{k=60 \times (l-1) + 301}^{300+60 \times l} x_{ijk}$, $l = 1, \dots, 30$. Considering the circumstances for every subject are not exactly the same, we focus on the ratio between X_{ijl} and X_{ij0} and assume $Y_{ijl} = X_{ijl}/X_{ij0}$, $Y_{jl} = \sum_{i=1}^{15} Y_{ijl}$, and $l = 1, \dots, 30$. For each j , the time series (Y_{j1}, \dots, Y_{j30}) stand for the trend of one group. Figure 6 shows the overall trend of the blood perfusion ratio in different groups.

From this we can see that all groups have similar changes, except the control group. First the PU rises obviously when the acupuncture stops; then it falls down in different periods of the trail. Furthermore, the trend of the PU falls down at different times in the four groups. The PU of the reinforcing manipulation by lifting and thrusting group is the most obvious because it could reach 2~2.5 times higher than the basic level. Then, the following groups: the reducing by lifting and thrusting group, the even reinforcing and reducing group, and the acupuncture were with no operation skills.

3.4. Single Factor Repeated Measurement ANOVA. When the same subject was measured at different times (p), $p \geq 3$, the repeated measurement ANOVA is appropriate, because of the possible high correlation between different times. We additionally need to check the covariance matrix sphericity besides the regular ANOVA requirements. Mauchly's test is used to examine sphericity. $P > 0.05$ indicates sphericity reliability, or, the DF of f -statistic should be adjusted. Table 1 is the single factor repeated measurement ANOVA based on the time factor. Table 2 is based on the manipulation-time factor.

This is the result of each group after the statistics of repeated measurement ANOVA. We can see that after Mauchly's test, the groups dissatisfied with the covariance matrix spherical properties. As we see except the control group, the adjusted $P < 0.01$ in all other groups. The result

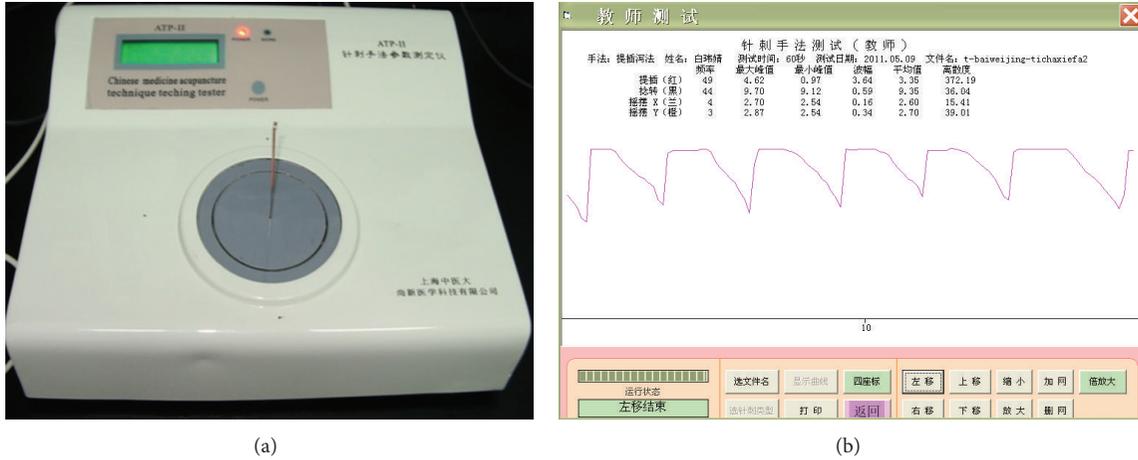


FIGURE 2: (a) ATP-II acupuncture manipulation parameter tester. (b) Output curve of acupuncture manipulation.

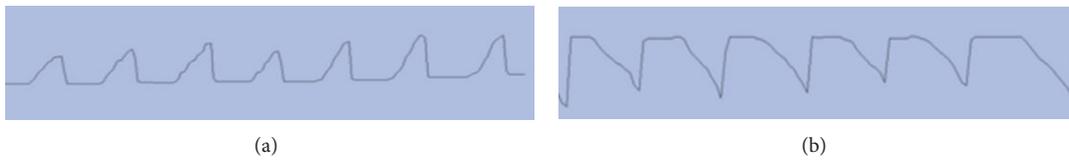


FIGURE 3: (a) Reinforcing manipulation by lifting and thrusting. (b) Reducing manipulation by lifting and thrusting.

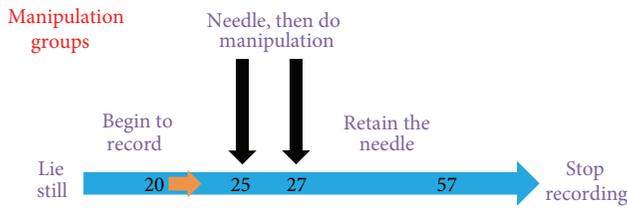


FIGURE 4: Procedure of acupuncture manipulation groups.

TABLE 1: Single factor repeated measurement ANOVA (time factor).

	P	Mauchly's test		Adjust P	
		P	G-G	H-F	
Reinforcing	<0.01	<0.01	<0.01	<0.01	<0.01
Reducing	<0.01	<0.01	<0.01	<0.01	<0.01
Even	<0.01	<0.01	<0.01	<0.01	<0.01
No manipulation	<0.01	<0.01	<0.01	<0.01	<0.01
Control	0.31	<0.01	0.390	0.404	

indicates that the blood perfusion in the other four groups changes with the time changed, and the factor of time plays a different role in different groups.

We can see in this chart that each group is compared with the others, and the operation skills show the difference ($P < 0.01$) expect between even group and no manipulation group. The result indicated that the blood perfusion changes with different acupuncture operation skills.

In Table 3 are the results of blood perfusion ratio of different manipulation groups at different periods.

TABLE 2: Two-factor repeated measurement ANOVA (manipulation-time factor).

	F value	$Pr > F$
Reinforcing-reducing	19.72	<0.01
Reinforcing-even	49.41	<0.01
Reinforcing-acupuncture	73.88	<0.01
Reinforcing-control	135.6	<0.01
Reducing-even	13.4	<0.01
Reducing-acupuncture	30.48	<0.01
Reducing-control	85.15	<0.01
Even-acupuncture	4.803	0.03
Even-control	47.74	<0.01
Acupuncture-control	37.16	<0.01

From Table 3, we can see that the values of PU in no manipulation group are higher than the control group expect 1 min after acupuncture. The values of PU in 3 different acupuncture manipulation groups increase at all time periods after acupuncture, which have significant statistics difference comparing with the control group. Among the 4 different acupuncture manipulation groups, the values of reinforcing lifting and thrusting group increase at all time periods after acupuncture comparing with the no manipulation group and reducing lifting and thrusting group, and there is a significant statistics difference between the reinforcing lifting and thrusting group and the even reinforcing-reducing group at all time periods after acupuncture. However, the values of reducing lifting and thrusting group are higher than the even

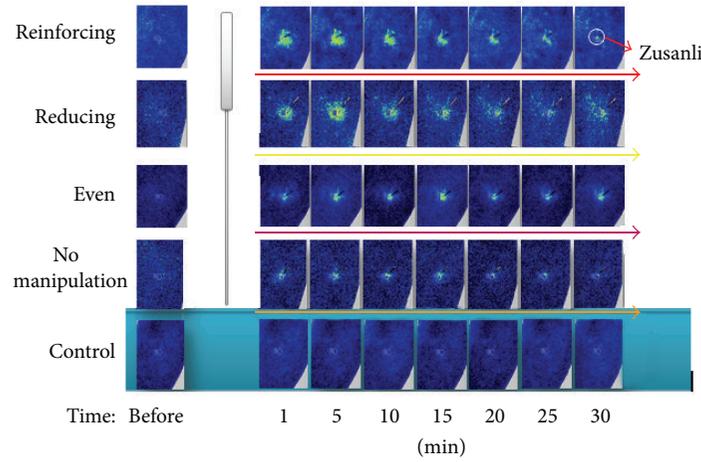


FIGURE 5: The blood perfusion images of different groups. As the blood perfusion is higher, the brighter areas indicate higher blood perfusion.

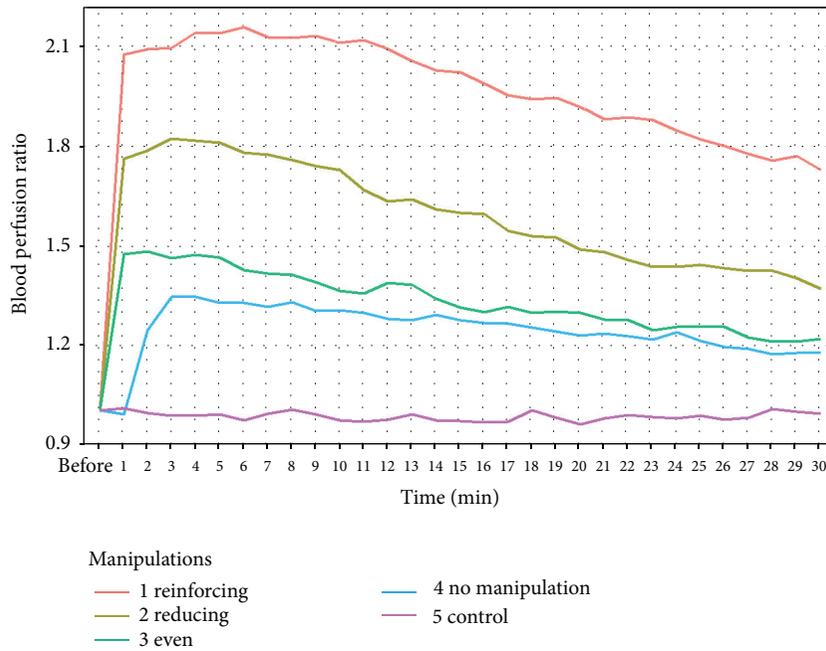


FIGURE 6: The overall trend of the blood perfusion ratio in different groups.

TABLE 3: Blood perfusion ratio of different manipulation groups at different periods.

Manipulation	Reinforcing (N = 15)	Reducing (N = 15)	Even (N = 15)	Acupuncture (N = 15)	Control (N = 15)
B N	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
1 min	2.08 ± 0.29 ^{*†}	1.71 ± 0.29 ^{**}	1.47 ± 0.22 ^{**†}	0.99 ± 0.20 [†]	1.01 ± 0.07 [†]
5 min	2.14 ± 0.38 ^{*†}	1.82 ± 0.36 ^{**}	1.47 ± 0.2 ^{**†}	1.34 ± 0.13 ^{**†}	0.98 ± 0.07 [†]
10 min	2.13 ± 0.37 ^{*†}	1.73 ± 0.34 ^{**}	1.39 ± 0.24 ^{**†}	1.30 ± 0.17 ^{**†}	0.99 ± 0.09 [†]
15 min	2.03 ± 0.35 ^{*†}	1.60 ± 0.29 ^{**}	1.34 ± 0.26 ^{**†}	1.29 ± 0.17 ^{**†}	0.97 ± 0.09 [†]
20 min	1.95 ± 0.35 ^{*†}	1.51 ± 0.29 ^{**}	1.30 ± 0.24 ^{**†}	1.24 ± 0.14 ^{**†}	1.00 ± 0.1 [†]
25 min	1.85 ± 0.35 ^{*†}	1.43 ± 0.25 ^{**}	1.25 ± 0.3 ^{**†}	1.24 ± 0.14 ^{**†}	0.98 ± 0.1 [†]
30 min	1.77 ± 0.38 ^{*†}	1.39 ± 0.24 ^{**}	1.21 ± 0.17 ^{**†}	1.17 ± 0.16 ^{**†}	1.00 ± 0.13 ^{*†}

Significant difference from control group ($P < 0.05$); ^{}significant difference from reinforcing group ($P < 0.05$); [†]significant difference from reducing group ($P < 0.05$).

reinforcing-reducing group and no manipulation group at all the moments after acupuncture.

4. Discussion

These results indicated that the blood perfusion of Zusanli point was increased after acupuncture. There were different influences on the blood perfusion according to different acupuncture manipulations while the reinforcing manipulation by lifting and thrusting is the most obvious. The reinforcing manipulation can make the blood perfusion kept at a higher level and suggest that this increase may be caused by local vasodilators.

The acupuncture manipulation is one of the most distinctive skills of the acupuncture and moxibustion. The mechanism underlying the acupuncture manipulation is currently unknown. Langevin et al. reported that needle grasp is a measurable biomechanical phenomenon associated with acupuncture manipulation [25] and demonstrated that subtle differences in acupuncture manipulations can affect cellular responses in mouse subcutaneous connective tissue [26]. Furthermore, a study [27] found manual acupuncture could change muscle blood flow locally at different doses and indicated this increase may be caused by local vasodilators. In our country, now most researches focus on the influences of blood biochemical criterions and the function of effect organ by different acupuncture manipulations [10–13].

Actually, acupuncture takes regulating effort by needling the acupoint to stimulate the body regulating system and the key factor is affecting the microenvironment. In our opinion, based on standardization and inheritance of acupuncture manipulation, we observe the mechanism of various manipulations. From our research results, we think acupuncture manipulations may change local acupoint peripheral tissue biophysical characteristics, which is the reason of different influences on the blood perfusion according to different acupuncture manipulations [28].

Whether such blood perfusion changes in acupoints can themselves result in the effectiveness of the acupuncture's therapeutic reinforcing or reducing is at the present unknown. Someone thought the mechanosturctural properties of soft connective tissues may affect their response to acupuncture therapy [29]. Dong et al. study indicated that effective acupuncture stimulation is induced mainly due to the receptor deformation [30]. In the future, we will continue our research on the different manipulations from the aspect of blood perfusion, so as to provide more evidence for the mechanism of the acupuncture manipulation.

Conflict of Interests

The authors declare that they have no conflict of interests.

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our lab in 2011 was done by her. They would like to thank all of the participants. X. M. Li and Y. M. Guo planned the study, recruited participants, and performed the experiments. Y. M. Guo did the acupuncture manipulation on the subjects. X. M. Li and Y. Q. Li performed data analysis and wrote the paper. X. M. Li and Y. Q. Li are cofirst authors. J. Z. Chen, D. Zhou, Y. Y. Liu, Y. H. Li, and J. W. Liu provided advice on the experiments and the paper. Y. Guo was the authority for the study planning as well as giving advice on the experiment. This work was supported by Grants no. 81001551 from the National Natural Science Foundation of China.

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Review Article

Acupuncture or Acupressure at the Sanyinjiao (SP6) Acupoint for the Treatment of Primary Dysmenorrhea: A Meta-Analysis

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This meta-analysis aimed to evaluate the effectiveness of acupuncture or acupressure at the Sanyinjiao (SP6) acupoint in relieving pain associated with primary dysmenorrhea. We searched the scientific literature databases to identify randomized controlled trials. The primary outcome was visual analogue scale (VAS) pain score. Three acupuncture and four acupressure trials were included in the meta-analyses. For the acupuncture analysis, there was no difference in the mean VAS score reduction between the SP6 acupoint and control (GB39 acupoint) groups (-4.935 ; lower limit = -15.757 , upper limit = 5.887 ; $P = 0.371$). For the acupressure analysis, there was a significant difference in the mean VAS score after intervention between the SP6 acupoint and control (rest/light touch at SP6/nonacupoint acupressure) groups, favoring the SP6 acupoint group (-1.011 ; lower limit = -1.622 , upper limit = -0.400 ; $P = 0.001$). Sensitivity analyses demonstrated good reliability of the meta-analyses findings. These findings suggest that acupuncture at SP6 is not more effective than acupuncture at an unrelated acupoint in the relief from primary dysmenorrhea. Acupressure at SP6 may be effective in the relief from primary dysmenorrhea. High-quality randomized controlled trials are needed to confirm these findings.

1. Introduction

Primary dysmenorrhea is a common gynecological condition, particularly in adolescents, that is characterized by cramping in the lower abdomen during menstruation [1]. Other symptoms may include nausea, vomiting, diarrhea, fatigue, fever, headache, and lightheadedness [2]. Reports on the incidence of primary dysmenorrhea vary considerably, ranging from 20% to up to 90% of menstruating females [1]. Approximately, 10% of women who experience primary dysmenorrhea suffer severe symptoms [3], which can disrupt activities of daily living, increase absenteeism, and reduce quality of life [2, 4–7].

Treatment for primary dysmenorrhea includes a variety of pharmacological, nonpharmacological, and surgical

options. Surgery, including uterine nerve ablation and hysterectomy, is typically reserved for severe refractory cases of primary dysmenorrhea [1]. Common pharmacological interventions include nonsteroidal anti-inflammatory drugs (NSAIDs) and oral contraceptives [1, 4]. NSAIDs are most frequently used and are effective in the relief from symptoms in many patients with primary dysmenorrhea [1, 4]. Although NSAIDs and other pharmacological treatments for dysmenorrhea generally provide pain relief, the use of these treatments can be costly and associated with adverse events [2, 8]. Hence, there is a need for effective nonpharmacological treatment options for this condition.

Nonpharmacological treatments for primary dysmenorrhea include bed rest, exercise, application of heat packs, and alternative treatments such as acupuncture and acupressure

[4, 9–11]. Of these treatments, acupuncture and acupressure have been widely investigated. Indeed, a number of systematic reviews published within the last decade have examined the use of acupuncture and/or acupressure for the treatment of dysmenorrhea. These reviews, however, have either included studies that involved the use of various acupoints [10, 11], multiple conditions (i.e., not just dysmenorrhea) [12], or moxibustion in addition to acupuncture/acupressure [13, 14]. To our knowledge, there has been no comprehensive review of acupuncture/acupressure studies involving the application of either of these approaches alone to a single, common acupoint for the treatment of primary dysmenorrhea.

The Sanyinjiao (SP6) acupoint, located medially four-finger wide above the ankle, is commonly used for both acupuncture and acupressure, and is thought to offer relief from gynecologic disorders, including dysmenorrhea [15, 16]. In recent years, a number of studies have evaluated the efficacy of acupuncture or acupressure at the SP6 acupoint for relieving pain associated with primary dysmenorrhea [16–24]. Many of these studies have included a relatively small number of participants and the findings have been somewhat inconsistent. Hence, we carried out a meta-analysis to evaluate the effectiveness of acupuncture or acupressure at the SP6 acupoint in relieving pain associated with primary dysmenorrhea.

2. Materials and Methods

2.1. Search Strategy. For acupuncture, we searched PubMed, the Cochrane Library, Google Scholar, and Current Controlled Trials databases (up to December 17, 2012) using combinations of the terms menstrual, dysmenorrhea, acupuncture, Sanyinjiao, and SP6. For acupressure, the above databases were searched (up to December 17, 2012) using combinations of the terms menstrual, dysmenorrhea, acupressure, Sanyinjiao, and SP6. EMBASE was not searched due to the lack of access.

2.2. Selection Criteria. Randomized controlled trials in which acupuncture or acupressure specifically at the SP6 acupoint were used for the treatment of primary dysmenorrhea with pain intensity as a measured outcome were eligible for inclusion in the meta-analysis. Trials involving simultaneous acupuncture or acupressure to combinations of acupoints were excluded. Trials involving acupuncture-like transcutaneous electrical nerve stimulation, moxibustion, and other acupuncture-related techniques (except acupressure) were also excluded. Only English-language articles were eligible for inclusion.

2.3. Data Extraction and Quality Assessment. Searches were performed and data extracted by two independent reviewers. Each trial identified in the search was evaluated for design, patient eligibility criteria, and outcome measures. Any disagreement between reviewers concerning the eligibility of a trial was resolved by consulting with a third review. Duplicate records were excluded based on review of titles. The abstracts

of all remaining articles were reviewed and any duplicate data sets were excluded. All remaining articles were reviewed in full.

Quality assessment of the trials included in the meta-analyses was performed by each reviewer according to previously described criteria [25].

2.4. Outcome Measure. The outcome measure of interest was the effect of treatment (acupuncture or acupressure) on pain as measured using the visual analogue scale (VAS), for which a score of 0 indicates no pain and a score of 10 indicates the worst pain imaginable.

2.5. Statistical Analyses. Mean and standard deviations were calculated for VAS scores and were compared among participants who were treated with acupuncture/acupressure and control. A χ^2 -based test of homogeneity was performed and the inconsistency index (I^2) statistic was determined. If I^2 was $>50\%$ or $>75\%$, the trials were considered to be heterogeneous or highly heterogeneous, respectively. If I^2 was $<25\%$, the studies were considered to be homogeneous. If the I^2 statistic ($>50\%$) that indicated heterogeneity existed between studies, a random-effects model was calculated. Otherwise, fixed-effects models were calculated. Pooled summary statistics of the difference in the mean for the individual studies are shown. Pooled differences in means were calculated and a two-sided P value < 0.05 was considered to indicate statistical significance. Moreover, sensitivity analysis was performed based on the leave-one-out approach. All analyses were performed using Comprehensive Meta-Analysis statistical software, version 2.0 (Biostat, Englewood, NJ, USA).

3. Results

3.1. Selection of Trials

3.1.1. Acupuncture. A total of 78 trials were identified in the literature search (Figure 1). Of these, 74 were subsequently excluded after abstract review for the following reasons: duplicate search results ($n = 35$), not in English language ($n = 15$), not the intervention of interest ($n = 14$), used a combination of acupoints ($n = 5$), not the outcome of interest ($n = 2$), duplicate patient set ($n = 2$), and case report ($n = 1$). Hence, a total of four trials met the inclusion criteria [17–20].

3.1.2. Acupressure. A total of 29 trials were identified in the literature search (Figure 1). Of these, 25 were subsequently excluded after abstract review for the following reasons: not the intervention of interest ($n = 11$), used combination of acupoints ($n = 2$), nonrandomized controlled trial ($n = 1$), and not in English language ($n = 1$). Hence, a total of four trials met the inclusion criteria [21–24].

3.2. Trial Characteristics

3.2.1. Acupuncture. The characteristics of the acupuncture trials are summarized in Table 1. The trials included a total of 358 participants (range: 40 to 200 participants) who

TABLE 1: Characteristics of acupuncture trials identified in the literature search.

First author, year (location)	Number of participants	Intervention	Control	Assessment	Outcomes
Shi, 2011 [19] (China)	40	Electroacupuncture at SP6 for 30 min	Electroacupuncture at unrelated acupoint (GB39), electroacupuncture at nonacupoint, and no electroacupuncture	Pain assessed by VAS before and after intervention	There were significant differences in VAS scores between the SP6 and no acupuncture groups after intervention (24.7 versus 48.2, $P < 0.05$). There were no significant differences in VAS scores between the 3 acupuncture groups
Liu, 2011 [17] (China)	200	Electroacupuncture at SP6 for 30 min for 2 menstrual cycles	Electroacupuncture at unrelated acupoint (GB39), electroacupuncture at nonacupoint, and no electroacupuncture	Pain assessed by VAS before intervention, 5, 10, 30 min during intervention, and 30 min after intervention	The mean decrease in VAS score was significantly greater in all acupuncture groups compared with the no acupuncture group (SP6: -15.56 , $P < 0.05$; GB39: -18.14 , $P < 0.05$; nonacupoint: -10.96 , $P < 0.05$) There were no significant differences in VAS scores between the 3 acupuncture groups
Ma, 2010 [18] (China)	52	Electroacupuncture at SP6 for 10 min on day 1, 30 min on days 2 and 3	Electroacupuncture at unrelated acupoint (GB39), electroacupuncture at nonacupoint, and no electroacupuncture	Pain assessed by VAS before intervention, 5, 10, and 30 min after intervention	There were significantly greater reductions in VAS scores in the SP6 group compared with the other groups at each time after intervention (all $P < 0.05$)
Yu, 2010 [20] (China)	66	Manual acupuncture at SP6 for 5 min	Manual acupuncture at unrelated acupoint (GB39)	Pain assessed before and after intervention according to dysmenorrhea score criteria	The pain score significantly decreased after intervention in the SP6 group (before = 11.20 versus after = 8.17, $P < 0.05$), but not in the GB39 group

VAS: visual analogue scale.

received either electroacupuncture ($n = 3$ trials) or manual acupuncture ($n = 1$) at the SP6 acupoint as a treatment for primary dysmenorrhea. Participants in all but one trial [17] were diagnosed with primary dysmenorrhea according to the Primary Dysmenorrhea Consensus Guideline [26]. Liu et al. did not specify how participants were diagnosed [17]. Each trial included at least one control group of participants who received acupuncture at the GB39 acupoint. Three trials also included a control group of participants who received acupuncture at a nonacupoint [17–19], and three trials included an additional control group of participants who did not receive acupuncture [17–19]. Acupuncture was applied from 5 to 30 min during menstruation and pain was assessed before and after (up to 30 minutes) treatment. Pain was also assessed during acupuncture in one study [17]. Pain was assessed using VAS in three of the four trials [17–19], whereas dysmenorrhea score criteria were used in one trial [20].

3.2.2. Acupressure. The characteristics of the acupressure trials are summarized in Table 2. The trials included a total of 231 participants (range: 30 to 86 participants) who received acupressure at the SP6 acupoint as a treatment for primary dysmenorrhea. None of the reports specified details about the diagnosis of dysmenorrhea. The control groups varied

between trials and included light touch at the SP6 acupoint in one trial [21], acupressure at a nonacupoint in another trial [22], and rest (i.e., no physical intervention) in the other two trials [23, 24]. In all trials, acupressure was initially applied by researchers for 20 to 30 minutes during menstruation. In two trials, acupressure was subsequently applied for 20 min by participants during menstruation [23, 24]. Pain was assessed before and immediately after acupressure in all trials, 30 min and 1, 2, and 3 h after treatment in two trials [21, 22], and after 3 months of ongoing treatment in two trials [23, 24]. Pain was assessed using the VAS in all four trials. The VAS for anxiety, the Short-Form McGill Pain Questionnaire (SF-MPQ), and the Short-Form Menstrual Distress Questionnaire (SF-MDQ) were also used in two trials [23, 24].

3.3. Effect of Treatment of Pain

3.3.1. Acupuncture. The key outcomes from each individual trial are summarized in Table 1. Of the trials comparing pain with acupuncture at the SP6 versus GB39 acupoint, one reported that the decrease in VAS pain was significantly more pronounced in the SP6 group compared with the GB39 group [18]. In one of the other trials, pain, assessed using dysmenorrhea score criteria, was significantly decreased after acupuncture at the SP6 acupoint, but not the GB39 acupoint

TABLE 2: Characteristics of acupressure trials identified in the literature search.

First author, year (location)	Number of participants	Intervention	Control	Assessment	Outcomes
Mirbagher-Ajorpaz, 2011 [21] (Iran)	30	Acupressure at SP6 for 20 min applied by researcher	Light touch at SP6 for 20 min applied by researcher	Dysmenorrhea severity measured using VAS before and immediately, 30 min, and 1, 2, and 3 h after treatment	There were significant differences in VAS scores between the acupressure and control groups immediately, 1, 2, and 3 h after intervention (3.50 versus 5.06, 3.30 versus 4.86, 2.40 versus 5.00, and 1.66 versus 4.80, resp., all $P < 0.05$)
Kashefi, 2010 [22] (Iran)	86	Acupressure at SP6 for 30 min applied by researcher during the first 24 h of menstrual cycle for 2 cycles	Acupressure at a nonacupoint 30 min applied by the researcher during the first 24 h of menstrual cycle for 2 cycles	Dysmenorrhea severity assessed by VAS before and immediately, 30 min, and 1, 2, and 3 h after intervention	For the first cycle, there were significant differences in VAS scores between the acupressure and control groups 30 min, 1, 2, and 3 h after intervention (4.90 versus 6.06, 4.38 versus 6.23, 4.55 versus 6.34, and 5.34 versus 6.81, resp., all $P < 0.05$). For the second cycle, there were significant differences in VAS scores between the acupressure and control groups immediately, 30 min, 1, 2, and 3 h after intervention (5 versus 6.16, 4.86 versus 6.04, 4.72 versus 6.04, 4.72 versus 6.04, 4.60 versus 6.58, and 5.67 versus 7.06, resp., all $P < 0.05$)
Wong, 2010 [23] (China)	46	Acupressure at SP6 for 20 min applied by researcher at initial intervention 20 min acupressure self-treatment upon waking and at bedtime during the first 3 days of the next 3 menstrual cycles	Rest for 20 min at initial intervention 20 min rest upon waking and at bedtime during the first 3 days of the next 3 menstrual cycles	Dysmenorrhea severity assessed immediately after first treatment and after 3 months using VAS, SF-MPQ, and SF-MDQ	There were significant differences in VAS scores and SF-MPQ between the acupressure and control groups immediately after initial intervention (VAS: 4.11 versus 5.81, $P < 0.05$; SF-MPQ: 5.26 versus 7.38, $P < 0.05$) There were significant differences in VAS scores of SF-MPQ and SF-MDQ between the acupressure and control groups after 3 months of self-care (VAS: 2.79 versus 4.30, $P < 0.05$; SF-MPQ: 3.53 versus 5.81, $P < 0.05$; SF-MDQ: 23.96 versus 26.61, $P < 0.05$)
Chen, 2004 [24] (Taiwan)	69	Acupressure at SP6 for 20 min applied by researcher at initial intervention 20 min acupressure self-treatment during next menstrual cycle	Rest for 20 min at initial intervention 20 min rest during next menstrual cycle	Dysmenorrhea severity assessed using VAS for pain and VAS for anxiety	There were differences in VAS pain and anxiety scores between the acupressure and control groups after the initial intervention (pain: 3.88 versus 4.79; anxiety: 3.13 versus 3.74) There were differences in VAS pain scores between the acupressure and control groups after self-treatment (2.92 versus 3.04)

VAS: visual analogue scale; SF-MPQ: Short-Form McGill Pain Questionnaire; SF-MDQ: Short-Form Menstrual Distress Questionnaire.

[20]. All three trials comparing acupuncture at the SP6 acupoint versus no acupuncture reported that the decrease in VAS pain was significantly more pronounced with acupuncture at the SP6 acupoint [17–19]. Of the three trials comparing acupuncture at the SP6 acupoint with acupuncture at a nonacupoint, one reported that the decrease in VAS pain was

significantly more pronounced with acupuncture at the SP6 acupoint [18] and two found no difference between the groups [17, 19].

One of the trials identified in the search [20] did not report mean VAS scores before and after treatment and could not be included in the meta-analysis examining the effect

TABLE 3: Quality assessment of trials identified in the literature search: risk of bias.

	Sequence generation adequate	Allocation concealment adequate	Blinding adequate	Incomplete outcome data addressed	Free of selective reporting	Free of other bias
Acupuncture trials						
Shi et al., 2011 [19]	+	+	+	+	?	?
Liu et al., 2011 [17]	+	+	+	+	+	?
Ma et al., 2010 [18]	+	+	+	+	?	?
Yu et al., 2010 [20]	+	+	-	+	?	?
Acupressure trials						
Mirbagher-Ajorpaz et al., 2011 [21]	+	-	-	+	?	?
Kashefi et al., 2010 [22]	-	-	+	+	?	?
Wong et al., 2010 [23]	-	-	-	+	?	?
H. M. Chen and C. H. Chen, 2004 [24]	-	-	-	+	?	?

+: low risk of bias; -: high risk of bias; ?: unclear risk of bias.

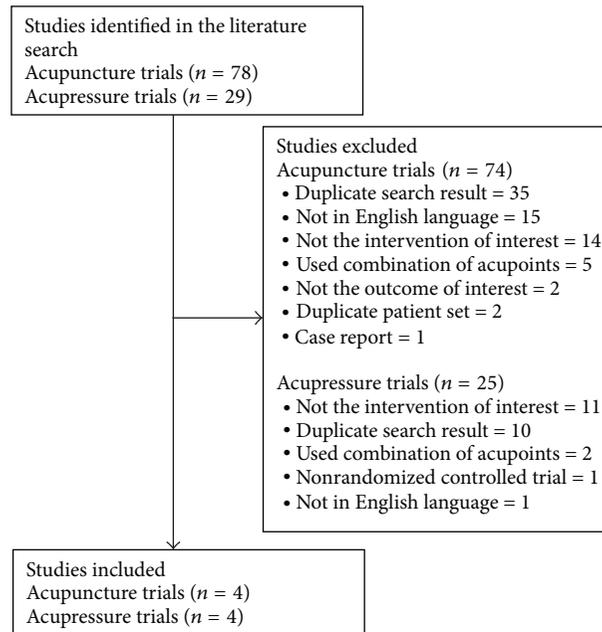


FIGURE 1: Flow diagram of trial selection.

of acupuncture on dysmenorrhea-associated pain. Hence, only three trials were included in the meta-analysis [17–19]. There was heterogeneity in the mean VAS score reduction among the three studies ($Q = 4.507$, $I^2 = 55.63\%$, and $P = 0.105$); therefore, a random-effects model of analysis was used. Pooled differences in mean VAS score reductions revealed that there was no significant difference between the SP6 and GB39 groups ($P = 0.371$, Figure 2). The pooled differences in mean VAS score reduction ranged from -15.757 to 5.887 ; the overall mean difference was -4.935 .

3.3.2. Acupressure. The key outcomes from each individual trial are summarized in Table 2. All trials found that pain

scores (VAS, SF-MPQ, or SF-MPQ) were significantly lower in the acupressure group compared with the control group after intervention. In one trial, VAS anxiety scores were also found to be significantly lower in the acupressure group compared with the control group after intervention [24].

All four acupressure trials were included in the meta-analysis examining the effect of acupressure on dysmenorrhea-associated pain. There was heterogeneity in the mean VAS score after intervention among the four studies ($Q = 6.169$, $I^2 = 51.37\%$, and $P = 0.104$); therefore, a random-effects model of analysis was used. Pooled differences in mean VAS scores after intervention revealed a significant difference between the SP6 and control groups

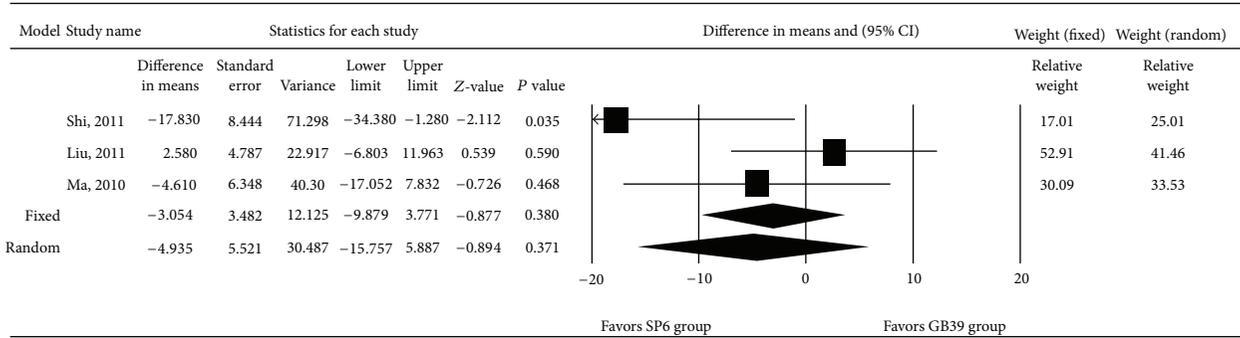


FIGURE 2: Forest plot showing differences in mean using the visual analogue scale pain score for trials in which women with dysmenorrhea received acupuncture at the SP6 or GB39 acupoint. Data are presented as the difference in means with the 95% confidence interval (CI). $P < 0.05$ indicates a statistically significant difference.

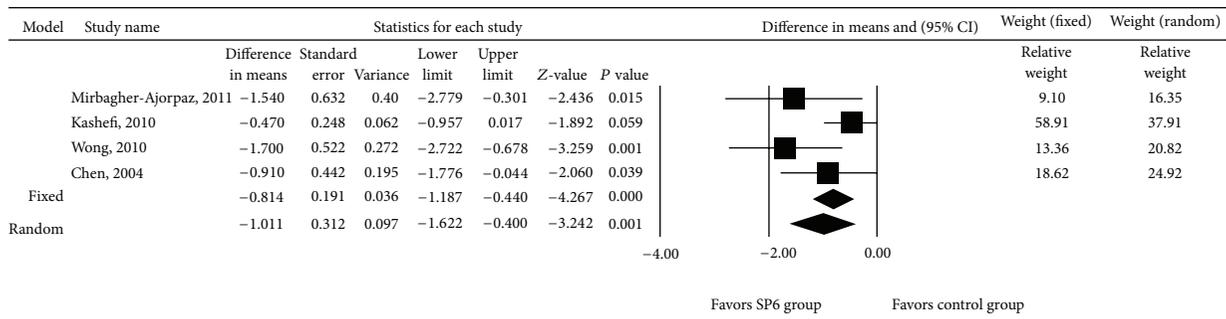


FIGURE 3: Forest plot showing differences in mean using the visual analogue scale pain score for trials in which women with dysmenorrhea received acupressure at the SP6 acupoint or control treatment. Data are presented as the difference in means with the 95% confidence interval (CI). $P < 0.05$ indicates a statistically significant difference.

($P = 0.001$, Figure 3). The pooled differences in the mean VAS score after intervention ranged from -1.622 to -0.400 , with the pooled mean difference being -1.011 .

3.4. Quality Assessment and Sensitivity Analysis

3.4.1. Acupuncture. Overall, the acupuncture trials identified had adequate sequence generation, allocation concealment, and addressed incomplete outcome data (Table 3). We were only able to determine that one of the acupuncture trials identified [17] was free of selective reporting. We were not able to confirm that any of the trials were free of other biases.

3.4.2. Acupressure. Overall, the acupressure trials identified had a high associated risk of bias due to sequence generation, allocation concealment, and blinding (Table 3). Incomplete outcome data were adequately addressed in all trials. We were unable to determine if any of the acupressure trials identified were free of selective reporting or other bias.

Figures 4 and 5 show the results of meta-analysis with one study removed in turns. These results demonstrate that even when each trial was excluded from the meta-analysis, the direction and magnitude of the pooled estimates did not vary markedly. Moreover, across all meta-analyses, we found that random-effects models provided similar estimates to those

of fixed models. These findings are indicative of good meta-analyses reliability.

4. Discussion

This is the first systematic review to evaluate the effectiveness of acupuncture or acupressure at the SP6 acupoint for relieving pain associated with primary dysmenorrhea. Our findings suggest that acupuncture at the SP6 acupoint may not be more effective than acupuncture at an unrelated (GB39) acupoint for the relief of dysmenorrhea-associated pain (assessed using a VAS). In contrast, our findings suggest that acupressure at the SP6 acupoint may provide more effective relief from dysmenorrhea-associated pain than control interventions.

Findings among the acupuncture trials identified in our literature search were generally inconsistent. The only consistent finding was that acupuncture at the SP6 acupoint resulted in a greater decrease in VAS pain than no acupuncture [17–19]. Other differences between treatment groups (SP6 versus GB39, SP6 versus nonacupoint acupressure) were apparent in some studies, but not others. This lack of consistency is reflected in our meta-analysis finding that acupuncture at the SP6 acupoint did not provide better pain relief from primary dysmenorrhea than did acupuncture at the GB39 acupoint.

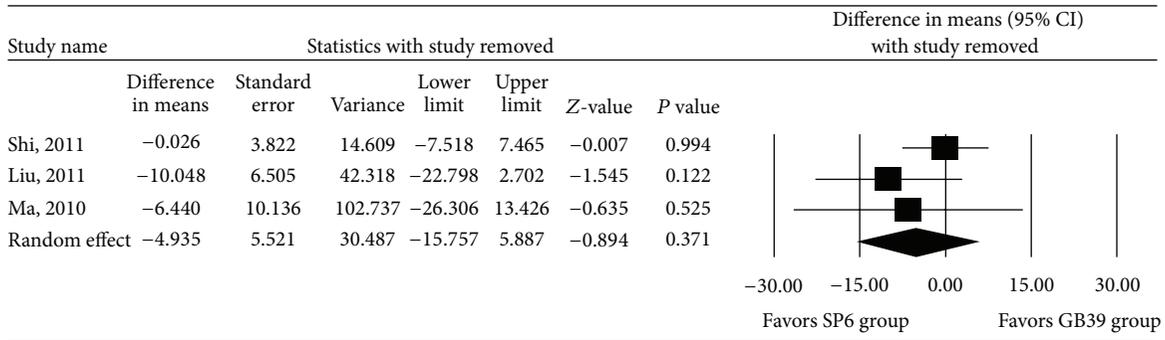


FIGURE 4: Sensitivity analysis for the influence of individual studies on the pooled estimate (as determined using the leave-one-out approach) of the visual analogue scale pain score for trials in which women with dysmenorrhea received acupuncture at the SP6 or GB39 acupoint. Data are presented as the difference in means with the 95% confidence interval (CI). $P < 0.05$ indicates a statistically significant difference.

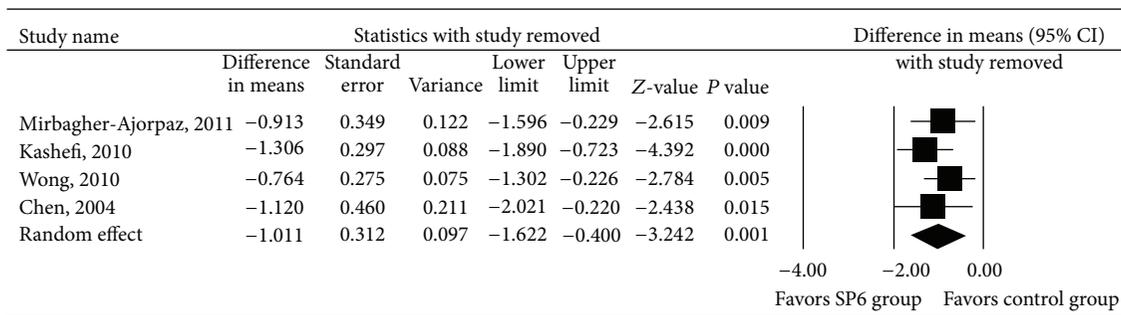


FIGURE 5: Sensitivity analysis for the influence of individual studies on the pooled estimate (as determined using the leave-one-out approach) of the visual analogue scale pain score for trials in which women with dysmenorrhea received acupressure at the SP6 acupoint or control treatment. Data are presented as the difference in means with the 95% confidence interval (CI). $P < 0.05$ indicates a statistically significant difference.

There was marked heterogeneity among the acupuncture trials that may have affected our meta-analysis findings and explain the between-trial disparity. Notably, the timing and length of acupuncture differed between some studies (see Table 1). Further, participants in three trials [17–19] were instructed/permitted to use aspirin for pain relief during the trial. Use of aspirin may have confounded any acupuncture-related pain relief.

The acupressure trials identified in our literature review consistently reported that acupressure at the SP6 acupoint resulted in better pain relief than that of control treatment, the nature of which varied between studies. Unsurprisingly, our meta-analysis also revealed that acupressure at the SP6 acupoint resulted in significantly better pain relief.

As with the acupuncture trials, there was distinct heterogeneity among the acupressure trials. There were between-trial differences in the timing and application of acupressure, and, as already highlighted, a lack of consistency in the types of control interventions used. Further, participants in some trials were allowed to take pain medication before acupressure; however, the timing of allowed medication differed, ranging between >3 hours before treatment [21], >4 hours before treatment [24], and >6 hours before treatment [23]. Another difference between studies that may have affected

the pain outcome is variable participant positioning during acupressure, that is, prone [22, 24], supine [21], or seated cross-legged [23]. Finally, none of the reports described how primary dysmenorrhea was diagnosed; hence, it is possible that some of the participants in the trials may have had secondary dysmenorrhea.

Our meta-analyses have several limitations that must be acknowledged. Firstly, and perhaps most notably, only a small number of trials met the criteria for inclusion, thus reducing the power of our analyses. Secondly, we only searched the English-language literature. It is possible that other relevant trials may have been identified if we had searched the literature in other languages. Finally, and as already discussed, there was clear heterogeneity among the studies identified, which may have affected the outcomes of the meta-analyses.

5. Conclusions

In conclusion, there is insufficient high-quality evidence available in the current literature regarding the effectiveness of acupuncture or acupressure at the SP6 acupoint for the treatment of pain associated with primary dysmenorrhea. Hence, the findings from our meta-analyses are by no

means definitive. Nevertheless, our findings do suggest that acupuncture at the SP6 acupoint may not be more effective in relieving pain than acupuncture at an unrelated acupoint. Further, our findings suggest that acupressure at the SP6 acupoint may provide more effective pain relief than that of control treatment. Clearly, there is a need for high-quality, randomized controlled trials to clarify the effectiveness of acupuncture/acupressure at the SP6 acupoint for the treatment of pain resulting from primary dysmenorrhea.

Conflict of Interests

All authors of the paper do not have a direct financial relation with the Computer Program mentioned in our paper that might lead to a conflict of interests.

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Research Article

Electroacupuncture Modulates Reproductive Hormone Levels in Patients with Primary Ovarian Insufficiency: Results from a Prospective Observational Study

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To investigate the effects of electroacupuncture (EA) on serum FSH, E2, and LH levels, women with primary ovarian insufficiency (POI) were treated with EA once a day, five times a week for the first four weeks and once every other day, three times a week, for the following two months, and then were followed up for three months. Serum E2, FSH, and LH levels were measured at baseline, at the end of treatment, and during followup. A total of 11 women with POI were included in this prospective consecutive case series study. Compared with baseline, patients' serum E2 increased, FSH decreased, and LH decreased ($P = 0.002, 0.001, \text{ and } 0.002$, resp.) after EA treatment, and these effects persisted during followup. With treatment, 10 patients resumed menstruation (10/11, 90.91%), whereas one patient remained amenorrhea. During followup, two patients, including the one with amenorrhea during treatment, reported absence of menstruation. Temporary pain occurred occasionally, and no other adverse events were found during treatment. The results suggest that EA could decrease serum FSH and LH levels and increase serum E2 level in women with POI with little or no side effects; however, further randomized control trials are needed.

1. Introduction

Primary ovarian insufficiency (POI) is a syndrome characterized by amenorrhoea, sex steroid deficiency, and elevated gonadotrophins occurring in women under the age of 40 years. Other terms describing the disease include premature ovarian failure, premature menopause, and premature ovarian dysfunction. Clinical symptoms observed are similar to those of menopause, which include hot flashes, vaginal dryness, dyspareunia, insomnia, vaginitis, and mood swings [1]; however, this condition differs from menopause in its existence of varying and unpredictable ovarian functions [2]. Rather than complete amenorrhea in menopausal women, these patients may present with intermittent and unpredictable menses. In addition, 5–10% of women with POI will be able to conceive and deliver a child after they have received the diagnosis [3]. Consequently, a more accurate and informative term for this condition,

as indicated by Nelson [2], tends to be “primary ovarian insufficiency.”

Formal diagnostic criteria of POI have not been established in any professional society; however, a commonly used definition refers to the development of amenorrhoea for four months or more due to cessation of ovarian function before the age of 40 years [4]. The diagnosis is based on elevated serum follicle-stimulating hormone (FSH) levels in menopausal range (usually above 40 IU/L) detected on at least two occasions with at least one month apart [5].

POI affects 1–2% of women younger than 40 years of age and 0.1% of women younger than 30 years of age [6]. Significant impacts including psychosocial sequelae and major health implications may result from POI. Long-term sequelae of POI include significant increase in all-cause mortality and decreased life expectancy, increased cardiovascular events, early onset of osteoporosis, increased risk of dementia or decreased cognitive function, devastating psychological

effects, sexual dysfunction, and infertility [7]. Given the significant impacts and long-term sequelae of POI on patients, appropriate management is crucial in relieving symptoms and improving quality of life. Besides daily intake of calcium and vitamin D and management addressing emotional well-being, use of hormone (estrogen and progestin) replacement therapy for young patients remains the major treatment [2]. However, not only evidence from randomized control trials is still lacking regarding specific hormone replacement therapy (HRT) regimen, but also the long-term risks of HRT in women with POI are still unclear [8]. HRT in menopausal women was found to be associated with increased risks of breast cancer, heart attacks, and strokes [9]. Although application of these results to young women with POI may be invalid, similar risks may still exist in women with POI receiving HRT.

Acupuncture, a major component of traditional Chinese medicine, has been used in eastern Asian countries for thousands of years for various symptoms that are or are similar to those of menopause. With little to no side effects, acupuncture has been found effective in relieving hot flashes of bilaterally ovariectomized women [10], women with breast cancer [11], and women undergoing perimenopause and menopause [12]. In a recent published meta-analysis, Zheng et al. [13] summarized several previously published meta-analyses and lots of clinical trials and found that acupuncture could improve pregnancy outcomes in women undergoing *in vitro* fertilization (IVF). In addition, for patients diagnosed with polycystic ovarian syndrome (PCOS), which is a disorder characterized by anovulation resulting in irregular menstruation, amenorrhea, infertility, and polycystic ovaries, Lim et al. [14] found that acupuncture may be an inexpensive effective intervention.

Although the therapeutic mechanisms of acupuncture in the above-mentioned disorders are yet to be fully investigated, a plausible hypothesis may be that acupuncture can modulate hypothalamic-pituitary-ovary axis (HPOA). Using ovariectomized rats, Chen et al. [15] found that repeated electroacupuncture (EA) increases serum E2 and reduces LH; Zhao et al. [16] found that EA stimulates hypothalamic aromatization which plays a key role in estrogen production from androgen; Ma et al. [17] reported that EA decreased the elevated estrogen receptor expression in hypothalamic preoptic area. This hypothesis is also concurred by the results from two clinical trials in which acupuncture decreased LH and increased E2 levels [10, 18]. Recent fMRI studies add further credence to the hypothesis in which acupuncture was found to modulate activities of the brain cortex including pituitary and hypothalamus [19, 20]. Nonetheless, inconsistent results were also found in other clinical trials. Dong et al. [21] found that acupuncture could significantly improve menopausal vasomotor symptoms but had no effects on serum FSH, LH, and E2 levels in menopausal patients. EA was found effective in the treatment of PCOS symptoms, but without significant change in these hormone levels [22, 23].

Similar to menopause, infertility, and PCOS, POI is also characterized by the dysfunction of HPOA. However, to our best knowledge, no studies regarding acupuncture effects on POI have been published in English; one study

performed in China found positive results of acupuncture on POI [24]. Nonetheless, the study had a small sample size, lacked detailed documentation on hormone measurement, acupuncture procedures, and methods used in the assessment of efficacy [24]. In the present study, we aimed to investigate the effects of acupuncture on serum FSH, E2, and LH levels in women with POI. In addition, changes related to the availability of menstruation were also reported.

2. Material and Methods

2.1. Study Design. This was a prospective consecutive case series study performed at the Acupuncture Department of Guang An Men Hospital, China Academy of Chinese Medical Sciences. The hospital ethics committee approved this treatment protocol for women with POI, and patients signed informed consent before study participation. Acupuncture procedures were implemented by a senior acupuncturist with more than 20 years' clinical experience. Data management and analysis were performed by graduates who were blinded to the treatment procedures.

2.2. Participants. For inclusion, the following criteria had to be fulfilled by the patients: amenorrhea for four months or longer and FSH above 40 IU/L as detected on at least two occasions with at least one month apart. Before treatment, all patients had gone through one month baseline evaluation period during which they stopped all medications influencing reproductive hormones. These medications include but are not limited to clomiphene, human chorionic gonadotropin (HCG), Letrozole, Premarin, and Provera (Medroxyprogesterone Acetate). Patients were advised and agreed not to use these medications during study.

2.3. Acupuncture Protocol. Hua Tuo brand needles (size 0.45 mm × 125 mm and 0.30 × 75 mm, manufactured by Suzhou Medical Appliance, Suzhou, Jiangsu Province, China) together with GB6805-2 Electro-Acu Stimulators (Medical Supply & Equipment Co., Ltd, Shanghai, China) were used. The parameters of electric stimulation were set as the following: continuous wave with electric current frequency of 20 Hz and intensity between 1 and 4 mA based on patients' tolerance. Based on the clinical experiences of the acupuncturist, published research studies [15, 23, 24], and anatomical knowledge (direct or indirect stimulation of T12-L2, S2-S4), two EA prescriptions were used on patients alternatively. Acupoint use in Prescription 1 consists of bilateral BL33, and acupoints in Prescription 2 consist of CV4, bilateral ST25, and bilateral ST29.

Acupoints were selected and localized according to WHO Standardized Acupuncture Points Location [25]. Paired alligator clips with negative and positive electrodes of the EA apparatus were attached to the needle holders at each pair of the same acupoints on each side during treatment; no electric stimulation was used at CV4. EA stimulation in each treatment lasted 20 minutes. All patients received EA treatment once a day, five times a week for the first four weeks, once every other day, and three times a week for the following two months. They were then followed up for three months.

Needles of the size of 0.45 mm × 125 mm were inserted obliquely into the bilateral third sacral foramina (BL33) with a depth of 70–80 mm. Using 0.30 mm × 75 mm size needles, needle insertion at bilateral ST25 was performed perpendicularly with a depth of 45–55 mm. 0.30 mm × 40 mm size needles were inserted obliquely at CV4 and bilateral ST29 with a depth of 25 mm. Upon acupuncture at BL33, patients should have a strong soreness sensation which radiates to the lower abdomen. For acupuncture at ST25, CV4, and ST29, needles should be inserted just deep enough to touch the parietal peritoneum where the acupuncturist may feel resistance under the needle tip and patients may have tingling and twisting sensations.

2.4. Outcome Measures. At baseline of initial evaluation, the dates of patients' last menstrual cycle were documented, and patients' serum E2, FSH, and LH levels were measured. Based on patients' last menstrual cycle prior to study participation, patients' E2, FSH, and LH levels were measured on the 2nd–4th day of the 1st menstrual cycle and on the 2nd–4th day of the 4th menstrual cycle after cessation of EA treatment. In addition, patients' vaginal bleeding conditions were recorded based on patients' reports. Adverse events were documented if available.

2.5. Statistical Analysis. Statistical analysis was performed with the SPSS software package (Version 17.0) for Windows XP. Quantitative data of serum E2, FSH, and LH levels were expressed with mean ± SEM. Paired samples *t*-test was used to measure the difference between values at baseline, at the end of treatment, and during followup. A 5% significance level ($P < 0.05$) and two-tailed tests were used for all tests. Qualitative data including menstrual bleeding conditions and adverse events during study were described.

3. Results

From February 2, 2010 to December 30, 2011, a total of 21 patients with POI, as diagnosed by department of gynecology at tertiary-level hospitals in China, visited the Outpatient Department of Acupuncture at the Guang An Men Hospital. Of these patients, 10 were excluded from the study for the following reasons: one patient did not meet the diagnostic criteria of POI; eight had incomplete data although various patient contacts had been made; one used estrogen medications during followup (Figure 1). Of the included 11 patients, four were 25 to 30 years old, three were 31 to 35 years old, and four were 36 to 39 years old. The shortest history of amenorrhea was four months, and the longest was 10 years.

3.1. Hormone Levels (Tables 1 and 2). During the one month baseline evaluation period, patients' serum E2, FSH, and LH values were 33.35 ± 10.83 pmol/L, 89.08 ± 11.97 IU/L, and 37.10 ± 3.47 IU/L, respectively. Serum E2 level increased to 223.82 ± 45.95 pmol/L after EA treatment and was maintained with a value of 217.53 ± 63.39 pmol/L during followup. Patients' serum FSH level decreased to 45.37 ± 7.07 IU/L after EA treatment and was maintained at 49.28 ± 8.85 IU/L during followup. Patients' serum LH level decreased to $22.08 \pm$

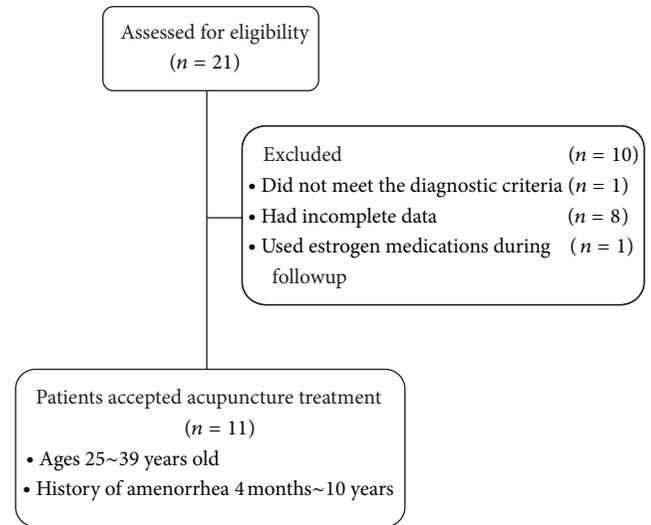


FIGURE 1: Flow chart of study participation.

3.66 IU/L after EA treatment and was maintained at 22.29 ± 4.42 IU/L during followup. Compared with baseline, patients' serum E2 increased, FSH decreased, and LH decreased ($P = 0.002, 0.001, \text{ and } 0.002$, resp.) after EA treatment, and these results persisted during followup ($P = 0.016$ for E2, 0.005 for FSH, and 0.023 for LH as compared with baseline). No difference was noticed in the hormone values between values at the end of treatment and during followup ($P > 0.05$).

3.2. Symptoms. At baseline, all patients had had no periods for more than four months and presented with varying degrees of menopausal symptoms including hot flashes, night sweats, vaginal dryness, and mood swings. With treatment, 10 patients resumed menstruation (10/11, 90.91%), whereas one patient remained amenorrhea. Of the 10 patients who regained menstruation, four patients had decreased menstrual flow but with normal color and duration as compared with normal menstrual bleeding and six regained normal duration, volume, and color of periods.

During followup, two patients reported amenorrhea including the one with amenorrhea during treatment; four patients maintained the normal menstrual cycle and flow; five patients had irregular menstrual cycles. Of the 10 patients who regained menstruation during treatment, their symptoms of night sweating, hot flashes, vaginal dryness, and mood swings, if presented, were all alleviated during treatment. During followup, their symptoms remained largely improved in six patients (6/10, 60%), but fluctuated in the other four patients (4/10, 40%). In the one patient who had had amenorrhea during the whole study, no obvious change was observed in symptoms.

3.3. Adverse Events. In the present study, pain which is considered normal occurred occasionally. The pain was either instant upon needle insertion or well tolerated during treatment and disappeared after needle removal. No other adverse events were reported during study.

TABLE 1: Demographic data and hormone values of patients during study.

Number	Age (y)	History of amenorrhea	Hormone level								
			Baseline			After treatment			Followup		
			E2 pmol/L	FSH IU/L	LH IU/L	E2 pmol/L	FSH IU/L	LH IU/L	E2 pmol/L	FSH IU/L	LH IU/L
1	39	4 years	7.34	54.88	41.34	418.38	23.30	19.33	18.35	59.29	4.12
2	32	4 months	51.75	47.20	32.10	319.29	9.40	2.30	418.38	5.00	2.10
3	30	3 years	52.52	148.00	43.10	29.36	89.10	26.19	22.02	89.00	18.00
4	30	10 years	18.83	73.40	35.80	90.14	70.23	26.83	337.64	5.50	10.94
5	31	7 years	0.37	72.57	25.40	253.23	46.90	15.40	352.32	29.30	16.02
6	37	1 year	22.02	138.03	49.10	232.31	63.30	37.83	649.59	37.20	18.24
7	32	5 years	17.10	63.12	37.47	374.34	27.04	15.4	44.04	63.60	38.38
8	27	15 months	14.68	43.70	15.90	18.35	48.00	13.09	14.68	57.90	23.68
9	38	4 months	128.45	86.34	47.56	407.37	24.90	45.48	282.59	36.70	43.20
10	39	2 years	13.47	147.83	54.06	264.24	42.10	15.01	205.52	67.83	26.07
11	28	1 year	40.37	104.80	26.32	55.05	54.80	26.05	47.71	90.80	44.47

E2: estradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone.

4. Discussion

In the present study, EA induced significant changes of serum E2, FSH, and LH and the effects were maintained during three-month followup when acupuncture treatment was stopped. The FSH level dropped 43.71 ± 9.49 IU/L from baseline to the end of treatment ($P = 0.001$) and the decrease was still significant during the three-month followup with a value of 39.80 ± 11.19 IU/L ($P = 0.005$ as compared with baseline). E2 level increased 190.47 ± 45.10 IU/L from baseline to the end of treatment ($P = 0.002$) and the increase was still significant during the three-month followup with a value of 184.18 ± 63.47 IU/L ($P = 0.016$ as compared with baseline). These results were similar to those report by Sha et al. [24]. Using tailored cupping and moxibustion with acupuncture therapy for patients with POI, Sha et al. [24] found that FSH and E2 levels increased 30 days and 90 days after cessation of treatment whereas LH had no change 30 days but decreased significantly 90 days after cessation of treatment. The slight difference may be due to date difference of hormone measurements within the menstrual cycle as Sha et al. [24] did not reported the time of hormone measurement. Nonetheless, in the present study, 10 patients resumed menstruation, and the majority (6/10) regained normal period during treatment. The effects persisted in the majority of patients during followup. The results of the present study supported the gaining of menstruation in a top athlete recently reported in a Japanese acupuncture study [26]. In addition, improvements of other symptoms including menstrual bleeding, night sweating, hot flashes, vaginal dryness, and mood swing in the present study may also indicate certain clinical effects of acupuncture for POI.

Modulation of FSH, E2, and LH levels may help explain the mechanism of acupuncture for POI, which may be similar to the use of acupuncture in other gynecological disorders. Using acupuncture and auricular acupuncture, Zhou et al. [10] found that acupuncture could significantly increase serum E2 while decrease FSH and LH in bilaterally

ovariectomized Chinese women. Although patients' exact diagnoses vary, our results match the results reported by Zhou et al. [10]. Patients included in the study by Zhou et al. [10] were 41.6 ± 5.8 years old. By referring to the diagnostic criteria of POI, we could infer that a great portion of patients included in their study may also be diagnosed with POI. Therefore, the results of the present research met the expectation as deduced from the study by Zhou et al. [10]. In addition, the results of the present study also support the effects of acupuncture in the modulation of HPOA as reported in rat studies [15–17].

Although measurement of sex hormones at a specific date of the menstrual cycle seems illogical in rats, specific measurement time of sex hormones during menstrual cycle may be accounted for the hormone differences of acupuncture effects observed in other clinical trials [21–23]. In both PCOS studies by Jadel et al. [22] and by Pastore et al. [23], hormone levels were measured during the follicular phase of the menstruation cycle when researchers did not find significant changes in E2, FSH, and LH. However, in the menopausal study, Dong et al. [21] found significant changes in these reproductive hormones with acupuncture. Our study confirmed the results reported by Dong et al. [21]. Although patients' diagnoses in these studies are different, PCOS, menopause, and POI all are characterized by dysfunctions of the HPOA and share similar physiological changes and similar clinical presentations. This is true especially for menopause and POI, and the present study of POI showed similar responses of acupuncture on reproductive hormone regulation as the study of menopause [21]. Consequently, the different results regarding reproductive hormone regulation in the PCOS studies may be due to different diagnosis or different hormone measurement time during the menstrual cycle. As patients' symptoms of PCOS were improved in both studies [22, 23], the difference may be more likely due to the later. Sex hormone was measured in the 2–4th day of menstrual phase in the present study, but measured in the follicular phase in the PCOS studies. In addition, the

TABLE 2: Serum E2, FSH, and LH values assessed during study (mean \pm SEM).

Serum hormone level	n	Baseline (A)	After treatment (B)	Followup (C)	Difference $x \pm s$			t value			P value		
					AB	AC	BC	AB	AC	BC	AB	AC	BC
E2 (pmol/L)	11	33.35 \pm 10.83	223.82 \pm 45.95	217.53 \pm 63.39	190.47 \pm 45.10	184.18 \pm 63.47	6.29 \pm 70.25	-4.223	-2.902	0.09	0.002	0.016	>0.05
FSH (IU/L)	11	89.08 \pm 11.97	45.37 \pm 7.07	49.28 \pm 8.85	43.71 \pm 9.49	39.80 \pm 11.19	3.91 \pm 9.47	4.606	3.557	-0.413	0.001	0.005	>0.05
LH (IU/L)	11	37.10 \pm 3.47	22.08 \pm 3.66	22.29 \pm 4.42	15.02 \pm 3.70	14.81 \pm 5.52	0.21 \pm 4.31	4.057	2.683	-0.049	0.002	0.023	>0.05

E2: estradiol; FSH: follicle-stimulating hormone; LH: luteinizing hormone.

results of acupuncture on menstruation and gynecological symptoms in the present study partially confirmed the therapeutic effects of acupuncture on gynecological symptoms. Therefore, we should believe that acupuncture could restore the normal function of hypothalamus-pituitary-ovary axis (HPOA) in body.

Besides the HPOA hypothesis, modulation of autonomic nerve function is also proposed as the reason of acupuncture effects on gynecological disorders [27, 28]. Acupuncture stimulation to the sacral segment was found to change the vigilance state of animals via GABAergic systems suppressing the activity of noradrenergic LC neurons [27]. In the study by Wang et al. [28], researchers found that acupuncture could enhance vagal activities and suppress sympathetic activities. Based on anatomical knowledge, acupuncture at BL33 stimulates sacral nerves, part of which form the pelvic nerve and other autonomic nerve structures innervating deep organs inside the lower abdominal areas. Thus, reproductive hormone modulation effects in the present study may also be caused by the effects of acupuncture in the autonomic system.

In addition, Stener-Victorin et al. [29] found that low-frequency (2 Hz) EA stimulation with a strong intensity (6 mA) increases ovarian blood flow. The visceral peritoneum is supplied by branches from somatic efferent and afferent nerves that also supply the muscles and skin, respectively. Acupuncture deep into the parietal peritoneum at CV4, ST25, and ST29 of the present study may thus provide stimulations to the abdominal muscles and the surrounding structures and thus modulate their function and improve blood circulation inside the lower abdomen. EA stimulation parameters of 20 Hz and 1 to 4 mA based on patients' tolerance were used in the present study. As 20 Hz is significantly different from the high frequency EA (80 Hz) used in the study by Stener-Victorin et al. [29] and largest tolerant EA intensity in patients can be considered as a strong intensity, the positive results of the present study may thus partially support that middle to low-frequency EA stimulation with strong intensity may be the optimal stimulation parameters for EA treatment [29].

4.1. Limitations. Nonetheless, this study only included 11 patients at only one tertiary level hospital in China; therefore, the result of this study may not well characterize the general response of women with POI undergoing acupuncture treatments. Although the significant changes of sex hormones in the present study were most likely due to acupuncture effects; it may also be partially due to the typical disease course of POI as 5–10% of women with POI will be able to conceive and deliver a child after they have received the diagnosis [3]. With an open label prospective study design and no control group, researcher could not eliminate these confounding factors. Furthermore, signs and symptoms of POI were subjectively reported by patients and documented by researchers rather than objectively measured with standard questionnaires and statistically analyzed. These may cause bias in the data management and documentation. Although EA at ST25, CV4, and ST29 using stimulation of 20 Hz and 1 to 4 mA based on patients' tolerance were applied in the present study, the optimal EA treatment regime with appropriate stimulations remains to be established. To test the

therapeutic effectiveness of acupuncture, further randomized control trials are needed.

5. Conclusion

The present study demonstrated that EA could decrease serum FSH and LH levels, increase serum E2 level, and help regain menstruation in women with POI with little or no side effects; however, further randomized control trials are needed.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Acupuncture for Spinal Cord Injury and Its Complications: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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To evaluate the evidence supporting the effectiveness of acupuncture treatment for SCI and its complications, we conducted search across 19 electronic databases to find all of the randomized controlled trials (RCTs) that used acupuncture as a treatment for SCI and its complications. The methodological quality of each RCT was assessed using the Cochrane risk of bias tool and the PEDro scale. Sixteen RCTs, including 2 high-quality RCTs, met our inclusion criteria (8 for functional recovery from SCI, 6 for bladder dysfunction, and 2 for pain control). The meta-analysis showed positive results for the use of acupuncture combined with conventional treatments for the functional recovery in terms of motor ASIA scores and total FIM scores when compared to conventional treatments alone. Positive results were also obtained for the treatment of bladder dysfunction, in terms of the total efficacy rate, when comparing acupuncture to conventional treatments. However, 2 RCTs for pain control reported conflicting results. Our systematic review found encouraging albeit limited evidence for functional recovery, bladder dysfunction, and pain in SCI. However, to obtain stronger evidence without the drawbacks of trial design and the quality of studies, we recommend sham-controlled RCTs or comparative effectiveness research for each condition to test the effectiveness of acupuncture.

1. Introduction

Spinal cord injury (SCI) affects approximately 900 to 1,000 individuals per million in the general population, and it is estimated that there are 12,000 new cases of SCI every year in the United States [1]. Nearly 80% of the individuals who experience SCI are male, and, since 2000, the average age at injury has increased to 39.5 years, with 11.5% of those injured greater than 60 years of age [2]. These statistics indicate that SCI may be a significant social and economic burden for patients and their families.

SCI causes lesions, damaged neurological structures, and secondary pathophysiological changes in spared tissue [19]. Thus, complete or partial loss of sensory and motor function is the most significant result of injury, and most patients

experience secondary complications, such as bladder and bowel dysfunction, chronic pain, infertility, and autonomic dysfunction [19].

There are many treatments for functional recovery from injury and the related complications that result from SCI; these include surgery [20], prescription drugs [21], behavioral therapy [22], physical therapy [23], and supportive treatment [24]. There are also a variety of treatments used for the secondary complications of SCI, such as intermittent catheterization for bladder dysfunction, analgesics for pain, and others [25]. These treatments tend to be administered over long periods of time, and because of the potential complications of treatment, there has been an increased interest in alternative medical treatments, including acupuncture and other related therapies (moxibustion and acupressure) [26]. Acupuncture

based on Traditional Chinese Medicine has been commonly used for pain or neurological problems in Chinese cultures [27]. Additionally, many studies [28, 29] have analyzed the use of acupuncture for these types of problems; these studies report a variety of outcomes regarding level of function, pain, and quality of life.

Three recently published reviews have examined the results of studies that support the use of treatments such as acupuncture for individuals with SCI [26, 30, 31]. One review [30] that was limited to a Chinese literature suggested that acupuncture treatments have a positive effect, while a second [26] did not systematically evaluate the available evidence and even failed to include several important trials [3, 5–13, 15, 16, 18]. The third review was restricted to the pain related to SCI [31]. Therefore, the purpose of this systematic review was to provide a qualitative analysis of all of the randomized controlled trials (RCTs) to date that were designed to determine the effectiveness of acupuncture for patients with SCI and its complications along PRISMA guidelines [32].

2. Methods

2.1. Data Sources. This systematic review included studies published in electronic databases over the time period ranging from their inception to December 2011. Relevant publications were found in the following databases: the Cochrane Central Register of Controlled Trials (CENTRAL), the Cochrane Database of Systematic Review (CDSR), PubMed, MEDLINE, EMBASE, CINAHL, nine Korean databases (Korean Studies Information, DBPIA, Korea Institute of Science Technology Information, Korean National Assembly Library, RISS4U, KoreaMed, Korean medical database, Korean Traditional Knowledge Portal, and Oasis), Chinese database (China National Knowledge Infrastructure), and two Japanese databases (J-STAGE and JAMAS). In addition, we searched databases that contained registered trials, such as ClinicalTrials.gov (<http://www.clinicaltrials.gov>).

The search keywords used were (acupuncture OR acup* OR “electroacupuncture” OR “auricular acupuncture” OR “scalp acupuncture”) AND (“spinal cord injury” OR “spinal injury” OR “spinal cord trauma” OR “spinal cord lesion” OR “spinal cord damage” OR “spinal cord fracture” OR “spinal cord contusion”) in each base language. This search strategy was adjusted for each database. In addition, the bibliographies of relevant systematic reviews and clinical guidelines were manually searched. We also searched the gray literature that included dissertations, letters, government documents, research reports, conference proceedings, and abstracts when available. The reference section for each study was also searched.

2.2. Study Selection

2.2.1. Types of Studies. We evaluated RCTs that studied the clinical effects of acupuncture as a treatment for SCI or its direct complications. Dissertations and abstracts examining

these topics were also included. The review included prospective, parallel RCTs, or cross-over RCTs that assessed the efficacy of acupuncture regardless of blinding, language, or the type of report. The title and abstract of each identified article were read by a single primary researcher (IH) who completed the screening process. If articles were not written in English, they were translated into English prior to screening. The articles that would then be potentially included in our analysis were carefully checked by 2 independent reviewers (IH, YDK).

2.2.2. Types of Participants. The clinical trials included in our review examined patients with SCI or complications secondary to SCI. There were no restrictions related to the amount of time after injury, the type of injury, the site of injury, or participant’s age.

2.2.3. Types of Interventions. Our study considered the effects of needle acupuncture. We included needle acupuncture with or without electrical stimulation or heating by moxa, auricular acupuncture, and scalp acupuncture. We excluded injection acupuncture, nonneedling acupuncture (e.g., laser acupuncture), acupuncture-like intervention (e.g., acupressure, moxibustion), and mixed treatments. Control conditions in the reviewed studies included sham acupuncture, no treatment, and conventional treatment for SCI (e.g., medication, rehabilitation). Studies using cointerventions were included only if cointerventions were given to both treatment groups.

2.2.4. Outcome Measures. The outcome measures we considered were neurologic status or score (i.e., the American Spinal Injury Association neurologic and functional score; the ASIA score), functional ability outcomes (i.e., the Fugl-Meyer score), activities of daily living (i.e., the Functional Independence Measure; FIM score), scores related to the efficacy rate (i.e., the rate of participants who demonstrated efficacy), and outcome measures related to the complications of SCI (i.e., the bladder function scale for bladder dysfunction, pain scores for the level of pain and range of motion; ROM). We also considered measures of general safety reported for acupuncture as a treatment.

2.3. Data Extraction and Quality Assessment. Two reviewers (IH, YDK) reviewed each article independently and were blinded to the findings of the other reviewer. The reviewers collected data relating to the methodology, outcome measures, results, and final conclusions of each study. The reviewers also quantitatively evaluated each study’s methodological quality through the use of the Cochrane risk of bias [33] and the Physiotherapy Evidence Database (PEDro) scale [34], which was developed to assess the methodological quality of RCTs specifically pertaining to physical therapy interventions [35]. The PEDro scale allows researchers to assess the quality of studies based on a cutoff score: studies that score fewer than six points are considered to be “low quality,” while studies with a score equal to or greater than six points are considered to be “high quality” [36]. Any discrepancies

between reviewers were resolved through discussion until a consensus was reached.

2.4. Data Synthesis. To summarize the effects of acupuncture on each outcome measure, we used Cochrane Collaboration software (Review Manager (RevMan) Version 5.1 for Windows. Copenhagen: The Nordic Cochrane Centre). We extrapolated the risk estimate (relative risk; RR) and the 95% confidence interval (CI) for dichotomous data. The standard mean difference (SMD) and the 95% CI were calculated for continuous data. The variance of the amount of change was imputed using a correlation factor of 0.4, which is the value suggested by the Cochrane Collaboration [33]. We pooled data across studies using random effect models if excessive statistical heterogeneity did not exist. The chi-squared test and the Higgins I^2 test were used to assess the heterogeneity of the data [33]. We planned to use a formal funnel plot to assess publication bias if more than 10 trials were included; however, we were unable to generate a funnel plot due to the small number of trials included in our meta-analysis.

3. Results

3.1. Study Description. We considered 960 potentially relevant articles. After screening the abstracts and titles, we excluded 557 studies (Figure 1). Forty-seven articles were fully evaluated. Thirty-one additional articles were subsequently excluded; 5 studies were not controlled and 13 studies were not randomized trials. Thirteen RCTs were also excluded: 3 of them compared 2 types of acupuncture [37–39], 2 analyzed injection acupuncture [40, 41], 3 included mixed treatments [42–44], 3 included patients other than those with SCI [45–47], 1 provided an insufficient report [48], and 1 used acupressure as opposed to acupuncture [49]. Consequently, 16 RCTs met our inclusion criteria [3–18]. Eight trials studied functional recovery in SCI (5 in traumatic SCI [4–6, 8, 10], 1 in nontraumatic SCI [7], and 2 were not reported [3, 9]), 6 trials studied bladder dysfunction secondary to SCI (1 in traumatic SCI [13], 1 in mixed SCI [15], and 4 were not reported [11, 12, 14, 16]), and 2 trials studied the use of acupuncture for pain control in SCI (2 trials did not report SCI type) [17, 18]. The main data are summarized in Table 1. There were 12 Chinese studies [3, 5–13, 15, 16], 2 Taiwanese studies [4, 14] and 2 American studies [17, 18]. Fifteen studies used 2-parallel-arm group designs [3–13, 15–18] and 1 used a 4-parallel-arm group design [14].

3.2. Study Quality. The mean PEDro score was 4.5; scores ranged from 4 to 8 points (Table 2). Only 2 of 16 RCTs met the PEDro criteria for high quality [9, 17]. The results of the Cochrane risk of bias analysis varied widely (Table 2). Two studies reported appropriate sequence generation [17, 18], in which the researchers used a coin toss method [18] and stratified block randomization [17]. Two of the trials reported inappropriate randomization procedures [8, 16], in which the researchers used hospital admission numbers for randomization. One trial blinded the participants and the assessors [17], and three blinded the assessors only [4, 9, 18]. Only

one trial mentioned that the outcomes were analyzed with an intention-to-treat analysis [17]. Incomplete outcome data were addressed adequately in three studies [14, 17, 18]. Only one trial implemented allocation concealment; however, a detailed description of the method was not reported [9].

3.3. Descriptions of Acupuncture Treatment. The majority of the included RCTs stated that the rationale for acupuncture point selection was drawn from Traditional Chinese Medicine theory (Table 3). Fourteen studies used electrical stimulation with acupuncture [3–16] and 2 studies used needling acupuncture alone [17, 18]. Ten trials used fixed acupuncture points [4, 5, 7, 9–14, 16], 5 trials used fixed plus individualized acupuncture points [6, 8, 15, 17, 18], and 1 trial chose individualized by symptoms acupuncture points [3]. A total of 48 acupuncture points were included in functional recovery (meridian points: 36, extra point: 1, ear acupuncture points: 8, scalp acupuncture points: 2, Ashi point: 1), and 62.5% were located in the upper and lower extremities, 37.5% in head and back. A total of 18 acupuncture points were included in bladder dysfunction (meridian points: 17, extra point: 1) and 77.8% were located in the lower back. A total of 30 acupuncture points were included in pain control (meridian points: 29, Ashi point: 1) and 93.3% were located in shoulder area for the shoulder pain control of SCI.

3.4. Outcomes

3.4.1. Functional Recovery

Acupuncture versus Conventional Treatment (1 RCT). One RCT evaluated the effect of electrical acupuncture compared to rehabilitation [3]. A significant difference was found between the two groups regarding the total efficacy rate ($P = 0.05$, Table 1).

Acupuncture Plus Conventional Treatment versus Conventional Treatment (7 RCTs). Five RCTs evaluated the effects of electrical acupuncture plus rehabilitation and compared the results to those of rehabilitation alone [4–7, 9]. Three RCTs described improved outcomes with electrical acupuncture plus rehabilitation over rehabilitation alone [4, 7, 9]. However, 2 RCTs did not show any significant differences between treatment groups (Table 1) [5, 6].

One RCT compared the effects of electrical acupuncture, rehabilitation, and oral neurotropic drugs to those of rehabilitation and oral neurotropic drugs [8]. This study showed a positive effect in terms of rehabilitation effectiveness only ($P = 0.05$, Table 1).

One RCT evaluated the effects of electrical acupuncture plus intravenous drugs compared to intravenous drugs alone [10]. This RCT showed that treatment consisting of electrical acupuncture plus intravenous drugs was more effective than intravenous drugs alone ($P = 0.003$).

The meta-analysis of acupuncture plus conventional treatment versus conventional treatment alone showed that treatment including acupuncture led to significantly more improved motor ASIA scores (2 studies [4, 7]; $n = 156$, SMD = 0.78, 95% CI of 0.36 to 1.20, $P = 0.0002$, heterogeneity:

TABLE 1: A summary of the randomized controlled trials of acupuncture for spinal cord injury.

First author (ref) (year) Country	Study design	Patient population Type of SCI Target state Duration of SCI (mean (range))	Experimental treatment (regimen)	Control treatment (regimen)	Main outcomes	Intergroup differences Experimental versus control
Functional recovery						
Chen [3] (1995), China	Parallel 2 arms	67 n.r. Lower extremity spasticity (A) 11.3 ± 10.0 (1-53) mo (B) 15.5 ± 16.7 (1-81) mo	(A) EA, (n = 32)	(B) Rehabilitation, (n = 35)	Total efficacy rate (Ashworth scale)	RR; P = 0.05, 1.86 (1.00, 3.45)
Wong [4] (2003), Taiwan	Parallel 2 arms Assessor blind	100 Traumatic SCI Complete motor paralysis n.r.	(A) EA + AA, plus (B), (n = 50)	(B) Rehabilitation, (n = 50)	(1) ASIA score (1) Motor (2) Sensory (3) Pain (2) Total FIM score	(1) (1) MD, P = 0.003, 0.61 (0.21, 1.01) (2) MD, P = 0.005, 0.58 (0.18, 0.98) (3) MD, P = 0.009, 0.54 (0.14, 0.94) (2) MD, P = 0.02, 0.49 (0.09, 0.89)
Cui [5] (2004), China	Parallel 2 arms	72 Traumatic SCI n.r. (A) 12.9 ± 5.1d (B) 13.4 ± 6.2d	(A) EA, plus (B), (n = 37)	(B) Rehabilitation, (n = 35)	(1) FIM score (complete independent rate) (1) 3 sessions (2) 6 sessions	(1) (1) RR, P = 0.36, 2.84 (0.31, 26.01) (2) RR, P = 0.34, 1.89 (0.51, 6.99)
Xu [6] (2004), China	Parallel 2 arms	62 Traumatic SCI n.r. n.r.	(A) EA, plus (B), (n = 32)	(B) Rehabilitation, (n = 30)	Total FIM score	MD, P = 0.08, 0.44 (-0.06, 0.95)
Chen [7] (2005), China	Parallel 2 arms	56 Nontraumatic SCI Acute SCI n.r.	(A) EA + AA, plus (B), (n = 28)	(B) Rehabilitation, (n = 28)	(1) ASIA score (1) Motor (2) Sensory (3) Pain (2) FIM score (locomotion ability)	(1) (1) MD, P = 0.0003, 1.05 (0.48, 1.61) (2) MD, P < 0.00001, 1.91 (1.27, 2.55) (3) MD, P < 0.00001, 1.85 (1.22, 2.48) (2) MD, P < 0.00001, 2.13 (1.46, 2.79)*

TABLE 1: Continued.

First author (ref) (year) Country	Study design	Patient population Type of SCI Target state Duration of SCI (mean (range))	Experimental treatment (regimen)	Control treatment (regimen)	Main outcomes	Intergroup differences Experimental versus control
Gu [8] (2005) ^a , China	Parallel 2 arms	62 Traumatic SCI n.r. (A) 30.3 ± 17.6 d (B) 28.8 ± 11.7 d	(A) EA, plus (B), (n = 32)	(B) Rehabilitation + neurotropic oral drugs, (n = 30)	(1) Total FIM score (2) Rehabilitation effectiveness (= (FIM discharge – FIM admission)/hospitalization day) (1) Fugl-Meyer's score (2) Lindmark's score	(1) MD, <i>P</i> = 0.08, 0.44 (–0.06, 0.95) (2) MD, <i>P</i> = 0.05, 0.51 (0.01, 1.02)
Ma [9] (2005), China	Parallel 2 arms Assessor blind	30 n.r. SCI (walking function) n.r.	(A) EA + AT, plus (B), (n = 15)	(B) Rehabilitation, (n = 15)	(1) Total efficacy rate	(1) MD, <i>P</i> = 0.008, 1.04 (0.27, 1.81) (2) MD, <i>P</i> < 0.00001, 8.55 (6.12, 10.98)
Sheng [10] (2009), China	Parallel 2 arms	48 Traumatic SCI n.r. n.r.	(A) EA, plus (B), (n = 24)	(B) IV (BPH 120 mg + 0.9% NaCl 250 mL, daily for 3 months), (n = 24)	(1) Total efficacy rate	(1) RR, <i>P</i> = 0.003, 2.10 (1.28, 3.45)
Bladder dysfunction						
Huang [11] (2002), China	Parallel 2 arms	64 n.r. Urinary retention (A) 11.0 (5–20) d (B) 10.5 (5–20) d	(A) EA, (n = 32)	(B) IM (Neostigmine methylsulfate, 1 mg/2 mL), 2 hours after catheter removal) + IC + BT (n = 32)	Total efficacy rate	RR, <i>P</i> = 0.02, 1.50 (1.07, 2.11)
Zhang [12] (2008), China	Parallel 2 arms	89 n.r. Neurogenic bladder (A) 2–3 m (B) n.r.	(A) EA, (n = 45)	(B) IM (Neostigmine 0.5–1 mg, once a day) + IC + BT, (n = 44)	Total efficacy rate	RR, <i>P</i> = 0.11, 1.12 (0.95, 1.32)
Zhou [13] (2007), China	Parallel 2 arms	111 Traumatic SCI Neurogenic bladder (A) 45.62 ± 6.23 d (B) 43.76 ± 8.23 d	(A) EA (n = 56)	(B) IC + BT, (n = 55)	(1) Total efficacy rate (2) Residual urine (mL)	(1) RR, <i>P</i> = 0.006, 1.47 (1.12, 1.94) (2) MD, <i>P</i> < 0.00001, –1.16 (–1.56, –0.76)

TABLE 1: Continued.

First author (ref) (year) Country	Study design	Patient population Type of SCI Target state Duration of SCI (mean (range))	Experimental treatment (regimen)	Control treatment (regimen)	Main outcomes	Intergroup differences Experimental versus control
Cheng [14] (1998), Taiwan	Parallel 4 arms	80 [†] n.r. Neurogenic bladder (A) 23.7 ± 12.8 d (B) 26.1 ± 12.1 d	(A) EA, plus (B) (1) above T11 (n = 18) (2) below T11 (n = 14)	(B) IC + BT, (1) above T11 (n = 16) (2) below T11 (n = 12)	Total days needed to reach bladder balance (1) Above T11 (2) Below T11	(1) MD, <i>P</i> = 0.003, 1.10 (0.37, 1.83) (2) MD, <i>P</i> = 0.009, 1.12 (0.28, 1.96)
Gu [15] (2005) ^b , China	Parallel 2 arms	64 mixed Bladder dysfunction n.r.	(A) EA, plus (B), (n = 32)	(B) IC, (n = 32)	Total efficacy rate	RR, <i>P</i> = 0.007, 1.53 (1.12, 2.08)
Liu [16] (2009), China	Parallel 2 arms	40 n.r. Bladder dysfunction 14 days~90 d	(A) EA, plus (B), (n = 20)	(B) IC + BT, (n = 20)	Bladder voiding function parameters (1) Frequency of urination (times) (2) Maximum voided volume (mL) (3) Bladder capacity (mL) (4) Residual urine (mL) (5) Quality of life score	(1) MD, <i>P</i> = 0.012, -0.49 (-1.12, 0.14) (2) MD, <i>P</i> = 0.024, 0.37 (-0.25, 1.00) (3) MD, <i>P</i> = 0.06, 0.61 (-0.02, 1.25) (4) MD, <i>P</i> = 0.32, -0.32 (-0.94, 0.31) (5) MD, <i>P</i> = 0.30, -0.33 (-0.96, 0.29)
Pain condition						
Dyson-Hudson [17] (2007), USA	Parallel 2 arms Patient blind Assessor blind	17 n.r. Chronic shoulder pain (A) 9.3 ± 10.5 y (B) 13.1 ± 7.7 y	(A) AT, (n = 8)	(B) Sham AT, (n = 9)	(1) PC-WUSPI (2) NRS (11 points scale, shoulder pain)	(1) MD, <i>P</i> = 0.19, -0.65 (-1.64, 0.33) (2) MD, <i>P</i> = 0.19, -0.67 (-1.69, 0.34)

TABLE 1: Continued.

First author (ref) (year) Country	Study design	Patient population Type of SCI Target state Duration of SCI (mean (range))	Experimental treatment (regimen)	Control treatment (regimen)	Main outcomes	Intergroup differences Experimental versus control
Dyson-Hudson [18] (2001), USA	Parallel 2 arms	18 n.r. Chronic SCI and shoulder pain (A) 16.2 ± 9.7 y (B) 13.4 ± 6.2 y	(A) AT, (n = 9)	(B) Trager Approach, (n = 9)	(1) PC-WUSPI (2) NRS (10 points VAS, shoulder pain) (3) VRS (shoulder pain)	(1) MD, $P = 0.91$, -0.05 (-0.98 , 0.87) (2) No significant difference ($P > 0.05$) [‡] (3) RR, $P = 0.46$, 0.89 (0.67 , 1.20)

AA: auricular acupuncture; ASIA score: American spinal injury association neurologic and functional score; AT: acupuncture; BPH: brain protein hydrolysate; BT: bladder training; EA: electrical AT; FIM score: functional independence measure score; IC: intermittent catheterization; IM: intramuscular; IV: intravenous; n.r.: not reported; NRS: numeric rating scale; PC-WUSPI score: performance-corrected wheelchair user's shoulder pain index score; SCI: spinal cord injury; VAS: visual analog scale; VRS: verbal response score.

*The author did not report total FIM score but did each of 6 domains.

[†] 80 patients were randomized, but 60 were analyzed.

[‡] Each group had a significant effect after therapy ($P < 0.01$).

TABLE 2: Quality assessment of included randomized clinical trials.

First author (year)	PEDro scale item															Cochrane risk of bias				
	A	B	C	D	E	F	G	H	I	J	K	Total	L	M	Patient	Therapists	Assessors	O	P	Q
Chen [3] (1995)	1	1	0	0	0	0	0	1	0	1	1	4	U	U	N	N	U	U	U	N
Wong [4] (2003)	1	1	0	0	0	0	1	1	0	1	1	5	U	U	N	N	Y	U	U	N
Cui [5] (2004)	1	1	0	1	0	0	0	1	0	1	1	5	U	U	N	N	U	U	U	N
Xu [6] (2004)	1	1	0	0	0	0	0	1	0	1	1	4	U	U	N	N	U	U	U	N
Chen [7] (2005)	1	1	0	0	0	0	0	1	0	1	1	4	U	U	N	N	U	U	U	N
Gu [8] (2005)a	1	1	0	1	0	0	0	1	0	1	1	5	N	U	N	N	U	U	U	N
Ma [9] (2005)	1	1	1	1	0	0	1	1	0	1	1	7	U	Y	N	N	Y	U	U	N
Sheng [10] (2009)	1	1	0	0	0	0	0	1	0	1	1	4	U	U	N	N	U	U	U	N
Huang [11] (2002)	1	1	0	1	0	0	0	1	0	1	1	5	U	U	N	N	U	U	U	N
Zhang [12] (2008)	1	1	0	1	0	0	0	0	0	1	1	4	U	U	N	N	U	U	U	N
Zhou [13] (2007)	1	1	0	1	0	0	0	0	0	1	1	4	U	U	N	N	U	U	U	N
Cheng [14] (1998)	1	1	0	1	0	0	0	0	0	1	1	4	U	U	N	N	U	Y	U	U
Gu [15] (2005)b	1	1	0	0	0	0	0	1	0	1	1	4	U	U	N	N	U	U	U	N
Liu [16] (2009)	1	1	0	0	0	0	0	1	0	1	1	4	N	U	N	N	U	U	U	N
Dyson-Hudson [17] (2007)	1	1	0	1	1	0	1	1	1	1	1	8	Y	U	Y	N	Y	Y	Y	N
Dyson-Hudson [18] (2001)	1	1	0	0	0	0	1	1	0	1	1	5	Y	U	N	N	Y	Y	U	N

PEDro scale items (each satisfied item except the first item contributes 1 point to the total PEDro score): A: eligibility criteria specified, B: randomization, C: allocation concealment, D: groups similar at baseline, E: blinded subjects, F: blinded therapist, G: blinded assessors, H: adequacy of followup, I: ITT analysis, J: between-group comparison, K: point and variability measures; L: item positive, 0: item negative or unknown. Cochrane risk of bias: L: was the allocation sequence adequately generated? M: was allocation adequately concealed? N: was knowledge of the allocated intervention adequately prevented during the study? O: were incomplete outcome data adequately addressed? P: are reports of the study free of suggestion of selective outcome reporting? Q: was the study apparently free of other problems that could put it at a high risk of bias? Yes (Y); low risk of bias, no (N); high risk of bias, and unclear (U); uncertain risk of bias.

TABLE 3: Summary of treatment acupuncture points and other information related to acupuncture.

First author (ref) (year)	Acupuncture method	Regime	Acupuncture points	Deqi	Rationales for acupuncture points	Number of CMSP	Adverse events
Functional recovery							
Chen [3] (1995)	Individualized by injured spinal level	EA (1-2 Hz, 1 session = once a day, 30 min, 6 times a week, total 48 treatments)	Injured spinal level, upper 1 point and lower 1 point of Governor vessel of injured level (inter spinous process)	n.r.	TCM theory	1	n.r.
Wong [4] (2003)	Fixed	EA + AA (75 Hz, 10 mV, 1 session = 30 min, 5 times a week, till discharge)	EA: bilateral SI3, BL62 AA: 4 acupoints related to the spinal cord at the antihelix, helix, and lower portion of the ear-back areas	Considered	n.r.	1	n.r.
Cui [5] (2004)	Fixed	EA (1 session = once a day, 30 min for 1 month, total 90 or 180 treatments)	Arm: HT1, LU5, PC3, HT3 Leg: SP12, BL37 or GB30, BL40 Other peroneal nerve stimulation point (no reports about where EA was applied)	Considered	TCM theory	1	n.r.
Xu [6] (2004)	Fixed + individualized by symptoms	EA (1 Hz, 1 session = once a day, 30 min for 1 month, 7 days' rest, total 150 treatments)	EX-B2 + LI4, LI11, LI15, TE5, GB30, GB31, GB34, GB39, ST36, ST41, BL60, LR3 (limb dyskinesia) or SP6, SP9, BL25, eight- <i>liao</i> (evacuation disorder)	n.r.	TCM theory	2	n.r.
Chen [7] (2005)	Fixed	EA + AA (EA; 1-5 Hz, 30 min, 6 days a week for 3 months, session interval 1-2 weeks, till discharge, AA; 1 session = once a day, 10 times, total 2-3 session, each ear alternately)	EA: bilateral SI3, BL62 AA: brain, subcortex, sympathetic, Shenmen	Impossible in SCI patients because of sensory impairment (+)	TCM theory	1	No adverse events (+)

TABLE 3: Continued.

First author (ref) (year)	Acupuncture method	Regime	Acupuncture points	Deqi	Rationales for acupuncture points	Number of CMSP	Adverse events
Gu [8] (2005)a	Fixed + individualized by symptoms	EA (1 Hz, 3–5 V, 1 session = once a day, 30 min for 1 month, 1 week rest, total 150 treatments)	EX-B2, LI15, LI11, TE5, LI4, GB30, GB31, GB34, ST36, GB39, BL60, ST41, LR3 (EA applied to major extremity points) + eight- <i>liao</i> , BL25, SP6, GB34 (evacuation disorders)	n.r.	TCM theory	2	n.r.
Ma [9] (2005)	Fixed	EA + AT (scalp EA; 5 min, body AT; 25 min, once a day, total 168 sessions)	Scalp EA: MS6 (motor area), MS14 (equilibrium area) Body AT: LI10, LI11, TE8, SI5 (arm), LR12, GB34, GB39, BL54, BL60, LR3 (leg)	Considered (authors did not describe, but might be considered)	TCM theory	1	n.r.
Sheng [10] (2009)	Fixed	EA (1 session = once a day, 30 min for 10 days, 2 days' rest, total 70 treatments)	EX-B2, LI4, LI11, LI15, TE5, ST31, ST32, ST36, GB34	n.r.	TCM theory	1	n.r.
Bladder dysfunction							
Huang [11] (2002)	Fixed	EA (continuous wave, 1 session = 30 min, for 5 days, 3 days' rest, total 5–20 treatments)	Bilateral BL54, ST28, BL32, BL34, T12-L2 Huatuojiayi (EX-B2), SP9, SP6	Considered (authors did not describe, but might be considered)	TCM theory	1	n.r.
Zhang [12] (2008)	Fixed	EA (2 Hz, 6 V, 1 session = once a day, 30 min for 2 weeks, total 12 treatments)	Bilateral BL23, BL35	n.r.	TCM theory	1	n.r.
Zhou [13] (2007)	Fixed	EA (continuous wave, 80 Hz, 20 mA, 1 session = once a day for 15 days, 5 days' rest, total 30 treatments)	Bilateral BL31, BL32, BL33, BL34, BL35	Considered	TCM theory	1	n.r.

TABLE 3: Continued.

First author (ref) (year)	Acupuncture method	Regime	Acupuncture points	Deqi	Rationales for acupuncture points	Number of CMSP	Adverse events
Cheng [14] (1998)	Fixed	EA (20–30 Hz, 30–50 mA, 1 session = 15 min, 4~5 sessions each week, till their bladders were balanced)	CV3, CV4, BL32 (bilateral)	Considered	TCM theory	2	n.r.
Gu [15] (2005)b	Fixed + individualized by symptoms	EA (continuous wave, 1 session = once a day, 30 min for 2 weeks, total 14–56 treatments)	eight- <i>liao</i> (EA) + BL23, CV6, BL20, BL22 (deficiency syndrome) or SP6, BL28, SP9, CV3 (excess syndrome)	Considered	TCM theory	1	n.r.
Liu [16] (2009)	Fixed	EA (1 session = once a day, 30 min for 15 days, total 60 treatments)	EX-B2	n.r.	TCM theory	1	n.r.
Pain condition							
Dyson-Hudson [17] (2007)	Fixed + individualized by symptoms	AT (1 session = 20 min, twice a week, total 10 treatments)	Local points (chosen 6 points according to shoulder pain symptoms): LI14, LI15, LI16, TE13, TE14, TE15, GB21, SI9, SI10, SI11, SI12, SI13, SI14, SI15, LU1, LU2, PC2 Distal points (chosen 2 points according to local points used): LI2, LI4, LI10, LI11, LI18, SJ3, SJ6, LU3, GV14, GB20, BL10, BL11 Ashi points (1~4 points per treatment)	Considered	TCM theory	1	Minimal adverse effect

TABLE 3: Continued.

First author (ref) (year)	Acupuncture method	Regime	Acupuncture points	Deqi	Rationales for acupuncture points	Number of CMSP	Adverse events
Dyson-Hudson [18] (2001)	Fixed + individualized by symptoms	AT (1 session = 20~30 min, twice a week, total 10 treatments)	Local points (chosen 6 points according to shoulder pain symptoms): LI4, LI5, LI6, TE13, TE14, TE15, GB21, SI9, SI10, SI11, SI12, SI13, SI14, SI15, LU1, LU2, PC2 Distal points (chosen 2 points according to local points used): LI2, LI4, LI10, LI11, LI18, SJ3, SI6, LU3, GV14, GB20, BL 10, BL11 Ashi points (1~4 points per treatment)	Considered	TCM theory	1	No adverse events (+)

AA: auricular acupuncture; AT: acupuncture; CMSP: Chinese medicine syndrome pattern; EA: electrical AT; n.r.: not reported; TCM: traditional Chinese medicine; (+): mentioned in text.

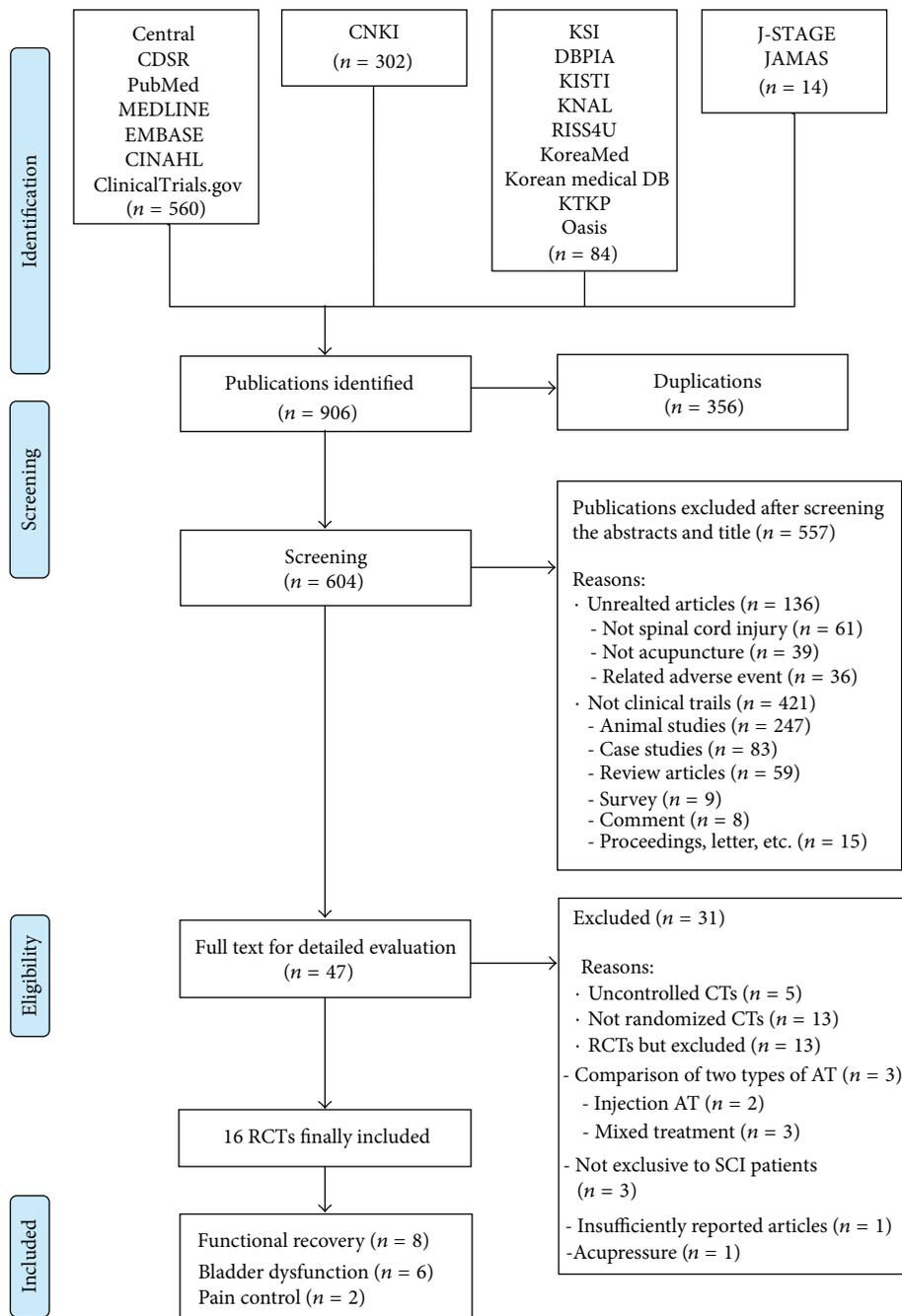
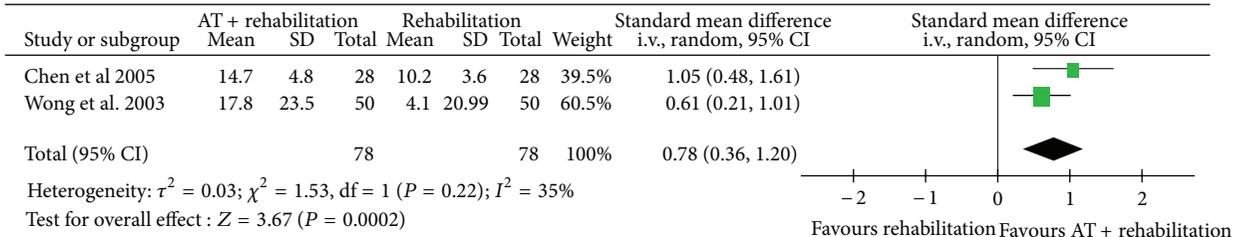


FIGURE 1: A flow chart describing the trial selection process. AT: acupuncture; CDSR: The Cochrane Database of Systematic Review; CENTRAL: The Cochrane Central Register of Controlled Trials; CNKI: China National Knowledge Infrastructure; CT: clinical trial; DB: database; KSI: Korean Studies Information; KISTI: Korea Institute of Science Technology Information; KNAL: Korean National Assembly Library; KTKP: Korean Traditional Knowledge Portal; RCT: randomized clinical trial; SCI: spinal cord injury.

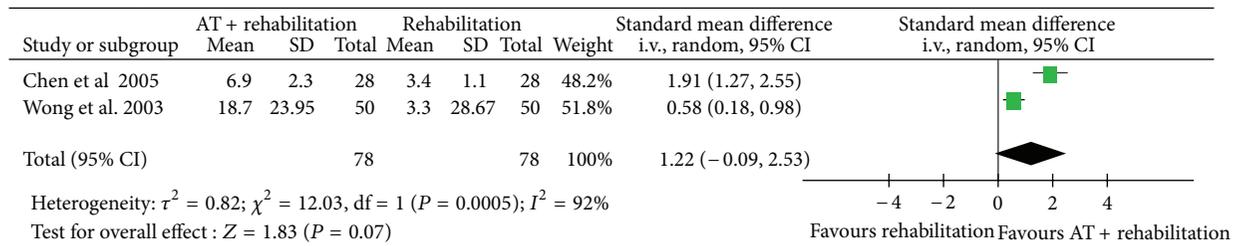
1. Functional recovery

1.1. ASIA score

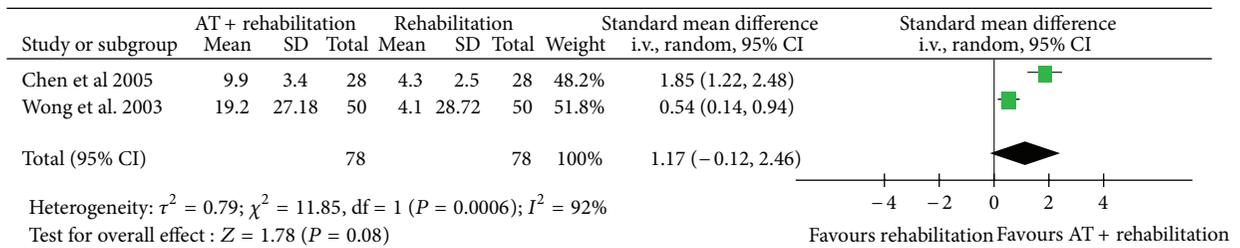
1.1.1. Motor



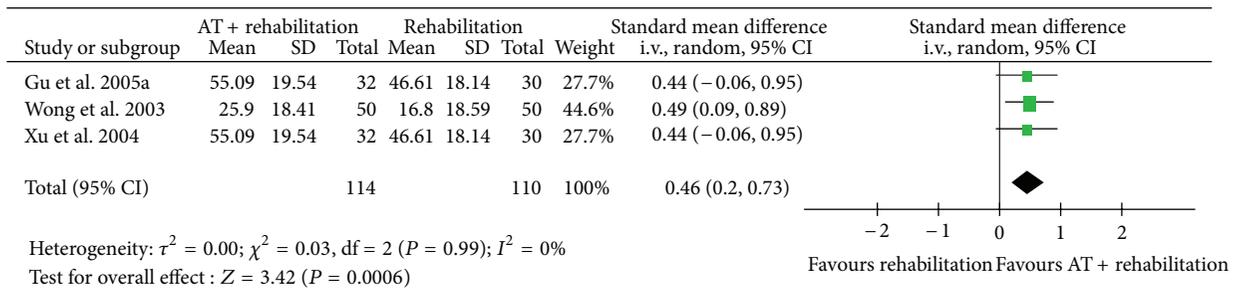
1.1.2. Sensory



1.1.3. Pain



1.2. Total FIM score



2. Bladder dysfunction

2.1. Total efficacy rate

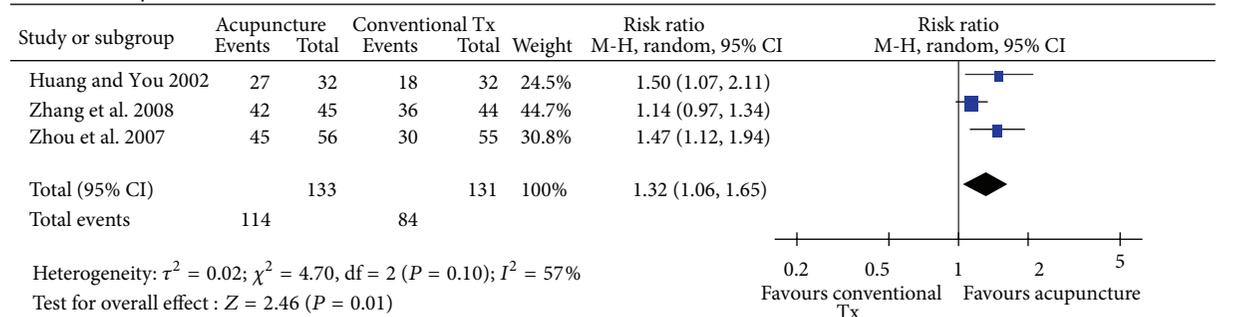


FIGURE 2: The meta-analysis of acupuncture for spinal cord injury and its complications. ASIA: American Spinal Injury Association; AT: acupuncture; FIM: functional independence measure; Tx: treatment.

$\chi^2 = 1.53$, $P = 0.22$, and $I^2 = 35\%$; Figure 2) and total FIM scores (3 studies [4, 6, 8]: $n = 224$, SMD = 0.46, 95% CI of 0.20 to 0.73, $P = 0.0006$, heterogeneity: $\chi^2 = 0.03$, $P = 0.99$, and $I^2 = 0\%$; Figure 2).

3.4.2. Bladder Dysfunction

Acupuncture versus Conventional Treatment (3 RCTs). Three RCTs evaluated the effects of electrical acupuncture compared to conventional treatment (intramuscular neostigmine methylsulfate, intermittent catheterization, bladder training, etc.) [11–13].

Two of the 3 RCTs used intramuscular neostigmine methylsulfate plus intermittent catheterization and bladder training [11, 12]. One study showed that electrical acupuncture induced significantly greater improvement when compared to conventional treatment alone in terms of the total efficacy rate ($P = 0.02$, Table 1) [11]. However, the other study did not show a significant difference between treatment conditions ($P = 0.11$, Table 1) [12].

One of the 3 RCTs compared the effects of electrical acupuncture with conventional treatment (intermittent catheterization and bladder training) [13]. This study showed a significant positive effect for electrical acupuncture in terms of the total efficacy rate ($P = 0.006$, Table 1) and the levels of residual urine ($P < 0.00001$, Table 1) [13].

The meta-analysis of acupuncture versus conventional treatment showed a significant positive effect of acupuncture in terms of the total efficacy rate (3 studies [11–13]: $n = 264$, RR = 1.32, 95% CI of 1.06 to 1.65, $P = 0.01$, heterogeneity: $\chi^2 = 4.70$, $P = 0.10$, and $I^2 = 57\%$; Figure 2).

Acupuncture plus Conventional Treatment versus Conventional Treatment Alone (3 RCTs). Two RCTs compared the effects of electrical acupuncture plus intermittent catheterization and bladder training to intermittent catheterization and bladder training alone [14, 16]. A recalculation of the mean difference (MD) revealed that there was a significant positive effect for electrical acupuncture in terms of the total days needed to reach bladder balance ($P < 0.05$, Table 1) [14]; however, there was not a positive effect in bladder voiding function parameters (the frequency of urination, the maximum voided volume, bladder capacity, residual urine, and quality of life scores, Table 1) [16].

One RCT compared the effects of electrical acupuncture plus intermittent catheterization versus intermittent catheterization alone [15]. This study showed a positive effect in terms of the total efficacy rate ($P = 0.007$, Table 1) [15].

3.4.3. Pain Control

Acupuncture versus Sham Acupuncture (1 RCT). One RCT evaluated the effects of manual acupuncture with no manipulation at sites located at least 1 cun (1 Chinese anatomic inch, approximately 2.5 cm) away from the established meridian and extra points [17]. The results showed that acupuncture did not significantly affect the PU-WUSPI and the NRS (Table 1) [17].

Acupuncture versus Trager Approach (1 RCT). One RCT assessed the effects of manual acupuncture compared to the Trager Approach (a form of bodywork and movement reeducation developed by Milton Trager) [18]. Our recalculation of the MD showed that there was no significant difference between the 2 treatments ($P > 0.05$, Table 1) [18].

3.4.4. The Safety Reporting of Acupuncture. Only three RCTs reported adverse events associated with acupuncture [7, 17, 18]. Two RCTs reported no adverse events [7, 18], and 1 trial reported minimal adverse effects without a detailed explanation [17].

4. Discussion

This is the first systematic review and meta-analysis of RCTs that fully evaluates the effectiveness of needle acupuncture for SCI and its complications. Of the 16 trials included in this paper, 8 trials studied the effects of acupuncture on functional recovery in SCI [3–10] and 8 trials that studied its effect on the secondary complications that follow SCI (6 trials for bladder dysfunction [11–16], and 2 trials for pain levels [17, 18]).

Based on the Cochrane risk of bias [33] and the PEDro scale [34], the methodological quality and design of the primary studies was mostly poor (only 2 of 16 were considered high quality, 12.5%). Of the 16 studies included in our review, 2 trials used appropriate sequence generation methods [17, 18]. Only one of the studies reported allocation concealment [9]. One RCT reported details of assessor- and patient-blinding procedures [17]. Trials with inappropriate randomization were threatened by selection bias [50] and inadequate blinding tended to exaggerate the effects of treatment [33]. Researchers should conduct their trials according to the CONSORT statement [51]. In addition, 14 of the 16 RCTs originated from Chinese sources [3–16]. Several groups have shown that the majority of Chinese acupuncture studies report positive results [52, 53]. Therefore, it is possible that a publication bias exists; although we searched extensively for all the studies that are relevant to this paper, we may have failed to conduct an analysis of publication bias [54]. This phenomenon casts a considerable doubt on the reliability of these data.

Ten trials compared acupuncture plus various conventional therapies to conventional therapies alone [4–10, 14–16], and 5 trials compared acupuncture to conventional therapies [3, 11–13, 18]. Such trial designs are open to bias because participants were not blinded. Sham-controlled trials could control nonspecific effects of acupuncture [55]. Only one trial adopted a penetrating sham control [17]. This sham-controlled trial had a small sample size, and our recalculated power for this study was 0.326. This finding indicates that the study lacked sufficient statistical power. Thus, these results limited our ability to evaluate the effectiveness of this treatment for various conditions in SCI. Additionally, nonpenetrating sham acupuncture may be more acceptable than penetrating sham [56, 57].

Although the effectiveness of acupuncture is unclear in SCI or its complications, acupuncture therapy is a relatively

convenient and safe treatment for some conditions [58]. SCI patients who receive long-term rehabilitation or medication treatments may occasionally need a safe nonpharmacological treatment [59]. In this regard, acupuncture can be useful to treat SCI and its complications if patients experience side effects or have no (or a weak) response to a conventional treatment. Thus, we carefully present several points that must be discussed in future research.

First, although several animal studies of SCI have reported that acupuncture induces neuronal function recovery [60], an analgesic effect [61], and anti-inflammatory responses [62], the RCTs included in this paper might have used specific treatment conditions that may not have fully drawn the maximal benefit from acupuncture. To evaluate the effects of acupuncture on functional recovery in SCI, an appropriate set of treatment conditions which can maximize the therapeutic effect of acupuncture is highly recommended. Also, the conditions have to include the appropriate duration of treatment resulted from comparison of duration of the course of acupuncture treatment. Acupuncture as a (sole or adjunct) treatment for bladder dysfunction demonstrated a positive effect over all conventional treatments except one; while this is encouraging, this result is inconclusive due to the small sample size and low methodological quality. The sham trial for pain control that was reviewed failed to show any specific positive analgesic effect of acupuncture; however, this trial lacked statistical power. It is possible that acupuncture is an effective treatment for pain in SCI, as indicated by a previous rigorous review [31]. Thus, future high-quality trials on secondary conditions of SCI should be conducted to evaluate the potential effects of acupuncture [63].

Second, the prescription of acupuncture points was not consistent across studies (Table 3). Also, the degree of stimulation and the duration and frequency of acupuncture treatment were very various (Table 3). And we could not find the consistency of the Chinese medicine pattern in included trials. So it was difficult to estimate the correlation between the difference of acupuncture treatment and its therapeutic effectiveness. They might not have been optimal or might even be considered an underdosage of treatment for SCI [64]. As a matter of fact, the optimal prescription of acupuncture treatment (acupuncture point, degree of stimulation, frequency of treatment, and a number of treatment sessions) for SCI is a controversial issue amongst acupuncture experts. A standardized prescription of acupuncture for SCI or its complications is necessary.

Third, sham controls in acupuncture research for SCI may represent an ethical issue [65]. Therefore, an adjunctive treatment of acupuncture to conventional treatments for SCI can be evaluated by RCTs comparing acupuncture plus conventional treatment to sham acupuncture plus conventional treatment or through comparative effectiveness research (CER) [66].

5. Conclusion

The results of our systematic review and meta-analysis suggest that the evidence for the effectiveness of acupuncture

as a symptomatic treatment for SCI and its complications is encouraging but limited. There is a great need to test the clinical implications of acupuncture for a number of SCI-related conditions. The efficacy of acupuncture for SCI and its complications must be studied using sham-controlled RCTs or CER with a standardized acupuncture procedure; such RCTs would conform to the recommendations of the CONSORT and STRICTA guidelines [51, 67].

Conflict of Interests

The authors report no financial or other relationships relevant to the subject of this paper.

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Research Article

The Influence of Different Acupuncture Manipulations on the Skin Temperature of an Acupoint

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This study was performed to observe the influence of sham and different verum acupuncture manipulations on skin temperature of the stimulated acupoint in healthy volunteers. Thirty-seven healthy volunteers with a mean age of 25.4 ± 2.2 years were enrolled in the study. All volunteers had experienced acupuncture before. They received sham acupuncture and two different kinds of verum acupuncture stimulation (lifting-thrusting and twisting-rotating) on Zusanli (ST36). The skin temperature of ST36 was measured before acupuncture, after needle insertion, after needle manipulation, immediately after removal of the needle, and as further control 5 minutes after removal of the needle using a FLIR i7 infrared thermal camera. During the measurement, the needling sensations of volunteers were enquired and recorded. During the sham acupuncture stimulation, the skin temperature of ST36 decreased in the first 5 minutes, when the point was exposed, and then increased gradually. During verum acupuncture stimulations, the skin temperature increased continually and then decreased in the last phase. The increase in temperature caused by lifting-thrusting stimulation was significantly higher than that of twisting-rotating manipulation, which may be related to the stimulation intensity.

1. Introduction

Already in ancient times, doctors realized that acupuncture can influence the skin temperature. This is already mentioned in the first Chinese medical book *Miraculous Pivot*, where it is stated that “the doctor could warm the body through stimulating foot Shaoyin and cool the body through stimulating foot Yangming channels.” So, even at that time it was well known that the skin temperature is an indicator of the reinforcing or reducing acupuncture manipulations. In *Plain Questions*, it is also stated that coldness and heating are part of the indexes of the acupuncture treatment principles.

Around the time of the Jin-Yuan dynasty, the compound manipulations like directional supplementation and draining method (迎随补泻, *yingsui buxie*), heat-producing needling (烧山火, *shao shan huo*), and cool-producing needling (透天凉, *tou tian liang*) were developed to treat diseases

aided by heat or coldness feeling after acupuncture manipulation stimulation. These complex manipulations, however, are rarely used in current acupuncture clinic routine; many researchers focus on simpler acupuncture needle manipulations in their experiments and achieve different results.

This experiment tries to observe the influence of Cheng's basic acupuncture manipulations—lifting-thrusting and twisting-rotating—on local acupoint skin temperature in a randomized study, which may lay a foundation for further research.

2. Materials and Methods

2.1. Selection of Study Participants. Through advertisements on the campuses, 37 healthy volunteers (30 female, 7 male) with a mean age of 25.4 ± 2.2 years (range: 20–35 years) were enrolled among students from the Beijing University of



FIGURE 1: The location of the Zusanli acupoint (ST36).

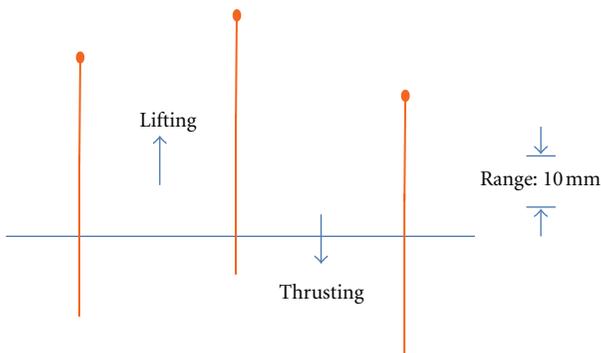


FIGURE 2: Lifting and thrusting acupuncture needle manipulation.

Traditional Chinese Medicine and Graduated School of the China Academy of Chinese Medical Sciences. All participants had received acupuncture before and gave informed consent. The experimental procedure was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion of China Academy of Chinese Medical Sciences.

2.2. Acupuncture. Each volunteer underwent three measurements (two different kinds of verum acupuncture, see below, and sham acupuncture, in randomized order) every other day. To avoid discrepancies in manipulation, all acupuncture operations were performed by the same medical practitioner. The volunteers lay down on the back and exposed the right lower leg, so the Zusanli (ST36) acupoint could be marked in accordance with a textbook on *Acupuncture and Moxibustion* [1] (see Figure 1). Verum acupuncture was performed according to Cheng [2].

Verum Acupuncture: Lifting and Thrusting Manipulation (提插, *ticha*). Acupuncture stimulation was done manually, using single-use acupuncture needles (0.30 × 40 mm, Zhongyan Taihe brand, Suzhou, China). The doctor inserted

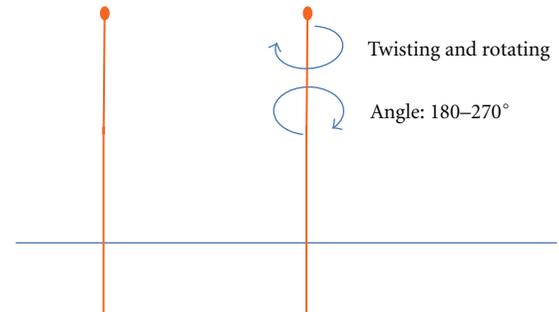


FIGURE 3: Twisting and rotating acupuncture needle manipulation.

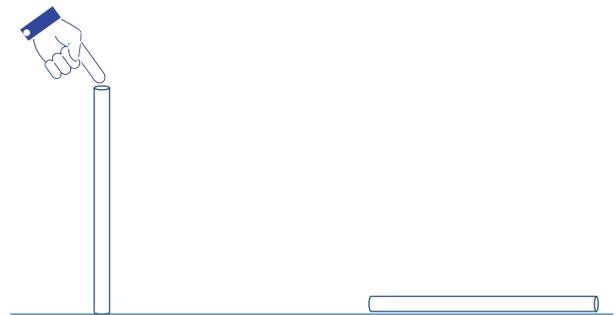


FIGURE 4: The operation of sham acupuncture.

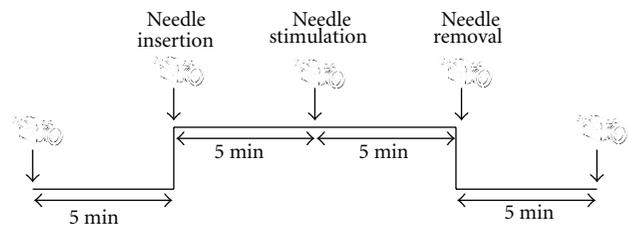


FIGURE 5: Measurement procedure.

the needle on ST36 through a tube to retain depth, until both the practitioner and the volunteer felt the qi arrival; then the insertion was stopped, and the needle remained in place. After 5 mins, the practitioner lifted and then thrust the needle evenly approximately 10 mm (see Figure 2), repeating this operation from 20 to 25 times in 20 seconds. The needle was left in place for 5 more mins and then removed.

Verum Acupuncture, Twisting and Rotating Manipulation (捻转, *nianzhuan*). Acupuncture stimulation was done manually, using the same single-use acupuncture needles as mentioned before. The doctor inserted the needle on ST36 through a tube to retain depth, until both the practitioner and the volunteer felt the qi arrival; then the insertion was stopped, and the needle remained in place (the same procedure as mentioned before). After 5 mins, the practitioner twisted the handle of the needle clockwise and counterclockwise evenly through 180° to 270° (see Figure 3), repeating this

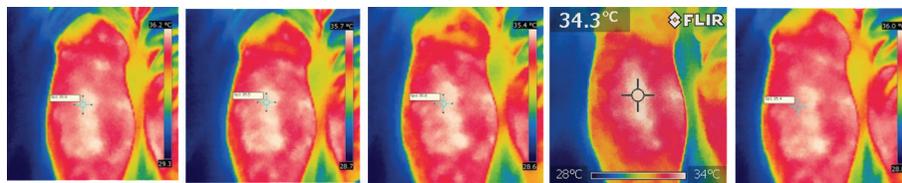


FIGURE 6: Temperature changes during the sham acupuncture procedure.

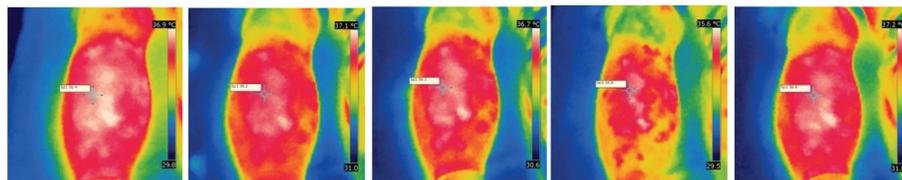


FIGURE 7: Temperature changes before, during, and after verum acupuncture—lifting-thrusting needle manipulation.

operation from 40 to 45 times in 20 seconds. The needle was left in place for 5 more mins and then removed.

Sham Acupuncture (Placebo). Sham acupuncture was performed using a single-use acupuncture needle tube (Zhongyan Taihe brand, Suzhou, China) which was tapped on ST36, but no needle was inserted and so the volunteers did not receive any stimulation (see Figure 4).

2.3. Measurement of Skin Temperature and Heart Rate. The temperature of the lab was kept at 26°C, and the volunteers were asked to come into the room 5–10 mins ahead of schedule to adapt to the temperature. The skin temperature at ST36 was measured using a FLIR i7 (Flir Systems, Portland, OR, USA) thermographic camera 5 mins before acupuncture, immediately after needle insertion, after manipulation, immediately after removing the needle, and 5 mins after the needle removal (see Figure 5). During the whole experiment, the volunteers' heart rates (HR) were measured with three electrodes on standard positions of the chest, using a Medilog AR12 system (Huntleigh Healthcare, Cardiff, UK).

2.4. Needling Sensation. The volunteers were asked about their needling sensation after needle insertion and manipulation, respectively, were assessed by visual analogue scale (VAS). 0 means “no sensation at all,” and 10 means “too much to bear.”

2.5. Statistical Analysis. Paired *t*-test was used to compare the temperature changes between the different manipulations, with $P < 0.05$ denoted as significant.

3. Results

3.1. Changes of HR. Sham as well as verum acupuncture caused changes in the volunteers' HR. After the 5 min phase of rest before acupuncture, HR decreased significantly. After verum acupuncture, needle manipulation, the lifting-thrusting as well as the twisting-rotating stimulation induced

a significant increase in HR (compared to the phase of needle insertion), whereas during the sham procedure HR continued to decrease (cf. Table 1).

3.2. Skin Temperature. After needle insertion, the skin temperature at ST36 decreased insignificantly following the sham procedure, but increased significantly in the two verum procedures. After manipulations, the temperature increase caused by lifting-thrusting stimulation was higher than that caused by sham acupuncture (see Figures 6, 7, and 8).

Table 2 shows the mean and standard deviation of the temperature values of all 37 volunteers.

3.3. Needling Sensations during Verum Acupuncture. All subjects could tell sham from verum acupuncture, but could not recognize the lifting-thrusting or twisting-rotating manipulations. After puncturing ST36, 71% of the subjects felt distension, 36% felt sourness, 16% felt pricking, and 10% felt numbness spreading. There was of course no statistical difference in the intensity of the needling sensation when the needle was inserted, but the intensity caused by lifting-thrusting stimulation was significantly higher than that of twisting-rotating manipulation (Table 3).

4. Discussion

Previous studies investigating acupuncture and skin temperature used compound reinforcing and reducing techniques to observe changes in skin temperature of the area along the meridian after puncturing the acupoint. A study by Li et al. showed that the skin temperature of acupoints changed according to the frequency of rotating acupuncture stimulation on ST36 [3]. A similar study from Tianjin in China showed that the skin temperature of the abdomen could be increased by reinforcing technique and decreased by reducing acupuncture stimulation technique on ST36 [4]. An experiment by Wang et al. in Shanghai showed that after twisting-rotating acupuncture stimulation with an angle $\leq 360^\circ$ on the acupoint SJ5 (Waiguan), the temperature of the ipsilateral acupoint PC1 (Zhongchong) increased [5]. A

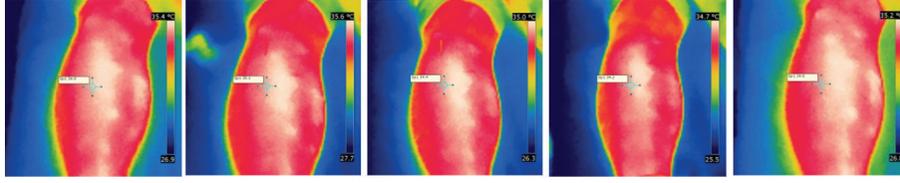


FIGURE 8: Temperature changes before, during, and after verum acupuncture—twisting-rotating needle manipulation.

TABLE 1: The changes of volunteers' HR (in [1/min]) during the experiment (* $P < 0.05$).

	Before acupuncture	Needle insertion	Needle manipulation	Needle removal	After acupuncture
Sham acupuncture	71.03 ± 9.04	68.50 ± 8.29*	68.15 ± 7.84	66.79 ± 7.32	67.82 ± 8.04
Lifting-thrusting	73.68 ± 8.88	69.84 ± 9.93*	69.43 ± 8.69	69.95 ± 9.71	71.22 ± 11.01*
Twisting-rotating	69.54 ± 11.47	65.27 ± 12.28*	66.89 ± 10.80	67.32 ± 9.63	67.32 ± 11.50*

measurement in Hebei in China showed that after acupuncture stimulation on LI11 (Quchi), the skin temperature of the ipsilateral point LI1 (Shangyang) increased, too [6]. Similar to this experiment, Dong and Che could show that, after electroacupuncture stimulation on LI4 (Hegu), the temperature of LI4 increased significantly, decreasing slowly afterwards [7].

The lifting-thrusting and twisting-rotating needle manipulations described and investigated in this experiment come from one of the authors' (Professor X. Cheng) clinical experience. His very important results are summarized in *Chinese Acupuncture and Moxibustion* [2]. The clinical technique from Professor X. Cheng has influenced tens of thousands of acupuncturists all over the world. He emphasizes simple and direct clinical acupuncture techniques and uses lifting-thrusting and twisting-rotating manipulations, stopping all operations when qi arrives. These techniques belong to middle and low dosages of stimulations. As his students, the authors adopt his ways in clinical routine and scientific experiments, which allows them to achieve good results [8, 9].

In one of these experiments [9], we used sham acupuncture as a control, considering it to represent the influence of environment, body position, and emotion. It was shown that, even without verum acupuncture stimulation, the skin temperature of the observed point changed significantly. This shows the necessity of a placebo control group in such investigations.

Skin is the only heat dissipation way of the human body; when the body energy metabolism and heat production increases, heat dissipation through the skin increases, too, and so the surface temperature rises [10]. There are three ways of skin heat dissipation: radiation, transmission, and evaporation. Thermal radiation is a procedure of living beings who emit heat in infrared rays to the surrounding environment; the higher the temperature compared to that of the surroundings, the higher the emission of infrared rays. Although we asked the volunteers to come to the lab early to adapt to the temperature, in the first 5 mins the temperature of the observational area decreased, following the exposure. The skin temperature of the acupoint then showed an increase

caused by verum acupuncture with deqi arrival, whereas during the sham acupuncture procedure skin temperature continued to decrease. This shows that deqi acupuncture could increase skin temperature, which corresponds to the findings of one of our previous studies [9]. However, 5 minutes are quite short for determining baseline values, and, in further studies, this period should be expanded.

Both twisting-rotating (although not reaching the level of significance) and lifting-thrusting manipulations have been shown to make the skin temperature of the stimulated acupoint increase at first and decrease slowly later on. So, one can say that the lifting-thrusting method causes a stronger needling sensation and a stronger stimulation and induces a higher temperature at the stimulated acupoint. The stronger acupuncture stimulation may be related to the increase in blood perfusion [11, 12].

5. Conclusion

Compared to sham acupuncture, deqi acupuncture can be able to increase the skin temperature of the stimulated acupoint. The range of temperature increase caused by lifting-thrusting stimulation is higher than that of the twisting-rotating method. This may be connected with the stimulus intensity. Further research is needed to verify these findings.

Conflict of Interests

The authors declare that they have no conflict of interests.

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TABLE 2: Changes in skin temperature (in [°C]) at ST36. The values in parentheses are the increases (decreases) of temperature with respect to the baseline values (**P* < 0.05).

	Before acupuncture	Needle insertion	Needle manipulation	Needle removal	After acupuncture
Sham acupuncture	34.87 ± 1.14	34.69 ± 1.02	35.18 ± 0.67	35.21 ± 1.06	35.23 ± 0.78
	—	(−0.18)	(+0.31)	(+0.34)	(+0.36)
Lifting-thrusting	34.53 ± 1.01	35.01 ± 0.85	35.37 ± 0.79	35.41 ± 1.05	35.35 ± 1.13
	—	(+0.48)	(+0.84)*	(+0.88)*	(+0.82)*
Twisting-rotating	34.49 ± 1.14	34.64 ± 1.38	34.86 ± 1.27	34.96 ± 1.09	34.91 ± 1.14
	—	(+0.15)	(+0.37)	(+0.47)	(+0.42)

TABLE 3: VAS scores expressing the intensity of the needling sensation caused by needle insertion and manipulations (mean ± SD).

	Needle insertion	Needle manipulations
Lifting-thrusting	3.93 ± 1.76	7.13 ± 1.90**
Twisting-rotating	4.72 ± 1.46	4.91 ± 2.32

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Research Article

Objectifying Specific and Nonspecific Effects of Acupuncture: A Double-Blinded Randomised Trial in Osteoarthritis of the Knee

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Introduction. Acupuncture was recently shown to be effective in the treatment of knee osteoarthritis. However, controversy persists whether the observed effects are specific to acupuncture or merely nonspecific consequences of needling. Therefore, the objective of this study is to determine the efficacy of different acupuncture treatment modalities. **Materials and Methods.** We compared between three different forms of acupuncture in a prospective randomised trial with a novel double-blinded study design. One-hundred and sixteen patients aged from 35 to 82 with osteoarthritis of the knee were enrolled in three study centres. Interventions were individualised classical/modern semistandardised acupuncture and non-specific needling. Blinded outcome assessment comprised knee flexibility and changes in pain according to the WOMAC score. **Results and Discussion.** Improvement in knee flexibility was significantly higher after classical Chinese acupuncture (10.3 degrees; 95% CI 8.9 to 11.7) as compared to modern acupuncture (4.7 degrees; 3.6 to 5.8). All methods achieved pain relief, with a patient response rate of 48 percent for non-specific needling, 64 percent for modern acupuncture, and 73 percent for classical acupuncture. **Conclusion.** This trial establishes a novel study design enabling double blinding in acupuncture studies. The data suggest a specific effect of acupuncture in knee mobility and both non-specific and specific effects of needling in pain relief.

1. Introduction

Knee osteoarthritis is a major cause of morbidity, disability, and health care utilisation, particularly in elderly patients [1]. The primary clinical manifestations are pain and joint stiffness [2]. Therapy recommendations aim to improve physical function and to relieve symptoms [3]. Unfortunately, pharmacological approaches often render limited effects and

also carry the burden of potentially serious side effects [4]. Hence, many patients try complementary medicine treatments [5–7]. Amongst the nonpharmacological approaches, the use of acupuncture has increased consistently during the past few decades [7].

Recent randomised controlled trials have produced rather contradictory results with respect to acupuncture's effects. Some trials have suggested a potential benefit of

acupuncture beyond that of sham or minimal acupuncture [8–10], whereas other studies have reached the opposite conclusion [11]. These inconsistent results have generated much discussion in the scientific community as to whether the effects in acupuncture were caused by mere skin penetration or by the stimulation of specific points [12–16]. In an attempt to clarify this issue, we found corresponding inconsistencies in the study designs themselves: the sham or minimal acupuncture procedures used as controls in the aforementioned trials differed systematically from the actual acupuncture groups regarding number, size and length of needles, and intensity and duration of the doctor-patient encounter. Moreover, the trials failed to achieve complete blinding [8–12]. Any attempt to clarify the issue of efficacy in acupuncture requires a more controlled study design.

The controversy over acupuncture extends to the issue of the most effective method of acupuncture [17]. Some practitioners favour a *modern acupuncture*, treating patients according to a semistandardised set of disease-specific points. Other practitioners adhere to an individualised *classical acupuncture*, which derives acupuncture points from an assessment of disease modalities and a physical examination, including Chinese tongue and pulse diagnosis and the localisation of paraesthetic pressure points [18, 19].

To elucidate these open questions, we conducted a repeated measures, double-blinded, and placebo-controlled, multicentre trial in patients with chronic osteoarthritis of the knee. The study compared the effects of three modalities of acupuncture (sham, semistandardised modern and individualised classical) within two parameters: joint mobility and pain [20, 21].

2. Materials and Methods

2.1. Patient Population. Patients aged 35 years or older were recruited by newspaper advertisements and from the outpatient clinics of the three participating centres. Potential participants were first screened by telephone interview, followed by a clinical examination to ascertain the satisfaction of the diagnostic criteria of the American College of Rheumatology and the presence of a severity grade of II or III according to the radiological Kellgren classification. Patients with congenital or traumatic deformations of the knee, malignant disease, autoimmune disorders, surgery or arthroscopy during the past 12 months, medication with steroids, physical therapy, or acupuncture within the last four weeks, as well as intake of opioids during the study period, were excluded from the study. Patients were allowed to continue their regular medication including NSAID or COX2-inhibitors while participating in the study, but changes in medication and dosage were not allowed. The local ethics committee approved the protocol. All patients provided written informed consent.

2.2. Intervention, Randomisation, and Double Blinding. Patients were informed that the study aimed to identify the most effective of three acupuncture techniques, including one sham technique. Participants were allocated in random order to (a) the needling of non-specific points (*sham*), (b)

a semistandardised selection of disease-specific acupuncture points as used in recent studies (*modern acupuncture*), and (c) an individualised selection of acupuncture points determined by the diagnosis according to the traditional Chinese medicine (*classical acupuncture*). Each patient received all three forms of acupuncture (a, b, and c) in a random order. Each session was spaced seven days apart resulting in one single treatment per week as well as one single treatment per form of acupuncture (a, b, and c). Prior to every acupuncture session, a fully qualified and experienced physician and acupuncturist established the Chinese medical diagnosis as defined by the Heidelberg Model of Chinese medicine [22]. Using three differently coloured pens at random choice, the first physician marked points for *classical*, *modern*, and *sham acupuncture*. Thereafter, the first physician informed the study-coordinating centre about the colour allocation. The study-coordinating centre compared these colour codings to the sequence of treatment modalities according to a computer-generated randomisation table and informed a second physician about the colour of the points to be needed. In all study centres, this second physician was a novice to acupuncture in order to minimise possible biases arising from the observation of points. This apprentice practitioner was instructed to maintain a standardised method as to needle insertion or needle stimulation throughout all three sessions. After acupuncture, the patients redressed with light garment to cover any potential marks from needling. Thereafter, the patient returned to the first physician, who was unaware of the used acupuncture method, for assessment of pain and knee flexibility.

2.3. Acupuncture Technique. Acupuncture was performed using 0.22×40 mm copper needles. Only one knee was treated in the study. Ear and hand acupuncture was not allowed. During all sessions, the number of needles, the type of needles, the depth of insertion, and the intensity of stimulation were kept identical. In each session, ten points \pm two points were allowed to be stimulated. The needles were rotated immediately after insertion and again after 15 minutes. Needles were then withdrawn after 30 minutes. Communication with the patient during the acupuncture procedure was minimised to an explanation of the procedure.

The only systematic difference across the treatment modalities was the location of needling points. *Modern acupuncture* adhered to previously recommended methods for selection of points for knee pain (ST36, ST34, EX32 twice, SP9, SP10, SP6, GB34, LI 4) [11, 23]. In addition, up to three further points were admissible (e.g., *ashi*, LI3, ST40). Non-specific needling used the points described in Table 1. The points for the *classical acupuncture* were determined individually for each patient according to the classical Chinese diagnosis, which assessed the modality of symptoms, complaints associated with certain movements, tissue tenderness along the postulated acupuncture channels, tongue diagnosis, and pulse quality. In contrast to the *modern acupuncture* treatment, the *classical acupuncture* resulted in a larger variation of needling points between patients with a certain overlap to the points selected in *modern acupuncture*.

(Data were not shown; statistics on the selected points are available from the authors.)

2.4. Outcome Measures. Reasoning that pain-related restrictions in knee flexibility are more direct external measure of pain than subjective self-reported measures, we a priori defined knee flexibility as the primary outcome measures and the WOMAC scale as the secondary outcome parameter [24, 25]. Knee flexion was measured in standardised fashion by using a universal goniometer, aligned with the greater trochanter, through the lateral joint line to the lateral malleolus. The first physician bent the knee to the point at which pain limited further flexion. Knee flexibility was measured before acupuncture, immediately thereafter, and after 7 days (for session two and three, the latter coincided with the baseline-measurement prior to the next treatment). The abbreviated WOMAC pain score was determined prior to acupuncture and immediately thereafter, as well as three and seven days after treatment. Change scores for either outcome were calculated by subtraction of post- from preacupuncture measurements, with a positive change score indicating improvement. For dichotomous outcomes, a treatment success was defined as an improvement of the knee flexibility by 10 degrees or more or a reduction of the WOMAC pain score by 50 percent or more, respectively.

2.5. Statistical Analysis. Knee flexibility as the primary outcome parameter served to determine the sample size. An improvement by 10 degrees was regarded as potentially clinically relevant, and a difference of 5 degrees was viewed as a marginal difference. Based on a pilot study, we estimated a required total of 100 patients to obtain a power of 90% at a type I error of less than 5% in order to demonstrate a difference between methods in knee flexibility change scores by 5 degrees (StateMate 2, Graphpad Software Inc., San Diego, CA, USA). We aimed to recruit 125 patients to allow for dropout and noncompliance. Knee flexibility was shown to be a reliable and valid parameter in several studies [26–29].

Fisher's exact test or the Kruskal-Wallis test was employed to compare baseline characteristics of the three groups resulting from the first randomisation. The main analysis comprised a two-factor analysis of variance (treatment modality and time) with repeated measures. Least square means, 95% confidence intervals for knee flexion, and WOMAC scores were estimated for each patient while taking into account the covariates of gender, premedication (yes or no), disease severity (Kellgren II versus Kellgren III), and number of needles applied. Within subject contrasts were adjusted using the Greenhouse-Geisser correction.

Repeated measures analysis of variance does not readily provide for explicit modelling of possible carry-over effects. We expected that the effect size of the intervention in weeks 2 or 3 might depend on the treatment of the preceding week. Therefore, we employed multilevel, hierarchical, random-intercept, and random-slope modelling of the change scores in knee flexion. In these models, we nested the six change scores (immediately after the treatment and 7 days after the treatment for all three modalities) within patients. The order

of treatment and the preceding treatment were entered as dummy variables. All possible interactions with the treatment modality were systematically explored with non-specific needling and the first treatment as the respective reference categories. Particular attention was given to the modelling of carry-over effects from *classical acupuncture* to *modern acupuncture* and vice versa. In a final step, we explored random intercepts/random slopes of the fixed effects model, as long as the -2 log-likelihood value significantly improved [30, 31]. Blinding was maintained during the statistical analysis.

All analyses were on an intention-to-treat basis. Analyses of variance were conducted using SPSS version 12 (SPSS Inc., Chicago, IL, USA), multilevel modelling employing MLwiN (Version 2.02, Multilevel Models Project, Institute of Education, London, UK).

3. Results and Discussion

One-hundred and sixteen patients (mean age 62.4 years, range = 40–83, 33% males) with chronic osteoarthritis of the knee completed the study between April 2004 and May 2005. Figure 1 displays the patient recruitment, allocation, losses to followup, and exclusions. Randomisation resulted in a similar distribution of gender, premedication, and disease severity across the allocation for the first treatment modality (Table 2).

Knee flexibility improved by 10 degrees or more immediately after the acupuncture procedure in 75 of 116 *classical acupuncture* sessions, giving rise to a number needed to treat (NNT) of 1.5 (95% confidence interval 1.4 to 1.8); this compared to 41 of 116 *modern acupuncture* sessions (NNT = 2.9, 95% CI 2.2 to 3.8) and to 6 of 116 non-specific needling sessions (NNT = 19, 95% CI 9.2 to 53, $P < 0.001$). *Classical acupuncture* resulted in a significantly larger improvement immediately after the treatment (Figure 2, mean change = 10.3 degrees, 95% CI 8.9 to 12) compared to *modern acupuncture* (4.7 degrees, 95% CI 3.6 to 5.8), while no effect was observed for non-specific needling (0.34 degrees, 95% CI -0.61 to 1.3; $F = 27.3$; $df = 3.1, 358$; $P < 0.001$). Adjusting for the Kellgren classification revealed that the difference between *classical acupuncture* and *modern acupuncture* was even larger in patients with more severe illness ($P = 0.02$).

The analysis of the change scores employing multilevel modelling revealed significant carry-over effects from the first to the second and from the second to the third treatment. When the first treatment consisted of *classical acupuncture* (estimated mean change = 9.1 degrees, 95% CI 6.2 to 13), the effects from *modern acupuncture* (mean change = 0.7 degrees, 95% CI -1.3 to 2.7) were negligible. However, when the first treatment consisted of *modern acupuncture* (mean change = 5.5 degrees, 95% CI 3.1 to 7.9), subsequent *classical acupuncture* resulted in a further flexibility gain (mean change = 4.3 degrees, 95% CI 2.0 to 6.6). The small differences from the values reported in the preceding paragraph arise from the adjustment for carry-over effects.

TABLE 1: Acupuncture points chosen for nonspecific needling.

-
- (i) A point between the gallbladder and stomach conduit at the posterior edge of the fibula 2 cun above the malleolus lateralis
(ii) A point 2 cun and 6 cun, respectively, above the malleolus medialis on the tibial surface (intracutaneous needling without contact to the periost with the needles pointing to the knee)
(iii) A point in the middle of the thigh on a line between the patella and the anterior iliac spine
(iv) A point at the top of the contracted biceps muscle

To equalise the number of needles employed between the different needling modalities, the following additional points were permitted:

- (i) A point 3 cun above and medial to the cleft of the knee joint between the spleen conduit and the renal conduit
(ii) A point in the middle of a line between liver 13 and liver 16
(iii) A point in the middle of a line between gallbladder 37 and vesical 58
(iv) A point 2 cun dorsal to gallbladder 32
(v) A point in the middle of a line between heart 2 and pericardium 3
-

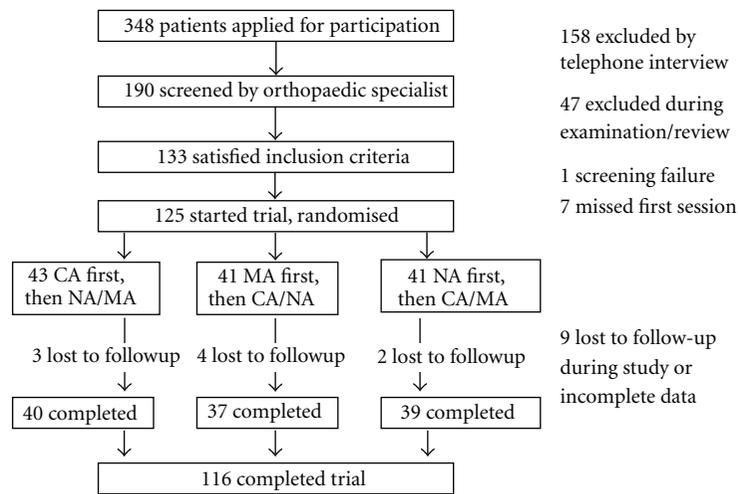


FIGURE 1: Patient recruitment, randomisation and followup.

The multilevel model also suggests that the substantial variation between patients in the effects of *classical acupuncture* is relatively independent of the variation in the effect of *modern acupuncture*—in other words, the extent of improvement after classical acupuncture is not correlated with the extent of improvement after modern acupuncture ($P = 0.43$ for the covariance in the random part of the model).

In contrast to the differences in efficacy for knee mobility, all three treatment forms resulted in some immediate improvement of pain scores (Figure 3). *Classical acupuncture* showed a significantly larger improvement immediately after treatment than non-specific needling did (post-hoc contrast, $F = 5.4$, $df = 1$, $P = 0.022$). Success rates defined as a WOMAC reduction by 50% were the largest immediately after *classical acupuncture* (85 of 116, NNT 1.4, 95% CI 1.23–1.56) as compared to *modern acupuncture* (74 of 116, NNT 1.56, 95% CI 1.38 to 1.84, nonsignificant difference) and non-specific needling (56 of 116, NNT 2.1, 95% CI 1.68–2.46, $P = 0.02$). The pain relieving effect of any needling rapidly declined. At the 7-day follow-up visit, pain scores were similar across the three methods (Figure 3).

3.1. Strengths and Weaknesses. The strength of the present study is its use of a novel study design for acupuncture which establishes blinding of both patients and the treating physicians. This design overcame major shortcomings of previous studies which failed to achieve adequate blinding and in which sham treatment usually differed substantially from acupuncture. The results of the present study offer an answer to the basic question of whether the effects in acupuncture are specific or caused by mere skin penetration. In our study, the needle location remained the only difference between the three treatment modalities, approximating for the first time the principles of randomised and double-blinded, controlled trials in acupuncture studies.

116 patients with osteoarthritis of the knee received three treatments in a random order: acupuncture according to an individualised diagnosis of Chinese medicine (classical acupuncture), a semistandardised modern version of acupuncture usually employed in acupuncture trials (modern acupuncture) and non-specific needling. The main findings were a twice as large improvement in knee flexibility immediately after classical acupuncture (10.3 degrees) as compared to

TABLE 2: Patient characteristics.

Characteristic	Total sample	CA as first treatment	MA as first treatment	NA as first treatment	P value
N	116	40	37	39	
Gender male (percent)	38 (33%)	8 (20%)	17 (46%)	13 (33%)	0.053
Age (years)	62.4	62.7	61.6	62.9	0.79
Kellgren Grade III (percent)	57 (49%)	22 (55%)	17 (46%)	18 (46%)	0.66
Left knee (percent)	54 (47%)	17 (43%)	20 (54%)	17 (44%)	0.54
Duration of pain (years)	4.7	4.9	5.3	3.9	0.50
Premedication (percent)	39 (34%)	13 (33%)	13 (35%)	13 (33%)	0.96

CA: classical acupuncture; MA: semistandardised modern acupuncture; NA: nonspecific needling. Columns describe the first treatment. All patients subsequently received the two remaining treatment modalities.

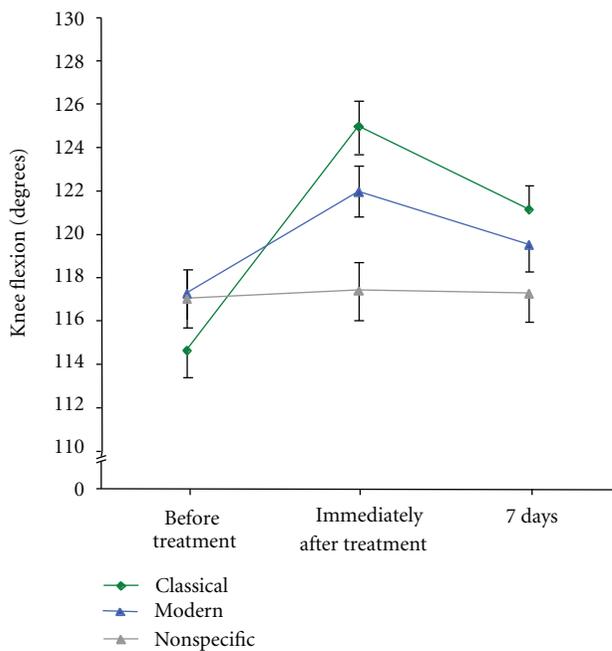


FIGURE 2: Knee flexion before and after acupuncture. The figure compares the maximum possible knee movement until further flexion was blocked by pain for classical acupuncture, semistandardised modern acupuncture, and non-specific needling. Flexion was assessed immediately prior to treatment, directly thereafter and at a recall visit after 7 days. Data display the means adjusted for Kellgren classification, prior intake of medication, and patient gender. Error bars indicate the standard error of the mean. Knee flexion is displayed in degrees according to the neutral-zero method.

modern acupuncture (4.7 degrees) and no change after non-specific needling (0.3 degrees). The largest improvements in pain were also seen immediately after classical acupuncture (a WOMAC score reduction by 50% or more in 85 of 116 patients); however, non-specific needling also achieved considerable effects (core reduction by 50% in 56 of 116 patients, approaching two-thirds of the maximum effect observed after classical acupuncture. Therefore, the present data suggest substantial non-specific effects in subjective pain relief. In contrast to subjective pain relief, however, improvements in knee flexibility as objective outcome measure were *only* seen after the needling of specifically selected points and not after

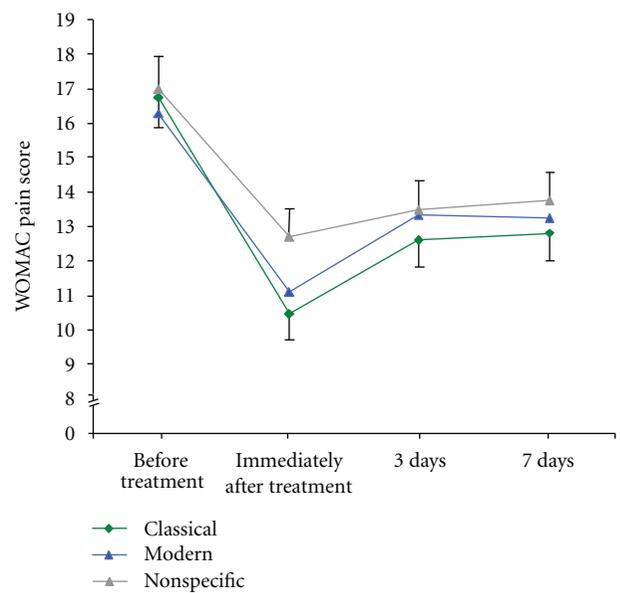


FIGURE 3: WOMAC pain scores before and after acupuncture. The figure compares the WOMAC pain scores for classical acupuncture, semistandardised modern acupuncture, and non-specific needling. Pain was assessed immediately prior to acupuncture, directly thereafter, by self-administered questionnaire at home at 3 days after acupuncture, and at a recall visit after 7 days. Data display the means adjusted for Kellgren classification, prior intake of medication, and patient gender. Error bars indicate the standard error of the mean.

non-specific sham needling. To our understanding, this is the first study to prove specific effects of acupuncture and the first to exclude bias caused by differences in the control arms.

With respect to pain relief, the present study corroborates earlier findings. The measure of effect observed for the sham acupuncture as well as for the semistandardised modern acupuncture was similar to those previously observed in multicentre trials. Pain relief of comparable effect can also be achieved by other methods such as transcutaneous electrical nerve stimulation, supporting the notion that neurogenic pain contributes to the symptoms in patients with degenerative changes in joints [32, 33]. However, the non-specific effects of acupuncture may exceed those of mere placebo effects [34], for reasons as yet unexplained.

Interestingly after seven days, no relevant difference in pain scale was reported, although we found the significant changes in knee motility to be persistent among the three treatment groups. This gain in function (knee flexibility) may be considered an indirect measure of pain relief as pain is the main limiting factor for knee motility.

Moreover, we observed a rapid improvement of knee flexibility immediately after classical acupuncture, which was twice the effect observed after modern acupuncture and absent after non-specific needling. Elucidating the physiological mechanisms [35–38] underlying this method-specific difference in effect was beyond the scope of the present study. Experimental data, however, offer some possible explanations: while the immediate effects on pain and knee flexibility exclude structural changes in the affected joints as the underlying mechanism of acupuncture in this experimental setting, they do, however, indicate an underlying neural mechanism [36]. It remains speculative as to whether this reflex-like effect involves functional changes within higher regions of the central nervous system or whether regional effects on musculoskeletal dynamics and connective tissue structures may be the dominant mechanism. The observed immediate effects, however, make a primarily systemic or humoral effect rather unlikely. As the systematic search for acupuncture points with altered perception is an integral part of history taking and work-up for the Chinese diagnosis, it is conceivable that the individualised diagnostic approach may enhance the chance to effectively identify needling points with the potential for reducing functional limitations. The present study suggests that the methodology of arriving at acupuncture points may matter. In the present study, classical acupuncture outperformed modern acupuncture. Future acupuncture studies should, therefore, consider potential differences arising from the modality of acupuncture techniques in the study design.

3.2. Limitations. Several caveats of the present investigation require consideration. Firstly, we studied each acupuncture technique only once in each patient, and treatments were usually one week apart. Thus, we are unable to infer the long-term or cumulative effects of repeated applications; the study should, therefore, be considered a proof of concept study.

The available data from the present study corroborate a rapid decline, particularly of the non-specific pain relief effect, within one week. Secondly, the present data suggest that effects on knee mobility are somewhat retained. However, the imperfect retest reliability of repeated knee-flexion measures after one week suggests viewing this result with caution and encourages repetition in other studies. Thirdly, crossover designs are prone to carry-over effects. We cannot rule out residual carry-over effects beyond those explicitly modelled within the multilevel statistical method. Finally, while the data support the notion that the choice of needling points matters, the relevant aspects of the Chinese diagnosis still remain to be elucidated. This, however, cannot be addressed in this work.

4. Conclusions

In summary, our double-blinded and randomised crossover study provides a novel study design for assessing efficacy in acupuncture and establishes a framework for addressing the question as to whether the specific choice of acupuncture points matters. The study was conducted in osteoarthritis of the knee, and the outcome measures are self-reported pain relief and knee motility. As to the first, non-specific needling achieved about two-thirds of the subjective pain relief achieved after classical acupuncture, suggesting considerable non-specific effects. With respect to knee motility, individualised classical acupuncture achieved twice the effect of semistandardised modern acupuncture. No change, however, was observed after non-specific needling. This suggests a considerable specific effect of acupuncture in objective knee flexibility, an effect that appears to be method-specific as well. In the scientific discussion about efficacy of acupuncture, our data suggests that it bears both specific and non-specific effects, and the selection of acupuncture points for treatment does appear to be relevant.

Abbreviations

NSAID: nonsteroidal anti-inflammatory drug
NNT: number needed to treat.

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Research Article

Observation of Microvascular Perfusion in the Hegu (LI4) Acupoint Area after Deqi Acupuncture at Quchi (LI11) Acupoint Using Speckle Laser Blood Flow Scanning Technology

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The aim of this study was to investigate the traditional meridian theory using speckle laser blood flow scanning technology to observe microcirculation of the Hegu acupoint area after acupuncture stimulation on distant points. An observational study was conducted to observe the microvascular perfusion of Hegu (LI4) and control points after acupuncture at Quchi (LI11). Thirty healthy volunteers (mean age 31.6 ± 8.7 years) received deqi acupuncture on Quchi (LI11, right side), and simultaneously changes in microvascular perfusion of Sanjian (LI3), Hegu (LI4), Yangxi (LI5), and two control points were observed before, during, and after needling using a MOOR speckle laser. The results showed that the changes in microvascular perfusion of the observed points are not regular. After correction, the experiment showed that the blood perfusion on 3 meridian acupoints was increased while the perfusion on 2 control points was decreased following acupuncture stimulation, the changes at Hegu (LI4) being the statistically most significant ones. Deqi acupuncture can help in regulating the body's blood flow, with a certain degree of meridian specificity.

1. Background

In accordance with traditional Chinese medicine (TCM), effective acupuncture could enhance people's health by clearing main and collateral channels, thus allowing the body's Yin and Yang to achieve a state of dynamic equilibrium. Usually, effective acupuncture is also called deqi acupuncture (arrival of Qi). Deqi/Qi arrival (with its uniquely human characteristics like Qigong and Yin-yang) is accepted by parts of the international academic community [1]. When inserting the needle to a certain depth, both the acupuncturist and the patient will feel something is changing, this means Qi arrival or deqi sensation. Comparing placebo and deqi acupuncture, it was found that after acupuncture

stimulation on one point, the former case saw a universal but insignificant increase of transcutaneous CO₂ emission, while the latter case showed a significant increase of transcutaneous CO₂ emission specifically at acupoints located on the same meridian [2].

The physiological changes caused by acupuncture are multifaceted, they occur on different levels and are aimed at different targets. In previous studies, our group observed changes of microcirculation in Hegu (LI4), Neiguan (PC6), and Weizhong (UB40) areas after deqi acupuncture stimulation using laser Doppler perfusion imaging, and we found that there are 3 kinds of effects: on the acupoint areas, on meridians, and on the whole body [3–5]. After deqi acupuncture at some acupoints, the microvascular skin

perfusion of some special distant acupoints showed specific changes with statistical difference compared to the control points, which is in keeping with the traditional Chinese medical acupuncture and meridian theory.

In order to further test the results described above, an experiment was conducted at the Institute of Acupuncture and Moxibustion (China Academy of Chinese Medical Sciences), to observe the blood flow changes on the Hegu (LI4) acupoint after acupuncturing Quchi (LI11) using speckle laser blood flow scanning technology.

2. Materials and Methods

2.1. Selection of Study Participants and Acupuncture Methods. All participants gave informed consent. The experimental procedure was approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion of China Academy of Chinese Medical Sciences. To avoid discrepancies in manipulation, all the acupuncture operations were performed by the same medical practitioner.

Thirty healthy volunteers (14 female, 16 male; mean age \pm SD 31.6 ± 8.7 years) came from the Beijing University of Traditional Chinese Medicine and the China Academy of Chinese Medical Sciences.

Acupuncture stimulation was done manually, using single-use acupuncture needles (0.30×40 mm, Huacheng brand, Suzhou, China). The acupoint Quchi (LI11); located in the midpoint between the lateral end of the transverse cubical crease and the lateral epicondyle of the humerus; right side), was punctured perpendicularly (needle insertion depth 15–20 mm), then needle stimulation was performed by lifting, thrusting, and rotating the needle till achieving Qi arrival. The needle was left in place for 10 min and then removed (see Figure 1).

2.2. Observation Methods. The temperature of the lab was kept at 26°C , and the volunteers were asked to come into the room 5–10 mins ahead of schedule to adapt to the temperature. Then we marked point 1 (Sanjian, LI3), point 2 (Hegu, LI4), point 3 (Yangxi, LI5), point 4 (control point, located on the Large Intestine channel/meridian), and point 5 (control point, not located on the Large Intestine channel/meridian); see Figure 2.

The principle of speckle laser blood flow scanning technology is that the speckle (basically the spot of light emitted by the instrument) is formatted at the tissue surface, and the speckle will change with the moving particles (such as red blood cells). Then the information about perfusion distribution can be obtained by assessing the intensity of fluctuations. The instrument we used was a multi-point synchronization scanner Moor FLPI (Moor Instruments Ltd., Millwey, UK). Measurement parameters: scanning distance about 11 cm; sample interval 40 ms; flux time constant 0.5 s; total scanning time 20 min. For the exact procedure of the experiment, see Figure 1. During the whole experiment, the volunteers were requested to be quiet unless being asked about the needle sensation.

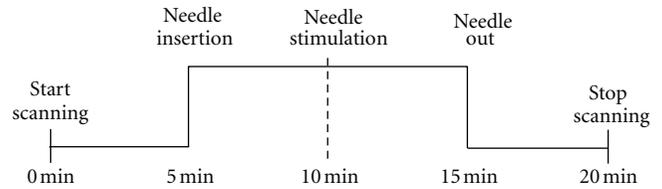


FIGURE 1: Flow diagram of acupuncture and scanning.

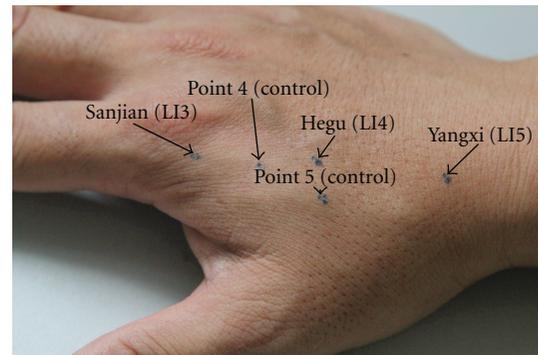


FIGURE 2: Marked points to be investigated.

2.3. Statistical Analysis. Thirty sets of average data of the skin microvascular perfusion (Flux, in arbitrary units) were analyzed by Moor Full-field Laser Perfusion Single Point Review 2.1 (provided by Moor Instruments Ltd.), comparing the average values of the different periods (before, during, and after acupuncture).

Further, according to our past research results and to eliminate the systemic effects of acupuncture, we processed the first 4 groups of original data referenced with point 5 as a control point. The correction formula was as follows.

During acupuncture-before acupuncture of point 1–4/during acupuncture of point 5.

After acupuncture-during acupuncture of point 1–4/after acupuncture of point 5.

Paired *t*-test was used to compare the differences between the blood flow per unit in different periods of acupuncture, with $P < 0.05$ denoted as significant.

3. Results

3.1. Analysis of the Original Data. When analyzing the average of original skin microvascular perfusion, it was noted that the blood flow of the observation points was different, with a large data standard deviation. The highest level of skin blood flow area was on Sanjian (LI3) which is situated foremost on the finger, and the lowest was on Hegu (LI4).

The blood flow of the observed points was influenced by needling Quchi (LI11), however, the changes did not reach the level of statistical significance (see Table 1).

3.2. The Corrected Ratio Analysis. From the past research results we know that every kind of acupuncture stimulation—including shallow acupuncture, but also deqi

TABLE 1: Changing of skin microvascular perfusion (in arbitrary units) of different points before, during, and after needling homolateral Quchi (LI11) in experiment 1 ($N = 30$).

Mean	Before acupuncture	During acupuncture	After acupuncture
Sanjian (LI3)	87.24 ± 55.20	91.99 ± 57.08	91.16 ± 51.75
Hegu (LI4)	57.57 ± 36.57	61.29 ± 38.55	61.28 ± 39.17
Yangxi (LI5)	63.24 ± 25.35	65.17 ± 25.32	65.48 ± 25.90
Point 4	71.20 ± 34.67	69.92 ± 32.80	68.25 ± 29.63
Point 5	65.96 ± 43.17	69.50 ± 52.69	66.03 ± 45.71

TABLE 2: The ratio of skin microvascular perfusion of Points 1–4 referenced to Point 5 before acupuncture in the experiment.

	Sanjian (LI3)	Hegu (LI4)	Yangxi (LI5)	Point 4	Point 5
During acupuncture	0.02	0.04	0.03	−0.03	0.01
After acupuncture	0.04	0.56	0.03	−0.04	−0.02

acupuncture—can bring about systemic reactions and changes of microvascular perfusion. To eliminate this influence, the original data was processed using the correction method described above [6], and results are shown in Table 2. After averaging the ratio during and after acupuncture, it was found that the skin microvascular perfusion of the point 1–3 areas all increased, while that of the control points decreased. But only the change at Hegu (LI4) had statistical significance ($P < 0.01$), while the changes in other acupoints did not reach the level of statistical significance.

Comparing the ratio of microvascular perfusion of points 1–4, it showed that there was no significant difference ($P > 0.05$) among 3 channel/meridian points (Point 1–3 (LI3, LI4, LI5)). However, there was a significant difference ($P < 0.01$) between Yangxi (LI5) and control point 4 as well as between Hegu (LI4) and control point 4 ($P = 0.0017$).

4. Discussion

This group of experiments focuses on the role of acupuncture on microvascular function.

Microvessels provide the environment for cell growth, thus having direct impact on the function of cells and tissues. The microcirculation directly participates in the flow present in tissue (cell, blood, lymph, and tissue fluid) and is considered as a breakthrough for researching meridian phenomenon and acupuncture mechanism. In 1988, Xiu et al. reported that after acupuncturing Chize (Lu5), the skin microvascular self-discipline movement frequency of Shaoshang (Lu11) remained unchanged while the amplitude increased by more than 60%. They concluded that acupuncture could regulate microcirculation such as the microvascular vasomotion [7]. Mu et al. also proposed that the blood flow in acupoints and non-acupoints was significantly different, and acupuncture could increase the amplitude of microvascular vasomotion as well as the blood flow velocity in the acupoint area [8]. With acupuncture stimulation on Neiguan (PC6), Yuan et al. observed that the blood flow of the acupoints on the Pericardium meridian increased compared with the 4 control points on the heart and spleen meridian [9]. Sa et al. also noticed that acupuncture had

a positive impact on the deep tissue of the points and control points on or on the left/right of the stomach channel/meridian [10].

For revealing the difference between acupoints and non-acupoints with regard to microcirculation, our group did a series of studies on skin microvascular perfusion after acupuncture stimulation using laser Doppler flowmetry, laser Doppler imaging, and laser speckle scanning technology [11, 12]. Previous studies in our laboratory showed that acupuncture could specifically enhance the related regional blood flow within a specific period of time. When needling Weizhong (UB40) and achieving Qi arrival, blood flow at the lumbar area of low back pain patients increased specifically, which is in concordance with the traditional Chinese medicinal old saying of “*Yao Bei Wei Zhong Qiu*, UB40 could solve all the problems of the lower back” [11]. When applying manual needling on Hegu (LI4) and getting Qi arrival, the volunteers’ facial blood flow specifically increased, in conformity with the traditional Chinese medicinal old saying of “*Mian Kou He Gu Shou*, LI4 could solve all the problems of the face and mouth” [12]. When acupuncturing Neiguan (PC6) with Qi arrival, the blood flow of Quze (LU5), which is located near the elbow, decreased instantly but then increased significantly [6]. This phenomenon is consistent with the results of Itaya et al. [13]. Itaya acupunctured Geshu (UB17) in rabbits, and the microvascular blood flow slightly decreased in the early stage, but when the needling stopped, the rhythmic movement of the microvessels increased quickly [13].

In previous studies at our laboratory, all experiments were conducted by stimulating acupoints in the extremities to observe the blood flow of acupoints in the proximal end on the same channel/meridian. The current experiment was conducted by stimulating the proximal end point Quchi (LI11) to observe the blood flow on the lower end of the same extremity, at Hegu (LI4), Sanjian (LI3), Yangxi (LI5), and the control points. In the current experiment and past experiments, only one point was stimulated at a time. The results showed that the increase of blood flow in 3 related meridian acupoints after needling Quchi (LI11) was statistically significant compared with that in the control points. Especially the blood flow of the Hegu (LI4) acupoint

obviously changed after acupuncture the point Quchi (LI11).

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