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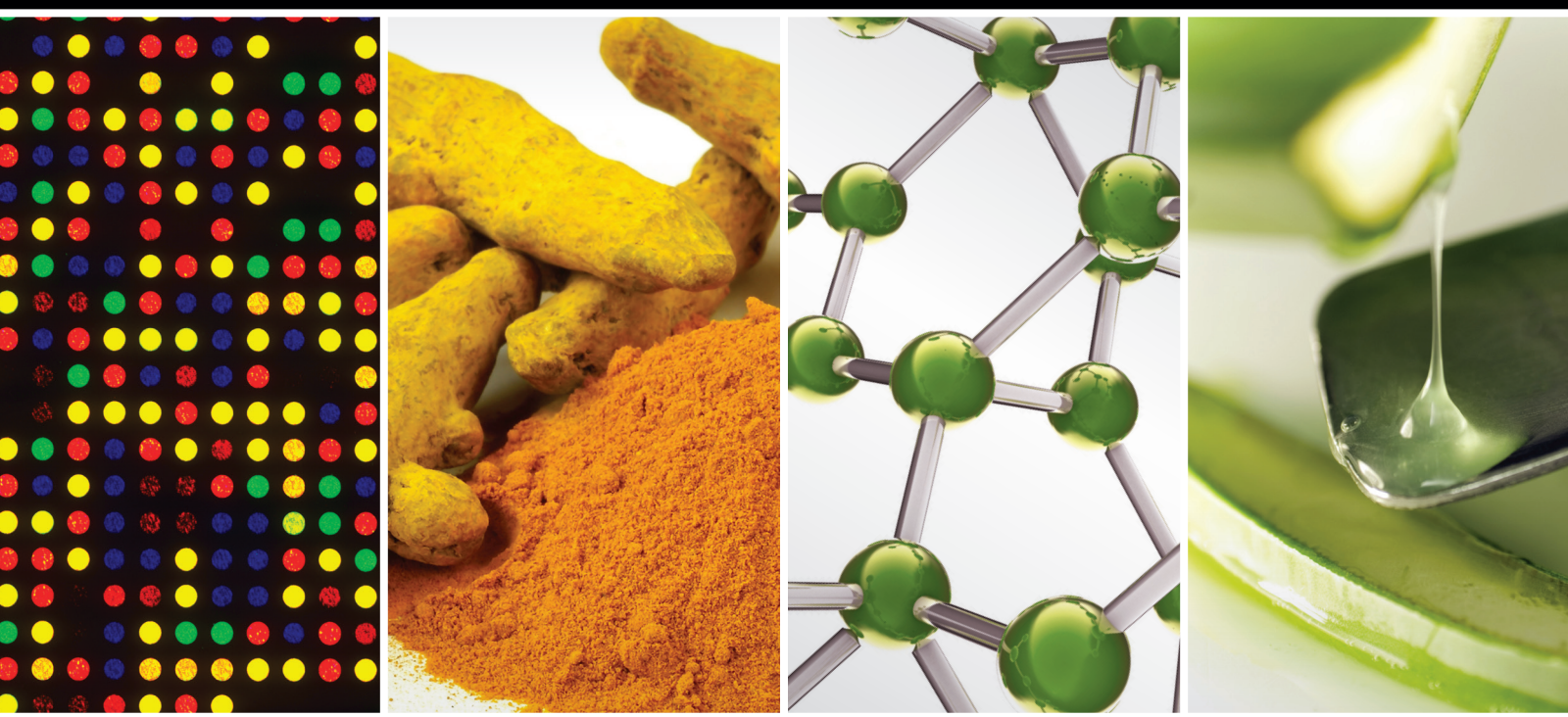
Advances on the Acupuncture Therapies and Neuroplasticity

Lead Guest Editor: Cun-Zhi Liu
Guest Editors: Jiande D. Z. Chen and Meng Zhang



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Editorial Board

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Editorial

Advances on the Acupuncture Therapies and Neuroplasticity

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Acupuncture as one of complementary therapies is widely used in pain and neurodegenerative diseases in clinical practice. Although many studies have tried to analyze the potential effects of acupuncture, the mechanisms are not fully elucidated yet. As a potent form of sensory peripheral stimulation, acupuncture may affect pain and neurodegenerative diseases via mediating on neural plasticity. Neuroplasticity, including dendritic remodeling, synapse turnover, long-term potentiation (LTP), and neurogenesis, is engaged in development of brain, skills learning, formation and extinction of memory, and self-repair of neural injuries.This special issue collects nine papers concerning acupuncture therapies and neuroplasticity in diverse disciplines (molecular biological technology and imaging technologies).

There are three manuscripts about the neuroprotection of acupuncture on neurologic disease. First, the paper by L. Guo et al. entitled "Electroacupuncture Ameliorates Cognitive Deficit and Improves Hippocampal Synaptic Plasticity in Adult Rat with Neonatal Maternal Separation" suggested that early life stress due to maternal separation may induce adult cognitive deficit associated with hippocampus, and the therapeutic effect of electroacupuncture in young adult may be via ameliorating deficit of hippocampal synaptic plasticity. The study by J. Jiang et al. showed that electroacupuncture treatment could inhibit the inflammation reaction in the hippocampus of Alzheimer's disease animal model. The possible mechanism of electroacupuncture reduced the expression of IL-1β and NLRP3 inflammasome relative protein. The paper by D. Lin et al. showed that the acupuncture stimulation on the acupoint (ST-36) could activate the brain-derived neurotropic factor (BDNF) signaling pathways in telomerase deficient mice. This study suggested that the neuroprotection and neuron regeneration maybe play a critical role in electroacupuncture-induced antiaging effect.

The paper by L. Cavalli et al. reported that the beneficial effects of acupuncture on acute severe acquired brain injuries may be related to neuroinflammation, intracranial oedema, oxidative stress, and neuronal regeneration. Acupuncture controlled the imbalance of IGF-1 hormone, decreased spasticity, pain, and the incidence of neurovegetative crisis. The paper by Y.-F. Li et al. indicated that the comprehensive therapy of electroacupuncture in rats with spinal cord injury can effectively enhance the growth of nerve fibers and improve the hindlimb motor function recovery, suggesting that combination therapies could become a powerful treatment for spinal cord injury.

There are two manuscripts using functional magnetic resonance imaging to explore the mechanism underlying acupuncture treatment. The aim of P. Wu et al. study was to observe the grey matter (GM) tissue changes of ischemic stroke in 24 patients. They found acupuncture could evoke pronounced structural reorganization in the frontal areas and the network of DMN areas, which may be the potential mechanism that acupuncture improved the motor and cognition recovery. In the paper by J. Li et al., they used 18F-2-fluorodeoxy-D-glucose positron emission tomography (18F-FDG-PET) to examine the effects of acupuncture at LR3 in spontaneously hypertensive rats. They suggested that acupuncture improves hypertension through a mechanism involving altered brain activation. Acupuncture could decrease the cerebral glucose metabolism in the hypothalamus, thalamus, medulla oblongata, and cerebellum.

In addition, K. Zhang et al. provided an updated state of the effectiveness of acupuncture in obesity by a meta-analysis. They summarized the available studies on exploring the mechanisms under the efficacy of acupuncture in obesity animals and found that acupuncture is an effective treatment for obesity and inferred neuroendocrine regulation might be involved. The study by Z. Ge et al. found home-based transcutaneous neuromodulation improved constipation via modulating gastrointestinal hormones and bile acids

In summary, this issue provides different varies evidences presented by diverse authors covering several topics related to advances in acupuncture for mediating neural plasticity. Neural plasticity could be a bridge between acupuncture and various neurological diseases. More in-depth researches are required to reveal the underlying mechanism of acupuncture.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of the special issue.

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Cun-Zhi Liu
Jian-De Chen
Meng Zhang

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Review Article

Role of Acupuncture in the Management of Severe Acquired Brain Injuries (sABIs)

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Acupuncture therapy has been used to treat several disorders in Asian countries and its use is increasing in Western countries as well. Current literature assessed the safety and efficacy of acupuncture in the acute management and rehabilitation of patients with neurologic disorders. In this paper, the role of acupuncture in the treatment of acute severe acquired brain injuries is described, acting on neuroinflammation, intracranial oedema, oxidative stress, and neuronal regeneration. Moreover, beneficial effects of acupuncture on subacute phase and chronic outcomes have been reported in controlling the imbalance of IGF-1 hormone and in decreasing spasticity, pain, and the incidence of neurovegetative crisis. Moreover, acupuncture may have a positive action on the arousal recovery. Further work is needed to understand the effects of specific acupoints on the brain. Allegedly concurrent neurophysiological measurements (e.g., EEG) may help in studying acupuncture-related changes in central nervous system activity and determining its potential as an add-on rehabilitative treatment for patients with consciousness disorders.

1. Introduction

Severe acquired brain injuries (sABIs) include a variety of acute brain lesions characterized by the occurrence of variably prolonged coma (24 hours), and simultaneous motor, sensory, cognitive, and/or behavioural impairment that causes a certain degree of disability. Congenital, perinatal onset, or degenerative-progressive brain injuries are excluded from this definition.

A common consequence of sABIs is disorders of consciousness (**DOCs**), a prolonged cognitive impairment including the loss of awareness of oneself and environment. DOCs represent one of the greatest challenges that modern medicine faces today, with a huge burden of care for families and facilities. On the basis of the current taxonomy of DOCs, a state of altered consciousness can be categorized into coma, vegetative state (VS), also referred to as unresponsive wakefulness syndrome (UWS), and minimally conscious state (MCS) [1].

The most common cause of sABI is traumatic brain injury (**TBI**), a major source of death and disability worldwide. Two further causes of sABI are anoxic encephalopathy (**AE**), usually due to cardiocirculatory arrest (secondary to extensive myocardial injury and/or malignant arrhythmias), and **ischemic or haemorrhagic stroke**. These conditions mostly affect subjects from the fifth decade onwards and represent about 40% of the sABIs. The main involvement of neurons leads to a worse prognosis than TBI. In AE, the neurons disruptions, with a low regenerative potential, cause a high risk of irreversibility of the consciousness disorder. Moreover, the time interval within which a recover is reasonable, up to 12 months from the event for TBI, is unlikely more than 3-6 months from the event for anoxic or vascular brain injuries. Late recoveries are possible, but rare. Further nontraumatic sABI arises from brain tumors, infections, and toxic-metabolic encephalopathy [2].

Despite the fact that steady progress has been made toward prolonging patients survival and several

Table 1: Physiopathology of DOCs.

Primary brain injuries	secondary brain injuries
Focal	
(i) contusion	(i) ionic imbalance (due to hypoxia and hypotension)
(ii) laceration	(ii) glutamate excitotoxicity
(iii) hemorrhage	(iii) oxidative stress (generated by mitochondrial dysfunction)
Diffuse	
(i) concussion	(iv) ischemic injury
(ii) diffuse axonal injury	(v) edema formation
	(vi) intracranial hypertension
	(vii) neuroinflammation
	(viii) blood-brain barrier disruption

pharmacologic and neuromodulating strategies have been proposed, results on functional recovery of DOCs are still scarce.

2. Physiopathology of DOCs

The immediate effect of the impact forces on intracranial tissues, i.e., the **primary brain injury,** can be focal or diffuse. Focal injuries, such as contusion, laceration, and haemorrhage, are early detectable upon imaging and their consequences depend on their location and severity. Diffuse brain injuries, like concussion or diffuse axonal injury, require magnetic resonance to be detected [3]. However, what is susceptible of treatment is **secondary brain injury** (SBI), the cascade of biochemical and cellular events developing minutes to months after the insult: ionic imbalance (due to hypoxia and hypotension), glutamate excitotoxicity, oxidative stress generated by mitochondrial dysfunction, ischemic injury, edema formation, intracranial hypertension, neuroinflammation (by both systemic and central neuron system immunoactivation), and blood-brain barrier (BEE) disruption [3, 4], as schematized in Table 1.

Several secondary processes are based on intracellular **calcium** overload, due to excitatory amino acids and inflammatory cytokines released. In absence of oxygen, glucose enters the glycolytic pathway, where it is converted to NADH and pyruvate; the latter becomes lactic acid just producing 2 ATP molecules, while Krebs cycle is precluded. Thus, a down-regulation of ATP-dependent Na^+/K^+-ATPase pump leads to sodium overload, which acting on Na^+/Ca^{2+} exchange pump provides calcium retention.

Analogously, the huge release of glutamate following a head trauma, in hypoxic conditions, is not counterbalanced by astrocyte reuptake of glutamate, which acts on N-methyl-d-aspartate (NMDA) receptor, thus increasing calcium influx. Calcium is therefore sequestered by mitochondria causing their dysfunction, lysis, and release of byproducts [3]. Moreover, calcium stimulates neuronal and endothelial cells overproduction of nitric oxide (NO), which can be associated with oxidative stress. A further effect of calcium overload is mitochondrial-mediated apoptosis following the release of cytochrome c.

In response to primary brain injury, an **inflammatory response** is provided by microglia and astrocytes, which attract leukocytes via cytokines, chemokines, and NO. The disruption of the BEE often leads to cerebral oedema and intracranial hypertension [3].

3. Reactions to Brain Injury

A series of mechanisms are activated at neuronal (neurogenesis, synaptogenesis, and dendritic remodelling) glial, and vascular level, referred to as***neuroplasticity*** [5]. These events are promoted by increased expression of growth factors involved in brain development, such as nerve growth factor (NGF), neurotrophin 4/5, basic fibroblast growth factor, and brain derived neurotrophic factor (BDNF); an increasing importance has also emerged for insulin-like growth factor 1 (IGF-1), regulating metabolic function, oligodendrocyte proliferation, and survival, angiogenesis, myelinisation, and neurite outgrowth [5] and receptors for IGF-1, virtually ubiquitary, are mainly expressed by mesenchymal origin-derived cells in the hippocampus, parahippocampus, amygdala, cerebellum, and cortex [6].

4. Available Strategies to Manage sABIs

New insights into the pathophysiology of sABIs initiated new therapeutical approaches (neuroprotective strategies) aimed at interrupting secondary brain injury development and promoting mechanisms of arousal (see Table 2). Among the neuroprotective strategies, mild hypothermia (which decreases cerebral metabolic demand, excitatory neurotransmitter release, inflammation, and oedema), hyperosmolar therapies (also known for their immune-modulating property), statin (reducing oxidative stress and inflammation), and cyclosporin A (ameliorating mitochondrial function) have been proposed [3]. For arousal recovery, dopaminergic, serotoninergic and GABAergic drugs have been explored, with occasional results. Several nonpharmacological strategies have been utilized. Early verticalization has been shown to increase the spectral power of the EEG higher frequencies and the subject's arousal [7, 8], probably enhancing vestibular afference on locus coeruleus, raphe, and thalamic intralaminar nuclei. Enriched environment, equipped with emotional stimuli and biographically meaningful objects, showed greater range of behavioural responses [9]. Finally neuromodulatory techniques, including deep brain stimulation (DBS), transcranial direct current stimulation (TDCS),

Table 2: Available strategies to manage sABIs and their mechanisms of action.

NEUROPROTECTIVE STRATEGIES	
Mild hypothermia	*decrease of cerebral metabolic demand, excitatory neurotransmitter release, inflammation and edema*
hyperosmolar therapies	*immune-modulating effects*
statins	*reducing oxidative stress and inflammation*
cyclosporin A	*ameliorating mitochondrial function*
NEUROMODULATIVE STRATEGIES	
Pharmacologic (i) dopaminergics (ii) serotoninergics (iii) gabaergics	*neurotransmitter modulation*
Non Pharmacologic postural verticalization	*potential enhancement of the vestibular effects on locus coeruleus, raphe and thalamic intralaminar nuclei*
Neuromodulative Techniques: (i) deep brain stimulation (DBS) (ii) transcranial direct current stimulation (TDCS) (iii) transcranial magnetic stimulation (TMS) (iv) spinal cord stimulation (v) median nerve stimulation (vi) vagus nerve stimulation	*increasing metabolism in the forebrain, thalamus and reticular formation. modulating neuronal networks and ANS*

transcranial magnetic stimulation (TMS), spinal cord stimulation, median nerve stimulation, or vagus nerve stimulation, have been proposed.

5. A Complementary Approach for the sABIs' Management Derivating from Ancient Chinese Medicine: *Acupuncture*

The aim of this review is to explore the potential role of acupuncture (1) in the acute treatment of sABIs, in containing secondary brain injury by acting on different pathophysiologic mechanisms, and (2) in the subacute/chronic management of sABI outcomes, through a limitation of spasticity, pain, dysautonomia, and a possible action on the arousal recovery.

Acupuncture is a traditional medicine traced back to over 3000 years ago in China [10], consisting in inserting needles into specific points of the patient's body ("acupoints") chosen on the basis of the Meridian theory of Traditional Chinese Medicine (TCM) [11]. It has been used in the treatment of stroke and its consequences for over 2,000 years in China.

Under the influence of neuroanatomy, neurophysiology, and bioholographic principle of modern medicine, in the early 1970s, scalp acupuncture, one of the several specialized acupuncture techniques in which a filiform needle is used to penetrate specific stimulation areas on the scalp mainly for the treatment of brain diseases, was set up and separated from traditional acupuncture system [12].

Scalp acupuncture has been reported to (1) improve cerebral blood circulation, promoting regional energy metabolism; (2) upregulate expression of glial cell-line derived neurotrophic factor (GDNF), possibly promoting proliferation and differentiation of neural stem cells in the focal cerebral cortex and hippocampus; (3) reduce contents of excitatory amino acid and increase level of GABA, thus lowering neurogenic toxicity; (4) ease cerebral vascular immunoinflammatory reactions; (5) regulate blood lipid metabolism to resist cerebral free radical damage; and (6) inhibit cerebral cortical apoptosis [13].

5.1. Acupuncture and Inflammation in DOCs. Few studies considered the diachronic assessment of inflammatory markers after a sABI in order to monitor the local and systemic stress response in DOCs. Amico and colleagues evaluated the subacute phase of DOCs in five patients in vegetative state (VS) and one in minimal consciousness state (MCS), by clinical assessment and biochemical analyses [14]. A positive correlation was found between the serum levels of **osteopontin** (OPN), a cytokine involved both in neuroinflammation and neurorepair, and prognosis, with the lowest level detected in the patient who then emerged from MCS, the highest in the one who then died. Moreover, the lymphocyte subset presented a general increase of CD4+/CD3+ ratio, with a suspect unbalance of CD4+ toward Th2; prolactin resulted to be the best endocrinological marker of sABI [14].

Applied at *Baihui* (**GV20**) and left *Zusanli* (**ST36**), acupuncture significantly reduced the infiltration of inflammatory cells and the expression of the proinflammatory enzyme MMP2 in cerebral ischemia/reperfusion injury (CIRI) model rats. In particular they attenuated the expression of the **water channel proteins P4 and AQP9** in the ischemic brain, leading to the mitigation of inflammation-related brain edema. Consistent with the smaller observed infarct size, acupuncture and EA both promoted significant improvements in the Modified Neurological Severity Score (mNSS) in CIRI model rats, indicative of enhanced neurological function [15].

Moreover, acupuncture successfully downregulates tumor necrosis factor alpha (TNF-α), which results in

anti-inflammatory responses. The neural pathways by which acupuncture signalling stimulates anti-inflammatory effects have been mapped. By testing the effects that a splenic neurectomy and vagotomy have on TNF-α levels in the spleen and the brain, Lim et al. found that the anti-inflammatory effects of manual acupuncture at **ST36** rely on the vagus nerve pathway. Moreover, both manual acupuncture stimulation (MAC) and electroacupuncture (EA) induce c-Fos protein generation [16].

5.2. Acupuncture and Redox Equilibrium. Oxidative stress, the imbalance between the production of reactive oxygen and nitrogen species (ROS/RNS) and the endogenous antioxidant system, causing a cascade of chain reactions resulting in cellular damage, is a critical feature in the pathological process of various diseases [17].

Recently, a large body of evidence demonstrated that acupuncture has antioxidative effect in various conditions [18–20], although the exact mechanism, especially the influence of acupuncture on signalling pathways, remains unclear. Through redox system, antioxidant system, anti-inflammatory system, and nervous system, acupuncture could make the oxidative damage and the antioxidant defence remain in a relatively constant redox state. However, the recent acupuncture researches about oxidative stress are sporadic and preliminary [21].

5.3. Acupuncture and Intracellular Calcium. The insertion of the needle represents an effective mechanical stimulus, leading to tissue displacement and to intracellular calcium increase and signalling. The modulation of calcium channels seems to be the primary mechanism for endorphin secretion and release from immunocytes and for the inhibitory effects of opioids on peripheral neurons [22]. Thus, **calcium** ion, whose increase is so crucial in the pathogenesis of SBI, may be taken as the carrier of the biological modulation system provided by acupuncture, where the mechanical wave onsets an acoustic shear wave, and this drives to calcium signalling [22].

5.4. Acupuncture and Neuron Regeneration. In the brain of human adults, neural stem cells (NSCs) have been demonstrated in the pallium, subependymal region, hippocampus, and corpus striatum, which have the ability of self-duplication, self-regeneration, and differentiation into neurons and glial cells. During cerebral ischemia reperfusion, astrocytes play a crucial role in limiting neuronal lesion, as they release epoxyeicosanoic acids in order to enlarge brain vessels, release Nerve Growth Factors to make neurons survive and axons grow, produce neurotransmitters, metabolize toxic molecules, and have also the potentiality to become NSCs [23, 24].

The astrocyte activation and proliferation marker is Glial Fibrillary Acidic Protein (**GFAP**), while Neuron Specific Enolase (NSE) is one of the neurons' markers. Acupuncture on the conception (CV) and governor vessels (GV) has been shown to inhibit excessive proliferation of astrocytes and promote NSCs differentiation in the ischemic brain [25, 26]. In particular, needling acupoints **GV20** and **GV26** could downregulate the number of GFAP+ cells, while increasing the GFAP/NSE double–labelled cells in the hippocampal dentate gyrus [25, 26]. Another study, which employed a rat TBI model, proved that during the early post-TBI stage, acupuncture (**GV20, GV26, GV15, GV16, and LI4**) can promote the proliferation and differentiation of NSCs and glial cells, which is crucial to control neuronal necrosis; in the late phase, it can inhibit glial proliferation and differentiation, driving to neuron and oligodendrocytes regeneration and tissue repair [27].

Moreover, needling **CV24, CV4,** and **CV3** has been shown to upregulate the expression of basic FGF, EGF, and NGF after cerebral ischemia reperfusion, activating nerve repair and proliferation of neuronal precursors [28, 29].

As regards human studies, recent evaluations used single-photon emission computed tomography (SPECT) and T2-weighted imaging (T_2WI) [30], or functional magnetic resonance imaging (fMRI) [31]. Shen and colleagues compared serial diffusion tensor imaging (DTI), fluid-attenuated inversion recovery (FLAIR), and T_2WI performed in 20 patients with recent cerebral infarction in the basal ganglia, randomly divided into an acupuncture group and a control group [32]. The apparent diffusion coefficient (ADC) of infarction lesions, decreased at stroke onset, was showed significantly elevated after the acute stage, while the ADC of the bilateral cerebral peduncle was reduced on the infarction side. Fractional anisotropy (FA) values of abnormal signals on DTI in the infarction areas and cerebral peduncles underwent a significant reduction from stroke onset to the chronic stage. Interestingly, a significant difference in ADC and FA values between the two groups was observed, with a higher FA value in the acupuncture group than the control group, thus suggesting the effectiveness of acupuncture for **protecting neurons by postponing Wallerian degeneration** of brain infarction, and facilitating recovery [32].

5.5. Acupuncture and Arousal. The pathology of **disorders of consciousness** can be represented by (A) damage of Reticular Ascending System (B) large-scale damage to cerebral cortex, (C) injury to links (e.g., thalamus) between cerebral cortex and brain stem, and (D) injury to connections (e.g., corpus callosum) within the cerebral cortex, i.e., severe diffuse axonal injury (DAI).

The production of inhibitors (including GABA) induced by brain injury generates a response resembling automatic shutdown, probably aimed at conserving energy and promoting cell survival, but causing a comatose state [33]. Therefore, any treatment affecting the reticular activating system may be worth trying, and, among the possible treatments, acupuncture has the highest potential [34].

Recent studies on resting state (RS) in DOC, by using functional magnetic resonance imaging (fMRI), showed that functional connectivity is severely impaired above all in the Default Mode Network (DMN). In the vegetative and minimally conscious state, DMN integrity seems to correlate with the level of remaining consciousness.

The DMN is among the most robust networks found with resting state fMRI and encompasses the posterior cingulate cortex (PCC)/precuneus, mediofrontal/anterior cingulate cortex, and temporoparietal cortex [35]. Activity in the

DMN diminishes when the brain is involved in attention-demanding cognitive tasks [36] and returns to its prominent presence when no such task is being performed.

The DMN seems to be of particular interest, as its connectivity has been shown to decrease during loss of consciousness, and PET studies have shown an increase in neuronal activity in DMN regions (especially in the PCC/precuneus) upon recovery from VS [37]. Indeed, Vanhaudenhuyse [38] and Fernández-Espejo [39] et al. observed a correlation between the DMN integrity and the level of consciousness in noncommunicative brain-damaged patients.

Imaging evidence has been provided to support that electroacupuncture at **GV20**, employed to treat major depressive disorders, may modulate the Default Mode Network (**DMN)**, the cerebral functional network encompassing the posterior and anterior cortical midline structures, which is considered to be involved in stimulus-independent thought, mind-wandering, and self-consciousness. EA at **GV20** would increase functional connectivity (FC) between the precuneus/posterior cingulate cortex (PC/PCC) and bilateral anterior cingulate cortex (ACC) and reduce FC between the PC/PCC and left middle prefrontal cortex, left angular gyrus, and bilateral hippocampus/parahippocampus [40]. These findings are of particular importance when considering DOCs, where resting state network activity reveals reduced interhemispheric connectivity and correlates with levels of consciousness.

The acupoint *Shuigou* (**GV26**), placed at the junction of the upper one-third and lower two-thirds of the philtrum midline, also has been described as promoting the function of GV meridian, closely related to brain function, decreasing cognitive impairment, and promoting neurogenesis in the APP/PS1 transgenic mice [41].

Interestingly, enhanced bodily attention can be triggered by genuine acupuncture at **PC6** and **HT7** acupoints, which were exhibited to activate the salience network (insula, ACC, secondary somatosensory cortex, and superior parietal cortex) and deactivate the DMN (medial prefrontal cortex, PCC, inferior parietal cortex, and parahippocampus) [42].

Combined with western medicine, electroacupuncture therapy at *Baihui* (**GV20**), *Shuigou* (**GV26**), and *Yongquan* (**KI1**), resulted effective in improving consciousness recovery of patients in coma due to TBI, both reducing awake time and increasing awake rate, compared to a control group receiving only western treatments [43].

5.5.1. Autonomic Dysfunction in sABI. Autonomic nervous system (ANS) deregulation and/or dysautonomia is another severe consequence of brain injury, not well cleared. Dysautonomia affects in particular ninety percent of TBI patients during the first week, leading to sleep and heart rhythm disorders, and increasing specific biomarkers of neural damage [6]. Clinically, patients suffering from DOC can show the so called "paroxysmal sympathetic hyperactivity" (PSH), episodic sudden increase in vital signs, particularly heart rate, blood pressure, respiratory rate, and temperature, with possible diaphoresis (i.e., excessive sweating) and abnormal, unintentional movements, spontaneously or in response to external painful stimuli [44, 45]. A growing body of evidence suggests that ANS may mediate large-scale brain activation, in an extreme attempt to preserve body system homeostasis and regain consciousness [46]. The primary and secondary brain lesions have the potential to compromise both cortical and subcortical control mechanisms of the ANS.

Most often, TBI leads to sustained sympathetic activation, contributing to the high morbidity, with oxidative stress in the ANS and activated hypothalamic-pituitary-adrenal axis and hypothalamic-sympathoadrenal medullary axis [4].

5.6. Acupuncture and ANS Modulation. An increasing clinical evidence demonstrates that acupuncture is helpful in treating ANS dysfunctions, such as nausea and vomiting [47]. For example, acupoints stimulation has been shown to change the sympathovagal balance toward vagal predominance [48, 49].

Abnormalities in the ANS, such as sympathetic overactivation and/or parasympathetic hypoactivation, may generate and sustain chronic pain [50, 51].

Acupuncture at certain points could reduce sympathetic nervous system activity associated with pain [52] or during mental stress in patients with heart failure [53]. However, the neurobiological basis of these effects is not yet clear [54]. In order to explore the regulative effect on ANS by acupuncture, Sakatani and colleagues monitored **heart rate** by placing photoelectrical sensor on the first finger of eighteen healthy male adults, and thus low frequency (LF) amplitude (0.04–0.15 Hz) and high frequency (HF) amplitude (0.15–0.4 Hz) were calculated by power spectral analysis [54]. Real acupuncture performed at point Large Intestine 4 (LI4) of the right hand (r-LI4) was shown to determine significant decreases of HR and LF/HF and a significant increase of HF, indicating a parasympathetic activation as well as sympathetic depression [55].

Moreover, vagus nerve stimulation (VNS) increases metabolism in the forebrain, thalamus, and reticular formation [56]. It also enhances neuronal firing in the locus coeruleus, which leads to massive release of norepinephrine in the thalamus and hippocampus, a noradrenergic pathway essential for arousal, alertness, and the fight-or-flight response [57]. Recently, based on this rationale, Transcutaneous Auricular VNS (**taVNS),** a noninvasive stimulation developed for treating epilepsy and depression without the surgery-related risks [58, 59], was firstly employed by Yutian Yu and colleagues on a patient in vegetative state [60]. A further case with implanted VNS recovered behavioural responsiveness and enhanced brain connectivity patterns [61].

5.7. Acupuncture against Neuroendocrine Dysfunction. An imbalance of the pituitary and hypothalamus hormones and their axes is often associated with sABI, due to compression, edema, skull base fracture, haemorrhage, intracranial hypertension, or hypoxia. In the acute phase of sABI, low IGF-1 with elevated GH levels have been detected, with increasing IGF-1 and normalizing GH concentrations in the following weeks. The IGF-1 upregulation and the disruption of BEE that persists until 7 days after injury, allowing a wide level of hormone to reach the surviving neurons, probably play a

role in promoting neurite overgrowth, inducing progenitor cell differentiation and inhibiting neuron apoptosis [6].

In rat model of renal failure- (RF-) induced hypertension, stimulation with acupuncture, and most significantly with EA, at **ST36** and **KI3**, not only attenuated glomerulosclerosis and tubulointerstitial fibrosis, but corrected the decreases in RF-induced IGF-1 mRNA and protein levels, thus counteracting oxidative stress [62]. These finding may suggest the ability of acupuncture in restoring IGF-1 function in any situations where its levels are reduced, including sABI, although no studies have been conducted on this purpose.

5.7.1. Pain and DOC. The experience of pain in disorders of consciousness is still debated. Neuroimaging studies, using functional magnetic resonance imaging (fMRI) [63, 64], Positron Emission Tomography (PET) [65], multichannel electroencephalography (EEG), and laser-evoked potentials [66], suggest that the perception of pain increases with the level of consciousness.

VS and MCS are by definition incompatible with a reliable and consistent ability to communicate about pain experiences, while the nature of these conditions is characterized by various factors that can give rise to pain (e.g., spasticity, contractures, etc.) [67].

5.8. Acupuncture and Pain Relief. Many different mechanisms may explain the analgesic effect of acupuncture. Among these is the***gate control theory*** of pain proposed by Melzack and Wall in 1965 [68]. Specific nerve fibers would transmit pain to the spinal cord, while other nerve fibers inhibit pain transmission. Both groups of fibers met at the *substantia gelatinosa* in the spinal cord, where pain and pain inhibitory stimuli were integrated. Pain would be perceived only if the noxious input exceeded the inhibition of pain. However, gate control theory cannot explain the full spectrum of acupuncture effects, and in particular the prolonged pain relief.

Since the 1970s, the secretion of a range of biochemicals or neurotransmitters has been considered among the mechanism of acupuncture analgesia, such as **adenosine, opioid peptides, cholecystokinin octapeptide, 5-hydroxytryptamine, noradrenalin, glutamate, GABA, substance P, calcium ions, angiotensin II, somatostatin, arginine vasopressin, and dopamine** [69–81].

Different subtypes of opioid receptor were also believed to mediate the frequency-dependent electroacupuncture analgesia [82, 83]. For example, EA at a low frequency of 2 Hz would facilitate the release of enkephalin, but not dynorphin, while a high frequency of 100 Hz would stimulate the dynorphin but not enkephalin release in rats [82], as well as in humans [83]. The primary foundations for acupuncture effects seem to be **bioelectromagnetic**, while biochemical factors would be secondary. By the way, the opioid peptide secretion was recently proposed as being due to mechanical acoustic shear wave activation and calcium signalling induced by **needle rotation** [22].

Recently, the inflammatory reflex (via the ANS) has been observed as potentially crucial for the antihyperalgesic effect of acupuncture: by regulating the immune system it can elucidate not only the analgesic, but also the anti-inflammatory mechanism of acupuncture [76].

5.9. Acupuncture and Control of Spasticity. Spasticity is a frequent consequence of sABIs, arising from an anarchic reorganization of the pyramidal and parapyramidal fibers, and leading to hypertonia and hyperreflexia of the affected muscular groups and, if untreated, to possible irreversible joint lesions. Current treatment options include intrathecal baclofen and soft splints, botulinum toxin, or cortical activation by thalamic stimulation [84]. There is a low quality evidence for rehabilitation programs, extracorporeal shock-wave therapy, transcranial direct current stimulation, transcranial magnetic stimulation, and transcutaneous electrical nerve stimulation targeting spasticity, while a moderate evidence has been shown for electroneuromuscular stimulation and acupuncture as an adjunct therapy to conventional routine care (pharmacological and rehabilitation) [85]. In patients with DOCs, acupuncture at **GV26, Ex-HN3, LI4, and ST36** was proven to reduce spastic muscle hypertonia by decreasing the excitability of the spinal motor neurons, both ten minutes after needles insertion and ten minutes after their removal [86].

6. Final Considerations and Conclusions

The World Health Organization has recommended acupuncture in 1980 as an effective complementary therapy for several diseases. Among the indications, neurologic disorders have been shown to benefit from acupuncture.

In this review, we analysed scientific studies and clinical reports that explored the acupuncture's effects in several acquired brain injuries, aiming to

(1) limit brain secondary injury, by acting on systemic and local inflammation, oxidative stress, intracellular calcium overload, neuron regeneration, and growth factors release;

(2) manage sABI consequences, such as neuroendocrine and autonomic dysfunction, muscle spasticity, and pain.

Research in this field has obtained significant improvement with the technical support of the life sciences, and the studies of acupuncture have in turn accelerated the development of biomedical science. However, intrinsic aspects of this medical approach make it difficult to run a clinical study, and several data derive from animal studies or from small-size and heterogeneous samples of patients. Moreover, the acupoints selected for treating sABI can differ between research groups.

In addition, patients with disorders of consciousness are *per se* difficult to study. Given the impossibility of communicating with the patient, the content of consciousness can be only inferred by response behaviour. Diagnostic errors may depend not only on the operator but also on wakefulness fluctuations of the patient, who may be drowsy or agitated, or have epileptic seizures or aphasia. The neurophysiologic evaluations are made difficult by the presence of sweat of the head (which worsens the EEG impedance), muscle hypertonus (that obstructs mobilization), and noise from electromedical equipment, while functional imaging techniques are expansive and not easily available.

Table 3: Acupuncture and neuroplasticity.

MAIN MECHANISMS BY WHICH ACUPUNCTURE MAY INFLUENCE THE PHYSIOLOGIC PLASTIC REACTIONS TO BRAIN INJURY	
(i) Inhibition of brain neuronal apoptosis	*(vii) Reduction of blood-brain barrier permeability in intracerebral haemorrhage (caveolin-1/matrix metalloproteinase)*
(ii) Inhibition of aberrant astrocyte activation	*(viii) Regulation of blood lipid metabolism to counteract cerebral free radical damage*
(iii) Upregulation of neurotrophins expression	*(ix) Promoting cerebral vascular immunoinflammatory reactions*
(iv) Upregulate expression of GDNF	*(x) Increase of GABA level*
(v) Increased functional connectivity	*(xi) Reduce contents of excitatory amino acids*
(vi) Enhanced neuroblast proliferation and differentiation	

Figure 1: Role of acupuncture in the management of sABIs.

Further studies are needed to identify the most efficient and customized therapeutical protocol, aiming in particular at eliciting arousal.

The available data suggest that, in patients with sABIs/DOCs, acupuncture may represent an interesting frontier in the years ahead, as it seems to limit the secondary brain injury development, modulate ANS, and ameliorate their quality of life (Table 3, Figure 1). The absence of side effects or drug interactions make it particularly indicated for such fragile subjects.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Electroacupuncture Could Influence the Expression of IL-1β and NLRP3 Inflammasome in Hippocampus of Alzheimer's Disease Animal Model

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Background. Effective therapies for Alzheimer's disease (AD) are still being explored. Electroacupuncture with traditional Chinese medicine theory may improve spatial learning and memory abilities and glucose metabolism rates in an animal model of AD. However, the mechanism of electroacupuncture in intervention of AD is still unclear. According to recent studies of AD mechanisms, the NLRP3 inflammasome regulated the expression of IL-1β in the brain which may mediate AD related processes. Therefore, in our study, we intend to explore the possible relation between electroacupuncture and the expression of NLRP 3 inflammasome in the hippocampus of an AD animal model. *Method*. In this study, 7.5-month-old male senescence-accelerated mouse prone 8 (SAMP8) mice were used as an AD animal model, which were randomly divided into two groups: Alzheimer's disease model group (AD group) and electroacupuncture group (EA group). In the control paradigm, 7.5-month-old male SAMR1 mice were used as the normal control group (N group). DU20, DU26, and EX-HN3 were selected as the acupuncture points, and after a 15-day treatment of electroacupuncture, we used immunohistochemistry and Western blotting to examine the expression of IL-1β and NLRP3, ASC, and Caspase-1 in the hippocampus of the AD animal model. *Results*. Compared with N group, IL-1β, NLRP3, ASC, and Caspase-1 positive cells in AD group were increased, and the relative expression of all above proteins significantly increased ($P < 0.01$). Compared with AD group, the expression of IL-1β, NLRP3, ASC, and Caspase-1 in EA group was significantly decreased ($P < 0.01$). *Conclusion*. Electroacupuncture treatment could inhibit the inflammation reaction in the hippocampus of SAMP8 mice. What is more, the possible mechanism of electroacupuncture reduced the expression of IL-1β and NLRP3 inflammasome relative protein.

1. Introduction

Alzheimer's disease (AD), as the most common type of dementia, represents about 60%-80% of these diseases [1]. "World Alzheimer's Disease Report" [2] reported that there were currently 47 million people worldwide suffering from Alzheimer's disease, with a diagnosis every 3.2 seconds. With the aging global population aging, the number of AD patients will be greater than 131 million by 2050. In addition, recent studies indicate that the prevalence of AD in developing countries is particularly high [3]: in 2015, 58% of AD patients were from developing countries, but this figure is expected to increase in 2030 and 2050 to 63% and 68%, respectively. As a result, AD has become one of the major challenges facing the world (especially in developing countries) in the public health sector.

A large number of studies have concluded that there is no currently effective treatment to terminate or reverse AD. Therefore, the goal of clinical treatment of AD is to prevent the occurrence of AD, delay the disease process, and/or stabilize or improve its symptoms [4]. In traditional Chinese medicine theory, acupuncture therapy based on meridian and acupoints plays an active role in the clinical treatment of AD [5]. We have found that electroacupuncture could improve the spatial learning and memory ability and glucose metabolism rate level in an AD animal model [6, 7]. We have also reported that electroacupuncture could alleviate the neuroinflammation reaction in brain of ischemic stroke [8],

post-stroke cognitive impairments [9], and cognitive deficits of CCI rats [10]. However, the mechanism of electroacupuncture is still being explored for expanding the use of this therapy in real clinical situations.

Researchers are actively investigating the mechanisms of AD, and the accumulation of insoluble amyloid-β, hyperphosphorylation of Tau protein, and neuroinflammation caused by the innate immune system in the brain have gained greater attention [11]. Recent data emerging from genetic studies, clinical imaging, and animal experimentation point to an intimate interaction of innate immune system in AD processes [12]. Researchers have reported that, in NOD-like receptor (NLR) family, the pyrin domain containing 3 (NLRP3) inflammasome, which regulates the expression of IL-1β in brain, is active in neurodegenerative disease, especially in AD [13]. Amyloid β in brain, as one of the triggers of NLRP3 inflammasome, can induce the activation of the NLRP3 inflammasome and induce overexpression of IL-1β and neuroinflammation, ultimately accelerating AD [14]. Therefore, inhibiting activation of the NLRP3 inflammasome and expression of IL-1β is regarded as one possible therapy for AD [15].

What is the mechanism of electroacupuncture in intervention on AD? Could electroacupuncture inhibit the activation of NLRP3 inflammasome and expression of IL-1β in AD animal model? To answer these questions, we designed the present study. 7.5-month-old male senescence-accelerated mouse prone 8 (SAMP8) mice were used as an AD animal model, and immunohistochemistry and Western blot were used to examine the expression of IL-1β, NLRP3, ASC, and Caspase-1 proteins in hippocampus of the brain.

2. Materials and Method

2.1. Animals. Senescence-accelerated mouse prone 8 (SAMP8) mice and cognate normal senescence-accelerated mouse-R1 (SAMR1) mouse breeding pairs were kindly provided by Professor Takeda at Kyoto University, Japan. All animals were male and specific pathogen free (SPF), weighing 30±2 g. They were housed in a barrier facility at the Experimental Animal Centre of First Teaching Hospital of Beijing University of Traditional Chinese Medicine, under controlled temperature (24 ± 2°C) and 12h/12h dark-light cycle, with sterile drinking water and standard pellet diet *ad libitum*. All experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications number 80-23). Twenty 7.5-month-old male SAMP8 mice were divided into two groups (n = 10 per group), including SAMP8 Alzheimer's disease control (AD group) and electroacupuncture (EA group). Ten 7.5-month-old male SAMR1 mice composed the normal control (N) group.

2.2. Electroacupuncture Manipulation. In the EA group, electroacupuncture treatment was performed 20 minutes per day, one time a day for 15 days (no treatment on 8th day). Acupuncture points included DU20 *Baihui*, DU 26 *Shuigou*, and EX-HN3 *Yintang* (significant extra points); the locations of these points were according to the National Acupuncture Society for Experimental Research developed by the "laboratory animal acupuncture atlas". 0.5-inch needles (*Huatuo, Beijing, China*) were used for treatment. The pricking method was used for DU 26 *Shuigou*, and the flat thorn method for DU20 *Baihui* and EX-HN3 *Yintang* (needle depth was 0.5 cm). The needles were taped and connected to the HANS- LH202 electroacupuncture device (*Peking University Institute of Science Nerve and Beijing Hua Wei Industrial Development Company, Beijing, China*), with sparse wave at 2 Hz, 2 V, and 0.6 mA.

In the N and AD groups, no treatment was carried out other than handling and fixing once daily for 15 days, except on the 8th day, to ensure the same treatment conditions.

2.3. Immunohistochemistry. After the treatment, 4 mice were chosen from each group for immunohistochemical examination. They were anesthetized by intraperitoneal injection of 10% chloral hydrate at 0.35 mL/100 g body weight. Three minutes later, the chest was opened and the heart was exposed; intubation was performed from the left ventricle to the ascending aorta with quick injection of 100 ml saline. Then, the right atrial appendage was cut, and 4% paraformaldehyde was injected until the liver turned white with clear fluid flowing out from the right atrial appendage. After the perfusion, the mouse was decapitated and the whole brain extracted and placed on ice. Brains were then placed into 4% paraformaldehyde for paraffin embedding.

For immunohistochemistry, paraffin embedded brain tissue sections were deparaffinized with xylene and hydrated with graded alcohol. Then, the sections were treated with citric acid antigen repair buffer and washed with PBS (pH 7.4) three times with shaking, 5 minutes apart. After incubation with 3% hydrogen peroxide for 20 min in the dark to quench endogenous peroxidase, the sections were separately incubated with anti-Aβ1-42 antibody (1:50, ab10148), anti-NLRP3 antibody (1:500, orb76179), anti-ASC antibody (1:900, NBP1-68187), anti-Caspase-1 antibody (1:600, ab108362), and anti-IL-1β antibody (1:50, ab9787) overnight. Then, secondary antibodies were added for 30 min at room temperature, and detection was performed with DAB. Finally, the sections were dehydrated with graded alcohol and mounted. Micrographs of brain tissue samples were obtained at 400x magnification, and integral optical density (IOD) values were calculated using Image-Pro Plus 6.0 software.

2.4. Western Blotting. At the end of the treatment, the remaining 6 mice of each group were sacrificed by caudal dislocation. After decapitation, the whole hippocampus was dissected on a sterile working table. This procedure was performed on ice. The removed hippocampal tissue was placed in a cryopreserved tube, numbered, and stored in liquid nitrogen. After the operation, the hippocampus tissue was transferred to a refrigerator at – 80°C.

Total protein was extracted using cell lysate. Protein quantification was performed using the Bradford method. A 40 μg sample of protein was added to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Incubation of primary antibodies was performed overnight at 4°C. Antibody dilution was as follows: NLRP3 (1:300,), ASC (1:500,),

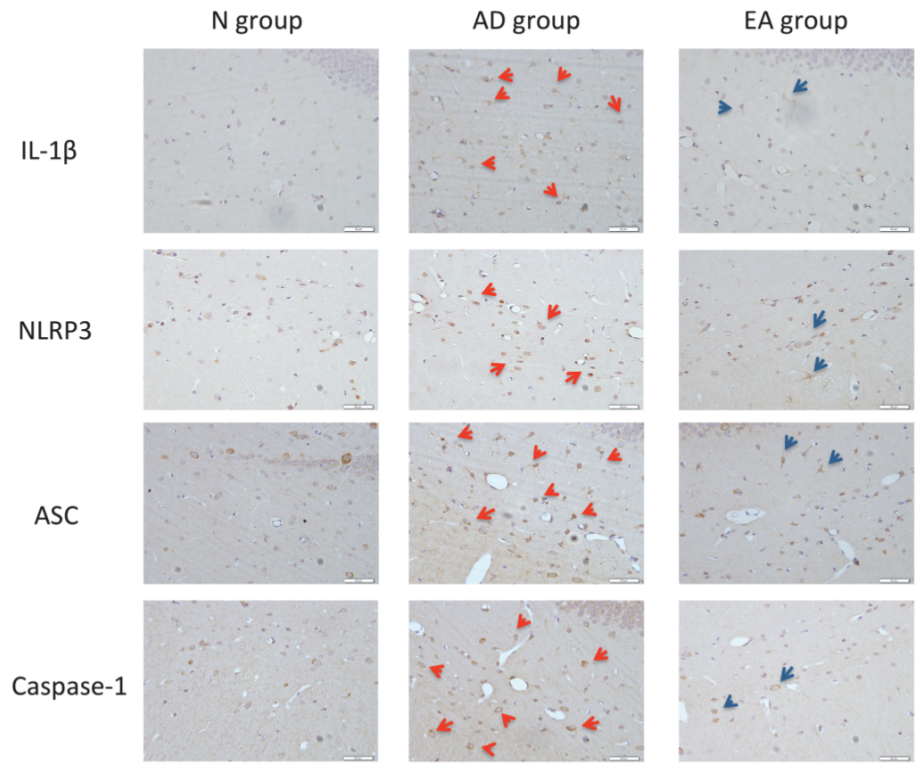

Figure 1: Expression of IL-1β and NLRP3 related proteins.

Caspase-1 (1:1000,), and IL-1β (1: 2,500,). This was followed by incubation with HRP-coupled anti-mouse or rabbit IgG antibody (1:5,000,) at 37°C for 1 hours. Target proteins on polyvinylidene fluoride membrane were visualized using ECL kit and captured using a BIO-RAD BioImaging System.

2.5. Statistical Analysis. All data are presented as mean ± SD for each group. One-way ANOVA was used with Western blot data. LSD tests were used to compare group pairs. Statistical significance was set at $p<0.05$. All statistical analyses were performed with the SPSS software V.17.0 (SPSS, USA).

3. Results

3.1. Expression of IL-1β and NLRP3 Related Proteins in Hippocampus: Seeing from Immunohistochemistry. In the immunohistochemical results (Figure 1), the number of positive cells expressing IL-1β protein in hippocampus of mice in N group was low, and occasional positive cells were also lighter in staining. Compared with N group, the number of IL-1β protein positive cells in the same field of view of the hippocampus of the AD group was significantly higher (highlighted by red arrows). Compared with AD group, the number of IL-1β positive cells in the same field of view of the EA group was reduced and the staining was lighter (highlighted by blue arrows).

In addition, the expression of NLRP3 and related proteins (ASC and Caspase-1) was also increased in the AD group (highlighted by red arrows). Interestingly, these proteins were associated with the expression of IL-1β. We could still visually distinguish that the expression of NLRP3 and related protein (ASC and Caspase-1) positive cells in the EA group was decreased compared with the AD group, with lighter staining (highlighted by blue arrows).

3.2. Expression of IL-1β and NLRP3 Related Proteins in Hippocampus from Western Blots. In the Western blot results (Figure 2), the relative expression of IL-1β and NLRP3 related proteins were obtained by comparing the gray value of each sample with β-actin, which was set as the internal reference.

Compared with N group, the expression of IL-1β protein in the hippocampus of the other two groups was significantly higher than that of N group (AD group: $P < 0.01$; EA group: $P < 0.01$). Compared with AD group, the expression of IL-1β protein in the hippocampus of the EA group was significantly decreased ($P < 0.01$, Figure 2(a)).

Compared with N group, the relative expression of NLRP3 protein in the hippocampus of other two groups was significantly higher (AD group: $P < 0.01$; EA group: $P < 0.05$). Compared with AD group, the expression of NLRP3 protein in the hippocampus of the EA group was significantly decreased ($P < 0.01$, Figure 2(b)).

Compared with N group, the relative expression of ASC protein in the hippocampus of other two groups was significantly higher (AD group: $P < 0.01$; EA group: $P < 0.05$). Compared with AD group, the expression of ASC protein in the hippocampus of the EA group was significantly decreased ($P < 0.01$, Figure 2(c)).

Compared with N group, the relative expression of Caspase-1 protein in hippocampus of AD group was

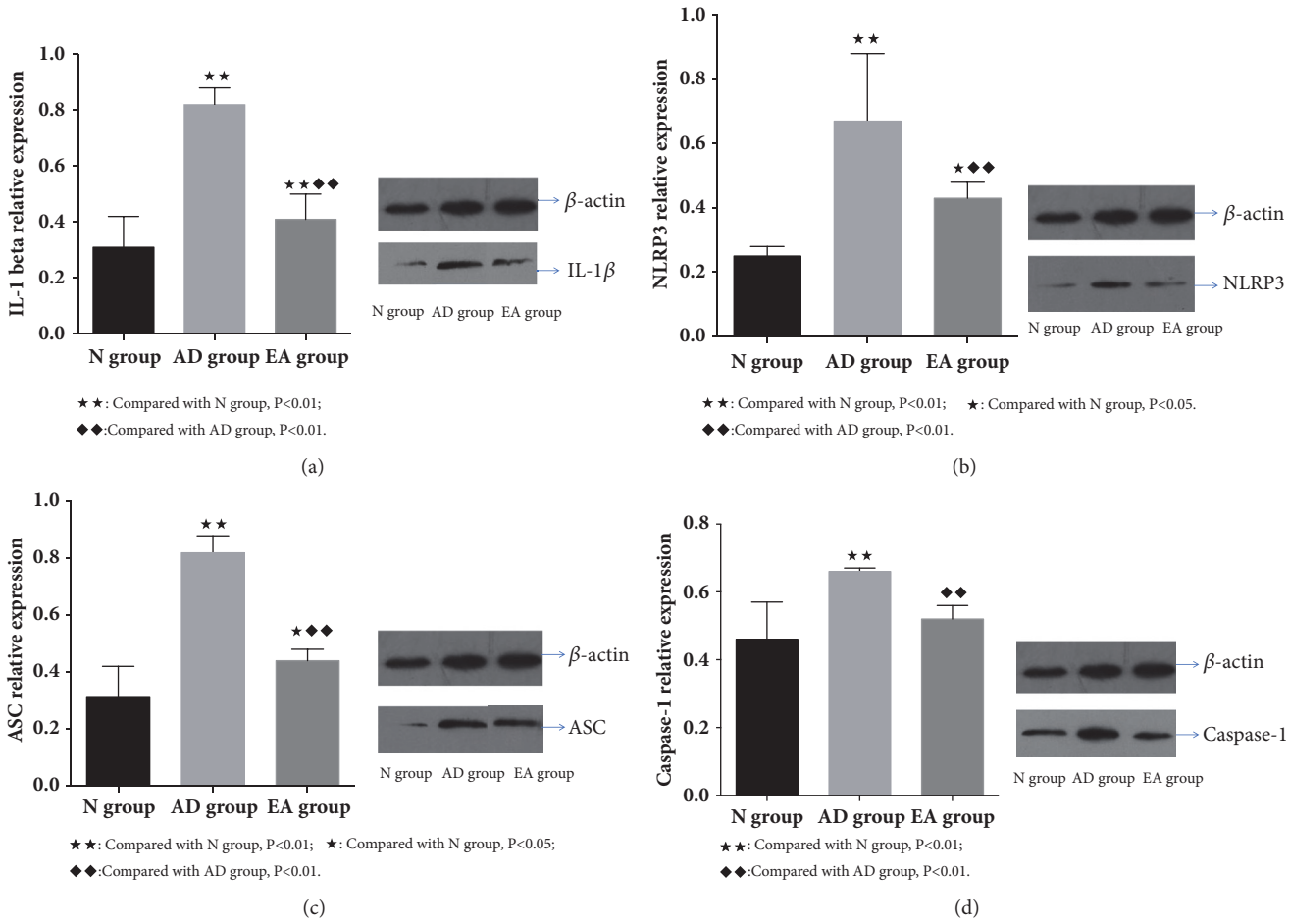

Figure 2

significantly higher ($P < 0.01$). Although the EA group was higher than N group, this was not statistically significant ($P = 0.212$). However, compared with AD group, the relative expression of Caspase-1 protein in the hippocampus of the EA group was significantly decreased ($P < 0.01$, Figure 2(d)).

4. Discussion

4.1. Neuroinflammatory Response with Alzheimer's Disease. Several pathological and molecular biology studies of AD have indicated that the brain inflammatory response was central to the pathological changes of AD [16]. This kind of inflammatory response is different from the classical symptoms of acute inflammation (swelling, discoloration, and pain); it is a chronic inflammatory response. The cells involved in this inflammatory response are astrocytes and microglia which can produce a variety of cytokines, such as interleukin 1α (IL-1α), interleukin 1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor (TNF), interleukin 8 (IL-8), macrophage inflammatory protein 1α, and monocyte chemo attractant protein 1, resulting in chronic inflammation, neuronal necrosis, and apoptosis, and eventually leading to impairment of cognitive function [17].

A large number of activated microglia and specific reactive astrocytes often accumulate around SPs [18]. Amyloid-β can have direct toxic effect on nerve cells and can also activate central nervous system microglia to release inflammatory mediators which trigger nerve cell injury signaling pathways, leading to neuronal damage [19]. Following activation of microglia cells, inflammatory factors can induce increases in amyloid-β precursor protein (APP) metabolism to increase amyloid-β, thereby increasing the inflammatory response reciprocally and accelerating the disease process [20]. Associated clinical studies have reported that long-term use of nonsteroidal anti-inflammatory drugs could reduce the incidence of Alzheimer's disease or delay its course [21]. Therefore, inhibition of the brain's neuroinflammatory response has become a novel target for treating Alzheimer's disease.

Previous work has concluded that the inflammatory mediator IL-1β is significantly increased in age-related brain diseases such as Alzheimer [22]. Our studies report a decreased expression of IL-1β following electroacupuncture in the hippocampus of the SAMP8 mouse brains. This suggests that electroacupuncture treatment could decrease central neuroinflammation via inhibition of IL-1β expression.

4.2. NLRP3 Inflammasome and Alzheimer's Disease. In the neurodegenerative progression of Alzheimer's disease, the deposition and aggregation of amyloid-β can activate a variety of receptors on the surface of microglia, stimulating

the releasing of inflammatory cytokines such as IL-1β [23]. Recent studies suggest that NLRP3 inflammasome in microglia could be a new target for the treatment of AD [24].

NLRP3 is a macromolecular protein complex with a molecular weight of about 700 kD that exerts an exogenous microbial or endogenous risk sensor in the cytoplasm [25]. It is a molecular platform for the activation of caspase-1, which regulates the maturation and secretion of inflammatory cytokines such as IL-1β, IL-18, and IL-33 [26, 27]. In recent years, studies have shown that endogenous risk signals for the activation of NALP3 inflammatory include ATP, uric acid, reactive oxygen species (ROS), Aβ [28], extracellular matrix components, and lysosomes. Therefore, it is likely that the NLRP3 inflammasome plays a key role in type II diabetes [29], Alzheimer's disease [30], and other noninfectious inflammatory diseases [31].

The NLRP3 inflammasome consists of a NLRP3 scaffold and three ASC and Caspase-1 precursor components which are involved in the regulation of IL-1β activation and secretion [32]. Amyloid-β stimulation of NALP3 inflammatory agents induces IL-1β maturation and release, causing neuroinflammation which may be involved in AD etiology [33–35].

Apoptosis-associated speck-like protein containing a CARD (ASC), an important class of cell adapter protein, is involved in the composition of the NLRP3 inflammasome. ASC has a two-domain structure, the oligomeric domain (PYD), and the effect domain (CARD). Through these two domains, NLRP3 and Caspase-1 precursors can be recruited to form the NLRP3 inflammasome [36]. Immunohistochemistry studies indicate that the expression of ASC in microglia is positively correlated with the severity of inflammation [37].

Caspase-1 can initiate and perform a series of cellular procedures that lead to inflammatory responses or apoptosis [38]. After the assembly of NLRP3 inflammasome, Caspase-1 precursor is used to catalyze the formation of active Caspase-1. Caspase-1, also known as IL-1β converting enzyme (ICE) [39], is an effector of inflammatory cells, mediating the transition of inactive IL-1β precursor into mature IL-1β [40].

4.3. The Possible Mechanism of Electroacupuncture in Alzheimer's Disease. The actions of the NLRP3 inflammasome and its regulation of IL-1β expression have been explored in animal model neural studies [35]. Furthermore, clinical studies report upregulation of mRNA for NLRP3 inflammasome and its downstream effector IL-1β in both mild and severe AD patients [14]. Therefore, our study focused on the NLRP3 inflammasome to investigate the mechanisms of electroacupuncture in the treatment of AD.

In this study, we qualitatively analyzed the expression of IL-1β, NLRP3, ASC, and Caspase-1 by immunohistochemistry. We report positive expression of the above proteins in the hippocampus of 7.5-month-old SAMP8 mice (AD group), with the expression of positive cells (microglia) in the cytoplasm. Combined with Western blot results, the relative expression of the proteins in the AD group was significantly higher than in the N group, indicating that NLRP3 inflammasome-mediated neuroinflammation was present in the hippocampus of the AD model animals.

After the 15 days of electroacupuncture treatment, the spatial learning and memory abilities and the glucose metabolism rates are improved in the AD model animal [41]. In addition, from the present immunohistochemistry and Western blot results, we also documented that the expression of the inflammatory mediator IL-1β in the EA group was significantly lower than the levels in the AD group ($P < 0.01$). We postulated that electroacupuncture may inhibit inflammation in the hippocampus of the brain. Interestingly, the relative expression of NLRP3, ASC, and Caspase-1 in the EA group was also significantly decreased compared with the AD group ($P < 0.01$). These data confirm our hypothesis that electroacupuncture can inhibit the neuroinflammatory response by modulating the NLRP3 inflammasome.

5. Conclusions

We report that electroacupuncture treatment can attenuate neuroinflammation in the hippocampus via inhibiting the expression of IL-1β, NLRP3, ASC, and Caspase-1. However, future genetic-level testing is needed to confirm and fully describe the beneficial effects of electroacupuncture.

Data Availability

All the data (tables and figures) used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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Review Article

Acupuncture on Obesity: Clinical Evidence and Possible Neuroendocrine Mechanisms

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Objective. Acupuncture, as one of the complementary and alternative medicines, represents an efficient therapeutic option for obesity control. We conducted a meta-analysis to investigate the effectiveness of acupuncture in obesity and also summarized the available studies on exploring the mechanisms. *Design*. We searched six databases from the inception to April 2017 without language restriction. Eligible studies consisted of acupuncture with comparative controls ((1) sham acupuncture, (2) no treatment, (3) diet and exercise, and (4) conventional medicine). The primary outcomes consisted of BMI, body weight reduction, and incidence of cardiovascular events (CVD). Secondary outcomes included waist circumference (WC), waist-to-hip ratio (WHR), body fat mass percent, body fat mass (kg), total cholesterol (TC), triglyceride (TG), glucose, low density lipoprotein cholesterol (LDL-c) reduction, high density lipoprotein cholesterol (HDL-c) increase, and adverse effects. The quality of RCTs was assessed by the Cochrane Risk of Bias Tool. Subgroup analyses were performed according to types of acupuncture. A random effects model was used to adjust for the heterogeneity of the included studies. Publication bias was assessed using funnel plots. *Main Results*. We included 21 studies with 1389 participants. When compared with sham acupuncture, significant reductions in BMI (*MD*=-1.22, *95%CI*=-1.87 to -0.56), weight (*MD*=-1.54, *95%CI*=-2.98 to -0.11), body fat mass (kg) (*MD*=-1.31, *95%CI*=-2.47 to -0.16), and TC (*SMD*=-0.63, *95%CI*=-1.00 to -0.25) were found. When compared with no treatment group, significant reductions of BMI (*MD*=-1.92, *95%CI*=-3.04 to -0.79), WHR (*MD*=-0.05, *95%CI*=-0.09 to -0.02), TC (*MD*=-0.26, *95%CI*=-0.48 to -0.03), and TG (*MD*=-0.29 *95%CI*=-0.39 to -0.18) were found. When compared with diet and exercise group, significant reduction in BMI (*MD*=-1.24, *95%CI*=-1.87 to -0.62) and weight (*MD*=-3.27 *95%CI*=-5.07 to -1.47) was found. Adverse effects were reported in 5 studies. *Conclusions*. We concluded that acupuncture is an effective treatment for obesity and inferred that neuroendocrine regulation might be involved.

1. Introduction

Obesity is a chronic disease characterized by the rise of body fat stores. It is caused by the interaction of genetic, dietary, lifestyle, and environment factors. The prevalence of obesity among children, adolescents, and adults has been dramatically increased during the last decades. Obesity and overweight are closely related to type 2 diabetes, hypertension, and coronary heart disease [1]. The World Health Organization (WHO) indicates that more than 1.9 billion adults, 18 years and older, were overweight. Of these, over 650 million were obese in 2016. According to a report in the JAMA Journal of Internal Medicine, more than two-thirds of people in the United States are considered to be overweight or obesity [2]. Data from China's fourth national physical fitness survey in 2014 showed that the morbidity of obesity in adult and the aged reached 10.5% and 13.9%, respectively, which showed 0.6% and 0.9% increase in comparison to the data in 2010. Moreover, the epidemics of obesity and overweight are not limited in developed countries, and the prevalence also increases among people in developing countries.

Obesity can be defined by BMI. According to the WHO definition, a BMI over 25kg/m^2 is taken as overweight and over 30kg/m^2 as obese. In terms of the physique of the Asia-Pacific population and the characteristics of obesity-related disease, the WHO obesity adviser group agrees that BMI over 23kg/m^2 is defined as overweight and over 25kg/m^2 as obese. In addition, other guidelines also include parameters such as WC and WHR to define obesity.

Numerous people cannot manage the weight only through dietary change and increasing physical activity. Although pharmaceutical treatments for obesity such as Fenfluramine and Sibutramine are effective, there exist various limits due to security reasons [3–6]. As an alternative intervention for obesity, acupuncture is relatively easy, cheap, and safe and has been widely used in clinical practice [5–8] in both China and other countries. Although efficacy of the acupuncture therapy has been reported, the underlying mechanisms have not been completely illustrated. Therefore, we conducted a systematic review and meta-analysis to evaluate the effectiveness of acupuncture in obesity and also summarized the present studies on exploring mechanisms under acupuncture treatment in obesity animals.

2. Methods

2.1. Search Strategy. To identify studies of acupuncture on obesity, retrievals were implemented in three English databases (PubMed, EMBASE, and Cochrane Library) and three Chinese databases (VIP information database, Chinese National Knowledge Infrastructure, and Wanfang Data Information Site) from the inception to April 2017. The search strategies were (weight loss OR overweight OR obesity OR weight control OR simple obesity OR weight reduction OR weight increase OR weight decrease OR weight watch OR overeat OR overfeed OR slim) AND (acupuncture and moxibustion OR acupuncture OR embedding therapy OR acupoint catgut embedding OR electro-acupuncture OR EA OR auricula-acupuncture OR ear seed pressure OR auricular plaster OR auricular acupuncture OR auricular acupressure OR fire needle OR moxibustion OR herbal acupuncture OR dermal needle OR aqua acupuncture OR body acupuncture OR meridians OR abdominal acupuncture) AND (clinical trial OR clinical study OR efficacy OR effectiveness) AND (random OR random$). Conference proceedings, dissertations, and reference lists of retrieved articles were also searched manually for additional relevant studies.

2.2. Inclusion and Exclusion Criteria

2.2.1. Types of Studies. Published randomized controlled trials (RCTs) compared acupuncture with control (no treatment, placebo acupuncture, western medicine, diet or exercise, etc.) and assessed the efficacy of acupuncture on obesity and overweight. We excluded quasi-randomized studies, such as those allocated by using alternate days of week. No restriction was imposed on blinding. Comments, case reports, technical reports, animal studies, self-control studies, or non-RCTs were excluded. No language restriction was made for selecting the studies.

2.2.2. Types of Participants. We included participants with no limitation of age and gender. All appropriate definitions of overweight or obesity including BMI, body weight, or percentage of weight excess compared with ideal weight were accepted. A diagnosis of simple obesity patients was included. The secondary obesity which was complicated with hypothalamus disease, anterior hypopituitarism, hypothyroidism, hypercriticism, hypogonadotropic hypogonadism, pregnancy, lactation, polycystic ovarian syndrome (PCOS), menstrual disorder, amenorrhea, or other serious medical conditions was excluded.

2.2.3. Types of Intervention. We recruited trials with various acupuncture therapies. The acupuncture therapy included classical acupuncture, electroacupuncture (EA), laser acupuncture, catgut embedding, auricular acupressure, and auricular acupuncture, which could be analyzed in subgroup. Studies that combined acupuncture with other therapies such as medication, moxibustion, or message were excluded; the studies with lifestyle intervention such as diet and exercise in treatment group were also included. Based on the different acupuncture therapies, we performed the subgroup analysis. The control inventions were divided into four types, sham acupuncture ((1) needle inserting into skin but not penetrating the exact acupoints; (2) needle inserted into an area where it is near the exact acupoints), no treatment, diet, and exercise therapy, medicine.

2.2.4. Types of Outcome Measures. The primary outcomes consisted of BMI, body weight reduction, and the incidence of CVD. Secondary outcomes included WC, WHR, body fat mass percent, body fat mass (kg), serum cholesterol (TC), triglyceride (TG), glucose, low density lipoprotein cholesterol (LDL-c) reduction, high density lipoprotein cholesterol (HDL-c) increase, and adverse effects.

2.3. Study Selection, Data Extraction, Management, and Analysis. According to the prespecified inclusion and exclusion criteria, two authors (Kepei Zhang and Chunyan Wang) separately identified the eligible studies by reading the title, abstract, and full text of every paper and then extracted the data. A discussion with the other authors was conducted to solve any discrepancies.

The following information was abstracted from all included publications: year, country, number of included patients, interventions of treatment and control groups, basic treatment, duration of treatment, adverse reactions, and outcomes. Authors of studies were contacted for clarification when necessary.

The quality of RCTs was assessed by the Cochrane Risk of Bias Tool, including seven domains: generation of a random sequence, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, completeness of outcome data, selectiveness of reporting, and other biases. A score of 1 or 0 was given for each item depending on the information provided by study (1, low risk of bias, the information of the domain was adequate in the text; 0, high risk of bias, the information of the domain was inadequate in the text). The studies with the cumulative score of at 3 or more were included in our study.

Cochrane Review Manager (RevMan 5.3) software was used for statistical analysis. Binary data were reported as risk ration (RR), and continuous data were reported as mean difference (MD) when the outcomes were measured in the same way among different trials. For trials reporting the same outcome measures but used different methods,

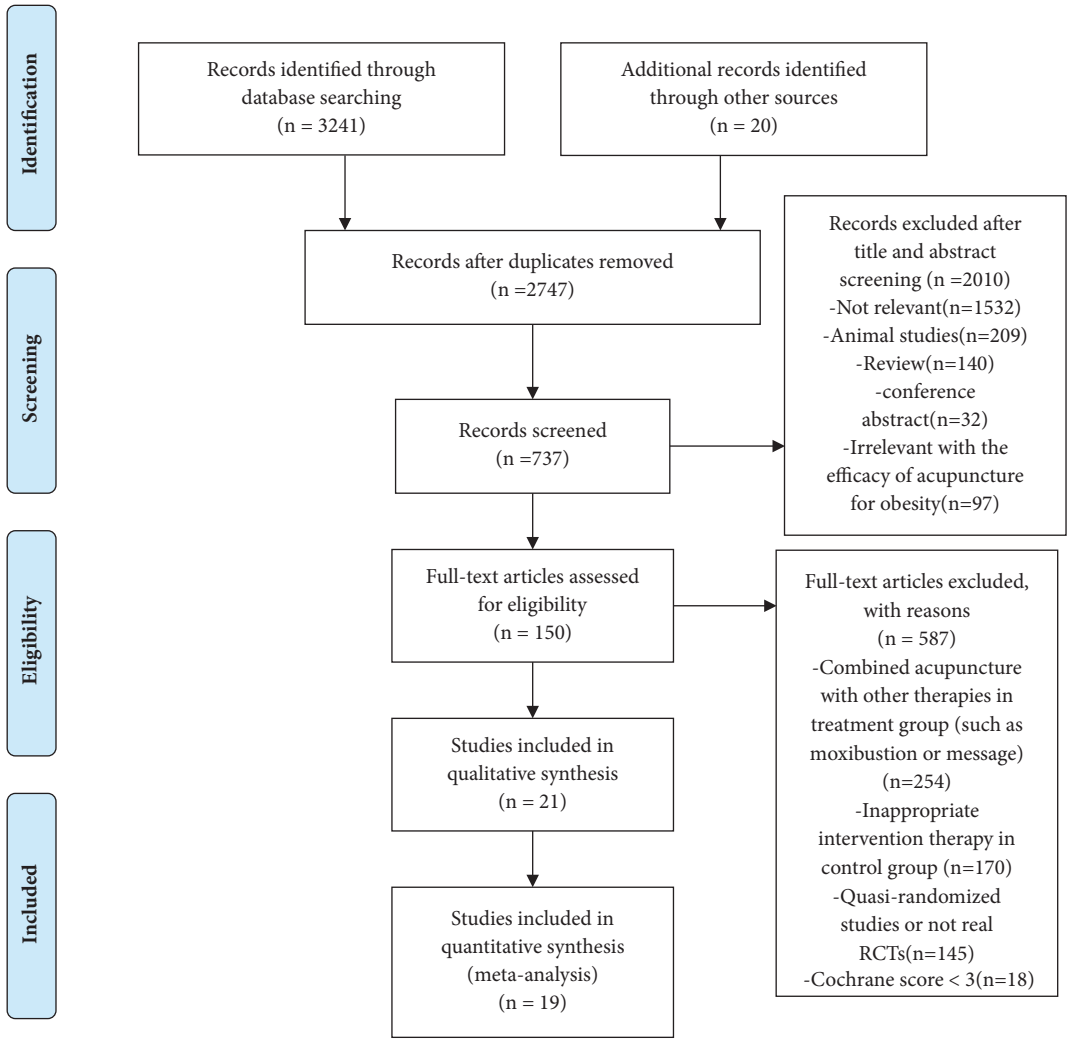


Figure 1: Flow diagram or the number of studies included and excluded.

the standardized mean difference (SMD) was reported. 95% confidence interval (95%CI) was used as an effective size for the combined analysis. A random effects model was conducted to analyze pooled effects. We tested heterogeneity using the Chi^2 statistic (with significance being set at $P<0.1$) and the I^2 statistic. I^2 value above 50% was set as substantial heterogeneity. Possible sources of heterogeneity were assessed by sensitivity and subgroup analysis. The existence of publication bias was checked using a funnel plot.

3. Results

3.1. Study Description and Quality Assessment. A total of 3261 potentially relevant papers were retrieved. 514 duplicate records were removed. 737 articles were remaining after the scan of titles and abstract. 2010 articles (including not relevant, animal studies, review, conference abstract, and irrelevant with the efficacy of acupuncture for obesity) were excluded. 150 articles were selected after screening full-text articles. 587 articles (including studies which combined acupuncture with other therapies in treatment group, inappropriate intervention therapy in control group, quasi-randomized studies or not real RCTs, and Cochrane score<3) were excluded. Finally, we included 21 studies [9–29]: 7 [9–12, 17, 20, 25] in English and 14 [13, 16, 18–21, 21, 22, 22, 23, 23, 24, 24–26] in Chinese. 19 studies [9, 10, 12, 13, 15–29] were included in meta-analysis. The screening process is summarized in **Figure 1**. The sample size of the included studies ranged from 9 to 43, enrolling a total of 1389 participants altogether, 760 patients in the treatment group and 629 patients as the control. Meanwhile, among these studies, the study by Han 2016 [13] included two independent experiments, so we divided this study into Han-1 2016 and Han-2 2016. The study by Darbandi et al. 2014 [11] included one experiment, which contributed four independent comparisons. Descriptive analysis was used in this study. The basic characteristics of studies included are summarized in **Table 1**. Of these 22 records, there were 12 records [9–12, 15, 16, 18, 21, 24–26, 29] reporting the effect of acupuncture versus sham acupuncture, 5 [13, 17, 19, 27] acupuncture versus no treatment, 4 [20, 22, 23, 29] acupunctures versus diet and exercise, and 1 [14] acupuncture versus medicine.

Table 1: Characteristics of the included studies.

Included studies	Country	Number of participants Trial	Control	Interventions Trial	Control	Basic treatment	Duration of treatment (week)	Adverse reactions	Outcomes
Darbandi et al., 2012 [9]	Iran	43	43	Auricular acupressure	Sham auricular	Low-calorie diet	6	None	BW, BMI, BFM, plasma leptin
Darbandi et al., 2013 [10]	Iran	42	44	Electro-acupuncture	Sham acupuncture	Low-calorie diet	6	None	BM, BMI, BFM, plasma leptin
Darbandi et al., 2014 [11]	Iran	20/20	20/20	Body electro-acupuncture/ Auricular acupuncture	Sham body electro-acupuncture/ sham Auricular acupuncture	Low-calorie diet	6	None	Height, WHR, BMI, trunk fat mass, Cr, Albumin, Uric acid, FBS, HLD-c, LDL-c, WBC, RBC
Gucel et al., 2012 [12]	Turkey	20	20	Body acupuncture	Sham acupuncture	None	5	Not reported	Weight, BMI, insulin, leptin, ghrelin, cholecystokinin
Han-1, 2016 [13]	China	36	37	Body electro-acupuncture	No treatment	Dietary and exercise	4	2 Subcutaneous bleeding and hematoma	BMI, WC, hip circumference, WHR and spleen dampness improve the situation of symptom score
Han-2, 2016 [13]	China	40	41	Body electro-acupuncture	No treatment	Dietary and exercise	4	3 Subcutaneous bleeding and hematoma	BMI, WC, hip circumference, WHR and spleen dampness improve the situation of symptom score
He et al., 2008 [14]	China	40	40	Body electro-acupuncture + Auricular acupuncture	Sibutramine	None	8	No reported	Body weight, BMI, waist and hip circumference and WHR
Hsu et al., 2009 [15]	Taiwan	23	22	Auricular acupuncture	Shame auricular acupuncture	None	6	1 Minor inflammation, 8 mild tenderness	Body weight, BMI, WC, obesity-related hormone peptides
Hung et al., 2016 [16]	Taiwan	32	30	Verum laser acupuncture	Sham laser acupuncture	None	3	None	BMI, BFP, waist-to-buttock ratio
Kim et al., 2014 [17]	Korea	25	24	Auricular acupressure	No treatment	None	4	None	Weight, BMI, body fat mass percentage, WHR
Lien et al., 2012 [18]	Taiwan	48	23	Auricular stimulation	Sham auricular acupuncture	None	8	1 dizziness	BMI, weight, obesity-related hormone peptids, life quality scores

Table 1: Continued.

Included studies	Country	Number of participants Trial	Control	Interventions Trial	Control	Basic treatment	Duration of treatment (week)	Adverse reactions	Outcomes
Luo, 2006 [19]	China	40	20	Electro-acupuncture / only acupuncture	No treatment	None	3	Not reported	WHR, MBI, TC, TG, HDL-c, LDL-c, LEP, Adiponectin(ADI)
Nourshahi et al., 2009 [20]	Iran	9	9	Acupuncture	No treatment/diet and exercise	Diet and exercise	8	Not reported	Body weight, skin fold thickness, BMI, fat mass
Tong, 2011 [21]	China	76	42	Acupuncture	Placebo-acupuncture	Diet	5	None	BMI, TC, TG, Glucose, BUN, Uric Acid and adverse reactions
Xing, 2009 [22]	China	31	30	Electroacupuncture	Diet and exercise	Diet and exercise	8	No reported	Body weight, BMI, TG, TC, LDL-c, HDL-c, WHR, Leptin
Xiong et al., 2016 [23]	China	29	21	Acupuncture	Exercise	None	4	No reported	Body weight, BMI
Yeh et al., 2015 [24]	Taiwan	36	34	Auricular electrical stimulation +auricular acupressure	Same manner but at sham acupoints	Diet	10	No reported	BMI, blood pressure, TC, TG, Leptin, Adiponectin
Yeo et al., 2014 [25]	Korean	43	15	Ear acupuncture	Sham acupuncture	None	8	No reported	BMI, WC, weight, body fat mass (kg), percentage body fat and blood pressure
Zhang et al., 2012 [26]	China	15	15	Electro-acupuncture	Sham Electro-acupuncture	None	4	1 Bleeding	Weight, BMI, body fat mass %
Zhang, 2012 [27]	China	29	28	Catgut embedded	No treatment	Diet and exercise	8	The bleeding rate was 18.75% in treatment group	Weight, BMI, waistline, hipline, circumference, quality of life
Zhao et al., 2011 [28]	China	35	35	Electro-acupuncture	Sham Electro-acupuncture	None	4	No reported	BMI, Weight
Zhao, 2010 [29]	China	30	30	Electro-acupuncture	Diet and exercise	Diet and exercise	8	No reported	BMI, waist, FBS, 2hPG, FINS, 2hFINS

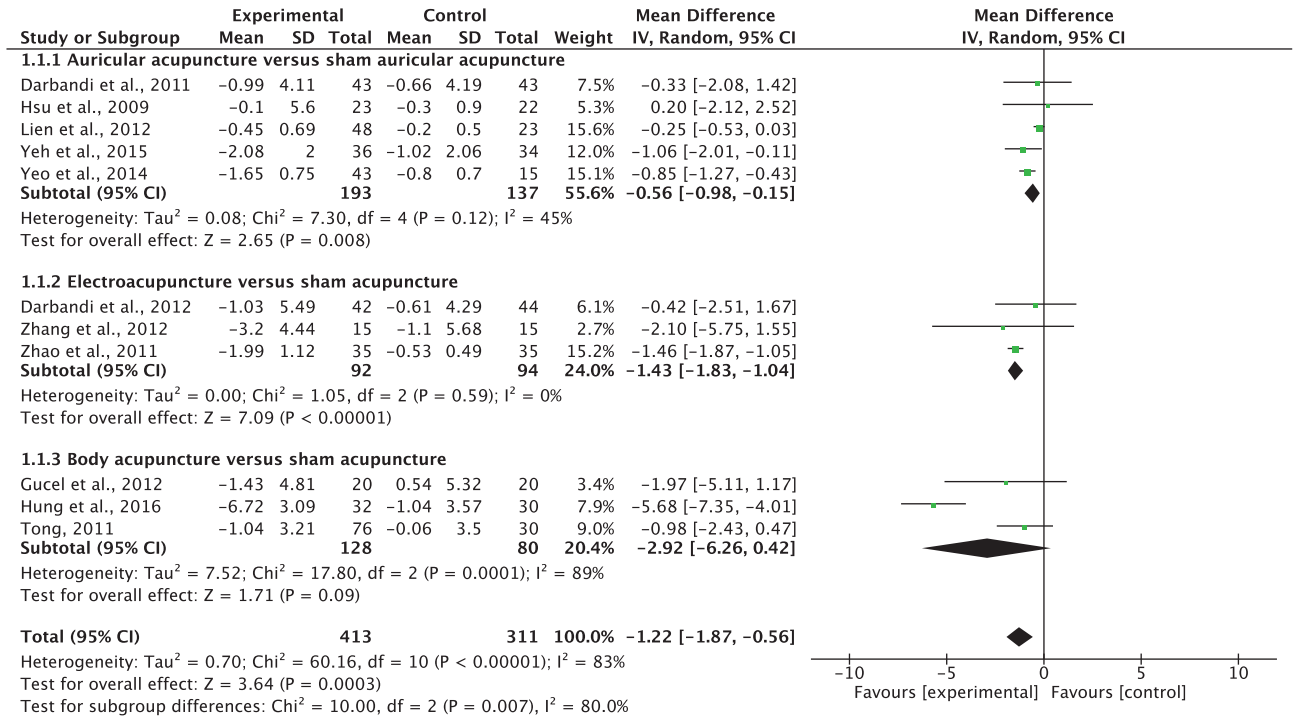
The quality assessment of the included studies is summarized in **Table 2**. The majority of studies included had more or less methodological weakness according to the quality criteria applied. Of the 22 records, 1 [20] fulfilled three, 11 [9–12, 15, 17–19, 23, 24, 28] fulfilled four, 4 [14, 22, 25, 28] fulfilled five, 3 [16, 21, 27] fulfilled six, and 3 [13, 26] fulfilled seven. All records had random allocation, 14 [9–11, 13, 15, 18, 20–28] in used random number table, 4 [14, 17, 19, 29] employed draw lots, 1 [12] used urn randomization, 1 [16] performed permuted block randomization, and 1 [20] randomly listed names and assigned them to three groups. Moreover, 10 [11, 13, 15, 16, 18, 21, 24–26] mentioned blinding of participants and personnel and 7 [13, 14, 21, 26, 27] mentioned blinding of outcomes. In addition, 8 [13, 16, 22, 24–28] reported the plan of allocation and concealment. Two [20, 28] had no information about withdraws but provided complete outcome data. Three [15, 24, 25] had a loss to follow-up more than 15%.

3.2. Effectiveness

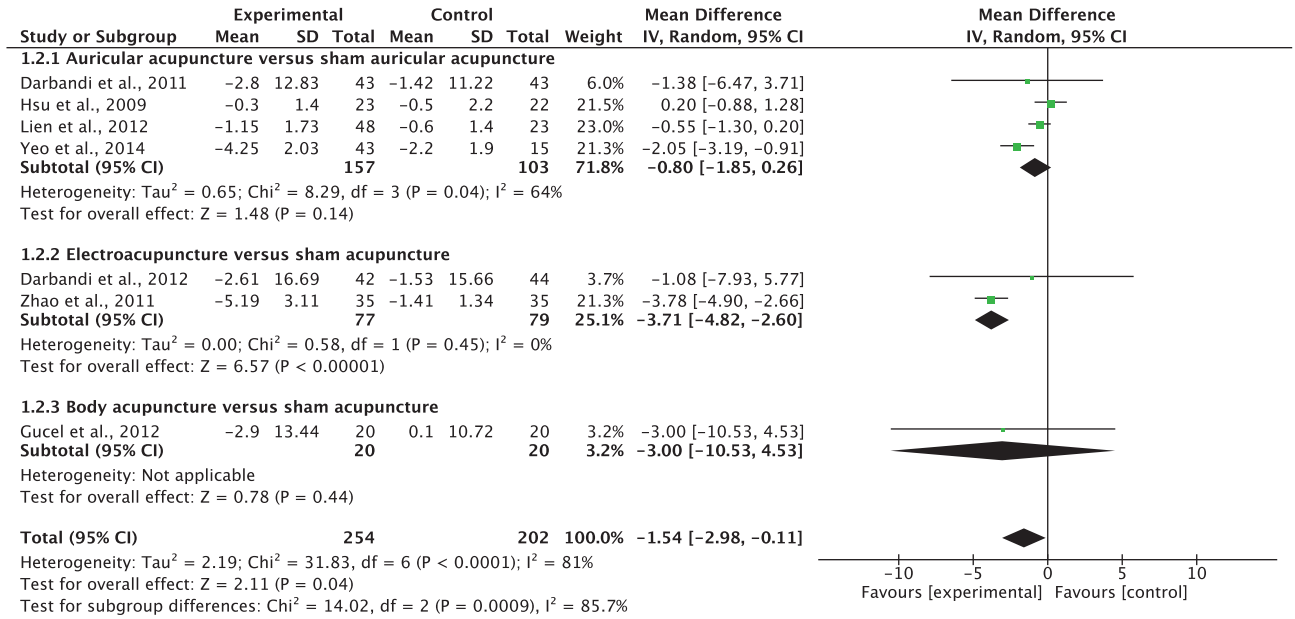
3.2.1. Acupuncture versus Sham Acupuncture. A total of 12 records [9–12, 15, 16, 18, 21, 24–26, 29] showed significant difference in BMI reduction between the acupuncture and sham acupuncture. The random effects model was used (*MD*=-1.22, *95%CI*=-1.87 to -0.56); the high heterogeneity was detected (heterogeneity: Chi^2 =60.16, *df* =10 (*P*<0.00001); I^2=83%). Sensitivity analysis was conducted to explore potential source of heterogeneity, which yielded I^2 ⩾50% results after the omission of each individual study. Subgroup analyses were performed based on different acupuncture therapies. Results showed that both auricular acupuncture and EA significantly reduced BMI compared with control group (*MD*=-0.56, *95%CI*=-0.98 to -0.15; *MD*=-1.43, *95%CI*=-1.83 to -1.04; **Figure 2(a)**). The funnel plots were bilateral asymmetry, suggesting the publication bias may exist. A total of 7 records [9, 10, 12, 15, 18, 25, 29] reported the data of weight loss. There was significant difference between two groups. The random effects model was used (*MD*=-1.54, *95%CI*=-2.98 to -0.11); high heterogeneity was detected (heterogeneity: Chi^2 =31.83, *df* =6 (*P*<0.00001); I^2=81%). Subgroup analyses showed that EA significantly reduced weight compared with control group (*MD*=-3.71, *95%CI*=-4.82 to -2.60; **Figure 2(b)**). Two records [16, 18] showed no difference in WHR loss between two groups. One record [26] showed that EA significantly reduced WHR compared with control group. Three records [15, 18, 25] showed no difference in WC loss between two groups. The random effects model was used (*MD*=-0.56, *95%CI*=-2.03 to 0.91; **Figure 2(c)**); high heterogeneity was detected (heterogeneity: Chi^2 =4.41, *df* =2 (*P*=0.11); I^2=55%). One record [11] showed auricular acupuncture and EA significantly reduced WC compared with control group. Three records [9, 10, 25] showed that the acupuncture significantly reduced body fat mass (kg). The random effects model was used (*MD*=-1.31, *95%CI*=-2.47 to -0.16; heterogeneity: Chi^2 =0.14, *df* =2 (*P*=0.93); I^2=0%). Subgroup analyses showed that auricular acupuncture significantly reduced body fat mass (kg) compared with control group (*MD*=-1.32, *95%CI*=-2.55 to -0.10; **Figure 2(d)**). Three records [16, 25, 26] showed no significant difference between two groups in body fat mass percentage reduction. Two records [15, 24] showed that the auricular acupuncture significantly reduced serum total cholesterol (TC) in patients. The random effects model was used (*SMD*=-0.63, *95%CI*=-1.00 to -0.25; heterogeneity: Chi^2 =0.05, *df* =1 (*P*=0.81); I^2=0%; **Figure 2(e)**). Two records [15, 24] showed no significant difference in reducing TG in patients between groups. The random effects model was used (*SMD*=-0.35, *95%CI*=-0.72 to 0.02; heterogeneity: Chi^2 =0.49, *df* =1 (*P*=0.48); I^2=0%; **Figure 2(f)**). Two records [15, 21] showed no significant difference in reducing glucose in patients. One record [15] showed no significant difference in HDL-c and LDL-c between two groups. One [26] case of bleeding after treatment was reported in 1 record. A few participants developed minor inflammation and had mild tenderness at the acupuncture sites during the treatment in 1 record [15]; no major adverse effects were seen during the study. A subject in treatment group experienced dizziness immediately after auricular acupuncture in 1 record [18]. Slight bleeding was observed in 1 record [27].

3.2.2. Acupuncture versus No Treatment. Five records [13, 17, 19, 27] showed acupuncture significantly reduced BMI. The random effects model was used (*MD*=-1.92, *95%CI*=-3.04 to -0.79); high heterogeneity in the data was detected (heterogeneity: Chi^2 =17.51, *df* =4 (*P*=0.002); I^2=77%). Subgroup analyses showed that EA significantly reduced BMI compared with control group (*MD*=-2.69, *95%CI*=-4.93 to -0.45; **Figures 2(g) and 3**). Three records [13, 27] reported the data of weight loss. There was no difference between two groups. The random effects model was used (*MD*=-3.08 *95%CI*=-6.91 to 0.74); heterogeneity in the data was detected (heterogeneity: Chi^2 =4.52, *df* =2 (*P*=0.10); I^2=56%). Subgroup analyses showed that there was no difference between EA and control group in weight loss (*MD*=-5.25, *95%CI*=-10.58 to 0.08; **Figure 2(h)**). Five records [13, 17, 19, 27] showed acupuncture significantly reduced WHR. The random effects model was used (*MD*=-0.05, *95%CI*=-0.09 to -0.02); high heterogeneity in the data was detected (heterogeneity: Chi^2 =22.16, *df* =4 (*P*=0.0002); I^2=82%). Subgroup analyses showed that EA significantly reduced WHR compared with control group (*MD*=-0.06, *95%CI*=-0.11 to -0.02; **Figure 2(i)**). One record [27] showed acupuncture significantly reduced WC compared with control group. Two records [13, 19] showed the acupuncture significantly reduced TC. The random effects model was used (*MD*=-0.26, *95%CI*=-0.48 to -0.03; heterogeneity: Chi^2 =0.64, *df* =1 (*P*=0.42); I^2=0%; **Figure 2(j)**). Two records [13, 19] showed the acupuncture significantly reduced TG. The random effects model was used (*MD*=-0.29 *95%CI*=-0.39 to -0.18; heterogeneity: Chi^2 =0.01, *df* =1 (*P*=0.90); I^2=0%; **Figure 2(k)**). One record [19] showed the acupuncture significantly changed LDL-c in patients. One record [19] showed no significant difference in HDL-c between two groups. Subcutaneous bleeding or hematoma after treatment was reported in 2 records [13].

3.2.3. Acupuncture versus Diet and Exercise. The efficacy of acupuncture was compared to diet and exercise in 4 records



(a)



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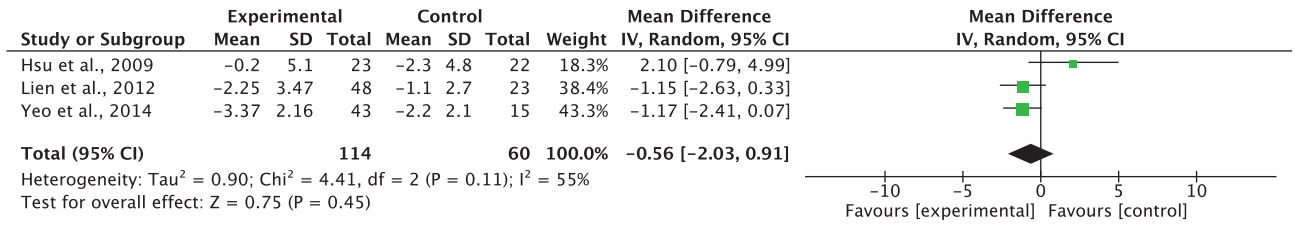

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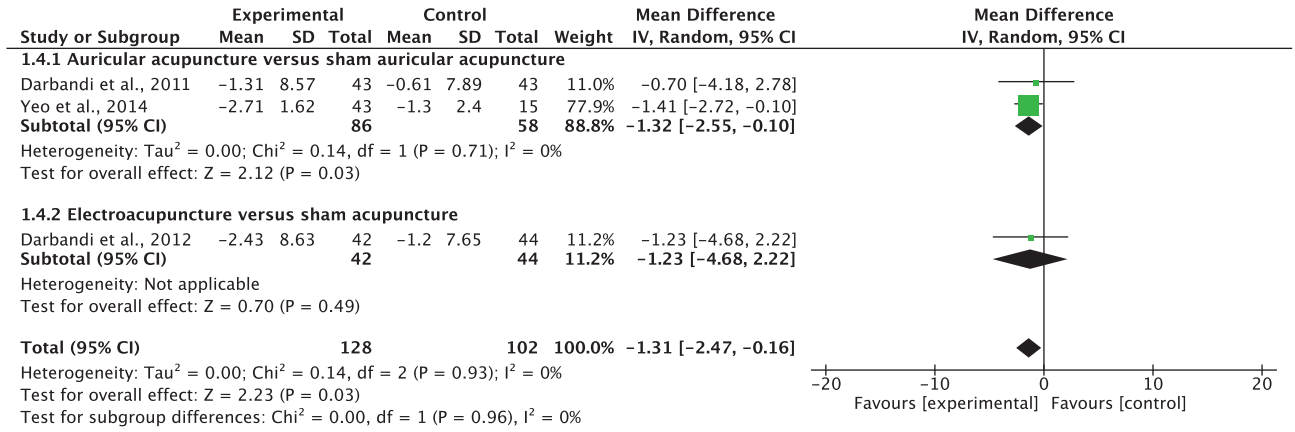

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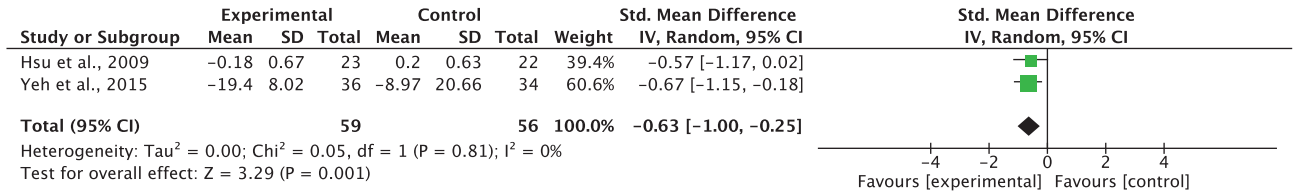

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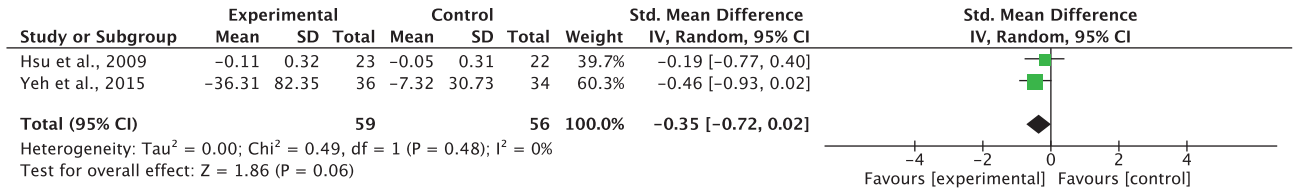

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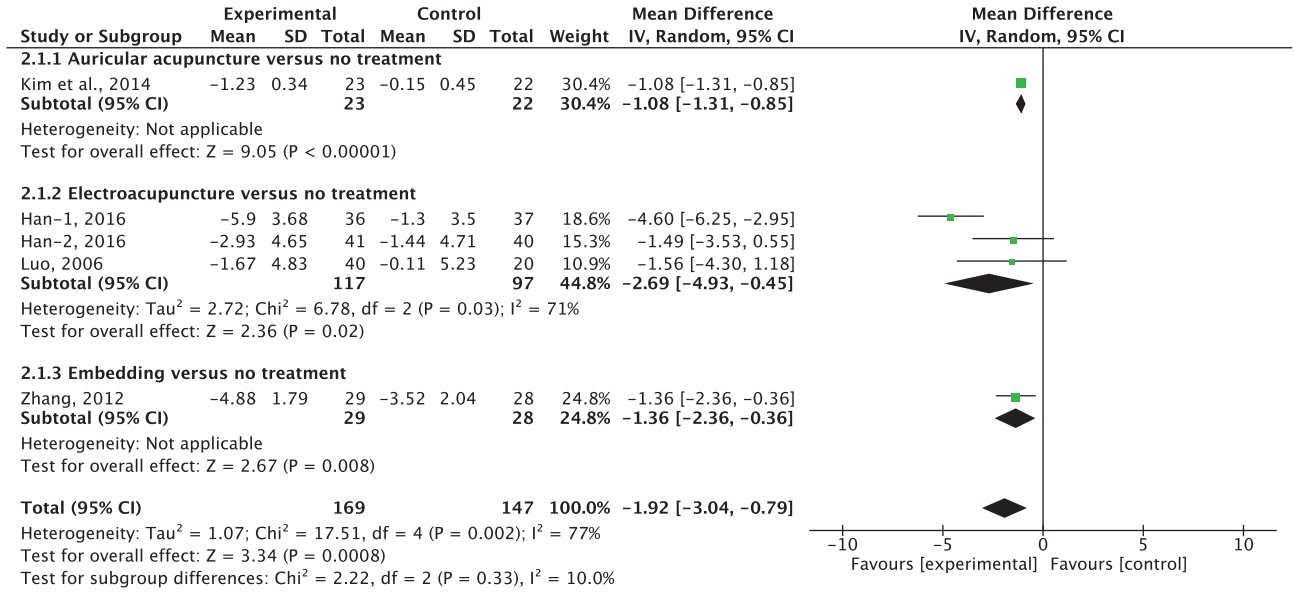

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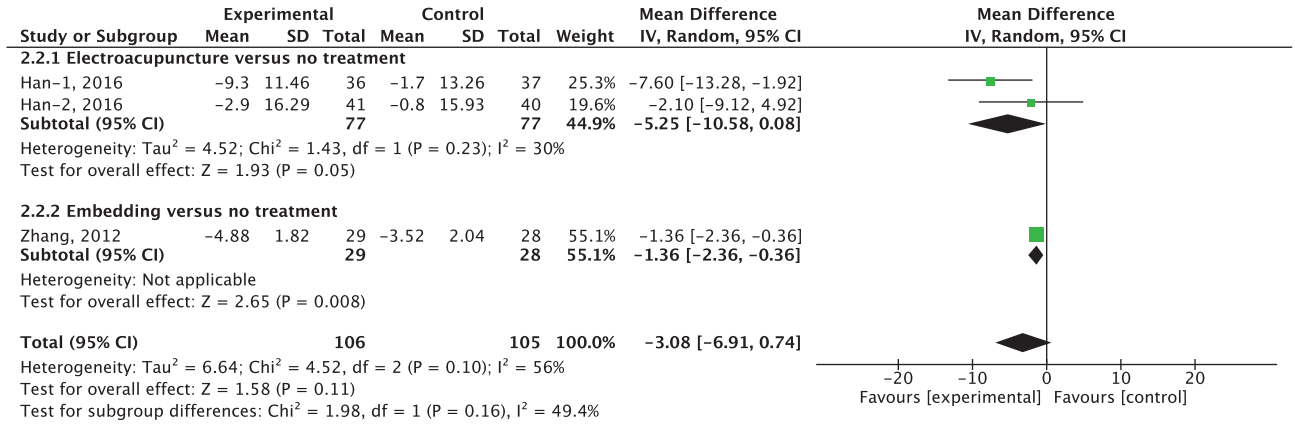

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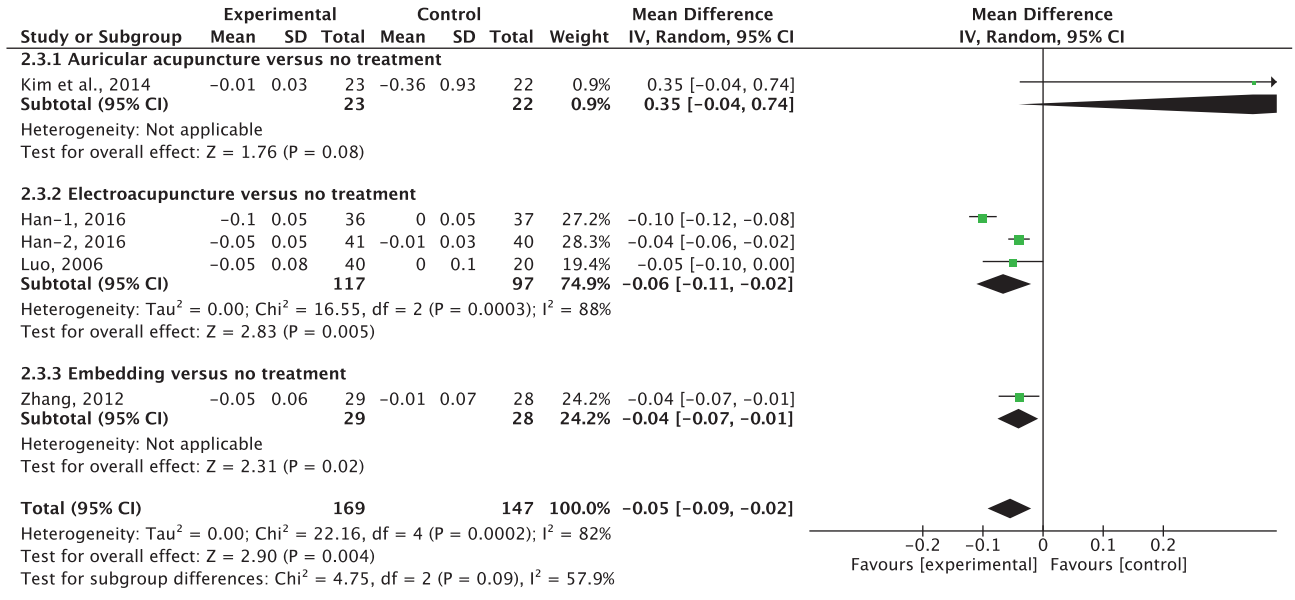
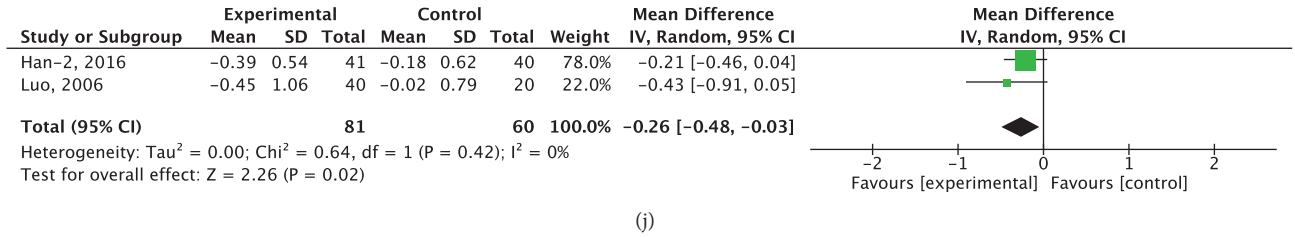


(i)



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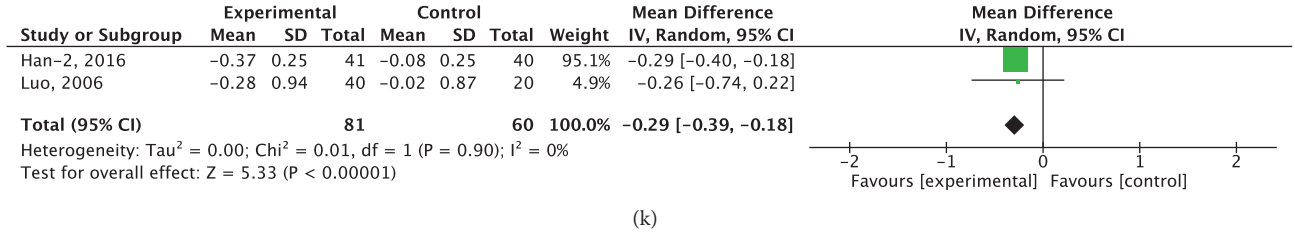

(k)

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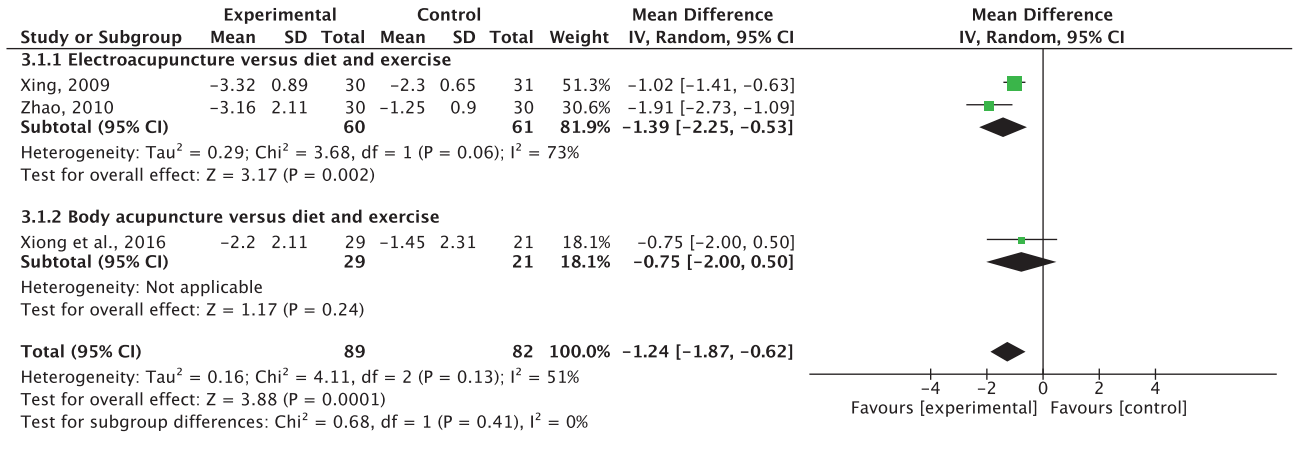
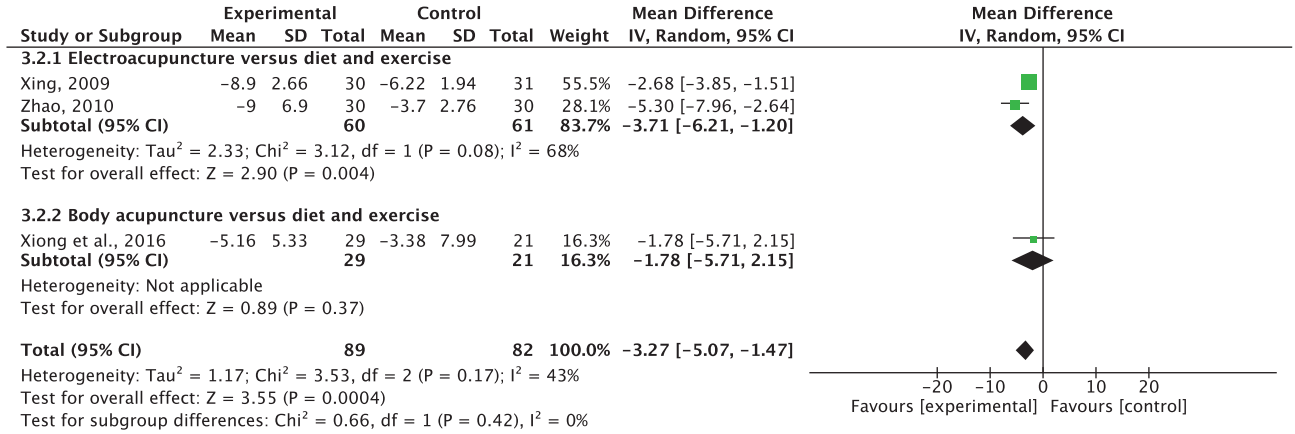


(l)



(m)

Figure 2: The forest plots of the efficacy of acupuncture for obesity. (1) Acupuncture versus sham acupuncture: (a) analysis of BMI in obesity patients; (b) analysis of weight loss in obesity patients; (c) analysis of WC in obesity patients; (d) analysis of body fat mass (kg) in obesity patients; (e) analysis of TC in obesity patients; and (f) analysis of TG in obesity patients. (2) Acupuncture versus no treatment: (g) analysis of BMI in obesity patients; (h) analysis of weight loss in obesity patients; (i) analysis of WHR in obesity patients; (j) analysis of TC in obesity patients and (k) analysis of TG in obesity patients. (3) Acupuncture versus diet and exercise: (l) analysis of BMI in obesity patients and (m) analysis of weight loss in obesity patients.

[20, 22, 23, 29]. The pooled effect on BMI outcome in 3 records [22, 23, 29] showed no significant difference in BMI decrease. The random effects model was used (*MD*=-1.24, *95%CI*=-1.87 to -0.62); heterogeneity was detected (heterogeneity: Chi^2 =4.11, *df* =2 (*P*=0.13); I^2=51%). Subgroup analyses showed that EA significantly reduced BMI compared with control group (*MD*=-1.39, *95%CI*=-2.25 to -0.53; **Figure 2(l)**). One record [20] reported BMI, but the data of control group was not given. Therefore, description analysis was used and it suggested that no significant difference was found between the two groups. Three records [22, 23, 29] showed the acupuncture significantly reduced weight. The random effects model was used (*MD*=-3.27 *95%CI*=-5.07 to -1.47); heterogeneity was detected (heterogeneity: Chi^2 =3.53, *df* = 2 (*P*=0.17); I^2=43%). Subgroup analyses showed that EA significantly reduced body weight compared with control group (*MD*=-3.71, *95%CI*=-6.21 to -1.20; **Figure 2(m)**). One record [22] showed the acupuncture significantly changed WHR in the first duration of treatment, but no significant difference was found in the last two durations between two groups. One record [29] showed the treatment group was more effective than the control group in WC loss. No significant difference in body fat mass (kg) was found between two groups in 1 record [29]. One record [22] showed no significant change between two groups in serum TC, TG, LDL-c, and HDL-c.

3.2.4. Acupuncture versus Medicine. Acupuncture was compared to medicine in 1 record [14]. There was no significantly different between two groups in BMI and weight decrease. Control group was more effective than the acupuncture group in WC and WHR reduction.

3.3. Possible Mechanisms of Acupuncture on Obesity. Acupuncture is believed to be involved in neuroendocrine axis regulation. Modulating eating habits and energy metabolism are the promising strategies for obesity, and it is an immensely complex process involving the gastrointestinal tract, many

Table 2: Risk of bias of the included studies.

Included studies	A	B	C	D	E	F	G	Total
Darbandi et al., 2012 [9]	1	0	0	0	1	1	1	4
Darbandi et al., 2013 [10]	1	0	0	0	1	1	1	4
Darbandi et al., 2014 [11]	1	0	1	0	1	0	1	4
Gucel et al., 2012 [12]	1	0	0	0	1	1	1	4
Han-1, 2016 [13]	1	1	1	1	1	1	1	7
Han-2, 2016 [13]	1	1	1	1	1	1	1	7
He et al., 2008 [14]	1	0	0	1	1	1	1	5
Hsu et al., 2009 [15]	1	0	1	0	1	0	1	4
Hung et al., 2016 [16]	1	1	1	0	1	1	1	6
Kim et al., 2014 [17]	1	0	0	0	1	1	1	4
Lien et al., 2012 [18]	1	0	1	0	1	0	1	4
Luo, 2006 [19]	1	0	0	0	1	1	1	4
Nourshahi et al., 2009 [20]	1	0	0	0	0	1	1	3
Tong, 2011 [21]	1	0	1	1	1	1	1	6
Xing, 2009 [22]	1	1	0	0	1	1	1	5
Xiong et al., 2016 [23]	1	0	0	0	1	1	1	4
Yeh et al., 2015 [24]	1	1	1	0	0	0	1	4
Yeo et al., 2014 [25]	1	1	1	0	1	0	1	5
Zhang et al., 2012 [26]	1	1	1	1	1	1	1	7
Zhang, 2012 [27]	1	1	0	1	1	1	1	6
Zhao et al., 2011 [28]	1	0	0	1	1	1	1	5
Zhao, 2010 [29]	1	0	0	0	1	1	1	4

Note: A, adequate sequence generation; B, concealment of allocation; C, blinding of participants and personnel; D, blinding of outcome assessment; E, incomplete outcome data; F, selective reporting; G, other bias; 1, low risk of bias, the information of the domain was adequate in the text; 0, high risk of bias, the information of the domain was inadequate in the text.

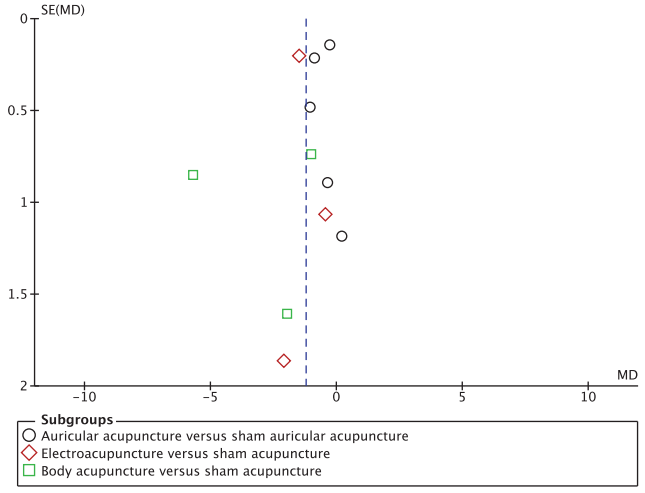


Figure 3: The funnel plots of the efficacy of acupuncture for obesity. Funnel plots of the effect of acupuncture on BMI between acupuncture and sham acupuncture.

hormones, and both the central and autonomic nervous systems (**Figure 4**).

The arcuate nucleus of the hypothalamus (ARH) is the main regulatory organ for appetite in human. In diet-induced obesity (DIO) rats, EA treatment significantly decreased food intake and reduced body weight compared with the untreated rats. Further analysis revealed that EA treatment increased peptide levels of α-MSH and mRNA expression of its precursor proopiomelanocortin (POMC) in ARH neurons. In addition, α-MSH in cerebral spinal fluid (CSF) elevated upon EA application. However, the lesion in ARH could abolish the inhibition effect of EA on food intake and body weight, suggesting the beneficial effects of EA treatment are acted through ARH, and that the stimulation of α-MSH expression and release might be involved in the process [30]. In 14-week high-fat diet feeding rats, 4-week EA treatment causes a reduction of both in body weight and energy intake, along with the upregulation of the cocaine and amphetamine-regulated transcript (CART) peptide, an anorexigenic peptide in the arcuate nucleus (ARC) [31].

Activating the satiety center tends to be one of the effective methods in preventing obesity. Su et al. [32] have shown that acupuncture can raise the frequency of neural discharge in the hypothalamic ventral medial nucleus (VMH), indicating acupuncture could improve the excitability of the medial nucleus in experimental obese animals. Liu et al. [33] have found that the frequency of spontaneous discharges of nerve cells in VMH and the levels of tyrosine (Tyr), dopamine (DA), tryptophan (Typ), and 5-hydroxytryptamine (5-HT)/5-hydroxyindole acetic acid (5-H1AA) ratio were elevated, along with the decrease of 5-HT level upon 12 days of consecutive acupuncture treatment. Lateral

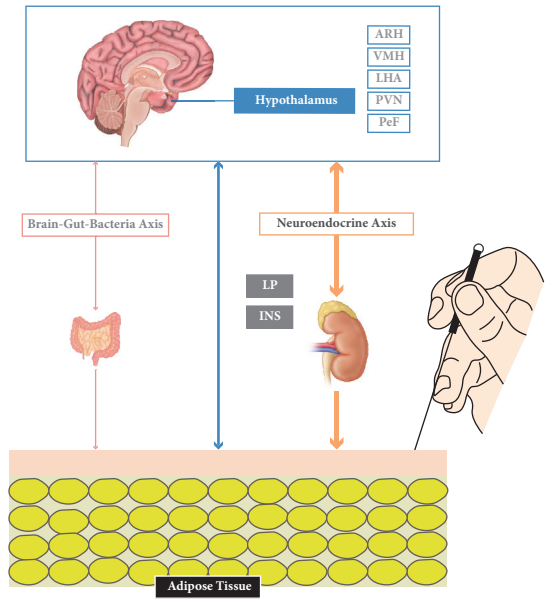


Figure 4: The potential neuroendocrine regulation under the efficacy of acupuncture in the animal studies.

hypothalamic area (LHA) is the main neuroregulator in triggering ingestion. Acupuncture is reported to reduce excitation of LHA, inhibit hyperorexia, and regulate the activity of 5-HT, the catecholamine neurotransmitter, and ATPase activity in the LHA [34, 35].

Some studies [36, 37] believed that acupuncture could improve the frequency of spontaneous discharges of nerve cell in the paraventricular nucleus (PVN) and reduced the activity of hypothalamic perifornical nucleus (PeF) neurons. Ji et al. [38] concluded that an upregulation of anorexigenic factor POMC production in the nucleus tractus solitarius (NTS) and hypoglossal nucleus (HN) regions were generated by EA Zusanli (ST36), thus preventing food intake and causing weight loss. Signal transduction of EA stimuli included expression of transient receptor potential vanilloid type-1 (TRPV1) and neuronal nitric oxide synthase (nNOS) in the ST36 and the NTS/gracile nucleus through somatosensory afferents-medulla pathways. Kim et al. [39] found that stimulation of auricular acupuncture point affected the expression of NPY expression in the ARN and PVN in rats. Fu et al. [40] suggested that transcription factor STAT5 in the central nervous system plays different roles in the hypothalamus and white fat tissue during gene transcription, and acupuncture could regulate a large amount of differentially expressed genes toward their normal expression, especially genes in the hypothalamus. Thus, the weight loss effect of acupuncture might be attributed to its functional gene regulatory mechanisms. Upregulation the transcription of adenosine 5′-monophosphate-activated protein kinaseα2 (AMPKα2), promotion protein expression of liver kinase B1 (LKB1) and AMPKα1, and inhibition acetyl-CoA carboxylase (ACC) protein expression in the hypothalamus were observed after 4 weeks of EA treatment [41]. However, auricular acupuncture stimulation is reported to be associated with satiation formation and preservation in the hypothalamus but fails to work on anorexia activity [42].

Certain hormones including insulin and ghrelin may influence appetite in the hypothalamus [43]. One study [44] showed that downregulation of ghrelin in the stomach and neuropeptide Y (NPY) in the hypothalamus was in line with the reduction in food intake in rats receiving EA stimulation once every day. Liu et al. [45] speculated that, in high-fat diet (HFD) animals, EA treatment (ST36 and LI11, 20 minutes per day for 28 days) could reduce the body weight, homeostasis model assessment-insulin resistance index, adipocyte diameters, and neuroprotein Y/agouti-related protein and protein tyrosine phosphatase 1B levels. In db/db mice, Liang et al. [46] found that EA treatment (five times per week for eight weeks) contracted the increase of fasting blood glucose, food intake, and body mass and maintained insulin levels via stimulation of skeletal muscle Sirtuin 1 (SIRT1)/peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), suggesting the role of EA in improving insulin resistance. Gong et al. [47] applied EA stimulation to diet-induced obese rats for four weeks and observed the reduced body weight, plasma levels of leptin, and increased expression of leptin receptor in the hypothalamus. In addition, Shen et al. [48] discovered that four weeks of EA treatment caused remodeling white adipose tissues (WAT) to brown adipose tissue (BAT) via inducing uncoupling protein-1 (UCP1) in EA group. Besides, acupuncture also adjusted the intestinal flora, achieving the balance of brain-gut-bacteria axis [49–51].

4. Discussion

Here we selected 21 RCTs including 1389 patients suffering from obesity to evaluate the efficacy of acupuncture. We found that acupuncture was more effective than shame

acupuncture in BMI, weight, body fat mass (kg), and TC; acupuncture was more effective than no treatment group in BMI, WHR, and TG. In addition, acupuncture is showed to be more effective than diet and exercise group in BMI and weight loss. To a limited extent, we concluded that acupuncture is an effective treatment for obesity.

Currently, the etiology of obesity has not been defined yet; many factors such as neuromodulation, viral, immune, endocrine, free radical, and genetics are reported to be involved [52–57]. Each indicator can affect a wide range of factors, hormones, and even genetic changes, so the mechanisms of acupuncture on obesity tend to be the simultaneous adjustment of multiple systems and targets. So, we reviewed the potential mechanisms under the efficacy of the animal studies and highlighted neuroendocrine regulation to be essential in the process.

The limitations of this work are as follows:

(1) Bias risk exists because most studies do not describe the allocation of hidden methods or use blind methods [58], which might result in performance bias and detection bias.

(2) The number of samples of each trial is relatively small, which might cause the insufficient sample size for analysis and test efficacy.

(3) The researchers are evaluated by different diagnostic criteria; inclusion and exclusion criteria, forms of acupuncture (acupuncture, EA, ear needles, ear pressure, and embedding), the course of treatment (different acupoints, duration of treatment), basic intervention (diet and exercise), and the confounding factors are different, which might increase heterogeneity.

(4) Some researchers do not mention the methods used in dealing with missing data although they have a loss to follow-up more than 15%. Most studies lack following course and fail to understand the long-term effects of auricular acupressure treatment, which might increase attrition bias.

(5) There are some objective factors like language and limited search resource, which may lead to the incomplete searching.

In conclusion, acupuncture is a reasonable and effective treatment for people who suffer from obesity. However, according to CONSORT Declaration and STRICTA Standard, some researchers point out that the efficacy of acupuncture on mild obesity is not significant, which is difficult for readers to understand the rationality of the study design, the correctness of the implementation, the authenticity of the results, and the clinical applications [59]. Obesity is a chronic condition, requiring long-term treatment. However, the treatment period of obesity is rather short in many studies, varying from 3-8 weeks. Follow-ups are needed to observe curative effect since the bodyweight might be easily rebound. In clinical, obesity is the major risk of cardiovascular events, so we put the incidence of CVD as the primary outcome; however, no included studies set CVD as primary outcome. More researches need to be done to evaluate CVD as the curative effect of acupuncture treatment for obesity in clinical. This systematic review was conducted to critically assess evidence from RCTs regarding the efficacy of various types of acupuncture therapies on obesity. We analyzed the outcomes which were related to obesity comprehensively. In the future, larger number of samples and higher-quality randomized controlled trials are required to verify the clinical effectiveness in treating obesity by acupuncture. Moreover, understanding the mechanisms under the efficacy of acupuncture on weight loss provides reliable experimental basis, thus convincing the patients with obesity with the application of acupuncture.

Conflicts of Interest

The authors declared no conflicts of interest.

Acknowledgments

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Research Article

Structural Changes Induced by Acupuncture in the Recovering Brain after Ischemic Stroke

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The aim of this study was to observe the grey matter (GM) tissue changes of ischemic stroke patients, to explore the therapy responses and possible mechanism of acupuncture. 21 stroke patients were randomly assigned to receive either acupuncture plus conventional (Group A) or only conventional (Group B) treatments for 4 weeks. All patients in both groups accepted resting-state functional magnetic resonance (fMRI) scan before and after treatment, and the voxel-based morphometry (VBM) analysis was performed to detect the cerebral grey structure changes. The modified Barthel index (MBI) was used to evaluate the therapeutic effect. Compared with the patients in Group B, the patients in Group A exhibited a more significant enhancement of the changes degree of MBI from pre- to post-treatment intervention. VBM analyses found that after treatment the patients in Group A showed extensive changes in GMV. In Group A, the left frontal lobe, precentral gyrus, superior parietal gyrus, anterior cingulate cortex, and middle temporal gyrus significantly increased, and the right frontal gyrus, inferior parietal gyrus, and middle cingulate cortex decreased ($P < 0.05$, corrected). In addition, left anterior cingulate cortex and left middle temporal gyrus are positively related to the increase in MBI score ($P < 0.05$, corrected). In Group B, right precentral gyrus and right inferior frontal gyrus increased ($P < 0.05$, corrected). In conclusion, acupuncture can evoke pronounced structural reorganization in the frontal areas and the network of DMN areas, which may be the potential therapy target and the potential mechanism where acupuncture improved the motor and cognition recovery.

1. Introduction

Stroke is the second major cause of death in the world [1] and the first leading cause of adult disability. It is reported that there are 1.5 to 2 million new strokes per year in China [2]. The symptoms caused by stroke, such as hemiplegia, cognitive disorder, aphasia, and dysphagia, greatly affect the ability of patients to perform activities of daily living (ADL), as well as social participation, imposing a great burden on families and communities in many developed countries [3]. Stroke became an important public health-care and social issue because of its high prevalence, unsatisfactory treatment options, large medical burden, and serious reduction in quality of life (QQL). Hence, both patients and practitioners desire effective alternative therapies.

Acupuncture, a major medical resource, has been extensively used to treat stroke for several millennia. Acupuncture is regarded as a very effective therapeutic intervention and is becoming more and more popular in western countries [4, 5]. During the past decades, a considerable number of clinical and experimental studies have shown its safety and potential beneficial effects in the poststroke rehabilitation [6]. The latest review in 2016 has indicated that acupuncture was demonstrated to be a promising tool for improving functional recovery in stroke patients [7]. However, we still have not been able to explore its exact so far.

In recent years, some studies suggested that structural neuroplastic changes in the brain, such as grey matter volume, might be closely related to both behavioral recovery and active rehabilitation after stroke [8–10]. Miao et al. [11] found that well-recovered stroke patients exhibited significantly increased grey matter volume (GMV) in contralesional supplementary motor area (SMA). In another motor rehabilitation therapy study, stroke patients have shown a high GMV in frontal and parietal sensory-motor areas and in the hippocampus [12]. Moreover, our previous research [13] also found that stroke patients showed some changes in cerebral GMV, including precentral gyrus, cerebellum, and middle frontal gyrus compared with healthy subjects. However, whether acupuncture can induce the structural changes was not addressed in the previous study. Hence, based on the converging evidence, we hypothesize that acupuncture therapy will change the GMV of the key regions by inducing the structure neuroplastic changes in the cerebral cortex and subcortex. Hence, the present study aimed to investigate the influence of acupuncture on cerebral grey matter volume in order to explore the potential central mechanism of acupuncture treatment.

2. Methods

2.1. Participants. In this study, we recruited the ischemic stroke patients from the 1st Teaching Hospital of Chengdu University of Traditional Chinese Medicine (CDUTCM). All the subjects need to meet the following criteria: (1) were diagnosed with ischemic stroke by CT or MRI [14]; (2) had first onset and course of disease in less than six months; (3) were right-handed and aged 35–80 years old; (4) were conscious and able to cooperate with the study; (5) meet the cognition assessment by Mini-Mental State Examination (MMSE) > 21; (6) signed the informed consent form. The exclusion criteria for this study were as follows: (1) with any MRI contraindications or other brain diseases; (2) with some severe comorbidities such as heart or renal function failure, pulmonary insufficiency, serious lung infection or liver dysfunction, and malignant tumor; (3) history of epilepsy or other neurological diseases and psychiatric disorders; (4) unable to complete the entire treatment and fear of acupuncture.

The eligible patients were randomized into either Group A or Group B through computer-generated randomization sequences. Opaque envelopes were used to hide the randomized data in this study. The group assignment was unknown for patients.

This study was based on the principles of the Declaration of Helsinki (Version Edinburgh 2000) [15] and obtained the approval of the Ethics Committee of the 1st Teaching Hospital of CDUTCM (No. 2011KL-002).

2.2. Acupuncture Interventions. Each subject of this study received basic standard treatments, including Anti-platelet aggregation therapy (100 mg aspirin once per day), neuroprotective treatment (500 mg citicoline per day), and other treatments according to the clinical symptoms.

In addition to standard treatment, the 11 patients in Group A received acupuncture treatment. The acupoints are as follows: Baihui (GV20), Fengchi (GB20), Quchi (LI11), Hegu (LI4), Yanglingquan (GB33), Zusanli (ST36), Sanyinjiao (SP6), and Xuanzhong (GB39). After needling, we used some Auxiliary techniques of acupuncture such as gentle manipulations of thrusting, lifting, and twirling to achieve de qi (including numbness, soreness, distention, heaviness, and other sensations), which is believed to be a crucial part of acupuncture efficacy. Participants were treated with 20 sessions in all, once per day, 5 consecutive sessions, and 2 days off in a week, with a duration of 30 min. The location of the acupoints is mainly determined by the national standard of the People's Republic of China (2006), Names and Locations of Acupoints (GB/T12346-2006). Moreover, Group B (10 patients) only received basic standard treatments.

2.3. Outcome Measurement. To assess clinical efficacy, modified Barthel Index (MBI), created by Shah et al. [16], has been used for symptom severity and quality of life among stroke patients. MBI is a daily life index that evaluates the ability of self-care independence, which consists of 10 items, including feeding, grooming, bathing, dressing, bowel and bladder care, using toilet, ambulation, transferring, and climbing stairs. Total score was from 0 to 100, with the higher score representing smaller nursing dependency [17]; on the contrary, the lower score indicated poor daily living ability. And it has obvious acceptability and similar psychometric characteristics for stroke patients during the rehabilitation process in hospital [18].

2.4. fMRI Scan. All brain images were obtained on the 3T Siemens MRI scanner (MAGNETOM Trio Tim, Siemens, Amberg, Germany) at the Huaxi MRI Center, West China Hospital of Sichuan University, China. The VBM protocol used a spin-echo planar image sequence with the following parameters: repetition time/echo time = 1,900 ms/2.26 ms, flip angle = 9°; in-plane matrix resolution = 256 × 256; slices = 176; field of view = 16 × 16 mm^2; voxel size = 1 × 1 × 1 mm^3. During the scan, each patient was blindfolded and their ears were plugged; moreover, in order to prevent the head from translating and rotating, all of them wore the foam cushions. Additionally, all female patients were scanned within one week after the menstrual cycle to avoid possible impact on brain activity and the size of the menstrual cycle [19, 20].

2.5. Statistical Analysis

2.5.1. Clinical Variables. SPSS 19.0 software (SPSS, Chicago, IL, USA) was used to analyze the clinical variables by two blinded evaluators. The numerical variables comparisons within and between-group were performed using analysis of variance and the Kruskal-Wallis test. A two-sided test was applied for all available data. Categorical variables were calculated by a X^2 test and described as n (percentage). Continuous variables were presented as the mean with 95% confidence intervals (CI). A P value < 0.05 was considered to be statistically significant.

2.5.2. Voxel-Based Morphometry Analysis. Voxel-based morphometry (VBM) with Diffeomorphic Anatomical Registration using Exponentiated Lie Algebra (DARTEL) was

Table 1: Demographic and clinical characteristics of patients ($n = 21$).

Characteristic	Group A	Group B	P value
No. of patients (n)	11	10	-
No. of women, n (%)	4 (36.364)	5 (50.000)	0.497
Age (y), mean (95% CI)	69.364 (61.245, 77.483)	61.300 (53.362, 69.238)	0.127
Course of disease (D), mean (95% CI)	52.818 (22.348, 83.289)	52.200 (18.001, 86.399)	0.976
MBI score, mean (95% CI)	32.386 (24.613, 40.160)	32.980 (26.378, 39.572)	0.898
MMSE score, mean (95% CI)	21.000 (19.725, 22.275)	21.800 (20.262, 23.338)	0.380

Notes. Group A received standard conventional treatment and acupuncture treatment; Group B only received standard conventional treatment. CI: confidence interval; MBI: modified Barthel index; MMSE: Mini-Mental State Examination; *N*: number; %: percent; y: year; D: days.

conducted [21]. DARTEL has been shown to produce a more accurate registration than the standard VBM procedure and enables increased sensitivity to findings such as the correlation between grey matter volume and several measures such as age. After image acquisition by MRI, all T1-weighted MR images were analyzed using Statistical Parametric Mapping 8 (SPM8) (Wellcome Department of Cognitive Neurology, London, UK) in Matlab (Math Works, Natick, MA, USA). First, the "New Segmentation" algorithm from SPM8 was applied to every T1-weighted MR image to extract tissue maps corresponding to grey matter, white matter, and cerebrospinal fluid (CSF). This algorithm, which is an improvement on the unified segmentation algorithm [22], uses a Bayesian framework to iteratively perform the probabilistic tissue classification and spatial non-linear deformation in terms of Montreal Neurological Institute (MNI) space. Next, these segmented tissue maps were used to create a customized, more population-specific template using the DARTEL template-creation tool [21]. DARTEL estimates the best set of smooth deformations working from every subject's tissues to their common average, applies the deformations to create a new average, and then reiterates the process until convergence is achieved. We used a set of standard MNI tissues maps and a multivariate tissue-affinity-registration algorithm provided by SPM and DARTEL for that process. At the end of the process, each subject's grey matter map was warped using its corresponding smooth, reversible deformation parameters to transform it to the custom template space and then to the MNI standard space. Finally, the warped modulated grey matter images were smoothed by convolving an $8 \times 8 \times 8\,\text{mm}^3$ full-width at half-maximum isotropic Gaussian kernel. After completing these image analyses, we obtained smoothed modulated grey matter images to be used for the statistical analysis. The significance of group differences was set at $P < 0.05$ using family-wise error correction.

2.5.3. Correlation Analysis between Scales Score and GMD. The peak voxel and neighboring 100 voxels for each subject exhibiting GMV changes ($P < 0.05$, TFCE FWE correction) were selected as the region of interest (ROI). Then, we performed ROI-wise correlation analyses to evaluate whether the changes in GMV would be associated with the changes in the clinical variables (MBI scores). After correcting the volume, Pearson correlation coefficients were calculated between the mean volumes of the GMV and clinical variables (MBI scores).

3. Results

From January 2011 and December 2013, 21 ischemic stroke patients were recruited and randomly assigned in this study. In all, 21 patients all finished the treatment and fMRI scans, the lesion locations of which were primarily in the left basal ganglia. Demographic and clinical characteristics of 21 patients (11 in Group A and 10 in Group B) were shown in the Table 1. There were no significant differences in the demographics, including age, sex, and disease status as indicated by, for example, duration of symptoms, MBI score, and MMSE score, which did not differ between the two groups ($P > 0.05$).

After treatment intervention, a significant difference was found in MBI scores between two groups (Group A: from 32.4 ± 7.8 to 44.9 ± 6.4; Group B: from 33.00 ± 6.6 to 38.3 ± 7.2; $P = 0.00$). Additionally, the difference in change degree of MBI scores showed a significant improvement in the Group A, compared with Group B (Group A: 13.0 ± 3.58; Group B: 5.275 ± 0.902; $P = 0.001$) (Figure 1).

3.1. Changes in Grey Matter Volume after Treatment. In Group A, an increase in the cerebral grey matter volume was observed after treatment in the left frontal gyrus, left precentral gyrus (BA6), left superior parietal gyrus, left anterior cingulate cortex (BA32), and left middle temporal gyrus. A decrease in the cerebral grey matter volume was detected in the right frontal gyrus, right inferior parietal gyrus, and right middle cingulate cortex ($P < 0.05$, family- wise error corrected with a minimal cluster size of 20 voxels) (Table 2) (Figure 2).

In Group B, an increase was observed after treatment in the right precentral gyrus (BA6), and right inferior frontal gyrus (BA10) ($P < 0.05$, family- wise error corrected with a minimal cluster size of 20 voxels) (Table 3) (Figure 3).

3.2. Correlations between GMV and Clinical Scale Scores. In Group A, the increase in MBI score was significantly related to the GMV increase in the left middle temporal gyrus ($r^2 = 0.597$, $P = 0.005$) and left anterior cingulate cortex ($r^2 = 0.680$, $P = 0.002$) (Figure 4) ($P < 0.01$, corrected).

4. Discussion

Previous studies have demonstrated positive effects on functional recovery by using acupuncture in stroke patients [7, 23,

Table 2: The cerebral GMV changes in Group A after treatment (end of treatment minus baseline).

Region	Side	Talairach			t value	BA	Voxel	Sign
		X	Y	Z				
frontal lobe	L	15	−10	61	7.00	-	1768	↑
	R	−8	39	31	6.24	-	674	↓
Precentral gyrus	L	−32	6	48	7.13	BA6	233	↑
Superior Parietal gyrus	L	−18	−57	48	6.98	-	358	↑
Inferior Parietal gyrus	R	50	−34	40	6.66	-	308	↓
Anterior Cingulate cortex	L	−10	44	7	6.44	BA32	378	↑
Middle Temporal gyrus	L	56	−12	−21	6.18	BA21-	230	↑

Notes. Group A received standard conventional treatment and acupuncture treatment. BA: Brodmann area; L: left; R: right; before treatment versus after treatment in group A, $P < 0.05$; family-wise error corrected with a minimal cluster size of 30 voxels.

Table 3: The cerebral GMV changes in Group B after treatment (end of treatment minus baseline).

Region	Side	Talairach			t value	BA	Voxel	Sign
		X	Y	Z				
precentral gyrus	R	15	−18	70	6.68	BA6	221	↑
Inferior frontal gyrus	R	−18	54	1	6.37	BA10	232	↑

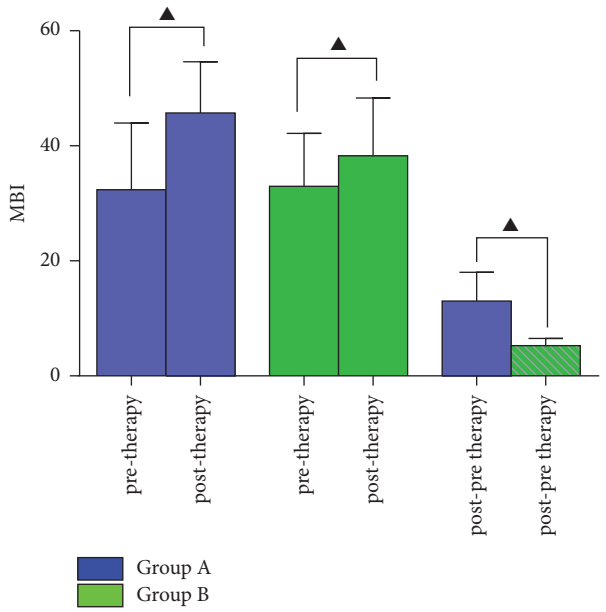

Figure 1: The changes of MBI performance with different intervention therapies. Paired t-test analyses showed significant increase of MBI scores from pre- to post-treatment in Group A and Group B. The change degree of MBI in patients with acupuncture treatment showed a significant enhancement comparing with the patients with conventional treatment (post-pre therapy in two groups). ▲$P < 0.05$.

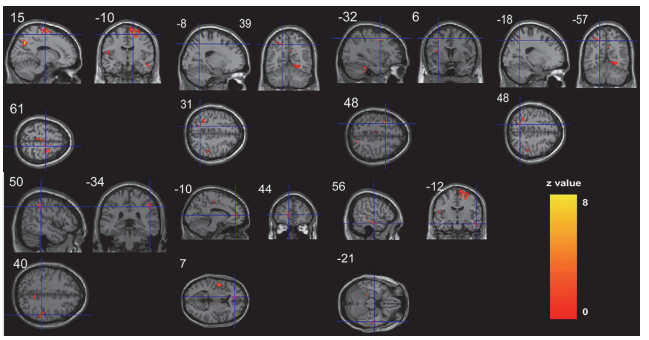

Figure 2: Cerebral GMV changes in ischemic stroke patients after acupuncture treatment. After acupuncture treatment in group A, ischemic stroke patients showed higher GMV in the left frontal lobe, precentral gyrus, Superior Parietal gyrus, Anterior Cingulate cortex, and Middle Temporal gyrus and lower GMV in the right frontal lobe and Inferior Parietal gyrus. before treatment versus after treatment in group A, $P < 0.05$; family-wise error corrected with a minimal cluster size of 30 voxel.

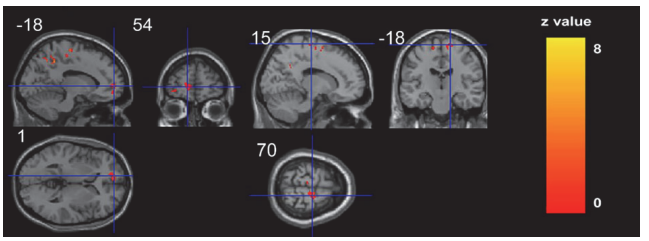

Figure 3: Cerebral GMV changes in ischemic stroke patients after conventional treatment. After conventional treatment in group B, ischemic stroke patients showed higher GMV in the right precentral gyrus (BA6) and inferior frontal gyrus (BA10). Before treatment versus after treatment in group B, $P < 0.05$; family-wise error corrected with a minimal cluster size of 30 voxels.

24]. Our present study also demonstrated acupuncture can improve the ability of daily life of stroke patients. Moreover, the novel key finding of the present neuroimaging study was that acupuncture can lead to structural reorganization in the recovering brain of stroke. Compared with patients who only received conventional treatment (Group B), the patients who

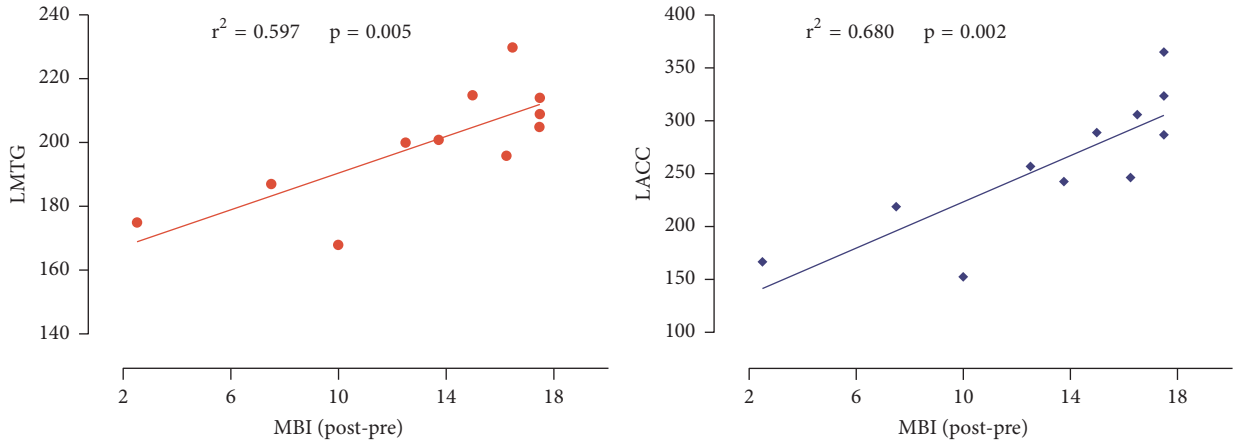

FIGURE 4: The correlation coefficients of brain grey structure changes and clinical variables. Note: LMTG: left middle temporal gyrus; LAGG: left anterior cingulate cortex; *r*: correlation coefficient; post-pre: posttreatment minus pretreatment.

received acupuncture treatment (Group A) showed extensive cerebral GMV changes in different regions. In Group A, the GMV was significantly increased in the left frontal lobe areas and left "default mode" network area, and the cerebral regions which GMV obviously decreased were found in the right frontal gyrus and right inferior parietal gyrus. In addition, the cerebral GMV changes in the Group B are right precentral gyrus and right inferior frontal gyrus. Moreover, we found the GMV changes in the left middle temporal gyrus (MTG) and left anterior cingulate cortex (ACC) positively are correlated with the behavioral changes of daily life. The different neuroplasty induced by acupuncture compared to conventional medicine indicates that acupuncture may enhance the role of conventional therapy. The grey matter (GM) tissue changes especially those involved in motor and cognition areas may be the potential therapy target and the potential mechanism of acupuncture.

4.1. Acupuncture Modulates the Motor Cortex Areas of Strokes. Numerous studies had demonstrated that both structural and functional reorganizations would occur in patiens following subcortical stroke, and the neuroplasticity changes in structural and functional levels were closely correlated to motor recovery of strokes [11, 25–27]. In the current study, the VBM analysis approach was used to the explore the effects of acupuncture on structural changes in the ischemic stroke patients. Our results showed significant alterations of grey matter structure in some motor-related regions, which probably interpret the mechanism of acupuncture on patients motor recovery from stroke. In the VBM analysis, there was a significant degeneration the contralesional frontal lobule; in contrast, the ipsilesional frontal lobule, precentral gyrus, and superior parietal gyrus increased obviously after four-week acupuncture treatments. The precentral gyrus plays a role in the relationship between motor function and the primary motor cortex. Additionally, the frontal and parietal lobe are also the key motor-related areas. The primary motor cortex (M1) and secondary motor areas (SMA) in the frontal and parietal lobe are recognised to the inter-regional corticocortical connectivity between key areas of the human motor network [28, 29]. Particularly, the premotor area of the frontal lobe and the supplementary motor area might act a potential substrate for brain reorganization after stroke as they have direct access to M1, as well as to the spinal cord [30]. Previous studies have reported that ipsilesional premotor areas such as ventral premotor cortex (PMv) and their interplay with M1 are contributing to motor function, spontaneous recovery [31], and also motor learning after stroke [32]. In monkeys, rehabilitation after stroke involves the primary motor cortex, as evidenced by changes in brain activity during recovery of hand function [33]. In addition, a recent fMRI research with multi-modality approach has identified the bi-hemispheric structural alterations after stroke and also reflected the increase in GMV of the contralesional SMA [25], suggesting that structural plasticity was associated with motor recovery. In our study, acupuncture treatments have induced the GMV decrease in contralesional hemisphere and GMV increase in the ipsilesional hemisphere. Another fMRI study with similar results considered that recovery of motor function after stroke is associated with normalization of activity in overactive brain regions [34]. Similar to the above study, the GMV of the patients in the group B mainly increased in the contralesional hemisphere and showed overactive in the present study. However, after receiving acupuncture treatment, the GMV in the contralesional frontal gyrus of strokers was induced to reduce. The result suggested that acupuncture might reduce the overactiveness of contralesional hemisphere. On the other hand, the GMV of ipsilesional motor-related area increased in the present study. Grefkes and Fink demonstrated the primary motor cortex of undamaged side in strokes had an interhemispheric inhibition on the injured side [35]. From this perspective, acupuncture might weaken the inhibitory effect by decreasing the GMV aggrandize of undamaged side, so the cerebral GMV of the damaged side increases, as a result of promoting the motor recovery.

4.2. Acupuncture Modulates the DMN of Strokers. Apart from the motor deficiency, emotion and cognition disorders, such as depression, confusion, and forgetfulness, are also the common complications of stroke patients and occur at a high

incidence. For instance, recent studies have shown that at least 30–60% of post-stroke patients present symptoms of depression, which seriously restricts their rehabilitation [36]. The previous behavioral results showed that acupuncture improved the depression [37] or cognitive impairments [38] better than conventional therapy. In this study, acupuncture treatment elicited more extensive and remarkable cerebral structural changes as compared with conventional treatment. The left ACC and left MTG were only found in the acupuncture group and not in the conventional group (Tables 2 and 3 and Figures 2 and 3). The majority of these regions in the acupuncture group belong to "default mode" network (DMN).

The DMN is a brain network that presents as deactivated regions at rest, and various goal-directed [39], as well as emotional stimuli; this network can be activated. Our previous study indicated that, as compared with healthy subjects, stroke patients showed lower GMV in MTG [13]. In addition, Shi et al. [40] also found decreased grey matter volume in prefrontal cortex and cingulate cortex in stroke patients, which were also the key regions of DMN. The results suggested that successful treatment should modulate this network.

The ACC and MTG, considered to as key parts of the DMN [41, 42], play important roles in processing and modulating episodic memory [43], depression, and anxiety [44]. The ACC has been regarded as a core region involved in generating emotional responses, and its abnormal functioning has been linked to many psychiatric conditions [45], including memory and cognitive processing and their interactions with other brain networks related to conscious awareness [46]. The middle temporal gyrus was involved in episodic memory processing [43]. In some other fMRI studies it was found that, following a stroke, patients presented with delayed memory dysfunction and reduced functional connectivity in the temporal regions, prefrontal cortex, and cingulate gyrus within the DMN compared with healthy subjects [47]. Therefore, the structure changes of ACC and MTG areas might be the potential mechanism and therapy responses of acupuncture treatment for emotion and cognition recovery of strokes.

Interestingly, the current study found that the GMV increases of ACC and MTG are positively related to the increase in MBI score. This means that the GMV increases induced by acupuncture in these regions were associated with the improvement in ability of daily life. Nowadays, the cognitive impairments that might contribute to poor executive function have been documented before. For example, recent studies [48] found the function connection of ACC and MTG impaired of cirrhotic patients. They demonstrated that the function connection reduction within cognitive networks including DMN, executive control (ECN), and salience (SN) and performed significantly worse as reflected by the longer time with more errors to complete the Stroop task. So they thought slower psychomotor speed and impaired cognitive flexibility could consequently lead to executive dysfunction. Besides, relative to emotion and cognition function, some researchers found the ACC also had some relationship with the motor network. Treserras et al. [49] proved the posterior cingulate cortex (PCC) and ACC played an important role on interaction between DMN and sensorimotor network (SMN) during movement-readiness state. They claimed that the two networks were functionally correlated through an interaction between the PCC and ACC during movement-readiness but not functionally correlated during rest, and the ACC would have a motivational role or could generate predictions about the movement. Additionally, ACC was found to be implicated in attentional control, the execution or inhibition of motor commands [50]. Another study on bipolar disorder also suggested impaired ACC might modulate between emotion dysregulation and motor processing in youths with bipolar disorder [51]. In the present study, after 4 weeks of acupuncture treatment, the left ACC and MTG significantly increased and showed positive relationship to the increase in MBI score. The results were partly in line with those of a study by Zhang et al. [52], who found that acupuncture at Yanglingquan (GB34) improved the motor function by increasing DMN connectivity in the ACC and posterior cingulate cortex (PCC). The results suggested that, compared with conventional treatment, acupuncture treatment might improve the ability of daily life by not only affecting the motor regions but also modulating the ACC and MTG.

Hence, we speculated that the modulatory effects of acupuncture on the DMN of stroke patients might partly be explicated as the recovery of the cognitive ability and motor recovery.

5. Conclusions

In summary, our study showed that acupuncture can evoke pronounced structural reorganization. The frontal areas and the network of DMN of brain areas which related to motor and cognition recovery may be the potential therapy target and the potential mechanism of acupuncture treatment for ischemic stroke.

Limitations. The sample size of this study was small, and future investigations need a larger sample size for statistically accurate analysis. In addition, we found some significantly obvious changes in the cerebral areas related to cognition or emotion. There is no doubt that these changes could seriously restricts the rehabilitation of patients. However, more professional scales would be used to evaluate the emotion and cognition recovery, and then research the relationship between scales and brain changes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ping Wu, Yu-mei Zhou, and Chen-xi Liao contributed equally to this paper. Fan-rong Liang, Fang Zeng, Ping Wu, and

Yu-mei Zhou conceived and designed the experiments. Yu-mei Zhou, Chen-xi Liao, and Yu-zhi Tang performed the experiments. Yong-xin Li, Li-hua Qiu, and Wei Qin analyzed the data. Fang Zeng and Fan-rong Liang revised the paper. All authors approved the final version of the paper.

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Research Article

The Comprehensive Therapy of Electroacupuncture Promotes Regeneration of Nerve Fibers and Motor Function Recovery in Rats after Spinal Cord Injury

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The present study aimed to evaluate the role of the combination treatment of methylprednisolone (MP) and electroacupuncture (EA) in regeneration of nerve fibers and functional recovery in rats with spinal cord injury (SCI). Female Wistar rats were used for an SCI model by using a weight-drop hammer at levels T_{10} (spinal cord segment corresponding to the 10th thoracic vertebra). Four groups received different treatments for the study: SCI control, MP, MP and EA, and Sham. The growth of nerve fibers was examined by counting fluorescein positive nerve fibers. The motor functional recovery was evaluated by Basso, Beattie, Bresnahan (BBB) score, and electrophysiology analysis. We found that, compared to MP groups, there were more well-oriented and paralleled fluorescein positive nerve fibers in MP and EA group. Both latencies and amplitudes of the Motor Evoked Potential (MEP) in the combination therapy of MP and EA were higher than MP group. Additionally, recovered hindlimb movements were sustained in most rats in the MP and EA group. Our study indicated that combination therapies could become a powerful treatment for SCI in rats.

1. Introduction

A spinal cord injury (SCI) is damage or trauma to the spinal cord. Patients with SCI usually have temporary or permanent neurologic deficits and disability, including motor deficit, sensory deficit, breathing difficulty, and bowel and/or bladder dysfunction. SCI can be caused by accident, diseases, or degeneration. It is estimated that the incidence of SCI around the world is between 250,000 and 500,000 people every year [1].

Steroids have been used in the management of acute SCI for decades due to anti-inflammatory effects and inhibition of lipid peroxidation [2]. Especially in 1990, the results of the Second National Acute Spinal Cord Injury Study (NASCIS II) showed that high-dose of methylprednisolone (MP) could improve neurological recovery of SCI patients [3]. In NASCIS III, post hoc analysis showed that the motor functions had been improved at least temporarily on MP-treated patients that received 48 h MP compared to 24 h administration [4, 5]. However, high-dose MP in acute SCI can lead to side effects including hyperglycemia, wound infections, delayed healing, gastrointestinal complications, and pulmonary embolism [6]. Therefore, the use of MP in acute SCI patients has been controversial in recent years.

Acupuncture is an ancient Chinese therapy by inserting the needles at certain points of the meridians to cure disease

and relieve pain [7]. Electroacupuncture (EA) is a form of acupuncture that involves the application of a gentle pulsating electrical current on the specific traditional acupuncture points on the body. The procedure can be done with or without the use of needles. Previous animal studies have demonstrated that EA can promote the differentiation of mesenchymal stem cells and regeneration of nerve fibers in rats with SCI [8]. Additionally, EA can improve neuronal function recovery and inhibits inflammation responses and microglial activation after SCI [9].

In the present study, we examined the role of combination treatment of MP and EA in axon growth and regeneration and hindlimb movement function recovery in rats with SCI.

2. Materials and Method

2.1. Animals Group. Adult female Wistar rats (200–250 g) were purchased from the Experimental Animal Center of Jilin University. Animals were housed in a standard cage with the temperature 22 ± 1°C and humidity of 50%–60%. Animal experiments related to the study were approved by the Local Ethics Committee for Animal Research at Jilin University and performed in accordance with international standards for animal welfare. Rats were randomly divided into four experimental groups, which each contained 18 rats; specifically (1) SCI control group: no treatment after the SCI surgery; (2) MP group: intravenous injection MP 30 mg·kg^{-1} immediately after SCI, repeated once 4 h after surgery, and then injected twice per day with 3 days; (3) MP and EA group: both MP and Hua Tuojiaji (EX-B2, the Hua Tuojiaji point is located in the first thoracic vertebra to the fifth lumbar vertebra, each vertebral spinous process by 0.5 inches), Ming Men (GV4, the Ming Men point is located between the spinous processes of the second and third lumbar vertebrae) and Da Zhui (GV14, the Da Zhui point is locate in that depression below the spinous process of the seventh cervical vertebra) acupoints were used in treatment 4 h after SCI; and (4) Sham group: vertebral plate was opened to expose spinal marrow without SCI. The acupuncture needles were 25 mm long and 0.35 mm in diameter. The 6805-II electroacupuncture therapeutic apparatus made in Shanghai is provided with a positive electrode and a negative electrode. EA parameters of 1-2 Hz at 0.3–1.0 mA were used in the present study. EA treatment was given once every day for 6 days, 15 minutes each time. After a 2-day interval, the second course started, with three courses in total. All surgical procedures were performed under general anesthesia with 3% pentobarbital sodium.

2.2. SCI Model. Adult female Wistar rats were anaesthetized with 3% pentobarbital sodium. A laminectomy was carried out at the T_{10} (the 10th thoracic vertebra) level to expose the spinal segment and then a hammer (20 g) was dropped from a height of 30 mm onto the exposed dura mater. After the induced SCI, all rats received extensive care, including penicillin (80,000 U/per rat) and gentamicin (2000 U/per rat), for 7 days and thick, soft bedding in individual cage. Manual emptying of the bladders was performed three times daily. All procedures were approved and in accordance with the Institutional Animal Care and Use Committee guidelines at Jilin University.

2.3. Behavioral Testing. Functional recovery was assessed by observers that were blind to groups of the experiment and graded each animal according to Basso, Beattie, Bresnahan (BBB) open field locomotion test [10]. The BBB score was determined by voluntary hindlimbs movement towards each group.

2.4. Electrophysiological Analysis. Thirty days after surgery, six rats from each group were used to study motor evoked potentials. Following anesthesia with 3% pentobarbital sodium, a midline incision was made on the rat's head skin, and the cranium was exposed. One hole was drilled for the skull by using a standard dental drill. The sciatic nerve was exposed to the left leg. The stimulating electrode was placed beneath the scalp, recording electrode was placed on the sciatic nerve, and reference electrode was placed below hard palate. The MEP was induced and registered for evoked potential instrument of Powerlab and biofunction experiment system of BL-410 (Taimeng, Chengdu, China) by appropriate stimulation parameters.

2.5. Fluorescein Injection. Thirty days after surgery, six rats from each group were anesthetized with 3% pentobarbital sodium. At the second spinal segment of SCI area, fluorescein (FR) (Invitrogen Company, USA) was slowly injected using a Hamilton microinjector (Dingguo, Beijing, China) at depths of 2.5 mm, 1.5 mm, and 0.5 mm away from the spinal dura mater, respectively, on both sides with the spinal cord. Four days after injection, the spinal tissue was removed, placed at 4°C in 4% paraformaldehyde overnight, and then replaced with 5%, 15%, and 30% cryoprotective sucrose for 90 min, respectively. Specimens were then quickly embedded in frozen OCT compound and stored at −80°C. Sections were prepared for a cryostat (Leica Company, Germany), cover-slipped with glycogelatin to preserve fluorescence, and observed under fluorescence microscopes.

2.6. Statistical Analysis. Statistical analyses of electrophysiology MEP results were performed by single factor analysis of variance (ANOVA), and least significant difference (LSD) was used for intergroup comparison. Statistical analyses of functional recovery BBB score results were performed by the ANOVA of repeated measurement design based on the original data by using the SPSS program (version 13.0) for Windows (SPSS, Chicago, IL, USA). Differences are considered statistically significant if $P < 0.01$.

3. Results

3.1. Fluorescein Positive Nerve Fibers. To evaluate the growth of nerve fibers after treatment for SCI rats, the FR-positive nerve fibers were counted in six rats for each group. As shown in Figure 1, no FR-positive nerve fibers were observed in the SCI control group. In the MP group, short FR-positive nerve fibers (red) were occasionally seen in the proximal SCI region, but none of them was distributed over the SCI region.

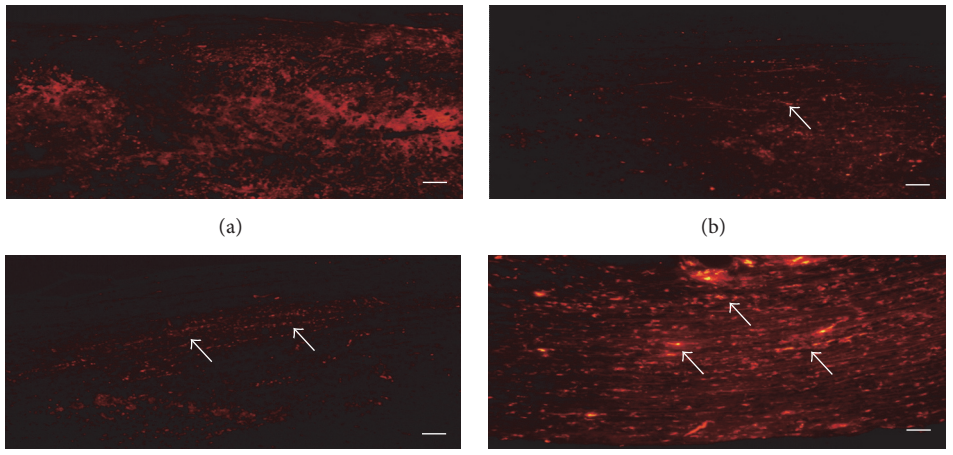

FIGURE 1: FR anterograde tagging for spinal cord of coronal plane. Arrow indicates the positive nerve fibers (red): (a) SCI control group; (b) MP group; (c) MP and EA group; and (d) Sham group (bar = 100 μm).

Many FR-positive nerve fibers were found in the MP and EA group, and the neural tracer agents were seen in distal SCI region, which indicated the regenerated nerve fibers extended to the distal SCI region. Compared with other groups, there were more well-oriented and paralleled FR-positive nerve fibers. Many FR-positive nerve fibers were well-oriented and paralleled with each other in sham group.

3.2. Electrophysiology Analyses. To further examine the function of the severed spinal cord after treatment, the MEP was measured. As shown in Figure 2, the rats in the SCI control group showed negligible signal of MEPs. However, a noticeable increase in peak-to-peak valued was observed in the MP group and MP and EA group. Additionally, the rats in the MP and EA group exhibited the highest degree of recovery from both latencies and amplitudes of the MEP, which is similar to what was observed in the sham group.

3.3. Functional Recovery. To evaluate the functional recovery, the BBB score was determined by voluntary hindlimb movement towards each group. As shown in Figure 3, voluntary hindlimb movement was not seen until two weeks after surgery in SCI control group. The rats in the SCI control group were unable to walk while bearing weight. In rats treated with MP, the recovery trend was seen from one week after surgery, but it did not reach significance compared with the SCI control group. In rats treated with MP and EA in the presence, voluntary hindlimb movement was significantly improved compared with the rats in the MP group. In the sham group, the hindlimb movement was seen on day 1 after surgery and almost achieved full recovery one week later.

4. Discussion

Recent studies have found that adult mammalian spinal cord injury leads to a series of neurological deficit symptoms [11, 12] and nerve conduction pathway interruption [13], affecting its metabolism and axonal transport function. Since axonal regeneration depends on this function, structural protein is supplied by the neuronal cell synthesis via axonal transport, and nerve conduction pathway recovery degree directly influences the nerve fiber regeneration situation, it is clear that how to restore the conduction pathway and promote nerve fiber regeneration is the key to the recovery of spinal cord function [14]. MEP mainly reflects the nerve conduction function after SCI. At present, as a more objective and sensitive detection method, it has been more and more used in clinical SCI nerve function evaluation [15]. MEP is a sensitive index to evaluate the functional state of motor conduction pathway, can directly reflect the functional state of spinal cord descending conduction bundle or peripheral motor nerve, and has a strong correlation with lower limb motor function [16, 17], and it can effectively detect the degree of spinal cord injury and functional recovery degree after injury [18].

In our study, FR anterograde tagging indicated that many FR-positive nerve fibers were distributed among SCI area and extended to the distal side. Additionally, the nerve fibers were well-oriented and paralleled in the combination therapy of MP and EA. Furthermore, the latencies and amplitudes of the MEP in the combination therapy of MP and EA were higher than those in all other groups. In addition, recovered hindlimb movements were sustained in most rats in MP and EA group. The combination therapy of MP and EA might function via the following mechanism: (1) MP provides multifarious neuroprotective effects, including improving microcirculation, inhibiting lipid oxidation, reducing calcium influxes in cells, and maintaining nervous system excitability; (2) the EA treatment decreases the production of free radicals, regulates neuropeptide secretion, and improves the blood circulation of SCI.

From the clinical and anatomical point of view, it is feasible to effectively restore the neural pathway in spinal cord injury by combining Chinese and western medicine [19, 20]. Electroacupuncture has its unique advantages. EA

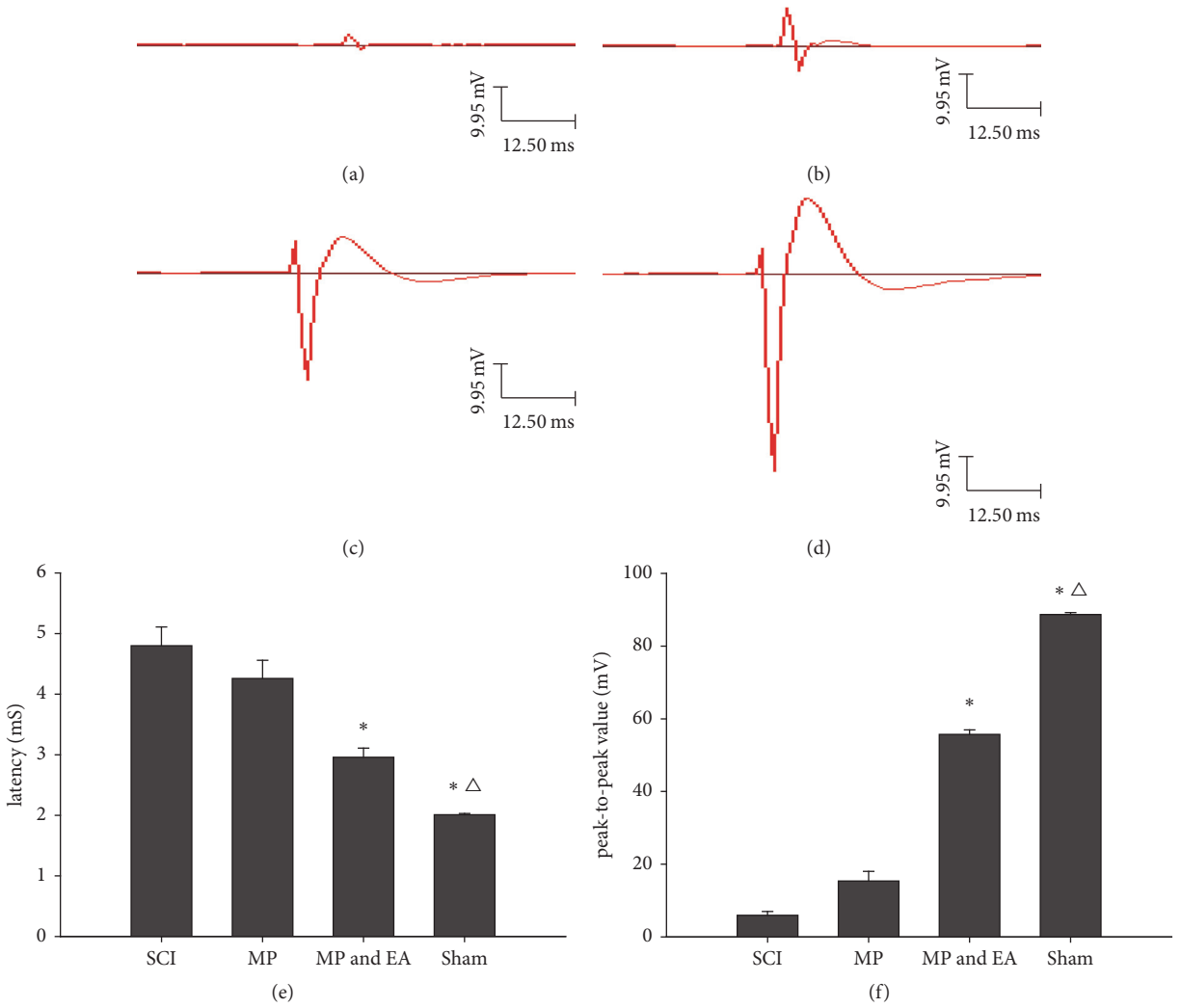

Figure 2: MEP wave shape. (a) SCI control group; (b) MP group; (c) MP and EA group; and (d) Sham group. (e) The latency of MEP ($n = 6$; $*$ versus MP group, $P < 0.01$; $\triangle$ versus MP and EA group, $P < 0.01$). (f) The peak-to-peak value of MEP ($n = 6$; $*$ versus MP group, $P < 0.01$; $\triangle$ versus MP and EA group, $P < 0.01$). Statistical analyses of MEP results were performed by single factor analysis of variance (ANOVA), and least significant difference (LSD) was used for intergroup comparison.

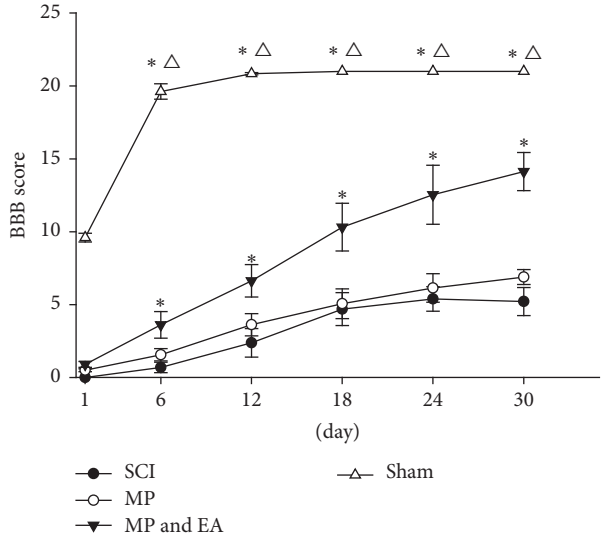

Figure 3: Comparison of BBB score of hind limb motor function. $n = 18$; $*$ versus MP group, $P < 0.01$; $\triangle$ versus MP and EA group, $P < 0.01$; statistical analyses of BBB score results were performed by the ANOVA of repeated measurement design based on the original data.

has been shown to be effective against improving functional recovery from SCI patients in traditional Chinese medicine [21]. Animal studies indicate that EA promotes the secretion of neurotrophin-3 and enhances the differentiation rate of exogenous neural stem cells. The therapy of EA promotes the survival and axonal regeneration in rat SCI model [8, 22]. Although there are many problems to be solved, the combination of traditional Chinese medicine and western medicine treatment method will become the focus of research, giving full play to its combined and complementary advantages [23, 24].

In summary, we have presented data that indicate that the comprehensive therapy of EA in rats with SCI can effectively enhance the growth of nerve fibers and improve the hindlimb motor function recovery, suggesting that combination therapies could become a powerful treatment for SCI.

Disclosure

Yi-Fan Li and Tie Li should be considered co-first authors.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Authors' Contributions

Chen Li and Fu-Chun Wang conceived and designed the experiments. Yi-Fan Li and Tie Li performed experiments and contributed equally to this work. Yi-Fan Li, Tie Li, Da-Wei Zhang, Hui Xue, and Dong Chen contributed reagents/materials/analysis tools. Yi-Fan Li and Tie Li analyzed the data. Yi-Fan Li, Tie Li, and Da-Wei Zhang drafted the manuscript and all authors approved the final version for publication.

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Research Article

Home-Based Transcutaneous Neuromodulation Improved Constipation via Modulating Gastrointestinal Hormones and Bile Acids

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This study aims to investigate the role of transcutaneous neuromodulation (TN) on the regulation of gastrointestinal hormones and bile acids in patients with functional constipation (FC). Twenty FC patients were treated with TN for four weeks. The effects of TN on symptoms were evaluated by questionnaires. Plasma levels of serotonin (5-HT), motilin, somatostatin, and vasoactive intestinal peptide (VIP) were measured by ELISA and 12 individual bile acids assayed by liquid chromatography tandem mass spectrometry. Results were as follows. (1) TN treatment increased the frequency of spontaneous bowel movement, improved the Bristol Stool Score, and reduced Patient Assessment of Constipation Symptom score and Patient Assessment of Constipation Quality of Life score. (2) FC patients showed decreased plasma levels of 5-HT, motilin, and VIP and an increased plasma level of somatostatin ($P < 0.05$). Four-week TN treatment increased plasma levels of 5-HT and motilin and decreased the plasma level of somatostatin in the FC patients ($P < 0.05$). (3) Taurocholic deoxycholate, taurocholic acid, and taurocholic lithocholic acid were increased in the FC patients ($P < 0.005$) but reduced by TN treatment ($P < 0.05$). This study has suggested that the therapy may improve the symptoms of FC by alleviating the disorders of gastrointestinal hormones and bile acids.

1. Introduction

Functional constipation (FC) is mainly characterized by a low defecation frequency, defecation difficulty, and incomplete defecation. The prevalence of FC has been high in female and elderly [1]. Based on the pathophysiology, FC is classified as follows: Slow Transit Constipation (STC), Defecatory Disorder (DD), and Normal Transit Constipation (NTC) [2]. Traditional treatment is mainly focused on drugs such as leavening agent, osmotic laxatives, stimulant laxatives, prosecretory agents, and prokinetic agents [3]. Recently, the bile acid regulator has become a widely applied medical

therapy [4]. However, there are still a large number of patients who are refractory to medical therapies and there is a need to develop effective therapies for FC.

Neuromodulation has recently been introduced for the treatment of FC, such as sacral nerve stimulation [5] and tibial nerve stimulation [6] with inconclusive results and largely unknown mechanisms. In a previous study, we reported a promising ameliorating effect of transcutaneous neuromodulation (TN) in FC patients [7]. In this method, electrical stimulation was delivered noninvasively via surface electrodes placed at both an acupoint ST36 and the posterior tibial nerve using an external watch-size stimulator. The therapy was home-based and self-administrated. The therapeutic effect of TN was reported to be mediated via the autonomic functions (enhancement of vagal activity and suppression of sympathetic activity) and hypothesized to improve gastrointestinal motility. However, its exact prokinetic mechanisms, especially the involvement of neurotransmitters and bile acids, were still unclear.

Acupuncture and electroacupuncture have been reported to alter various neurotransmitters in both patients with functional gastrointestinal diseases and animal models of constipation, such as serotonin (5-HT) [8, 9], motilin [10], and vasoactive intestinal peptide (VIP) [11, 12]. While little is known about the direct effects of acupuncture or electroacupuncture on bile acid metabolism, which is involved in 5-HT activation [13]. Meanwhile, the abnormal metabolism of bile acids was found in patients with FC [14, 15], indicating their potential roles in colon motility and hormone secretion to influence intestinal transit [16].

Accordingly, this experiment aims to investigate the therapeutic effect of the home-based, noninvasive, and self-administrated TN at ST36 and posterior tibial nerve on FC and to explore its mechanisms involving neurotransmitters associated with colon motility and bile acids in patients with FC.

2. Materials and Methods

2.1. Study Subjects. This study included 20 FC patients and 20 healthy volunteers, recruited from the Department of Physical Examination, the First Affiliated Hospital of Dalian Medical University from December 2016 to April 2017. The study protocol was approved by the hospital ethics committee (number LCKY2016-31) and registered in Chinese Clinical Trial Registry (number ChiCTR-OOC-16010259). All participants in the study signed the informed consent form and were free to quit the study at any time for any reasons.

2.1.1. Inclusion Criteria for Patients. Inclusion criteria for patients were as follows: (1) aged 18–80 years; (2) met Rome IV criteria [2] for FC; (3) no organic diseases by colonoscopy; (4) no acupuncture treatment in the preceding 3 months; (5) no participation in any clinical trials in the preceding 3 months; and (6) being capable of conducting the treatment at home.

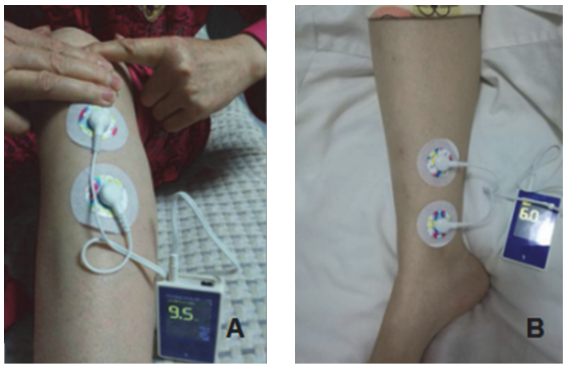
2.1.2. Exclusion Criteria. Exclusion criteria were as follows: (1) abdominal surgery history; (2) metabolic diseases such as diabetes and hypothyroidism; (3) neurologic diseases or any organic diseases causing constipation such as multiple sclerosis, rachischisis, Parkinson's disease, or spinal cord injury; (4) any organic diseases, such as liver, gallbladder, pancreas, or intestines; (5) pregnancy or intention to become pregnant during the trial; (6) allergic to skin preparation or electrodes; (7) being with an implanted pacemaker; (8) Self-Rating Anxiety Scale (SAS) score > 60 or/and Self-rating Depression Scale (SDS) score > 63; and (9) refusal of blood withdrawing.

2.1.3. Inclusion Criteria for Healthy Volunteers. Inclusion criteria for healthy volunteers were as follows: (1) aged 18–80 years; (2) no diagnosis of gastrointestinal diseases in the preceding 3 months and no constipation and other gastrointestinal symptoms; (3) no abdominal surgery, metabolic disease, neurologic diseases, or other organ diseases; (4) no medications affecting bowel movement in the preceding 3 months; (5) willingness to sign the informed consent; and (6) willingness for blood withdrawing.

2.2. Experimental Methods

2.2.1. TN Treatment. A watch-size microstimulator, called Neuromodulator for Gastrointestinal Functions (SNM-FDC01, Ningbo Maida Medical Device Inc., Ningbo, China), was used for the TN treatment via electrodes placed at the posterior tibial nerve and the acupoint ST36 (Zusanli, either right leg or left leg) according to a previous study [7]. For the ST36, one electrode was placed at ST36 and the other at 4 cm below ST36 along the same meridian; for the posterior tibial nerve, one electrode was placed at approximately two fingers' breadth up to the malleolus medialis and posterior to the tibia and the other at 4 cm above the first electrode (Figure 1). The following parameters were used for the TN: train on-time of 2 sec and off-time of 3 sec, pulse width of 0.5 ms, pulse frequency of 25 Hz, and amplitude of 2–10 mA (at the maximum level tolerated by the subject) [7].

2.2.2. Experimental Protocols. The TN treatment was performed at home and administrated by the patient. Before the initiation of the treatment, each patient was trained for the use of the therapy, including identification of stimulation locations, preparation of the skin, operation of the device, and recharging of the device as well as the completion of questionnaires. Before the TN treatment, there was a phase-in period of one week during which the patient was required to stop taking any medications affecting gastrointestinal motility or defecation. The TN treatment was given twice a day: 6–8 am before breakfast and 6–8 pm after dinner, each lasting 1 hour. Various questionnaires were completed by the patient at the end of each week, including the phase-in period (Figure 2). At the end of the 4-week treatment, the patients were informed to come to the hospital to return the stimulation device and at the meantime blood samples were taken for the assessment of gastrointestinal hormones and bile acids. Four more weeks (no TN treatment) later, the patients were informed to complete the symptom



(a) (b)

Figure 1: *The location of electrode*. (a) ST36: one electrode was placed at ST36 and the other at 4 cm below ST36 along the same meridian. (b) Posterior tibial nerve: one electrode placed at approximately two fingers' breadth up to the malleolus and posterior to the tibia and the other at 4 cm above the first electrode.

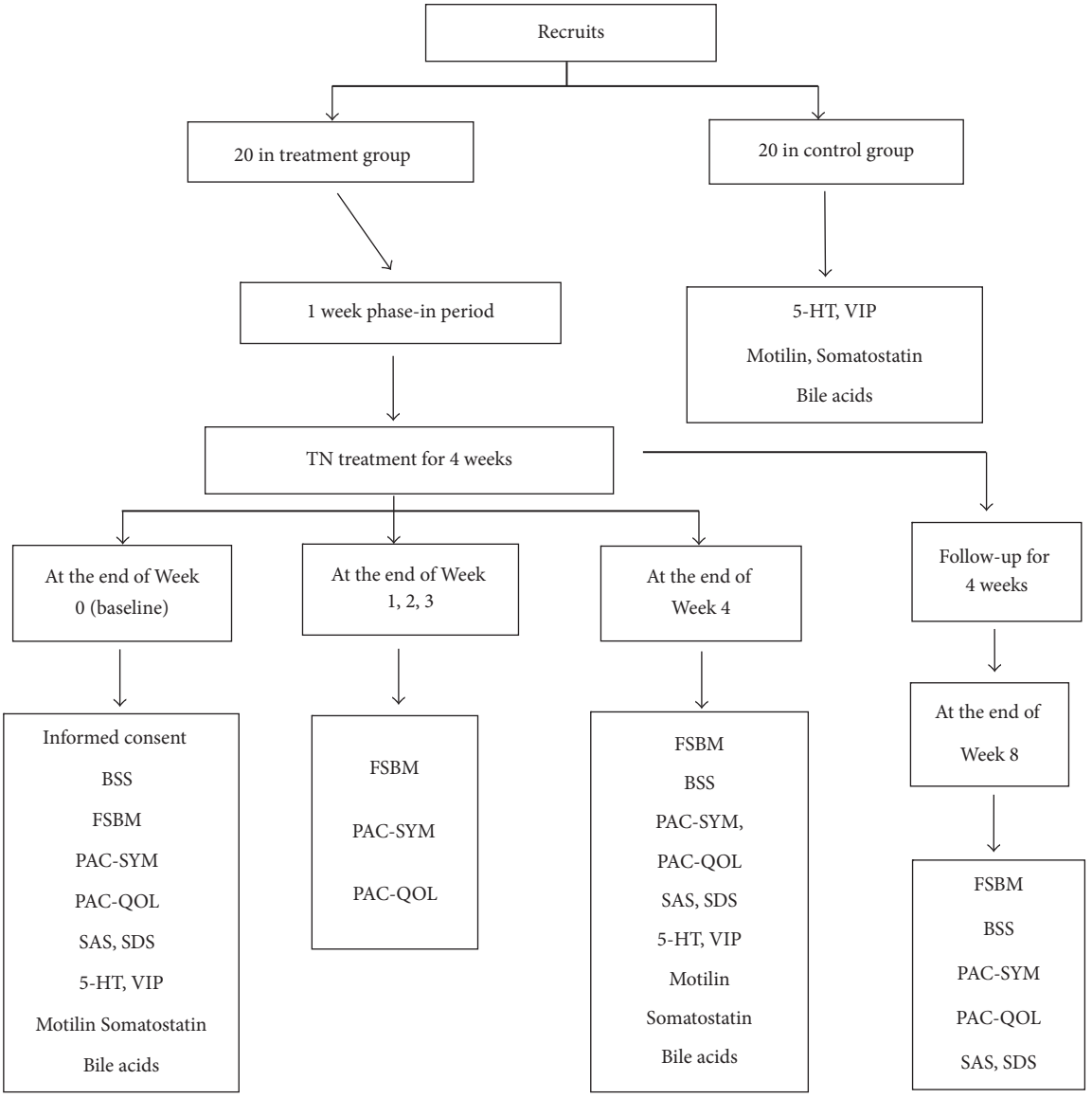


Figure 2: Experimental protocols.

questionnaire for the assessment of possible sustained effects of TN treatment; then all the questionnaires were collected.

During the treatment, participants should avoid using other cathartic in principle, unless no defecation lasted 3 days or more. Under this condition, Enema Glycerini was allowed to assist defecation once or twice and had to be recorded in the defecation diary. Emergency medicine could also be applied during the phase-in period. Participants were free to withdraw from the experiment for further treatment if they kept suffering from constipation.

No sham treatment was given in this study because firstly the ameliorating effect of the TN on constipation was previously established in a previous placebo-controlled study [7] and the major aim was to investigate the underlying mechanisms of TN treatment; secondly, it is well-known that the placebo effect is rare in objective physiological or mechanistic measurements.

2.2.3. Assessment of Constipation-Related Symptoms. Constipation-related symptoms were assessed by patient completed questionnaires which included (a) Bristol Stool Score (BSS) [17], (b) the frequency of spontaneous bowel movement per week (FSBM), (c) Patient Assessment of Constipation Symptom (PAC-SYM) [6]; (d) Patient Assessment of Constipation Quality of Life (PAC-QOL) [6], (e) SAS [18], and (f) SDS [18]. Before the treatment (baseline), items (a)–(f) were completed. During the first 3 weeks of treatment, items (b)–(d) updated the defecation diary and the time and frequency of the use of medications were recorded. At the end of the 4-week treatment, items (a)–(f) were completed. Four more weeks (no TN treatment) later, items (a)–(f) were completed.

2.2.4. Quantification of Gastrointestinal Hormones and Bile Acids. Blood samples were taken from FC patients and healthy volunteers for the measurement of levels of plasma gastrointestinal hormones and bile acids. Healthy volunteers were not treated with TN. A 4 ml blood sample was taken in fasting state (7-8 am) from the patients before and at the end of the 4-week treatment. A 4 ml blood sample was also taken from each healthy volunteer in fasting state. The sample was collected in a test tube containing EDTA, placed for 30 minutes under room temperature and centrifuged for 10 minutes at 4000 r/min; then 1.5 ml upper plasma were transferred into an EP tube and saved in a −80°C refrigerator.

The enzyme-linked immunosorbent assay method was applied to measure the plasma levels of gastrointestinal hormones, including 5-HT, motilin, somatostatin, and VIP according to the manufacturers instructions (USCN Life Science Inc., Wuhan, China).

Liquid chromatography tandem mass spectrometry [19] was used to assay 12 subtypes of bile acids (Dalian Meilun Biotech Co., Ltd., Dalian, China), including tauroursodeoxycholic acid (TUDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurocholic deoxycholate (TDCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), cholic acid (CA), lithocholic acid (LCA), taurocholic lithocholic acid (TLCA), glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), and glycodeoxycholic acid (GDCA).

2.3. Statistical Analysis. Statistical analysis was conducted using SPSS16.0 Software. Data with a normal distribution are presented as mean ± SE. Data that did not have a normal distribution are presented as median (quartile range). The Independent Samples t-test was used to compare general characteristics of groups. Wilcoxon Signed Ranks Test was used to compare the measurements before and after the treatment, while Mann-Whitney U Test was used to compare the measurements between treatment group and control group. The correlation analysis was performed using the Spearman correlation method. Statistical significance was assigned for $P < 0.05$.

3. Results

A total of 20 FC patients (14 female, 6 male) were enrolled in the study. Three patients were dropped out during the study: two of them attributed to allergic reaction to the stimulation electrodes and the other due to poor compliance (incomplete questionnaires). The remaining 17 patients underwent the trial without any uncomfortable complaints. The ratio of female/male, age, and body mass index of the patients were 14/6, (49.30 ± 11.94), and (23.17 ± 3.64), respectively. Those of the healthy controls were 12/8, (47.70 ± 14.71), and (23.31 ± 2.44), respectively. No ratio and mass index difference was noted between the patients and controls ($P > 0.05$).

3.1. Improvement in Constipation by TN. The 4-week TN treatment significantly improved the frequency of spontaneous bowel movement per week. The FSBM was significantly increased in comparison with the baseline at the end of the 3rd and 4th weeks of the treatment ($P < 0.001$). Most interestingly, the FSBM increase remained significant at 4-week follow-up without stimulation ($P < 0.001$), suggesting a sustained effect after TN which was not previously reported [7].

The stool characteristics assessed by the Bristol Stool Score was also significantly improved with the treatment. Types 1 and 2 in the BSS are hard and suggestive of constipation, types 3–5 are considered normal, and types 6 and 7 represent loose and liquid stools associated with diarrhea [17]. After the 4-week TN treatment, the score of BSS was increased from a median of 2.0 (1.0~2.0) at the baseline level to a median of 3.0 (3.0~4.0) ($P < 0.001$; see Table 1) and remained at a median of 3.0 (3.0~3.0) after 4 weeks of follow up without TN treatment ($P < 0.001$ versus baseline).

The TN treatment also significantly improved constipation symptoms and the quality of life. Compared with the baseline, the PAC-SYM and the PAC-QOL were significantly decreased at the end of the 3rd and 4th week of the treatment ($P < 0.001$) and remained reduced after 4 weeks of follow-up without the treatment ($P < 0.001$ versus baseline; see Table 1). The PAC-SYM score was decreased from a median of 26.0 (24.5~32.0) at the baseline level to a median of 16.0 (14.0~18.5) ($P < 0.001$) after the 4-week treatment and a median of 18.0 (14.5~18.5) at the end of 4-week follow-up without

Table 1: Comparisons of FC patients' questionnaires. Median (quartile range), Wilcoxon Signed Ranks Test.

	0th week (baseline)	1st week	2nd week	3rd week	4th week	8th week
FSBM	2.0 (1.0~2.0)	2.0 (1.0~2.0)	2.00 (2.0~2.0)	3.0 (2.5~3.0)*	4.0 (3.0~4.0)*	3.0 (3.0~4.0)*
BSS	2.0 (1.0~2.0)	- -	- -	- -	3.0 (3.0~4.0)*	3.0 (3.0~3.0)*
PAC-SYM	26.0 (24.5~32.0)	26.0 (24.0~29.0)	26.0 (24.0~31.0)	22.0 (20.0~24.0)*	16.0 (14.0~18.5)*	18.0 (14.5~18.5)*
PAC-QOL	52.0 (48.0~58.0)	52.0 (48.0~57.0)	52.0 (48.0~55.0)	39.0 (35.0~43.0)*	31.0 (27.0~36.0)*	33.0 (26.5~38.0)*
SAS	44.0 (37.5~51.5)	- -	- -	- -	30.0 (28.0~34.5)*	28.0 (26.0~28.5)*
SDS	44.0 (37.5~49.5)	- -	- -	- -	30.0 (28.0~32.5)*	29.0 (27.0~30.5)*

$n = 17$; * $P < 0.001$ versus baseline.

Table 2: The comparison of plasma bile acids among FC patients and control group. Median (quartile range), nonparametric test.

Bile acids (ng/ml)	Before TN treatment (treatment group, $n = 17$)	After TN treatment (treatment group, $n = 17$)	Control group ($n = 20$)
TUDCA	0.00 (0.00~1.65)	0.00 (0.00~6.22)	1.02 (0.18~2.10)
TCDCA	22.80 (7.33~55.60)	36.80 (14.90~58.95)	13.65 (7.38~47.45)
UDCA	11.80 (5.23~36.30)	26.00 (12.45~44.75)	8.74 (2.68~33.78)
CDCA	44.70 (19.15~44.70)	98.00 (19.95~273.00)	87.80 (21.60~283.35)
LCA	ND	ND	<5
GCA	43.50 (17.85~147.35)	52.60 (23.30~106.00)	16.95 (10.83~70.80)
GCDCA	118.70 (56.05~491.10)	188.20 (78.60~255.20)	400.00 (90.25~677.50)
GDCA	34.80 (9.41~89.05)	20.60 (12.40~38.50)	28.95 (11.33~52.43)
TDCA	31.30 (11.85~60.05)#	12.64 (9.29~19.95)#*	2.40 (1.85~4.63)
TCA	67.60 (30.71~178.90)#	31.70 (12.54~72.90)#*	2.45 (1.03~26.57)
TLCA	4.10 (3.23~4.95)#	3.20 (2.09~3.70)#*	1.05 (0.26~2.26)
CA	18.70 (7.35~31.00)	21.10 (13.60~33.00)	28.70 (3.83~92.35)

# $P < 0.05$ versus control group (Mann-Whitney U Test); * $P < 0.05$ versus treatment group before TN treatment (Wilcoxon Signed Ranks Test); ND, not detectable.

the treatment ($P < 0.001$ versus baseline). The PAC-QOL score was decreased from a median of 52.0 (48.0~58.0) at the baseline level to a median of 31.0 (27.0~36.0) ($P < 0.001$) after the 4 weeks' treatment and a median of 33.0 (26.5~38.0) at the end of 4 weeks' follow-up after stopping the treatment ($P < 0.001$ versus baseline).

3.2. Alteration in Plasma Gastrointestinal Hormones. A number of major gastrointestinal hormones associated with motilin were altered in FC patients and improved after 4-week TN treatment. As shown in Figure 3, (1) the plasma level of 5-HT in the FC patients before TN therapy was lower than that of the healthy volunteer control group ($P = 0.009$) but increased significantly after the treatment ($P = 0.004$ versus baseline; $P = 0.180$ versus controls), (2) the concentration of the plasma motilin in FC patients was lower than that of the control group ($P < 0.001$) but increased significantly after the treatment ($P < 0.001$) although being still lower than that of the control group ($P = 0.003$), (3) the plasma level of VIP in FC patients was lower than that of the control group ($P = 0.037$) and not altered by TN treatment ($P = 0.093$ versus baseline; $P = 0.006$ versus controls), and (4) the plasma level of somatostatin in the FC patients was higher than that of the control group before the TN therapy ($P = 0.011$) but increased after the 4-week therapy ($P = 0.031$ versus baseline; $P = 0.563$ versus controls).

3.3. Modulation of Bile Acid Metabolism. Plasma levels of three of 12 subtypes of bile acids, TDCA, TCA, and TLCA were significantly higher in FC patients before treatment than that of healthy volunteers (control group) (all, $P < 0.005$ versus controls) but decreased obviously after 4-week TN treatment, with TDCA and TCA close to the control levels (all; $P < 0.05$ versus baseline) (Figure 4). For other bile acids, TUDCA, TCDCA, UDCA, CDCA, GCA, GCDCA, GDCA, and CA, we did not see any difference between FC patients and healthy controls and any change after TN treatment (Table 2).

3.4. Correlation Analysis of the Plasma Gastrointestinal Hormones and Bile Acids (Table 3). There was no correlation between the plasma gastrointestinal hormones (5-HT, motilin, VIP, and somatostatin) and bile acids (TDCA, TCA, and TLCA) in the control group and treatment group, neither before nor after TN treatment ($P > 0.05$).

3.5. Correlation Analysis of the Plasma Gastrointestinal Hormones, Bile Acids, and the Scores of Questionnaires (Table 4). There was no correlation between the plasma gastrointestinal hormones (5-HT, motilin, VIP, and somatostatin), bile acids (TDCA, TCA, and TLCA), and the scores of questionnaires (FSBM, BSS, PAC-SYM, and PAC-QOL) in FC patients before TN therapy ($P > 0.05$).

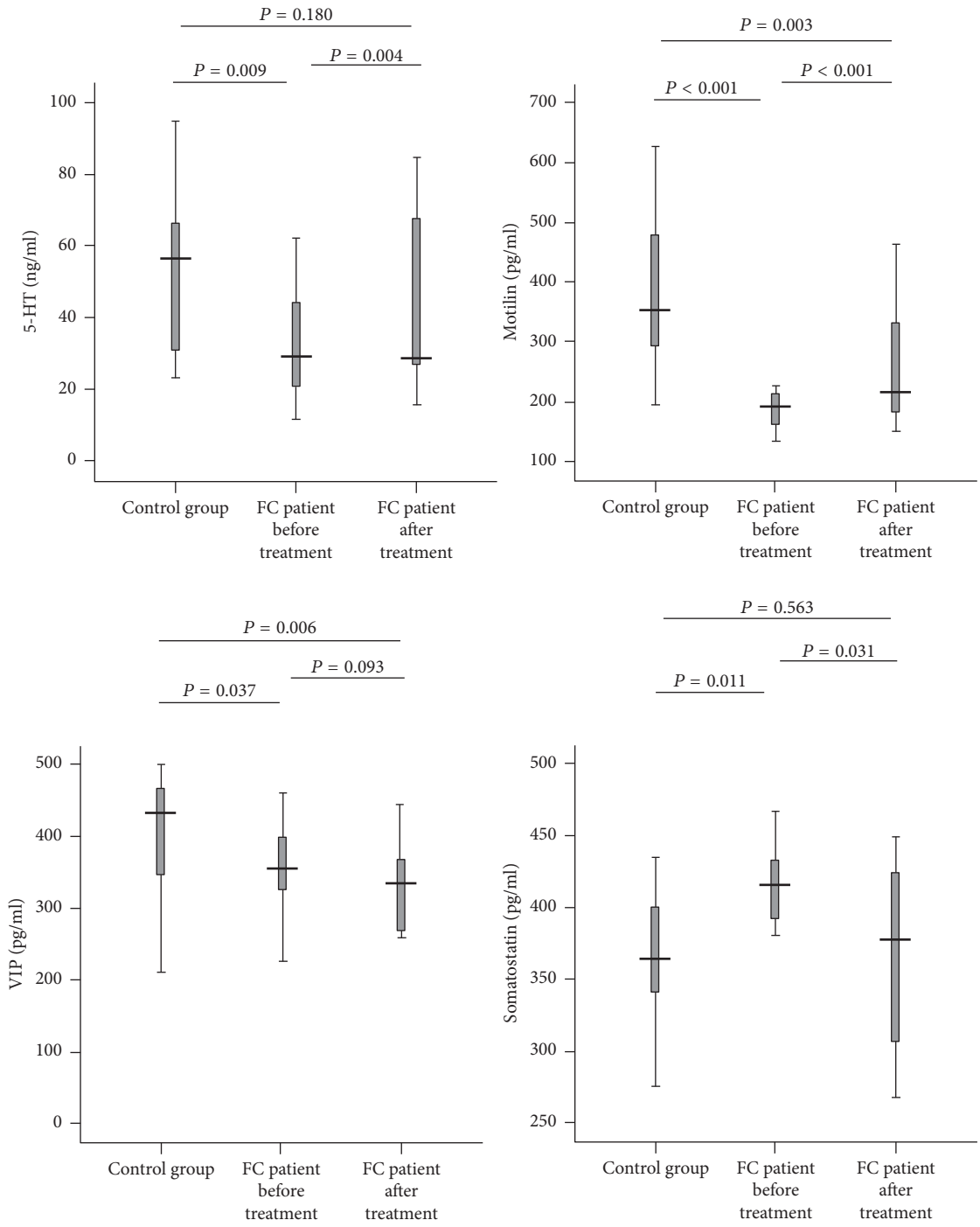


Figure 3: *The levels of the plasma gastrointestinal hormones in FC patients and control group*. FC patients showed a decreased plasma level of 5-HT, motilin, and VIP and an increased plasma level of somatostatin (all; $P < 0.05$ versus control group). The 4-week TN treatment increased plasma levels of 5-HT and motilin and decreased the plasma level of somatostatin in FC patients ($P < 0.05$ versus before treatment).

4. Discussion

Demonstrated by questionnaires of PAC-SYM and PAC-QOL TN treatment significant improved constipation symptoms and life qualities, which is consistent with the previous study [7]. More interestingly, we found that TN increased FC patients' plasma levels of 5-HT and motilin and decreased the plasma level of somatostatin. Furthermore, plasma levels of TDCA, TCA, and TLCA were downregulated by TN. Nevertheless, we did not find significant correlations between the gastrointestinal hormones and subtype bile acids in FC patients or between the above indexes and the scores of questionnaires.

It has been reported that patients with FC have a trend to suffer from mental or psychogenic diseases, such as anxiety-depression status [20–22]. Thus, in this study we introduced

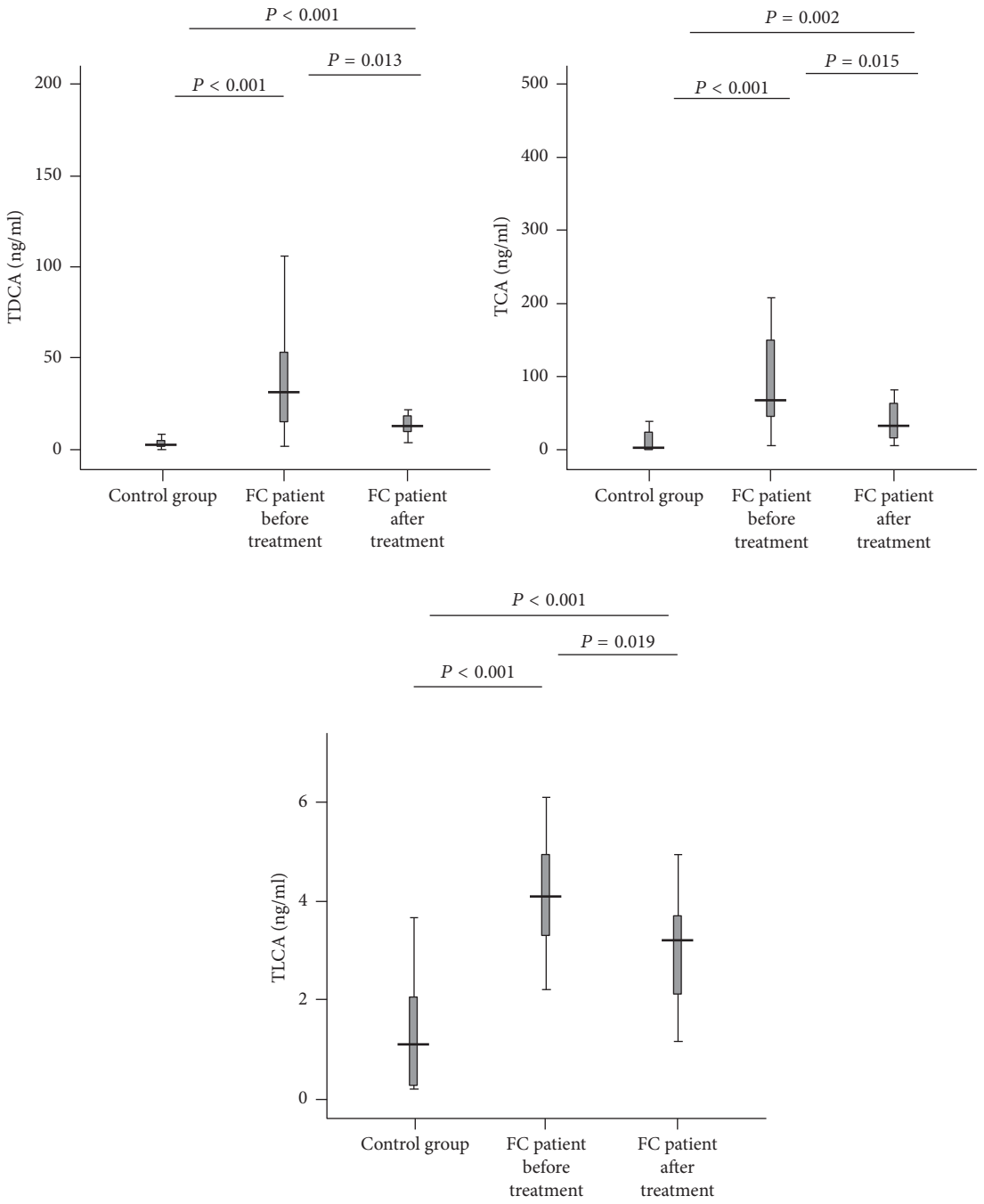


Figure 4: *The levels of the plasma bile acids in FC patients and control group*. TDCA, TCA, and TLCA were increased in FC patients (all; $P < 0.005$ versus control group) but reduced by TN treatment (all; $P < 0.05$ versus before treatment).

questionnaires of SAS and SDS and found that the FC patients were indeed in various degrees of anxiety-depression status. Therefore "SAS < 60, SDS < 63" was chosen as inclusion criteria to exclude the FC patients with serious mental or psychogenic disorders. The results showed that TN therapy did not aggravate the anxiety-depression.

TN has gained much attention due to being noninvasive, home-based, and self-administred. Our previous study has included sham treatment as control, and the effectiveness of TN treatment on FC was confirmed. Our major aim in this study was to investigate the underlying mechanisms of TN in terms of gastrointestinal hormones and bile acids. So, we do not establish sham treatment.

It is clear that the plasma levels of gastrointestinal hormones secreted by enteroendocrine cells were changed by the TN. Enteroendocrine cells are controlled by enteric neurons, which govern all gastrointestinal functions, including motility. The enteric nervous system receives information from

Table 3

(a) The Spearman correlation coefficient of the plasma gastrointestinal hormones and bile acids in FC patients before TN treatment (r-s, P)

	5-HT		Motilin		VIP		Somatostatin	
TDCA	−0.38	0.14	−0.05	0.86	0.09	0.72	−0.02	0.94
TCA	−0.26	0.32	0.01	0.98	0.21	0.42	−0.26	0.32
TLCA	−0.12	0.65	0.04	0.88	0.25	0.33	−0.13	0.63

$n = 17$; r-s, Spearman correlation coefficient.

(b) The Spearman correlation coefficient of the plasma gastrointestinal hormones and bile acids in FC patients after TN treatment (r-s, P)

	5-HT		Motilin		VIP		Somatostatin	
TDCA	0.03	0.90	−0.19	0.46	−0.06	0.81	−0.05	0.85
TCA	−0.41	0.10	−0.29	0.25	0.20	0.44	0.41	0.10
TLCA	−0.23	0.37	−0.12	0.66	0.06	0.82	−0.19	0.46

$n = 17$; r-s, Spearman correlation coefficient.

(c) The Spearman correlation coefficient of the plasma gastrointestinal hormones and bile acids in healthy volunteers (r-s, P)

	5-HT		Motilin		VIP		Somatostatin	
TDCA	−0.07	0.76	0.11	0.65	0.23	0.32	0.19	0.43
TCA	−0.33	0.15	−0.02	0.93	0.01	0.96	0.09	0.71
TLCA	−0.06	0.81	−0.10	0.69	0.06	0.80	−0.17	0.48

$n = 20$; r-s, Spearman correlation coefficient.

Table 4: The Spearman correlation coefficient of the level of plasma gastrointestinal hormones, the levels of plasma bile acids, and the scores of questionnaires of the FC patients before TN treatment (r-s, P).

	FSBM		BSS		PAC-SYM		PAC-QOL	
5-HT	−0.20	0.45	0.02	0.93	0.23	0.38	−0.12	0.65
Motilin	−0.20	0.45	0.02	0.94	0.00	0.99	−0.15	0.56
VIP	−0.01	0.96	0.40	0.11	0.06	0.83	0.10	0.71
Somatostatin	−0.07	0.78	0.21	0.41	−0.12	0.64	0.33	0.19
TDCA	0.15	0.58	−0.21	0.43	−0.33	0.19	−0.10	0.71
TCA	0.10	0.71	0.11	0.67	−0.31	0.22	−0.19	0.47
TLCA	−0.01	0.96	−0.30	0.24	0.00	1.00	−0.41	0.11

$n = 17$; r-s, Spearman correlation coefficient.

the parasympathetic and orthosympathetic nervous systems [23–25]. Our previous study reported that TN might improve the symptoms of FC patients by increasing vagal activity and concurrently decreasing sympathetic activity [7]. The animal experiment also demonstrated that both electroacupuncture and transcutaneous electroacupuncture at ST-36 were able to ameliorate intestinal hypomotility by increasing vagal activity [26]. Therefore, we believed that gastrointestinal hormones played an important role in the regulation of gastrointestinal motility.

5-HT is important to regulate gut motility and locates in the subsets of mucosal cells and neurons of gut [27]. 5-HT has many receptors expressed widely in gut, and the receptors of 5-HT3 and 5-HT4 their clinical value in regulating the gut motility has been proved [28]. Prucalopride is a 5-HT4 agonist which was found effective and safe for constipation patients [29, 30]. Researchers found that patients with STC may have 5-HT transporter gene which interferes with 5-HT reuptake [31]. In this study we found that the concentration of 5-HT in FC patients was lower and increased significantly after 4 weeks of TN treatment. Xu et al. [8] used electroacupuncture to stimulate Tianshu, Fujie, and Shangjuxu to treat serious FC. They found that plasma 5-HT increased and NOS decreased significantly after therapy, which is consistent with our results, and the similar effects were also shown when the therapy was applied on animals [9].

Motilin is secreted by Mo cells mainly distributed in digestive system [32], which is considered to contribute to gastrointestinal tract contraction [33]. We found that motilin's concentration in FC patients is lower than normal but increased after the TN therapy. The early study indicated that motilin level in STC group was higher than normal [34]. However, more researches suggested motilin level in FC patients is lower than that in healthy individuals, which is consistent with our study [35, 36]. Interestingly, Fei et al. [37] detected the motilin level in descending colon and rectal mucosa and also found it significantly decreased in STC group.

VIP is a kind of neural polypeptide scattering in the nervous system, immune system, and digestive system, which is composed of 28 acid residues and secreted by neurons,

endocrine, and immune cells. It can regulate gastrointestinal physiological functions [38]. King et al. [39] found that 1/3 of STC children had lower VIP nerve fiber density in the proximal colon. Tomita [40] found that STC patients' colon had a weaker response to VIP than normal colon. Xiong et al. [41] found that different frequency of electroacupuncture at Quchi point and ST36 could elevate the VIP level in serum of FC patients and relieve the symptoms. This study showed that the plasma level of VIP in FC patients was lower than that of control group, and 4-week therapy did not cause significant change. In this study, the cases could not be subtyped based on constipation characteristics due to small sample size. In addition, animal experiments indicated that the distribution of VIP positive nerve fibers had no difference between STC and normal mice [42].

Somatostatin is a kind of cyclic peptides distributing in nervous system and digestive system. Previous researches mainly focused on its influences on central nervous system. Recent reports implicate a negative effect on gastrointestinal peristalsis and hormones secretion [43]. In this study, we found that somatostatin in FC patients was higher than that in control group and decreased after TN therapy. Xi et al. [44] found somatostatin level in plasma increased as colon movement weakened in 90 healthy individuals. Further study showed serum somatostatin in STC patients raised obviously compared to normal controls [45]. Zhao et al. [46] found that *Lactobacillus* could relieve constipation in mice and decrease the somatostatin level, which was in line with findings of other researches [24, 47]. It was also reported that acupuncture and electroacupuncture improved symptoms of patients with constipation, with significantly decreased serum somatostatin [48].

It is well-known that bile acid in small intestine is mainly responsible for the fat emulsion, lipid digestion, and absorption, while some other physiological functions such as antimicrobial activity, gastrointestinal movement regulation, water and electrolyte absorption, intestinal epithelium growth, and epithelial gene expression are gradually unveiled [49]. It has been demonstrated that the abnormal metabolism of bile acid is involved in the pathophysiological mechanism of chronic constipation [50, 51]. Bile acids can connect the G-protein coupled plasma membrane receptor on colonic epithelial cell and induce the secretion of electrolyte and water, which can also reinforce the colonic movement and stimulation of defecation [52]. Oddsson et al. [53] found that the bile acids induced secretion of more rectal fluids in irritable bowel syndrome (IBS) patients than healthy volunteers. The phasic contraction of colon was stimulated by infusion of 5 mmol/L or higher concentration of bile acids, but such high concentration cannot exist in colon physiologically unless the ileum is removed [54, 55]. It has been suggested that CDCA in rectum could effectively stimulate the proximal colon to produce pressure wave [56]. Furthermore bile acids have been shown to stimulate intestinal chromaffin cells, releasing 5-HT [13] which affects gastrointestinal dynamics. Bile acid regulators such as elobixibat have been emerging as novel therapeutics for chronic constipation. Thus, we further examined the subtypes of bile acids in the plasma and explored their relationship with gastrointestinal hormones.

We detected 12 subtypes of bile acids in the plasma and found that only the levels of TDCA, TCA, and TLCA in FC patients were higher than that of control group and decreased after TN therapy. We speculate that TN might regulate the bile acid metabolism in patients with constipation. Hofmann et al. [14] found that fecal bile acids of most FC children had a normal metabolism. However, some of them had abnormal bile acids metabolism. Their major fecal bile acid is 3-sulfate of CDCA, which can abolish CDCA secretory activity and may contribute to constipation. which implicated that abnormal metabolism of bile acids might be a cause of childhood FC. Shin et al. [15] have shown that IBS with diarrhea (IBS-D) patients had a higher fecal level of CA/CDCA in comparison with that of healthy control, whereas in IBS with constipation (IBS-C) patients lower fecal CDCA and DCA were found in feces than that in healthy control. The percentage of LCA in fecal excretion increased significantly in IBS-C group, which might be associated with slow transit. Abrahamsson et al. [57] found that the changes in bile acid synthesis might be associated with transmission of colon. Fast transit of colon could reduce the absorption of bile acid, while slow transit of colon could increase the absorption of bile acid. The different absorption led to more bile acid synthesis in IBS-D and less in IBS-C, suggesting a significant positive correlation between fecal bile acid and fecal weight [58]. However, in this trial, LCA in the plasma of FC patients was not detected which might be due to the excessive excretion and subsequently low absorption. In our study, the level of CDCA and CA were lower in FC patients but there was no statistical difference before and after the TN therapy. We have detected that TDCA, TCA, and TLCA were significantly higher in the FC patients before treatment than that of healthy volunteers control group and decreased obviously after TN treatment. These results indicate that TDCA, TCA, and TLCA were to be reabsorbed more in the bowel when patients suffered from constipation; then the absorption decreased as the constipation symptoms improved by TN therapy. Furthermore, the plasma bile acid spectrum may reflect the levels of the fecal bile acids partly, which deserve to be explored further. In addition, there was no obvious correlation between the plasma gastrointestinal hormones and bile acids. The exact link between bile acids and gastrointestinal hormones remains to be further investigated.

In conclusion, TN may improve the symptoms of FC patients by alleviating the disorders of gastrointestinal hormones and bile acids metabolism. Strategies aiming at modulating such disorders may emerge as novel therapeutics for chronic constipation.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Authors' Contributions

Zhenyang Ge performed the research and wrote the paper; Zhijun Duan, Qingyong Chang, and Jiande D. Z. Chen

designed the research study and revised the manuscript; Shuang Zhang, Lixia Wang, Dong Yang, Liping Su, Xiaoyu Sun, and Zhifeng Zhang completed volunteer recruitment; Hong Zhu and Dongdong Zhou completed blood test; Hang Yang, Shengai Zhang, Honggang Shi, Jun Yu, and Hui Yang completed the material collection; Bojia Liu and Dongsheng Wu directed the data analysis; Nina Zhang directed the experiment.

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Research Article

Effect of Acupuncture at LR3 on Cerebral Glucose Metabolism in a Rat Model of Hypertension: A ^{18}F-FDG-PET Study

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Our objective was to investigate the effect of acupuncture at LR3 on cerebral glucose metabolism in spontaneously hypertensive rats (SHRs). We used ^{18}F-2-fluoro-deoxy-D-glucose positron emission tomography (^{18}F-FDG-PET) to examine the effects of acupuncture at LR3 on cerebral glucose metabolism in SHRs. SHRs were randomly allocated to receive no treatment (SHR group), needling at LR3 (SHR + LR3 group), or sham needling (SHR + sham group). Rats received 10 min acupuncture once per day for 7 days and were compared to normotensive Wistar Kyoto (WKY) rats. Blood pressure (BP) measurement and PET were performed after the first needling and the 7-day treatment period. BP was lower in the SHR + LR3 group compared to the other SHR groups between 30 and 60 min after the first needling and at 24 and 48 h after the 7-day treatment period. Glucose metabolism in the motor, sensory, and visual cortices was decreased in SHR group compared to WKY group. Needling at LR3 was associated with decreased glucose metabolism in the dorsal thalamus, thalamus, and hypothalamus and with increased metabolism in the cerebellar anterior and posterior lobes, medulla oblongata, and sensory cortex compared to the SHR group. These findings suggest that LR3 acupuncture improves hypertension through a mechanism involving altered brain activation in SHRs.

1. Introduction

Hypertension is characterised by a sustained increase in arterial BP and is usually associated with heart disease, peripheral vascular disease, and stroke [1]. Pharmacological therapy for hypertension has some disadvantages, such as costs and adverse effects [2].

Acupuncture is a complementary approach to BP management. It is an ancient treatment technique in traditional Chinese medicine which has been used for more than 3000 years [3, 4]. In recent years, many randomized controlled trials have demonstrated that needling at LR3 regulates essential hypertension in patients [5–7], and animal experiments have also indicated that moderate stimulation of LR3 has a significant effect on BP of SHRs [8–11]. Additionally, our previous study found that needling at LR3 altered the expression of six proteins in the medulla of SHRs [12]. A fMRI study found that acupuncture regulated the cardiovascular system through a neural network involving the hypothalamus, cortex, and brainstem [13]. Yet, the exact mechanisms underlying effects of acupuncture on BP remain unclear.

Positron emission tomography-computer tomography (PET-CT) is an advanced brain functional imaging technique that is used to measure regional glucose metabolism as an indicator of brain activity. PET-CT is characterised by high sensitivity, improved resolution, and the ability to provide semiquantitative data compared to fMRI [14, 15]. Acupuncture stimulation of specific acupoints such as LR3 affects the cardiovascular system and accordingly may have effects on specific brain regions receiving input from somatic afferent stimulation to affect BP [8, 10, 16]. Therefore, we hypothesised that acupuncture at LR3 would alter cerebral glucose metabolism as measured by PET-CT in a manner associated with lowered BP.

2. Methods

2.1. Experimental Animals. Forty-eight 10-week-old spontaneous hypertensive rats (SHRs) and 12 WKY rats weighing 200–250 g were provided by Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats were maintained in an environment with controlled temperature (20–24°C) and 40–60% relative humidity on a 12-hour light/dark cycle (lights on at 07:00) at the Experimental Animal Centre of Guangzhou University of Chinese Medicine in Guangzhou, China. Rats had free access to standard diet and distilled water. All experimental procedures were conducted in accordance with the People's Republic of China Ministry of Science and Technology Laboratory Animal Care and Use Guidelines and were approved by the Committee on the Ethical Use of Animals.

2.2. Grouping and Acupuncture Treatment. Healthy WKY rats were used as normotensive controls (WKY group, $n = 12$). SHRs were randomly divided into 3 groups: an untreated group (SHR group, $n = 12$), a group treated with acupuncture needling at LR3 (SHR + LR3 group, $n = 12$), and a sham acupuncture group (SHR + sham group, $n = 12$). The SHR + LR3 group received acupuncture at bilateral LR3 located on the back of the foot. We selected the position of the sham point as between the 3rd and 4th toes on the back of the foot (a nonacupoint) for the SHR + sham group. Briefly, acupuncture needles (stainless steel; 13 mm in length, 0.25 mm in diameter; SUIXIN, Suzhou Hualun Medical Appliance Co., Ltd., China) were inserted at LR3 and twirled at a frequency of 90 ± 5 rotations/min with an angle of 120 ± 5°. Each treatment lasted for 10 min (5 min per side) and rats were treated once per day for 7 days. The same acupuncturist performed all acupuncture and sham treatments.

2.3. BP Measurement. The Kent Scientific CODA noninvasive BP measurement system was used to screen rats. Each measurement had 15 cycles and the average BP value was used. The standard for hypertension was SBP ≥ 140 mmHg or diastolic blood pressure (DBP) ≥ 90 mmHg. SHRs that did not meet the high criteria were excluded from the experiment. BP was measured between 9:30 am and 2:30 pm when it was stable. BP was measured before treatment, at 30, 60, and 90 min after acupuncture, and at 24, 48, and 72 h after the 7-day treatment period.

2.4. ^{18}FDG-PET Imaging. All imaging was performed at the animal molecular imaging platform of Sun Yat-sen Medical College. Cerebral PET images were acquired 60 min after acupuncture and 24 h after the 7-day treatment period. Blood glucose values were measured from tail blood after 24 h of fasting using the glucose oxidase method (Glucose Kit, bioMérieux, normal standard 3.95 ± 1.31 mmol/L) prior to PET. A tracer (fludeoxyglucose [^{18}F]) ^{18}F-FDG synthesised with the Mini Tracer accelerator was injected (1.5 mci/kg) intravenously via the tail vein 45 min after acupuncture treatment and scanned within 15 min. Rats were anesthetised with 5% isoflurane in 100% oxygen 5 min before scanning. We used a Siemens Inveon PET system (Siemens Medical Solutions) with a radial spatial resolution of 1.4 mm full-width at half-maximum at the centre of the field of view to capture FDG-PET images. We then reconstructed images on the 128128159 matrix using the filtered backprojection algorithm, where the voxel size is equal to 1.461.460.79. All scans were saved in the Analyze 7.5 format.

2.5. Statistical Analysis. Data are presented as the mean ± SD. A one-way analysis of variance was used to evaluate between-group differences in BP. Analyses were performed with SPSS version 17.0 and the threshold for statistical significance was $p < 0.05$.

PET images were analysed using spmratIHEP in the SPM8 toolbox. Images were preprocessed with spatial normalisation and smoothing. Two-sample t-tests were used to identify differences in FDG signal between two groups. Brain regions demonstrating significant between-group differences in FDG were identified based on a voxel-level height threshold of $p < 0.001$ (uncorrected) and a cluster-extent threshold of 20 voxels.

3. Results

Before treatment, SBP and DBP were higher in the SHR groups than in the WKY group ($p < 0.05$) (Figure 1). Compared to sham or no treatment, acupuncture at LR3 reduced elevated SBP and DBP values in SHRs from 30 to 60 min after initial needling ($p < 0.05$) (Figures 1(a) and 1(b)). SBP and DBP values remained lower in the SHR + LR3 group compared to the SHR and SHR + sham groups at 24 and 48 h after completion of the 7-day treatment period ($p < 0.05$) (Figures 1(c) and 1(d)).

3.1. Brain Regional Glucose Metabolism. Tables 1–3 summarise the brain regions with significant differences in glucose metabolism between the SHR and WKY groups, SHR + LR3 and SHR groups, and SHR + LR3 and SHR + sham groups. Significant differences in maximum t-values (Max_T) in each cluster reflect positions as per Paxinos and Watson space maximum effects. The x-axis from left to right is negative to positive, the y-axis from low to high is dorsal to ventral, and the z-axis from negative to positive is direction from the cerebellum to the olfactory bulb. Using the Paxinos and Watson atlas, brain regions with significant differences in glucose activity were fused on structural sections of the rat brain (Figure 2). The warm colour represents increased glucose metabolism, whereas the cold colour reflects decreased glucose metabolism.

On days 1 and 8, glucose metabolism was higher in the SHR group than in the WKY group in the thalamus, dorsal thalamus, hypothalamus, and orbital cortex. Glucose metabolism was lower in the SHR group than in the WKY group in the motor cortex, sensory cortex, and visual cortex (Table 1 and Figures 2(a) and 2(b)).

On days 1 and 8, glucose metabolism was higher in the SHR + LR3 group than in the SHR group in the cerebellum

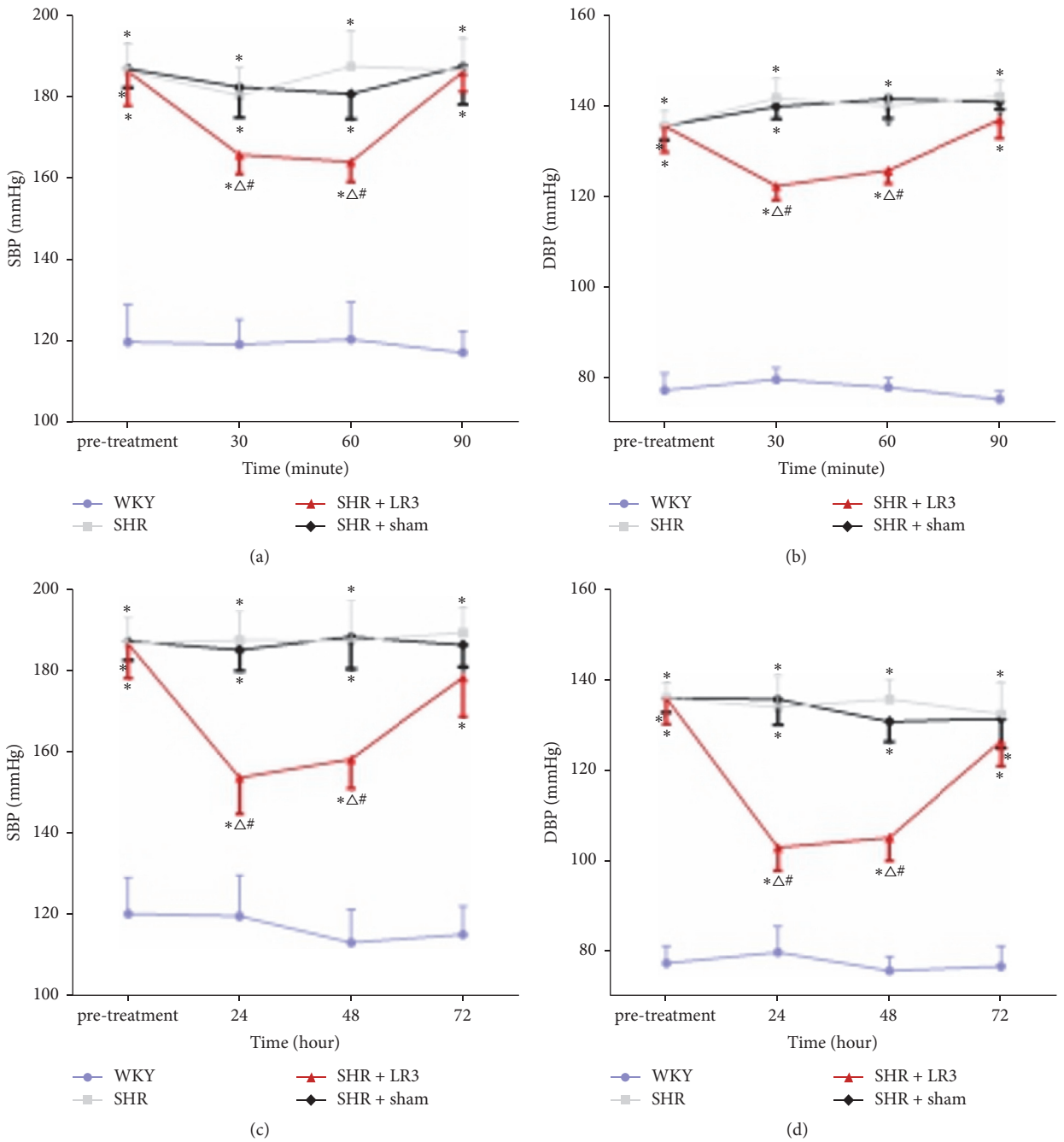

Figure 1: Effects of needling at LR3 in spontaneous hypertension rats (SHR). Between-group differences in systolic blood pressure (SBP; (a) and (c)) and diastolic blood pressure (DBP; (b) and (d)) were detected at 30, 60, and 90 min after initial acupuncture and at 24, 48, and 72 h after completion of the 7-day treatment period. $^{*}p < 0.05$ versus the WKY group; $^{\triangle}p < 0.05$ versus the SHR group; $^{\#}p < 0.05$ versus the SHR + sham group.

anterior lobe, cerebellum posterior lobe, medulla oblongata, and sensory cortex. Glucose metabolism was lower in the SHR + LR3 group than in the SHR group in the thalamus, dorsal thalamus, and hypothalamus (Table 2 and Figures 2(c) and 2(d)).

On days 1 and 8, glucose metabolism was higher in the SHR + LR3 group than in the SHR + sham group in the fimbria of hippocampus and caudate putamen. Glucose metabolism was lower in the SHR + LR3 group than in the SHR + sham group in the sensory cortex (Table 3 and Figures 2(e) and 2(f)).

4. Discussion

In the present study, we found that needling at LR3 for 10 min reduced in SHRs over a period of 60 min. This finding

Table 1: Sites of changed brain glucose metabolism in the SHR group versus the WKY group at 60 min after initial acupuncture or 24 h after completion of the 7-day treatment period.

Anatomical structure	Max_T	Peak coordinates (mm)		
		X	Y	Z
Day 1 (↓)				
Motor cortex	6.60	1.76	−0.04	−2.52
Sensory cortex	6.06	3.33	0.86	−0.12
Visual cortex	5.68	5.28	2.33	−5.64
Day 1 (↑)				
Infralimbic cortex	5.79	−1.27	4.78	3.72
Thalamus	7.66	−1.97	6.79	−3.00
Ventral orbital cortex	6.49	−1.68	3.96	4.44
Dorsal thalamus lateral nucleus group	7.83	−1.97	6.81	−3.24
Dorsal thalamus	7.83	−1.97	6.81	−3.24
Orbital cortex	6.49	−1.68	3.96	4.44
Hypothalamus	5.31	5.14	3.18	−5.40
Day 8 (↓)				
Cingulate cortex	6.17	1.19	2.15	0.36
Basal ganglia	7.79	1.99	2.83	−0.12
Caudate putamen	7.79	1.99	2.83	−0.12
Corpus callosum	7.62	1.86	2.65	0.12
Cingulate gyrus	6.20	1.05	2.44	0.36
Motor cortex	8.03	1.36	−0.19	−2.76
Sensory cortex	8.15	4.16	0.49	−2.04
Visual cortex	6.06	4.06	0.55	−4.44
Day 8 (↑)				
Infralimbic cortex	7.02	−0.87	4.64	3.96
Thalamus	5.01	−3.14	5.08	−5.88
Ventral orbital cortex	6.67	−1.27	4.47	3.96
Cerebellum anterior lobe	5.22	−3.76	5.03	−9.00
Dorsal thalamus lateral nucleus group	5.01	−3.14	5.08	−5.88
Dorsal thalamus	5.01	−3.14	5.08	−5.88
Orbital cortex	6.98	−1.00	4.63	3.96

The upward arrow (↑) indicates increased glucose metabolism, and the downward arrow (↓) indicates decreased glucose metabolism.

Table 2: Sites of changed brain glucose metabolism in the SHR + LR3 group versus the SHR group at 60 min after initial acupuncture or 24 h after completion of the 7-day treatment period.

Anatomical structure	Max_T	Peak coordinates (mm)		
		X	Y	Z
Day 1 (↓)				
Agranular insular cortex	4.00	−2.87	3.50	3.96
Anterior olfactory nucleus	4.90	−1.13	6.00	3.24
Accumbens nucleus	5.51	−1.39	5.57	3.00
Basal ganglia	5.24	−2.24	7.55	−3.48
Caudate putamen	4.60	−1.65	5.28	2.76
Infralimbic cortex	4.78	−1.27	4.78	3.72
Lateral orbital cortex	4.76	−1.94	3.80	4.44
Medial orbital cortex	3.44	−0.61	4.90	4.44
Medial amygdaloid nucleus	3.60	−2.93	8.24	−1.80
Olfactory bulb	4.09	−1.58	4.01	7.32
Prelimbic cortex	3.69	−1.14	4.02	4.20
Thalamus	7.46	−2.11	7.35	−2.76
Ventral orbital cortex	5.57	−1.26	5.55	3.24
Ventral pallidum	3.58	−0.98	7.09	2.52
Anterior commissure	3.90	−1.15	5.41	4.92
Amygdaloid body	3.51	−3.05	8.27	−2.28
Dorsal thalamus lateral nucleus group	7.11	−1.97	7.08	−3.00
Dorsal thalamus	7.11	−1.97	7.08	−3.00
Hypothalamus tuberal region	7.19	−2.11	7.49	−2.76
Internal capsule	5.64	−2.51	7.62	−2.76
Nucleus around the septal area	5.51	−1.39	5.57	3.00
Orbital cortex	5.21	−1.67	4.76	3.72
Day 1 (↑)				
Cerebellum anterior lobe	5.17	−3.60	5.10	−11.40
Cerebellum posterior lobe	4.47	−1.44	6.50	−12.60
Medulla oblongata	4.39	−1.72	6.88	−12.12
Sensory cortex	4.43	4.95	3.96	−1.08
Day 8 (↓)				
Lateral dorsal thalamus Nucleus group	3.49	2.55	5.03	−1.56
Dorsal thalamus	3.49	2.55	5.03	−1.56
Striatum	3.64	3.08	4.43	−1.08
Day 8 (↑)				
Medulla oblongata	5.51	−1.71	7.80	−12.60

The upward arrow (↑) indicates increased glucose metabolism, and the downward arrow (↓) indicates decreased glucose metabolism.

is consistent with other researches describing the ability of needling at LR3 to reduce (especially SBP) in hypertensive rat models and patients with hypertension [7, 17]. As acupuncture is a general treatment with "pleiotropic" responses, the simultaneous activation of multiple therapeutic mechanisms is expected [12]. Clinical acupuncture often involves repeated acupuncture treatments; indeed, repetitive acupuncture produces molecular changes and long-term cardiovascular effects that far outweigh the effects of one-time acupuncture stimulation [18]. In our study, BP was reduced for 48 h after completion of the 7-day acupuncture treatment period. Previous studies have described two possible mechanisms for the long-term cardiovascular effects of acupuncture. First, acupuncture may affect a reciprocating reinforcing circuit between the ventral hypothalamic arcuate nucleus and the midbrain ventrolateral periaqueductal gray. Second, acupuncture elicits the prolonged release of neurotransmitters and neuropeptides such as γ-aminobutyric acid (GABA)

Table 3: Sites of changed brain glucose metabolism in the SHR + LR3 group versus the SHR + sham group at 60 min after initial acupuncture or 24 h after completion of the 7-day treatment period.

Anatomical structure	Max_T	Peak coordinates (mm)		
		X	Y	Z
Day 1 (↑)				
Fimbria of hippocampus	3.23	−4.52	3.56	−2.76
Internal capsule	3.21	−4.52	3.54	−2.52
Day 8 (↓)				
Sensory cortex	4.85	6.14	4.23	−0.12
Day 8 (↑)				
Accumbens nucleus	6.68	1.96	7.12	2.52
Basal ganglia	6.29	1.96	7.56	2.52
Caudate putamen	5.01	2.23	7.03	2.04
Thalamus	4.51	1.80	8.70	−5.64
Ventral pallidum	6.29	1.96	7.56	−2.52

The upward arrow (↑) indicates increased glucose metabolism, and the downward arrow (↓) indicates decrease glucose metabolism.

and opioids in the rostral ventrolateral medulla (rVLM) and other regions [18]. Therefore, while the mechanisms by which acupuncture at LR3 improves hypertension remain unclear, several studies [10] have described the antihypertensive effect and mechanism.

In this study, we used PET-CT as a novel approach to investigate the effects of needling at LR3 on various brain regions. In the SHR groups, ^{18}F-FDG metabolism was lower at baseline in the medulla oblongata, cerebellum, and several cortical areas compared to the WKY group but increased after needling at LR3. It is well known that neurons are unable to synthesize or store glucose; therefore they are only dependent on glucose import. In human tissues, glucose is metabolized through glycolysis in the cytosol [19, 20]. As neurons present relatively weak expression of glycolytic enzymes, they favor another important metabolic pathway in glucose oxidation, the pentose phosphate pathway (PPP) [21]. Thus, we speculate that changes of glucose metabolism in different brain regions of rats after needling may be related to the regulation of PPP by acupuncture. Increased reactive oxygen species (ROS) formation (termed "oxidative stress") precedes development of hypertension in SHRs, suggesting that ROS participate in the development and maintenance of hypertension [22]. Several studies have shown that the PPP is upregulated when the brain is subjected to abnormal oxidative stress, and the PPP may play a role of protecting brain against oxidative stress in neurological diseases by reducing the amounts of ROS [23]. PPP largely contributes to neuronal protection against oxidative injury by reducing NADP to NADPH [21]. Therefore, we speculate that acupuncture may reduce BP by inhibiting oxidative stress response, which is regulated by ROS formation and PPP. In our previous study, the proteomic response that contributes to the SHR phenotype and reduction of hypertension in LR3-needled rats includes the modulation of seven proteins related to oxidative stress in the medulla oblongata, including SOD, ALDH2, GSTM5, GLUD1, protein DJ-1, Hsp90a, and a-ETF [12]. The above results indicated that acupuncture may exert antihypertensive effect directly or indirectly by inhibiting oxidative stress.

Additionally, several medulla neurotransmitters are involved in cardiovascular regulation. NO is one such molecule that serves a variety of physiological functions, which is a potent vasodilator [24]. There are 3 isoforms of NO synthase (NOS): calcium-dependent endothelial NOS (eNOS), neuronal NOS (nNOS), and calcium-independent inducible NOS (iNOS). One study reported that the therapeutic effects of acupuncture in experimental renovascular hypertension were related to decreases in NOS expression. nNOS-derived NO in the rVLM induced sympathetic activation by receptor activation, whereas iNOS-derived NO induced sympathetic blockade mediated by GABA. Acupuncture reduced and downregulated nNOS protein and mRNA expression while it upregulated iNOS expression in the medulla oblongata of a stress-induced hypertensive rat model. These results suggest that acupuncture affects hypertension by activating the central sympathetic inhibitory pathway and attenuating the central sympathetic excitatory pathway through a mechanism partly involving NO [25].

It is well known that the hypothalamus plays an important role in regulation. The hypothalamus is the most advanced integration centre below the cerebral cortex which regulates the activity of the autonomic nervous system; its afferent impulses arrive from the marginal forebrain, thalamus, and brainstem reticular structures, and these efferent impulses reach these areas. The hypothalamus affects the autonomic nervous system by modulating the activity of these regions through neurotransmitter and neuroendocrine communication. A number of regions in the hypothalamus, midbrain, and medulla receive somatic input during electroacupuncture or acupuncture. Neurons in these regions are activated through a network of projections extending from the hypothalamus to more caudal regions such as the ventrolateral periaqueductal gray and rVLM through neurotransmitters including opioids, GABA, nociception, and serotonin which act postsynaptically to directly or indirectly modulate autonomic outflow [18]. Repeated needling at PC5-6 and the underlying median nerve in normotensive rats increase proenkephalin release in the rVLM. Angiotensin II (Ang II) is a key mediator in the development and the maintenance of hypertension which exerts effects on brain circulation by regulating vascular structures and two main mechanisms that control cerebral blood flow [26]. Most central actions of Ang II are mediated by activation of the Ang II type 1 receptor (AT1R). AT1Rs are expressed in the sensory circumventricular organs, hypothalamus, and brainstem in several mammals including humans [27]. Cerebral angiotensinogen, which is primarily produced by astrocytes in the thalamus, hypothalamus, and brain stem as well as cortical neurons, can convert Ang I to Ang II and elicit Ang II secretion [26]. Furthermore, studies have shown that Ang II and oxidative stress increase neuronal activity in the

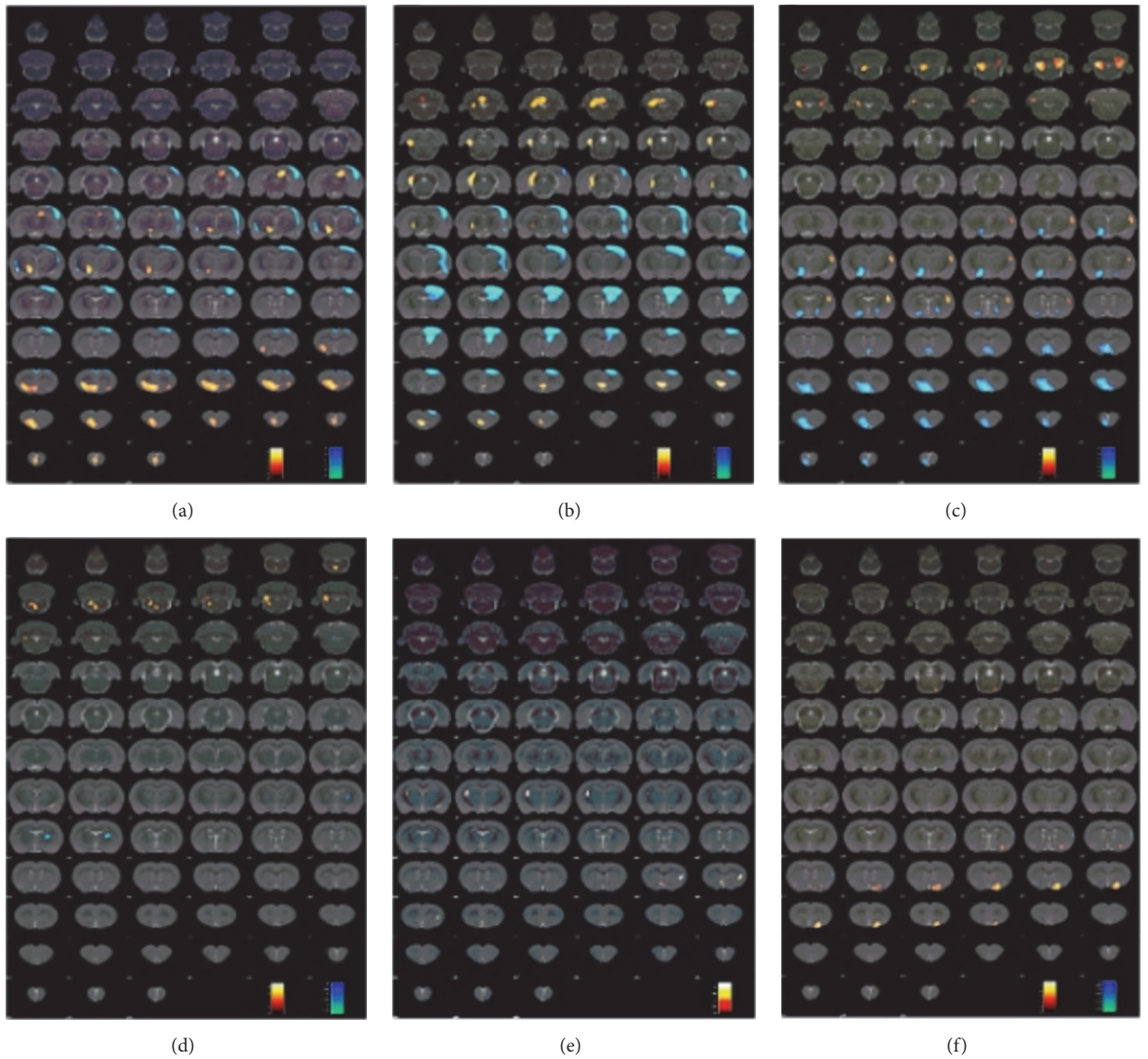

Figure 2: Regional glucose metabolism in the rat brain. Regional glucose metabolism was scanned at 60 min after initial acupuncture ((a), (c), and (e)) or 24 h after completion of the 7-day treatment period ((b), (d), and (f)). Results are overlaid on a coronal view of the rat brain and mapped to the Paxinos and Watson rat brain atlas. ((a) and (b)) SHR group versus WKY group. ((c) and (d)) SHR + LR3 group versus SHR group. ((e) and (f)) SHR + LR3 group versus SHR + sham group. Colour bars represent the t-value of each significant voxel.

paraventricular nucleus and rVLM as a mechanism maintaining sympathetic activation of the cardiovascular system in patients with renovascular hypertension [28]. Moreover, the δ-opioid system in the rVLM partly mediates the long-term antihypertensive effects of electroacupuncture; needling at ST36-ST37 in rats with cold-induced hypertension increased the mRNA expression of preproenkephalin in the rVLM for up to 48 h after treatment [29]. These mechanisms may also be involved in the LR3-needled effect in the hypothalamus and thalamus of SHRs.

Finally, it is important to note that our research demonstrates a point-specific effect of acupuncture in hypertension by using the SHR + sham group as a nonacupuncture control. Glucose metabolism was differentially altered in the SHR + LR3 group compared to the SHR + sham group in several brain regions including the sensory cortex and thalamus. Additionally, LR3 needling produced a better antihypertensive effect than sham needling.

The present study had several limitations. We cannot directly explain the relationship between brain glucose metabolism and prognosis in hypertensive animals. Future studies should independently investigate brain regions of interest using gene chip technology in order to better inform the therapeutic mechanism of acupuncture in hypertension. Our current research focuses on the effect of acupuncture on BP and the identification of changes of glucose metabolism of target brain regions in SHRs, and the results showed that there was no antihypertensive effect at 72 hours after 7-day

acupuncture. In order to study the molecular mechanism of acupuncture on the regulation in the target brain region, we sacrificed the rats quickly to perform DNA sequencing after scanning. Therefore, we cannot afford to track these rats and perform long-term longitudinal studies of acupuncture efficacy, but we are clear that it is meaningful. In the future, we will conduct further studies on the mechanism of long-term efficacy of acupuncture for the treatment of hypertension. Indeed, there are many other valuable acupoints to reduce BP, such as KI3, ST36, or acupoint compatibility [6, 9, 30], which is also one of our future research directions.

5. Conclusion

Using the SHR model of hypertension, we demonstrated that acupuncture at LR3 not only decreased but also altered cerebral glucose metabolism in the hypothalamus, thalamus, medulla oblongata, and cerebellum. Further investigation is needed to clarify the significance of these alterations and the mechanisms of action underlying the therapeutic effects of acupuncture in hypertension.

Data Availability

Readers can access the data underlying the findings of the study by contacting the corresponding author's email address (lai1023@163.com).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Research Article

The Effect of Electroacupuncture versus Manual Acupuncture through the Expression of TrkB/NF-κB in the Subgranular Zone of the Dentate Gyrus of Telomerase-Deficient Mice

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Our previous study showed that the acupuncture stimulation on the acupoint (ST-36) could activate the brain-derived neurotropic factor (BDNF) signaling pathways in telomerase-deficient mice. Recently, we set out to investigate whether the manual acupuncture (MA) or electroacupuncture (EA) displays a therapeutic advantage on age-related deterioration of learning and memory. Both telomerase-deficient mice (Terc$^{-/-}$ group, $n = 24$) and wild-type mice (WT group, $n = 24$) were randomly assigned to 3 subgroups (CON, controls with no treatment; MA, mice receiving manual acupuncture; EA, mice receiving electric acupuncture). The mice were subjected to behavior test, and EA/MA were applied at bilateral acupoints (ST36) 30 min daily for 7 successive days. The brain tissues were collected after the last Morris water maze (MWM) test and were subjected to the immunohistochemistry and western blot analysis. The MWM test showed that EA can significantly increase the time in target quadrant ($P \leq 0.01$) and frequency of locating platform for Terc$^{-/-}$ mice ($P \leq 0.05$), while nothing changed in WT mice. Furthermore, western blotting and immunohistochemistry suggested that EA could also specifically increase the expression of TrkB and NF-κB in Terc$^{-/-}$ mice but not in wild-type mice ($P \leq 0.05$). Meanwhile, the expression level and ratio of ERK/p-ERK did not exhibit significant changes in each subgroup. These results indicated that, compared with MA, the application of EA could specifically ameliorate the spatial learning and memory capability for telomerase-deficient mice through the activation of TrkB and NF-κB.

1. Introduction

Aging related neurodegeneration diseases are currently the mostly studied area in neuroscience. It is well known that aging is a multifactorial complex process that leads to the deterioration of biological functions, and the telomeres and telomerase may play a key role in this biological aging [1]. It is previously known that telomere protects chromosomes and plays important role in the cell life for its prolonged persistence. The telomerase is a DNA polymerase that plays an important role in telomere synthesis [2, 3]. In the nervous system, the neurons during embryonic and early postnatal life have high levels of telomerase activity, while in adult brain the level rapidly decreases, and at the same time the apoptosis of neurons occurs naturally during development. Therefore, some researchers believed that the reducing telomeres appear to be essential for the aging process in different organism [4, 5]. Previous research suggested that adult neurogenesis declines with age, and the age-related neurodegeneration could be due to dysfunctional telomeres, especially the telomerase with deficiency [3, 6, 7].

It is well known that acupuncture treatment taking as an traditional Chinese medicine has been widely used in some neurological disorders. Furthermore, some studies

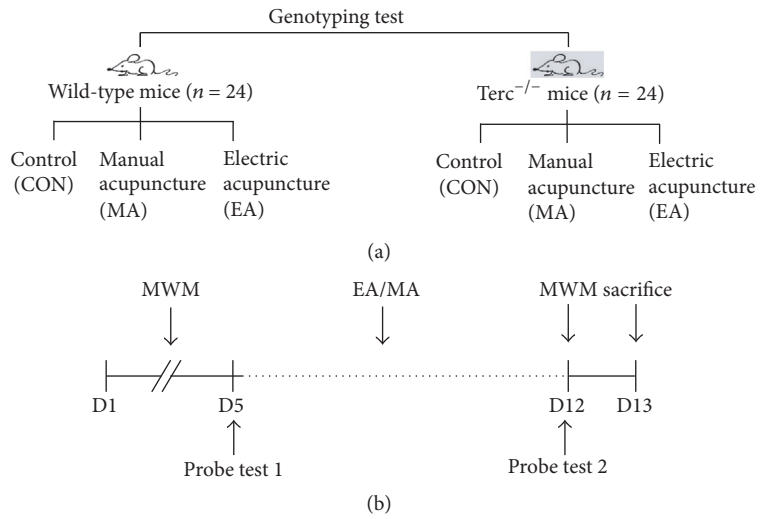

Figure 1: Experiment design on grouping, schematic representation of the methodology used. (a) All of the WT group mice and Terc$^{-/-}$ group mice were randomly distributed to 3 subgroups ($n = 8$ per subgroup): (1) controls with no treatment, (2) mice receiving manual acupuncture (MA), (3) mice receiving electric acupuncture (EA). (b) The mice received MWM test. In the 5th day, the MWM test was taken, and then MA/EA treatment was performed for 7 days. After the last MA/EA treatment administration, the last probe test was carried out. And all of the mice were sacrificed after the last behavioral observations (24 h later).

have demonstrated that acupuncture or electroacupuncture exerted a vital function in the treatment of Alzheimer disease (AD), even proven a great efficiency in improving intelligence [8]. Some researches indicated that acupuncture treatment targeted the acupoints in surface and finally resulted in particularly neuroprotective effects in nervous system [9, 10]. Among the nonpharmacological techniques, manual acupuncture (MA) and electroacupuncture (EA) were the basic two categories to acupuncture [11]. Compared with the different patterns of stimulation, EA is more repeatable and adjustable, while MA is more flexible and suitable in many diseases. Furthermore, there were more and more research that revealed that both EA and MA could improve cognitive deficits in AD animal models. Meanwhile, our previous studies have indicated that manual acupuncture could activate the brain-derived neurotropic factor (BDNF) and its downstream signaling pathways for neuroprotection [12, 13]. However, accumulating evidence has demonstrated that the therapeutic effect of EA was focused on attenuating cognitive deficits and increasing pyramidal neuron number in hippocampal [14, 15]. In the present study, we investigated the difference of effect induced by EA and MA on telomerase-deficient mice. In addition, we further explored the expression of TrkB (tropomyosin receptor kinase B)/NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)/ERK (extracellular regulated protein kinases)/p-ERK (phosphorylated-extracellular regulated protein kinases) protein in the subgranular zone (SGZ) of the dentate gyrus (DG) of telomerase-deficient mice.

2. Materials and Methods

2.1. Animals. The mice deficient for TERC genes were provided by the Jackson Laboratory in United States (Stock #004132). The experiments were approved by the Institutional Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine, China, and performed according to the NIH Guideline for the Care and Use of Laboratory Animals. The mice were housed in an environmentally controlled vivarium under a 12 h light–dark cycle and temperature 23 ± 2°C, humidity 50–60%. Food and water were available for freedom usage. All the animals were generated by inbreed crossing of heterozygous knockout mice that were backcrossed to naïve C57BL/6J mice for more than 3 generations. The mice were genotyped using Polymerase Chain Reaction to confirm the genetic modifications. Two strains of 7-month-old adult mice were used for the current study (i.e., wild-type mice [WT, $n = 24$] and telomerase-deficient mice [Terc$^{-/-}$, $n = 24$], with 8 mice each in 3 subgroups).

2.2. Experimental Protocol. The mice in both the WT group ($n = 24$) and the Terc$^{-/-}$ group ($n = 24$) were randomly assigned to 3 subgroups, 8 mice to each subgroup per group (Figure 1): (1) the control subgroup (CON) without any treatment; (2) the manual acupuncture subgroup (MA) that received manual acupuncture at the acupoint ST-36; (3) the electroacupuncture (EA) subgroup that received an electrical acupuncture stimulation on acupoint ST-36. All animals were observed while the acupuncture was performed, and if anyone looked uncomfortable, it was stroked gently on the back until it became calm again [16].

2.3. Morris Water Maze (MWM) Behavioral Test. For the purpose of evaluating the ability of learning and memory, Morris water maze procedure was performed as described [17, 18]. The water maze consisted of a circular tank (120 cm in diameter, 50 cm in height) filled with water to a depth of 28.5 cm, maintained at 22 ± 2°C. The area of the pool was conceptually divided into four quadrants (NE, NW, SW, and SE) of equal size. In the center of the 3rd quadrant,

we placed a hidden escape platform as the target quadrant with 12.5 cm in diameter. The mice were given 60 s to locate the hidden platform. Once the mice found the submerged platform, it could remain on it for 10 s, and the latency to escape was recorded. Any mouse that failed to locate the platform within 60 s was placed on the platform by hand. Each mouse was subjected to 4 training trials per day for 4 consecutive days. Twenty-four hours after the final trial, the assessing spatial memory was taken in probe test. In this test, the mice need to swim freely for 60 s without the platform in the tank. Time spent in the target quadrant and the frequencies of locating platform were taken to indicate the degree of memory consolidation after learning. All data were collected by a video camera (TOTA-450III, Japan) and analyzed by an automated analyzing system (Dig-Behav, Jiliang Co. Ltd., Shanghai, China). Considering the following 7-day acupuncture treatment, we designed two probe tests after 4-day training time [19]. The probe test 1 was arranged before acupuncture intervention, and the probe test 2 was carried out after the last day of treatment.

2.4. Acupuncture Intervention. The control subgroup did not receive any treatment but were immobilized by hand with gentle plastic restraints just as the treatment groups. In the treatment group, acupuncture stimulation was performed by a small acupuncture needle (13 mm in length, 0.3 mm in diameter, from Suzhou Hua Tuo Medical Instrument Co., Suzhou, China). Because of the effectiveness in improving the brain function, the point of bilateral ST-36 was chosen to be used. The locations of ST-36 and acupuncture manipulation were chosen following our previously described protocol [13, 14]. In the MA subgroup, the manual acupuncture on the point of ST-36 was applied for 30 mins. The needles were inserted into acupoint for a depth of 1.5–2 mm, and twirling manipulation was applied every 5 min and lasted 20 s each time. Each needle was rotated bidirectionally within 90° at a speed of 180°/s. For EA subgroup, a pair of needles were tightly tied together and inserted to bilateral ST-36 just as reported previously [20]. The needles were was also inserted into ST-36 acupoint for the same depth just as the MA group and connected to a Han's acupoint nerve stimulator (HANS, Han's Acupoint Nerve Stimulator, Model LH 202H, Beijing Huawei Ltd., Beijing, China). The parameters were as follows: sparse-dense wave with a frequency of 2/50 Hz, current of 2 mA, 30 min/stimulation, and one stimulation per day, for 7 consecutive days.

2.5. Tissue Preparation and Immunohistochemistry. After behavioral test and acupuncture intervention all the animals were sacrificed under 10% chloral hydrate (0.35 ml/100 g, intraperitoneal [i.p.]), and the brain tissues were collected after intracardial perfusion with saline. The brain samples were halved for each of the subjects; the left-half was separated out for protein preparation, and the right was fixed with 4% (w/v) paraformaldehyde for next immunohistochemistry analysis. The tissue blocks containing hippocampus were dehydrated and embedded in paraffin. Fixed brains were cut in 5 μm sagittal sections. The sections was mounted on 0.1% polylysine reagent (Sigma) coated slides. Subsequently, the sections were dewaxed and hydrated and incubated in 0.01 mol/L of citrate buffer for antigen thermal remediation for 5 min by being treated with microwave (700 W), and then for 10 min with 3% H_2O_2 at room temperature, and washed in phosphate-buffered saline (PBS) for 3 × 5 min. Next, the sections were blocked in 2% BSA for 10 min and incubated with primary antibody diluent (rabbit anti-TrkB 1 : 500, Cell Signalling Technology; rabbit anti-NF-κB 1 : 200, Cell Signalling Technology) for 12 h at 4°C. Then, the sections were rinsed by PBS and next incubated with secondary antibody diluent (biotinylated goat anti-rabbit IgG, diluted 1 : 1000, Vector Laboratories) for 30 min at room temperature. After wash by PBS for 3 × 5 min, the diaminobenzidine (DAB) kit (Vector Laboratories, Burlingame, USA) was used for color development for 5 min. After being redyed with hematoxylin, the brain slices were dehydrated and observed under a light microscope, BX53 (BX-51 Olympus, Tokyo, Japan), and analyzed using Image J software.

2.6. Western Blot Analysis. The frozen hippocampus tissues were obtained after behavior test and were homogenized on ice in 1.5 ml RIPA protein lysis buffer supplemented with 500 g PMSF. After centrifugation for 15 minutes at 12,000 ×g at 4°C, the protein in cleared supernatant was quantified and adjusted to 5 mg/ml. Equivalent amounts of protein (30 μg/lane) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% (w/v) bovine serum albumin in Tris-buffered saline with Tween 20 for 1 hour and then incubated with primary antibody (rabbit anti-mouse TrkB [1 : 1000], ERK [1 : 2000], P-ERK [1 : 1000], NF-κB [1 : 500], Santa Cruz Biotechnology,) overnight at 4°C. The immunoblots were then incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG for 2 hours at room temperature (1 : 1,000), we applied the chemiluminescent to develop the films, and the protein bands were quantified by Quantity One. The protein expression level was controlled by the protein of β-actin. All the data were expressed as the ratio relative after normalization to the β-actin levels.

2.7. Statistical Analysis. All data is presented as mean ± SEM for each group. For the Morris water maze test the escape latency time of the hidden platform trial was analyzed by two-way ANOVA of repeated measures, and the probe trial including escape latencies and original angle was conducted in the form of a multifactorial analysis of variance (ANOVA). The immunohistochemistry and western blot assay data were also analyzed by one-way ANOVA analysis of variance followed by LSD (equal variances assumed) or Dunnett's T3 (equal variances not assumed) post hoc test. All the analysis was performed with Prism 6.0 (GraphPad Software Inc., San Diego, USA), and the P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of Electroacupuncture on Spatial Learning and Memory. The results of the Morris water maze test are presented in Figure 2. In the hidden platform trial, the escape latency time in each group showed a downward trend in

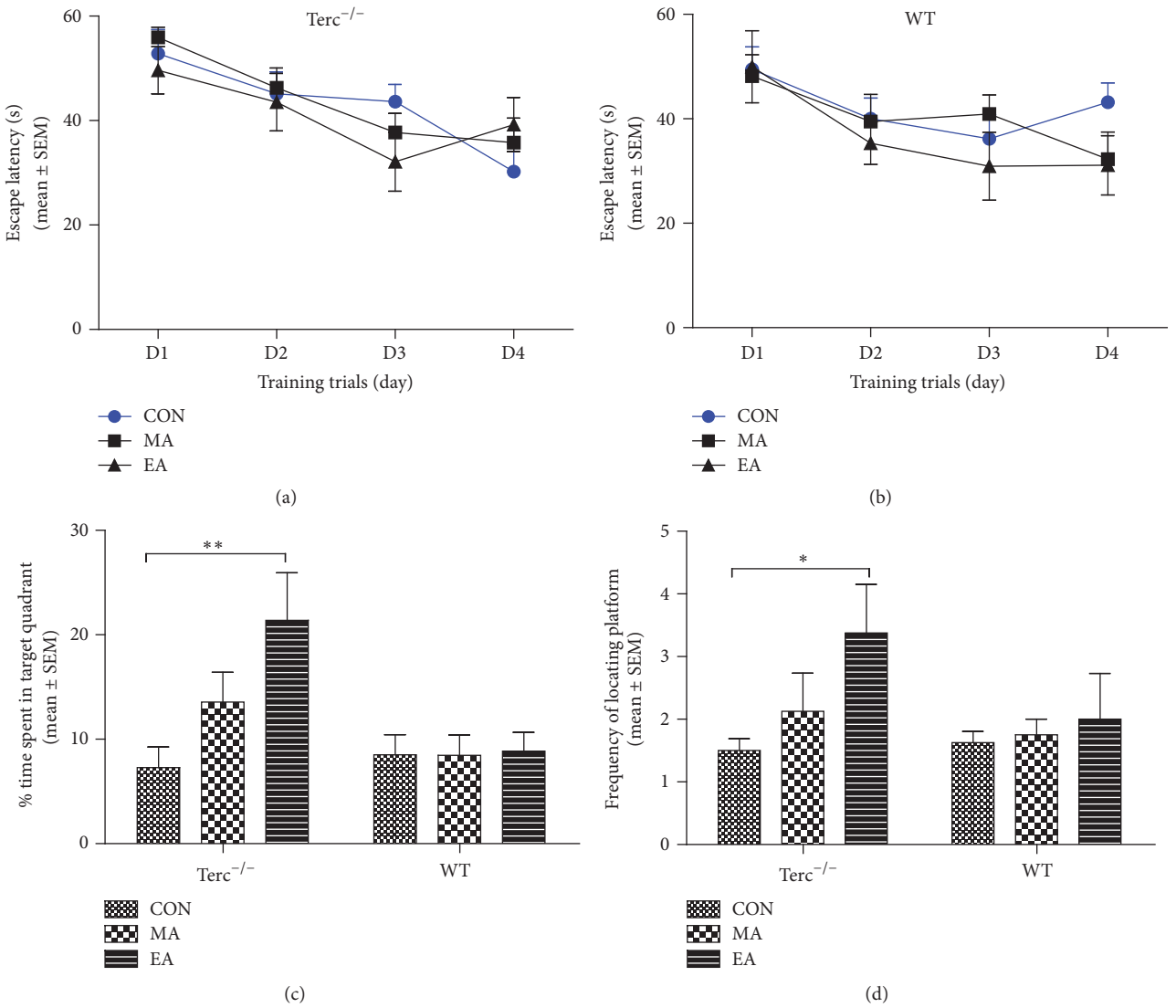

FIGURE 2: Acupuncture intervention prevented spatial and memory impairment in telomerase-deficient mice ($Terc^{-/-}$) in Morris water maze task. (a) Performance in training trial of $Terc^{-/-}$ mice ($n = 8$ for each subgroup) and (b) wild-type mice ($n = 8$ for each subgroup), during 4-day hidden platform trial. The data shows that escape latency to reach the hidden platform before acupuncture intervention in three subgroups. (c) In the probe test, the difference value of time spent in the target quadrant between before and after treatment was calculated. It was interesting that the EA stimulation can significantly increase the time in target quadrant for $Terc^{-/-}$ mice ($^{**}P \leq 0.01$), while nothing changed in WT mice. (d) There was also no significant difference among each subgroup for WT mice, and, compared with CON group, EA stimulation could significantly increase the D-value of frequency of locating platform between before and after treatment ($^{*}P \leq 0.05$).

4-day training time extension (Figures 2(a) and 2(b)). To analyze the effect of acupuncture treatment, the two probe trials were designed. The percentage of time spent in the target quadrant and the frequency of locating platform were used for statistical analysis. At the same time, the different values of the percentage of time and the frequency between pretreatment and posttreatment were further calculated to evaluate the significance of change after acupuncture. The results of probe trial showed that, compared with CON group, $Terc^{-/-}$ mice in EA subgroup had significantly more variation to the time in quadrant where the platform used to be ($P \leq 0.01$, Figure 2(c)), while there was almost no difference among three subgroups for WT mice. Meanwhile the D-value of frequency of locating platform between before and after treatment in EA group appeared significantly increased compared to CON group ($P \leq 0.05$, Figure 2(d)), whereas the variation among WT mice shows no difference among three subgroups. These results demonstrated that EA acupuncture could ameliorate the cognitive deficits in the $Terc^{-/-}$ mice, while it did not affect WT mice. Fortunately, these findings were consistent with our previous report [12].

3.2. Effects of EA Treatment Improved the Levels of TrkB Protein in the Hippocampus for $Terc^{-/-}$ Group. Brain tissue samples from the subjects were analyzed using immunohistochemistry and western blot analysis to investigate the effect of acupuncture stimulation in the two strains of mice (Figures 3(a) and 3(c)). The TrkB protein is mainly distributed in

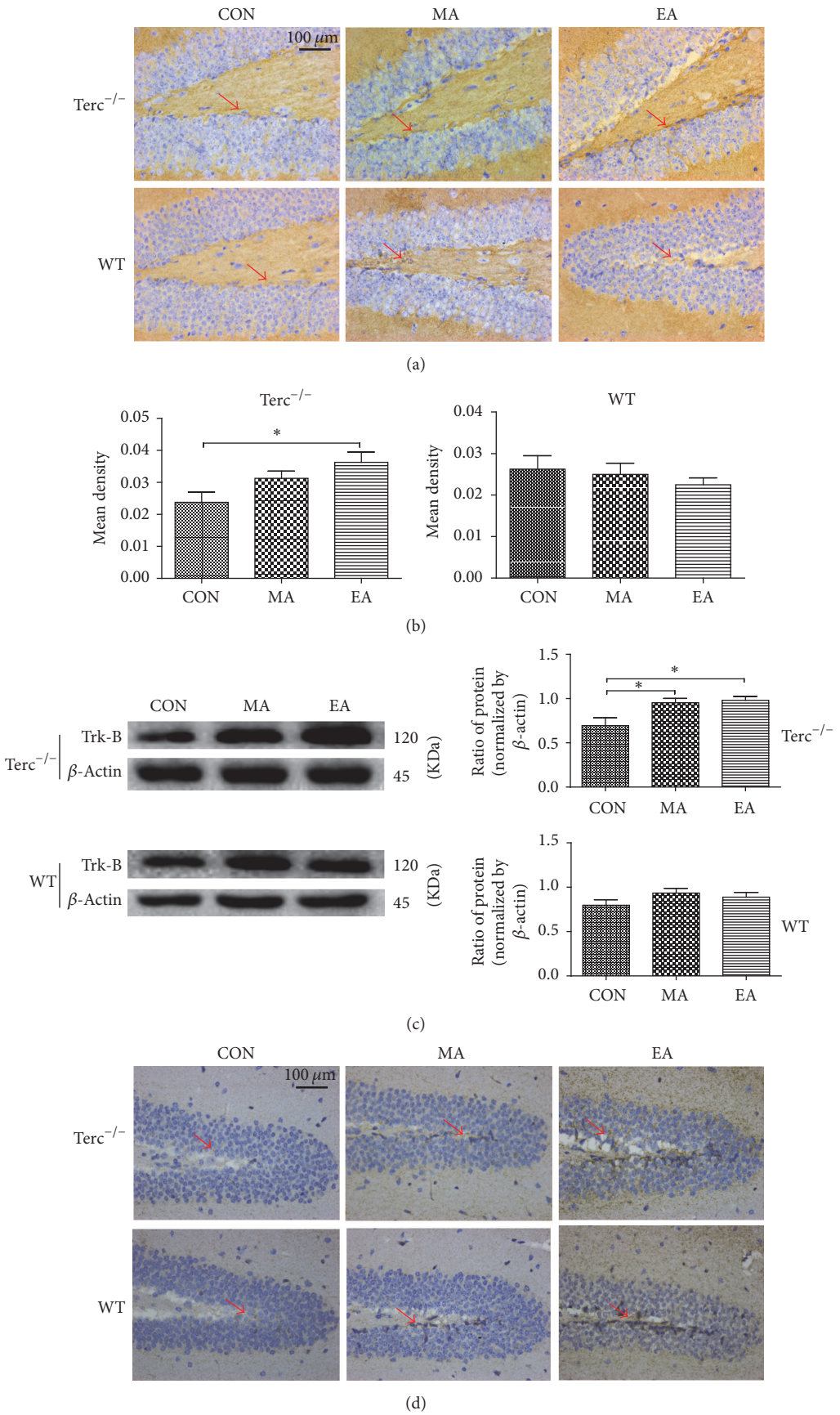

Figure 3: Continued.

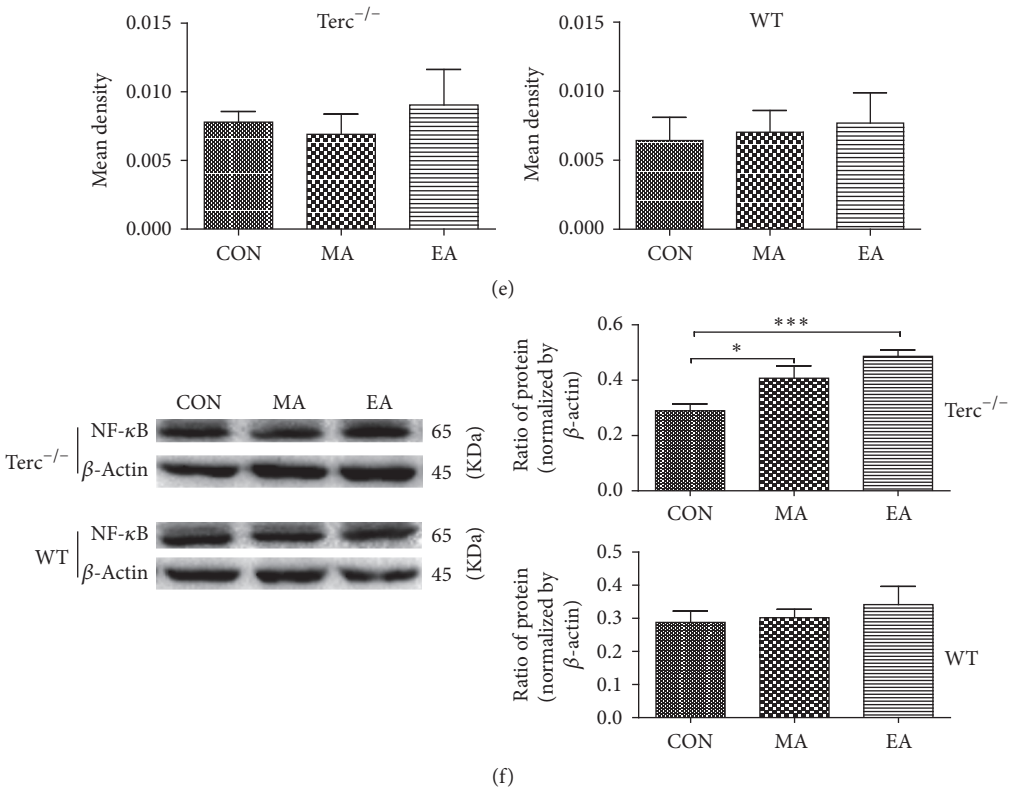

Figure 3: Immunohistochemistry and western blot analysis of TrkB/NF-κB in hippocampus of the WT and Terc$^{-/-}$ mice ($n = 8$ per subgroup). The brain samples were sliced sagittally into 5 μm sections, and the representative photographs and the mean optical density of positive cell values are, respectively, shown in a and d; b and e. Data were expressed as mean ± SEM, and the data were analyzed using one-way ANOVA followed by Turkey's test of multiple comparisons. (a and d) The mice brain slices were hematoxylin-stained in the same region of the hippocampus among the three subgroups. The TrkB/NF-κB positively stained cells appear brown (red arrow) in the subgranular zone (SGZ) around the dentate gyrus area (DG), and the scale bar = 100 μm. (b) Compared with CON subgroup, the mean optical density of positive TrkB protein in EA subgroup was significantly increased in Terc$^{-/-}$ mice ($^{*}P \leq 0.05$), and there was no difference among the three subgroups for WT mice. Although there was obviously more NF-κB positively strained cell observed in the picture, (e) no significantly differences were observed in NF-κB immunoreactivity in any subgroups in both strains. (c and f) Expression of TrkB/NF-κB in WT and Terc$^{-/-}$ mice ($n = 8$ per subgroup) was detected by western blotting assay. Data are represented as the ratio of TrkB (NF-κB)/β-actin. The bar graphs represent the levels of TrkB/NF-κB in hippocampus in both strains. In the Terc$^{-/-}$ mice, both electroacupuncture and manual acupuncture significantly increased the expression of TrkB/NF-κB ($^{*}P \leq 0.05$; $^{***}P \leq 0.001$), and nothing changed in WT mice.

the membrane in the subgranular zone (SGZ) around the dentate gyrus (DG) of the hippocampus. Even there were fewer weakly stains, they were still some positively stained cells. The result from the immunohistochemical evaluation indicated that only in Terc$^{-/-}$ mice the stimulation of electroacupuncture (EA group) could significantly increase the expression of TrkB protein ($^{*}P \leq 0.05$) compared with CON subgroup, and there was no obvious difference among any subgroup in WT mice (Figure 3(b)). The western blotting results of TrkB in the hippocampus were also shown that EA could promote the expression of TrkB compared with the other subgroup (Figure 3(c)), which was also consistent with our former reports [12].

3.3. Effects of EA Treatment Increased the NF-κB Expression in the Hippocampus for Terc$^{-/-}$ Group. To investigate whether EA or MA can alter the expression of TrkB downstream signal pathway, the expression of NF-κB/ERK/p-ERK was measured in tissue sample. From the photograph, the positively stained NF-κB appears brown mainly in the subgranular zone (SGZ) around the dentate gyrus area (DG) of hippocampus (Figures 3(d) and 3(e)), and there were no significant differences among any groups in the two strains of mice (Terc$^{-/-}$ and WT mice). Meanwhile, the western blotting results of NF-κB showed that, compared with CON group, the relative expressions of NF-κB significantly increased in EA ($^{***}P \leq 0.001$) and MA ($^{*}P \leq 0.05$) subgroups for Terc$^{-/-}$ mice (Figure 3(f)).

3.4. The Effects of Acupuncture Treatment on the Phosphorylation Levels of ERK. In order to further explore the mechanisms of acupuncture, the protein levels of p-ERK and ERK were measured by western blot to evaluate the activation of ERK. As p-ERK is a marker of ERK activation, the ratio of them was also calculated (Figure 4). The result demonstrated that neither electroacupuncture nor manual

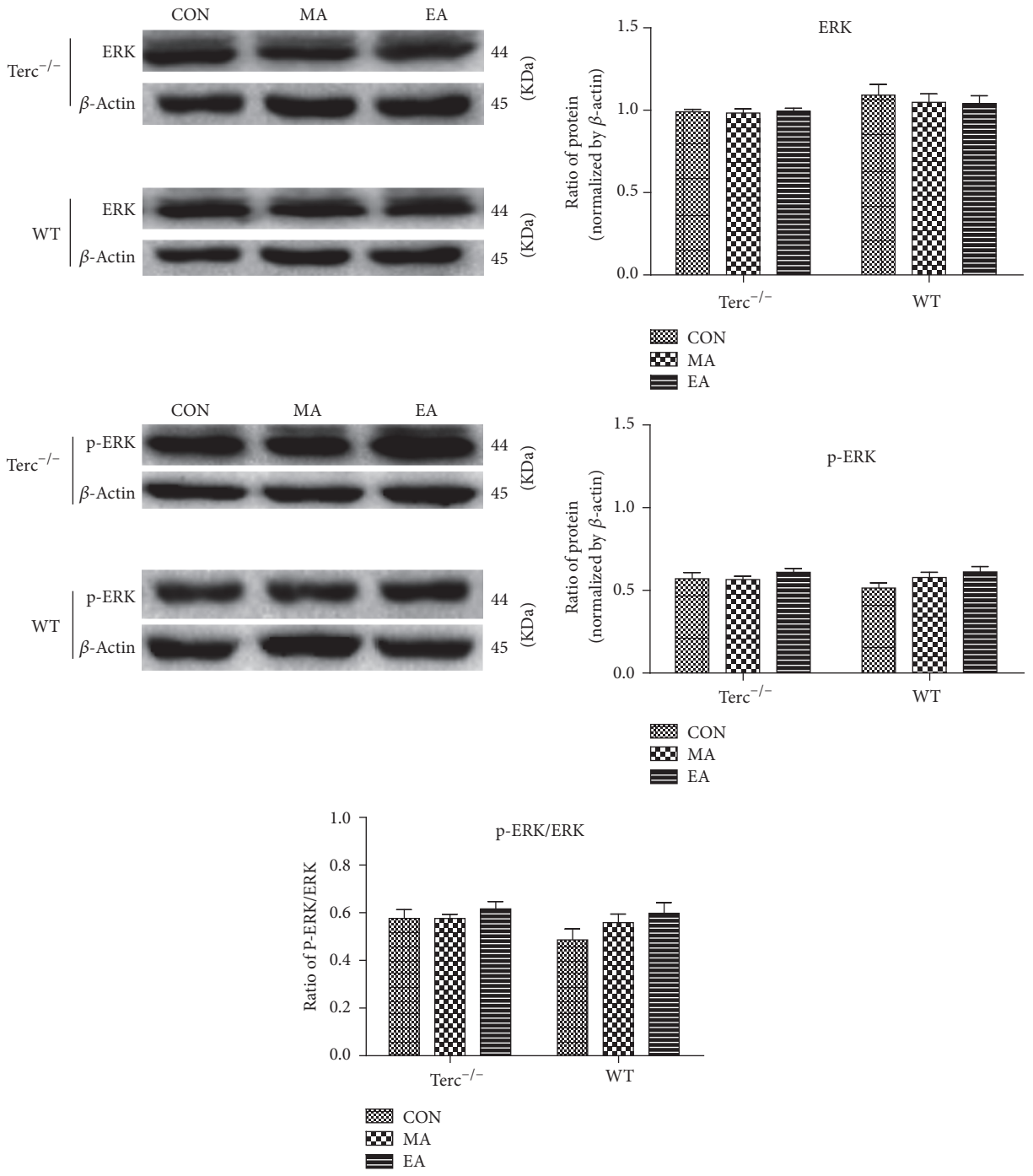

Figure 4: Expression level of ERK in the hippocampus of WT and Terc$^{-/-}$ mice ($n = 8$ per subgroup) was detected by western blot assay. Blots were reprobed for expression of β-actin to control for loading and transfer. Data were expressed as mean ± SEM. The degree of ERK activation was represented as the ratio of p-ERK/ERK. The result demonstrated that the expression of ERK/p-ERK shows nothing significantly changed in the subgroups for the 2 types of mice, even in the ratio of p-ERK/ERK.

acupuncture showed significant differences in ERK/p-ERK expression among any subgroups in Terc$^{-/-}$ mice ($P \geq 0.05$) and likewise in the subgroups of WT mice. Furthermore, the ratio of p-ERK/ERK shows no significance in any subgroups for the 2 types of mice ($P \geq 0.05$).

4. Discussion

As one of the most common tasks used to assess spatial learning and memory ability, the Morris water maze (MWM) was used in this study. The hidden platform trial and probe trial were used to assess the capabilities in spatial learning

and memory, respectively. The abilities of spatial learning and memory were observed in the two strains of mice for 4 consecutive days [19]. The results of training period showed no significant difference among the various groups of mice, suggesting that all mice had the same learning and memory capacity before treatment (Figure 2).

Even the acupuncture has been widely applied for different kinds of nervous system, but there were still few studies that described whether the acupuncture intervention had different effects in different strains. In our present study, the Terc$^{-/-}$ mice showed a better response to electroacupuncture. It implies the stimulation of acupuncture only produced therapeutic effects on animals at the pathological state. Some studies have reported that, in healthy animals [21], both electroacupuncture and manual acupuncture can lead to a significant increase in cell proliferation just in the SGZ of the DG. However, in our studies, only electroacupuncture can play a therapeutic role in the amelioration of learning and memory abilities for Terc$^{-/-}$ mice [15, 22].

Recently, it has been reported that aging related neurodegenerative diseases are characterized by imbalance between neurogenesis and neurodegenerative diseases. And interestingly, some researches demonstrated that the stimulation of neurogenesis may be beneficial to patients with those diseases [23, 24]. In the current study, our research team found that, only in Terc$^{-/-}$ mice, after acupuncture treatment, the TrkB/NF-κB proteins were exhibited in the SGZ around the dentate gyrus area of hippocampus. We also found that EA administration showed more amelioration of reference memory impairment in Terc$^{-/-}$ mice [2]. This suggests that EA administration alleviates aging risk by inhibiting reference memory decline. On the other hand, the hippocampus, which plays an important role in learning and memory, demonstrates a high degree of neurogenesis, and only the DG of hippocampus continues to develop through adulthood [25]. Presently more and more research demonstrated that there are only two neurogenic areas in the brain including subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the DG in hippocampus [26]. So it is obviously that the ability of undifferentiated and rapidly proliferating for the progenitor cells that could differentiate into granule in the SGZ of DG throughout life. In our study, the NF-κB and TrkB positively strained cell could be found in SGZ area in hippocampus, and they showed significantly higher level compared with CON subgroup or MA subgroup. This indicates the electroacupuncture treatment can activate some protein signal pathways in the cell around DG for Terc$^{-/-}$ mice. And the WT mice were not affected after the acupuncture stimulation [15, 27, 28].

For the difference between the effect of electroacupuncture and manual acupuncture, it is commonly accepted that EA stimulation shows a beneficial effect on neurodegeneration diseases. For manual acupuncture, the "De-Qi" feeling is essential to induce action. In clinical, acupuncture needles were repetitively penetrated up and down in different directions just for the purpose of "De-Qi" feeling [29, 30]. Consequently, some people believed that the effect of MA depends upon stimulating intensity (mild or strong) and duration of manipulation, even when the needle was tightly wound around by muscle fibers [31]. Some researches demonstrated that EA may cause electrical twitching of surrounding tissues and induce MA-like stimulation through mechanoreceptors [32]. The previous studies showed that manual acupuncture at ST36 significantly increased the number of BrdU-positive cells after ischemic injury [33, 34]. Subsequently, electroacupuncture stimulation at ST36 was reported to enhance cell proliferation in the DG a rat model of diabetes [9]. In our research, after electroacupuncture treatment, the hippocampal expression of TrkB was significantly increased in Terc$^{-/-}$ group compared with the WT group. These results may indicate that the stimulation of acupuncture may have a close relationship with the neurogenesis in the hippocampus. For acupuncture, the acupoint was ST36, which is located on the anterior tibia muscle, and is one of the most important acupoints in clinical acupuncture for antiaging. Simulation of ST36 is carried out for a wide range of conditions affecting digestive system, cardiovascular system, immune system, and nervous system. Furthermore, ST36 is one of the seven acupoints used for stroke treatment. As the high-affinity BDNF receptor, the tyrosine protein kinase receptor B (TrkB) just the same as the BDNF was expressed in different kinds of neurons in the brain. [35–37]. Our previous studies demonstrated that manual acupuncture stimulation can activate the BDNF and its downstream signaling pathways [2].

Our study has shown that EA causes an increase in the positive cell of TrkB and NF-κB signal pathway, but there was no evidence supporting that acupuncture can activate downstream protein of TrkB through ERK signal pathway. Several studies support that the activation of TrkB can prevent cell death by activating the ERK pathway in cortical neurons and cerebellar neurons [38]. And some researchers suggested that the ability of BDNF-TrkB to stimulate telomerase activity can be partially decreased through the total inhibition of the extracellular signal-regulated protein kinase (ERK) [6]. However, our study indicated that acupuncture can only specifically increase the expression of TrkB and NF-κB in Terc$^{-/-}$ mice instead of via the activation of ERK/p-ERK (Figures 3 and 4) protein. From the result, we found that even the ERK signal pathway plays an important role in the overall effects of electroacupuncture, but there was nothing changed in neurons of hippocampus [39]. As such, based on our result, it can be inferred that EA stimulation increased the ability of spatial learning and memory in Terc$^{-/-}$ mice, and it might stem from the activation of NF-κB.

Some studies demonstrated that NF-κB is proinflammatory transcription factor which is increased in aging brain, and the activation of NF-κB can protect neurons against death induced by neurodegeneration [40]. Therefore, the upregulated of NF-κB could show an neuroprotective effect in our brain [41]. At the same time, reactive oxygen species (ROS) have been implicated in many aspects of aging and in neurodegenerative diseases [42]. And NF-κB is oxygen sensitive and also is a precursor to VEGF

(vascular endothelial growth factor) gene expression that leads to angiogenesis, it can regulate the proinflammatory response in endothelial cells [43]. Several studies supported that the role of NF-κB depends on the types of axoneuron, and the activation of NF-κB in ischemic dementia caused the neuron degeneration to microglia in cortex. However the neuroprotection effect was shown in the hippocampal neuron cell [43–45]. Our result indicated that NF-κB could be specifically increased by electroacupuncture in Terc$^{-/-}$ mice rather than WT mice, and the positive cells were exhibited in the SGZ around the dentate gyrus area of hippocampus. It suggests that the electroacupuncture may be involved in the nerve regeneration in SGZ; furthermore the positive increasing expression of TrkB and NF-κB in the subgranular zone (SGZ) around the dentate gyrus (DG) area may be a possible mechanism of EA in the treatment of aging in telomerase-deficient mice.

5. Conclusions

In summary, our key findings suggest that, compared with MA, the application of EA could ameliorate the spatial learning and memory ability for telomerase-deficient mice; furthermore, it could also increase the expression of TrkB and NF-κB in the subgranular zone (SGZ) around the dentate gyrus (DG) area. Based on this result, it is also suggested that the neuroprotection and neuron regeneration may play a critical role in electroacupuncture-induced antiaging effect. At the same time, the mechanisms of EA and MA effects on telomerase-deficient mice further provide the theoretical basis for antiaging clinical applications.

Conflicts of Interest

The authors declare that they have no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Dong Lin designed the study and performed fundraising and wrote the paper. Jie Zhang and Xiaodan Yan performed acupuncture. Wanyu Zhuang and Xiaoting Yang performed immunohistochemistry and western blots. Shen Lin provided critical revision of the paper for intellectual content. Lili Lin performed parts of the experiment and data analysis and fundraising. All authors approved the final version of this paper.

Acknowledgments

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Research Article

Electroacupuncture Ameliorates Cognitive Deficit and Improves Hippocampal Synaptic Plasticity in Adult Rat with Neonatal Maternal Separation

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Exposure to adverse early-life events is thought to be the risk factors for the development of psychiatric and altered cognitive function in adulthood. The purpose of this study was to investigate whether electroacupuncture (EA) treatment in young adult rat would improve impaired cognitive function and synaptic plasticity in adult rat with neonatal maternal separation (MS). Wistar rats were randomly divided into four groups: control group, MS group, MS with EA treatment (MS + EA) group, and MS with Sham-EA treatment (MS + Sham-EA) group. We evaluated the cognitive function by using Morris water maze and fear conditioning tests. Electrophysiology experiment used in vivo long-term potentiation (LTP) at Schaffer Collateral-CA1 synapses was detected to assess extent of synaptic plasticity. Repeated EA stimulation at *Baihui* (GV 20) and *Yintang* (GV 29) during postnatal 9 to 11 weeks was identified to significantly ameliorate poor performance in behavior tests and improve the impaired LTP induction detected at Schaffer Collateral-CA1 synapse in hippocampus. Collectively, the findings suggested that early-life stress due to MS may induce adult cognitive deficit associated with hippocampus, and EA in young adult demonstrated that its therapeutic efficacy may be via ameliorating deficit of hippocampal synaptic plasticity.

1. Introduction

Since the early 90s in 20th century, mother-infant separation shortly after birth in hospitals has become routine to human [1] and mother-newborn repeat separation has been also widespread for a number of postpartum women who devoted themselves to work after delivery under social pressure or other social issues [2, 3]. In China, "left behind children" (LBC) living apart from their parents annually is quite a prevalent phenomenon in the last decade; the prevalence of LBC is 37.7% as reported nationally in 2013 [4]. Increasing evidences show that maternal separation (MS), as an early-life adverse event in humans, has long-term negative repercussions on child neuron development and increases vulnerability to stress-related psychopathology in adult life [5, 6].

Neonatal MS of rat is a successfully neurodevelopmental model used to mimic physiology and behavior responsiveness to early-life stress in human [7–9]. Previous studies showed that early MS may perturb neurotransmitter system in rat brain [10], increase stress reactivity in neuroendocrine signs [11], alter function of hypothalamic-pituitary-adrenal (HPA) axis [12], and express abnormal behavior phenotypes including anxiety, depression, autism, and social deficits [10, 13, 14], and impair cognitive function associated with hippocampal or prefrontal cortex [15–17], whose effects may persist and endure into adulthood.

Currently, no drugs have been proven effective in the treatment of cognitive impairment, except for encouraging patients to engage in mental or physical activity [18]. Therefore, complementary and alternative therapies for the

treatment of various cognitive deficits are urgently required. Acupuncture, as a traditional therapy originating from ancient China, has become popular and been widely accepted in many countries around the world. Acupuncture therapy has been used to treat cognitive impairment of stroke and some neurodegenerative disorders including Alzheimer disease and dementia [19–23]. *Baihui* (GV 20) is located above the apex auriculate, on the midline of the head, and *Yintang* (GV 29) is located at the midpoint between the two eyes [24]. In traditional Chinese medicine, GV 20 and GV 29 are the two acupoints which have been used in neurological diseases like depression, mania, epilepsy, and headache [25–27]. However, we do not know whether the cognitive impairment resulting from maternal separation in early-life could be attenuated by EA treatment in young adults. In the present study, we investigated the therapeutic efficacy of EA against cognitive impairment of young adult rats exposed neonatal MS by behavior studies including Morris water maze and fearing conditioning test.

Hippocampal synaptic plasticity is thought to be a cellular mechanism of memory formation and impaired long-term potentiation (LTP) of excitatory synaptic transmission contributes to cognitive deficits [28–30]. In the current study, electrophysiological study by using LTP recording in vivo at Schaffer Collateral-CA1 (SC-CA1) synapses in the hippocampus was to explore synaptic plasticity for the underlying mechanisms. To the best of our knowledge, our study is the first to provide evidence that EA ameliorates cognitive deficit and improves synaptic plasticity in adult rat with neonatal MS experience.

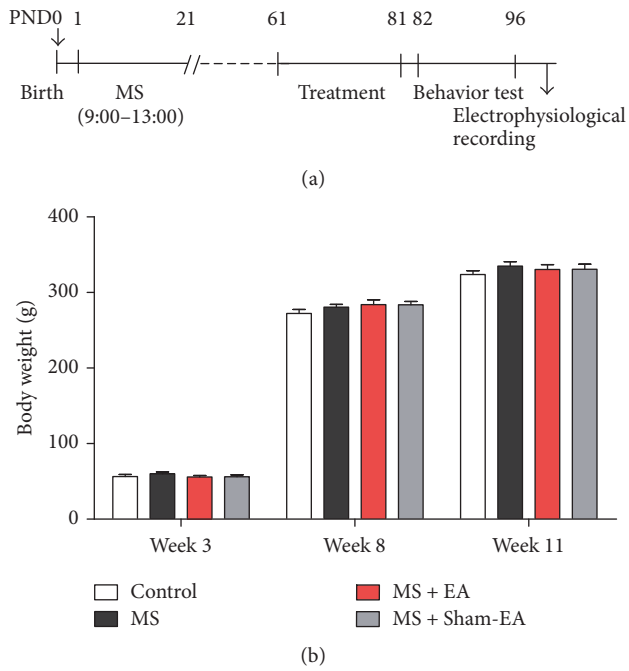

Figure 1: Experimental schedules and body weight gain of rats in all subgroups. (a) The experimental schedule of MS, acupuncture stimulation, behavioral tests, and electrophysiology recording. (b) Animals in all subgroups were weighted on the weaning day in the postnatal 3rd week, the day before EA treatment in the postnatal 8th week, and the day after treatment in the postnatal 11th week. There was no difference on weight among groups observed, $F_{(3,40)} = 0.711$; $P > 0.05$; $n = 11$/group; two-way ANOVA.

2. Materials and Methods

2.1. Experimental Animals. Male and nulliparous female Wistar rats of 2 months old were obtained from the Guangdong Medical Laboratory Animal Center. All rats were housed in groups of four with the same gender in home cages made from Plexiglas with sawdust (42 × 26 × 15 cm), in a controlled environment with temperature rooms (23 ± 2°C) and humidity maintained (50 ± 5%). The rats were under a controlled 12/12 h light/dark cycle with light on from 7:00 a.m. to 19:00 p.m. After a week of adaptation, male and nulliparous female Wistar rats were put together in standard cages with access of food and water ad libitum. After two weeks, the female rats were checked twice daily for delivery with male rats removed away.

2.2. Experimental Design. Wistar dams and their litters were assigned either to control group (Control) or to the mother separation group. For each litter, the day of birth was named as postnatal day 0 (PND 0). Maternal separation procedure: maternal separation took place according to the pervious protocol [31–33] (Figure 1(a)). In brief, the mothers were daily removed into another cage for four hours (9:00–13:00) and the litters remained in the home cage. After 4 hours, the mothers were returned back to their home cage. During the separation, litters were maintained in heating plate and water was provided to maintain temperature and humidity. The separation procedure last from PND1 to PND20. The mothers and litters in control were not disturbed until weaning, except for cage cleaning at PND10. All cages were cleaned firstly at PND10. Pups were weaned at PND 21 when female pups were removed, and male pups were left for experiment and housed by four or five per cage until adult age. Food and water were available ad libitum. The experimental procedure was approved by the Animals Care and Use Committee of Guangzhou University of Traditional Chinese Medicine. All efforts were made to minimize animals suffering and reduce the number of animals used for experiments.

At the PND60, the maternal separation group rats were assigned randomly into three groups: maternal separation group (MS), electroacupuncture treatment group (MS + EA), and sham electroacupuncture treatment group (MS + Sham-EA). There were forty-four rats in total, $n = 11$ rats/group.

2.3. Treatment. All the treatments were performed from PND61 to PND81.

2.3.1. EA Stimulation. Rats in the MS + EA were anesthetized with isoflurane (RWD, Shenzhen, China). Anesthetized concentration was maintained at 2% and positioned on a stereotaxic frame (RWD, Shenzhen, China). Disposable acupuncture needles (0.25 × 13 mm, Suzhou Medical Appliance Factory, Suzhou, China) were inserted to a depth of

5 mm at the GV 20 and GV 29 after skin had been cleaned with alcohol swabs. And then a Master-8 Stimulator (Master-8, AMPI, Israel) was connected, and the electrical current was delivered to the needles. The output parameters were set as follows: frequency was held constant at 2 Hz and intensities of 2 mA, for 20 min. EA stimulation was administered every other day for 20 min starting at 8:30 a.m.

2.3.2. Sham-EA Procedure. Rats in MS + Sham-EA group were anesthetized with isoflurane as MS + EA group. After the skin was cleaned with alcohol swabs, rats in MS + Sham-EA group received no electrical stimulation: disposable acupuncture needle was pasted at the surface of GV 20 and GV 29 and the needle touched the skin but not inserted the acupoints. Control group rats were anesthetized with isoflurane as MS + EA group.

2.4. Body Weight Measurements. To assess the effect of EA and MS on rat, body weight measurements were taken on the weaning day (week 3), the day prior to treatment (week 8), and the day after treatment (week 11) by balances (MS3002ts/00, Mettler Toledo).

2.5. Behavioral Tests. Behavioral tests were started on the day after all treatments were over. All behavioral tests were performed during an active period of animals' light cycle (07:00–19:00). The behavior of rats was recorded by video analysis system (Shanghai Jiliang Software Technology Co., Ltd., Shanghai, China). The investigators for behavior test were blinded for groups. To avoid effects of learning and memory, rats were tested for the same paradigms only one time [34].

2.5.1. Morris Water Maze (MWM). The purpose of MWM test was to measure hippocampus-dependent spatial memory, as described before [35]. The diameter of swimming pool was 1.6 m and the height was 0.6 m. The pool was filled with water with a depth of 48 cm with 24 ± 1°C. The water was made opaque with the addition of a small quantity of nontoxic, water-based black paint. An escape platform (10 cm in diameter) was placed in the pool and the top of the platform was 1 cm submerged under the surface of the water. The swimming behavior of rats was recorded by a video camera located above the center of the swimming pool and was analyzed. Four visual cues used as spatial references for rats were located on the center of the 4 quadrant walls of the pool, including square, round, triangular, and star. The starting position, at which subjects were placed in the pool facing the wall, differed randomly across trials. The rats were trained for 5 consecutive days (4 trials/day) and they were allowed to swim until they found the platform or until 60 sec elapsed. If a rat found the platform within 60 sec, it was allowed to stay on the platform for 10 sec. If a rat failed to find the platform, it was guided to the platform and permitted to remain there for 10 sec. The trail interval among each quadrant of a rat was 15 minutes. At the end of the trial, rat was dried and replaced to the home cage. After 5 days of training, the escape platform was removed from the swimming pool. The test was on the sixth day. Every rat started swimming at the side of the pool opposite to the platform with the head facing the pool wall and was allowed to swim for 60 sec freely. The latency during the training days and the percent of distance spent in the target quadrant on test day were recorded.

2.5.2. Fear Conditioning Test. Test procedure was used to measure fear conditioning to tone and context as described in [36]. On the first day, rats were initially placed into Context A made of an operant chamber, metal walls, and bars on floor to habituate for 2 minutes without being disturbed. During the 2 minutes, the baseline of freezing behavior was recorded. And then they were presented with a 10 sec 80-dB white noise which served as the conditioned stimulus (CS), and a 2 sec shock (1.0 mA) was followed. Five tone-shock pairings were repeated every 70 sec. 60 sec following the final shock, the rats were taken out of the chamber and returned to their home cage. On the second day, each rat was placed into Context B, a novel context made of black plastic walls, bedding on the floor, unscented. After 2 min of habituation, they were presented with an 8 min tone (80-dB), with no shock, and scored for freezing behavior, as a measure of fear. 48 hours later, the rats were returned to Context A and freezing behavior was recorded for a 5 min trial (no shock, no tone) to assess its response to the original conditioning context. The chamber of fear conditioning was cleaned with 70% ethanol before each rat test. Fear conditioning to tone is percentage of freezing during the first 4 min exposed to the tone/percentage of freezing during the Context B habituation period. Fear conditioning to context is percentage of freezing for the total time exposed to Context A on day 3/the baseline of freezing percentage.

2.6. In Vivo Electrophysiological Recordings for Long-Term Potentiation. Adult male rats were anesthetized with isoflurane (RWD, Shenzhen, China). Induced anesthesia concentration was 4%, and concentration was maintained at 2-3% and positioned on a stereotaxic frame (RWD, Shenzhen, China). The rats were placed on a heating pad to maintain body temperature. Two holes were drilled in the skull by using a dental drill for recording and stimulating electrodes. According to the rat brain atlas (Paxinos, Watson, 1986), the bipolar stimulating electrode was implanted into the CA3 area (3.8 mm posterior to bregma, 2.1 mm lateral to midline, and 1.5 mm ventral below dura) of the hippocampus, and the recording electrode was placed in the CA1 area (3.4 mm posterior to bregma, 2.5 mm lateral to midline, and 3.0 mm ventral below dura). Field excitatory postsynaptic potentials (fEPSPs) were recorded in hippocampus of the CA1 area through current stimuli to the stimulating electrode in CA3 area. Baseline stimuli were delivered at 0.1 Hz, and the evoked response was digitized (10K Hz) and analyzed by using the Cambridge Electronic Design 1401 (Cambridge, UK) and the software Spike2 (Cambridge, UK).The stimuli ranged from 0.05 to 1 mA to find the optimal stimulus intensity that evokes a response of half of its maximum amplitude. The baseline fEPSPs were recorded under single-pulse (monopolar pulses, 0.2 ms duration) stimulation for 30 minutes, and the LTP

was induced by high frequency stimulation (HFS; two trains consisting of 100 pulses at 100 Hz; pulses interval, 10 ms; train interval, 30 s). The amplitudes of fEPSPs were recorded every 30 s for 60 mins after HFS. LTP was measured as the percentage of change of average fEPSPs slope at the last 10 minutes after HFS induction, comparing with the average fEPSPs slope of baseline.

2.7. Statistics. Data were presented as mean ± standard error of mean (SEM) and were analyzed by using SPSS 20.0 (Chicago, IL, USA) and GraphPad Prism version 5.0 (San Diego, CA, USA). One-way ANOVA was used to analyze multiple comparisons, and two-way ANOVA was used to analyze the results from MWM and fearing conditioning. Values of $P < 0.05$ were considered statistically significant.

3. Result

3.1. Both MS and Repeated EA Stimulation Did Not Alter Body Weight. To determine whether neonatal MS or repeated EA stimulation in young adult affects the nutritional development, animals in all subgroups were weighted on postnatal 3rd week (the weaning day), 8th week (the day before EA treatment), and the 11th week (the day after EA treatment). As shown in Figure 1(b), body weight at different time did not differ in control, MS, MS + EA, and MS + Sham-EA group ($P > 0.05$). These results demonstrated that both neonatal MS and repeat EA stimulation in this study had no effect on overall normal nutritional development of rats.

3.2. EA Improves Spatial Learning and Memory in Young Adult Rats with Neonatal MS. To assess effect of EA treatment on cognitive deficit in young adult rats that suffered maternal separation, rats were tested for spatial learning and memory with MWM, a widely used test in rodents known to require hippocampal function [37]. As shown in Figure 2(b), control group showed normal learning ability by showing gradually shorter escape latency to find the hidden platform during the MWM test; however, the latency was increased in MS group and MS + Sham-EA group compared to control group ($P < 0.01$, $P < 0.05$, resp.), indicating that ability of learning was compromised. In contrast, the latency in MS + EA group decreased significantly when compared to MS group ($P < 0.01$, Figure 2(b)). In probe trial after five days of training, rats were assessed regarding reference memory with target quadrant (Figures 2(a) and 2(c)) on the sixth day. Times spent in target quadrant of rats in MS group and MS + Sham-EA group were both less than control group ($P < 0.05$, $P < 0.05$, resp.), but time spent in target quadrant of rats in MS + EA group was increased compared with MS group ($P < 0.05$) and had no difference with control group ($P > 0.05$). These results indicated that repeated EA stimulation improved spatial learning and reference memory in young adult rats with neonatal MS.

3.3. EA Attenuated Contextual Fear Memory Impairment in Rats with Neonatal MS. To further demonstrate that EA could attenuate hippocampal-dependent cognitive impairment in rats with neonatal MS, we then explored performance of rat in the contextual fear conditioning task. As shown in Figure 3(a), we found that rats in MS group and MS + Sham-EA group exhibited decreased levels of freezing compared to controls ($P < 0.01$, $P < 0.05$, resp.). However, the level of freezing in MS + EA group was similar to control group ($P > 0.05$), demonstrating normal contextual fear memory. We next assessed cued fear conditioning task, which was generally believed to be largely dependent on amygdala, but not hippocampus [38]. As shown in Figure 3(b), no differences were found in percentage of freezing to the tone among all groups ($P > 0.05$). Together, the results from fear conditioning and MWM tests suggested that neonatal MS may specifically impair the hippocampal-dependent cognitive function, while EA ameliorated the deficit in young adult period.

3.4. EA Improved the Impaired LTP at SC-CA1 Synapse of Rats with Neonatal MS. To better directly assess the role of EA treatment in altering hippocampal synaptic plasticity of cognitive deficit rat, electrophysiological study used to record in vivo LTP induced by HFS at SC-CA1 synapses in the hippocampus was performed. As shown in Figure 4, fEPSPs were measured in the CA1 area of the dorsal hippocampus and the LTP magnitude was quantified in groups. We found that the LTP amplitude was largely suppressed in MS and MS + Sham-EA group compared with control group ($P < 0.05$, $P < 0.05$, resp.). Intriguingly, there was no difference between MS + EA group and control group.

4. Discussion

In this study, we modeled rat maternal separation which was observed commonly in society. The major findings of our study are as follows. First, we found that rat with neonatal MS exhibited normal weight gain and significant cognitive deficit in MWM and contextual fear conditioning test in adulthood (Figures 2 and 3). Intriguingly, after 3-week EA intervention in young adulthood, behavior deficits of young adult rats with maternal separation in early-life stage were ameliorated significantly (Figures 2 and 3). This indicated the efficacy of EA intervention on cognitive deficits in rats exposed to the neonatal MS. Moreover, this paper provided evidence that in vivo LTP induced by the HFS at hippocampal SC-CA1 synapses was suppressed in rat with neonatal MS, and EA intervention could restore LTP induction significantly (Figure 4), which identified straightforward function of EA intervention in regulating hippocampal synaptic plasticity in vivo.

The present study showed normal weight gain of young adult rats with MS. This result agreed with previous studies examining maternal separation on rats during PND2–9 or PND10–17 [39]. Some previous studies showed that repeated maternal separation altered body weight in offspring [40, 41], which may be related to variation of MS protocol (longer versus shorter time separation), species of animals, or the different postnatal periods of MS [42]. Moreover, EA stimulation in young adulthood does not affect the weight gain of rats with MS, compared to the MS or control group (Figure 1(b)). Thus, all the interpretation to the following

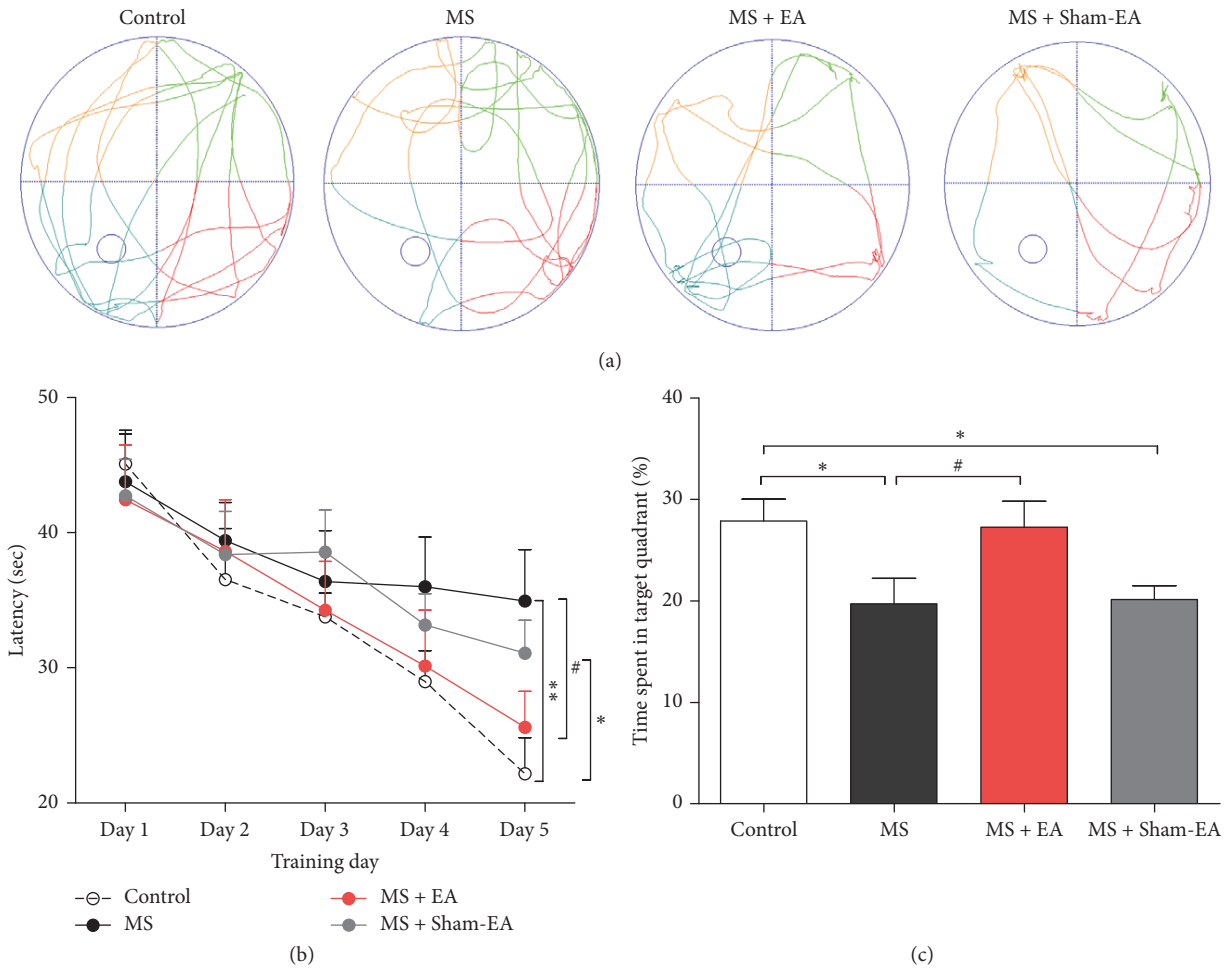

FIGURE 2: EA ameliorated spatial learning and memory deficits in rats with neonatal MS. (a) Representative swim paths in target quadrant from each group during the probe trials. The third quadrant is the target quadrant. (b) Increased latency in MS rats to reach the hidden platform in MWM, compared to control, and latency was reduced by repeating EA stimulation, but not MS + Sham-EA. $n = 11$/group; $F_{(3,40)} = 3.731$; $P < 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus MS group; two-way ANOVA. (c) Reduced percentage of time spent in target quadrant of rats in MS group, compared to rats in control, and percentage of time spent in platform quadrant was increased after EA treatment. $n = 11$/group. $F_{(3,40)} = 4.047$; $P < 0.05$, $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus MS group; one-way ANOVA.

results in this study may not be secondary to nutritional development by MS or EA intervention.

Hippocampus plays an important role in spatial information processing in both rodents and humans [29]. Morris water maze and other tasks were used to assess spatial memory related to hippocampus [43, 44]. In the present study, we demonstrated that rats with neonatal MS exhibited significant spatial learning and memory deficits which agree with previous studies [39, 45, 46]. It is generally believed that contextual fear conditioning is dependent on hippocampus, while cued fear conditioning largely involves amygdala [47]. We found cognitive deficit significantly in contextual fear conditioning test, but not cured fear conditioning test (Figure 3). The result supported the point that MS in early age impaired hippocampal-dependent cognitive function in adulthood.

Our result provided evidence for the first time that EA intervention at GV 20 and GV 29 significantly enhanced the impaired learning and memory ability in rat model exposed to the neonatal MS, which was similar to studies by using EA intervention in other cognitive deficit researches. A study found that EA intervention ameliorated ethanol-induced impairments of spatial learning and memory [48]. Huang et al. proved that ischemic stroke which accompanied memory in rat was improved by EA treatment at GV 20 and *Shenting* (DU24) acupoints [49]. Ye et al. reported that acupuncture at *Zusanli* (ST36) and GV 20 remarkably reversed cognitive deficits in 2-vessel occlusion model rats [50]. EA improved memory was also observed in neurological diseases such as MCI, Parkinson's disease, and Alzheimer disease [51]. These studies in animal models support the clinical data that acupuncture is promising for the treatment of cognitive deficits in various diseases [19–23].

The present results also showed that significant impaired LTP in rats with neonatal MS and EA treatment, but not Sham-EA, significantly enhanced the increasing of the slope

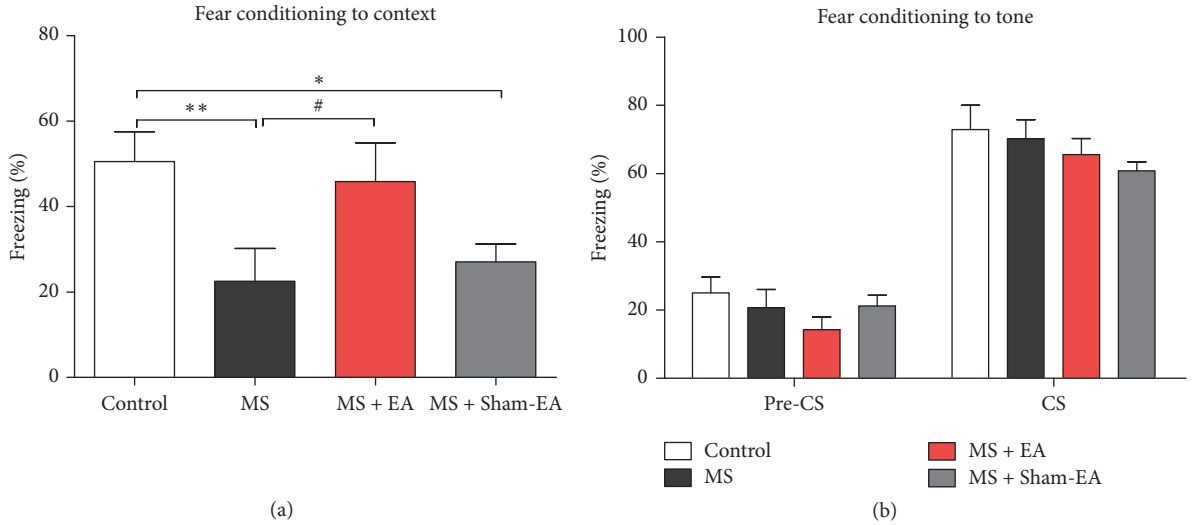

FIGURE 3: Effect of EA on performance in the fear conditioning task. (a) EA ameliorated impairment in contextual fear memory in rats with neonatal MS, $n = 11$/group; $F_{(3,40)} = 3.660$; $P < 0.05$, $^{*}P < 0.05$, and $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus MS group; one-way ANOVA. (b) There was no impairment in fear conditioning to tone in rats suffered maternal separation. CS represents condition stimulus, such as sound combined with an aversive unconditioned stimulus (foot shock); pre-CS represents a 120 sec pause without stimulation. $n = 11$/group; $F_{(3,40)} = 0.9171$; $P > 0.05$; two-way ANOVA.

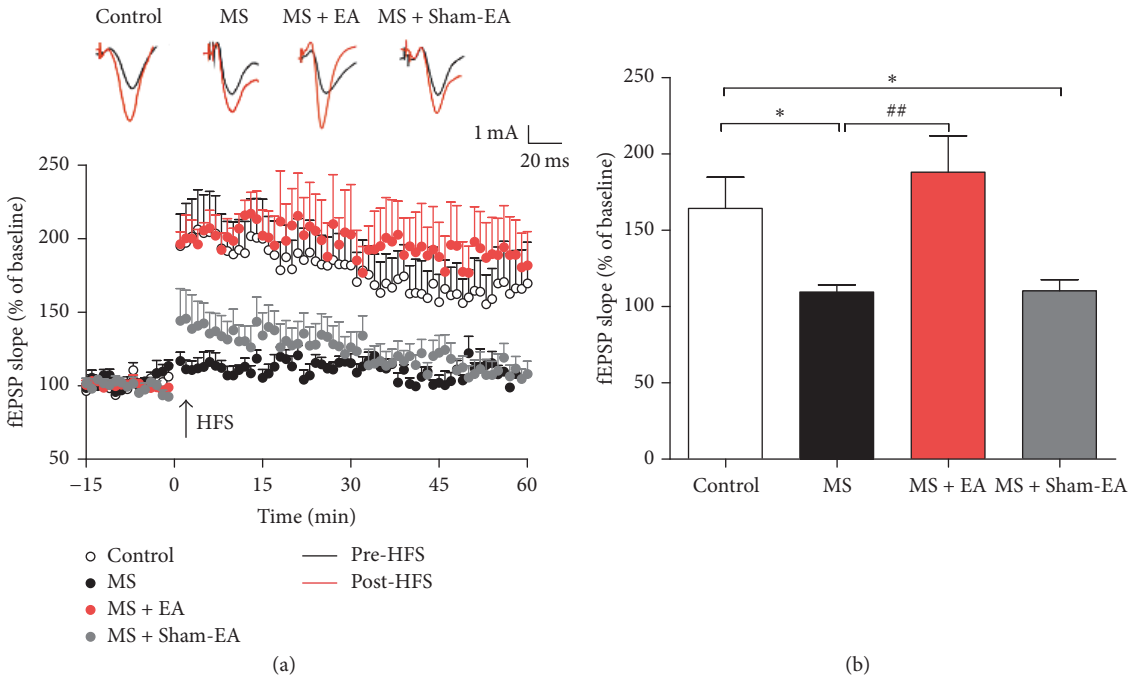

FIGURE 4: EA enhanced LTP in the hippocampus of young adult rats with neonatal MS. (a) Averaged time course changes in fEPSPs slope induced by HFS in hippocampal of rats. (b) Percentage of change in magnitude of LTP (fEPSPs slope average at last 10 minutes after HFS). $n = 5$-6 rats/group; $F_{(3,19)} = 6.282$; $P < 0.01$, $^{*}P < 0.05$ versus control group; $^{\#\#}P < 0.01$ versus MS group; one-way ANOVA.

of fEPSPs. LTP at hippocampal SC-CA1 synapse is widely considered the cellular mechanism that underlies learning and memory [28]. Previous studies suggest that chronic stress in early life was proven to impair LTP, resulting in spatial and fear related learning and memory [45, 52]. And it has been shown that EA could enhance LTP at SC-CA1 synapse in the hippocampus to improve cognitive deficits [27, 53]. These suggest that EA may improve spatial or fear learning and memory via enhancing LTP ability. However, the mechanisms of EA on learning and memory ability are not understood. In recent years, converging evidence has shown that one of the most important underlying

mechanisms for improving learning memory is via modulating hippocampus synaptic plasticity [29, 54]. In ischemic stroke animals model, manual acupuncture at ST 36 and EA at GV 20 improves cognitive hippocampus function by modulating cAMP/PKA/CREB [55] signaling pathway and by reducing the expression of NR1-TRPV1 [56], thus reducing deficits related to LTP. In vascular dementia rat model, cognition and hippocampal synaptic plasticity were improved by acupuncture via activating D1/D5 receptors [21]. These results agree with our observations in this study, which suggests that EA ameliorated learning and memory deficit in maternal separation animals via restoration of hippocampal LTP induction at SC-CA1 synapses. However, confirmation of the underlying molecular mechanisms of EA enhanced hippocampal neural plasticity and behavior deficit in adult induced by neonatal MS still await the results of further studies.

5. Conclusions

In conclusion, EA ameliorated learning and memory deficit in young adult rats that experienced neonatal maternal separation stress via restoration of hippocampal LTP induction at SC-CA1 synapses. EA at GV 20 and GV 29 may be of therapeutic value for its importance for the memory and cognitive dysfunction resulting from early stage stress.

Conflicts of Interest

The authors declare no conflicts of interest.

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