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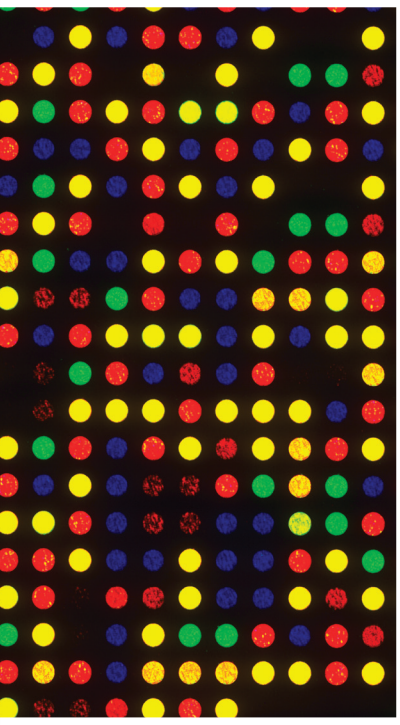
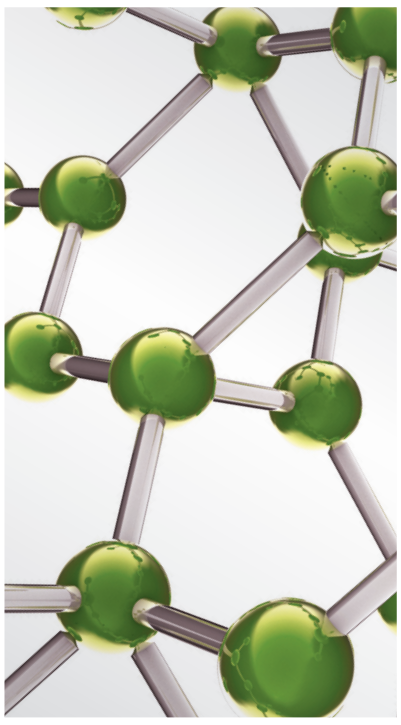
Acupuncture and Oxidative Stress

Guest Editors: Cun-Zhi Liu, Shu-Feng Zhou, Sergio-Botelho Guimarães, William Chi-shing Cho, and Guang-Xia Shi



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Editorial Board

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Editorial

Acupuncture and Oxidative Stress

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The current special issue is the 2014 issue which includes 7 interesting papers.

As one of the modalities of traditional oriental medicine, acupuncture has been widely used to treat many disorders and diseases including chronic pain, stroke, and insomnia as well as depression, while its mechanisms remain unclear. Enhanced production of reactive oxygen species causes oxidative stress leading to damage in lipids, proteins, and nucleic acids. Recent experimental studies have demonstrated that acupuncture could attenuate oxidative stress, which seems possible to explore the physiological antioxidative mechanism of acupuncture in various diseases.

This special issue contains 7 papers, of which 5 articles study the antioxidative mechanism of acupuncture in some diseases by animal models. These studies suggested that acupuncture may result from antioxidation, anti-inflammation, and antiapoptosis effects in several kinds of diseases. Among these, one study is related to the effect of laser acupuncture on memory impairment, oxidative stress status, and the functions of both cholinergic and dopaminergic systems in hippocampus of animal model of Parkinson's disease. One study explores whether electroacupuncture reduces myocardial ischemia-reperfusion (I/R) injury and inflammatory responses through inhibiting early growth response (Egr)-1 expression via the extracellular signal-regulated protein kinase-1 and kinase-2 (ERK1/2) pathway in a mouse model of myocardial ischemia reperfusion. Besides, one study compares the effects of antioxidant interventions on the electrical potential difference between acupoints along the stomach meridian on human. This paper suggests a possible underlying mechanism of acupuncture involving superoxide removal. One study focuses on the emerging links between acupuncture and redox modulation in vascular dementia, Alzheimer's vascular dementia, Parkinson's disease and hypertension, which represents an important step forward in the research of acupuncture antioxidative effect.

We are excited to explore the studies on the specific oxidative stress biomarkers and redox signaling cascades using oxidative stress-related assessments techniques should be particularly useful in generating new hypotheses to enhance our understanding of the mechanism of antioxidative effects of acupuncture.

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Shu-Feng Zhou
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William Chi-shing Cho
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Research Article

MicroRNA Profiling Response to Acupuncture Therapy in Spontaneously Hypertensive Rats

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MicroRNAs (miRNAs) are a group of endogenous noncoding RNAs that play important roles in many biological processes. This study aimed to check if miRNAs were involved in the response to acupuncture in rats. Microarray analysis was performed to compare the miRNA expression profiles of medulla in spontaneously hypertensive rats (SHRs) treated with or without acupuncture. Our microarray analysis identified 222 differentially expressed miRNAs in the medulla of SHRs treated with acupuncture at taichong acupoint. Among these miRNAs, 23 miRNAs with a significant difference were found in acupuncture-treated SHRs compared to untreated rats. These 23 miRNAs could regulate 2963 target genes which were enriched in at least 14 pathways based on our bioinformatic analysis. miRNA-339, miR-223, and miR-145 were downregulated in the medulla of SHRs compared to normotensive rats. Notably, these miRNAs were upregulated to basal levels in the medulla of SHRs treated with acupuncture at taichong in comparison with SHRs receiving acupuncture at nonacupoint group or SHRs without any treatment. Our findings have revealed significant changes of a panel of selective miRNAs in hypertensive rats treated at taichong acupoint. These data provide insights into how acupuncture elicits beneficial effects on hypertension.

1. Introduction

Hypertension, a major risk factor for cardiovascular disease, affects approximately one billion individuals worldwide [1], including 78 million (33%) American adults ≥20 years of age (i.e., it affects one in three adults in the United States) [2]. Although a number of treatment strategies have been developed for this disease, only 75% of hypertensive patients received pharmacologic treatment, and only 53% of those with documented hypertension have their condition controlled to target levels [2]. The major barriers to successful pharmacotherapeutic control of hypertension are poor drug response, adverse side effects, and low patient compliance [3].

Acupuncture originated in ancient China at least 2,500 years ago. Although there is some controversy in mainstream Western medicine, it has become one of the most widely practiced forms of alternative medicine in the world [4]. For example, estimated 3 million American adults receive acupuncture treatment each year [5]. The US Internal Revenue Service approved acupuncture as deductible medical expense in 1973. The National Institute of Health has sponsored a Consensus Development Conference on Acupuncture in 1997 [6]. WHO has approved acupuncture as an alternative therapy for the treatment of at least 64 types of diseases. On November 16, 2010, acupuncture has been listed

by the United Nations Educational, Scientific, and Cultural Organization as "Intangible Cultural Heritage."

Since the 1970s, a number of animal and clinical studies have demonstrated the effectiveness of acupuncture at specific acupoints to lower blood pressure in essential hypertension [7–10]. Unfortunately, the underlying mechanisms through which acupuncture lowers blood pressure remain unclear. Studies have suggested the involvement of nitric oxide (NO) [11], neurotransmitters [12], aldosterone, endothelin, and angiotensin II [13, 14], and acetylcholine, opioids, γ-aminobutyric acid, serotonin, and endocannabinoids in the brain all appear to contribute to the acupuncture's antihypertensive effect [15, 16].

The small noncoding microRNAs (miRNAs) have critical functions in the regulation of various critical biological processes such as cell metabolism, proliferation, death, and development [17, 18]. To date, there are more than 2,000 miRNAs reported in humans. A number of studies have found that miRNAs respond to various therapeutic interventions such as pharmacotherapy, physical therapy, and radiotherapy. However, there is no information on the effect of acupuncture therapy on miRNA profiles in animals and humans. The objective of the present study was to determine whether miRNAs were involved in the response to acupuncture therapy in rats. This study is the first to examine the miRNA response in the medulla of rats to acupuncture at the taichong (LR3) point compared to stimulation at a nonacupoint in spontaneously hypertensive rats (SHRs).

2. Materials and Methods

2.1. Ethics Statement. All animal experiments were performed at the Laboratory Animal Center of Guangzhou University of Chinese Medicine, Guangzhou, China. The procedure was approved by the Ethics Committee of Guangzhou University of Chinese Medicine, Guangzhou, China [permit number: SYXK (Yue) 2008-0085].

2.2. Chemicals and Reagents. The 6th generation of miRCURYTM LNA Array (v.16.0) was obtained from KangChen Bio-tech (Shanghai, China), which contains more than 1891 capture probes, covering all human, mouse, and rat microRNAs annotated in miRBase 16.0, as well as all viral microRNAs related to these species. In addition, this array contains capture probes for 66 new miRPlus human microRNAs. All chemicals and reagents used were of analytical grade.

2.3. Animals. The experiments were performed with SHRs and Sprague-Dawley (SD) rats provided by Beijing Vital River Laboratory Animals Co., Ltd (Beijing, China). The rats were housed in cages maintained in a temperature- and humidity-controlled room at the Laboratory Animal Center of Guangzhou University of Chinese Medicine, Guangzhou, China. The animals were given a standard diet. The SHRs with confirmed blood pressure $\geq$ 140 mmHg were included and randomly divided into three groups: (1) acupuncture at taichong group ($n = 6$) treated with acupuncture at LR3 point, (2) acupuncture at nonacupoint group ($n = 6$) treated with acupuncture at nonacupoints, and (3) model group ($n = 6$) untreated with acupuncture throughout the duration of the experiment. In the literature, SD rats have been used as the normotensive controls in comparison to SHRs [19, 20] and thus they were used as normal control group ($n = 6$) in this study.

2.4. Acupuncture Procedure. A number of clinical and animal studies have reported the efficacy of acupuncture at taichong point in reducing hypertension [21–23]. The acupuncture treatment procedure was the same as described previously by us [22]. Briefly, in acupuncture taichong group, acupuncture was performed at bilateral taichong points (LR3) located between the 1st and the 2nd metatarsal of dorsal foot, while, in acupuncture nonacupoint group, acupuncture was done at bilateral nonacupoint located at the fossa between the 3rd and 4th metatarsal of dorsal foot (Figure 1). As previously described [22, 24], the rats were lightly immobilized and the acupuncture needles were inserted to a depth of 3 mm at the appropriate location bilaterally, twisted at a rate of 80 spins per min, and removed afterward. This treatment was given daily (treatment lasting 5 min/day) for 7 consecutive days. After the last acupuncture treatment, rats were anesthetized and their medullas were quickly dissected. The medullas were preserved in liquid nitrogen until analysis.

2.5. Microarray Analysis. Total RNA was isolated using TRIzol (Invitrogen, Grand Island, NY, USA) and miRNeasy minikit (QIAGEN) according to manufacturer's instruction. After RNA isolation from the samples, the miRCURY Hy3/Hy5 Power labeling kit (Exiqon, Vedbaek, Denmark) was used according to the manufacturer's guideline for miRNA labelling. One microgram of each sample was 3′-end-labeled with Hy3 fluorescent label, using T4 RNA ligase. After stopping the labeling procedure, the Hy3-labeled samples were hybridized on the miRCURY LNA Array (v.16.0) (Exiqon) according to the manufacturer's manual. The slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA). Scanned images were then imported into GenePix Pro 6.0 software (Axon) for grid alignment and data extraction. Replicated miRNAs were averaged and miRNAs in which intensities >50 in all samples were chosen for calculating the normalization factor. The data were normalized using the Median normalization method and after normalization differentially expressed miRNAs were identified through Volcano Plot filtering. In addition, hierarchical clustering was performed using MEV software (v4.6, TIGR).

2.6. Bioinformatics Studies. Computational target prediction of miRNAs was conducted by miRDB online searching program (http://mirdb.org/miRDB/). The enriched KEGG pathway for these targets was analyzed in DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/).

2.7. Quantitative Real-Time PCR (qRT-PCR). The rat medulla was homogenized in 400 μL of TRIzol reagent (Invitrogen, Grand Island, NY, USA). To avoid contamination with

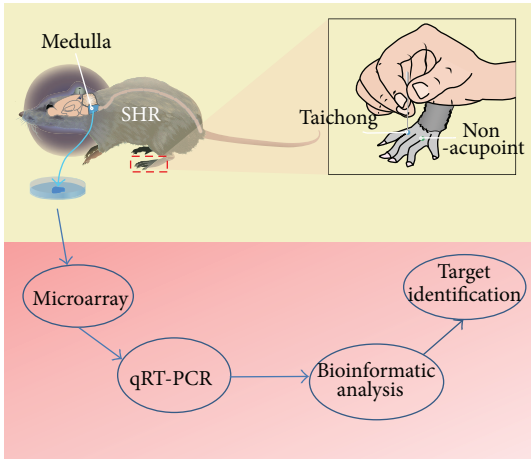

Figure 1: The acupuncture procedure at taichong point and nonacupoint in SHRs. In taichong group of the SHRs, acupuncture was performed at bilateral LR3 located between the 1st and the 2nd metatarsal of dorsal foot, while, in nonacupoint group, acupuncture was done at bilateral nonacupoint located at fossa between the 3rd and 4th metatarsal of dorsal foot.

genomic DNA, the RNA samples were treated with RNase-free DNase (Promega, Madison, WI, USA). Reverse transcription was performed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Real-time RT-PCRs were carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) and the SYBR Green PCR Master Mix kit (Toyobo Co., Ltd., Osaka, Japan). The $2^{-\Delta\Delta Ct}$ method was used to analyze the RT-PCR data and the $^{\Delta Ct}$ of each miRNA was determined relative to U6 RNA and endogenous control miRNA that is robustly and invariantly expressed in all cell types. The experiments were performed in triplicate, and consistent results were obtained.

2.8. Statistical Analysis. Data are presented as the mean ± SD. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure, with $P < 0.05$ considered significant. All statistical tests were performed using Prism software version 6.0 (GraphPad Software Inc., Chicago, IL, USA).

3. Results

3.1. miRNAs Profiling Response to Acupuncture Therapy in SHRs. To reveal miRNAs profiling response to acupuncture therapy in SHRs, microarrays containing 1891 human, rat, and mouse miRNAs were used to examine the expression of miRNAs in medullas after acupuncture treatment at taichong point in SHRs. The analyses were performed using total RNA collected from three rat medullas. Statistical analysis of the miRNA expression data identified 222 miRNAs with significant changes in the expression, with 23 miRNAs having expression levels greater or equal to a 1.5-fold change in SHRs with or without acupuncture treatment (Tables 1 and 2). Among these 23 differentially expressed miRNAs, 8 of 23 miRNAs were significantly upregulated, while 15 of 23 miRNAs were significantly downregulated after acupuncture treatment.

Table 1: miRNAs upregulated by acupuncture in the medulla of SHRs.

Mature miRNAs (rno-miR-)	Fold-change (mean ± SEM)	*P* value	miRBase accession number
339	2.13	0.0214	MIMAT0000583
223	3.34	0.0011	MI0000963
145	3.02	0.0059	MI0000918
451	3.32	0.0097	MI0001731
193	2.88	0.0300	MI0000936
378	2.30	0.0135	MI0003719
423	1.97	0.0277	MI0006145
let-7b*	1.70	0.0443	MIMAT0004705

Each experiment was performed in triplicate. The fold change is presented as the mean ± SEM. *Indicates antisense mature miRNA.

Table 2: miRNAs downregulated by acupuncture in the medulla of SHRs.

Mature miRNAs (rno-miR-)	Fold-change (mean ± SEM)	*P* value	miRBase accession number
7a	0.52	0.0254	MI0000641
9	0.48	0.0491	MI0000838
128	0.45	0.0229	MI0000900
132	0.42	0.031	MI0000905
134	0.59	0.0037	MI0000907
182	0.24	0.0093	MI0006133
335	0.6	0.0475	MI0000612
382	0.44	0.0381	MI0003548
383	0.51	0.0324	MI0003478
434	0.44	0.0155	MI0006147
496	0.59	0.011	MI0012622
135a	0.45	0.0414	MI0000908
136*	0.58	0.0131	MIMAT0004733
376b-5p	0.54	0.0052	MIMAT0003195
384-3p	0.57	0.0225	MIMAT0005310

Each experiment was performed in triplicate. The fold change is presented as the mean ± SEM. *Indicates antisense mature miRNA.

3.2. Confirmation of Microarray Profiling Data by qRT-PCR. To validate the microarray profiling data, qRT-PCR was used to confirm the upregulated miRNAs including miRNA-339, miR-223, miR-145, and miR-451. The data showed that miR-339, miR-223, and miR-145 were significantly upregulated in medullas of SHRs treated with acupuncture at taichong point in contrast to the model group untreated with acupuncture (Figure 2). While being compared to the normal control group (healthy SD rats), miR-339, miR-223, and miR-145 were significantly downregulated in model group. There was no significant increase in miR-451 between acupuncture group and model group or normal group (data not shown).

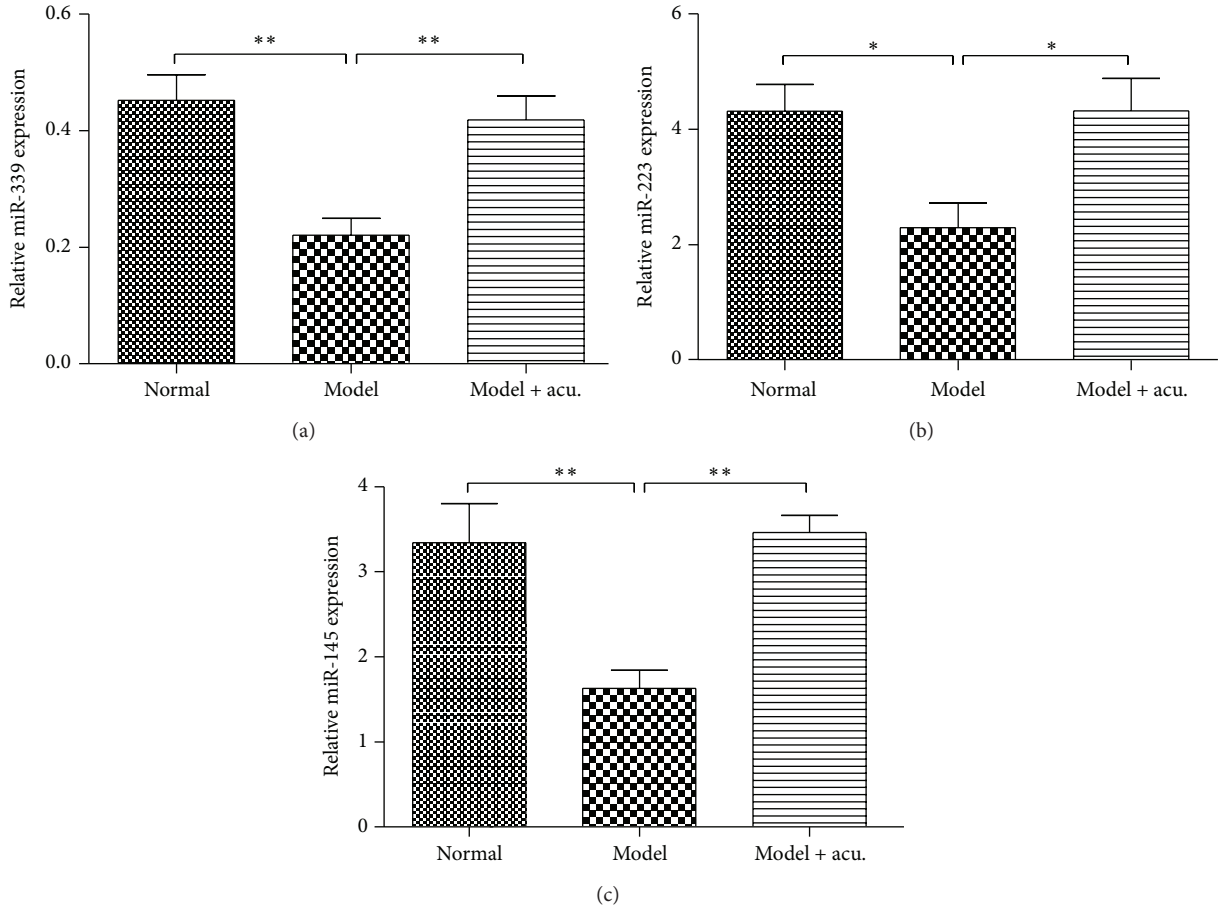

Figure 2: Confirmation of miR-339 (a), miR-223 (b), and miR-145 (c) expression changes in the medulla of SHRs treated with acupuncture at taichong point (model + acu.). The expression level of miR-339, miR-223, and miR-145 in rats was detected using qRT-PCR. The relative expression of these miRNAs was calculated in relation to levels of U6 RNA using the $2^{-\Delta\Delta Ct}$ method. The result was repeated in triplicate independent experiments. $^{*}P < 0.05$; $^{**}P < 0.001$.

3.3. Acupuncture Treatment at Taichong Acupoint Elicits Specific miRNA Expression Changes in SHRs. Next, we compared the effect of acupuncture at taichong acupoint or at nonacupoint on miRNA expression changes in SHR medulla. The data showed that acupuncture at taichong point significantly increased miRNA-339, miR-223, and miR-145 levels in medullas of SHRs in contrast to the model group, while acupuncture at nonacupoint did not significantly alter miR-339, miR-223, and miR-145 levels compared to the model group. Moreover, compared to acupuncture at nonacupoint, acupuncture at taichong point significantly upregulated the expression of miRNA-339, miR-223, and miR-145 in medullas of SHRs (Figure 3). These data showed significantly different miRNA response to real and sham acupuncture treatments in rats.

3.4. Possible Targets and Their Enriched Pathway of Acupuncture-Regulated miRNAs by Bioinformatics Analysis. To explore possible targets and signaling pathways involved in response to acupuncture, we used miRDB to predict the targets of the differentially expressed microRNAs responsive to acupuncture and used DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/) to analyze the enriched pathways. From the miRDB online database (http://mirdb.org/miRDB/), we identified 5223 targets for all 23 acupuncture-regulated miRNAs when the target score was set to ≥50. When the target score cut-off was set at ≥60, 2963 possible targets were predicted. We further analyzed the enriched KEGG pathways for these 2963 targets and found 14 pathways, including neurotrophin signaling pathway, pathways in cancer, MAPK signaling pathway, Wnt signaling pathway, Ubiquitin mediated proteolysis, cell cycle, chemokine signaling pathway, oocyte meiosis, tight junction, T cell receptor signaling pathway, axon guidance, and TGF-β signaling pathway (Table 3).

4. Discussion

In this study, we first found that miRNAs responded to acupuncture treatment in SHRs. Previous studies have demonstrated that physical therapy elicited remarkable miRNA profiling changes in humans [25–28] and mice [29]. Similarly, we have revealed that 222 medullar miRNAs were involved in acupuncture therapy in SHRs. Of these miRNAs,

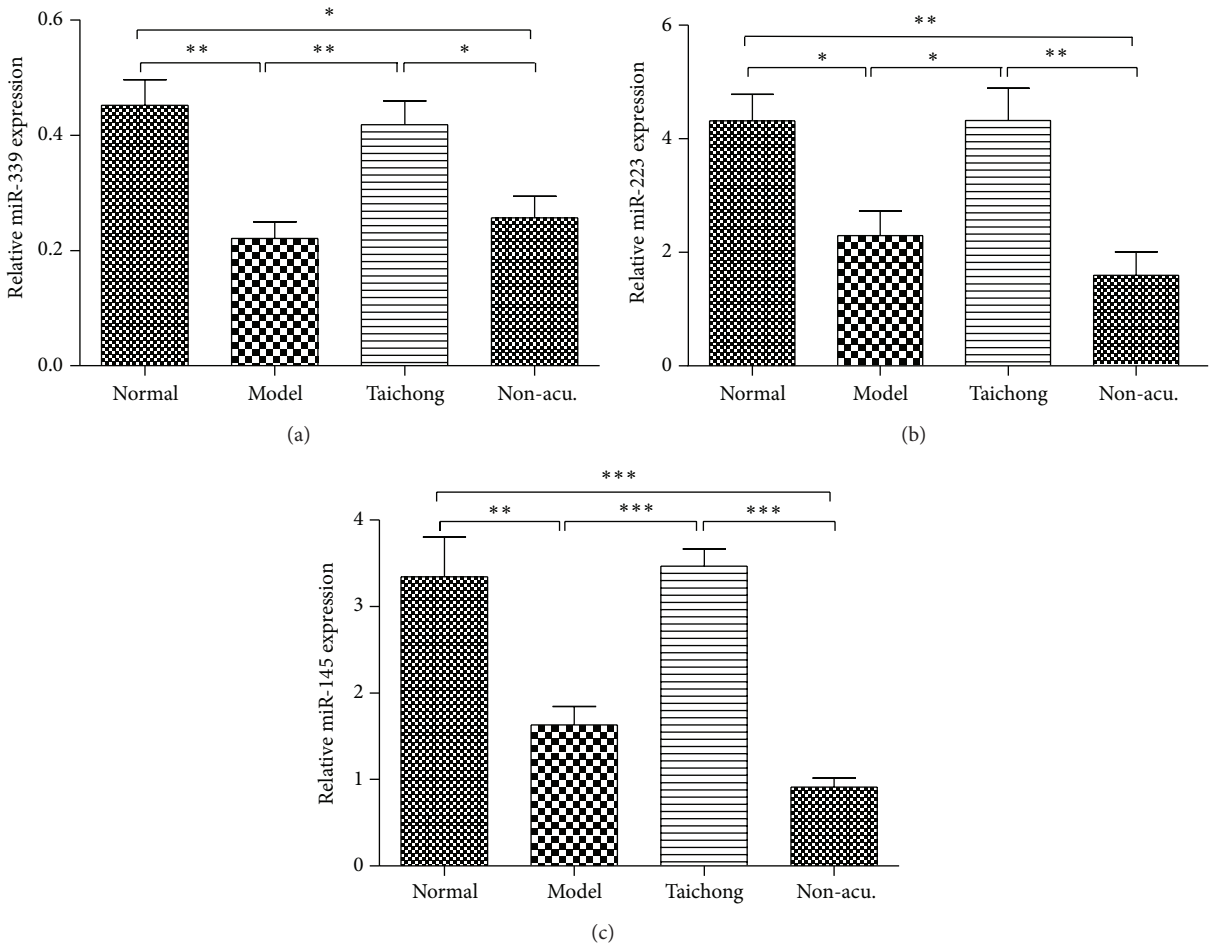

Figure 3: Acupuncture treatment at taichong acupoint elicits specific miRNA expression changes in SHRs. Acupuncture at taichong point (Taichong) significantly increased miR-339 (a), miR-223 (b), and miR-145 (c) expression levels in the medullas of SHRs compared to SHRs with nonacupoint treatment (non-acu.). $^{*}P < 0.05$; $^{**}P < 0.001$, $^{***}P < 0.001$.

Table 3: The enriched KEGG pathways of acupuncture-responsive miRNAs in SHRs.

Category	Term	Count	%	P value	Benjamini
KEGG_PATHWAY	Neurotrophin signaling pathway	21	0.1	$6.60E-02$	$6.50E-01$
KEGG_PATHWAY	Pathways in cancer	51	0.2	$6.50E-03$	$1.60E-01$
KEGG_PATHWAY	MAPK signaling pathway	42	0.2	$1.90E-02$	$3.20E-01$
KEGG_PATHWAY	Wnt signaling pathway	38	0.2	$7.80E-07$	$1.40E-04$
KEGG_PATHWAY	Ubiquitin mediated proteolysis	31	0.1	$3.70E-05$	$3.40E-03$
KEGG_PATHWAY	Cell cycle	29	0.1	$2.40E-04$	$8.80E-03$
KEGG_PATHWAY	Chemokine signaling pathway	29	0.1	$2.40E-02$	$3.50E-01$
KEGG_PATHWAY	Calcium signaling pathway	28	0.1	$7.50E-02$	$6.40E-01$
KEGG_PATHWAY	Oocyte meiosis	27	0.1	$1.60E-04$	$7.40E-03$
KEGG_PATHWAY	Tight junction	22	0.1	$5.20E-02$	$5.90E-01$
KEGG_PATHWAY	T cell receptor signaling pathway	21	0.1	$1.70E-02$	$3.20E-01$
KEGG_PATHWAY	Endocytosis	42	0.2	$7.70E-05$	$4.70E-03$
KEGG_PATHWAY	Axon guidance	21	0.1	$7.00E-02$	$6.40E-01$
KEGG_PATHWAY	TGF-beta signaling pathway	20	0.1	$2.50E-03$	$7.40E-02$

Thresholds: count cut-off ≥20; EASE <0.1.

although screened by fold change ≥1.5 and P value < 0.05, 23 miRNAs were significantly regulated by acupuncture treatment (Tables 1 and 2). These microarray data were further validated using qRT-PCR. The findings from this study suggest that miRNAs are involved in the therapeutic effects of acupuncture.

Our finding further showed that acupuncture treatment at specific acupoint elicits selective miRNA expression changes in SHRs (Figure 3). Acupoint specificity is the theoretical basis for meridian and acupuncture theory and is also the key factor for the application of effective and individualized acupuncture treatment [30, 31]. However, there is still some controversy about the existence of acupoint specificity [32–37]. Our data showed that acupuncture at taichong acupoint regulated miR-339, miR-223, and miR-145 expression, while acupuncture at nonacupoint failed to affect these miRNAs' expression. Other researches have observed acupoint-specific effect at taichong point in human brain by functional nuclear magnetic resonance imaging technique [38].

Our bioinformatics analysis showed that 23 miRNAs responded to acupuncture and these miRNAs might regulate 2963 targets. These miRNAs have many important physiological and pathological functions. miRNA-339, miR-223, and miR-145 are highly conserved and have multiple targets predicted for them in both humans and rats [39–41]. In addition, many studies have demonstrated that these miRNAs are involved in cell proliferation, apoptosis, and tumor suppressor. For example, miRNA-339 has been shown to have a central role in regulating the expression of intercellular cell adhesion molecule-1 [39]. Similarly, miR-223 has been found to negatively regulate progenitor proliferation and granulocyte differentiation and activation [42]. miR-145 has been proposed to inhibit human embryonic stem cell self-renewal, represses expression of pluripotency genes, and induces lineage-restricted differentiation [41]. miR-145 has also been proposed as a tumor suppressor that can target the 3′-untranslated region of the insulin receptor substrate-1 gene and dramatically inhibit the growth of colon cancer cells [43]. Therefore, acupuncture-responsive miRNAs may contribute to the therapeutic effects of acupuncture in hypertension through various pathways.

Our bioinformatic data also showed that there were 14 pathways regulated by the miRNAs responsive to acupuncture therapy (Table 3). Among these pathways, the neurotrophin signaling pathway plays critical roles in the regulation of brain activities and blood pressure control. Neurotrophins are a family of growth factors that induce the survival, development, and function of neurons [44, 45], which also play an important role in pathogenesis of hypertension [46] and in the neuroprotective activity of acupuncture treatment. The cardiovascular system is tightly controlled by the nervous system; neurons, cardiomyocytes, endothelial cells, and vascular smooth muscle cells are all well regulated by neurotrophins [46–49]. Our data suggest that acupuncture might regulate blood pressure through microRNA-neurotrophin signaling pathway. Studies by us and others have demonstrated that acupuncture activated neurotrophin signaling pathway and elicited neuroprotective activity [50–52]. Recent studies have showed that microRNA can not only regulate neurotrophin expression, but also target their receptors and downstream signaling proteins [53, 54]. For example, miR-206 was found to decrease brain-derived neurotrophic factor level [53, 54] and miR-21 regulated neuron growth factor signaling pathway in rats [55]. miR-592 was shown to modulate the induction of p75 neurotrophin receptor in neuronal ischemic injury [56]. Our data together with data from other groups suggest that acupuncture could modulate various miRNAs which consequently altered the function and activities of critical neuronal and cardiovascular factors contributing to the regulation of blood pressure (Figure 4). The acupuncture-responsive miRNAs are supposed to play important roles in the therapeutic effects of acupuncture treatment for hypertension, but further mechanistic studies are warranted to elicit how these responsive miRNAs contribute to the antihypertensive activity of acupuncture.

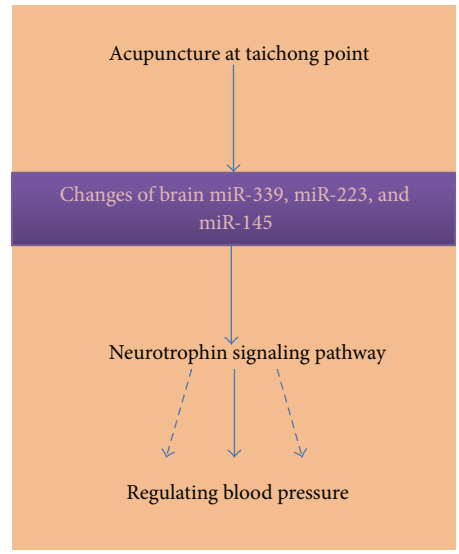

Figure 4: Proposed role of miRNAs in the antihypertensive effects of acupuncture.

In addition, our bioinformatics data predicted that mitogen-activated protein kinase (MAPK) signaling pathway was possibly involved in the antihypertensive activity of acupuncture in rats. The MAPK signaling molecules have been recognized as important mediators in directing cellular responses to a diverse array of stimuli, such as proinflammatory cytokines and exposure to environmental compounds. They regulate gene expression, differentiation, apoptosis and many other cellular processes [57]. Studies have showed that MAPK signaling pathway was activated by acupuncture in the brain [58]. Further functional studies are needed to dissect the role of MAPK signalling pathway in the therapeutic effects of acupuncture.

In summary, our microarray study for the first time identified 222 differentially expressed miRNAs in the medulla of SHRs treated with acupuncture at the taichong acupoint. Among these miRNAs, 23 miRNAs were found to be differentially expressed in acupuncture-treated SHRs compared

to untreated control rats. These 23 miRNAs could regulate 2963 target genes based on our bioinformatic analysis. Importantly, our RT-PCR assay has confirmed that miRNA-339, miR-223, and miR-145 were upregulated in SHRs treated with acupuncture at taichong acupoint in comparison with the nonacupoint group. Our findings have demonstrated significant changes of specific and selective miRNAs in rats when taichong acupoint was stimulated. Our data have revealed the specific miRNA profile changes in response to acupuncture treatment and strongly suggest that a selective panel of miRNAs play an important role in the antihypertensive activity of acupuncture therapy.

Conflict of Interests

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

Authors' Contribution

Jia-You Wang and Hui Li equally contributed to this work as joint first authors.

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Research Article

Monitoring the Effects of Acupoint Antioxidant Intervention by Measuring Electrical Potential Difference along the Meridian

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Previous studies suggest that superoxide anions are possibly traveling along acupuncture meridians. The electrical potential difference (EPD) between acupoints may be related to the movement. To test the above hypothesis, we conducted a study investigating the effects of acupoint antioxidant interventions on the meridian EPD. Firstly, ST39 (L) and ST44 (L) were screened out for the EPD detection along the stomach meridian, and ST36 (L) was selected for interventions including acumassage with the control cream, as well as the TAT-SOD cream for 30 minutes, or injection with reduced glutathione sodium. The EPD between ST39 and ST44 was recorded for 80 minutes and measured again 48 h later. While the EPD increased during the acumassage, the acumassage with TAT-SOD cream and the glutathione injection generated waves of EPD increased, indicating the migration or removal from the visceral organ of a greater quantity of superoxide. Remarkably lower EPD readings 48 h later with both antioxidant acupoint interventions than the mere acumassage imply a more complete superoxide flushing out due to the restored superoxide pathway at the acupoint after interventions. The results confirm superoxide transportation along the meridians and demonstrate a possibility of acupoint EPD measurement as a tool to monitor changes in the meridians and acupoints.

1. Introduction

Reactive oxygen species (ROS), molecules or ions formed by the incomplete one-electron reduction of oxygen, have drawn considerable interest because of their regulations ranging from cell signaling to inflammatory response and cell death [1, 2]. An imbalance between ROS and cell defense systems results in the oxidative stress which is intricately connected to ageing and life span [3, 4]. While investigating the ROS distribution in living SD-rats in 2008, we accidentally discovered that there existed a special ROS-containing cellular network which was perfectly superimposable on a standard human acupuncture meridian network, as to the phenomenon that conception vessel meridian, spleen meridian, stomach meridian, and kidney meridian emitted intense green fluorescence corresponding to the intracellular ROS indicator [5]. It suggests that acupuncture meridian could be a channel in which ROS are either localized or transported.

Acupoints lie along the meridians. What acupoints will be in relation to this "ROS network"? In the issue of Free Radical Biology and Medicine, a controlled study [6] has shown that topical application of superoxide dismutase (SOD) fused with the TAT peptide (TAT-SOD), to various acupoints along the meridian lines used in acupuncture to treat obesity, leads to significant weight loss. Similarly, topical application of TAT-SOD on acupoint LI20 (Yingxiang) alleviated allergic rhinitis [7]. It indicates a possibility of the new method as a simple substitute to acupuncture and an insight of superoxide modulation along meridians for acupuncture mechanism. Therefore, it could possibly be assumed that acupoints would be the important sites that can store ROS, more specifically, superoxide as implied by the effect of TAT-SOD, and also be the gates through which ROS are transported. While

the sites pile up with superoxide, "superoxide network" would be clogged with traffic. Thus, we can clear out the drains by way of the enzymatic removal of the intracellular superoxide at acupoints.

Within the acupuncture community, it is a commonly held opinion that acupuncture points have distinct electrical properties, for example, increased conductance [8, 9], reduced impedance and resistance [10, 11], and increased capacitance [8] compared to nonacupuncture points. Electrical measurements to study acupuncture points and meridians have become universal and internationally recognized. Recently, a clinical research [12] demonstrated that the EPD between ST39 and ST44 of essential hypertension group was between −60.00 mV and 60.00 mV, while the normal group was between −30.00 mV and 30.00 mV. The study revealed that the values of the EPD between acupoints can also reflect health status for subjects' organs.

Superoxide is negatively charged, and its migration is driven by the voltage difference. Therefore, any antioxidant intervention at acupoints is possible to be monitored by the electrical potential difference between acupoints along the meridians. To test the hypothesis, the effects of antioxidant interventions at one acupoint on the EPD between another two acupoints along the stomach meridian are investigated in hope to confirm superoxide's involvement in meridians and establish a new and effective tool for the studies of meridians and superoxide.

2. Materials and Methods

2.1. Materials. The vehicle cream for TAT-SOD cream was also used as control cream. The vehicle cream was baby lotion (Johnson & Johnson, Shanghai, China), which contains water, propylene glycol, myristyl myristate, glyceryl stearate, oleic acid, and stearic acid. 3000 U SOD/mL TAT-SOD cream was prepared by the homogenization of membrane permeable TAT-SOD with the vehicle cream. TAT-SOD was prepared by recombinant expression of a fusion protein of human Cu, Zn-SOD fused with TAT peptide in *E. coli* as follows: constructs preparation: the nucleic acid sequence encoding TAT-SOD fusion protein was constructed by DNA recombinant technology and inserted into expression vector pGEX-2 T; cell culture and transfections: *E. coli* (BL21, DH5α) cells were transformed with the expression vector pGEX-2 T containing the inserted TAT-SOD; TAT-SOD fusion protein preparation: TAT-SOD was expressed in the *E. coli* by the induction of IPTG. After purification of affinity chromatography, electrophoretically pure TAT-SOD protein was obtained [13]. 0.12 g/mL reduced glutathione sodium (Laboratorio Farmaceutico C.T.S.R.L., Strada Solaro, Villa Sayonara, Sanremo, Italy) was prepared by injecting 5 mL 0.9% (w/v) saline into a germ-free bottle equipped with 0.6 g freeze-dried powder of reduced glutathione sodium. Disposable sterilized acupuncture needles of 300 μm diameter and 40 mm length stainless steel (Huacheng, China) were used for acupuncture.

2.2. Subject. A total of 30 healthy volunteers were recruited in this study after giving full informed consent. Participants should meet the following criteria: (1) 20 and 35 years of age; (2) no history or physical examination suggestive of renal, hepatic, or cardiovascular diseases or any other severe organic diseases; (3) having regular diet; minimal liquor, tobacco, tea, and coffee; normal sleeping patterns (before 12 a.m.); (4) no long-term medications; (5) no history of drug abuse; (6) having not undergone acupuncture or other acupoint interventions within 1 month before the test. This study was approved by the Medical Research Ethics Committee and Institutional Review Board of Fujian Institute of Traditional Chinese Medicine.

2.3. Experimental Protocol. Before the trial, several acupoints along the ST meridian were selected for their convenience to operate. The subjects first underwent the measurement of EPD between acupoints. Based on the stability and individual difference of the EPD between acupoints, two acupoints were screened out for the EPD detection along the ST meridian. Meanwhile, one downstream acupoint along the ST meridian for interventions was selected. Subsequently, 30 subjects were divided into 6 groups. The treatments applied to different groups on acupoint were as follows: (A) no treatment, (B) acumassage without cream, (C) acumassage with control cream, (D) acumassage with TAT-SOD cream (3000 U/mL), (E) injection of reduced glutathione sodium injection (100 μL, 0.12 g/mL), and (F) acupuncture. When the EPD between the two selected detection acupoints was relatively stable after a balance of about 20 min, the cream was applied on the intervention acupoint assisted by massage stick for 30 min, reduced glutathione sodium was injected into the intervention acupoint quickly, and acupuncture was conducted, respectively. Make a record of the EPD between the two detection acupoints for 60 min, since the start-time of acupoint intervention. Besides, the EPD between the same acupoints was monitored again 48 h after treatments.

2.4. Selection of EPD Detection Points of Stomach Meridian. Stomach meridian has 45 acupoints each side. Ruling out the acupoints in the face, head, torso, and fingertip that are inconvenient to operate, 5 acupoints as EPD detection acupoints were selected (Table 1).

2.5. EPD Monitoring Procedure. The subjects were conscious, placed in a supine position, and asked to breathe calmly. The acupoints were localized according to name and location of acupoints: Chinese National Standards GB/T12346 [14]. The hair on the selected detection acupoints was trimmed. After previously disinfected with medical alcohol, the point sites were connected to a digital potentiometer via Ag/AgCl disposable ECG electrodes. After 15–25 min of equilibrium, data of the EPD between two acupoints in 10 min was collected and recorded for selection of two acupoints for the EPD detection along the ST meridian. During the monitoring of acupoint intervention, the EPD was constantly recorded for about 80 min.

2.6. Acumassage Methods. Massage stick (YJ-8, Bailing, China) was used for acupoint massage. Acupoint for intervention was localized according to name and location of acupoints: Chinese National Standards GB/T12346 [14].

Table 1: EPD detection acupoints of ST and their anatomical positions.

Acupoints	Location
ST36: Zusanli	On the anterior lateral side of the leg, 3 Cun below ST35 Dubi*, one finger breadth (middle finger) from the anterior crest of the tibia
ST37: Shangjuxu	On the anterolateral side of the leg, 6 Cun below ST35 Dubi, one finger breadth (middle finger) from the anterior crest of the tibia
ST39: Xiajuxu	On the anterolateral side of the leg, 9 Cun below ST35 Dubi, one finger breadth (middle finger) from the anterior crest of the tibia
ST42: Chongyang	On the dome of the instep of the foot, between the tendons of the long extensor muscle of the big toe and the long extensor muscle of the toes, where the pulsation of the dorsal artery of the foot is palpable
ST44: Neiting	On the instep of the foot, in the depression distal to the commissure of the 2nd and 3rd metatarsal bones

*Location of ST35 Dubi: with the knee flexed, on the knee, in the depression lateral to the patella and its ligament.

B group was immediately massaged assisted by massage stick. C and D groups have first applied 0.2 mL of the control cream and TAT-SOD cream, respectively, in an area of 1 cm^2 to acupoint for intervention and then massaged assisted by massage stick in a minute. During the 30 min, physician repeated this manipulation for 3 min every 5 min.

2.7. Acupuncture Methods. Disposable sterilized acupuncture needles of 300 μm diameter and 40 mm length stainless steel (Huacheng, China) were used for acupuncture in this study. Acupoint for intervention was localized according to name and location of acupoints: Chinese National Standards GB/T12346 [14]. Skin was disinfected with medical alcohol. The acupuncture procedures were referred to in Chunxiao Wu's paper [15]. After manipulating the needle for 10 min, the needle was held in place for another 20 min. After that, the needle was removed quickly.

2.8. Statistical Analysis. Data are reported as means (SEM). All statistical analyses were carried out using SPSS version 21.0 software. Results in left EPD and right EPD of the two same detection acupoints were compared using two-sample t-test, and paired t-test was used to analyze interclass variance. A one-factor ANOVA (SPSS version 21.0), followed by Duncan's test, was used to test for significant differences of the reduction of the EPD of each group 48 h after treatments.

3. Results

3.1. Selection of Two Detection Points for the EPD of Stomach Meridian and One Point for Interventions. As shown in Table 2, the stability of the EPD between acupoints varied in different combinations. Of all detection points, the EPD between ST37 and ST42 (R) and that between ST42 and ST44 (L) fluctuated relatively larger, and the EPD between ST39 and ST44 (L) was most stable. As to T_1 test (paired t-test), the EPD of most acupoint combinations showed significant individual difference; only the EPD between ST39 and ST44 (L) showed no significances ($P > 0.05$). There are also generally existing differences between the left side and the right side of the same two acupoints, regarding T_2 test (two-sample t-test).

Table 2: The interclass variance and between-cluster variance of the EPD of acupoints along the ST meridian.

Acupoints	Interclass variance	Between-cluster variance	T_1	T_2
ST36-ST37 (L)	2.07	6.48	**	*
ST36-ST37 (R)	1.31	7.19	**	
ST36–ST39 (L)	1.02	8.31	**	**
ST36–ST39 (R)	1.38	7.22	**	
ST36–ST42 (L)	2.54	5.68	**	**
ST36–ST42 (R)	1.26	6.28	**	
ST36–ST44 (L)	1.33	7.10	**	**
ST36–ST44 (R)	1.79	4.31	**	
ST37–ST39 (L)	1.91	7.79	**	**
ST37–ST39 (R)	1.46	7.71	**	
ST37–ST42 (L)	1.92	8.18	**	**
ST37–ST42 (R)	3.90	6.16	**	
ST37–ST44 (L)	1.29	6.27	**	**
ST37–ST44 (R)	1.14	6.18	**	
ST39–ST42 (L)	2.30	7.20	**	*
ST39–ST42 (R)	1.29	7.46	**	
ST39–ST44 (L)	**1.01**	**1.08**		**
ST39–ST44 (R)	2.15	5.41	**	
ST42–ST44 (L)	3.18	6.21	**	**
ST42–ST44 (R)	2.48	5.20	**	

T_1 (paired t-test): comparing the EPD of the same points of different subjects.
T_2 (two-sample t-test): comparing the left EPD and right EPD of the same detection points.
**($P < 0.01$): regarded as very significant.
*($P < 0.05$): regarded as significant.

Therefore, ST39 and ST44 (L) were screened out for the EPD detection along the ST meridian for the stable and reliable voltage reading. In the meantime, the downstream acupoint ST36, admittedly an important and effective acupoint along the ST meridian in dredging meridian channels and relieving fatigue [16–18], was selected for interventions.

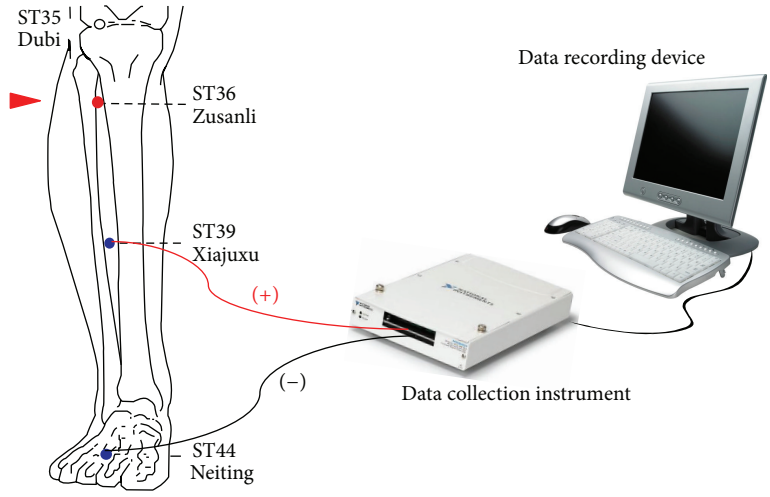

Figure 1: A diagrammatic sketch of how to monitor the variation of the EPD between ST39 and ST44 caused by different acupoint interventions on ST36.

3.2. The Effect of Acupoint Intervention on the EPD between Acupoints. A diagrammatic sketch of how to monitor the variation of the EPD between ST39 and ST44 caused by different acupoint interventions on ST36 was shown in Figure 1.

ST36 was subject to acumassage alone, acumassage with the control cream, and the cream containing membrane permeable TAT-superoxide dismutase for 30 min or injected with reduced glutathione sodium (100 μL, 0.12 g/mL), and the EPD between ST39 and ST44 was recorded for 80 min and measured again 48 h later. The results of the variation trends of different interventions during the first 80 min were presented in Figure 2.

After 15–25 min of equilibrium, the EPD of the control group, group A, displayed a slight decrease in the next 60 min. Groups other than A group showed a significant increase in EPD reading right after ST36 was stimulated with different modes. These groups all fluctuated during a 30-min period of intervention. However, the EPD readings of B and C groups gradually descended when interventions ended, while D, E, and F groups still fluctuated at high levels in the next 30 min follow-up. The variation trend of B and C groups was similar.

The reduction of EPD between ST39 and ST44 of each group 48 h after the intervention was demonstrated in Table 3. Compared to A group of no treatment on acupoint, B group of acumassage without cream and C group of acumassage with control cream showed almost no decrease, while D group of acumassage with TAT-SOD cream, E group of reduced glutathione sodium injection on acupoint, and F group of acupuncture reported a very significant decline ($P < 0.01$). The fall of the EPD readings of D and F groups was similar without significance ($P < 0.01$). E group had the largest decline between all groups ($P < 0.01$).

Table 3: The reduction of EPD 48 h after treatments.

Treatment		The reduction of EPD (mV)
A	No treatment on acupoint	0.34 ± 1.67a[#]
B	Acumassage without cream	1.05 ± 1.96a
C	Acumassage with control cream	1.00 ± 1.75a
D	Acumassage with TAT-SOD cream	4.51 ± 0.51b
E	Reduced glutathione sodium injection on acupoint	7.79 ± 0.64c
F	Acupuncture	4.35 ± 0.38b

[#]Values in the same column with different letters were significantly different by Duncan's test ($P < 0.01$).

4. Discussion

TAT-SOD, a fusion protein of human Cu, Zn-SOD fused with TAT peptide, is permeable membrane and well capable of eliminating intracellular superoxide. As a negatively charged anion, superoxide migration is caused by the EPD. Therefore, the EPD between acupoints may reflect the migration of superoxide along the meridian. The phenomenon that the EPD readings of B and C groups gradually descended when interventions ended, while D and E groups still fluctuated at high levels in the next 30 min follow-up, indicates that ROS, or more specifically superoxide anion, may be traveling along meridians.

All the interventions at ST36 in this study resulted in the immediate increase in EPD reading. The elevation of EPD reading stopped almost right after the interventions stopped with the acumassage alone and the acumassage with the control lotion, indicating that the intervention at ST36 could cause the increased flow of superoxide anion along the meridians. With both antioxidant oxidant intervention and acupuncture, it is clear that the increased superoxide flow could last for some time even after the intervention stopped. The continuation of the elevated flow of superoxide resulted in a lower EPD reading 48 h later, implying a lower level of superoxide migration as the result of the intervention. ST36 is like a gating point modulating the superoxide flow from its source organ. It turned on while acumassage intervention

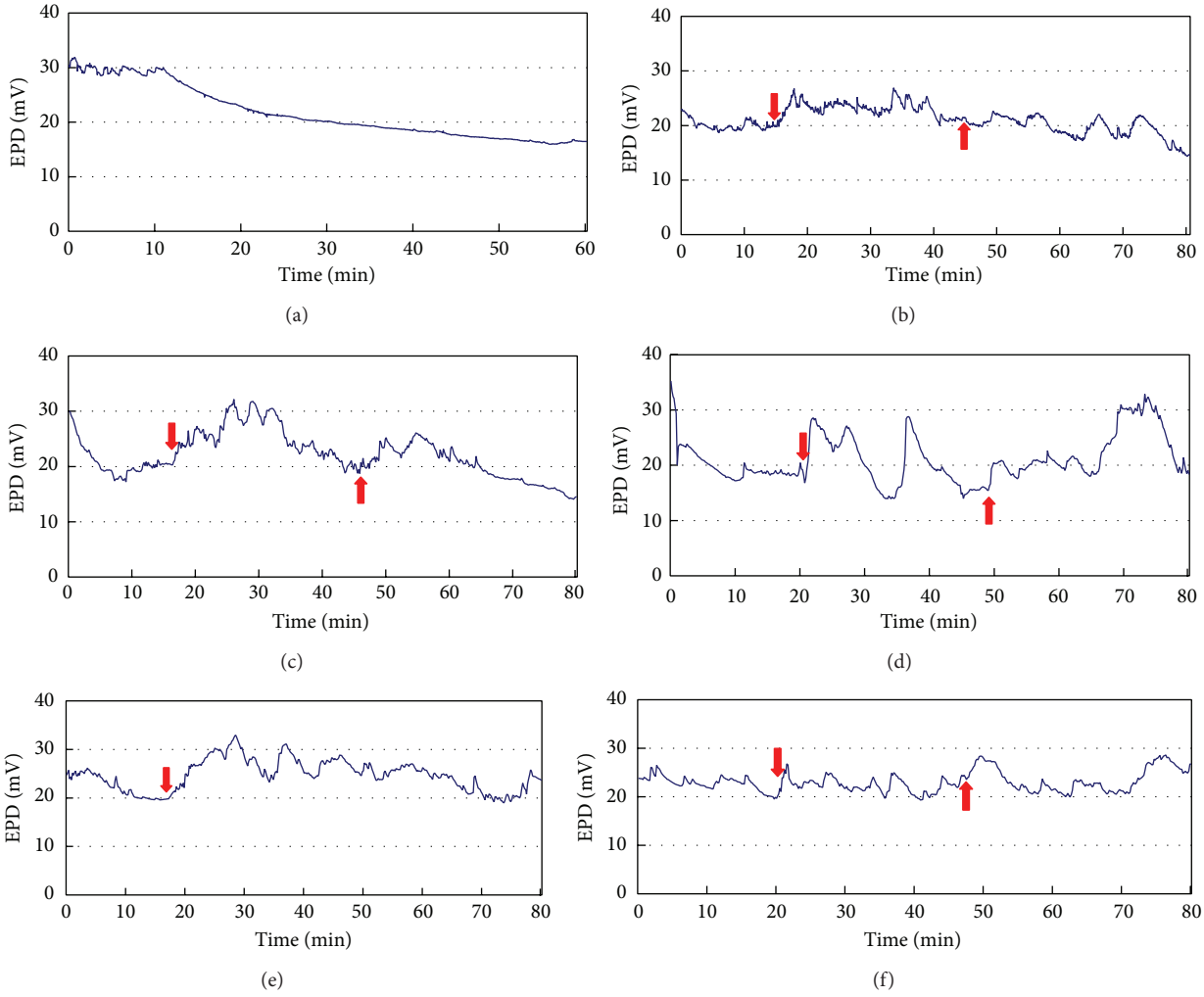

Figure 2: Monitoring the EPD caused by acupoint antioxidant interventions and acupuncture. (a) group A: no treatment; (b) group B: acumassage without cream; (c) group C: acumassage with control cream; (d) group D: acumassage with TAT-SOD cream (3000 U/mL); (e) group E: injection of reduced glutathione sodium (100 μL, 0.12 g/mL); and (f) group F: acupuncture. Downward arrow: start-time of intervention; upward arrow: end-time of intervention.

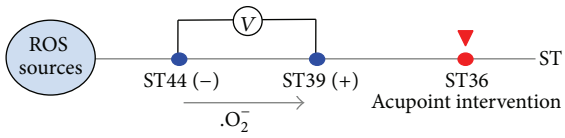

Figure 3: Schematics of a possible involvement of the acupoint superoxide removal.

lasted but went off as soon as it stopped. Antioxidant interventions and acupuncture seemed to generate a lasting effect to turn on the connection so as to enable the flow of waves of superoxide even after the intervention stopped, so much as to clear up the storage of superoxide at its source, as indicated by the remarkable lower EPD level 48 h later.

Figure 3 depicts schematics of a possible involvement of the acupoint superoxide removal. The positive electrode and the negative electrode were attached to ST39 and ST44, respectively. The EPD readings between ST39 and ST44 were positive, which meant that superoxide migrated from ST44 to ST39. When ST36 was subject to acupoint intervention, the EPD readings increased rapidly and then fluctuated at high levels; that is to say, more superoxide from upstream ROS sources migrated from ST44 to ST39 when a portion of superoxide piled up at ST36 was cleared out or flowed out by acupoint intervention, for example, mere acumassage, acumassage with control cream, or acumassage with TAT-SOD cream.

In addition, the EPD readings were much lower with both antioxidant acupoint interventions than the mere acumassage 48 h later, implying a more potent superoxide flushing out due to the restored superoxide pathway at the acupoint after the interventions. The effect of acumassage with TAT-SOD cream was similar to that of acupuncture intervention, as shown in Figure 3 and Table 2. The acupuncture at ST36 resulted in similar EPD patterns and 48 h EPD drop with the antioxidant interventions, suggesting a possible involvement of the acupoint superoxide removal.

Moreover, acupoint EPD measurement can quickly respond synchronously, when the status of intervention changes. For example, after acupuncture intervention at ST36, the needle was removed quickly; in the meantime, the EPD reading between ST39 and ST44 sharply ascended. There the same thing happened at the start-time of interventions. Therefore, acupoint EPD measurement hold great potential as a powerful tool to monitor changes in the meridian and acupoints.

Different responses of superoxide flow along the meridian caused by different interventions may be attributed to the biological nature of acupoints. It is reported that acupoints are high in mast cells which will respond to pressures such as acumassage [19]. Meridians are considered to anatomically reside in the fascia, connective tissues containing fibroblasts [20]. Fibroblasts in fascia are known to convert from network status with high conductivity to disconnected globular status under ROS stimulation. It is possible that the antioxidant interventions at acupoints lower than the intracellular superoxide level and restore the fibroblasts to the network status to recover the connectivity of superoxide flow. Much work is necessary to verify the reasoning and to reveal cellular mechanism of antioxidant interventions.

5. Conclusions

The results confirm superoxide transportation along the meridian and demonstrate a great potential of acupoint EPD measurement as a powerful tool to monitor changes in the meridians and acupoints.

Conflict of Interests

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

Acknowledgments

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Research Article

Laser Acupuncture at HT7 Acupoint Improves Cognitive Deficit, Neuronal Loss, Oxidative Stress, and Functions of Cholinergic and Dopaminergic Systems in Animal Model of Parkinson's Disease

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To date, the therapeutic strategy against cognitive impairment in Parkinson's disease (PD) is still not in satisfaction level and requires novel effective intervention. Based the oxidative stress reduction and cognitive enhancement induced by laser acupuncture at HT7, the beneficial effect of laser acupuncture at HT7 against cognitive impairment in PD has been focused. In this study, we aimed to determine the effect of laser acupuncture at HT7 on memory impairment, oxidative stress status, and the functions of both cholinergic and dopaminergic systems in hippocampus of animal model of PD. Male Wistar rats, weighing 180–220 g, were induced unilateral lesion at right substantianigra by 6-OHDA and were treated with laser acupuncture continuously at a period of 14 days. The results showed that laser acupuncture at HT7 enhanced memory and neuron density in CA3 and dentate gyrus. The decreased AChE, MAO-B, and MDA together with increased GSH-Px in hippocampus of a 6-OHDA lesion rats were also observed. In conclusion, laser acupuncture at HT7 can improve neuron degeneration and memory impairment in animal model of PD partly via the decreased oxidative stress and the improved cholinergic and dopaminergic functions. More researches concerning effect of treatment duration are still required.

1. Introduction

Cognitive deficit, a common nonmotor feature of Parkinson's disease (PD), produces a great impact on the quality of life of patients and caregivers as well as annual healthcare cost [1–3]. It has been reported that approximate one-fifth of newly diagnosed PD patients develop the fulfilled clinical criteria for mild cognitive impairment (PD-MCI) [4] and around one-sixth develop dementia after 5 years [5].

To date, the exact mechanism of cognitive impairment in PD is still unclearly known. However, recent substantial evidence has demonstrated that cholinergic and dopaminergic systems play the crucial roles on the pathophysiology of cognitive deficit in PD [6, 7]. In addition, the disturbance of oxidative stress homeostasis in hippocampus also plays a role on this condition [6]. The current pharmacological interventions against this condition are still not in satisfaction level and the novel effective therapeutic strategy is still required.

Acupuncture has been long-term treating various disorders including neuropsychological disorders. HT7, an acupoint located at the ulnar end of the transverse crease of the writs in the depression on the radial side of the tendon flexor carpi ulnaris, has been long-term used for treating many neuropsychological impairments such as amnesia, insomnia, mania, epilepsy, and stupor. In addition, it also regulates the physical response to emotional stimuli such as anxiety, fear, and panic [8, 9]. TE5, an acupoint located at the posterior aspect of the forearm and midpoint of the interosseous space between the radius and the ulna, is also claimed for the central nervous system effect. It has been used for treating headache,

stroke-related motor, and neurological and autonomic nerve problems in clinical practice [10]. However, it has been clearly demonstrated that the single acupoint stimulation only at HT7 can produce significant neuroprotective activity against the neuronal impairment and memory dysfunction induced by corticosterone, a stress hormone partly via the improved cholinergic function [11]. The stimulation acupoint can occur not only via needle stimulation but also via laser stimulation [12–18]. Therefore, various wavelengths of laser have been implemented in medicine for various purposes. It is believed that the stimuli must elicit "De Qi" which involves the stimulation and transmission of mechanical signal to connective tissue cells via mechanotransduction [19]. Since most of mechanoreceptors are located in the dermis especially at the superficial area of this layer and the skin of the rats did not contain abundant of pigment cells, the stimulation with laser with low penetration power such as blue laser at the wave length of 405 nm is enough to stimulate this group of receptor [20]. Moreover, it has been reported that the stimulation at HT7 acupoint either via manual or via laser can improve spatial memory impairment in various animal models [21, 22]. In addition, laser acupuncture at HT7 acupoint also improves neurodegeneration and cholinergic function in hippocampus [22]. Based on the these pieces of information, the beneficial effect of laser acupuncture at HT7, a noninvasive intervention, on cognitive deficit condition in Parkinson's disease has been considered. To the best of our knowledge, no scientific evidence concerning this issue is available until now. Thus, this study aimed to determine the effect of laser acupuncture at HT7 acupoint on memory impairment, oxidative stress status, and the function of both cholinergic and dopaminergic systems in 6-OHDA lesion rat, a validated animal model of Parkinson's disease.

2. Materials and Method

2.1. Animals. Young adult male Wistar rats, 8-week old, were used as experimental animals. They were obtained from National Laboratory Animal Center, Salaya, Nakorn Pathom. The weights of the animals on the first day of experiment are 180–220 grams. They were housed 6 per cage and maintained in 12 : 12 light : dark cycle and given access to food and water ad libutum. The experiments were performed to minimize animal suffering and the experimental protocols were approved by the Institutional Animal Care and Use Committee Khon Kaen University, Thailand (AEKKU 41/2554). All treatments in this study were performed once daily between 8.00 a.m., and 5.00 p.m.

2.2. Drugs and Chemicals. 6-Hydroxydopamine hydrochloride (6-OHDA) was purchased from Sigma-Aldrich Co., USA. Sodium pentobarbital was obtained from Jagsonpal Pharmaceuticals Ltd., Haryana, India. All other chemical substances were analytical grade and purchased from Sigma Chemical Company, St. Louis, MO.

2.3. Experimental Protocol. All rats were randomly assigned to 4 groups of 12 animals each as follows:

Group I Control group: rats in this group were exposed to sham operation and received no treatment (no laser treatment).

Group II 6-OHDA: rats were induced partial lesion in substantia nigra by injecting 6-OHDA into the right substantia nigra.

Group III 6-OHDA + sham acupoint + laser: rats received the administration of 6-OHDA into the right substantia nigra and laser acupuncture treatment at nonacupoint.

Group IV 6-OHDA + HT7 + laser: rats received the administration of 6-OHDA into the right substantia nigra and laser acupuncture treatment at HT7 acupoint.

Rats had been treated with laser acupuncture once daily for 14 days after the administration of 6-OHDA. Then, they were assessed spatial memory using Morris water maze test at 1-day, 7-day, and 14-day periods after 6-OHDA injection. At the end of experiment, half of the rats were used for the histological study whereas the other half of the rats were used for biochemical assays including the determination of oxidative stress markers and the activities of acetylcholinesterase (AChE) and monoamine oxidase type B (MAO-B) in hippocampus. To determine the oxidative stress, AChE and MAO-B, hippocampus of each rat was isolated and determined the density of survival neurons, oxidative markers including malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) enzymes in hippocampus. To perform histological study, rats were transcardially perfused, prepared as a coronal section, and stained with cresyl violet.

2.4. Substantia Nigra Lesion. The animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Jagsonpal Pharmaceuticals Ltd., Haryana, India) at dose of 60 $mg \cdot kg^{-1}$ BW. Each animal was mounted on a stereotaxic stand, the skin overlying the skull was cut to expose the skull and the coordinates for the substantia nigra par compacta (SNpc) were accurately measured (anteroposterior −0.5 mm from bregma, mediolateral 2.1 mm from midline, and dorsoventral −7.7 mm from the skull). Total 6 μg of 6-OHDA was dissolved in 2 μL 0.2% ascorbic acid saline [23] and was perfused into SNpc through a 30-gauge stainless needle. After the surgery, animals were allowed to recover from anesthesia and then placed in their cages.

2.5. Laser Acupuncture Treatment. Fifteen minutes before laser acupuncture treatment, all rats were anesthetized with sodium pentobarbital (40 $mg \cdot kg^{-1}$, i.p.) to minimize stress. Laser acupuncture treatment via HT7 acupoint was performed once daily for 14 days. The rats were treated with a laser instrument that operated with a continuous blue laser beam at wavelength of 405 nm, output power 100 mW and a spot diameter of 500 μm at HT7 acupoint (the transverse crease of the wrist of the forepaw, radial to the tendon of the muscle flexor carpi ulnaris) or at 2–4 mm lateral to the HT7 acupoint for 10 minutes [24–26].

2.6. Determination of Spatial Memory. Spatial memory was evaluated via the Morris water maze. The water maze consists of a metal pool (170 cm in diameter × 58 cm tall) filled with tap water (25°C, 40 cm deep). The pool was divided into 4 quadrants (Northeast, Southeast, Southwest, and Northwest). The water surface was covered with nontoxic milk. The removable platform was placed below the water level at the center of one quadrant. For each animal, the location of the invisible platform was placed at the center of one quadrant and remained there throughout training. The times which animals spent to climb on the hidden platform were recorded as escape latency. In order to determine the capability of the animals to retrieve and retain information, the platform was removed 24 hr later and the rats were released into the quadrant diagonally opposite to that which contained the platform. Time spent in the region that previously contained the platform was recorded as retention time [27].

2.7. Histological Procedure. After the anesthesia with sodium pentobarbital (60 mg/kg BW), brains were subjected to transcardial perfusion with fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.3. After the perfusion, brains were removed and stored over a night in a fixative solution that used for perfusion. Then, they were infiltrated with 30% sucrose solution at 4°C. The specimens were frozen rapidly and 10 μM thick sections were cut on cryostat. The selected sections were rinsed in the phosphate buffer and picked up on slides coated with 0.01% of aqueous solution of a high molecular weight poly L-lysine.

2.8. Morphological Analysis. Five coronal sections of each rat in each group were studied quantitatively. Neuronal counts in hippocampus were performed by eye using a 40x magnification with final field 255 μm^2. The observer was blind to the treatment at the time of analysis. Viable stained neurons were identified on the basis of a stained soma with at least two visible processes. Counts were made in five adjacent fields and the mean number extrapolated to give total number of neurons per 255 μm^2. All data are represented as number of neurons per 255 μm^2.

2.9. Determination of Acetylcholinesterase and Monoamine Oxidase-B Activities. The rats were divided into various groups as previously described in the experimental protocol. At the end of experiment, all rats were sacrificed. The hippocampus was isolated and prepared as a homogenate to determine the activities of AChE and MAO-B enzymes. The activities of AChE and MAO-B were determined by using the colorimetric method [28, 29].

2.10. Determination of Oxidative Stress Markers. Hippocampus was isolated and prepared as hippocampal homogenate and the determination of the oxidative stress markers in hippocampus was performed. Malondialdehyde (MDA) level was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) [30]. In order to determine the activities of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), hippocampus of each rat was weighed and homogenized with a buffer consisting of 10 mM sucrose, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.4). Then, a hippocampal homogenate was centrifuged at 3000 g at 4°C for 15 min. The supernatant was separated and used for bioassays. The activity of SOD was determined using a xanthine/xanthine oxidase system for the production of superoxide radical and subsequent measurement of cytochrome *c* as a scavenger of the radicals. Optical density was determined using a spectrometer (UV-1601, Shimadzu) at 550 nm [31]. SOD activity was presented as units per milligram of protein (U mg^{-1} protein). One unit of enzyme activity was defined as the quantity of SOD required to inhibit the reduction rate of cytochrome *c* by 50%. CAT activity in the supernatant was measured by recording the reduction rate of H_2O_2 absorbance at 240 nm [32]. The activity of CAT was expressed as μmol $H_2O_2 \cdot min^{-1}$ mg^{-1} protein. GSH-Px was determined using *t*-butyl hydroperoxide as a substrate. The optical density was spectrophotometrically recorded at 340 nm and expressed as U/mg protein [33]. One unit of the enzyme was defined as micromole (μmol) of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidized per minute.

2.11. Statistical Analysis. Data were expressed as means ± S.E.M. and analyzed statistically by one-way ANOVA, followed by post-hoc (LSD) test. The results were considered statistically significant at P value < 0.05.

3. Results

3.1. Effect of Laser Acupuncture at HT7 on Spatial Memory of 6-OHDA Lesion Rats. Figures 1(a) and 1(b) showed that the administration of 6-OHDA into right substantia nigra significantly enhanced escape latency (P value < 0.001 all; compared to control group) but decreased retention time (P value < 0.001 and 0.01, respectively; compared to control group) at 7-day and 14-day period after the 6-OHDA. It was found that sham laser acupuncture failed to improve the alteration of both escape latency and retention time induced by 6-OHDA. Interestingly, laser acupuncture at HT7 acupoint significantly improved the reduction of escape latency induced by 6-OHDA both at 7-day and 14-day intervention period (P value < 0.05 and 0.001, respectively; compared to sham laser acupuncture group) while it showed the significant increase in retention time in 6-OHDA lesion rats only at 14-day day intervention period (P value < 0.05; compared to sham laser acupuncture group).

3.2. Effect of Laser Acupuncture at HT7 on Hippocampal Neurons. The administration of 6-OHDA into right substantia nigra induced the decreased survival neuron density in CA1, CA2, and CA3 and dentate gyrus of hippocampus (P value < 0.001 all; compared to control group). Sham laser acupuncture failed to mitigate the reduction of survival neuron density in all subregions of hippocampus mentioned earlier. However, laser acupuncture at HT7 acupoint significantly attenuated the decreased neuron density induced by 6-OHDA in CA3 and dentate gyrus (P value < 0.05 all; compared to sham laser acupuncture group) as shown in Figures 2 and 3.

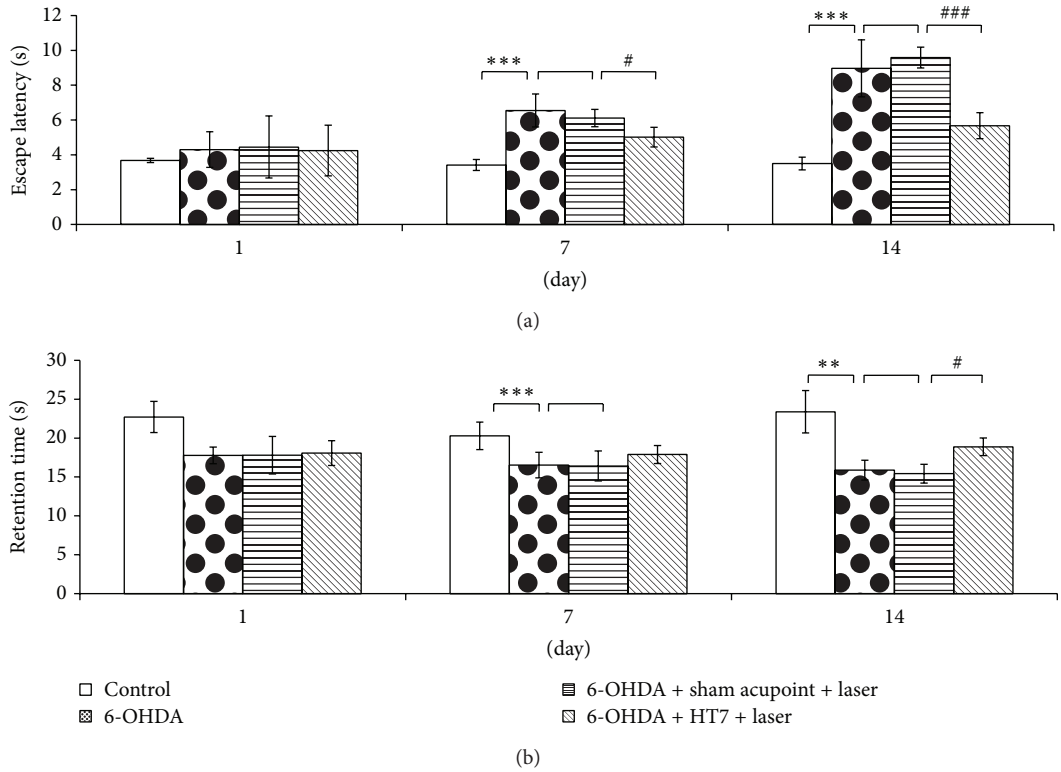

Figure 1: Effect of laser acupuncture on spatial memory using the Morris water maze test in Parkinson's disease rats (a) escape latency and (b) retention time. Values given are the mean ± S.D. ($n = 6$) $^{***}P < 0.001$ as compared with control group and $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ as compared with sham laser acupuncture group.

3.3. Effect of Laser Acupuncture at HT7 on AChE and MAO-B. In this study, the activity of AChE was used as indirect index to reflect the function of cholinergic whereas the activity of MAO-B was used as indirect index to reflect the function of monoaminergic especially dopaminergic system. The rats subjected to the unilateral lesion of substantia nigra induced by 6-OHDA showed the elevation of AChE in hippocampus (P value < 0.001; compared to control group). This change was mitigated by laser acupuncture at HT7 acupoint (P value < 0.05; compared to sham laser acupuncture group) while no significant change was observed in sham laser acupuncture treated group as shown in Figure 4.

Figure 5 showed the effect of laser acupuncture on MAO-B activity in hippocampus. Rats with the unilateral lesion of substantia nigra induced by 6-OHDA demonstrated the significant reduction of MAO-B in the mentioned area (P value < 0.001; compared to control group). Sham laser acupuncture failed to mitigate the elevation of MAO-B activity whereas laser acupuncture at HT7 significantly decreased the elevation of MAO-B activity in hippocampus (P value < 0.05; compared to sham laser acupuncture group).

3.4. Effect of Laser Acupuncture at HT7 on Oxidative Stress Markers. It was found that the administration of 6-OHDA into right substantia nigra significantly decreased CAT and GSH-Px activities but increased MDA level in hippocampus (P value < 0.05, 0.001 and 0.001, respectively; compared to control group). Sham laser acupuncture failed to produce significant changes of CAT and GSH-Px activities and MDA level induced by 6-OHDA in hippocampus. However, laser acupuncture at HT7 could significantly mitigate the decreased GSH-Px activity (P value < 0.05; compared to sham laser acupuncture group) and the elevation of MDA level (P value < 0.01; compared to sham laser acupuncture group) as shown in Table 1.

4. Discussion

The current study has clearly demonstrated that the administration of 6-OHDA into substantia nigra induced the elevation of MAO-B, AChE, and oxidative stress in hippocampus together with the enhanced spatial memory. The possible explanation for this phenomenon might be attributed to the disturbance of dopaminergic function in substantia nigra induced by 6-OHDA produced the disturbance in function of striatum via nigrostriatal pathway and striatum in turn induced the functional disturbance of hippocampus via the connection between ventral striatum and hippocampal pathway which plays a critical role on the association contextual-position information [34]. In addition, several lines of evidence have demonstrated that dentate gyrus of the hippocampus received the dopaminergic projection form substantia nigra (A9) [35, 36] and ventral tegmental area (A10), a structure nearby substantia nigra, via mesolimbic

Table 1: Effect of laser acupuncture on oxidative stress markers including MDA level and the activities of SOD, CAT, and GSH-Px enzymes.

Group	MDA (μ/mg protein)	SOD (μ/mg protein)	GSH-Px (μ/mg protein)	CAT (μ/mg protein)
Control	$0.0004 \pm 0.0001^{\#\#\#}$	4.007 ± 0.334	$0.387 \pm 0.040^{\#\#\#}$	$140.108 \pm 8.691^{\#}$
6-OHDA	$0.0011 \pm 0.0003^{***}$	3.457 ± 0.608	$0.232 \pm 0.057^{***}$	$114.708 \pm 9.873^{*}$
6-OHDA + sham acupoint + laser	$0.0010 \pm 0.0002^{***}$	3.505 ± 0.373	$0.217 \pm 0.035^{***}$	$111.688 \pm 8.716^{**}$
6-OHDA + HT7 + laser	$0.0007 \pm 0.0002^{**\#\#\#}$	3.615 ± 0.453	$0.297 \pm 0.058^{***\#}$	132.512 ± 7.356

Values given are the mean ± S.D. ($n = 6$) $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ as compared with control group and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ as compared with sham laser acupuncture group.

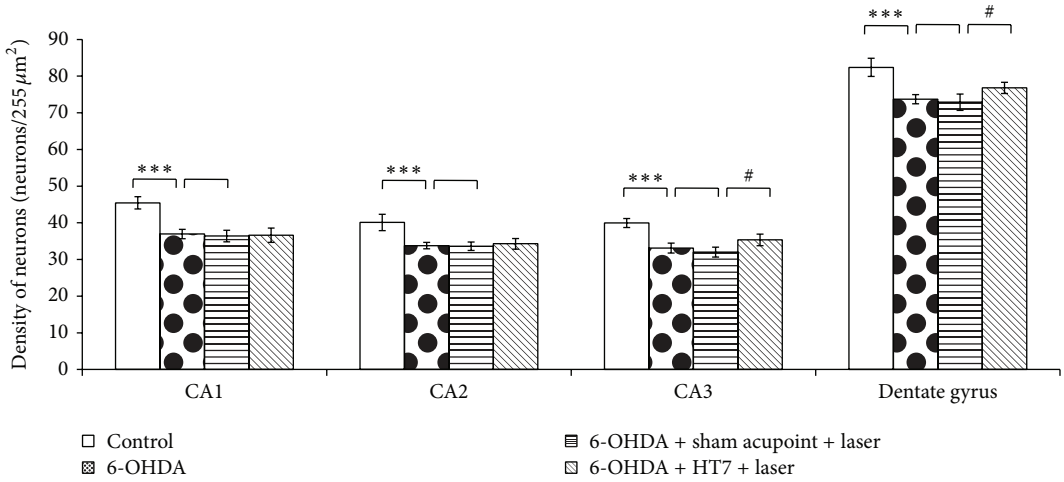

Figure 2: Effect of laser acupuncture on the neurons density in various subregions of hippocampus after treatments. Values given are the mean ± S.D. ($n = 6$) $^{***}P < 0.001$ as compared with control group and $^{\#}P < 0.05$ as compared with sham laser acupuncture group.

pathway. Therefore, the injected 6-OHDA might be transported into the nerve terminals in the area of both substantia nigra and ventral tegmental area and induced the disturbance of hippocampus via the mesolimbic connection mentioned earlier [6]. However, the precise underlying mechanism is still unclearly known and required further investigation.

Laser acupuncture at HT7 is demonstrated to suppress AChE in hippocampus together with the improved memory impairment in animal model of Alzheimer's disease [37]. Our findings are also in agreement with this study; the stimulation of HT7 acupoint can produce significant suppression of AChE and memory improvement even in animal model of PD. In addition to the suppression of AChE, the suppression of MAO-B in hippocampus was also observed in this study. It has been reported that laser beam could suppress MAO-B in erythrocyte of patients attacked with PD [38]. Although, laser can suppress MAO-B, sham laser acupuncture failed to suppress this enzyme activity in hippocampus. Therefore, the suppression MAO-B observed in this study might be associated with the stimulation of HT7.

In this study, the decreased MDA together with the increased GSH-Px enzyme activity induced by laser acupuncture at HT7 was observed. The discrepancy between our findings and the previous findings of Sutalangka et al. [37] which showed that no significant change of MDA was observed even the elevations of SOD and CAT were presented might be due to the different conditions of animal. Our study focused on the hypodopaminergic function induced by 6-OHDA whereas the previous study focused on the hypocholinergic condition induced by cholinotoxin, AF64A. Therefore, these data suggested that the effect of the stimulation of meridian and laser beam was varied depending on the pathological condition.

Both dopamine and cholinergic are reported to play a crucial role on memory impairment in PD [39–41]. In addition, the impairment of spatial memory is associated with the degeneration of hippocampus [42, 43] which is under the influence of the elevation of oxidative stress [35, 36]. Therefore, we did suggest that laser acupuncture at HT7 improved memory impairment in animal model of PD via the suppression of AChE and MAO-B which in turn increased the function of cholinergic and dopaminergic systems. In addition it also decreased oxidative stress via the enhanced GSH-Px enzyme activity in hippocampus giving rise to the increased oxidative stress buffering capacity resulting in the decreased neurodegeneration in the mentioned area and finally improved memory impairment as shown in Figure 6.

5. Conclusion

This study clearly demonstrates that the stimulation at HT7 acupoint with laser beam, a noninvasive tool, successfully improves the disturbances of neurotransmitters especially ACh and DA and oxidative stress resulting in the improved

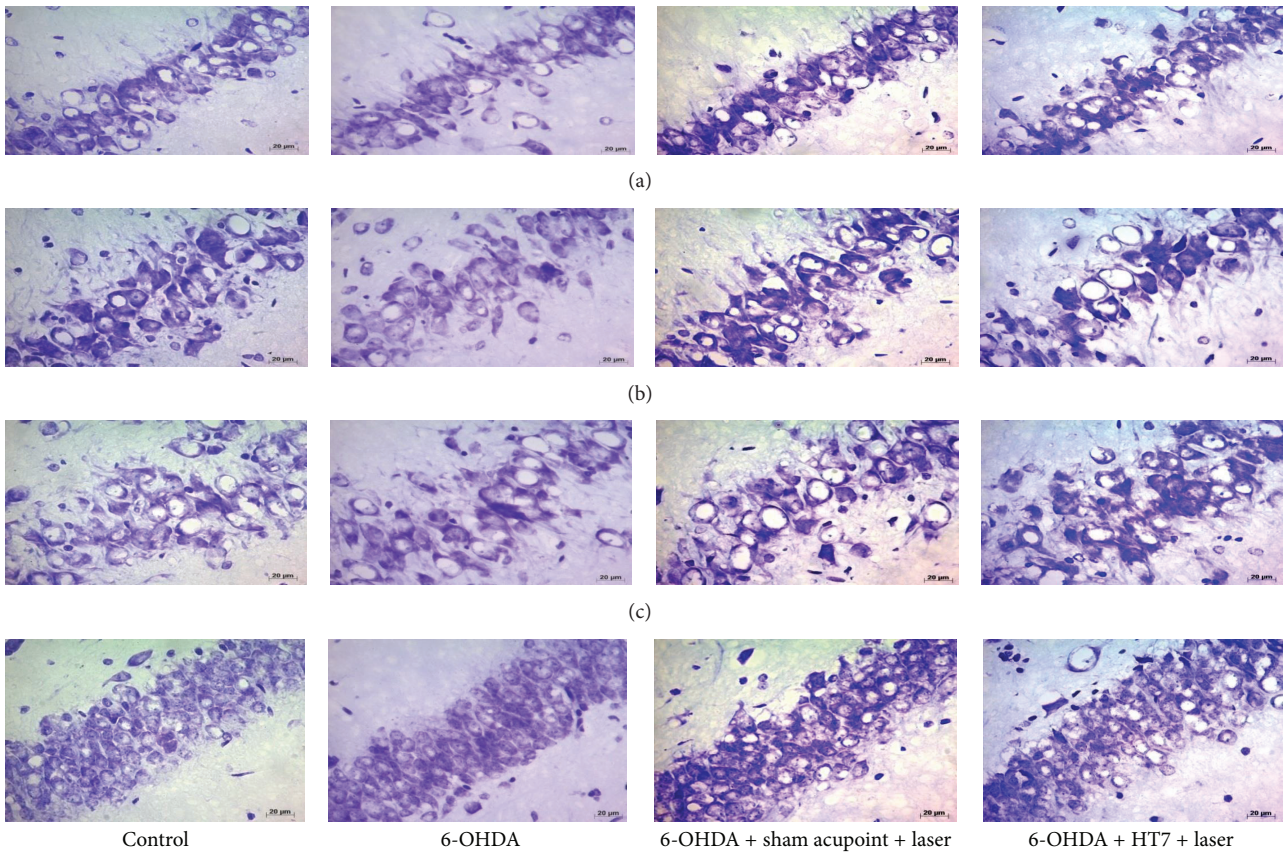

Figure 3: Photographic image of neurons with cresyl violet stained in various subregions of hippocampus. (a) CA1, (b) CA2, (c) CA3, and (d) dentate gyrus.

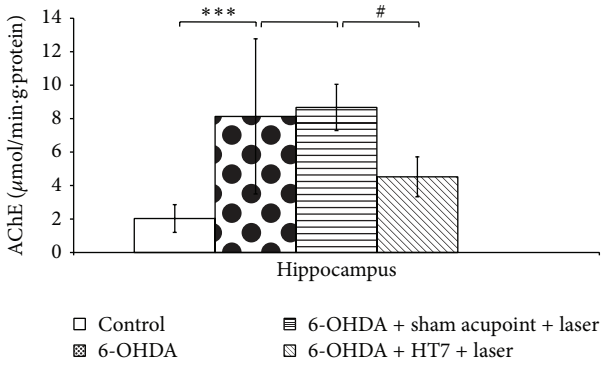

Figure 4: Effect of laser acupuncture on the activity of acetylcholinesterase (AChE) in the hippocampus. Values given are the mean ± S.D. ($n = 6$) $^{***}P < 0.001$ as compared with control group and $^{\#}P < 0.05$ as compared with sham laser acupuncture group.

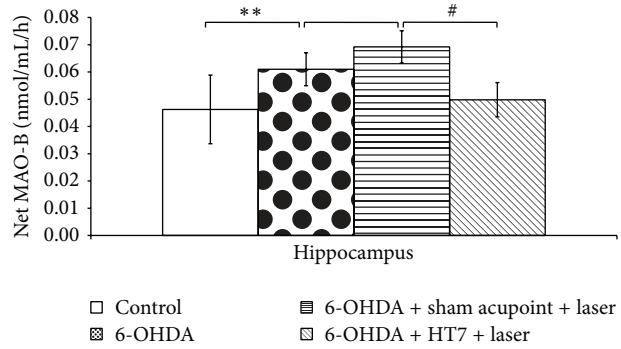

Figure 5: Effect of laser acupuncture on the activity of monoamine oxidase-B (MAO-B) in the hippocampus. Values given are the mean ± S.D. ($n = 6$) $^{**}P < 0.01$ as compared with control group and $^{\#}P < 0.05$ as compared with sham laser acupuncture group.

memory deficit in 6-OHDA lesion rats, a validated animal model of PD. Therefore, laser acupuncture may be a beneficial tool which is noninvasive for treating cognitive impairment in PD. The other benefit on motor symptoms of PD may also possible. However, further researches are necessary.

Abbreviation

PD: Parkinson's disease
6-OHDA: 6-Hydroxydopamine
SNpc: Substantia nigra par compacta
AChE: Acetylcholinesterase
MAO-B: Monoamine oxidase type B

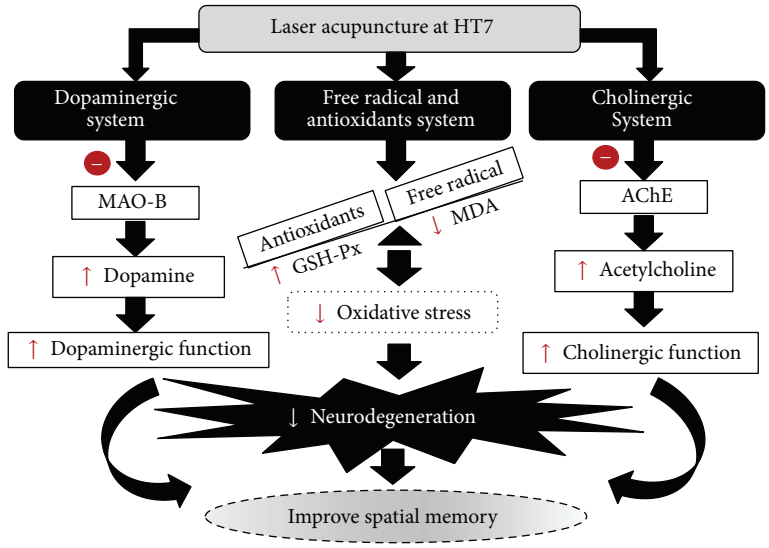

Figure 6: Schematic diagram illustrates the possible underlying mechanism of laser acupuncture at HT7 acupoint.

MDA: Malondialdehyde
SOD: Superoxide dismutase
CAT: Catalase
GSH-Px: Glutathione peroxidase
μ: Micro
NADPH: Nicotinamide adenine dinucleotide phosphate
u: Unit
H_2O_2: Hydrogen peroxide
M: Mole
m: Milli
ACh: Acetylcholine
DA: Dopamine.

Conflict of Interests

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

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Review Article

Acupuncture Mechanism and Redox Equilibrium

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Oxidative stress participates in the pathological process of various diseases. Acupuncture is a component of the health care system in China that can be traced back for at least 3000 years. Recently, increased evidences indicate that acupuncture stimulation could reduce oxidative damage in organisms under pathological state, but the exact mechanism remains unclear. This review focuses on the emerging links between acupuncture and redox modulation in various disorders, such as vascular dementia, Parkinson's disease, and hypertension, ranging from redox system, antioxidant system, anti-inflammatory system, and nervous system to signaling pathway. Although the molecular and cellular pathways studies of acupuncture effect on oxidative stress are preliminary, they represent an important step forward in the research of acupuncture antioxidative effect.

1. Introduction

Oxidative stress is defined as 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 and disease. Under physiological conditions, several related oxidative pathways contribute to ROS/RNS productions, while several intra- and extracellular antioxidant enzymatic systems account for ROS/RNS elimination [1]. Therefore, oxidative stress is a critical feature in the pathological process of various diseases. ROS/RNS is responsible for direct damage to cellular structures, while it also triggers a shift in the redox state of the biological compartment towards one that is more oxidizing [2, 3].

As one of the traditional oriental medicines, acupuncture has been widely used for more than 3000 years as a treatment for many diseases [4]. Recently, a large body of evidences demonstrated that acupuncture has antioxidative effect in various diseases [5–7], but the exact mechanism remains unclear. A literature review was conducted using Pubmed. Keywords were "acupuncture," "electric acupuncture (EA)," "acupoint," or "moxibustion" in combination with "antioxidative," "oxidative stress," "reactive oxygen species (ROS)," "reactive nitrogen species (RNS)," "redox," or "free radicals." The records were collected from December 2008 to present in each database. A total of 117 publications were identified as a result of the search which was related to acupuncture study and redox modulation. Eighty-four articles met the criteria; 79 of the articles in English and 5 articles in Chinese. In this review, the underlying mechanism of acupuncture-induced antioxidative effect is discussed based on the studies that have been published in the last 5 years. We will, in particular, focus on the antioxidative effect of acupuncture on (1) vascular dementia (VD); (2) Alzheimer's disease (AD); (3) Parkinson's disease (PD); and (4) hypertension.

2. Vascular Dementia

Changes in free radical generation and consequent oxidative stress may have a role in the pathogenesis of ischemic lesions. It has now been well established that generation and accumulation of ROS are detrimental to cells *in vitro* and *in vivo* [8] and promote cell death [9]. VD-induced

damage of neural tissues has been proved to produce excessive ROS [10]. It has been reported that acupuncture could improve memory impairment in VD patient [11]. The cognitive enhancing effect of acupuncture is likely to be at least partially attributable to decreased oxidative stress [12]. Shi et al. [13] found that oxidative stress marker 8-hydroxydeoxyguanosine (8-OHdG) increased significantly in the urine of VD patients. Meanwhile, the content of 8-OHdG could be decreased and cognitive function and quality of life were improved after acupuncture treatment [14]. Experimental studies reported that EA could effectively attenuate lipid peroxidation and malondialdehyde (MDA) content through increasing the antioxidant enzyme activities, such as superoxide dismutase (SOD) and glutathione peroxidases (GSH-Px), in hippocampal CA1 of VD rats [15, 16]. Consisting in these results, Liu et al. [7, 17] found that acupuncture could improve memory impairment through increasing antioxidant system ability, especially the expressions of CuZnSOD, and redox effector factor (Ref-1) in the hippocampus of VD rats. Furthermore, Zhang et al. [18] found that acupuncture's improvement of cognitive abilities was contributed to elevate MnSOD activities and the ratio of reduced glutathione (GSH) and oxidized glutathione (GSSG) in mitochondria in MID rats. These observations indicate that acupuncture-induced antioxidative effect may be related to GSH system and antioxidant enzyme in hippocampus which is crucial to learning and memory formation in vascular dementia patients and models.

Besides GSH system, a huge production of ROS during ischemia reperfusion alters a properly balanced thiol-redox environment, resulting in the oxidation of protein thiols of some enzymes and a loss of their normal biological activities [19, 20]. Thioredoxin (Trx) system, consisting of thioredoxin reductase (TR), Trx, and Nicotinamide Adenine Dinucleotide Phosphate (NADPH), could prevent susceptible proteins from this oxidative modification [21, 22]. Siu et al. [23] suggested that electroacupuncture (EA) treatment at *Zusanli* (ST36) could increase Trx expression in ischemic-reperfused brain tissues, which in turn increase the activity of antioxidase, shifting the intracellular more oxidative state to redox balance, and subsequently suppress ROS production. However, there are limited researches about the acupuncture effect on TR and NADPH, and further studies need to be performed.

During the subacute phase of ischemic brain injury (1–7 d after the onset of ischemia), astrocytes become activated and accumulate in the peri-infarct area, leading to glial scar formation. Complex neuron-glial interactions at high concentration could upregulate inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in glial cells, causing NO diffusion and neurotoxicity which exacerbate delayed infarct expansion and play a key pathological role in ischemic injury [24]. Increasing evidences have suggested that the neurotoxic effects of iNOS-derived NO could be attributed to its combination with the superoxide anion, leading to the formation of peroxynitrite, a strong oxidative/nitrative molecule that aggravates cerebral ischemia/reperfusion (I/R) injury [25]. EA could effectively downregulate astrocytic S100B expression to provide neuroprotection against delayed infarct expansion by modulating p38 MAP kinase-mediated NF-κB expression. These effects could subsequently reduce oxidative/nitrative stress and inhibit the TNF-α/TRADD/FADD/cleaved caspase-8/cleaved caspase-3 apoptotic pathway in the ischemic cortical penumbra 7 d after reperfusion [26]. This suggests that EA could reduce oxidative/nitrative stress and NF-κB-mediated inflammation during the later stages of cerebral I/R injury.

3. Alzheimer's Disease

AD is the most common form of dementia, which is characterized by the deposition of the amyloid β (Aβ) peptide and microtubule-associated protein tau in the brain [27, 28]. It has been proved that Aβ has capacity to interact with transition metals generating redox active ions, which precipitate in lipid peroxidation and cellular oxidative stress [29]. In other words, Aβ promotes cellular oxyradicals accumulation in neurons and glial cells in vulnerable regions of AD brain. Such oxidative stress may lead to many of the metabolic and neurodegenerative alterations observed in this disease. A variety of markers of oxidative stress are increased in postmortem brain tissues of AD patients, with a clear relationship with Aβ deposition and neurofibrillary degeneration [30]. It has been reported that the activity and/or protein levels of several antioxidant enzymes were altered in AD brain regions, consistent with ongoing oxidative stress [31].

Acupuncture has been reported to improve intelligence and ameliorate depression and anxiety in AD patients [32, 33]. This effect may be through decreasing the Aβ proteins level and increasing antioxidant system SOD and GSH-Px activities in the hippocampus of AD rats [34, 35]. APP transgenic mice study showed that EA stimulation at 2 Hz/100 Hz could significantly improve learning-memory capacity by reducing the expression of Aβ precursor protein and Aβ protein in the cerebral cortex and minimizing neuronal mitochondrial damages in hippocampal CA1 region [36]. Besides, EA at specific acupoints could improve the cognitive function of senescence-accelerated mouse (SAMP10) through reversing age-related protein and gene expression profiles, such as Hsp84, Hsp86, and YB-1, which are closely involved in oxidative stress-induced damage in the hippocampus [37]. Another research suggested that Choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities of hippocampal tissues were decreased in AD rat and acupuncture stimulation could reverse the decrease in both ChAT and AChE [38]. IPF2a, a sensitive and specific marker of lipid peroxidation, has been considered to be correlated with the cognitive functional impairment in AD patients [39]. The clinical research has reported that acupuncture stimulation could decrease iPF2a level in the cerebrospinal fluid, blood, and urine in AD patients [40].

Microglia is functionally polarized into different phenotypic activation states, referred as classical and alternative. The balance of the two phenotypes may be critical to ensure proper brain homeostasis and may be altered in brain pathological states of Alzheimer's disease. Inhibition of

NADPH oxidase or gene deletion of its functional p47phox subunit switched microglial activation from a classical to an alternative state in response to an inflammatory challenge, representing a promising neuroprotective approach to reduce oxidative stress and modulate microglial phenotype towards an alternative state [41]. Previous studies suggested that acupuncture could regulate microglial activation and attenuate oxidative stress in Limb ischemia reperfusion [42] and spinal cord injury [43], but that is not reported in Alzheimer's disease, which is worthy of further research. These observations suggest that the effects of acupuncture on learning-memory capacity in AD rats/patients may be related to neuronal mitochondrial integration, Aβ plaques, and acetylcholine neurotransmission.

4. Parkinson's Disease (PD)

Oxidative stress is thought to be one of the primary mechanisms behind the onset and progression of the neurodegeneration in PD [44], as highly neurotoxic free radicals are generated through the metabolism of dopamine and its own autooxidation [45]. Increased oxidative stress and mitochondrial dysfunction have been shown in PD patients. In particular, patients have disrupted iron (Fe) metabolism, as well as altered mitochondrial energetics, with a decrease in mitochondrial complex I levels and overall oxidative phosphorylation in the substantia nigra (SN) [46]. Moreover, depletion of the antioxidant glutathione (GSH) is also a prominent molecular consequence in PD, with several recent studies focusing on the potential therapeutic benefits of GSH administration [47].

Recent studies suggested acupuncture could attenuate oxidative stress and inhibit cell death in SN dopaminergic neurons [48]. The antioxidative effect of acupuncture is mediated by the activity of antioxidant system, inhibiting the production of H_2O_2 and MDA in 6-OHDA-lesioned rats or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse [49, 50]. Further study displayed that acupuncture could counteract MPTP-induced increase of oxidative stress-related proteins, such as cytosolic malate dehydrogenase, hydroxyacylglutathione hydrolase, and cytochrome c oxidase subunit Vb [51].

The iron redox system in SN may contribute to the vulnerability of dopaminergic neurons [52, 53]. Some reports have also shown that Fe3+ accumulated in the SN region of PD patients [54], MPTP-treated mice [55], and 6-OHDA-treated rats [56]. Acupuncture stimulation at *Taichong* (LR3) and *Yanglingquan* (GB34) acupoints for 15 days could attenuate MPTP-elicited dopaminergic neuronal degeneration through lowering levels of Fe3+ and ferritin-heavy chain, suggesting that acupuncture treatment could attenuate iron-related oxidative damage to dopaminergic neurons [57].

Phosphatidylinositol-3-kinase/protein kinase B (PI3K/Akt) signaling pathway is necessary for neuroprotection against MPTP-induced oxidative damage [58]. Acupuncture could restore the MPTP-induced impairment of Akt activation in SN dopaminergic neurons [59]. Furthermore, acupuncture-induced dopaminergic neuron protection and motor function improvement were significantly blocked by administration of LY294002, a specific inhibitor of PI3K/Akt signaling pathway [60]. These studies provide evidences that PI3 K/Akt signaling pathway may play an important role in the mechanism underlying acupuncture-induced neuroprotection in PD mouse.

Acupuncture has been proved to significantly reduce rotational motor deficit in Parkinson model through increasing expression of brain-derived neurotrophic factor (BDNF) receptor [61] and reducing TNF/IL-1 beta mRNAs and macrophages in the ventral midbrains [62]. These results concluded that neuroprotection by acupuncture treatment comes from the collaboration of its anti-inflammatory actions. Reactive microglia was present in the brains of Parkinson's disease [63]. It will be of interest to see whether acupuncture treatment prevents microglial activation for neuronal degeneration.

5. Hypertension

Oxidative stress in the central nervous system has an important role in the neural mechanisms of hypertension [64]. Brain stem ROS acts on the rostral ventrolateral medulla (RVLM) or nucleus tractus solitarii (NTS), augmenting central sympathetic outflow and suppressing baroreflex regulation of blood pressure [65]. Significance of the NADPH oxidase-derived ROS in pathogenesis of hypertension was comprehensively discussed in several recent reviews [66, 67]. In hypertension, neurohumoral stimuli such as Ang II, NE, and ET-1 activate receptors located on cell membrane, namely, AT1, α-AR, and ET receptors. The function of these receptors is coupled to G proteins, which activate the source of ROS, NADPH oxidase. The activated NADPH oxidase will produce ROS (e.g., O_2^-), and these, in turn, activate cell phosphorylation pathways: the mitogen-activated protein kinases (MAPKs), tyrosine kinases, and phosphoinositol-3-kinase/Akt kinase (PI3K/Akt). The activated phosphorylation pathways activate transcription factors, such as activated protein-1 (AP-1), p53, nuclear factor kappa B (NF-κB), and nuclear E2-related factor 2 (Nrf2), which stimulate transcription of genes after moving into nucleus. Proteins encoded by these target genes in turn mediate cellular consequences leading to changes in the phenotypes, such as hypertrophy, inflammation, necrosis, and apoptosis of cells and, on the other hand, stimulate the production of antioxidants involved in antioxidant defense [68].

Emerging evidences indicate that acupuncture could regulate blood pressure in hypertensive patients [69, 70]. The antihypertensive effect of acupuncture is related to the expression of different NOS, especially eNOS and iNOS in the RVLM of stress-induced hypertensive rats. Acupuncture could decrease blood pressure by increasing antioxidant enzymes, such as glutamate dehydrogenase 1, aldehyde dehydrogenase 2, glutathione S-transferase M5, and SOD in the medulla of the SHRs [71]. The antihypertensive effects of EA might be associated with the attenuation of apelin expression in the RVLM, exerting its anti-inflammatory effects, and then downregulated the apelin-induced oxidative stress [72]. Besides, an animal model of renal failure- (RF-) induced hypertension study showed that the antihypertensive

mechanism of EA may be related to the effects of oxidative stress on insulin-like growth factor-I (IGF-I), inducible nitric oxide synthase, heme oxygenase, and thiobarbituric acid-reactive substance expression [73]. These views suggest that antihypertensive effects of acupuncture may be mediated by antioxidant enzymes and anti-inflammatory effects, which could modulate the renal sympathetic nerve activity and nitric oxide levels, leading to decreased blood pressure.

6. Other Diseases

In fact, the antioxidative effect of acupuncture has been verified in other diseases. Hyperglycemia and oxidative damage were relieved after preventive acupuncture by reducing LPO level and enhancing SOD activity in the serum and the pancreas of streptozotocin-induced hyperglycemia rats [74]. More studies confirmed that EA exerts its antioxidative effect through inducing nNOS and iNOS expressions which are involved in NO signal transduction and increasing total NO concentration in hypercholesterolemia rats [75], acetylsalicylic acid-induced acute gastritis rats [76], and LPS-induced kidney injury rats [77].

Through detecting the activity of antioxidant system, many researches suggest that acupuncture could reduce oxidative damage in different organs and tissues, such as plasma and ovary [78], liver and kidneys [79], hypothalamus [80], and random skin flaps [81] in estradiol-induced inflammation and oxidative stress rats. The analgesic effect of acupuncture was mediated by inhibiting the production of superoxide anion (O_2^-) and ROS-induced p38MAPK and extracellular signal-regulated kinase (ERK) activation in microglia of spinal cord injury rats [82]. Furthermore, Moore and Roberts II [83] demonstrated that the removal of intracellular superoxide at acupoints may be an important process in reduced simple adiposity.

7. Conclusions

In conclusion, oxidative stress is an essential pathophysiological change of various diseases, but it serves as a potential treatment target. The above accumulating evidences demonstrates that acupuncture plays an antioxidant effect on these diseases. Through redox system, antioxidant system, anti-inflammatory system, nervous system, and signaling pathway, acupuncture could make the oxidative damage and antioxidant defense remain relatively constant redox state. However, the recent acupuncture researches about oxidative stress are sporadic and preliminary, and further thorough studies on possible antioxidative actions of acupuncture are highly recommended, especially the influence of acupuncture on signaling pathways. The corresponding research on new therapeutic targets may be helpful to our understanding about the mechanism of acupuncture.

Conflict of Interests

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

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Research Article

ERK1/2-Egr-1 Signaling Pathway-Mediated Protective Effects of Electroacupuncture in a Mouse Model of Myocardial Ischemia-Reperfusion

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Early growth response- (Egr-) 1 is an upstream master switch in controlling inflammatory responses following myocardial ischemia-reperfusion (I/R). Activation of extracellular signal-regulated protein kinase-1 and kinase-2 (ERK1/2) signaling is known to upregulate Egr-1. ERK1/2 pathway has been previously shown to mediate the therapeutic action of electroacupucture (EA). Thus, we hypothesized that EA would reduce myocardial I/R injury and inflammatory responses through inhibiting Egr-1 expression via the ERK1/2 pathway. Mice were pretreated with EA, U0126, or combination of EA and U0126 and then underwent 1 h myocardial ischemia and 3 h reperfusion. We investigated that EA significantly attenuated the I/R-induced upregulation of both Egr-1 and phosporylated-ERK1/2 (p-ERK1/2), decreased myocardial inflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and reduced the infarct size and the release of cardiac troponin I (cTnI). U0126 treatment also exhibited the same effect as EA on Egr-1 level and subsequent cardioprotective effects. There was no additive effect of cotreatment with EA and U0126 on the expression of Egr-1 and its downstream target genes (TNF-α, IL-1β) or serum cTnI level. Collectively, these observations suggested that EA attenuates myocardial I/R injury, possibly through inhibiting the ERK1/2-Egr-1 signaling pathway and reducing the release of proinflammatory cytokines.

1. Introduction

Acupuncture is a therapeutic technique that originated in China more than five thousand years ago [1]. Comparing with traditional manual acupuncture, electroacupuncture (EA) is more repeatable and adjustable. Accumulating evidences from experimental studies indicated that EA at selected acupoints [e.g., Neiguan (PC6)] can reduce myocardial ischemia-reperfusion (I/R) injury, as reflected by reducing release of myocardial enzyme such as cTnI and creatine phosphokinase (CPK) [2, 3], attenuating the frequency and severity of arrhythmias [4, 5] and decreasing infarct size [6–8]. More importantly, the beneficial effects of EA have also been observed in clinical settings, where it has resulted in reduced cTnI release, decreased C-reactive protein level, and shorter intensive care unit stay in both adult and pediatric patients receiving heart surgeries [2, 9]. Despite these visible benefits of EA, the underlying molecular mechanisms of EA-mediated cardiac protection remain unclear.

Early growth response- (Egr-) 1, a transcription factor, has been shown to be upregulated in the heart [10, 11] and initiate inflammation following I/R [12]. Using an Egr-1 antisense oligodeoxyribonucleotide or a catalytic deoxyribonucleic acid molecule (DNAzyme) to inhibit Egr-1 has been shown to reduce myocardial inflammation and protect heart function against the I/R [11, 13]. Studies using other disease models have also pointed to the regulation of Egr-1 by the ERK1/2 pathway. In lung I/R, Egr-1 requires the

activation of ERK1/2 to exert its proinflammatory effects [14]. Upregulation of Egr-1 via the ERK1/2 pathway also contributes to the damage to pulmonary artery smooth muscle cells in a model of chronic hypoxia [15]. Recently, the ERK1/2 pathway has been implicated in the therapeutic effects of EA. It is reported that EA at PC6 acupoints alleviates cardiac hypertrophy after myocardial infarction by inhibiting the activation of ERK1/2 pathway [16]. Collectively, it suggests a link among EA, Egr-1, and ERK1/2 signaling pathway. Thus, we tested the hypothesis that EA attenuates myocardial I/R injury by inhibiting the ERK1/2-Egr-1 pathway.

2. Materials and Methods

2.1. Ethics Statements. All experimental protocols were approved by Animal Care Committee of Shanghai Jiao Tong University, Shanghai, China. All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). For the experiments described here, we used a total of 153 male C57BL6 mice (8–10 weeks of age) from Sino-British SIPPR/BK Lab Animals (Shanghai, China). Animals were maintained under a 12/12 h light/dark cycle at 24°C ± 1°C, with unrestricted access to standard food and water.

2.2. Experiment Design. The hypothesis schematic diagram and study protocol diagram are depicted in Figures S1 and S2 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/253075. Three sets of experiments were conducted to investigate whether the ERK1/2-Egr-1 pathway is involved in the protective effects of EA against myocardial I/R injury. In experiment 1, thirty mice were used to examine the temporal profile of Egr-1 and p-ERK1/2 after 1 h ischemia followed by reperfusion of varying lengths of time (0, 3, 6, and 24 h; $n = 6$ for each time point). Based on the maximal expression of both Egr-1 and ERK1/2 observed in this experiment, reperfusion duration was set at 3 h for subsequent experiments (except for infarct size determination). Then we evaluated the effects of EA on Egr-1 and ERK1/2 expression during myocardial I/R, and mice were exposed to sham surgery (SHAM), I/R alone (IR), or EA prior to I/R (EA + IR, EA was performed 30 min prior to the surgery and lasted until the start of surgery) ($n = 12$/group). In experiment 2, the effects of U0126 [a highly selective inhibitor of ERK kinase; Cell Signaling Technology, Danvers, USA; 20 mg/kg, 1 h prior to the surgery, i.p. [17]] were compared to vehicle treatment (0.1% v/v DMSO) as well as I/R alone ($n = 12$/group). In experiment 3, mice received EA, U0126, or both treatments prior to the surgery ($n = 9$/group). Areas at risk (AAR) from the left ventricles (LV) were collected to measure the content of Egr-1, p-ERK1/2, and ERK1/2 using western blot, real-time PCR, and immunohistochemical staining. Myocardial levels of TNF-α and IL-1β were also measured. Serum was collected for the cTnI assay. Six mice per group were used to determine infarct size at 24 h after reperfusion.

2.3. Myocardial I/R Injury. Mice were anesthetized with 2% isoflurane (in 100% oxygen) under artificial ventilation using a rodent ventilator (Kent Scientific Co., Torrington, Connecticut, USA). The adequacy of anesthesia was verified using tail pinch. The heart was exposed at the fourth intercostal space, and the left anterior descending coronary artery (LAD) was occluded transiently using a 6–0 suture and reperfused for varying duration as described previously [18]. The incision was closed after the procedure, and the mice were allowed to recover from the anesthesia. Before the mice were sacrificed, AAR from the LV and blood from the inferior vena cava were collected for biochemical analyses. The body temperature was maintained at 37°C throughout the study.

2.4. Electroacupuncture. EA was delivered to PC6 acupoints bilaterally, at 1 mm above the wrist joint between the radius and ulna on the ventral surface of the forelimb [19]. Briefly, stainless needles were inserted to a depth of 3 mm and secured using plastic adhesive tape. Electrical stimulation (current of 1 mA, alternating dense and disperse mode, 2 Hz [0.6-ms pulse width] versus 100 Hz stimulation [0.2-ms pulse width], each lasting for 3 s) was delivered using an electrical stimulation device (HANS LH-202, Huawei Co., Beijing, China) for 30 min [20].

2.5. Determination of Infarct Size. Infarct size was evaluated by Evans blue and triphenyltetrazolium chloride (TTC) (Sigma-Aldrich Co., St. Louis, MO, USA) staining as described previously [21]. Briefly, LAD was religated at 24 h after the reperfusion followed by injection of 2% Evans blue into the aortic arch. The heart was sliced transversely into five blocks of equal thickness, incubated in 1% TTC for 15 min at 37°C, and fixed in 10% formalin overnight. Images were digitally captured using a microscope (DFC500, LEICA, Solms, Germany) and a digital camera (C-DSD230, Nikon, Tokyo, Japan). The LV area, AAR and infarct area (IA) were determined with planimetry software (Image J; National Institute of Health, Bethesda, USA) and adjusted for the weight. The ratio of AAR to LV area and IA to AAR was calculated.

2.6. Western Blot Analysis. The LV was frozen in liquid nitrogen and stored at −80°C until homogenization on ice using an EDTA-free buffer containing protease inhibitor cocktail (Roche Ltd., Basel, Switzerland). The homogenate was centrifuged for 15 min at 12,000 rpm at 4°C. Protein concentration of the supernatant was determined using a BCA kit (Thermo Scientific, Middletown, USA). Equal amounts of protein (40 μg) were fractionated in 12% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk and 0.01% Tween-20 in Tris-buffered saline (TBS) at pH 7.6 prior to incubation with monoclonal antibodies against Egr-1, p-ERK1/2, ERK1/2 (Cell Signaling Technology, Danvers, USA), or GAPDH (Kangcheng, Shanghai, China) at 4°C overnight. After incubation with an anti-rabbit secondary antibody (Biotime, Shanghai, China), the bands were visualized and analyzed using a BIO-RAD system (Molecular

Imager ChemiDoc XPS+, Hercules, USA). GAPDH was used as an internal control.

2.7. Quantitative Real-Time Polymerase Chain Reaction. Egr-1 mRNA in the LV was measured using quantitative real-time polymerase chain reaction (qRT-PCR) with SYBER Premix Ex Taq and Primescript RT reagent Kit (Takara, Otsu, Japan) and expressed as $2^{-\Delta\Delta Ct}$ (relative fold change). GAPDH was used as an internal control. The primers for Egr-1 were forward 5′-GCCTTAAGGGGGTAGGAGTG-3′ and reverse 5′-CCTCTTCCTCATCGTGCTCT-3′. The primers for GAPDH were forward 5′-GGTTGTCTCCTGCGACTTC-3′ and reverse 5′-CCTGTTGCTGTAGCCGTATTCAT-3′. The PCR was conducted using a standard cycle: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds.

2.8. Immunohistochemical Staining. The LV was fixed in 4% paraformaldehyde and embedded in paraffin for sectioning into 4 μm sections. After antigen retrieval, the sections were incubated with a primary antibody against Egr-1 followed by incubation with a biotin-conjugated secondary antibody and color reaction with avidin-peroxidase (ABC kit) and a DAB substrate kit (Vector Laboratories, Burlingame, USA). The sections were counterstained with hematoxylin. Images were digitized using a microscope (BX-51; Olympus, Tokyo, Japan) and analyzed using Image J software. The measurement was performed by two researchers blinded to the treatment condition.

2.9. ELISA. TNF-α and IL-1β in myocardial homogenate were examined using ELISA reagent kits (R&D systems, Minneapolis, USA). Serum cTnI was determined using an ELISA kit (Life Diagnostics, West Chester, USA).

2.10. Statistical Analysis. Data are expressed as the mean ± SEM and statistically analyzed by the Statistical Package for the Social Sciences (SPSS, Version 16.0; SPSS Inc., Chicago, USA). Independent Student's t-test was used when comparing the infarct size between IR and EA + IR group. All the other data were analyzed with one-way ANOVA followed by Turkey's post-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. EA Pretreatment Inhibited Myocardial Egr-1 and p-ERK1/2 Expression, Decreased Inflammatory Cytokines, and Reduced Infarct Size. Varying reperfusion time points demonstrated changes in mRNA and protein levels of Egr-1 paralleled that of p-ERK1/2. Nearly all time points exhibiting significant increases from Sham controls and peak levels were observed after 3 h of reperfusion ($P < 0.001$ versus SHAM; Figures 1(a)–1(d)). Using 3 h of reperfusion, we tested the effects of EA on myocardial Egr-1 and p-ERK1/2 levels. EA treatment significantly attenuated the I/R-induced increase in Egr-1 ($P = 0.036$ for protein, $P < 0.001$ for mRNA; EA + IR versus IR) and p-ERK1/2 ($P = 0.030$ for p-ERK1, $P = 0.037$ for p-ERK2; EA + IR versus IR; Figures 2(a)–2(d)). Immunohistochemical staining also revealed that EA attenuated Egr-1 expression in response to I/R (Figures 2(e)-2(f)). In addition, the infarct size (IA/AAR) is significantly smaller in EA pretreatment group than the I/R alone group (28.15 ± 1.59% versus 42.64 ± 2.83%, $P = 0.019$; Figures 3(a)-3(b)). EA decreased the cTnI release in the serum (23.16 ± 1.25 versus 13.03 ± 1.89 ng/mL for I/R controls, $P = 0.006$; Figure 3(c)) and led to a reduction in myocardial TNF-α (14.66 ± 1.67 versus 9.78 ± 1.19 pg/mL for I/R controls, $P = 0.043$, Figure 3(d)) and IL-1β levels (18.62 ± 1.95 versus 12.97 ± 1.18 pg/mL for I/R controls, $P = 0.035$, Figure 3(e)). These findings indicated a protective effect of EA against the myocardial damage and inflammatory injury.

3.2. Activation of the ERK1/2 Pathway Is Responsible for Egr-1 Upregulation and Myocardial Injury following I/R. In order to determine the effects of ERK1/2 inhibition on I/R injury, we used U0126 to block the activation of ERK1/2 in animals undergoing I/R surgery. U0126 treatment significantly inhibited p-ERK1/2 ($P = 0.003$, U0126 + IR versus DMSO + IR; Figures 4(a) and 4(c)). Additionally, U0126 treatment significantly decreased both Egr-1 protein ($P = 0.026$) and mRNA levels ($P < 0.001$, U0126 + IR versus DMSO + IR; Figures 4(a), 4(b), and 4(d)). Immunohistochemical analysis revealed fewer myocardial Egr-1-positive cells in mice receiving U0126 in comparison to the vehicle control (Figures 4(e)-4(f)). In addition, the infarct size (IA/AAR) in the U0126 + IR group was significantly reduced when compared with the DMSO + IR group ($P = 0.029$, 23.62 ± 1.43% versus 41.03 ± 2.03%; Figures 5(a)-5(b)) as well as the serum cTnI levels ($P = 0.034$, 23.24 ± 0.74 versus 17.67 ± 2.33 ng/mL; Figure 5(c)). The myocardial TNF-α and IL-1β levels in U0126 + IR group were attenuated when compared with DMSO + IR group ($P = 0.035$ for TNF-α: 13.66 ± 1.56 versus 9.65 ± 0.94 pg/mL; $P = 0.043$for IL-1β: 17.62 ± 1.59 versus 12.97 ± 0.54 pg/mL; Figures 5(d)-5(e)).

3.3. Combination of EA and ERK1/2 Inhibition Did Not Further Enhance Cardiac Protection against I/R Injury. As shown previously, both EA and U0126 treatment had favorable effects on myocardial damage and inflammation processes when given separately. However, effects of combined treatment of EA and U0126 did not differ from either EA or U0126 alone when comparing myocardial Egr-1 and p-ERK1/2 expression ($P > 0.05$; Figures 6(a)–6(c)). Additionally, EA + U0126 did not further affect the myocardial level of TNF-α, IL-1β, or serum cTnI levels in comparison to EA or U0126 alone ($P > 0.05$; Figures 6(d)–6(f)).

4. Discussion

The current study confirmed the cardiac protection of EA against I/R reflected by reduced infarct size and lower serum cTnI level, which is consistent with work done by our group and others [3, 22, 23]. Moreover, we demonstrated that EA at PC6 acupoints significantly attenuated I/R-induced upregulation of Egr-1 as well as ERK1/2 activation in the myocardium. Additionally, blocking ERK1/2 activation with

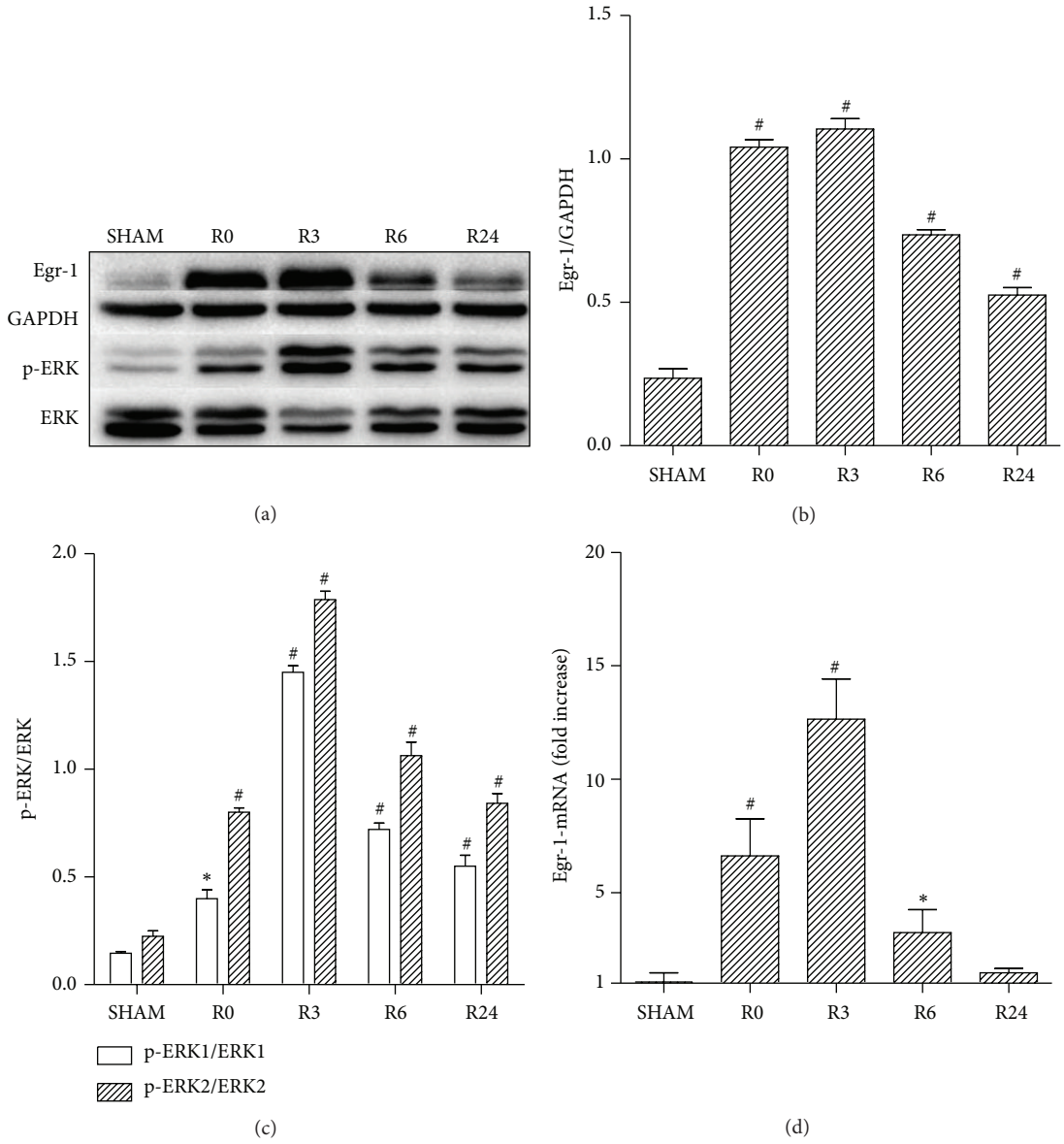

Figure 1: Comparison of the myocardial Egr-1and p-ERK1/2 expression at different time points after I/R. The protein levels of Egr-1and p-ERK1/2 at varying reperfusion time points (R0, R3, R6, and R24) were determined by western blot (a) and the corresponding densitometric analysis is shown in (b, c). Besides, the mRNA levels of Egr-1 were measured by qRT-PCR with data presented as relative fold increase versus sham control (d). For Egr-1 and p-ERK1/2, nearly all time points exhibit significant increase comparing with sham controls and peak levels are observed at R3 time point ($P < 0.001$, R3 versus SHAM). Six mice were sacrificed at each time points for protein and mRNA determination. R0 = ischemia for 1 h; R3 = ischemia for 1 h + reperfusion for 3 h; R6 = ischemia for 1 h + reperfusion for 6 h; R24 = ischemia for 1 h + reperfusion for 24 h. Data are presented as mean ± SEM; $^{*}P < 0.05$ and $^{\#}P < 0.01$ versus sham control.

U0126 elicited a significant decrease in Egr-1 expression and myocardial I/R injury. These data indicated that the cardiac protective effect of EA on myocardial I/R may be partially mediated by the attenuation of ERK1/2 activation and decrease in the downstream Egr-1expression in the myocardium.

Egr-1 is initially linked to the control of cell growth, survival, and transformation [24]. Recently, it has been implicated as a "master switch" in the injury response in a variety of models including vascular restenosis as well as I/R injury of many organs such as lung, heart, gut, and kidney [14, 25–28]. However, the upstream components capable of activating Egr-1 in myocardial I/R is still unclear. In a mouse lung I/R model, the ERK1/2 pathway was demonstrated to trigger Egr-1 expression and subsequent inflammatory damage [14]. In the current study, the change in Egr-1 after I/R parallels that of p-ERK1/2 at both mRNA and protein levels. After blocking the activation of ERK1/2 with U0126, the Egr-1 expression was significantly reduced and the myocardial injury was attenuated as well. Thus, our findings suggest that

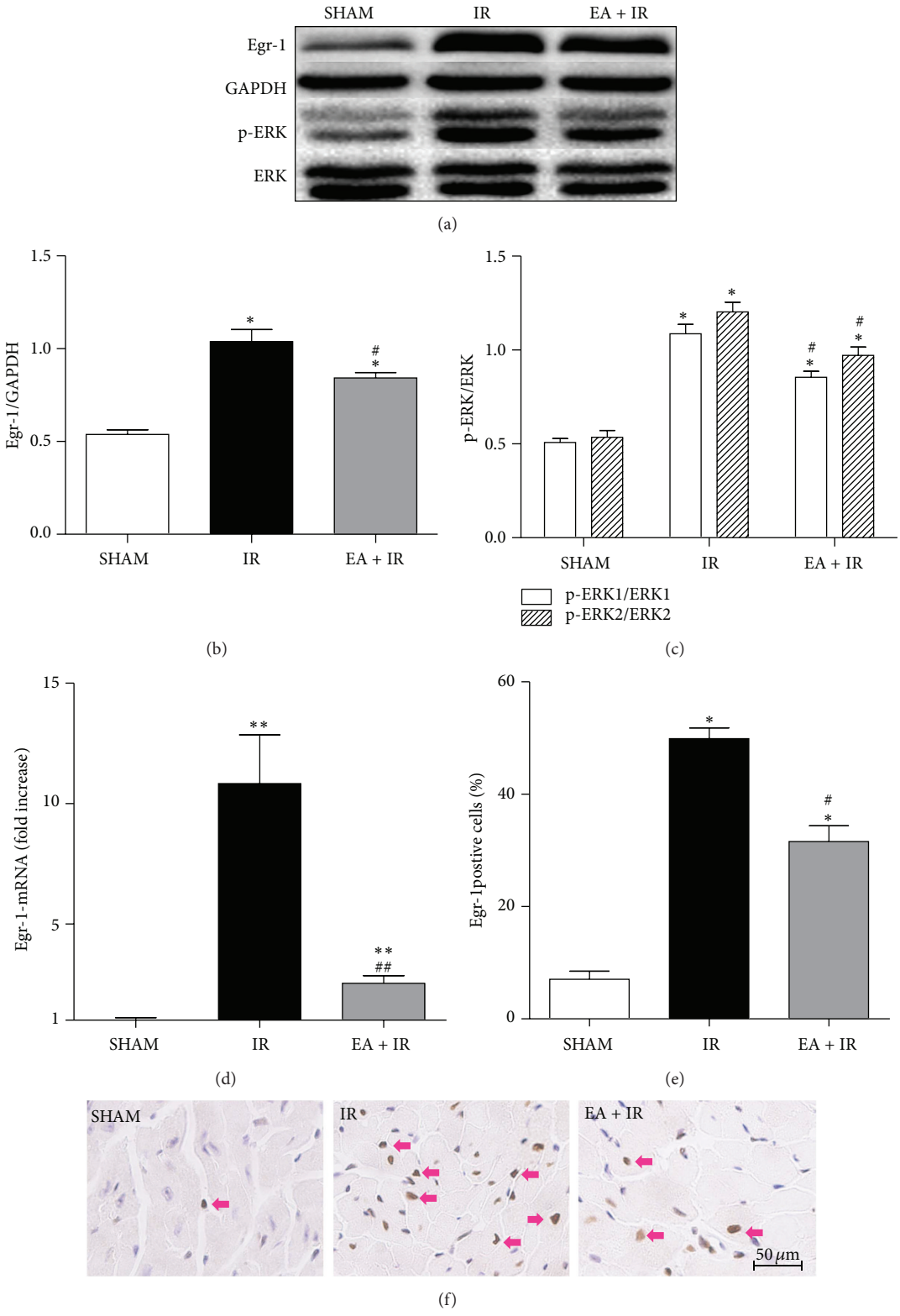

FIGURE 2: EA inhibited Egr-1 expression and ERK1/2 activation in myocardium undergoing myocardial I/R. Mice were divided into 3 groups: SHAM, IR (myocardial I/R), and EA + IR (EA stimulation was performed 30 min before myocardial I/R surgery and lasted for 30 min). After 3 h of reperfusion, the animals were sacrificed and the protein levels of Egr-1and p-ERK1/2 were measured by western blot (a) and densitometric analysis is shown in panel (b-c) (n = 3/group). The mRNA levels of Egr-1 in these three groups are represented as the relative fold increase versus sham controls (d, n = 3/group). Immunohistochemical staining of Egr-1 was performed and the quantitation results of Egr-1positive cells were shown in panel (e). Representative images were shown in panel (f). Pink arrows indicate Egr-1positive cells. Scale bar = 50 μm. $^{*}P < 0.05$ versus sham control; $^{\#}P < 0.05$ versus IR; $^{**}P < 0.01$ versus sham control; $^{\#\#}P < 0.01$ versus IR.

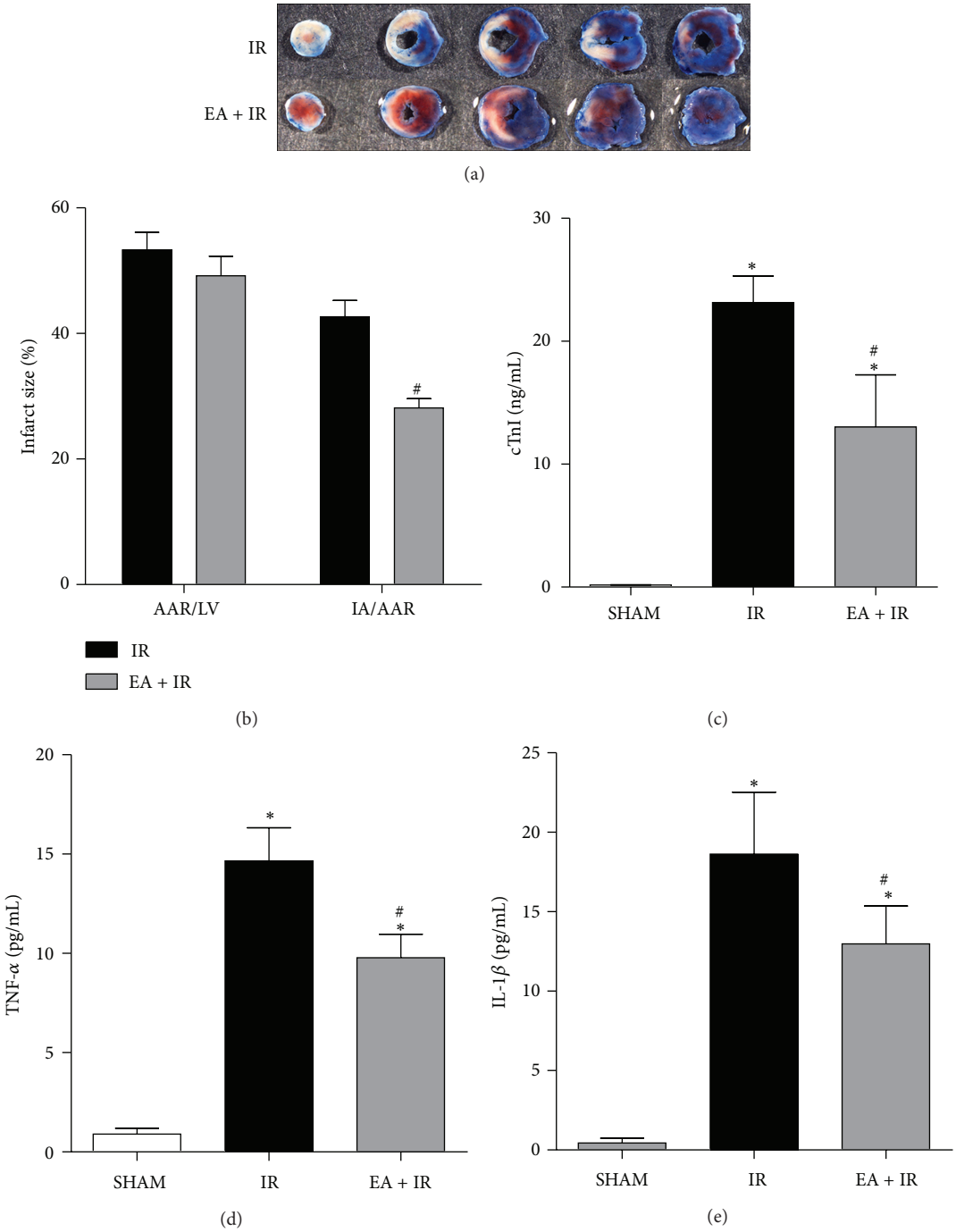

Figure 3: EA attenuated I/R-induced myocardial tissue damage and inflammatory response. After 24 h of reperfusion, Evans blue/TTC staining was applied to measure the infarct size ((a-b) $n = 6$/group). AAR/LV: area at risk/left ventricle area; IA/AAR: infarct area/area at risk. After 3 h of reperfusion, the serum cTnI level (c, $n = 6$/group) and the myocardial levels of TNF-α and IL-1β ((d-e), $n = 3$/group) were determined using ELISA. $^{*}P < 0.01$ versus sham control; $^{\#}P < 0.05$ versus IR alone.

ERK1/2 is an important upstream signal for modulating Egr-1 expression underlying myocardial ischemia stress.

Although ERK1/2 has been generally reported to be an important member of prosurvival kinases in ischemic preconditioning [29], its role in myocardial ischemia stress remains controversial. In both I/R-injured rat hearts and hypoxia-reoxygenation-injured cardiomyocytes, inhibiting ERK1/2 activation reverses the reactive oxygen species production and intracellular Ca^{2+} overload [30]. Furthermore, treating hypertrophic H9c2 cells with U0126 reduces the DNA fragmentation and nuclear condensation [31]. These findings are consistent with our data that blocking the activation of ERK1/2 with U0126 alleviates myocardial I/R injury.

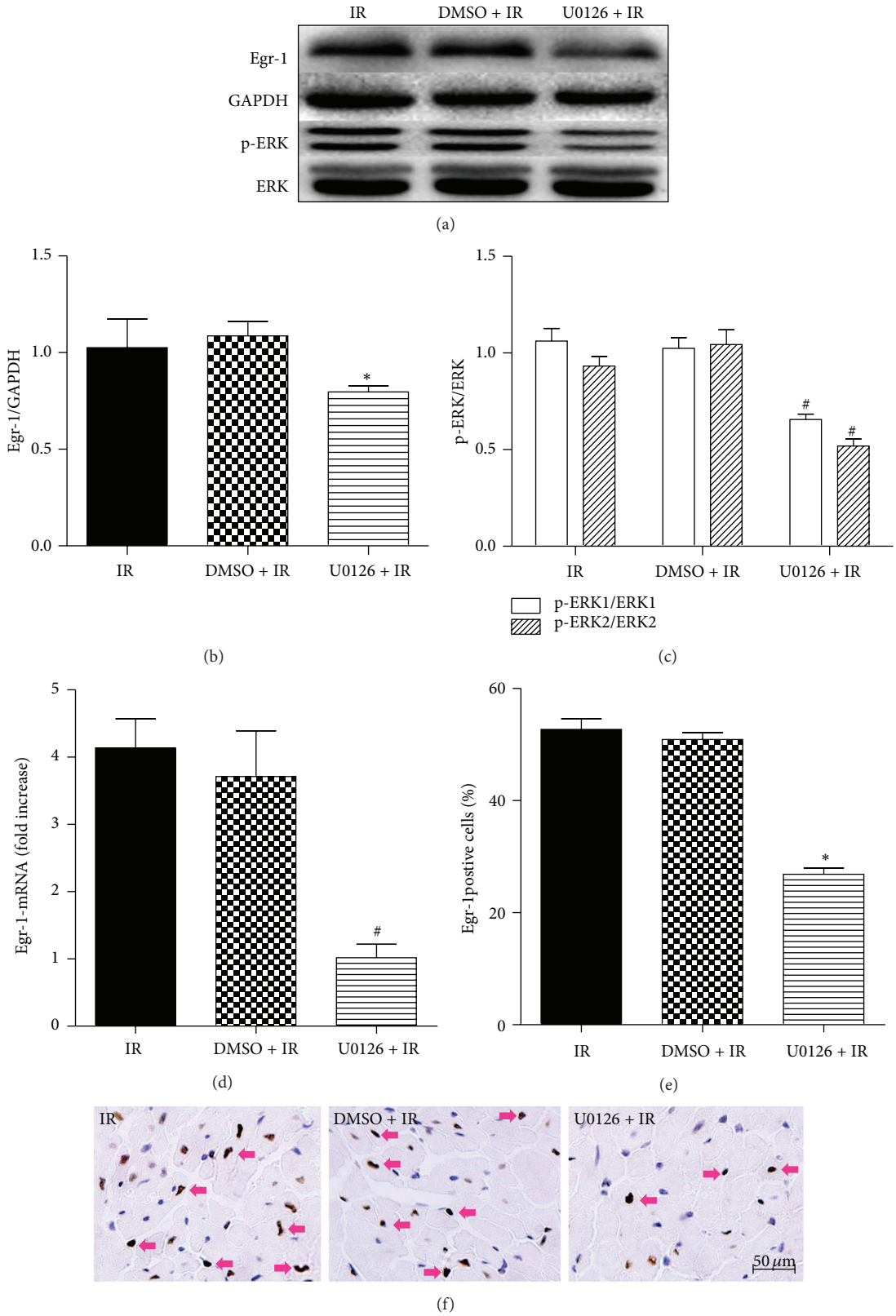

FIGURE 4: ERK1/2 activation is responsible for Egr-1expression during myocardial I/R injury. Mice received U0126 (an inhibitor of ERK1/2 kinase, 20 mg/kg, i.p.) or its vehicle 0.1% v/v DMSO treatment before surgery. As described previously, myocardial expression of Egr-1 and p-ERK1/2 was measured using western blot (a). The corresponding densitometric analysis is shown in (b-c) (n = 3/group). The mRNA levels of Egr-1 are shown as fold increase versus U0126 + IR (d, n = 3/group). Immunohistochemical staining of Egr-1 was performed and the quantitation results of Egr-1positive cells were shown in panel (e). Representative images were shown in panel (f) (3 mice /group). Pink arrows indicate Egr-1positive cells. Scale bar = 50 μm. $^{*}P < 0.05$ and $^{\#}P < 0.01$ versus DMSO + IR control.

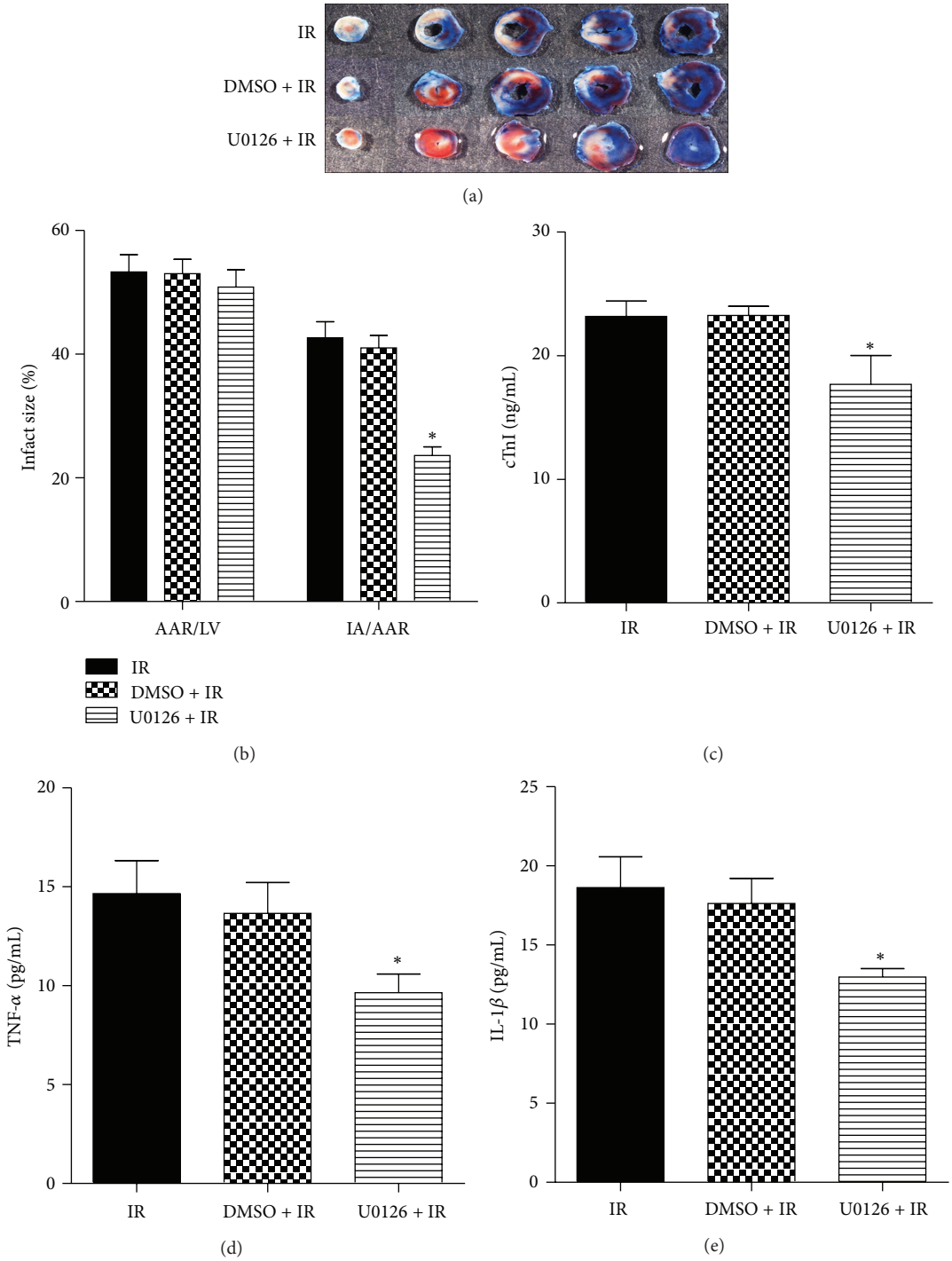

Figure 5: Inhibiting ERK1/2 activation with U0126 protected the myocardium against I/R injury. After 24 h of reperfusion, Evans blue/TTC staining was applied to measure the infarct size ((a-b) $n = 6$/group). AAR/LV: area at risk/left ventricle area; IA/AAR: infarct area/area at risk. After 3 h of reperfusion, the serum cTnI level (c, $n = 6$/group) and the myocardial levels of TNF-α and IL-1β ((d-e) $n = 3$/group) were determined using ELISA. $^*P < 0.05$ versus DMSO + IR.

EA can regulate the activation of ERK1/2 to produce anti-inflammatory [32] and analgesic effects [33, 34]. In a middle cerebral artery occlusion (MCAO) model, EA at either GV20 (Baihui) or DU26 (Renzhong) acupoints increases the phosphorylation of ERK1/2 in the ischemic cortex and hippocampus [35]. In contrast, in animal models of both chronic constriction injury (CCI) and cardiac hypertrophy [16, 33], EA reduced the expression of p-ERK1/2. Consistently, in the current study, we found the I/R-induced myocardial expression of both p-ERK1/2 and Egr-1 was downregulated by

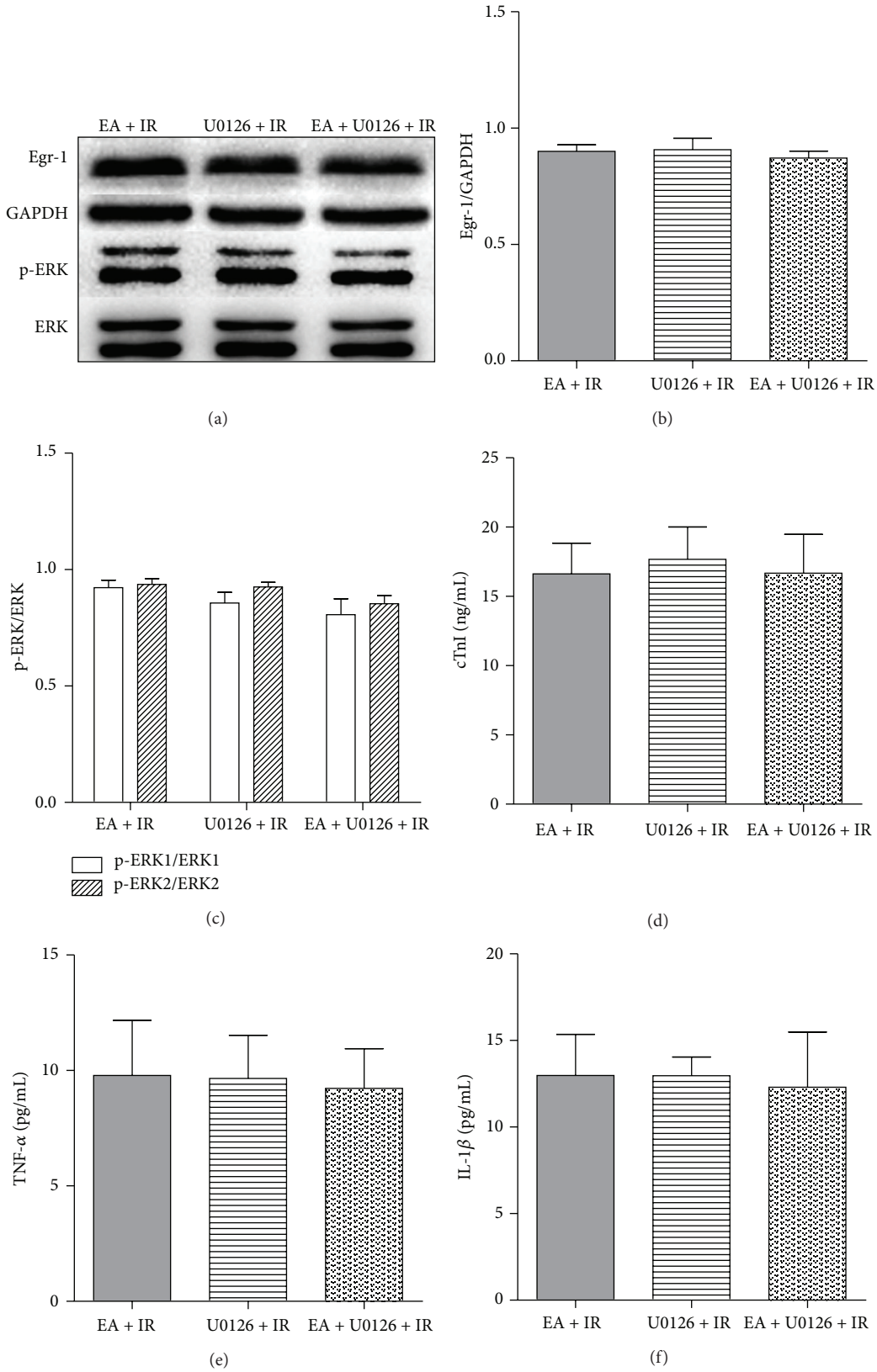

Figure 6: Combination of EA with ERK1/2 inhibitor did not produce more protection against myocardial I/R injury. Combination of EA and U0126 treatment was conducted to investigate the additive effects on ERK1/2/Egr-1 downregulation or the cardiac protective role. Western blot bands and corresponding densitometric analysis of Egr-1 and p-ERK1/2 are shown in (a–c) (n = 3/group). The myocardial levels of TNF-α and IL-1β ((d-e), n = 3/group) as well as the serum level of cTnI (f, n = 6/group) were determined.

EA at PC6 acupoints. Then we combined EA with the ERK1/2 inhibitor U0126 to investigate if they have an additive effect. However, the combined protocol did not further decrease p-ERK1/2 or Egr-1 expression or protect the myocardium. We speculate that ERK1/2-Egr-1 pathway might be a common target for both EA and U0126, and either EA or U0126 may have already exerted their maximal effects in our present experiment conditions.

In summary, EA treatment at PC6 (Neiguan) acupoints decreased infarct size, reduced the release of proinflammatory cytokines, and inhibited ERK1/2 activation and Egr-1expression in a mouse myocardial I/R injury model. The inhibition of ERK1/2-Egr-1signaling pathway may be, at least in part, responsible for the cardioprotective effects of EA against I/R injury.

Conflict of Interests

The authors declared no conflict of interests.

Authors' Contribution

Juan Zhang and Jiangang Song contributed equally.

Acknowledgments

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Research Article

Electroacupuncture Ameliorates Acute Lung Injury through Promoting Gastrointestinal Motility in Rats with Acute Pancreatitis

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Objective. Gastrointestinal disfunction and acute lung injury (ALI) were common in acute pancreatitis (AP). The effect of electroacupuncture (EA) on gastrointestinal motility and ALI in rats with AP was investigated to verify the theory of "lung and large intestine are interior exteriorly related" in traditional Chinese medicine. *Methods*. Male Sprague-Dawley rats were randomly divided into the normal group, model group, and EA group. AP model was established by three injections of 20% L-arginine at 1 h intervals. EA were applied to bilateral ST-25 and ST-36 for 30 minutes twice a day after modeling for 3 days. Arterial blood, pancreas, lung, and intestinal tissues were collected for detecting the inflammatory factors and histopathology. Intestinal propulsion rate (IPR) was also measured at 72 h. *Results*. EA treatment improved IPR and increased CCK-8 level compared with model group ($P < 0.05$). It lowered the serum levels of TNF-α and IL-6 and increased the level of IL-4 with no effect on IL-10. EA treatment reduced serum vasoactive intestinal peptide (VIP) and myeloperoxidase (MPO) level in the lung and the pathologic scores of pancreas, lung and intestine were decreased ($P < 0.05$). *Conclusion*. EA treatment could promote gastrointestinal motility through inhibiting VIP, and promoting CCK expression and regulate pro- and anti-inflammatory mediators to ameliorate ALI in AP.

1. Introduction

Acute pancreatitis (AP) is the inflammation of the pancreas with high morbidity and mortality. The overproduction of cytokines [1] and inflammatory mediators may account for systemic inflammatory response once the onset of disease of AP, which might cause multiple organ dysfunctions and/or failures if the inflammatory response was out of control, including the gastrointestinal dysfunction and acute lung injury (ALI) at the early stage [2]. Gastrointestinal dysfunction in AP, the trigger of multiple organ failure [1], was related to the intra-abdominal hypertension (IAH) and/or abdominal compartment syndrome (ACS). This commonly leads to ALI or acute respiratory distress syndrome (ARDS), which is difficult to manage up to date with high morbidity and mortality [3, 4]. The approaches to decrease IAH, sustain the intestinal barrier and gastrointestinal function would help inhibit the inflammatory response, enhance the blood SpO2, and improve the clinical results [5]. The optional decompression of ACS in a porcine model of AP incorporating IAH/ACS was associated with significantly reduced mortality, improved systemic hemodynamics, and organ function, as well as alleviated histologic injury and inflammatory intensity of the intestine and lung [6]. So, the decompression of IAH and the control of inflammation would ameliorate the severity of ALI in AP. Unfortunately, there is no specific treatment to control the inflammation and decease the IAH in AP except the invasive surgical decompression all over the world until today with high morbidity and mortality [6].

Traditional Chinese medicine has been adapted for AP for more than 30 years, including Chinese herbal formula and acupuncture. We found that the modified Chinese herbal

formula of Dachengqi decoction (DCQD) could relieve IAH and increase the oxygenation index significantly with shorter length of hospital stay for patients with severe AP [7]. Furthermore, DCQD could ameliorate ALI through decreasing IAH and inhibiting the inflammatory response in rats with AP [8]. As well as herbal formula, the effect of acupuncture on the inflammatory response and gastrointestinal motility was also explored. The electroacupuncture (EA) treatment could ameliorate the intestinal paralysis in patients with severe AP [9] and regulate the pro- and anti-inflammatory cytokines in rats with AP [10]. It was found that acupuncture could remarkably reduce the severity of ALI in rats with AP in the acute phase through suppressing the overexpression of serum macrophage inflammatory protein-2 (MIP-2) mRNA in the lung and large intestine tissues, lowering the level of serum MIP-2 [11].

Along these studies, we know that acupuncture could regulate the inflammatory response, promote the gastrointestinal motility, and ameliorate the lung injury in AP. However, it is still unclear whether acupuncture could ameliorate ALI through promoting gastrointestinal function and related inflammation based on the theory in traditional Chinese medicine of "Lung and Large intestinal exterior-interiorly related." The present study aimed to explore the effect of EA on ALI through regulating the gastrointestinal dysfunction and inflammatory response in rats with AP.

2. Material and Methods

2.1. Animal Experiment. Eighteen male SD rats of 160–200 g, 8–10 weeks old and clearing were obtained from the animal center of West China hospital of Sichuan University. They were randomly divided into normal group, model group, and EA group. The animal study was performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethics Committee for Animal Experiments of our hospital. Rats were intraperitoneally injected with 20% L-arginine (100 mg/100 g) at 1 h intervals for three times to induce AP [12].

2.2. EA Treatment Protocol. EA group rats were binding against slipping and turning. Bilateral ST-25 and ST-36 were pierced with acupuncture needles of type 32, 0.23 mm∗13 mm, and stimulated by SDZ-II-type EA treatment instrument (2 Hz/100 Hz, 2 mA) for 30 minutes twice a day after modeling for 2 days [13, 14]. Normal control group and model group were binding for the same time.

2.3. Sample Collection and Intestinal Propulsion Rate Measurement. Phenolsulfonphthalein 0.5 mL (2 mg/mL) was dosed to rats by intragastric infusion 30 min before scarification. Intestinal propulsion rate (IPR) was identified as ratio of the phenolsulfonphthalein promoting distance and the total length of the small intestine [15]. Rats were sacrificed 72 h after modeling and blood was obtained from heart. Atrial blood, pancreas, lung, and intestinal tissues were dissected immediately and collected for biomarkers and histopathology.

2.4. Serum and Tissue Measurement. Blood was centrifuged at 3000 rpm for 15 min and the serum was stored at −20°C. Serum CCK, VIP, TNF-α, IL-4, IL-6, and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA, Kits from Nanjing Jiancheng Bioengineering Institute). As previously described, the accumulation of neutrophils in the lungs was assessed by determination of myeloperoxidase (MPO) activity [16]. Briefly, the frozen tissue samples were thawed and suspended in 10% phosphate buffer (pH 6.0) containing 1% hexadecyltrimethylammonium bromide. The samples were sonicated on ice and centrifuged at 12000 rpm for 15 min at 4°C. An aliquot (30 μL) was transferred into 180 μL of phosphate buffer (pH6.0) containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (Sigma-Aldrich). The change in absorbance was read at 490 nm.

2.5. Pathological Assessment of Tissues. Parts of the tissues were fixed and embedded in paraffin wax for histological analysis. Pancreas, lung, and small intestine were scored by an experienced pathologist from West China Hospital of Sichuan University in a blinded fashion. The pathological scoring standard of pancreas includes edema, bleeding, inflammatory cell infiltration, and necrosis according to Schmidt's report [17].

2.6. Statistics. Statistical analysis was performed with the PEMS3.1 for Windows (Sichuan University, China). Data are expressed as mean ± SEM. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test for each paired experiment value of $P < 0.05$ which was considered to be significant.

3. Results

3.1. Electroacupuncture Treatment Regulates Acute Pancreatitis-Induced Serum CCK and VIP and Improves Intestinal Propulsion. As shown in Figure 1, IPR in model group was significantly slower than that in normal rats. After EA treatment, IPR in EA group was much faster than that in the model group (Figure 1). IPR in EA group and normal group was similar. This demonstrated that EA management could restore the intestinal motility affected in the AP.

3.2. EA Treatment Decreases Serum Level of CCK-8 and Increases VIP Level. As shown in Figure 2, the serum level of CCK in AP 72 h after modeling was obviously lower than that in normal rats. After 2 days treatment, CCK in EA group increased significantly, but it is still lower than that in the normal group, which meant that EA treatment could increase the serum level of CCK. To the contrary, the serum level of VIP in L-arginine induced AP rates was much higher than that in normal group. Compared to the model rats, 2 days of EA treatment significantly lowered the serum VIP (Figure 2). All these showed that EA could promote the

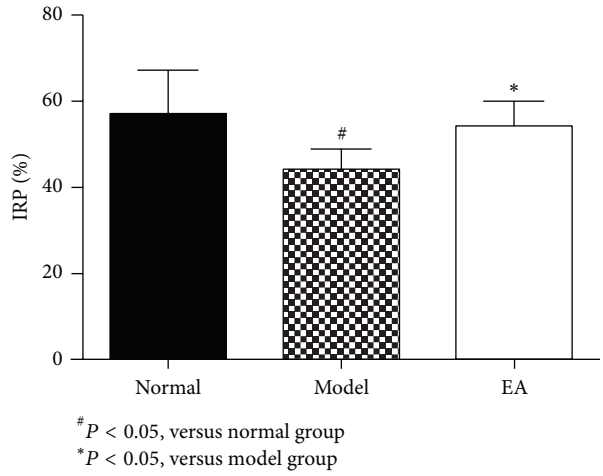

Figure 1: Comparison of intestinal propulsion rate (IRP) ($n = 6$).

intestinal motility through regulating the expression of CCK and VIP.

3.3. EA Treatment Attenuates Acute Pancreatitis-Induced Increases in Serum TNF-α, IL-6 and Increases IL-4. As shown in Figure 3, serum levels of proinflammatory TNF-α and IL-6 were increased (Figures 3(a) and 3(b)) and anti-inflammatory IL-4 and IL-10 were decreased after induction of AP in rates (Figures 3(c) and 3(d)). Compared to the model rats, EA could decrease the serum levels of TNF-α and IL-6 significantly after 2 days of treatment (Figures 3(a) and 3(b)). EA treatment could also increase IL-4 levels in rates with SAP significantly (Figure 3(c)), with no effect on IL-10 (Figure 3(d)). All these data displayed that EA treatment could regulate the inflammatory response in rats with AP via inhibiting the proinflammatory mediators and promoting the anti-inflammatory mediator.

3.4. EA Treatment Ameliorates Acute Pancreatitis-Induced Changes in Histopathology and MPO. As shown in Figure 4(a), MPO activities in the lung of AP model group were markedly higher than those in the normal group. The level of MPO in EA group was significantly lower than that in the model group after 2-day treatment in rats with AP. This showed that EA treatment could ameliorate ALI induced by AP, which is further identified by the pathological assessment.

Administration of intraperitoneally injections L-arginineat 1 h intervals for three times showed features of typical AP characterized by unclear pancreas acinar structure caused by moderate to severe interstitial edema, extensive inflammatory cell infiltration, parenchymal necrosis, and hemorrhage (Figure 5(h)) [12]. There were also marked pulmonary interstitial edema and inflammatory infiltration with alveolar collapse in the lungs (Figure 5(e)), which was similar to Dawra's research [18]. Moreover, the damage of intestinal mucosa was obvious, while the intestinal structural is relatively complete in the EA group (Figure 5(b)). After EA treatment, three groups of pathologic score ofpancreatic, lung, and intestinal tissues were much lower than model group (Figure 4(b)). All these data showed that 2-day EA treatment could improve the tissue pathological insult of intestine, lung, and at last pancreas.

In Figure 5: (a), (b), and (c) represent small intestine; (d), (e), and (f) represent lung tissue; (g), (h), and (i) represent pancreas tissue; (a), (d), and (g) represent normal group; (b), (e), (h) represent model group; (c), (f), and (i) represent EA group.

4. Discussion and Conclusion

The present study found that EA treatment could promote the intestinal propulsion rate through regulating the expression of CCK and VIP, decreasing the serum levels of TNF-α and IL-6, and increasing IL-4 levels in rates with SAP significantly after 2 days of treatment (Figures 3(a) and 3(b)). At the same time, two-day EA treatment could improve the tissue pathological injury of intestine, lung, and at last pancreas. It was concluded that EA could promote the intestinal motility and regulate the related inflammatory response, which lead to the amelioration of lung injury in rats with SAP.

Gastrointestinal dysfunction, the common clinical symptom in acute pancreatitis, can cause IAH and be the trigger of multiple organ failures [3, 4]. The first injured organ of lung commonly demonstrated with acute respiratory distress syndrome (ARDS), which is a typical index of the revised Atlanta classification for SAP. It is important to prevent the occurrence of ARDS in the early stage of acute pancreatitis, including the management of IAH and ACS with gastrointestinal dysfunction. In this study, the EA treatment was used to promote the intestinal motility, decrease IAH, and then ameliorate the related lung injury in SAP based on the theory of "lung and large intestine are interior exteriorly related" in traditional Chinese medicine. First, we identified that EA could promote the intestinal motility by increasing the IPR and regulating the expression of CCK and VIP. Recent studies found that disorder of gastrointestinal hormone such as CCK, VIP, and motilin (MTL) played an important role in gastrointestinal dysfunction [16, 19]. Blood CCK mainly comes from the intestinal secretory endothelial cells, promoting the contraction of the gallbladder and relaxation of the sphincter of Oddi and protecting the gastric mucosa [20]. All these showed that EA could promote the gastrointestinal motility via regulating the expression of related hormones.

Second, the injured intestinal tissue evoke and worsen the inflammatory response in SAP, including the bacterial translocation. The levels of proinflammatory mediators are elevated in the course of acute pancreatitis and are involved in the inflammatory cascade reaction to the pancreatic acinar cell damage, including TNF-α, IL-1, and IL-6 [21]. In this study, we found that EA increase the expression of IL-4 and inhibit the expression of TNF-α and IL-6 as well as increase the IPR in rats with SAP, which displayed that EA might regulate the inflammatory response while it promoted the intestinal motility. Former study reported that EA at ST 36 could downregulate the serums TNF-α and IL-6 in rates with sodium taurocholate-induced acute pancreatitis

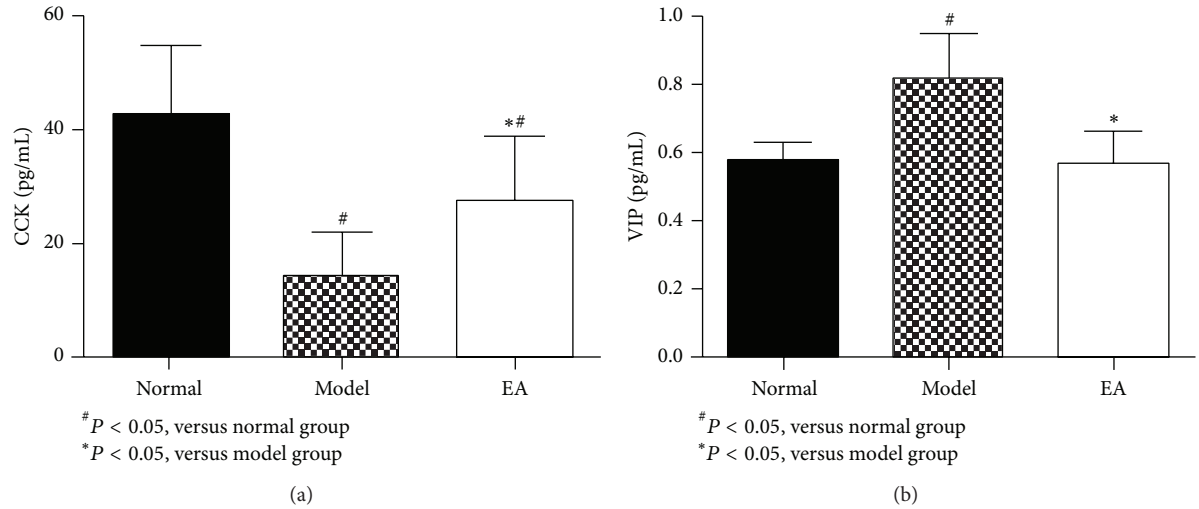

Figure 2: Comparison of serum CCK and VIP among rats of the three groups (mean ± SD) pg/mL.

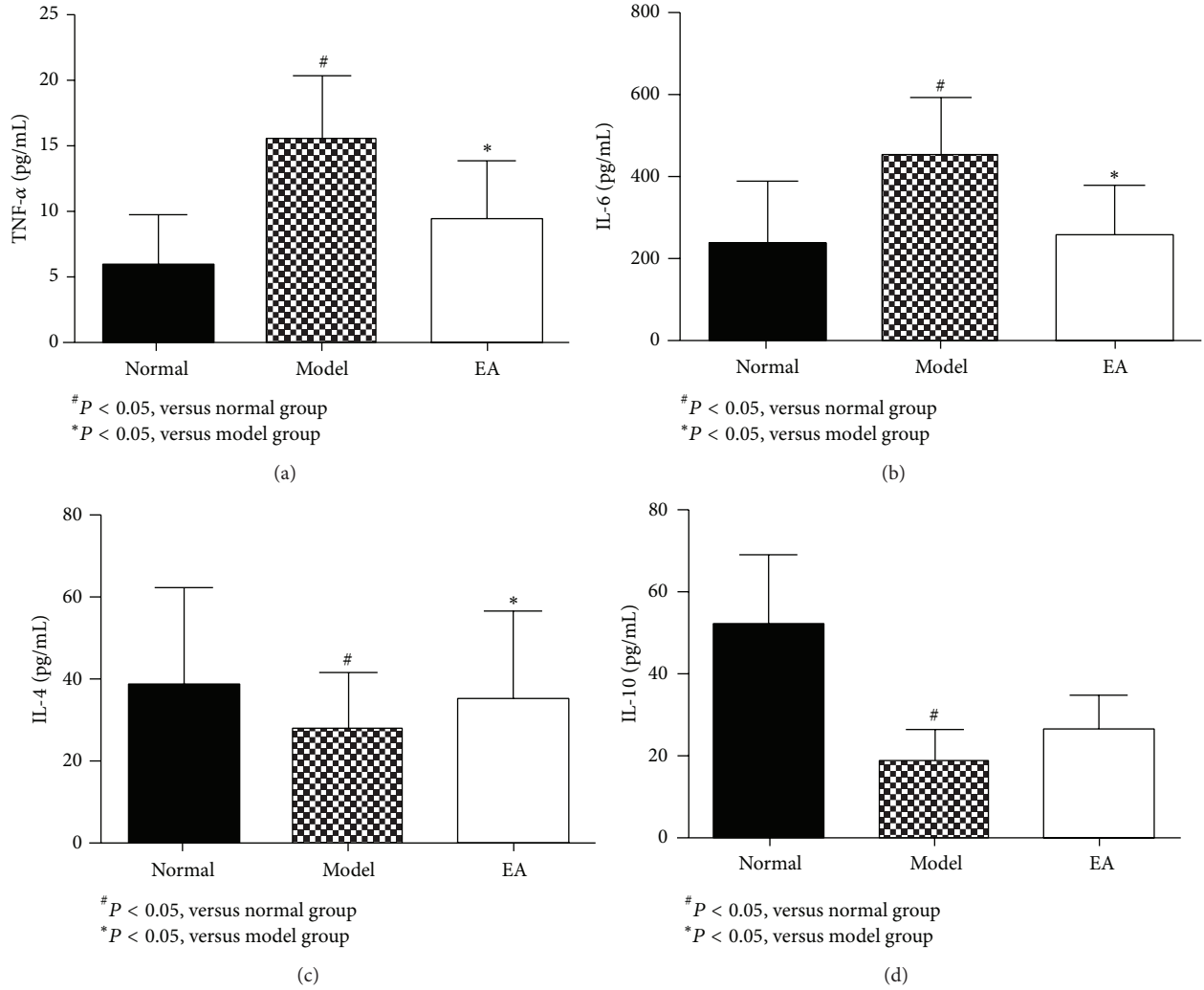

Figure 3: Comparison of serum TNF-α, IL-6, IL-4, and IL-10 among groups (mean ± SD) pg/mL.

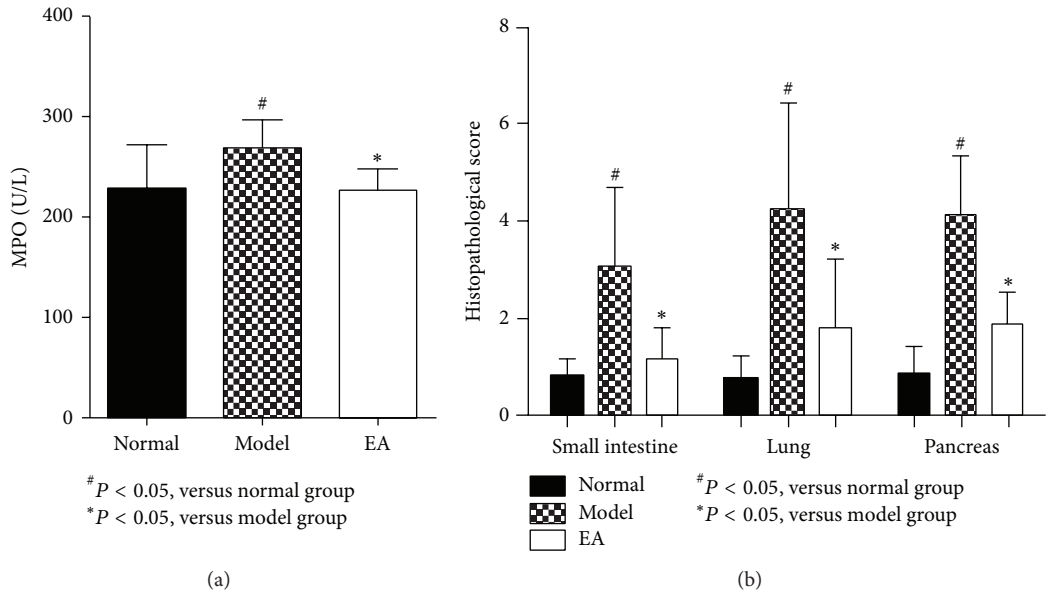

FIGURE 4: MPO in lung (a), pathological changes of pancreas, lung, and small intestine (b).

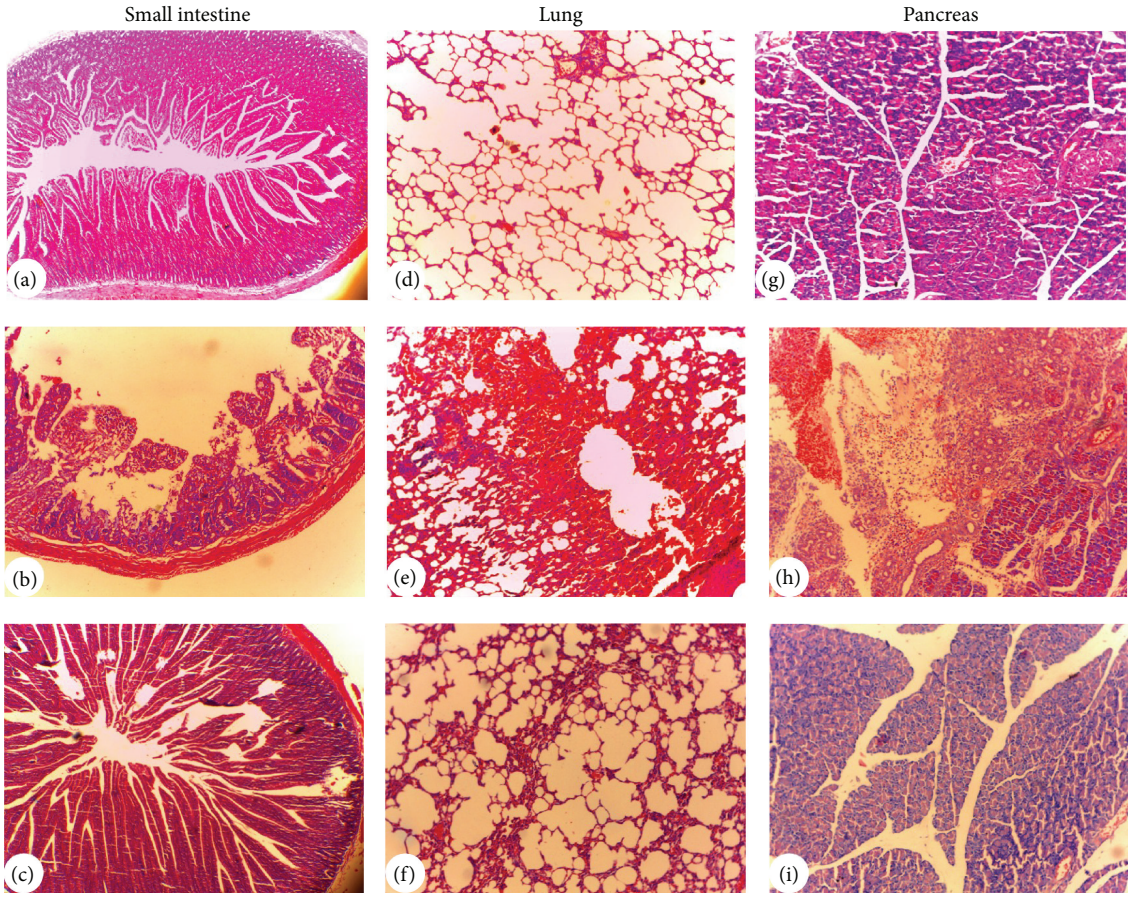

FIGURE 5: Histopathological findings of pancreatic, lung, and small intestinal tissues observed by hematoxylin and eosin staining (pancreatic and lung tissues: light microscopy, ×100; small intestinal tissue: light microscopy, ×40): in the photos of small intestinal tissue, the damage of intestinal mucosa was significant, in the photos of lung tissue, pulmonary interstitial edema and infiltration of large quantity of inflammatory cells were found in the model group, while the situation was much lighter in the EA group; and in the photos of pancreatic tissue, infiltration of large quantity of inflammatory cells was found in the model group, while the situation was much lighter in the EA group.

[22], which is similar to our results. All these showed that EA could inhibit the related inflammatory response. On the other hand, our study found that EA treatment might increase the level of anti-inflammatory cytokines IL-4 to relieve inflammation response in rats with acute pancreatitis. This is similar to the study that EA treatment of Zusanli (ST-36), Shangjuxu (ST-37), Quchi (LI-11), and other points could reduce the expression of IL-1β in intestine and elevate the serum level of IL-4 in rats with ulcerative colitis [23], which demonstrated that EA could promote the expression of anti-inflammatory mediators. It was deduced that EA could promote the intestinal motility through regulating the related inflammatory response in gastrointestinal.

Regarding IL-10, Pezzilli et al. and Myer et al. showed that IL-10 concentrations in SAP patients were significantly higher than those in mild AP. In this study, we found no difference in IL-10 concentration between EA group and model group rates. This is contrary to the result that acupuncture Tianshu (ST-25) point could increase the serum level of IL-10 in rats with sodium taurocholate-induced SAP [10]. This may be due to the different modeling method with sodium taurocholate or 20% L-arginine (100 mg/100 g) at 1 h intervals for three times, which lead to different disease severity of acute pancreatitis.

In addition, CCK-8 can inhibit the increase of TNF-α, IL-1, IL-6, and other proinflammatory cytokines [24, 25]. Xu et al. found that serums MTL and CCK decreased while VIP increased significantly in acute pancreatitis patients [16]. Wang et al. have found that EA treatment could increase colonic transit time (CTT) and serum CCK-8 and decrease serum VIP [26]. In the present study, EA treatment could increase serum CCK-8 concentrations and decrease serum VIP concentration significantly to regulate intestinal propulsion in L-arginine induced acute pancreatitis rates. All those showed that disorders of gastrointestinal hormone and related inflammatory response were partly on account of gastrointestinal dysfunction in acute pancreatitis and acupuncture is able to enhance the gastrointestinal dynamics and improve its motor activity through regulating the gastrointestinal hormone and their related inflammatory response mediators.

Third, the histopathology of lung was improved when EA promoted the intestinal motility and regulated the inflammatory response, with the decrease of MPO in the lung of AP and the pathological amelioration of lung. It was concluded that EA might ameliorate the lung injury via inhibiting the intestinal inflammatory response and promoting the gastrointestinal motility. Paralysis of intestine can aggravate the intestinal barrier resulting in "bacterial translocation" and "endogenous intestinal endotoxemia" and cause a "second strike" on body [27, 28]. The worse the intestinal dysfunction was, the more the neutrophil accumulated in the lung and the higher the levels of MPO increased, which displayed that the lung injury in the course of acute pancreatitis is associated with the motility and inflammatory response of the gut [29]. Our previous study found that improving gastrointestinal motility could relieve the IAH and reduce water content and MPO of the lung in rats with acute pancreatitis [8]. Other studies have suggested that acupuncture Zusanli (ST-36) could significantly reduce intestinal permeability in patients with acute pancreatitis and decrease endogenous inflammatory mediators and vasoactive substances in the intestinal mucosa membrane to ameliorate the intestinal epithelial cell necrosis for protecting gastrointestinal mucosa barrier [30]. This is similar to the theory in traditional Chinese medicine of "Lung and Large intestinal exterior-interiorly related." In the present study, our results indicated that EA treatment promotes gastrointestinal propulsion and ameliorates acute pancreatitis-induced intestinal histopathology, which might finally relieve the lung injury in SAP.

What is more, studies have found that CCK-8 could stimulate the tracheal respiration, make the trachea relaxed, and relieve the endotoxemia in rats with pulmonary hypertension. CCK-8 could also restrain the in vitro lipopolysaccharide (LPS) and activate pulmonary interstitial macrophages (PIM) to reduce the endotoxemia related inflammatory changes in the lung [31]. VIP, a straight-chain peptides, is widely distributed in the gastrointestinal tract, lung, and intestinal tract which mainly promote glandular secretion. It also has a negative relationship with gastrointestinal movement. The present study found that EA could promote intestinal motility through downregulation of VIP [32]. On the other hand, VIP is a vasodilator effect of vasoactive compounds, which has potent pulmonary vascular expansion and inhibits pulmonary artery smooth muscle cell hyperplastic biological activity [33]. It has been demonstrated in animal experiments that VIP could reduce the effect of pulmonary vascular resistance to decrease the pulmonary hypertension [34, 35]. VIP has a similar effect on expansion pulmonary vascular resistance in nitric oxide (NO) and activates adenylate cyclase to relax pulmonary artery smooth muscle cell [36]. It showed that CCK and VIP had some protective effects on the lung. In this study, serum VIP in EA group was lower than that in the model group, which suggested that acupuncture may facilitate the recovery of gastrointestinal function and relieve the lung injury through reducing the serum level of VIP.

In summary, the present findings demonstrated that EA treatment may be capable of attenuating the severity of acute pancreatitis and associated lung injury in rates. The potential mechanism might be that EA treatment improves the gastrointestinal dysfunction through regulating gastrointestinal hormone and related inflammatory mediators, which is in accord with the theory of "Lung and Large intestinal exterior-interiorly related."

Abbreviations

AP: Acute pancreatitis
EA: Electro-acupuncture
ALI: Acute lung injury
IPR: Intestinal propulsion rate
VIP: Vasoactive intestinal peptide
MPO: Myeloperoxidase
IAH: Intra-abdominal hypertension
ACS: Abdominal compartment syndrome
ARDS: Acute respiratory distress syndrome
DCQD: Dachengqi decoction.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Acupuncture Relieves the Excessive Excitation of Hypothalamic-Pituitary-Adrenal Cortex Axis Function and Correlates with the Regulatory Mechanism of GR, CRH, and ACTHR

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It had been indicated in the previous studies that acupuncture relieved the excessive excitation of hypothalamic-pituitary-adrenal cortex axis (HPAA) function induced by stress stimulation. But the changes in glucocorticoid receptor (GR) induced by acupuncture have not been detected clearly. The objective of the study was to observe the impacts of acupuncture on the protein expressions of corticotrophin releasing hormone (CRH), adrenocorticotropic hormone receptor (ACTHR), and GR under the physiological and stress states. The results showed that under the stress state, acupuncture upregulated the protein expression of GR in the hippocampus, hypothalamic paraventricular nucleus (PVN), and pituitary gland, downregulated the protein expression of GR in the adrenal cortex, and obviously reduced the protein expressions of CRH and ACTHR. Under the physiological state, acupuncture promoted GR protein expression in the hippocampus and CRH protein expression in the hippocampus and PVN. The results explained that acupuncture regulated the stress reaction via promoting the combination of glucocorticoids (GC) with GR, and GR protein expression. The increase of GR protein expression induced feedback inhibition on the overexpression of CRH and ACTHR, likely decreased GC level, and caused the reduction of GR protein expression in the adrenal cortex.

1. Introduction

The normal expression of glucocorticoid receptor (GR) plays an important role in maintaining the normal physiological condition in the body [1]. A large number of studies had shown that the stresses induced by various reasons may cause the decrease of GR expression in the human body, rat, and dog, which is relevant to the stress intensity [2–4].

When the stressors arrive to the paraventricular nucleus of the hypothalamic paraventricular nucleus (PVN), corticotrophin releasing hormone (CRH) neurons synthesize and release CRH and transport it to the adenohypophysis. Afterward, CRH enables the adenohypophysis to secrete adrenocorticotropic hormone (ACTH). In blood circulation, ACTH acts on the adrenal cortex and accelerates the synthesis and the secretion of GC in the adrenal cortex. Being the negative feedback regulator, the ectogenic GC adjusts CRH secretion in the PVN through hypothalamic-pituitary-adrenal cortex axis (HPAA), inhibits the stress reaction, modifies the structure of the hippocampus and other limbic systems, and impacts awakening, cognitive, and emotional functions [5, 6]. The negative feedback regulation of GC on HPAA is achieved by the action on GR and mineralocorticoid receptor (MR) extensively distributed in the central nervous system [7].

Acupuncture is a procedure in which fine needles are inserted into an individual at discrete points and then manipulated, with the intent of regulating HPAA function [8–10]. The clinical practice of acupuncture is growing in popularity worldwide. More than 40 disorders have been endorsed by the World Health Organization (WHO) as conditions that can benefit from acupuncture treatment [11–13]. Acupuncture at Shenshu (BL23), Qimen (LR14), Sanyinjiao (SP6), Zhubin (KI9), or the other points regulates HPAA function, but the effects of different acupoints are different [14–17].

In this study, we discussed the impacts of acupuncture at different acupoints on GR expressions in the hippocampus, PVN, pituitary gland, and adrenal cortex of the rats induced by unpredictable chronic mild stress (UCMS) stimulation as well as the associated changes in the expressions of CRH and ACTHR so as to explore the acupoint specificity and the mechanism of acupuncture on the regulation of HPAA function.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats in 150–170 g were obtained from the Laboratory Animal Resources Center, National Institute for the Control of Pharmaceutical and Biological Products, Beijing (Certificate no. SCXK (jing) 2009–0017). These animals were individually caged on a 12 h light/dark cycle (lights on at 8:00 am, lights off at 8:00 pm) under controlled temperature (22 ± 1°C) and humidity (50% ± 5%) conditions. Standard rat chow and water were given ad libitum. Animals were allowed to acclimatize for seven days before the study. All experiment procedures comply with the guidelines of the "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) and the legislation of the People's Republic of China for the use and care of laboratory animals. The experimental protocols were approved by the Animal Experimentation Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences. Efforts were made to minimize the number of animals used and the suffering of the experimental animals.

2.2. Behavior Test

Open Field Test. The open field apparatus was constructed of black plywood and measured 80 × 80 cm with 40 cm walls. White lines were drawn on the floor. The lines divided the floor into twenty-five 16 × 16 cm squares. A central square (16 cm × 16 cm) was drawn in the middle of the open field. Rats were put on the central square; at the same time the video camera was turned on for video recording from the top of the open field apparatus. Behaviors of rats were recorded for 3 minutes, with the grid number being counted as the horizontal score and the time of both frontal claws uplifting from the ground as the vertical score, one score counted after every 10 cm moving along the specific lines [18, 19]. Only one animal was used in one determination. Each determination lasted 3 min. The next determination with another rat began after thoroughly cleaning the box. Each animal was determined before and after modeling, as well as after acupuncture separately.

Sucrose Consumption. The 24-hour water fasting was required in all the rats before and after modeling, as well as after acupuncture (after open field test), but 1% sucrose water was allowed freely for 24 h. The sucrose consumption was observed in 24 h for the animals of each group.

2.3. Establishment of Unpredictable Chronic Mild Stress (UCMS) Model Rat. Fifty-eight of one hundred rats were recruited with the total score of 60~120 in the open field test [18] and evenly randomized into 5 groups. A successful UCMS model rat was created with the score of the open field test equal to or minus 60. Qualified rats were distributed into 5 groups: a normal group (N group, no acupuncture, $n = 10$), a Qimen (LR14) and Shenshu (BL23) group (NEA group, $n = 12$), an UCMS group (M group, no acupuncture, $n = 12$), an UCMS Sanyinjiao (SP6) and Zhubin (KI9) group (MNEA group, $n = 12$), and an UCMS Qimen (LR14) and Shenshu (BL23) group (MEA group, $n = 12$). Every five rats in the N and NEA group were housed in one cage. However, rats in the M, MEA, and MNEA groups were caged individually. Depression model was established by 21 days of UCMS combined with isolation. UCMS procedures were based on published studies [19, 20], including 7 kinds of stressors: food deprivation, water deprivation, cage tilt 45°C (Ugo Basile s.r.l. hot/cold plate, Model 35100-001, Italy), swimming in 4°C ice water, clipping tail 3 min, 50 V electric shock (Electronic stimulator, NIHON KOHDEN, Japan), and overnight illumination. The stressors were given randomly 3 times daily for 21 continuous days. The rats in the N and NEA group were housed without any external stimuli except for necessary procedures such as routine cage cleaning.

2.4. Experimental Procedures (Figure 1). The open field test on all rats was conducted on the day before the study (−1st day) , the 21st day (after UCMS), and the 29th day (after treatment) in the study course. After the models of UCMS were established on the 21st day, the electroacupuncture (EA) treatment of 8 days was applied to bilateral SP6 and KI9 of rats in the MNEA group once daily for 30 min. For the MEA group, EA was applied to bilateral BL23 and LR14 following the same procedure and EA parameters as the MNEA group. For the NEA group, EA was applied to the same procedure as the MEA group. M group and N group in the EA groups with the inhaled anesthesia accepted during the treatment (Figure 1). EA was applied at the frequency of 2 Hz and the intensity of 2 mA by using the EA stimulator (HANS-100A, Nanjing Gensun Medical Technology Co., Ltd., China). The inhaled anesthesia was conducted on the Isoflurane Vaporizer (Matrx VIP 3000, Midmark corporation, USA) with isoflurane (Hebei Nine Sent Pharmaceutical Co., Ltd., Hebei, China). Rats were sacrificed immediately after the last EA (on the 30th day), and the fresh tissues of brain (hippocampus and PVN area), pituitary, and adrenal gland were collected. Acupuncture, inhalation anesthesia process, or surgical procedure was the same in the NEA and the N group (Figure 1).

2.5. Western Blot Analysis. Western blot analysis was performed as follows [21]. Rats tissues of brain (hippocampus and PVN area), pituitary, and adrenal gland were homogenized on ice in RIPA buffer (50 mol/L Tris-Cl, pH 7.6, 5 mol/L ethylenediaminetetraacetic acid, 150 mol/L NaCl, 0.5% Nonidet P-40, and 0.5% Triton X-100) containing protease inhibitor cocktail and phosphatase inhibitor cocktails I/II (Sigma-Aldrich). The homogenate was centrifuged at 12,000 ×g for 30 minutes at 4°C. The supernatant was collected and the protein concentration was measured using the

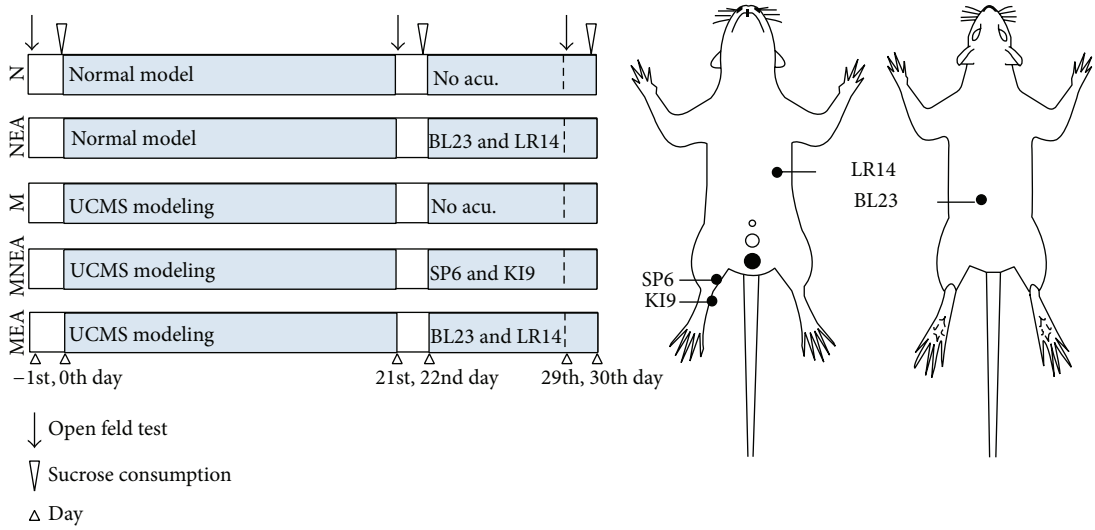

Figure 1: Experimental protocol. There were 5 groups. N: the normal group, no acupuncture; NEA: the normal acupuncture group, acupuncture at Qimen (LR14) and Shenshu (BL23); M: UCMS group, no acupuncture; MNEA: the group of UCMS acupuncture at Sanyinjiao (SP6) and Zhubin (KI9); MEA: the group of UCMS acupuncture at Qimen (LR14) and Shenshu (BL23) group.

Bradford assay. Twenty micrograms of the sample was separated with 10% polyacrylamide gel blotted on a PVDF film (Millipore Corp.). The blotted film was blocked for 2 hours at 4°C in blocking solution (1×TBS with 5% nonfat milk and 0.02% Tween 20). The blocked film was shaken overnight at 4°C using primary antibodies in blocking solution. Following three times washes with TBST (1×TBS with 0.02% Tween 20), the film was shaken for 1 hour at room temperature with peroxidase-conjugated secondary antibody and then washed three times with TBST. Detection was performed using an ECL kit (Santa Cruz Biotech) according to the manufacturer's instructions. The western blots shown are representative of at least three independent experiments.

The antibodies used included the following: anti-CRH (Datashect, #10944-1-AP) (1 : 500), anti-ACTHR and anti-GR (Santa Cruz, #H-70 and #H-300) (1 : 1000), anti-β-Actin (Sigma, A5316) (1 : 10000), and HRP-conjugated IgG secondary antibodies (1 : 2000) (GE Healthcare Life Sciences). All western blot data were analyzed by ImageJ software.

2.6. Statistical Analysis. The statistical analysis was performed by using one-way analysis of variance (ANOVA) with software GraphPad Prism (California), and the data were expressed as means ± SEM. All results with P values less than 0.05 were considered statistically significant.

3. Results

3.1. Effects of Acupuncture on Behaviors in UCMS Model Rats

3.1.1. Open Field Test (Figures 2(a), 2(b), and 2(c)). The activity of the animals in the five groups was gradually decreased on the 21st day (after modeling) and on the 29th day (after treatment) as compared with that before modeling (on −1st day). After modeling, the horizontal, vertical, and the along-the-line activities in the open field test in M, MNEA, and MEA groups were all reduced obviously as compared with N and NEA groups ($P < 0.05$, 0.01, and 0.001). After acupuncture, the difference in the horizontal activity was the most obvious as compared with that before acupuncture among the five groups. The decrease of the horizontal activity in M group was the most obvious, followed by MNEA, MEA, N, and NEA groups successively. The difference was extremely significant in M and MNEA groups as compared with N and NEA groups ($P < 0.01$). The difference was significant in MEA group as compared with N and NEA groups ($P < 0.05$) and between MEA and M groups ($P < 0.05$). The difference in the vertical activity was not significant among the groups. Concerning the along-the-line activity, the differences in M, MNEA, and MEA groups were significant extremely as compared with N and NEA groups ($P < 0.001$). There was no improvement after acupuncture.

3.1.2. Sucrose Consumption (Figure 2(d)). The sucrose consumption was done on the 0th day (before modeling), on the 22nd day (after modeling), and on the 30th day (after acupuncture). After modeling, the results in M, MNEA, and MEA groups were different significantly as compared with N and NEA groups. After acupuncture, it was significantly reduced in M group, indicating the extremely significant differences as compared with N and NEA groups ($P < 0.001$). The difference was significant in MNEA group as compared with N and NEA groups ($P < 0.05$). The difference was not significant in MEA group as compared with N and NEA groups. The difference was extremely significant between MEA and M groups ($P < 0.001$). The difference was significant between MNEA and M group ($P < 0.05$) as well as between MEA and MNEA group.

There are results that showed that the stress reduced sucrose consumption (a symptom of anhedonia and considered a depressive-like behavior in rats) and 8 days of

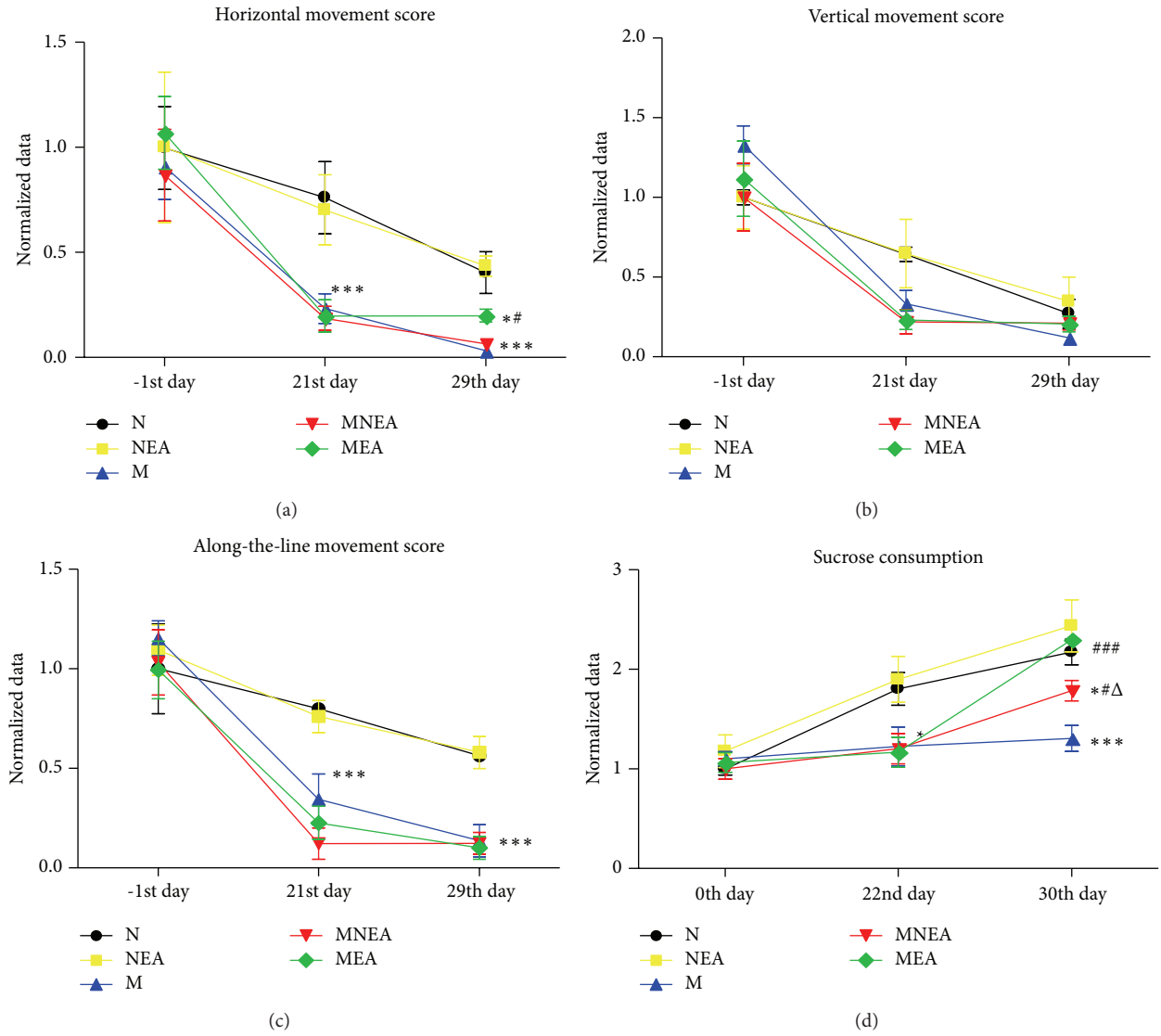

FIGURE 2: The open field test score and sucrose consumption. (a, b, and c) showed the normalized data of the horizontal, vertical, and along-the-line movement test on the −1st day, 21st day, and 29th day. (d) showed the normalized data of sucrose consumption detected on the 0th day, 22nd day, and 30th day. Normalized data [21] = tested value on the 21st, 22nd, 29th, or 30th day/tested value on the −1st day or 0th day in same animal separately. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus N group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus M Group. $^{\Delta}P < 0.05$, MNEA versus MEA (n = 10–12).

acupuncture, especially the MEA treatment, reduced anhedonia and restored the sucrose consumption of M animals to the levels of control animals. This is a very significant behavioral finding and should be discussed and properly emphasized.

3.2. Effect of Acupuncture on the Protein Expression of GR Induced by UCMS Stimulation (Figure 3). Western blot analysis showed that there was a protein expression of GR detected in the hippocampus, PVN, pituitary gland, and adrenal cortex. GR protein expression was increased or inhibited in response to acupuncture in NEA group. And it was decreased obviously in the hippocampus, PVN, and pituitary gland and increased obviously in the adrenal cortex in M group. Compared with the results in N group, GR protein expressions were either increased or decreased in the hippocampus, PVN, pituitary gland, and adrenal cortex by 95.99%, −23.61%, −5.2%, and 80.76% separately in NEA group. And the results were reduced significantly by 75.85%, 62.23%, and 28.04%, respectively, in the hippocampus, PVN, and pituitary gland, and the result was increased significantly by 265.01% in adrenal cortex in M group. Acupuncture induced the dual-directional regulation of GR protein expression in the hippocampus, PVN, pituitary gland, and adrenal cortex. Compared with M group, in MNEA and MEA groups, GR protein expressions were increased by 22.61% and 109.57%, 180.06% and 362.6%, and 52.9% and 246.38% separately in the hippocampus, PVN, and pituitary gland, but they

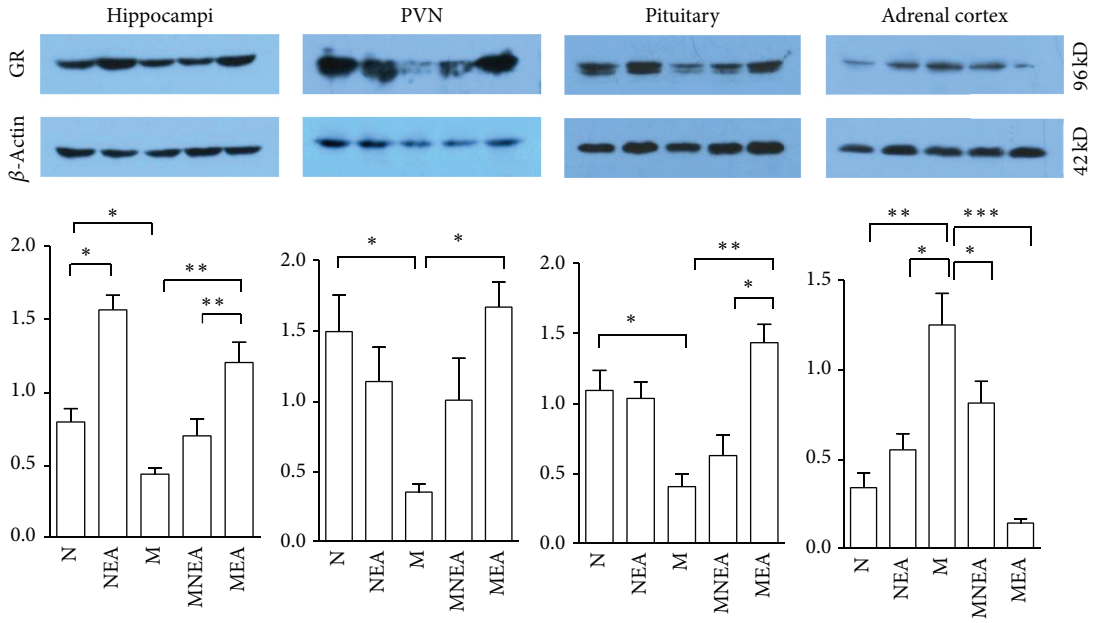

Figure 3: Effect of acupuncture on GR protein expression. GR protein expression was reduced obviously in the hippocampus, PVN, and pituitary gland and was dramatically increased in the adrenal cortex in M group as compared with N group. Compared with M group, GR protein expression was upregulated in the hippocampus, PVN, and pituitary gland and was downregulated in the adrenal cortex to different extents in MEAN and MNEA groups, but the results in MEA group were superior to MNEA group. The corresponding protein expression was increased obviously in the hippocampus in NEA group, which was significantly different as compared with N group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ ($n = 10$–12) (GR: #H-300, Santa Cruz, 96KD, dilution: 1 : 1000).

were decreased by 34.82% and 88.34% in the adrenal cortex in MNEA and MEA groups. These data suggested that acupuncture promoted GR protein expression in the hippocampus, PVN, and pituitary gland but inhibited the expression in the adrenal cortex. The results in MEA were superior to MNEA group.

3.3. Effect of Acupuncture on CRH Protein Expression Induced by UCMS Stimulation (Figure 4). Western blot analysis showed that there was a detectable signal of CRH in the hippocampus, PVN, and pituitary gland. CRH protein expression was slightly increased in response to acupuncture in NEA group. And the remarkable increase of CRH protein expression was discovered in M group. Obviously, acupuncture downregulated CRH protein overexpression induced by UCMS stimulation. Compared with N group, CRH protein expressions were increased or inhibited in NEA group in the hippocampus, PVN, and pituitary gland by 109.03%, 67.31%, and −23.15% separately. And the results were increased significantly in the hippocampus, PVN, and pituitary gland in M group by 543.81%, 307.66%, and 89.18%. The UCMS-induced overexpression of CRH protein could be inhibited by acupuncture. Compared with M group, CRH protein expression was reduced remarkably in the hippocampus, PVN, and pituitary gland in MNEA and MEA groups by 36.91% and 86.95%, 20.86% and 53.67%, 24.64% and 65.03% successively. Those data indicated that acupuncture inhibited CRH protein expression in the hippocampus, PVN, and pituitary gland. The results in MEA group were superior to MNEA group.

3.4. Effect of Acupuncture on ACTHR Protein Expression Induced by UCMS Stimulation (Figure 5). ACTHR is ACTH receptor. Western blot analysis showed that there was a detectable signal of ACTHR in the pituitary gland and adrenal cortex. ACTHR protein expression was slightly inhibited in response to acupuncture in NEA group. The remarkable increase of ACTHR protein expression was discovered in M group, and acupuncture downregulated obviously ACTHR protein overexpression induced by UCMS stimulation. Compared with N group, ACTHR protein expression was slightly inhibited in NEA group in the pituitary gland and adrenal cortex by 10.6% and 29.61% separately; ACTHR expression was increased significantly in the pituitary gland and adrenal cortex in M group by 95.78% and 123.79%. Compared with M group, ACTHR protein expression was reduced remarkably in pituitary gland and adrenal cortex in MNEA and MEA groups by 32.9% and 69.38% and 51.52% and 71.58% successively. Those data indicated that acupuncture inhibited ACTHR expression in the pituitary gland and adrenal cortex. The results in MEA group were superior to MNEA group.

4. Discussion

The main results of the study were as follows. (1) The UCMS model rats showed the decrease of the activity and sucrose consumption, explaining the success of stress model in the study. (2) UCMS induced the increase of GR protein expression in adrenal cortex and the decrease in the hippocampus, PVN, and pituitary gland. Acupuncture induced

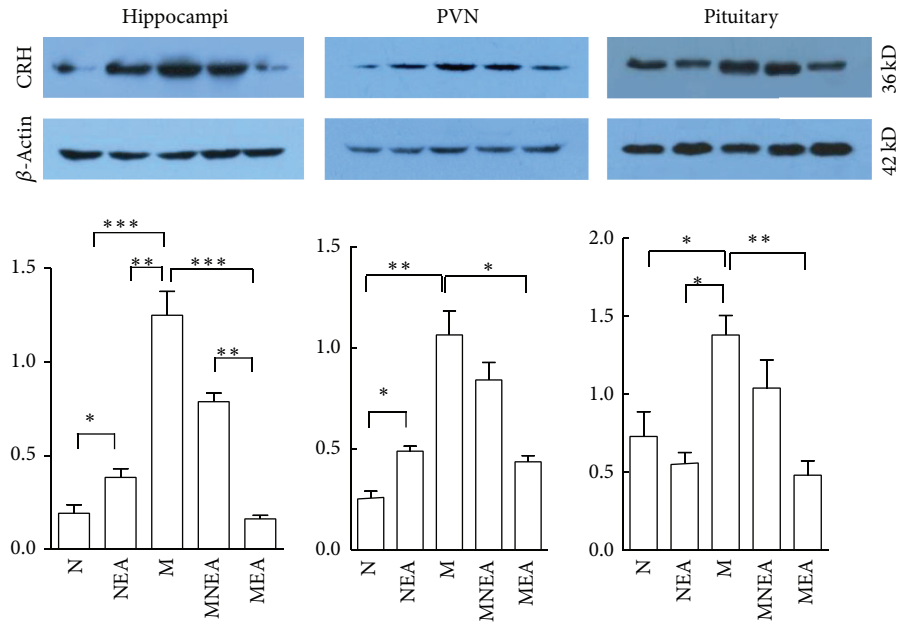

Figure 4: Effect of acupuncture on CRH protein expression. In M group, CRH protein expression was increased obviously in the hippocampus, PVN, and pituitary gland, which was significantly different as compared with N group. In MEA and MNEA groups, CRH protein expression was downregulated in hippocampus, PVN, and pituitary gland to different extents as compared with M group, but the results in MEA group were superior to MNEA group. The protein expression was increased significantly in the hippocampus and PVN in NEA group, which was different significantly as compared with N group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ ($n = 10$–12) (#10944-1-AP, Datashect, 36KD, dilution: 1 : 500).

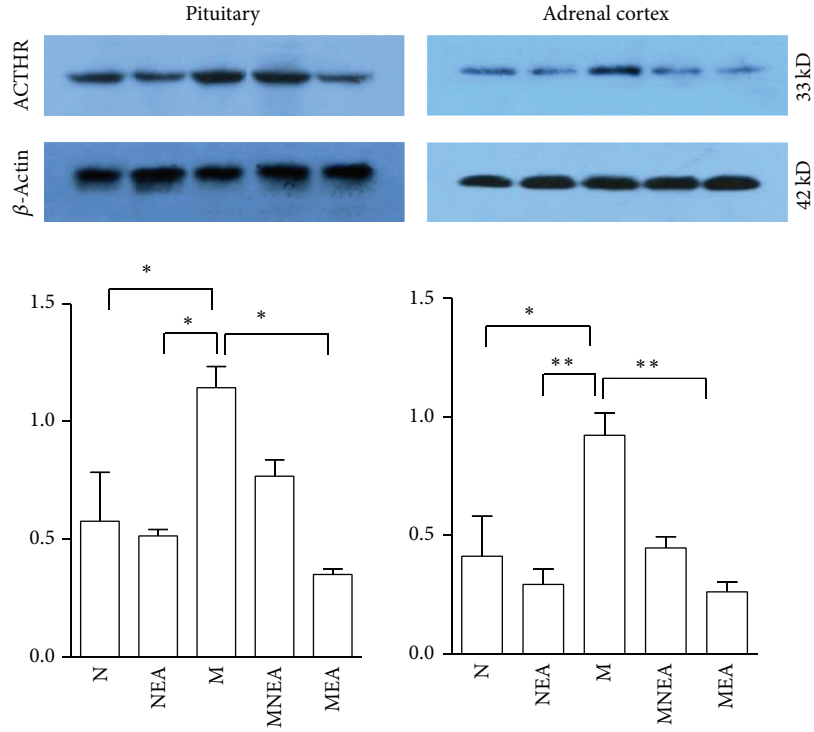

Figure 5: Effect of acupuncture on ACTHR protein expression. ACTHR protein expression was increased obviously in the pituitary gland and adrenal cortex in M group as compared with N group. ACTHR protein expression was downregulated in the pituitary gland and adrenal cortex in MEA and MNEA groups to different extents as compared with M group, but the results in MEA group were superior to MNEA group. The difference was not significant in comparison between NEA and N groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ ($n = 10$–12) (#H-70, Santa Cruz, 33KD, dilution: 1 : 1000).

the dual-directional regulation on GR protein expression in the hippocampus, PVN, pituitary gland, and adrenal cortex. And the effects were different in acupuncture at the different acupoints. (3) UCMS induced the increase of CRH protein expression in the hippocampus, PVN, and pituitary gland and the increase of ACTHR expression in the pituitary gland and adrenal cortex. All of those could be inhibited by acupuncture and the effects were different at different acupoints. (4) GR protein expression had been detected in the hippocampus, PVN, pituitary gland, and adrenal cortex; CRH expression had been detected in the hippocampus, PVN, and pituitary gland; and ACTHR protein expression had been detected in the pituitary gland and adrenal cortex in N and NEA groups. But the above corresponding expressions could be either increased or decreased in NEA group as compared with N group. Except that there were significant differences in GR and CRH protein expressions in hippocampus and CRH protein expression in the PVN between the two groups, the differences were not significant in the rest aspects.

4.1. Behavior Changes of UCMS Model Rats. After modeling, the horizontal, vertical, and along-the-line movements of the UCMS model rats were decreased apparently as compared with the normal model rats and the sucrose consumption was reduced significantly. These results were similar to the behavior changes of the chronic stress model rats in the previous studies [22]. It was explained that the stress model set up in this study was successful.

4.2. The Dual-Directional Regulation of Acupuncture on GR Protein Expression Induced by UCMS Stimulation. The chronic stress induced the decrease of GR expression in many organs of the body and the increase of adrenal weight. Acupuncture relieved the overexcitation of HPAA function induced by chronic stress [23, 24]. But the changes in GR protein expression caused by acupuncture were not clear completely in terms of physiology and pathology. The hazard caused by stress reaction is due to the higher concentration of GC in the body. The affinity of GR was very low with GR, and GR was only activated under the high concentration of GC [25]. GR participates in mainly the negative feedback regulation on HPAA [26].

The adrenal cortex is the target organ of the synthesis and secretion of GC. The long-term chronic stress stimulation can cause the excessive secretion of GC in the adrenal cortex of the rat. This study observed that UCMS induced the significant decrease of GR protein expression in the hippocampus, PVN, and pituitary gland. Acupuncture upregulated GR protein expression in the hippocampus, PVN, and pituitary gland. Perhaps, through promoting the connection of high concentration GC with GR in the hippocampus, PVN, and pituitary gland, the function of GR was activated and the negative feedback regulation of GR was intensified on HPAA. The decrease of GC level caused the decrease of HPAA excitability. The decrease of GC level lowered the combination with GR in adrenal cortex; as a result, GR activity as well as its protein expression was decreased. The results above were caused by the inhibition of acupuncture on GR protein expression induced by UCMS stimulation in the adrenal cortex. It was indicated that acupuncture presented the dual-directional regulation on GR protein expression induced by UCMS stimulation, which was achieved via HPAA. Our preliminary work had observed the impacts of acupuncture on the peripheral blood CORT in the rats with the spinal cord injury [15] and it was discovered that acupuncture promoted the increase of CORT level via the spinal reflex. Acupuncture inhibited the increase of GR level in UCMS model rats via the regulation of HPAA function. The acupoint specificity effect had been verified molecularly via the different expressions of GR protein in the hippocampus, PVN, and pituitary gland induced by acupuncture at different acupoints.

4.3. Inhibition of Acupuncture on CRH Protein Expressions Induced by UCMS Stimulation. Being the most superior hormone in HPAA, CRH launches the response of HPAA to the corresponding stressor. Simultaneously, CRH is also involved in the activation in the sympathetic-adrenal-medullary system. Therefore, it is recognized generally that CRH is one of the important initiating factors in the stress process. The increase of CRH secretion may be used as an objective index to reflect the body's stress state [27, 28]. This study observed that acupuncture decreased UCMS-induced overexpression of CRH protein. Possibly, acupuncture promoted GR protein expression of CRH neurons in the hippocampus, PVN, and pituitary gland and downregulated the excitability of CRH neurons so as to reduce the protein expression of CRH. The acupoints specificity effect of the acupuncture regulation on HPAA function via the difference protein expressions of CRH in the hippocampus, PVN, and pituitary gland by acupuncture at different acupoints was further verified.

4.4. Inhibition of Acupuncture on the ACTHR Protein Expression Induced by UCMS Stimulation. Lian et al. had made the model fitting to explain the stress reaction impact on the interaction of GR and ACTHR gene with stress factors. The results indicated that GR and ACTHR gene variants were the contributing factors of the decline of psychological stress reaction, physiological stress reaction, and work ability. It showed that ACTHR expression was closely related to the stress reaction [29].

This study had detected the protein expression of ACTHR in the pituitary gland and adrenal cortex and observed that UCMS induced the increase of ACTHR protein expression in the pituitary gland and adrenal cortex, which was downregulated by acupuncture. Additionally, the effects were different on the different acupoints, which was likely related to the downregulation of acupuncture on CRH protein expression and the inhibition of ACTHR protein expression via HPAA.

This study observed that acupuncture impacts the protein expressions of CRH, ACTHR, and GR in the normal model rats. Except for the significant differences in the protein expressions of GR and CRH in the hippocampus and the protein expression of CRH in the PVN between the N group and NEA group, the differences in the rest aspects were not significant. The stress is the comprehensive reaction of the body to the stressor [30]. Under the stimulation by

stressor, the body generates a series of neural endocrinal reactions via HPAA excitation so as to enable the body to enhance the resistance and maintain and recover the internal stability under the specific situation [31]. Being a kind of stress stimulation, acupuncture activated the activity of brain central CRH neurons of the normal model rats. But since HPAA function was of the normal state in the normal animal, the stress state can be relieved rapidly. Therefore, this stress state was just manifested in the relevant molecules of the brain center. Additionally, the increase of GR protein expression in the hippocampus could promote the growth and development of the hippocampal neuron [32]. It was explained that acupuncture presented the protective effect on the central nerve in the normal model.

5. Conclusion

Acupuncture improved the behavior changes induced by UCMS stimulation, which was related to the promotion of acupuncture on the combination of GC and GR in the hippocampus, PVN, and pituitary gland, the activation of GR, and promotion of GR protein expression. The increase of GR protein expression induced the negative feedback inhibition on CRH protein expression, downregulated ACTHR overexpression in the pituitary gland and adrenal cortex, decreased GC level, and reduced GR activity in the adrenal cortex and the protein expression. Molecularly, it had been verified that the regulation of acupuncture on stress reaction was achieved via the regulation of HPAA function, and the effects were different at different acupoints.

Abbreviations

EA: Electroacupuncture
PVN: Hypothalamic paraventricular nucleus
HPAA: Hypothalamic-pituitary-adrenal cortex axis
UCMS: Unpredictable chronic mild stress
GR: Glucocorticoid receptor
CRH: Corticotrophin releasing hormone
ACTHR: Adrenocorticotropic hormone receptor.

Conflict of Interests

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

Authors' Contribution

Shao-Jun Wang conceived the whole procedure of the study, wrote the paper, and conducted the statistical analysis. Jiao-Jiao Zhang, Shao-Jun Wang, and Li-Li Qie carried out the experiment. All authors discussed and approved the final paper.

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