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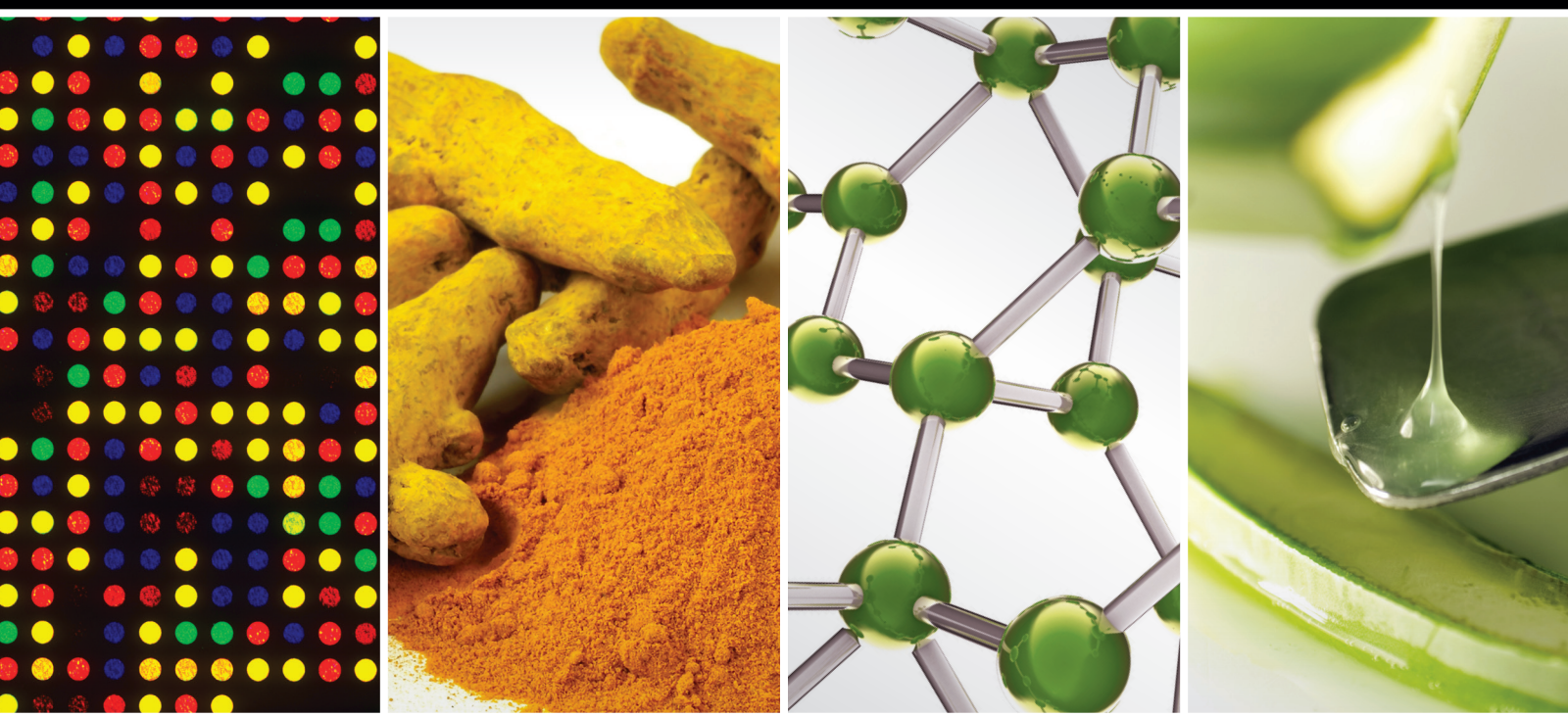
Modern Tools of Traditional Oriental Medicine

Lead Guest Editor: Gihyun Lee
Guest Editors: Kyung-Hwa Jung, Sang-Hoon Shin, and Hanbing Li



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Editorial Board

Contents

Hindawi
Evidence-Based Complementary and Alternative Medicine
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Editorial

Modern Tools of Traditional Oriental Medicine

Gihyun Lee,[1] **Kyung-Hwa Jung**,[2] **Sang-Hoon Shin**,[3] **and Hanbing Li**[4]

[1]*Dongshin University, Naju, Republic of Korea*
[2]*University of Pittsburgh, Pittsburgh, PA, USA*
[3]*Sangji University, Wonju, Republic of Korea*
[4]*Zhejiang University of Technology, Hangzhou, China*

Correspondence should be addressed to Gihyun Lee; glee@khu.ac.kr

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Decades ago, Oriental Medicine doctors could use just traditional tools including acupuncture, moxibustion, and herbal medicine despite its long history. However, technology improves and modern medical devices are invented to assist the Oriental Medicine doctors in performing diagnosis and treatment. Now, they can use exclusive medical devices such as pulse meter, tongue diagnosis, and face system with their own sensors and diagnostic skills to get information of body and disease from patients. They also can use modern therapeutic tools including electroacupuncture, pharmacopuncture, and electric-moxibustion as well as traditional ones. Experimental and translational studies on these new tools are underway.

For this special issue, we invited manuscripts with the following topics: improvement of tools in oriental medicine, pharmacopuncture (Chinese medicine injection), electroacupuncture and electric-moxibustion, validation of treatment efficacy, development of pulse meter and tongue diagnosis system, and experimental and translational studies on modern device of Oriental Traditional Medicine. We received 105 manuscripts from various labs for six months and 22 manuscripts were accepted for publication. Here we highlight some of the key ongoing challenges published in this special issue.

The efficacy of medical devices using radiofrequency is demonstrated in the papers "Moxibustion-Simulating Bipolar Radiofrequency Suppresses Weight Gain and Induces Adipose Tissue Browning via Activation of UCP1 and FGF21 in a Mouse Model of Diet-Induced Obesity" and "Short-Term Efficacy of Pulsed Radiofrequency Thermal Stimulation on Acupoints for Chronic Low Back Pain: A Preliminary Study of a Randomized, Single-Blinded, Placebo-Controlled Trial".

Granule, one of modern forms of herbal medicine, is used in the studies "Study of the Treatment Effects of Compound Tufuling Granules in Hyperuricemic Rats Using Serum Metabolomics" and "Randomized, Double-Blind, Placebo-Controlled Study of Modified Erzhi Granules in the Treatment of Menopause-Related Vulvovaginal Atrophy".

The article "Cinobufacini Injection Improves the Efficacy of Chemotherapy on Advanced Stage Gastric Cancer: A Systemic Review and Meta-Analysis" reviews the literature on the efficacy comparison between Cinobufacini injection (one of Chinese medicine injections) combined with chemotherapy and chemotherapy solely used in advanced gastric cancer treatment.

The protective effect of patches on acupoints against electromagnetic fields is shown in "Evaluation of the Effectiveness of Protective Patches on Acupoints to Preserve the Bioenergetic Status against Magnetic Fields".

The clinical application of the low voltage Meridian Energy Detection System is evaluated in assessing the electrodermal activity in the "The Development and Application Evaluation of Meridian Energy Detection System in Traditional Oriental Medicine: A Preliminary Study" and the safety of auto manipulation device for acupuncture which can help Oriental Medicine doctors is shown in the "Safety Assessment of the Auto Manipulation Device for Acupuncture in Sprague-Dawley Rats: Preclinical Evaluation of the Prototype".

Different face diagnosis system is used in "An Herbal Medicine, Yukgunja-Tang is more Effective in a Type of

Functional Dyspepsia Categorized by Facial Shape Diagnosis: A Placebo-Controlled, Double-Blind, Randomized Trial" and "Difference between Right and Left Facial Surface Electromyography in Healthy People".

In this special issue, there are more valuable manuscripts besides above. We hope the readers will be interested in improvement of diagnostic and remedial tools of Oriental Medicine.

Conflicts of Interest

The guest editorial team declare that no member of the team have any possible conflicts of interest or private agreements with companies. In case, the guest editor has a conflict with a manuscript, he will refuse to handle the manuscript.

Gihyun Lee
Kyung-Hwa Jung
Sang-Hoon Shin
Hanbing Li

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Research Article

The Efficacy and Safety of a Herbal Toothpaste in Reducing Gingivitis: A Double-Blind, Randomized, Placebo-Controlled, Parallel Allocation Clinical Trial

Jinfeng He, Yalan Deng, Fangzhi Zhu, Ting Zhong, Nanyu Luo, Lei Lei, Li Cheng, and Tao Hu

State Key Laboratory of Oral Diseases & National Clinical Research Center for Oral Diseases & Department of Preventive Dentistry, West China Hospital of Stomatology, Sichuan University, Chengdu, China

Correspondence should be addressed to Li Cheng; dentistcl@foxmail.com

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Aim. To examine the efficacy and safety of the toothpaste containing Rhizoma Chuanxiong and Rhizoma Imperatae extracts in reducing gingivitis. *Method*. A double-blind clinical trial was conducted, in which 120 volunteers were randomly assigned to the test group ($N = 60$) or the control group ($N = 60$). Tetramethylpyrazine, senkyunolide A, ferulic acid, and ligustilide are the main effective components of Rhizoma Chuanxiong and Rhizoma Imperatae contains the main components of cylindrin, carotene, 5-hydroxytryptamine, potassium, and calcium. The control group used placebo toothpaste containing neither Rhizoma Chuanxiong extract nor Rhizoma Imperatae extract. Plaque, gingivitis, and bleeding were assessed at the baseline, prior to the supragingival scaling, and at 4, 8, and 12 weeks. *Results*. During the trial, both test and control groups showed a decreasing trend compared to the baseline. At the end of 12 weeks, with respect to Gingival Index (GI), Bleeding Index (BI), and Bleeding on Probing percentage (BOP%) scores, there were significant differences between test and control groups (GI, $P<0.001$, BI, $P<0.001$, and BOP%, $P<0.001$, resp.). After 4 weeks of usage, there were no statistically significant differences in all of GI, BI, and BOP% scores between the two groups. However, the decrease became statistically significant at next two intervals (GI, $P<0.001$, BI, $P<0.001$, and BOP%, $P<0.001$, resp.) in the efficiency of GI, BI, and BOP% which was 8.04%, 11.02%, and 37.16%, respectively. There were no treatment-related adverse events reported. *Conclusion*. The toothpaste containing Rhizoma Chuanxiong and Rhizoma Imperatae extracts was well tolerated and significantly reduced gingivitis and bleeding after usage for 12 weeks. There was better improvement at molars, and the more serious the baseline status was, the better the efficacy was.

1. Introduction

Chronic gingivitis is one of the most common oral diseases with high prevalence around the world [1]. A survey of the prevalence and severity of gingivitis in American adults shows that the prevalence of gingivitis among adults ranged from 56% to 94% [2]. Even though the factor causing gingival lesions to be converted into periodontitis has not been well understood, current theory holds that the gingival lesion is the precursor of periodontitis [3]. Recent studies have also found that gingivitis is associated with a number of systemic diseases [4]. Therefore, the prevention and elimination of gum inflammation are essential for maintaining oral health and overall health [5].

Dental plaque is the major etiological and initiating factor for the development of gingivitis [6]. Therefore, the ideal plaque control is the basis for the prevention and control of gingivitis. It is considered that individual continuous removal of dental plaque is the most effective means of preventing and controlling gingivitis, in which brushing teeth and other mechanical methods to remove plaque are generally recognized as effective strategies [7]. However, due to the limitation of mechanical methods, the addition of some safe and effective drugs to prevent gingivitis in toothpaste is also considered to be a good supplementary to the control of mechanical plaque [8–10]. Studies have shown that certain chemicals, such as chlorhexidine or triclosan, are added to the toothpaste to directly inhibit the formation of plaque [11, 12],

nevertheless with the side effects of antimicrobial resistance, teeth coloring, taste changes, and so on [11, 13]. Recently, Chinese herbal medicinal ingredients have become the focus of research because of their natural, relatively low toxicity and cultural background [14–16].

Rhizoma Chuanxiong is the dried rhizome of *Ligusticum chuanxiong* Hort, known as a famous medicinal herb from Sichuan, and its main components include volatile oil, acid composition, and nitrogenous compounds [17]. In the past 50 years, pharmaceutical researchers have carried out a large number of studies on the pharmacodynamic basis of Rhizoma Chuanxiong. About 174 compounds have been isolated and identified from this herb [18]. The main effective components of Rhizoma Chuanxiong include tetramethylpyrazine, senkyunolide A, ferulic acid, and ligustilide [19]. Chemical structures of monomers are reported in Figure 1 [19]. Traditional Chinese medicine holds that Rhizoma Chuanxiong has the effect of invigorating blood and expelling wind-damp and relieving pain [20, 21]. Rhizoma Chuanxiong has been commonly used as traditional medicine for the treatment of various kinds of diseases including cerebrovascular diseases, migraine, maxillary sinusitis, pharyngitis, arthritis, and nephritis [22, 23]. However, the applications regarding Rhizoma Chuanxiong in the field of oral medicine have been poorly understood, especially in the prevention and treatment of periodontal diseases.

Rhizoma Imperatae is the rhizome of *Imperata cylindrica* (L.) Beauv. var. *major* (Nees) C.E.Hubb., which is a common perennial grass [24, 25]. As a traditional Chinese herb, Imperata Rhizoma has been reported to have the functions of clearing heat and cooling blood, as well as hemostatic effect [26]. The main components of Rhizoma Imperatae include cylindrin, carotene, 5-hydroxytryptamine, potassium, and calcium. Modern pharmacological research shows that Rhizoma Imperatae mainly has functions of hemostatic, diuresis antihypertensive, bacteriostasis, anti-inflammatory, analgesic, and anti-tumor, as well as reducing hydroxyl radical, antioxidant, and enhancing immunity [26, 27]. At present, there is a potential use of its hemostatic principle, and the Rhizoma Imperatae extract has been added to oral care products, which shows excellent effect on reducing gingival bleeding [28].

According to Chinese Pharmacopoeia (2015 edition), the rhizomas of *Ligusticum chuanxiong* Hort and *Imperata cylindrica* (L.) Beauv. var. *major* (Nees) C.E.Hubb. are the main medicinal parts and have been commonly used in traditional medicines and even added to the diet. Rhizoma Chuanxiong has been recorded on the herbal list declared by Ministry of Health of China, in which 101 herbs can be used for medicinal drugs and health products. In addition, the material from the toothpaste producer shows that the effective components of rhizomes of Chuanxiong and Imperatae are higher than those of stems and leaves, and the medicinal value is also greater. Therefore, the rhizomes were chosen to make the extract.

The compatibility of Rhizoma Chuanxiong and Rhizoma Imperatae is also used in traditional medicine. It has been reported that this compatibility can promote blood circulation, protect the vasculature, and inhibit bacteria, so as to achieve the effect of hemostasis, anti-inflammation, pain relief, and so on [29]. Taken together, the compatibility of Rhizoma Chuanxiong and Rhizoma Imperatae indicates they could be potentially used in the prevention and treatment of gingival bleeding, gingivitis, and periodontitis. The purpose of this study was to investigate the clinical efficacy and safety of Chinese herbal toothpaste containing the extracts of Rhizoma Chuanxiong and Rhizoma Imperatae in supragingival plaque formation and gingivitis progress when compared to placebo toothpaste over a period of 3 months.

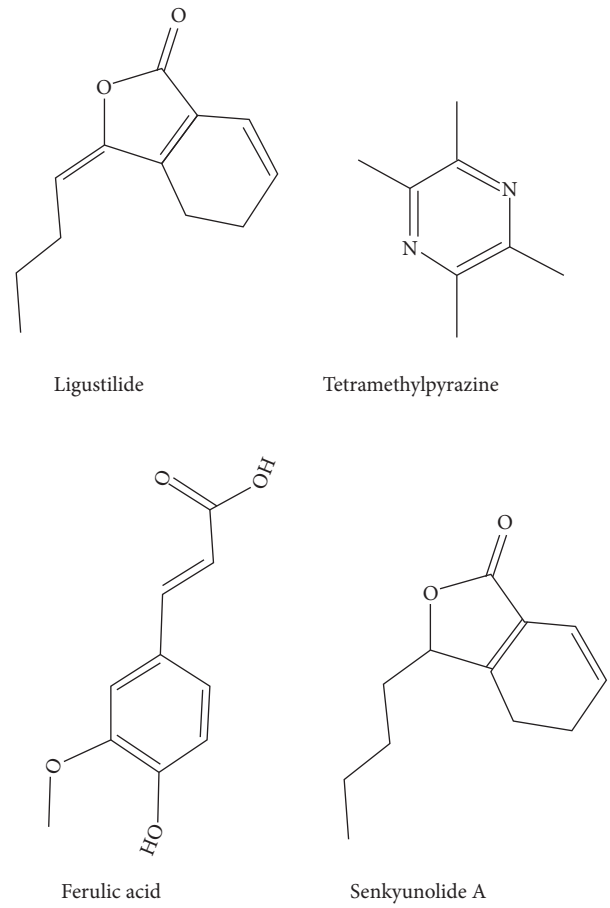

Figure 1: Chemical structures of main active monomers in Rhizoma Chuanxiong [19].

2. Materials and Methods

2.1. Study Design and Population. A 12-week randomized, double-blind, placebo-controlled clinical trial was conducted at West China Hospital of Stomatology, Sichuan University, Chengdu, China. The study protocol was approved by the institutional ethical board at West China College of Stomatology, Sichuan University (WCHSIRB-D-2017-078), and was in good accordance with the World Medical Association Declaration of Helsinki on ethical aspects and related regulations for clinical studies in China. This trial was registered as a clinical study (registration number: ChiCTR1800015742).

All participants in the study were volunteers. All voluntary participants were informed of the outline, purpose,

and duration of the study and signed an informed consent form before enrolment. 120 participants were enrolled in this clinical research based on the inclusion and exclusion criteria.

2.1.1. Inclusion Criteria. To be included in the trial, volunteers must be

(i) aged 18 to 70 years, male and female,

(ii) of good general health, having daily tooth-brushing habit,

(iii) possessing >20 natural permanent teeth that are uncrowned and at least 5 natural teeth in each quadrant,

(iv) diagnosed with gingivitis, GI ≥ 1 in 60% of the sites according to the Loe-Silness GI, having a whole-mouth mean PI ≥ 1.0 according to the modified Quigley and Hein index,

(v) signing the written consent before the initiation of the study and completing this clinical trial as required.

2.1.2. Exclusion Criteria. (i) Advanced periodontal disease, pulpitis, or open caries or soft tissue lesions

(ii) Allergy to the study product components

(iii) Usage of antibiotics, anticoagulant drugs, anti-inflammatory medication, or other drugs that may affect the results of the trial within the preceding 1 month

(iv) Wearing orthodontic bands or partial or removable dentures

(v) Pregnancy or breast-feeding

(vi) Receiving oral prophylaxis in the past 2 weeks

(vii) Participation in other similar tests in the past 3 months

2.1.3. Treatment Method. The toothpaste used in the both study groups was produced by Sichuan Green Herb Technology Development Co., Ltd. (Century City South Road, Hi-Tech Zone, Chengdu, Sichuan, China). The experimental toothpaste contained Rhizoma Chuanxiong extract, Rhizoma Imperatae extract, sorbitol, hydrated silica, water, glycerin, polyethylene glycol 400, sodium lauryl sulfate, carrageenan, xanthan gum, hydroxyethyl cellulose, saccharin, sodium benzoate, pigment CI42053, and edible saccharin. The extracts were extracted from the rhizomes by decocting method. 40-60 pieces of Rhizoma Chuanxiong and 35-70 pieces of Rhizoma Imperatae were mixed and crushed; after being heating-extracted by distilled water, the mixture was extracted by vacuum filtration. The extract was concentrated, vacuum-frozen, or spray-dried, and the traditional Chinese medicine composition with hemostatic, anti-inflammatory, and antibacterial effects was obtained. The best formulation was obtained by screening different formulations, and the results were verified by pharmacodynamic tests, so as to select the prescription.

Through random codes produced by SAS software, eligible subjects were block-randomized into the test group or the control group; allocation ratio was 1:1. Each group comprised 60 people. The baseline data were recorded and each participant was given a thorough scale using ultrasonic instruments to remove supragingival plaque. Then every patient was provided with the assigned toothpaste and the same adult soft-bristled toothbrush. The placebo toothpaste contained the same ingredients as the experimental toothpaste except for Rhizoma Chuanxiong extract and Rhizoma Imperatae extract. Toothpaste was in identical tubes; the labels were not revealed until the end of the study. Toothpaste was dispensed by an investigator not involved with clinical examinations. The participants were also not aware of the type of the toothpaste allocated to them. Every four weeks, the subjects were provided the new toothpaste and toothbrush according to number, and the used toothpaste would be collected. All participants who refrained from all other oral hygiene procedures during the study period were given professional brushing instructions and instructed to brush their teeth twice daily for 2-3 minutes.

At baseline, 4 weeks, 8 weeks, and 12 weeks, patients came to the clinical research center and were examined. All oral hygiene practices, such as brushing, flossing, and mouth-rinsing, were prohibited for 12 hours before examinations. Eating, drinking, and smoking were also prohibited for 4 hours before examinations. For clinical examinations, participants were instructed to refrain from brushing for about 12 hours before the clinic visits.

The examination was performed with CPI probe in the same clinical room. At baseline and the subsequent visits, three indicators were used to assess clinical efficacy. The Bleeding Index (BI) [30, 31] and the Gingival Index (GI) [32] were performed to assess the inflammatory state of the gums by an investigator. Then the Turesky modification of the Quigley-Hein Plaque Index (PI) [33, 34] was performed under the assistance of plaque indexes (GERMIPHENE, USA) to evaluate dental plaque by other investigators.

Safety observation indexes include (1) vital signs, such as blood pressure, respiration, body temperature, and heart rate, (2) adverse events and/or reactions that may occur, focusing on the presence of allergies and irritation (lips, buccal, tongue, and other soft tissue conditions), nausea, and so forth, and (3) suitability indicators of taste and tolerance.

2.2. Statistical Analysis. The therapeutic effect was evaluated by per-protocol set (PPS), and safety data set (SS) was used for safety evaluation. The management of the data was performed using the SAS 9.2 statistical program.

3. Results

According to the formula $n1=n2 = 2[(Z_{\alpha/2}+Z_{\beta})S/\delta]^2$ [35], considering the loss rate at 20%, sampling error, our manpower, and material resources, also referring to the standards of health industry of China and other toothpaste trails [36, 37], 120 participants eventually entered clinical trial and were equally allocated to the experimental group and the control group. A total of 108 cases entered the final statistical analysis, including 52 patients in the test group and 56 patients in the control group. The subjects failed to complete the study for some reason, which has nothing to do with the dentifrices. The study flow diagram is shown in Figure 2.

There were no statistically significant differences neither in age nor in gender between the two groups. There were also no statistically significant differences on PI, GI, BI, or BOP%

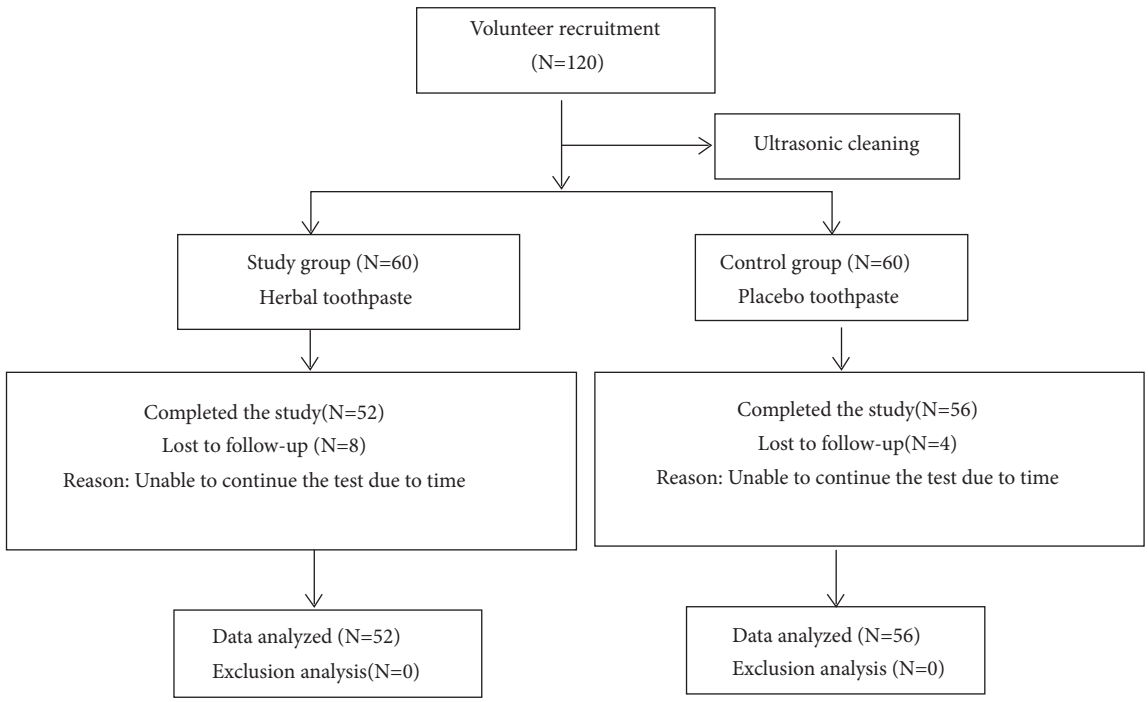

Figure 2: Flow of participants through each stage of the trial.

between the two groups (Table 1), which demonstrates that the group assignment was appropriate.

During the trial, both test and control groups showed a decreasing trend compared to the baseline. At the end of 12 weeks, with respect to GI, BI, and BOP% scores, there was a significant difference between test and control groups (GI, $P<0.001$, BI, $P<0.001$, and BOP%, $P<0.001$, resp.). However, the differences between two groups were not statistically significant with respect to all indexes at the 4-week and 8-week time intervals and the PI score between test and control groups at any time interval (Table 2).

The reduction percentage (mean ± Std.) for all groups and all parameters are given in Table 3. The difference between two groups was statistically significant with respect to GI, BI, and BOP% by the 8-12-, 4-12-week time intervals, and the reduction percentage of the test group was greater than that of the control group. However, there was no significant difference between test and control groups with respect to PI scores.

It was reported that there were significant variations in plaque accumulation within dentition, and tooth position within the alveolar bony strongly correlated with gingival recession [38, 39]. The efficiency differences among tooth types were evaluated when the trial was finished (Table 4). All three indexes were normally distributed ($P>0.05$) and their variances were also homogeneous ($P>0.1$). The results of ANOVA showed that there were significant differences between molar and premolar or anterior teeth with respect to GI and BI scores.

Furthermore, it was noticed that the severity at baseline throughout dentition varied at baseline examination. As a result, the correlation between the effect of toothpaste and the degree of gingivitis at baseline was examined, and significant positive correlation was found with respect to GI ($r=0.478$, $P=0.010$) and BI ($r=0.554$, $P=0.002$) scores, revealing that the more serious the baseline status, the better the effect of toothpaste (Figure 3).

As strict principles were undertaken, 42 cases of adverse events were reported, which included 25 cases in the control group and 17 cases in the test group. However, the researchers determined that their relationship with the use of toothpaste was "probably irrelevant" and not correlated with toothpaste adverse reactions. There was no statistically significant difference in the compliance (%) between the two groups of subjects (Table 5).

No bad/terrible-taste or hard-to-accept was reported in both groups, and the difference of the constituent ratio between two groups was not statistically significant with respect to taste and tolerance degrees ($P=0.648$, $P=0.829$).

4. Discussion

In the current study, the toothpaste containing the herbal ingredients Rhizoma Chuanxiong and Rhizoma Imperatae was tested for its efficacy and safety during 12 weeks of twice daily use in improving gingival and oral hygiene.

After 4 weeks of use, there were no statistically significant differences in the reduction of all parameters between the two groups ($P>0.1$). However, the reductions became statistically significant in the next two intervals (GI, $P<0.001$, BI, $P<0.001$, and BOP%, $P<0.001$, resp.). The efficiency of GI, BI, and BOP% was 8.04%, 11.02%, and 37.16%, respectively. The results demonstrated that the herbal toothpaste was effective in inhibiting gingivitis and oral health maintenance when

Table 1: Demographic data and examination status at the baseline.

Index		Control	Test	P value
Gender n (%)	Male	20 (35.7%)	20 (38.5%)	0.768[a]
	Female	36 (64.3%)	32 (61.5%)	
Age ($\overline{X}$±SD)		40.91±13.16	36.94±13.43	0.174[b]
PI ($\overline{X}$±SD)		2.66±0.54	2.69±0.47	0.797[c]
GI ($\overline{X}$±SD)		1.58±0.15	1.61±0.17	0.371[c]
BI ($\overline{X}$±SD)		2.15±0.35	2.24±0.43	0.252[c]
BOP (%)[d]($\overline{X}$±SD)		58.58±14.46	61.32±16.98	0.369[c]

[a] Chi-squared test.
[b] Wilcoxon rank-sum test.
[c] Independent samples *t* test.
[d] BOP% = bleeding sites on probing/total sites × 100%.

Table 2: PI, GI, BI, and BOP% at follow-up.

Index	Time point	Group	Mean	P25	P50	P75	Std.	Mean efficiency[c]	*P* value
Pi	4 weeks	Control	2.37	1.92	2.35	2.75	0.62	1.27%	0.411[a]
		Test	2.40	1.97	2.45	2.70	0.48		
	8 weeks	Control	2.27	1.91	2.35	2.71	0.60	4.85%	0.170[a]
		Test	2.38	1.93	2.34	2.83	0.52		
	12 weeks	Control	2.29	1.92	2.21	2.63	0.57	3.49%	0.232[a]
		Test	2.37	2.04	2.42	2.73	0.48		
GI	4 weeks	Control	1.12	1.03	1.10	1.16	0.12	3.57%	0.190[b]
		Test	1.16	1.04	1.12	1.24	0.15		
	8 weeks	Control	1.07	1.00	1.05	1.10	0.09	12.15%	0.487[b]
		Test	1.06	1.00	1.06	1.11	0.16		
	12 weeks	Control	1.12	1.04	1.10	1.19	0.10	-8.03%	<0.001*[b]
		Test	1.03	0.96	1.00	1.08	0.10		
BI	4 weeks	Control	1.20	1.05	1.15	1.27	0.19	4.17%	0.309[b]
		Test	1.25	1.07	1.18	1.36	0.25		
	8 weeks	Control	1.10	1.01	1.08	1.15	0.13	-0.91%	0.414[b]
		Test	1.09	1.00	1.08	1.15	0.11		
	12 weeks	Control	1.18	1.06	1.15	1.28	0.15	-11.02%	<0.001*[b]
		Test	1.05	0.97	1.01	1.12	0.13		
BOP%	4 weeks	Control	14.36	5.80	11.65	20.23	10.92	22.14%	0.199[b]
		Test	17.54	7.44	12.66	24.01	14.46		
	8 weeks	Control	9.55	4.32	7.14	12.79	8.03	-2.09%	0.409[b]
		Test	9.35	3.72	8.33	12.50	6.71		
	12 weeks	Control	15.61	8.48	14.88	21.40	8.76	-37.16%	<0.001*[b]
		Test	9.81	4.43	7.71	12.50	7.79		

∗ indicates statistical significance.
[a] Independent samples *t* test.
[b] Wilcoxon rank-sum test.
[c] Mean efficiency = (test - control)/control ∗ 100%.

compared to the negative control toothpaste. Moreover, a better efficacy was also found in the molars than the premolars and the anterior teeth. The effect has strong correlation with the severity at baseline with respect to GI (r=0.478, *P*=0.010) and BI (r=0.554, *P*=0.002) scores; the more serious the baseline status is, the better the effect is. It is indicated that more serious gingivitis may achieve a better improvement when treated with herbal toothpaste.

As an authentic herbal medicine of Sichuan, Rhizoma Chuanxiong was first recorded in the Divine Husbandman's Classic of the Materia Medica (*Shen Nong Ben Cao Jing*) more than 400 years ago. In traditional Chinese medicine beliefs, Rhizoma Chuanxiong has the effect of invigorating blood and expelling wind-damp as well as relieving pain. During the past years, valuable information has been obtained on its pharmacology. Rhizoma Chuanxiong mainly

Table 3: Percentage reductions in clinical indices at every follow-up.

Index	Time point	Group	Mean	P25	P50	P75	Std.	Mean difference[c]	*P* value
PI	8 weeks -4 weeks	Control	-3.88	-11.27	-7.00	4.69	13.47	3.24	0.105[a]
		Test	-0.67	-8.41	-3.96	6.64	12.90		
	12 weeks -8 weeks	Control	2.44	-7.04	2.97	12.18	13.64	-1.53	0.286[a]
		Test	0.91	-9.40	-2.08	11.96	14.33		
	12 weeks -4 weeks	Control	-2.22	-10.90	-4.25	7.74	14.96	1.28	0.307[a]
		Test	-0.94	-6.97	-1.73	4.56	11.18		
GI	8 weeks -4 weeks	Control	-4.41	-8.06	-4.14	1.61	7.30	-3.37	0.011[a]
		Test	-7.78	-14.22	-6.91	-1.27	7.68		
	12 weeks -8 weeks	Control	5.26	1.73	3.91	8.16	5.73	-8.55	<0.001*[b]
		Test	-3.28	-7.87	-3.99	0.60	6.03		
	12 weeks -4 weeks	Control	0.42	-4.62	0.28	3.91	7.16	-11.46	<0.001*[b]
		Test	-11.04	-15.46	-10.95	-5.83	6.70		
BI	8 weeks -4 weeks	Control	-6.44	-11.85	-4.93	-1.54	9.30	-4.04	0.019[a]
		Test	-10.50	-20.87	-9.65	-1.60	10.57		
	12 weeks -8 weeks	Control	7.44	2.23	5.47	11.71	8.34	-11.49	<0.001*[b]
		Test	-4.05	-8.42	-5.17	0.00	6.67		
	12 weeks -4 weeks	Control	0.18	-6.63	0.84	6.88	9.63	-14.57	<0.001*[b]
		Test	-14.39	-22.27	-13.36	-7.11	9.52		
BOP%	8 weeks 4 weeks	Control	-20.62	-65.68	-33.33	5.69	60.43	-7.78	0.257[b]
		Test	-28.40	-59.02	-43.65	-8.57	53.41		
	12 weeks -8 weeks	Control	158.66	21.37	68.11	167.80	231.47	-118.81	<0.001*[b]
		Test	39.85	-31.26	8.11	57.50	124.37		
	12 weeks -4 weeks	Control	48.33	-18.97	15.14	67.17	96.18	-73.14	<0.001*[b]
		Test	-24.81	-62.15	-40.27	-1.56	59.45		

∗ indicates statistical significance at p<0.05.
[a]Independent samples *t* test.
[b]Wilcoxon rank-sum test.
[c]Mean difference = test – control.

Table 4: Efficiency differences between tooth types at 12 weeks[a].

Index	Tooth types	Mean efficiency[b]	Efficiency difference[c]	*P* value
GI	Premolar	8.7%	-5.12%	0.013*
	Molar	13.8%		
	Anterior	7.1%	-6.73%	<0.001*
	Molar	13.8%		
	Anterior	7.1%	-1.61%	0.546
	Premolar	8.7%		
BI	Premolar	12.6%	-7.83%	0.003*
	Molar	20.5%		
	Anterior	9.6%	-10.88%	<0.001*
	Molar	20.5%		
	Anterior	9.6%	-3.04%	0.291
	Premolar	12.6%		

∗ indicates statistical significance at p<0.05.
[a]ANOVA.
[b]Mean efficiency = (test - control)/control ∗ 100%.
[c]Efficiency difference means the difference between two groups.

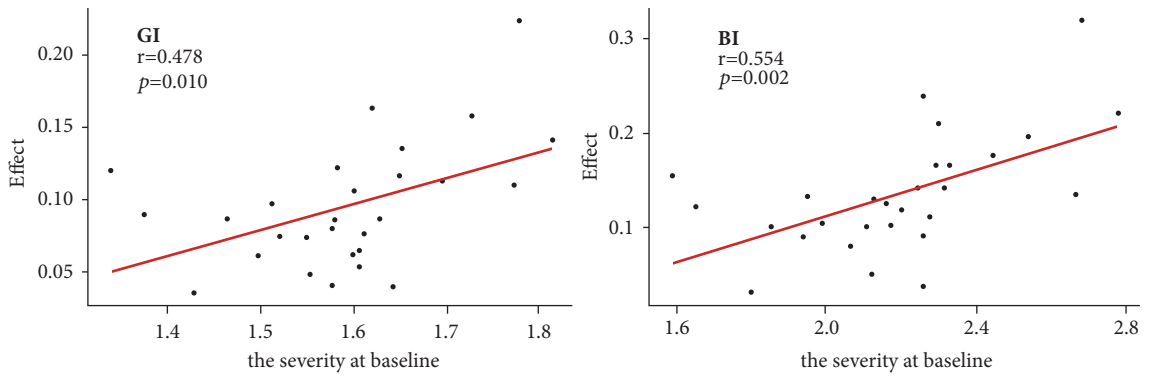

Figure 3: The correlation between effect of toothpaste and the severity at baseline. Efficiency = (control-test)/control∗100%.

Table 5: Comparison of adverse events and compliance (SS)[a].

Index		Test	Control	*P* value
Adverse event	No	42 (71.2%)	33 (56.9%)	0.107
	Yes	17 (28.8%)	25 (43.1%)	
<80%		1 (1.9%)	3 (5.4%)	0.664
80%~120		51 (98.1%)	53 (94.6%)	

[a] Chi-squared test.

contains organic acid, essential oils, and nitrogenous compounds. Tetramethylpyrazine, senkyunolide A, ferulic acid, and ligustilide are the main effective components [19]. Their main pharmacological effects include removal of oxygen free radical, antibiosis and anti-inflammatory, which was reported to increase of immune function, anti-platelet and can promot blood circulation, etc [40–46]. For anti-inflammatory activity, the modern pharmacological research discovered that Z-ligustilide and senkyunolide I exerted a potential anti-inflammatory effect on microglia through inhibition of NF-kappa B pathway [41, 47, 48]. Another study showed that the Rhizoma Chuanxiong essential oil fraction, senkyunolide H and senkyunolide O, inhibited significantly the production of nitric oxide and proinflammatory mediators such as IL-1β, IL-6, and TNF-α and also reduced the expression levels of cyclooxygenase-2 (COX-2) as well as inducible nitric oxide synthase (iNOS) [45, 49, 50]. For antioxidant activity, Z-ligustilide was reported to show a comprehensive antioxidant effect on the spontaneous oxidation of linoleic acid, mitochondrial oxidation, homogenate spontaneous oxidation, and oxidation induced by H_2O_2 [51]. Besides, tetramethylpyrazine was reported to significantly remove free radicals, effectively alleviate the oxidative stress, and decrease the reactive oxygen species formation induced by gentamicin [52, 53]. In addition, it was reported that senkyunolide H and senkyunolide I possibly attenuated oxidative damage by activating the HO-1 pathway and enhanced the cell resistance to oxidative damage related to hydrogen peroxide [54]. The blood circulation improvement effect of Rhizoma Chuanxiong was reported to be related with tetramethylpyrazine because it ameliorated platelet activation, aggregation, and adhesion. This procedure induced sustained infiltration and activation of various inflammatory cells, including lymphocytes and eosinophils [46].

Rhizoma Imperatae is also a common Chinese herbal medicine, of which the main components include cylindrin, carotene, 5-hydroxytryptamine, potassium, and calcium. Modern pharmacological research shows that Rhizoma Imperatae mainly has functions of hemostatic, diuresis, antihypertensive, bacteriostasis, anti-inflammatory, analgesic, and antitumor, reducing hydroxyl radical, antioxidant, and enhancing immunity [26, 27]. The extract of dry *Imperata cylindrica* (L.) was reported to show antimicrobial activity on *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [55]. The methanol extracts from the leaves of *Imperata cylindrica* (L.) were reported to show the anticancer activity in human oral cancer cell line [56]. Isoeugenin revealed the anti-inflammatory effects on LPS-activated RAW264.7 macrophages by inhibiting nitric oxide (NO) formation [57]. The polysaccharides from Rhizoma Imperatae showed high antioxidant activity including hydroxyl radical scavenging activity and 2,2-diphenyl-beta-picrylhydrazyl radical scavenging activity [58]. At present, there is a potential use of its hemostatic principle, and the Rhizoma Imperatae extract has been added to oral care products, which showed excellent effect on reducing gingival bleeding [28].

Existing theories show that Rhizoma Chuanxiong companied with Rhizoma Imperatae has great application potential and value in the treatment of gingivitis. The efficacy of the variety of Chinese herbal toothpaste has been studied previously. However, there are few standardized clinical studies on Rhizoma Chuanxiong or Rhizoma Imperatae regarding the extract-containing toothpaste at present. The

results from the current clinical study could not be directly compared with similar studies. And this is the first study reported in the medical literature which explores the effects of toothpaste containing Rhizoma Chuanxiong and Rhizoma Imperatae extracts on dental plaque and gingivitis. Effects of herbal toothpaste on reducing plaque formation and gingival inflammation were investigated in clinical studies and results were equivocal, which demonstrated significant plaque reductions ranging from 7.17% to 61.2% and gingivitis reductions ranging from 5.20% to 70.6% [59–61].

When the study ended, both types of toothpaste were effective in decreasing plaque and gingivitis parameters compared with the baseline. And the experimental group showed a decreased trend, while the control group went up at the end of 12 weeks. The reduction in indexes in the control group may be attributed to ultrasonic cleaning and repeated oral education, which probably improve oral health a lot. However, this influence was temporary, and the herbal toothpaste can help maintain this improvement. Moreover, the current study showed a better improvement in the molars. And the more serious the baseline status is, the better the effect is. It could be speculated that the molar is not easy to be cleaned resulting in worse health condition, and the herbal toothpaste has a broad application prospect in the treatment of gingivitis, especially severe inflammation. The reduction of dental plaque between test and control groups has no significant difference, while the bleeding improved a lot in test group. These phenomena may be attributed to the anti-inflammatory blood circulation, which improves the functions of Rhizoma Chuanxiong and Rhizoma Imperatae; even no obvious antibacterial effect was observed or reported. All of these may provide the theoretical basis of promoting blood circulation and removing stasis in traditional Chinese medicine. In addition, the observed effect in present study may be attributed to participants' awareness of enrolling in oral hygiene study—Hawthorne effect, no matter what toothpaste they receive. Therefore, extending observation time to a longer period will provide a more powerful comparison.

As the placebo-controlled setting can minimize the subjective expectation effect and bias of subjects and researchers and directly measure the difference in efficacy and safety between the test drug and placebo, it can give the appropriate conclusion of the test drug with a smaller sample. For the positive control, there is little difference between the efficacy of the test drug and that of the positive control drug, so a larger sample is needed to achieve the same efficacy in order to detect the difference between the two drugs. It would be better to set both placebo control and positive control, but, considering our study purpose, the test conditions, manpower, and material resources, only placebo control was chosen, which was the limitation of our study. We would add "positive control" group in our similar clinical trials in the future.

5. Conclusion

Within the limits of this clinical study, regular use of the herbal toothpaste containing Rhizoma Chuanxiong and Rhizoma Imperatae extracts could effectively and safely reduce gingivitis of the study subjects. There is a better improvement at molars, and the more serious the baseline status is, the better the effect is. Further long-term studies are needed to confirm its benefits.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

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

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Supplementary Materials

In the Supplementary Materials, we present the adverse events report which could not be included in the manuscript because of space limitations. The adverse events report showed the details of every adverse event reported including the description, duration, and any treatment undertaken. The relationship between the adverse events and the use of toothpaste was confirmed to be "probably irrelevant" and not correlated with toothpaste adverse reactions. *(Supplementary Materials)*

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Research Article

Snakehead Consumption Enhances Wound Healing? From Tradition to Modern Clinical Practice: A Prospective Randomized Controlled Trial

Nik Amin Sahid,[1] **Firdaus Hayati**,[1] **Challa Venkata Rao**,[1] **Rosnelifaizur Ramely**,[2] **Ikhwan Sani**,[2] **Andee Dzulkarnaen**,[2] **Zaidi Zakaria**,[2] **Syed Hassan**,[2] **Arman Zahari**,[3] **and Aishath Azna Ali**[4]

[1]*Surgery Department, Faculty of Medicine and Health Sciences, Universiti Malaysia Sabah, 88800 Kota Kinabalu, Sabah, Malaysia*
[2]*Surgery Department, School of Medical Sciences, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*
[3]*Plastic & Reconstructive Surgery Department, School of Medical Sciences, Hospital Universiti Sains Malaysia, 16150, Kubang Kerian, Kelantan, Malaysia*
[4]*Surgery Department, Indra Gandhi Memorial Hospital, Kanbaa Aisa Rani Higun, Malé, Maldives*

Correspondence should be addressed to Nik Amin Sahid; nike_opo@ums.edu.my and Rosnelifaizur Ramely; faizur@usm.my

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Background. Snakehead fish (*Channa striatus)* is a fresh water fish indigenous to many Asia countries and believed to have medical value. Studies showed that it contains all the essential amino acids and fatty acids able to accelerate wound healing and it has antinociceptive effect. However, little human study has been done to assess the effectiveness of *Channa striatus* in wound healing. A prospective RCT has been conducted on the effect of *Channa striatus* spray versus placebo on clean wound to assess its pain control effect and cosmetic outcome. *Methodology.* One hundred and two patients (102) underwent clean elective surgery; postoperatively they were randomized into two group. One group received *Channa striatus* extract spray (n=51) another group received placebo (n=51) on daily basis for 2 weeks. They were followed up on 2nd, 4th, and 6th weeks. Pain control effect was assessed based on Visual Analog Pain Score (VAPS) and cosmetic outcome based on Visual Analog Cosmetic Scale (VACS), Wound Evaluation Scale (WES), and Vancouver Scar Scale (VSS). *Result.* The patient treated with *Channa striatus* spray displayed a better outcome in terms of pain control compared to placebo. During analysis using repeated measure ANOVA, there was significant difference of patient's pain score based on VAPS between *Channa striatus* spray and placebo (F-stat (df) = 4.80 (2), p-value = 0.010). For cosmetic outcome it showed a better result in *Channa striatus* spray group for all the 3-scoring system, VACS, (F-stat (df) = 2.68 (2) , p-value <0.001), WES (F-stat (df) = 3.09 (2), p-value = 0.048), and VSS (F-stat (df) = 1.72 (2) , p-value = 0.011). *Conclusion.* Our study suggest that application of *Channa striatus* extract spray on clean wound has shown a significant better pain score result and cosmetic outcome on week 2, week 4, and week 6 comparatively with placebo.

1. Introduction

Snakehead fish (*Channa striatus)* flesh is claimed to be rejuvenating, particularly in recuperation from serious illness and in a postnatal diet. In Malaysia, it has always been a strong belief that *Channa striatus* enhances wound healing and is a very powerful tool for recovery of health and injury. Since 1931 there has been in Malaysian literature discussion about wound treatment using *Channa striatus*. Several studies showed that it contained all the essential amino acids and fatty acids (Table 1) which uniquely are capable of accelerating the wound healing [1, 2].

Despite the wide-spread uses of this fish for medicinal purposes, very little studies to establish the scientific basis for it claimed wound healing effects. In an animal study, *Channa striatus* extract has been shown to increase the tensile strength of the surgically stitched wound. It also has been formulated into aerosol/spray for drug delivery system to

Table 1: Composition of amino acids and fatty acid in *Channa striatus* extract.

	Fillet	Roe	Mucus
Amino acids	Glycine Glutamic acid Arginine Aspartic acid	(No study)	(No study)
Fatty acids	Eicosapentaenoic Acid (EPA) Docosahexaenoic Acid (DHA) Palmitic acid Oleic acid Stearic acid Arachidonic acid	Eicosapentaenoic Acid (EPA) Docosahexaenoic Acid (DHA) Hexadecanoid acid Oleic acid Linoleic acid	Oleic acid Linoleic acid

Adopted from [12].

wound and burn treatment [3, 4]. Evaluation of the film properties from concentrate of aerosol had been done in other study [5]. But the effect on human has not been done yet. Therefore the effects of *Channa striatus* extract in aerosol form on clean surgical wounds are evaluated in this study.

1.1. Snakehead Fish (Channa striatus). *Channa striatus* is a fresh water species which is also known as snake head fish or known as Haruan in Malay. It belongs to Channidae family and it is carnivorous fish.

1.2. Traditional Belief on Channa striatus. Traditionally Chinese and Malay community believes that eating *Channa striatus* during postpartum period is enhancing wound healing. It also believe that *Channa striatus* acts as energy booster meal based on a study done among Chinese respondents in a Kuala Lumpur maternity hospital involved questions on the consumption of *Channa striatus* [6].

1.3. Nutrition Composition of Channa striatus. Study found that *Channa striatus* extract is rich in amino acids, a nonessential amino acid which is glutamic acids, arginine, and aspartic acid [7]. Others were listed in the table below.

1.4. Composition of Channa striatus Spray. Snakehead fish (*Channa striatus)* water extract has been formulated in an aerosol system which can produce a film for wound dressing. It was manufactured by Skin Fix Company. Snakehead fish (*Channa striatus)* spray has been evaluated for the possibility of causing irritation reaction or toxic response; however from three experiments carried out to evaluate the safety of Snakehead fish (*Channa striatus)* spray which are Primary Skin Irritation test, Intracutaneous test, and Systemic Injection test, the result shows that Snakehead fish (*Channa striatus)* spray gave no significant responses to all the above tests [5, 8]. In 2011, Febriyenti has formulated an aerosol concentrate containing a mixture of Snakehead fish (*Channa striatus)* extract and a film-forming polymer. The concentrate when sprayed on the wound formed a thin layer of dressing and the added Snakehead fish (*Channa striatus)* extract proved to enhance the healing process as proven by Baie and Sheikh who studied the wound healing effect of *C. striatus* on *Sprague-Dawley* rats [9].

1.5. Channa striatus in as Antimicrobial. As a part of the wound healing process, antimicrobial activity is equally important. The antimicrobial properties of the skin and intestinal mucus of different *Channa* sp., *namely, C. striatus, Channa micropeltes, Channa marulius, Channa punctatus,* and *Channa gachua* have been studied by CARE research team. The investigation showed a broad spectrum of antibacterial activity of skin mucus against *Aeromonas hydrophila*, *Pseudomonas aeruginosa,* and *Vibrio anguillarum* [10].

1.6. Antinociceptive Properties Snakehead Fish (Channa striatus). The analgesic or antinociceptive effect were being studied by a few researchers. For instance, Mat Jais et al. (1997) investigated the antinociceptive effects in mice with a view to establishing the scientific basis of pain-relieving activities where the study showed that both the fillet and mucus of *Channa striatus* were found to exhibit a concentration dependent antinociceptive activity [11]. There are evidences for arachidonic acid of haruan enhancing the activity of other antinociceptive agents such as morphine [11].

1.7. Rationale of Study. Clean surgery procedure is one of major bulks of general surgery work load. It occupies almost 40% of all elective case. Postoperative pain and cosmetic outcome are of major concern for the patient and potentially debilitating. Previous animal study has proven that Snakehead fish (*Channa striatus)* extract has improved tissue healing. However there are only few human studies done regarding the effect of Snakehead fish (*Channa striatus)* spray on cosmetic outcome and pain control. It is clinically useful if we can identify the effectiveness of Snakehead fish (*Channa striatus)* extract spray on clean wound, which can be potentially extended to clean contaminated wound in future.

2. Methodology

2.1. Study Subject/Source Population. This is a randomized, prospective, clinical study to evaluate the effect of topical administered Snakehead fish (*Channa striatus)* extract in aerosol/spray form with the effect of placebo (spray without Snakehead extract). Subjects were recruited from Clinic of General Surgery (SOPD), Universiti Sains Malaysia (convenience based sampling). Patients were scheduled for an elective operation with clean incisional wounds that were primarily sutured. They were subjected to face to face interview to enquire about their suitability of the study. Eligible subjects consented to participate were randomly assigned to one of the two groups:

Group 1: subjects received Snakehead fish (*Channa striatus)* spray.

Group 2: subjects received placebo spray.

Subject must fulfill each of the following criteria.

Inclusion Criteria

(a) Age ≥ 18 and ≤ 50 years

(b) Subject who has given written informed consent to participate in the study and understand the nature of the study

Exclusion Criteria

(a) Taking any form of herbal extract in the last 3 months before study entry and during the study period

(b) History of drug or alcohol abuse

(c) Patient taking warfarin or heparin

(d) Clinical relevant cardiovascular, gastrointestinal, hepatic, neurologic, endocrine, hematologic, connective tissue disease or other major systemic diseases that would influence the interpretation of results

(e) Patients with medical disorder requiring steroid or immunosuppressive therapy with delay wound healing

(f) Patient with chronic cough or other condition which may cause a rise in intra-abdominal pressure

(g) Presence of any congenital anterior abdominal wall defects

(h) Patient with evidence of secondary infection after treatment

(i) Mental condition rendering the subject unable to understand the nature, scope, and possible consequences of the study

(j) Evidence of uncooperative attitude, including poor compliance including inability to attend follow-up visit

2.2. Method of Assigning Subjects to Treatment and Placebo Groups. Subject eligibility was established before treatment randomization. Subjects' number was allocated strictly sequentially, as subjects were eligible for randomization. A randomization method using randomization software is at www.randomization.com. Number that has been chosen by the software will determine whether the patient will get either treatment A or treatment B. None of the investigators knows the randomization scheme.

2.3. Blinding and Procedures for Breaking the Blind. This was double blinded study and once a subject has been randomized, the study treatment that they received was not be known by both the subject and the investigator.

2.4. Patient's Withdrawal. The investigator may cease study treatment and withdrew the subject or the subject may withdraw herself from participation in the study at any time. The reason for the withdrawal of a patient will be recorded in the case report form. Subject were followed-up for a minimum of 42 days (6 weeks) following the last dose of study drug.

Possible reasons for patient withdrawal include the following:

(a) The need to take medication may interfere with study measurements.

(b) Patient experiences an intolerable/unacceptable adverse event.

(c) Patient exhibits noncompliance with the protocol.

(d) Patient unwilling to proceed and/or consent is withdrawn.

(e) Investigator withdraws patient for reasons unrelated to the study drug (e.g., undercurrent illness)

3. Materials

3.1. Investigational Products. The topical administered Snakehead fish (Channa striatus) spray and placebo (spray without Snakehead fish (Channa striatus) extract) were prepared in GMP Laboratory, School of Pharmacy Universiti Sains Malaysia. The preparation of the concentrates followed the method that was described in detail in previous study [5].

3.2. Doses and Treatment Regimens. The treatment group were sprayed with Snakehead fish (*Channa striatus)* spray once a day while the placebo group were sprayed with placebo spray (spray without Snakehead fish) (*Channa striatus)* extract once a day.

3.3. Ethical Clearance. Ethical clearance has been obtained from Human Research Ethical Committee USM (HREC), USM/JEPeM/1403124

3.4. Data Collection Procedure. Basic demographic data were collected from the patient and surgical procedure, indication, and method of wound closure were gathered. The wound assessment was performed by clinical assessment using

Visual Analog Cosmetic Scale (VACS), Wound Evaluation Scale (WES), and Vancouver Scar Scale (VSS) by the investigators and also by the patient using Visual Analog Pain Score (VAPS). A photo of the wound was taken serially at every visit and assess by two independent investigators using VACS, WES, and VSS. Snakehead fish (*Channa striatus)* spray or placebo spray were used to protect the wound after postoperative wound inspection. All subjects were instructed to take normal diet during the study period and were not be allowed to take any other herbal products orally or consume *C. striatus*. The cosmetic assessments of the wound were done by the investigator who is part of the Clinical Trial Team. It was done on week 2, week 4, and week 6 after operations. Subjects were thoroughly examined by medical specialists or medical officers who are part of the Clinical Trial Team at every visit.

3.5. Sample Size Determination. The sample sizes are calculated based on two means formula (using G Power software), the power of the study taken at 90% and alpha (type one error) as 0.5%. The calculations are based on previous study [2],

Power = 90%

Type 1 error (α) = 0.5%

SD = 14mm

Expected detectable of mean difference between group = 10mm

The sample size required for both study limb = 92

Assuming 10% dropped out rate = 10

Total number participants required for the study = 102

Computer calculation

F tests-ANOVA: repeated measures, between factors

Actual power = 0.901176

4. Results

4.1. Description of Demographic Data. The demographic data of our study patient were summarized in Table 2 . Out of 102 patients, only 81 patients completed follow-up and were analyzed. The mean age was 39 years (SD 8.89) for Snakehead fish (*Channa striatus)* spray and 41 years (SD 8.95) for placebo. 63 (77.7%) were male patient. Majority of the patient were Malay ethnic (98.8%).

There are 3 types of surgery involved in this study which are hernioplasty, excision biopsy, and thyroidectomy. Majority of our patients undergo hernioplasty (n 61, 76.5%), while 17.2% undergo excision biopsy (n=14) and 6.1% undergo thyroidectomy (n=5).

4.2. Comparison of Visual Analog Pain Score (VAPS) between Snakehead Fish (Channa striatus) Spray and Placebo Based on Time. Comparison of Visual Analog Pain Score (VAPS) between Snakehead fish (*Channa striatus)* spray and Placebo based on time is measured by repeated measure ANOVA shown in Table 3 . There was significant difference of patient's pain and score based on VAPS between Snakehead fish (*Channa striatus)* spray and placebo (F-stat (df) = 4.80 (2), p-value = 0.010)

Table 2: Demographic distribution (n=81).

	Haruan Spray (n=41)	Placebo (n=40)
Variable	Frequency (%)	
Gender		
Male	30 (73.2)	33 (82.5)
Female	11 (26.8)	7 (17.5)
Age (years)*	39.4 (8.89)	41.2 (8.95)
Race		
Malay	40 (97.6)	40 (100.0)
Chinese	1 (2.4)	0 (0.0)
Type of surgery		
Hernioplasty	29 (70.7)	33 (82.5)
Excision biopsy	9 (22.0)	5 (12.5)
Thyroidectomy	3 (7.3)	2 (5.0)

Table 3: Comparison of VAPS between Snakehead fish (*Channa striatus)* spray and Placebo based on time (n=81).

Time	Group	Mean	95% Confidence Interval	
Week 2	*Channa striatus* spray	0.79	0.54	1.05
	Placebo	1.33	1.09	1.56
Week 4	*Channa striatus* spray	0.12	0.04	0.28
	Placebo	0.45	0.30	0.60
Week 6	*Channa striatus* spray	0.03	0.01	0.10
	Placebo	0.10	0.01	0.19

RM Anova: F-stat (df) = 4.80 (2), p-value = 0.010.

Estimated marginal means of visual analog score on weeks 2, 4, and 6 were plotted and it shows significant difference between Snakehead fish (*Channa striatus)* spray (mean=0.80, CI 0.61-0.99) and placebo (mean 1.26, CI 1.07-1.45) (Figure 2).

4.3. Comparison of Visual Analog Cosmetic Score (VACS) between Snakehead Fish (Channa striatus) Spray and Placebo Based on Time. The change in visual analog score between the initial, mid, and final follow-up attempted was analyzed using repeated measures ANOVA controlling for sample (placebo or trial); the interaction between both groups was significant: F-stat (df) = 2.68 (2), p-value <0.001 (Table 4). Mean parameter estimates are shown in Figure 3. Improvement of the score increased as the time of follow-up increased from 2^{nd} to 4^{th} to 6^{th} week. There was significant difference in estimated improvement between 4^{th} and 6^{th} week of follow-up completed. Figure 3 shows the estimated marginal means for the first mid and final follow-up score.

4.4. Comparison of Vancouver Scar Scale (VSS) between Snakehead Fish (Channa striatus) Spray and Placebo Based on Time. According to Vancouver Scar Scale, the worse

Table 4: Comparison of VACS between Snakehead fish (*Channa striatus)* spray and placebo based on time (n=81).

Time	Group	Mean	95% Confidence Interval	
Week 2	*Channa striatus* spray	6.65	6.28	7.02
	Placebo	6.39	6.04	6.73
Week 4	*Channa striatus* spray	7.65	7.29	8.00
	Placebo	6.74	6.41	7.08
Week 6	*Channa striatus* spray	8.41	8.08	8.74
	Placebo	7.46	7.15	7.77

RM ANOVA: F-stat (df) = 2.68 (2), p-value <0.001.
Repeated measure ANOVA between group analyses with regard to time was applied.

Table 5: Comparison of VSS between Snakehead fish (*Channa striatus)* spray and placebo based on time (n=81).

Time	Group	Mean	95% Confidence Interval	
Week 2	*Channa striatus* spray	3.97	3.39	4.56
	Placebo	4.35	3.81	4.89
Week 4	*Channa striatus* spray	2.50	1.97	3.03
	Placebo	3.48	2.98	3.97
Week 6	*Channa striatus* spray	1.68	1.24	2.12
	Placebo	2.48	2.07	2.88

RM ANOVA: F-stat (df) = 1.72 (2), p-value = 0.011.
Repeated measure ANOVA between group analyses with regard to time was applied.
Assumptions of normality, homogeneity of variances, and compound symmetry were checked and fulfilled.

cosmetic outcome score is 13. In our study, we found the mean score for Snakehead fish (*Channa striatus)* spray in week 2 is 3.97 (CI 3.39-4.56) which is lower compared to placebo 4.35, (CI 3.81-4.89). Overall, there was significant difference of mean resultant scars based on VSS between Snakehead fish (*Channa striatus)* spray and placebo (F-stat (df) = 1.72 (2), p-value = 0.011) as in Table 5 and Figure 4.

4.5. Comparison of Wound Evaluation Scale (WES) between Snakehead Fish (Channa striatus) Spray and Placebo Based on Time. There was significant difference of mean wound healing based on WES between Snakehead fish (*Channa striatus)* spray and placebo (F-stat (df) = 3.09 (2), p-value = 0.048) as in Table 6 and Figure 5.

5. Discussion

Snakehead fish (*Channa striatus)* has been used in traditional wound healing remedy for decades ago until today. It has been utilized not only in Malaysia, but also in most of country in South East Asia. In Malaysia, Malays believe that eating Snakehead fish (*Channa striatus)* during postdelivery period will enhance the recovery of the wound.

The effectiveness of topical application of Snakehead fish (*Channa striatus)* cream has been reported before. It shows

Table 6: Comparison of WES between Snakehead fish (*Channa striatus)* spray and Placebo based on time (n=81).

Time	Group	Mean	95% Confidence Interval	
Week 2	*Channa striatus* spray	4.06	3.68	4.44
	Placebo	3.75	3.40	4.10
Week 4	*Channa striatus* spray	5.06	4.78	5.33
	Placebo	4.33	4.07	4.58
Week 6	*Channa striatus* spray	5.62	5.38	5.86
	Placebo	4.95	4.73	5.17

RM ANOVA: F-stat (df) = 3.09 (2), p-value = 0.048.
Repeated measures ANOVA between group analyses with regard to time was applied. Assumptions of normality, homogeneity of variances, and compound symmetry were checked and fulfilled.

that it does enhance wound healing by increasing tensile strength and increases fibroblast count and hydroxyproline level [9]. However, there is no article that describes the effectiveness of topical Snakehead fish (*Channa striatus)* spray on human. Thus, we decided to conduct a clinical trial on the effectiveness of Snakehead fish (*Channa striatus)* extract spray on clean surgical wound and we observed its pain control and cosmetic outcome.

In our study, we recruited 102 patients randomized into two group: treatment group A (n=51) and group B placebo (n=51). Patient was subsequently followed up on second, fourth, and sixth week in our surgical clinic for photographic and pain score assessment. We utilize the local product of Snakehead fish (*Channa striatus)* extract spray in our study. It has been manufactured by Skin Fix Company. Meanwhile we used Opsite spray as a placebo. Both has transparent and odorless spray droplet. Twenty-one patients were excluded due to not compliance to medication and defaulted follow-up. We then evaluated remaining 81 patients who completed follow-up on the effect of Snakehead fish (*Channa striatus)* extract spray on clean wound particularly pain and cosmetic effect postoperatively. Group A, the treatment group (n=41), and group B, the placebo group (n=40), receive Opsite spray.

For pain assessment we use Visual Analog Pain Score (VAPS). In our study we have proved that topical application of Snakehead fish (*Channa striatus)* extract does improve local analgesic effect in second, fourth, and sixth week postoperative period. The patients treated with Snakehead fish (*Channa striatus)* spray display a better outcome in terms of pain control compared to placebo. During analysis using repeated measure ANOVA, there was significant difference of patient's pain score based on VAPS between Snakehead fish (*Channa striatus)* spray and placebo (F-stat(df) = 4.80 (2), p-value = 0.010). Initial mean of visual analog score on week 2 shows significant difference between Snakehead fish (*Channa striatus)* spray (mean=0.80, CI 0.61-0.99) and placebo (mean 1.26, CI 1.07-1.45).

This result also shows similar mean pattern for the week 4 and week 6 as shown in Figure 1. This is consistent with previous study [11, 13]. The proposed mechanism is that lipoamino acid and n-arachidonoyl glycine suppress the pain sensation by modulating the pain transmitter in the synaptic cleft [14, 15]. It was believed that extracts also

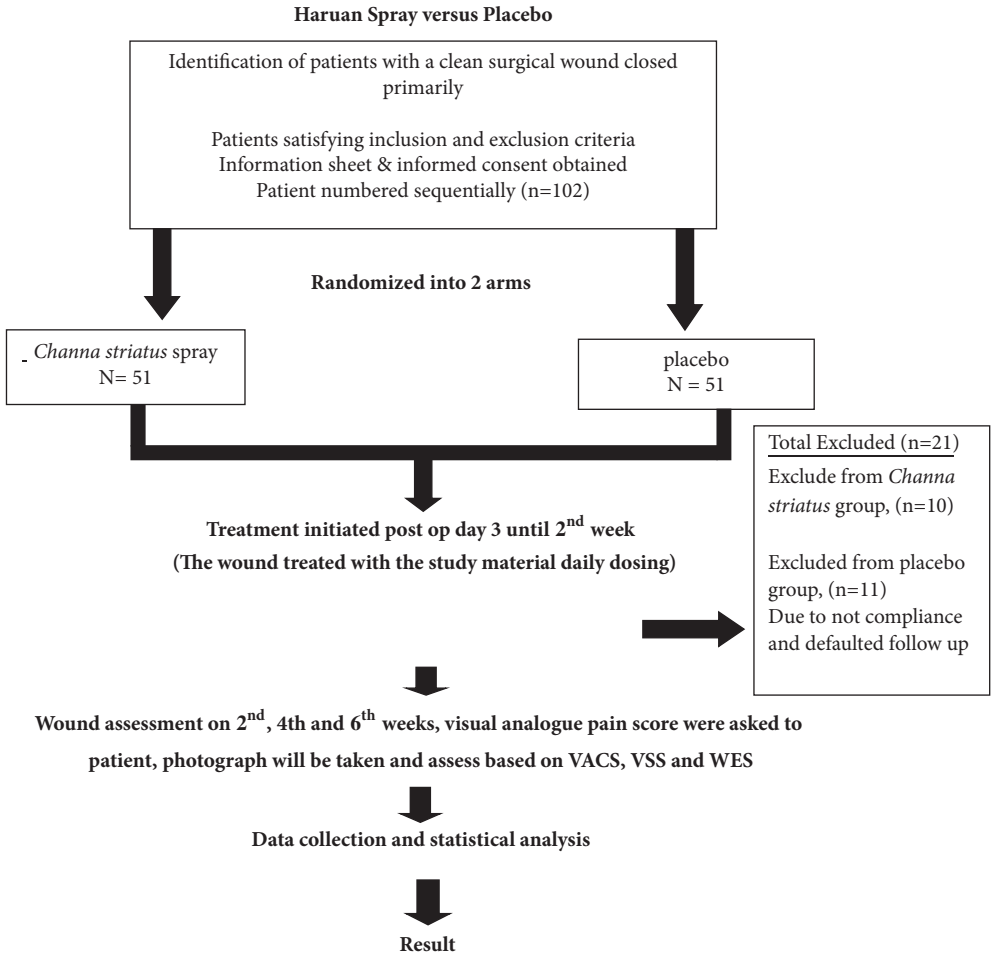

Figure 1: Flowchart of the study.

enhance the activity of morphine [11, 15]. Apart from that, high concentration of arachidonic acid in Snakehead fish (*Channa striatus)* extract which also has functions in the antinociceptive pathways, was found in many studies [16, 17]. Previous study shows that application of Snakehead fish (*Channa striatus)* in post-C-section patient shows that it improves the pain control outcome [18].

In terms of cosmetic outcome, there are various methods of assessing the wound. In our study we used Visual Analog Cosmetic Scale (VACS), Wound Evaluation Scale (WES), and Vancouver Scar Scale (VSS) which have been validated in previous study [19, 20]. In our study, we found that the cosmetic outcome shows a consistent significant better cosmetic result in Snakehead fish (*Channa striatus)* spray group for all the 3-scoring system which is VACS (F-stat(df) = 2.68 (2), p-value <0.001), WES (F-stat(df) = 3.09 (2), p-value = 0.048), and VSS (F-stat(df) = 1.72 (2), p-value = 0.011). In our study also we observe a consistent result between WES and VAS as both show similar result pattern with minimal clinical important different (MIDC) as was describe in previous study [19]

Many studies have been published regarding the specific element of Snakehead fish (*Channa striatus)* especially its meat and roe. The high content of essential amino acid and fatty acid is the main factor that contributes to speedy recovery of the wound [10, 16, 21]. These two components are reported to promote wound healing. It initiates collagen synthesis and reepithelialization in the healing wound. *Arginine* is also one of the potent amino acids that promote wound healing [22]. The polyunsaturated fatty acid (PUFA) has an important role in immune respond in healing process [23, 24]. It is an important component of cell plasma membrane synthesis (biphospholipid layer) [25]. PUFA is also an important substrate of production of *prostaglandin, thromboxane, leukotrienes,* and *lipoxin* synthesis [25]. Deficiency of this component will slow down the healing process [17].

We have 1 case of hypertrophic scar in Snakehead fish (*Channa striatus)* spray group, and none was seen in placebo group. However there is no case of keloid seen in either group. Hypertrophic scars are define as raised fibrous connective tissue in the dermis and adjacent subcutaneous tissue after traumatic or burn wound healing [26]. It is due to excessive accumulation of scar collagen and presence of abundance myofibroblast cell, a contractile cell [6]. Few studies have shown that *Channa striatus* application to the wound increases the tensile strength [1, 5].

Out of 81 patients, none was found to have surgical site infection (SSI). The rate for SSI in previous study is up to 2.1%

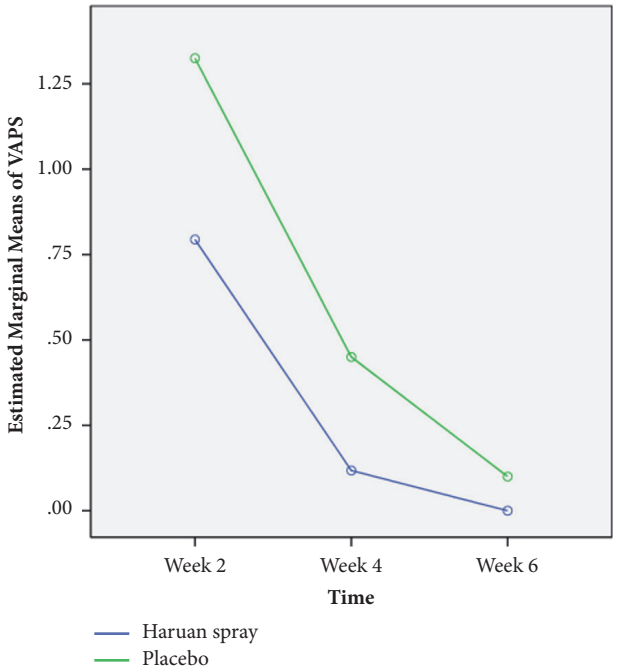

Figure 2: Comparison of estimated mean (estimated marginal means) of VASP for week 2, week 4, and week 6 interventions between Snakehead fish (*Channa striatus)* spray and placebo by comparing repeated measures ANOVA (n=81).

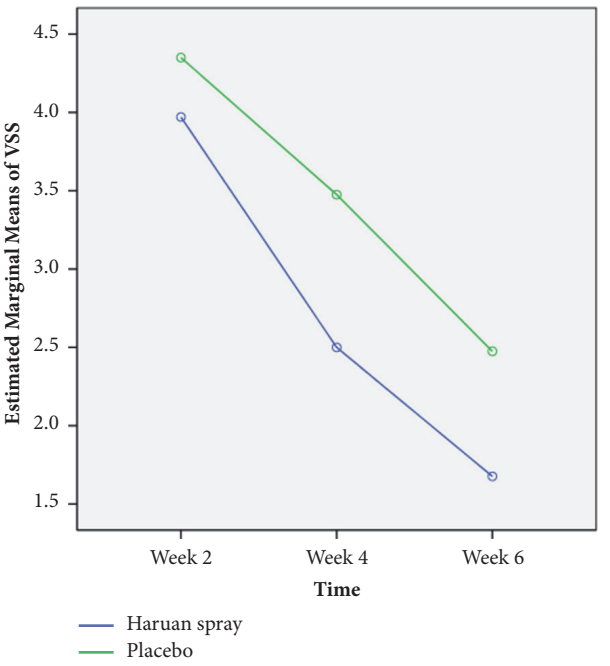

Figure 4: Comparison of estimated mean (estimated marginal means) of VSS for week 2, week 4, and week 6 interventions between Snakehead fish (*Channa striatus)* spray and placebo by comparing repeated measures ANOVA (n=81).

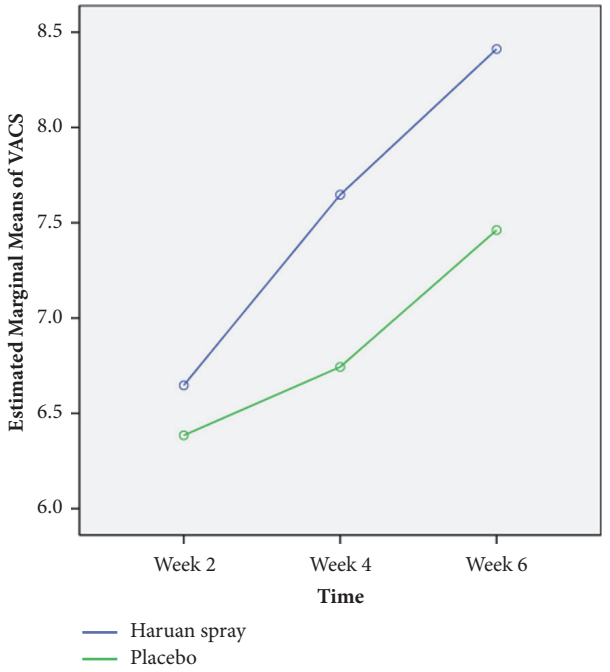

Figure 3: Comparison of estimated mean (estimated marginal means) of VACS for week 2, week 4, and week 6 interventions between Snakehead fish (*Channa striatus)* spray and placebo by comparing repeated measures ANOVA (n=81).

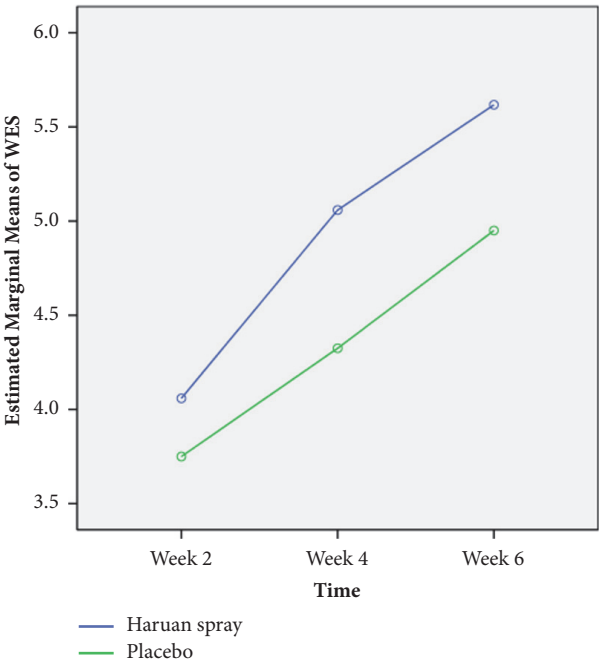

Figure 5: Comparison of estimated mean (estimated marginal means) of WES for week 2, week 4, and week 6 interventions between Snakehead fish (*Channa striatus)* spray and placebo by comparing repeated measures ANOVA (n=81).

[27]. Although this study did not specifically look at this issue, it shows that application of Snakehead fish (*Channa striatus)* extract did not increase the risk to get surgical site infection. This is postulated due to the antimicrobial effect of Snakehead fish (*Channa striatus)* extract.

As part of the wound healing process, antimicrobial activity is equally important. The antimicrobial properties have been studied by CARE research team. The investigation showed a broad spectrum of antibacterial activity of skin mucus against *Aeromonas hydrophila, Pseudomonas aeruginosa,* and *Vibrio anguillarum* and intestinal mucus against *A. hydrophila* [11]

The present study has several limitations. Difference in long-term effect of Snakehead fish (*Channa striatus)* extract spray on clean wound is not investigated. Longer clinical trials involving more patients are warranted. This is not single surgeon based study; therefore there is experience bias in terms of operating skill. Some patient defaulted after discharge from ward and outcome cannot be assessed.

Since our study once again proves that Snakehead fish (*Channa striatus)* extract *(Channa striata)* spray has shown a significant better pain score result and cosmetic outcome on clean wound, it has opened a window of opportunity to study the long term outcome. The future study on the use of Snakehead fish (*Channa striatus)* extract spray can also be extended on the clean-contaminated or contaminated wound. The other potential study is the effect of Snakehead fish (*Channa striatus)* pill and topical application of Snakehead fish (*Channa striatus)* cream as a dressing and its effect on wound healing.

6. Conclusion

In current study, it is clearly demonstrated that application of Snakehead fish (*Channa striatus)* extract *spray* on clean wound has shown a significant better pain score result and cosmetic outcome on week 2, week 4, and week 6 comparatively with placebo. It was not associated with additional morbidity in terms of its cosmetic outcome postoperatively at second, forth, and sixth week.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Study of the Treatment Effects of Compound Tufuling Granules in Hyperuricemic Rats Using Serum Metabolomics

Peng Wu,[1,2] Jing Li,[1] Xianxian Zhang,[1] Fuling Zeng,[1,2] Yingwan Liu,[1] and Weifeng Sun[1]

[1]*Department of Traditional Chinese Medicine, General Hospital of Guangzhou Military Command of PLA, Guangzhou 510010, China*
[2]*Guangzhou University of Chinese Medicine, Guangzhou 510006, China*

Correspondence should be addressed to Weifeng Sun; gzjqgzzyyzyk@126.com

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The study aimed to investigate the mechanism of the effect of Compound Tufuling Granules (CTG) to lower the serum uric acid level in a rat model of hyperuricemia. The rat model was established by administering hypoxanthine through oral gavage and potassium oxonate through intraperitoneal injection. Rats were divided into the normal group, model group, CTG group, and allopurinol group. Serum uric acid, creatinine, urea nitrogen, and inflammatory cytokine levels were determined in each group. In the model group, ultrahigh performance liquid chromatography-mass spectrometry was used to analyze the metabolic profiles and delineate the action mechanism of CTG; in addition, the orthogonal projection method was used to perform latent structure-discrimination analysis to screen the related metabolites. The results indicated significant differences in the metabolic profiles between the model and normal groups. A total of seven related metabolites were identified through screening in the model group, mainly related to the pathways of bile secretion, pyrimidine, purine, and phenylalanine metabolism, pantothenate and CoA biosynthesis, and pentose and glucuronate interconversions; these related pathways were reversed in the CTG group. In the metabolic networks, uracil and acetyl-coenzyme A were the nodal molecules. In addition, the test results of the evaluation of serum biochemical and inflammatory factors confirmed that CTG had significant effect in reducing the levels of serum uric acid and protecting renal function. These results confirmed that CTG primarily regulated the recruitment of nodal molecules to achieve anti-inflammatory effects, reduced uric acid level, and renal protection.

1. Introduction

Hyperuricemia is characterized by persistent increase in the uric acid level in the circulating blood. Oversaturated uric acid in the body may be due to increased uric acid synthesis or decreased excretion, which is the most important factor in the onset of gout [1, 2]. In addition, hyperuricemia is an independent risk factor for other diseases, such as hypertension, diabetes, obesity, hyperlipidemia, and heart disease [3–5]. At present, the incidence of hyperuricemia is increasing worldwide with increased occurrence in younger individuals, leading to a huge economic and social burden [6]. Since humans lack the corresponding enzymes, uricase, and allantoin, in vivo metabolism of purines involves xanthine oxidase-mediated decomposition to xanthines and finally conversion to uric acid [7, 8]. Therefore, lowering the uric acid level is a key to treating patients with hyperuricemia.

At present, the uric acid lowering drugs are mainly classified into two major categories: inhibitors of uric acid production and promoters of uric acid excretion. The drugs that inhibit the production of uric acid mainly include allopurinol and febuxostat, and the main drugs that promote its excretion include benzbromarone and probenecid. Due to their serious side effects, such as serious allergic reactions and rashes caused by allopurinol, the drugs that promote excretion have higher requirement for adequate renal function, with exacerbation under combined usage, which limits the use of these drugs, especially in patients with asymptomatic hyperuricemia [9, 10].Therefore, studies to determine safer and more effective treatments to reduce uric acid levels are needed.

As an effective alternative treatment method, traditional Chinese medicine (TCM) has advantages in the treatment of patients with gout and hyperuricemia. Compound Tufuling

Granules (CTG) is one of the representative treatments using TCM [11, 12]. It has been simplified by the experience party Xie-Zhuo-Chu-Bi-Fang and obtained the national invention patent (Patent no.: ZL 200710032363.3). It was formulated into nonstandard military preparation of CTG (lot no: Guang L2009001), consisting of *Rhizoma Smilacis Glabrae*, Rhizoma Dioscoreae Hypoglaucae, Pseudobulbus Cremastrae Seu Pleiones, Radix Achyranthis Bidentatae, and Semen Vaccariae, which has the functions of clearing heat and dampness and activating blood circulation to relieve pain. Previous studies have shown that it lowers the level of uric acid, preventing the recurrence of gout and suppressing the expression of inflammatory factors [13, 14]. However, the pharmacological mechanism of CTG-induced reduction in the uric acid level is unknown to date.

Metabolomics is the study of changes in metabolites produced by biological systems (the cell, tissue, or organism) after they have been stimulated. Small molecules are its target research objective, including their production and metabolism as end result of a series of events; therefore, metabolomics defines the state of biological systems more accurately [8, 12, 15, 16]. Currently, metabolomics is widely used in the field of TCM [7, 11, 17–19]. Commonly used metabolomics techniques include nuclear magnetic resonance (NMR), gas chromatography with mass spectrometry (GC-MS), liquid chromatography with mass spectrometry (LC-MS), ultrahigh performance liquid chromatography-mass spectrometry (UPLC-MS), and other such techniques, with unique advantages and disadvantages. Urine metabolome is more dependent on exogenous factors, such as fluid intake, as compared to serum metabolome, which is less affected by interfering factors and is more stable than the urine metabolome. In general, UPLC-MS is focused on identifying large molecular-weight polar metabolites; moreover, identification of metabolites through UPLC-MS is more effective than that through GC-MS, which does not allow gasification due to the presence of high molecular-weight metabolites; hence, for the purpose of our study, we selected the UPLC-MS detection method [7, 20]. In this study, UPLC-MS technology was used to investigate the serum metabonomics aspect of lowered uric acid levels in rats undergoing treatment with CTG.

In this study, oral hypoxanthine and intraperitoneal injection of potassium oxonate were used to establish the hyperuricemia rat model; the rats were divided into the normal group (N), model group (M), CTG group (Fa), and allopurinol group (B) (positive control group). In each group, the serum creatinine (Scr), serum uric acid (SUA), and blood urea nitrogen (BUN) levels were determined using biochemical tests; the levels of inflammatory cytokines TNF-α and IL-1β were determined using ELISA method, and for the purpose of serum metabolome study, HPLC-MS was used. The first aim was to elucidate the abnormal metabolic mechanism of hyperuricemia, and the second aim was to identify the relevant specific metabolic markers of uric acid metabolic pathway and other possible metabolic pathways in rats undergoing treatment with CTG, which will enable early diagnosis of patients with hyperuricemia.

Table 1: Constituents of Compound Tufuling Granules.

Components	Part used	Amount used (g)
Rhizoma Smilacis Glabrae	Root	35
Rhizoma Dioscoreae Hypoglaucae	Root	18
Pseudobulbus Cremastrae Seu Pleiones	Root	15
Semen Vaccariae	Seed	10
Radix Achyranthis Bidentatae	Root	10

2. Materials and Methods

2.1. Reagents and Instruments. For the purpose of study, 97% oxonic acid potassium salt and 99% hypoxanthine were purchased from Sigma (St. Louis, MO, USA); soluble starch from Shanghai Macklin Biochemical Science and Technology Co., Ltd. (Shanghai, China); ELISA kits for TNF-α and IL-1β from Beijing Cheng Lin Biological Technology Co., Ltd (Beijing, China); and methanol, chloroform, formic acid, L-2-chlorobenzene alanine, and acetonitrile from Shanghai Heng Chuang Biological Technology Co., Ltd. In addition, we used the following equipment: ultrasonic cell pulverizer and ultrasonic cleaning machine (Scientz-IID and SB-5200DT, respectively; Ningbo Xin Zhi Biologic Multi Tube Technology Co., Ltd. Ningbo, Zhejiang, China); vortex (TYXH-I; Shanghai Khan Novo Instrument Co. Ltd. Shanghai, China); high speed refrigerated centrifuge (TGL-16MS; Shanghai Luxiang Centrifuge Instrument Co., Ltd. Shanghai, China); and UPLC (ACQUITY UPLC I-Class), High resolution mass spectrometry (VION IMS Q-Tof), and chromatographic column (ACQUITY UPLC BEH C18) (100 mm × 2.1 mm, 1.7 um) from Voight World Science and Technology Co., Ltd. (Waters) (Shanghai, China).

2.2. CTG Preparation. CTG comprises the granules of the Xiezhuo Chubi formulation, which consists of *Rhizoma Smilacis Glabrae*, Rhizoma Dioscoreae Hypoglaucae, Pseudobulbus Cremastrae Seu Pleiones, Radix Achyranthis Bidentatae, and Semen Vaccariae (Table 1). As per requirement of nonstandard military preparation, CTG was made by the Department of Pharmacy of the Guangzhou Military Command General Hospital (batch number: Guang L2009001), in packets of 10-g weight each (batch No. F01009) [14]. The formulation was extracted and purified by means of water extraction and alcohol sedimentation process [21]. The excipient of the granules included soluble starch (Xiangtan County Starch Products Co., Ltd., batch number: 20110116). The quality of the granules was measured using thin layer chromatography (TLC) and HPLC [22]. Allopurinol tablets provided by the Western Pharmacy Department of the Guangzhou Military Command General Hospital were purchased from Chongqing Qingyang Pharmaceutical Co., Ltd. (Chongqing, China).

2.3. Animal Models and Experimental Study Design. The experimental protocols were approved by the ethics committee of the General Hospital of Guangzhou Military Command; all experimental procedures were conducted in

accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Thirty-two SPF-grade SD rats with body weight of 200±20 g provided by the Experimental Animal Center of Southern Medical University were divided into eight cages, of four rats per cage. All included animals were housed and fed in the SPF Animal Experimental Center of the General Hospital in Guangzhou Military Command; the room temperature was maintained at 23±2°C and the humidity was maintained at 55%±5; the rats were maintained on a 12-h light cycle. After adaptive feeding for 7 days, the rats were randomly divided into the N, M, Fa, and B groups at eight rats per group. Except for the N group, oral hypoxanthine 500 mg/kg and intraperitoneal morning injection of potassium oxonate 100 mg/kg were administered to each group for a total of 10 days. The CTG and allopurinol solution was freshly prepared for administration through gavage. The allopurinol tablets and CTG were crushed to powder and subsequently dissolved in distilled water to achieve concentration of 500 mg/ml and 1.25 mg/ml, respectively. The injection volume was 8 ml/kg. The Fa and B groups were administered orally with specific volume of the indicated drugs at 4 g/kg and 5 mg/kg, respectively; the N and M groups were administered orally with the same volume of distilled water. On day 4 of the experiment, the drug-groups were administered CTG or allopurinol solution through gavage at 30 min after injection of the modeling agent. Dosage of all modeling agents, drugs, and distilled water were converted according to the body's surface area [23]. Each administration group received gavage once a day for a total of 7 days; the entire experiment lasted for 10 days.

2.4. Metabolomic Sample Collection. At 2 hours after last administration of the drug, blood was collected from the orbital vein of the rat under anesthesia; subsequently, the blood sample was centrifuged at 3000 rpm at 4°C for 5 minutes. The resulting supernatant was centrifuged at 12,000 rpm for 5 minutes to remove insoluble proteins and then stored at -80°C until use in the metabolomic assay.

2.5. Serum Biochemical and TNF-α and IL-1β Assay. Anesthesia was performed through abdominal injection of choral hydrate (350 mg/kg). The blood sample (3 ml) was collected from the abdominal aorta, placed in a red tube (non-anticoagulation tube), allowed to stand at room temperature for 30 minutes, and centrifuged at 3000 rpm for 20 minutes; the obtained serum was sent to the General Hospital of Guangzhou Military Region for laboratory tests including SUA, Scr, and BUN detection. The remaining supernatant was collected for use in ELISA for TNF-α and IL-1β performed using the manufacturers' instructions.

2.6. Sample Preparation for Metabolomics Study. The internal standard (10 μl) (L-2-chlorophenylalanine, 0.3 mg/ml, methanol configuration) was added to serum (100 μl) and the solution was mixed by vortexing for 10 s; subsequently, methanol-acetonitrile (300 μl) (2:1, v/v) was added followed by vortexing for 1 min. Extraction was performed in an ice water bath, and the sample was maintained at -20°C for 30 min; centrifugation was performed at 13,000 rpm for 15 min using a 0.22 μm organic phase pinhole filter to obtain filtrate (supernatant, 200 μl) which was transferred to the liquid chromatography injection vial. The quality control sample (QC) was prepared by mixing equal volume of the extracts of all samples, to obtain the same volume as that of the sample.

2.7. UPLC-MS Process. UPLC conditions are as follows: column, ACQUITY BEHC18 column (100 mm × 2.1 mm i.d., 1.7 μm; Waters, Milford, USA); solvent, the column was maintained at 45°C and separation was achieved using the following gradient: one to 30% B over 0–1 min, 30–60% B over 1–2.5 min, 60–90% B over 2.5–6.5 min, and 90–100% B over 6.5–8.5 min; the composition was held at 100 % B for 2.2 min, followed by 10.7–10.8 min, 100% to 1% B, and 10.8–13 min holding at 1% B at flow rate of 0.40 ml/min, where B is acetonitrile/methanol 2/3 (v/v)(0.1% (v/v) formic acid) and A is aqueous formic acid (0.1% (v/v) formic acid). Injection volume was 1 μl and the column temperature was set at 45°C.

The mass spectrometric data was collected using Waters VION IMS Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The capillary voltages, DP, and CE were 2.5 kV, 40 V, and 6 eV, respectively. Source temperature and desolvation temperature were set at 115°C and 450°C, respectively, with desolvation gas flow at 900 l/h. Centroid data was collected from 50 to 1,000 m/z with scan time of 0.2 s and interscan delay of 0.02 s over a 13-min analysis time-period. The QCs were injected at regular intervals (every 10 samples) throughout the analytical run to provide the data set used for repeatability assessment.

2.8. Data Processing and Statistical Analysis. The acquired UPLC-MS raw data were analyzed using the Progenesis QI software (Waters Corporation, Milford, USA) with the following parameters: precursor tolerance of 5 ppm, fragment tolerance of 10 ppm, and retention time (RT) tolerance of 0.02 min. Internal standard detection parameters were deselected for peak RT alignment, isotopic peaks were excluded from the analysis, noise elimination level was set at 10.00, and minimum intensity was set to 15% of base peak intensity. The excel file with three-dimension data sets was obtained, including m/z, peak RT, and peak intensities; RT–m/z pairs were used as the identifier for each ion. The resulting matrix was further reduced by removing any peaks with missing value (ion intensity=0) in more than 60 % samples. The positive and negative data were combined to obtain a combined data set, which was imported into the SIMCA software (version 14.0, Umetrics, Umeå, Sweden). Principal components analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were conducted to visualize the metabolic alterations among the experimental groups. Variable importance in the projection (VIP) ranks the overall contribution of each variable to the OPLS-DA model, and those variables with VIP > 1 are considered as relevant for group discrimination. Other data were analyzed using one-way ANOVA followed by the least significant difference (LSD) test. All statistical analyses were performed using SPSS

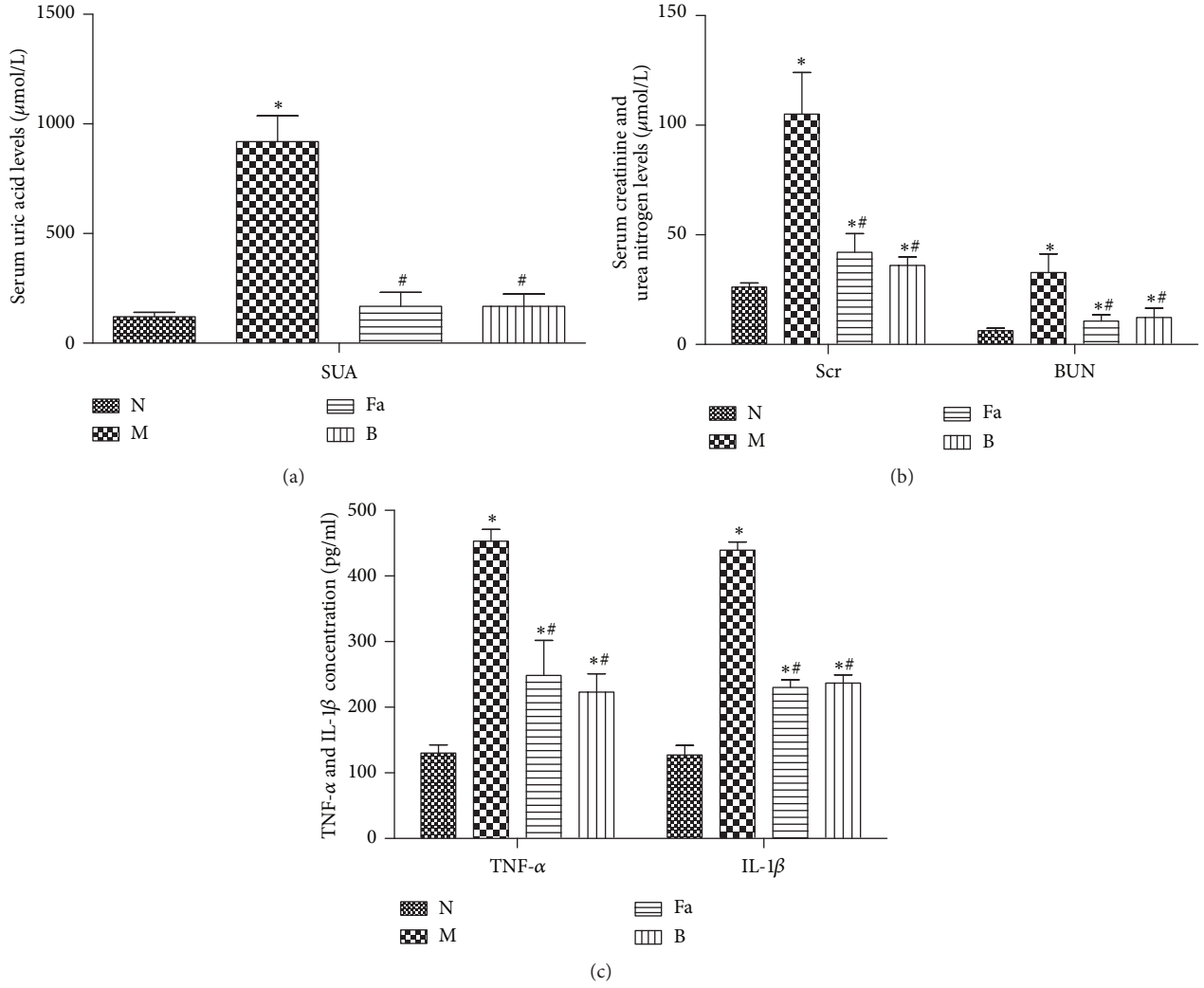


Figure 1: Concentration of SUA (a), Scr, BUN (b), and TNF-α and IL-1β (c). Compared with the N group, $^{*}p<0.05$; compared with the M group, $^{\#}p<0.05$.

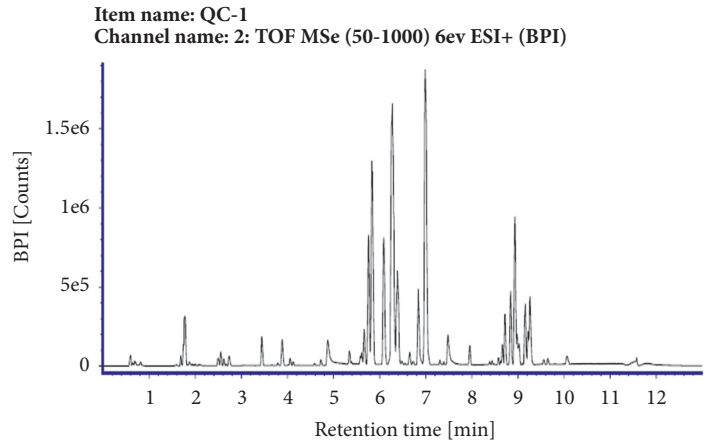
version 20.0 (IBM, Armonk, NY, USA). The results were assumed to be statistically significant at $P<0.05$.

2.9. Related Metabolite Identification. The related metabolites were identified as follows: First, potential metabolites were separated in the loading scatter plot of OPLS-DA (the N and M group). Second, the related metabolites were further screened by limiting VIP (VIP > 1) and performing Student's t-test ($p<0.05$). The information of these metabolites was obtained through searches conducted in the Kyoto encyclopedia of genes and genomes (http://www.kegg.jp [24]) and HMDB (www.hmdb.ca). Finally, commercial standards were adopted to support the metabolites' identification.

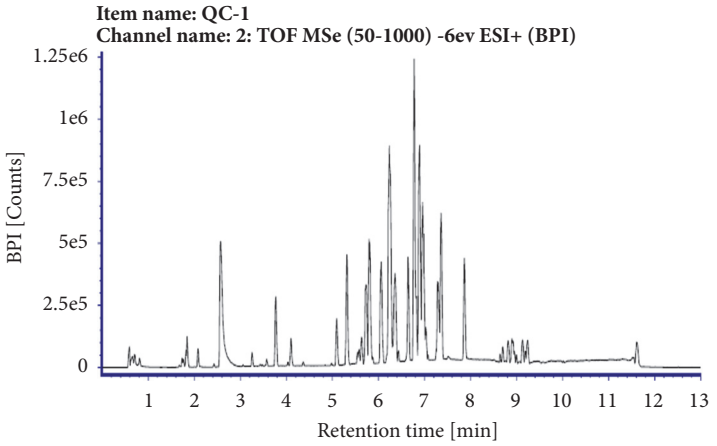
3. Results

3.1. Serum Biochemical Analysis and ELISA. As shown in Figure 1, significant increase in the SUA level is an important biochemical basis for the development and detection of hyperuricemia, and impaired kidney function is effectively reflected by the level of Scr and BUN. The involvement of inflammatory factor(s) is an important basis for the onset of gout and hyperuricemia. Compared with the N group, the levels of SUA, Scr, BUN, TNF-α, and IL-1β in the M group were significantly elevated *($p<0.05$)*. Compared with the M group, the levels of SUA, Scr, BUN, TNF-α, and IL-1β in the Fa and B groups were lower than those in the M group *($p<0.05$)*, without significant difference between the Fa and B groups *($p>0.05$)*. These results indicated that the Fa group had reduced level of uric acid, improved renal function, and anti-inflammatory effects.

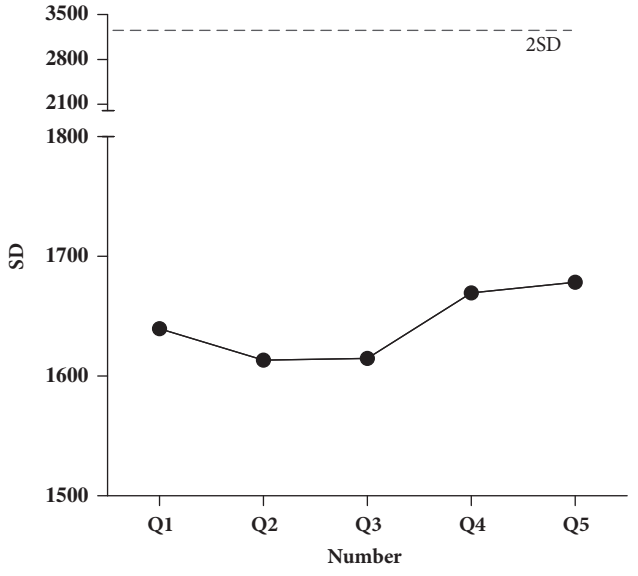
3.2. Serum Metabolic Profiles. In all samples, the UPLC-MS method was used to detect the base peak ion current (BPI); the instrument analyses were characterized by strong signal, large peak capacity, and good reproducibility of retention time (Figures 2(a) and 2(b)). In the analysis sequence, one QC sample was inserted into every 10 analysis samples. The



(a)



(b)



(c)

Figure 2: Quality control sample (QC) base peak ion chromatogram and PCA analysis results. (a) Base-band ion flow diagram at positive ion mode of the control sample BPI (+). (b) Base-level ion flow diagram at negative ion mode of the control sample BPI (-). (c) QC plot of five repeat UPLC-MS analyses. X-axis, number, y-axis, standard deviation (SD).

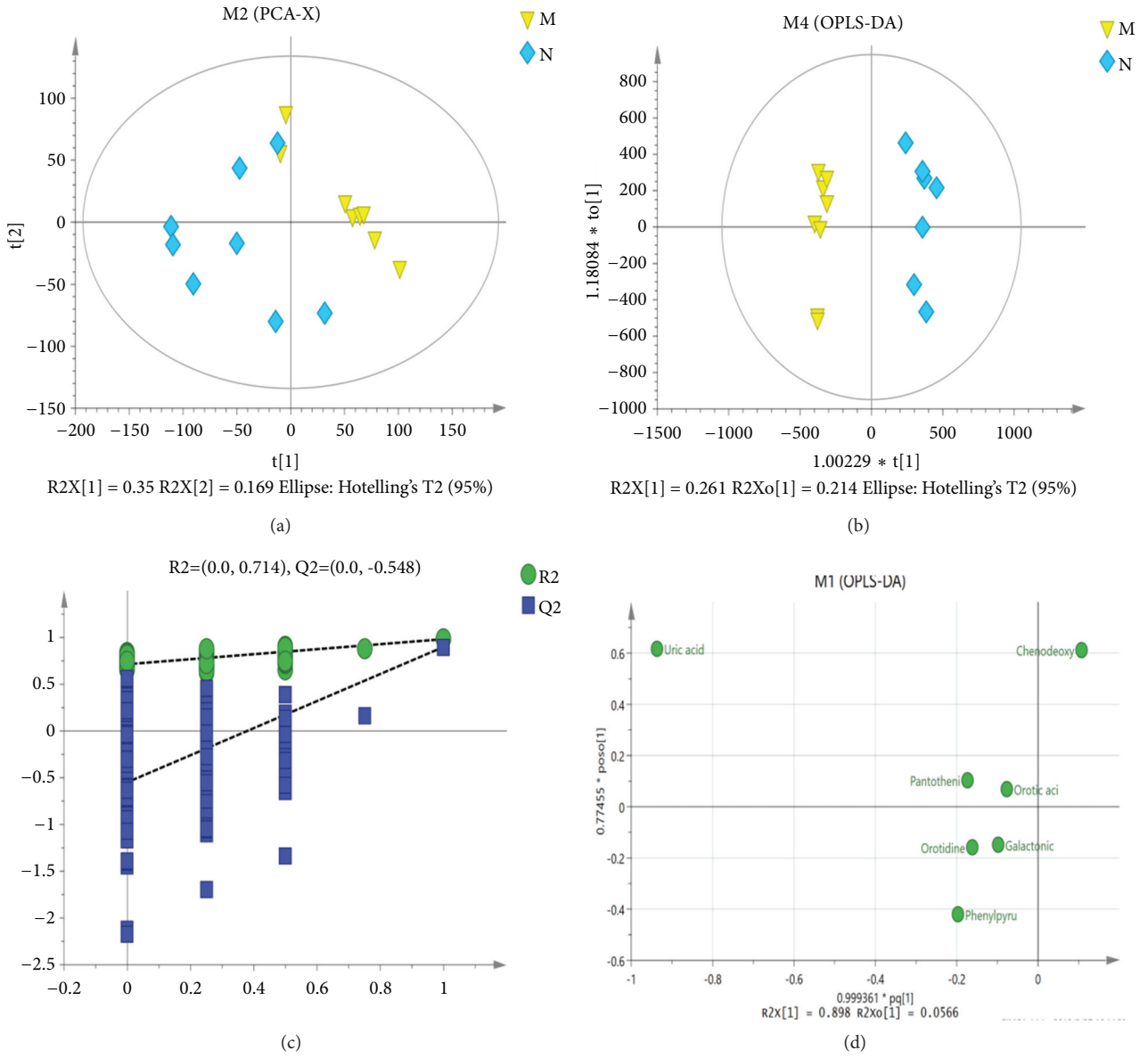


Figure 3: Multivariate data analysis. (a) Principal component analysis (PCA) score map derived from UPLC-MS in the normal rats (◇) and hyperuricemia model rats (▽). (b) Orthogonal projections to latent structures-discriminant analysis (OPLS-DA) score. (c) Validation plot obtained from 200 permutation tests. (d) The loading scatter plot of OPLS-DA.

analyses showed that the method had good repeatability and met the analysis requirements of the metabolic group (Figure 2(c)).

3.3. Potential Biomarkers Related to Hyperuricemia. First, PCA was used to analyze rats in the N and M groups. In the PCA model, there were significant differences between the N and M groups (R2X=0.704, Q2=0.49), indicating that the model was reliable (Figure 3(a)). To obtain more reliable related metabolites, we used OPLS-DA to filter the model-independent signals and obtain the final OPLS-DA model. As a result, two categories, R2Y (0.982) and Q2 (0.9), were obtained, indicating that the two groups of samples had significant differences in the OPLS-DA score map (Figure 3(b)).

We performed 200 response sequencing tests on the OPLS-DA model (Figure 3(c)) and obtained intercepts of R2=0.879, Q2=−0.331, indicating that the OPLS-DA model was without overfit and reliable. The relevant metabolites were screened by defining VIP>1 for the first principal component of the OPLS-DA model and Student's t-test ($p < 0.05$). The loading scatter plot of OPLS-DA (Figure 3(d)) and statistical analysis (Table 2) indicated increased levels of galactonic acid, pantothenic acid, orotidine, orotic acid, uric acid, phenylpyruvic acid, and decreased levels of chenodeoxycholic acid.

3.4. Effects of Allopurinol and CTG on Metabolite Profiling. For purpose of identifying potential drug-treatment target, we established a new PCA model including seven different

Table 2: Potential related metabolites and their metabolic pathways.

VIP	Compounds	Formula	Retention time	Measured	Adducts type	M∗	Fa#	B#	Related pathway
1.26	Galactonic acid	C6H1207	0.65	195.0505	M-H	↑	↓		Pentose and glucuronate interconversions
2.11	Pantothenic acid	C9H17N05	1.71	218.1029	M-H	↑	↓	↓	Pantothenate and CoA biosynthesis
2.13	Orotidine	C10H13N2011P	0.68	287.0516	M-H,2M-H	↑	↓	↓	Pyrimidine metabolism
1.02	Orotic acid	C5H4N204	0.71	155.0094	M-H	↑	↓		Pyrimidine metabolism
12.17	Uric acid	C5H4N403	0.71	167.0207	M-H, 2M-H	↑	↓	↓	Purine metabolism
2.58	Phenylpyruvic acid	C9H803	1.79	163.0397	M-H	↑	↓	↓	Phenylalanine metabolism
4.72	Chenodeoxycholic acid	C02528	4.99	391.285	M-H, 2M-H	↓	↑	↑	Bile secretion

M: model group; N: normal group; Fa: CGT group; B: allopurinol group.
Variable importance in the projection (VIP) was obtained from the OPLS-DA model.
∗Compared with the N group, $^{*}p<0.05$.
#Compared with the M group, #$p<0.05$. ↑, Relative increase in signal; ↓, relative decrease in signal.

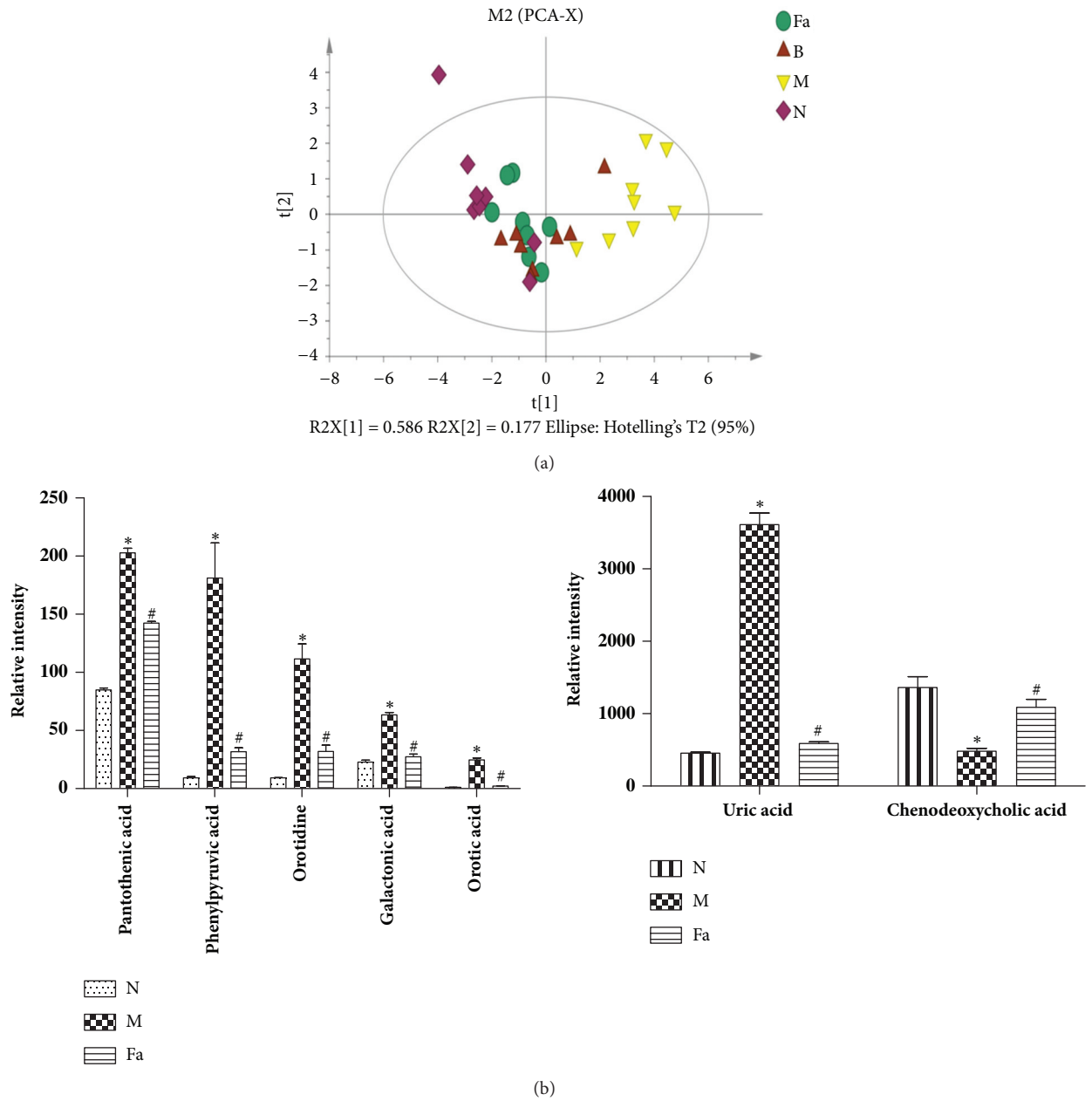


FIGURE 4: Multivariate data analysis. (a) PCA scores' plot derived from the serum levels of seven metabolites in the normal group (◇), hyperuricemia model group (▽), Fa group (○), B group (Δ). (b) Bar plots show UPLC-MS relative intensities for seven metabolites in the normal, model, and Fa groups. Data are expressed as the mean ± SD using one-way ANOVA. Compared with the normal group, $^{*}p<0.05$; compared with the model group, $^{\#}p<0.05$.

metabolites. Compared with the model, the B and Fa groups were more similar to the N group (Figure 4(a)), indicating that allopurinol and CTG had capability to reverse the pathological process of hyperuricemia. To further explain the different degree of improvement of the CTG-related seven metabolites, we used one-way analysis of variance *($p<0.05$)*. Compared with the model, CTG reduced the expression level of galactonic acid, pantothenic acid, orotidine, orotic acid, uric acid, and phenylpyruvic acid to varying degree and increased that of chenodeoxycholic acid (Figure 4(b)) *($p<0.05$)*.

3.5. Interpretation of Metabolic Networks. In this experiment, CTG showed significant effects of anti-inflammation, decreased level of uric acid, and improved renal function at varying degrees. Compared with the N group, the M group included seven related metabolites through the UPLC-MS method. These substances are mainly related to bile secretion, pyrimidine, purine, and phenylalanine metabolism, pantothenate and CoA biosynthesis, and pentose and glucuronate interconversions pathways, which are directly or indirectly related pathways. Uracil, as a nodal molecule,

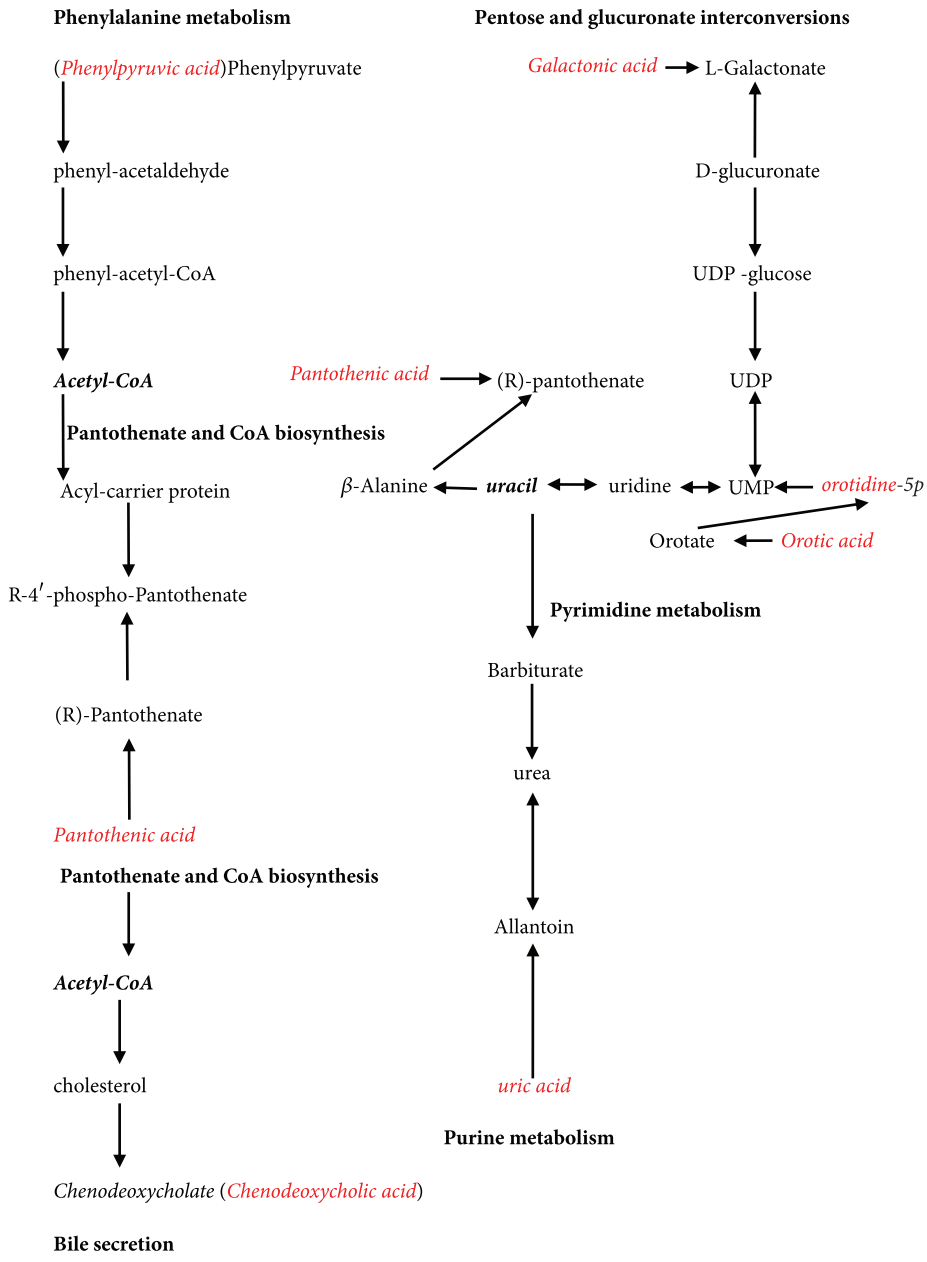


FIGURE 5: Diagram of the metabolic pathway networks involved in the CTG intervention. The nodal molecules are represented in bold italic font. The seven related metabolites are represented in red font. The pathway names are in bold font.

is associated with pyrimidine and purine metabolism, pantothenate and CoA biosynthesis, and pentose and glucuronate interconversions; in addition, acetyl-coenzyme A (acetyl-CoA) is another nodal molecule in the pathways of bile secretion, pantothenate and CoA biosynthesis, and phenylalanine metabolism. In metabolic networks (Figure 5), the core pathways included pyrimidine metabolism and pantothenate and CoA biosynthesis. In this study, the Fa group showed reversed expression levels of the seven related metabolites, indicating that CTG can potentiate the role of other pathways by regulating the nodal molecules in the networks.

4. Discussion

Hyperuricemia is closely related to the occurrence of gout, as well as several metabolic diseases such as obesity, diabetes, and hyperlipidemia. Allopurinol inhibits both the synthesis and metabolism of uric acid. By inhibiting xanthine oxidase, it prevents the conversion of hypoxanthine and xanthine to uric acid, thereby reducing the concentration of uric acid in the blood. Previous studies using the rat model of hyperuricemia have shown that CTG inhibited xanthine hydrogenase activity (XO) and xanthine oxidase (XDH) mRNA expression in the liver and anti-inflammation, promoted

uric acid excretion by regulating the expression of miR-34a and miR-146a, and inhibited URAT1 and GLUT9 mRNA transcription and protein expression [13, 14, 25, 26]. However, the metabolic mechanism involved remains unclear. In the present study, we conducted serum metabonomics' study in rats with hyperuricemia by means of UPLC-MS.

Increased synthesis or decreased excretion of uric acid in the body can lead to the occurrence of hyperuricemia. Therefore, currently, animal models of hyperuricemia are established using mainly in vitro xanthine/hypoxanthine infusion and in vivo inhibition of the uricase activity [17, 19]. In this study, we successfully prepared the hyperuricemia model by oral gavage of hypoxanthine and intraperitoneal injection of potassium oxonate. A large number of studies have confirmed that long-term increase in the SUA level triggered the acute onset of gout, as well as the deposition of urate crystals in the internal organs, causing renal insufficiency [2, 4]. Secondly, the acute onset of gout was additionally associated with many inflammatory factors; of these, IL-1β, a key cytokine of gout, can act on many cell types to initiate inflammatory responses, whereas TNF-α, a type of macrophage and pro-inflammatory cytokines produced by monocytes, participates in the inflammatory response of gout. Moreover, studies have confirmed that elevated levels of IL-1β and TNF-α can be detected in the blood of rats with gout and those of the hyperuricemia model [27, 28]. CTG consists of five herbs. Among them, *Rhizoma Smilacis Glabrae* has the functions of clearing heat and detoxication and promotion of blood flow of the joints and kidney. The main function of Radix Achyranthis Bidentatae is to replenish qi, activate blood circulation, and promote water and uric acid excretion. Rhizoma Dioscoreae Hypoglaucae has the function of dispelling wind and removing dampness and diuresis. Semen Vaccariae has the function of promoting blood circulation and removing blood stasis, promoting metabolism, and improving microcirculation. Colchicine is the active ingredient of Pseudobulbus Cremastrae Seu Pleiones, which significantly reduced the inflammatory reaction [13, 14, 25, 26]. In this study, as compared to the normal range for this species, the levels of SUA, Scr, BUN, IL-1β, and TNF-α were significantly increased in the rat model, while those in the Fa group were significantly lower, indicating that CTG can reduce the level of uric acid through anti-inflammatory action and improving the renal function.

In clinical studies, the diagnosis of hyperuricemia is based on the SUA level alone, which limits early detection; therefore, identifying significant related metabolites is crucial. In our study, results of screening indicated seven metabolites, mainly related to bile secretion, pyrimidine, purine, and phenylalanine metabolism, pantothenate and CoA biosynthesis, and pentose and glucuronate interconversions.

In metabolic networks, galactonic acid is the product of pentose and glucuronate interconversion through the oxidation of hydroxy groups in galactose to form shuttle groups. Galactose is a type of monosaccharide that can be catalyzed by β-glycosylase in the intestine. Galactose for anabolism can be produced from uridine diphosphate glucose (UDPG) in the absence of food substrate. It can be found in dairy products, and plant mucin and bacterial polysaccharides, which is the component of lactose in mammalian milk [29]. More importantly, galactonic acid can be associated with D-glucuronate through L-galactonate and subsequently with pyrimidine metabolism through UDPG. Studies have shown that glucose metabolism is involved in the development of gout and hyperuricemia and hyperuricemia exacerbates the disorder of glucose metabolism [30, 31]. In our study, the level of galactonic acid was upregulated in the M group versus the N group; in addition, the related pathway of pentose and glucuronate interconversions and pyrimidine metabolism were dysregulated. However, as compared with the M group, these effects were reversed in the Fa group, with no significant difference compared with the N group, indicating that CTG can inhibit abnormal glucose transformation in vivo to lower the level of uric acid.

Uric acid is the final metabolic product of purine, one of the most important components of DNA and RNA. Purine is decomposed into uric acid through a series of catalytic reactions; in some animals, under the effect of uricase, it is further decomposed to allantoin and urea through association with the pyrimidine metabolism via uracil [19]. Pyrimidine metabolism comprising orotic acid and orotidine was first core of the hyperuricemia metabolic networks. Orotidine can be decomposed into uridine monophosphate (UMP) and uridine diphosphate (UDP). UMP is further decomposed into uridine and uracil which is a unique base of RNA. In transcription of DNA, thymine (T) in DNA is substituted and paired with adenine to methylate uracil which is an important component of nucleic acids. The results of previous studies confirmed that uridine, uracil, and pyrimidine metabolism had a pathogenic role in the development of hyperuricemia and exacerbation of acute onset gout [16, 32]. In our study, the level of orotic acid and orotidine was increased in the M group relative to the N group, indicating that pyrimidine metabolism was an influencing factor in hyperuricemia; moreover, uric acid, pantothenic acid, and galactonic acid were significantly increased in the M group. Contrary to expectation, the Fa group showed significantly reduced level of orotic acid, orotidine, uric acid, pantothenic acid, and galactonic acid, indicating that pyrimidine metabolism is related to pantothenate and CoA biosynthesis, pentose and glucuronate interconversions, and purinergic metabolic pathways via uracil. These results indicated that CTG can regulate uracil, a nodal molecule in the metabolic networks, to mediate other pathways' regulation with final outcome of decrease in the uric acid level.

Phenylpyruvic acid is a dicarbonyl compound, a product of phenylalanine metabolism. Phenylalanine is an essential amino acid, comprising mostly of tyrosine catalyzed by phenylalanine hydroxylase, and is involved in the synthesis of important neurotransmitters and hormones together with tyrosine as part of the body's glucose and lipid metabolism. Under physiological conditions, only a small proportion of phenylalanine is converted to phenylpyruvic acid under the action of aminotransferases. In case of decreased or lost phenylalanine hydroxylase activity, a large amount of phenylpyruvic acid is generated, which may lead to phenylketonuria, affecting brain development, causing mental retardation and nervous system symptoms such as microcephaly and

convulsions. Jiang and Liu reported that both hyperuricemia and gout were associated with the phenylalanine metabolism [7, 16]. In our study, the level of phenylpyruvic acid was significantly increased in the M group, while the disordered phenylalanine metabolism was reversed in the Fa group, indicating that CTG can be used to lower the level of uric acid by regulating the phenylalanine metabolism. Phenylpyruvate can further produce phenyl-acetaldehyde, which subsequently generates phenyl-acetal-CoA, which is associated with pantothenate and CoA biosynthesis via acetyl-CoA.

Chenodeoxycholic acid (CDCA) is closely related to cholesterol. The combination of CDCA with glycine or taurine in the liver can inhibit the cholesterol synthesis, increase the bile secretion in patients with gallstone disease, and be used to treat cholesterol gallstone disease. Previous studies have shown that CDCA has a wide range of effects on the lipid metabolism; hence, it may be associated with the onset of hyperuricemia and gout [33]. In our study, the CDCA content of the model group was lower than that of the normal group, while that of the Fa group was significantly increased. Collectively, these results indicated that hypercholesterolemia can increase the risk of hyperuricemia and gout, while CTG can reduce the level of uric acid through its cholesterol lowering effect.

In our study, pantothenate and CoA biosynthesis was second core of the hyperuricemia metabolic networks. Pantothenic acid, also known as vitamin B5, is one of the 13 essential vitamins in humans. It consists of pantoic acid and β-alanine, which is an acyl transporter and participates in the metabolism process, regulating energy and fat metabolism [34]. Acetyl-CoA, an acetylated form of CoA, is a precursor of substances such as fatty acids and ketone bodies and plays a pivotal role in the energy metabolism in vivo [35], whereas CoA can form acyl carrier protein and further produce R-4-'phospho-pantothenate which is associated with pantothenic acid. Acetyl-CoA may be associated with the bile secretion and phenylalanine metabolism. Both vitamin B and acetyl-CoA are involved in the metabolism of sugars, proteins, and lipids in vivo and, thus, may be involved in the development of hyperuricemia; therefore, we hypothesized that the onset of hyperuricemia is associated with vitamin B5 and acetyl-CoA metabolism. In this study, we observed an increase in the level of pantothenic acid in the model group, indicating dysregulated pantothenate and CoA biosynthesis. Moreover, in the M group, the level of chenodeoxycholic acid was decreased and that of phenylpyruvic acid was increased. After CTG intervention, in the Fa group, the level of phenylpyruvic acid was significantly reduced, and that of chenodeoxycholic acid was significantly increased, indicating intersection of pantothenate and CoA biosynthesis with the pathways of pyrimidine metabolism, bile secretion, and phenylalanine metabolism through acetyl-CoA. Thus, CTG affects acetyl-CoA, the nodal molecule in the metabolic networks, to regulate the other pathways with the final outcome of decreased uric acid level.

Among the metabolic diseases such as hypertension, diabetes, obesity, and hyperlipidemia, hyperuricemia is an independent risk factor for several other metabolic diseases. Studies have demonstrated involvement of other inflammatory factors in their pathogenesis. CTG contains Pseudobulbus Cremastrae Seu Pleiones, whose effective ingredient is colchicine, and can significantly reduce inflammation. It is likely that CTG may regulate the metabolism of carbohydrates, amino acids, lipids, vitamins, purines, and pyrimidines to achieve anti-inflammatory effects, reduced uric acid levels, and renal protection.

5. Conclusion

In this study, the hyperuricemia rat model was successfully prepared by means of oral gavage of hypoxanthine and intraperitoneal injection of oxonic acid potassium for 10 consecutive days. The levels of SUA, Scr, BUN, and inflammatory cytokines such as TNF-α and IL-1β were evaluated; the result of serum metabonomics study through UPLC-MS indicated total seven related metabolites including chenodeoxycholic acid, orotic acid, orotidine, uric acid, phenylpyruvic acid, pantothenic acid, and galactonic acid. Hyperuricemia is mainly associated with metabolism of cholesterol, amino acid, lipids, vitamin, and glucose. By regulating the bile secretion, pyrimidine, purine, and phenylalanine metabolism, pantothenate and CoA biosynthesis, and pentose and glucuronate interconversions pathways at various degrees, CTG showed effectiveness to achieve anti-inflammation, lowered level of uric acid in the blood, and protection of the renal function.

Data Availability

The raw data of this article is reliable. Anyone can find the original data through this link https://figshare.com/s/5b323dcee664cbfc8d6d.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Jing Li and Peng Wu contributed equally to this work.

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Research Article

Randomized, Double-Blind, Placebo-Controlled Study of Modified Erzhi Granules in the Treatment of Menopause-Related Vulvovaginal Atrophy

Ranran Chen,[1] **Dianrong Song**,[2] **Wei Zhang**,[2] **Guanwei Fan**,[1] **Yingqiang Zhao**,[2] **and Xiumei Gao**[1]

[1]*Tianjin University of Traditional Chinese Medicine, China*
[2]*The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, China*

Correspondence should be addressed to Dianrong Song; songdr58@126.com

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Objective. To evaluate the clinical therapeutic efficacy and safety of modified Erzhi granules (MEG) in patients with menopause-related vulvovaginal atrophy (VVA). *Methods*. This randomized, double-blind, placebo-controlled study comprised two groups, including the treatment and control groups. Patients receive MEG and placebo for 12 weeks, respectively. Vaginal health score (VHS), vaginitis score, vaginal maturation index (VMI), female sexual function index (FSFI), and modified Kupperman Index (modified KI) were used as efficacy endpoints and assessed at baseline, 4, 8, and 12 weeks during administration, and 4 weeks after drug withdrawal. At baseline and 12 weeks, serum estradiol (E_2), follicle stimulating hormone (FSH), pelvic ultrasound, breast ultrasound, and other safety parameters were measured, recording adverse events. *Results*. At 12 weeks, VHS, percentage of superficial cells in the vaginal epithelium and FSFI were significantly increased, while vaginitis score, percentage of basal cells in the vaginal epithelium, and modified KI were significantly decreased in comparison with baseline and control group (all $P<0.05$); these differences persisted for up to 4 weeks after drug withdrawal. The placebo group showed no significant change during treatment compared with baseline values ($p>0.05$). Serum E_2 and FSH levels, endometrial thickness, and breast thickness in all patients were within the normal ranges before and after treatment, with no serious adverse reactions observed. *Conclusion*. MEG significantly alleviates menopause-related vulvovaginal atrophy, with no overt adverse effects on the endometrium, breast, hepatic, and renal functions.

1. Introduction

Vulvovaginal atrophy (VVA), which often occurs 2-5 years after menopause [1], is a common disease in postmenopausal women, with an incidence of up to 50% [2]. VVA is mainly due to decreased estrogen levels after menopause, which reduces the thickness of epithelial tissues in the female reproductive tract, smooth muscle function, and collagen and hyaluronic acid levels; this results in decreased vaginal wall elasticity, increased mucosal fragility, imbalanced local flora, and increased pH. The clinical manifestations include vaginal dryness, painful sexual intercourse, vaginal burning, postcoital bleeding [3]. Vaginal wall atrophy, flattened villi, loss of folds, and pale pink petechiae are occasionally found in gynecologic examination. Because pelvic floor muscles, the urethra, and the urinary bladder's trigone are also rich in estrogen receptors, connective tissue relaxation and sphincter dysfunction occur with declined body hormone levels, often accompanied by frequent urination, urinary urgency, urinary pain, enuresis, recurrent urinary tract infection, and other symptoms. According to a survey, 44.4% of women with VVA have moderate or severe vaginal dryness, with 30.2% of women with VVA experiencing severe pain during intercourse [4].

Another study in the United States found that VVA not only causes local discomfort in the vagina, but also significantly affects the patient's sexual function, sleep pattern, and personality [5]. A survey of women whose ages

are between 55 and 65 revealed that 58% of patients with vaginal discomfort avoid intimacy [6]. Moreover, vaginal itching, leukorrhea, and sexual problems caused by VVA seriously affect the quality of life of postmenopausal women [7].

Hormone replacement is currently considered the most effective treatment [8]. Meanwhile, lubricants, vaginal flora regulators, and androgen preparations are also widely used in clinical practice. However, the safety of hormone replacement therapy remains controversial, especially for patients with a history of breast or endometrial cancer, or those with atherosclerotic heart disease, venous thromboembolism, or active liver disease. In addition, no consensus has been reached regarding the optimal treatment scheme and dose in hormone replacement therapy [9]. Vaginal lubricant treatment increases the rate of sexually transmitted diseases (p=0.006) and adversely affects vaginal epithelial cells and flora [10, 11]. Moreover, it also tends to cause mucosal irritation (p=0.001), resulting in further aggravation of long-term symptoms [12]. The efficacy and safety of other drugs in the treatment of VVA remain largely undefined.

Traditional Chinese medicine suggests that the relationship between VVA occurrence and the kidney is the closest; the syndrome of Yin deficiency of kidney is mostly in the perimenopausal and early menopausal periods; with the prolongation of menopausal time, the innate essence is further exhausted, Yang cannot support Yin, and Yin impairment affects Yang, leading to kidney Yang debilitation. The main clinical manifestations are cold, declined sexual desire, soreness and weakness of waist and knees, vaginal dryness, and dysuria.

Our previous studies [13, 14] found that, compared with the placebo group, treatment with the formulation comprising Jiawei Qing'e Fang, Danzhi Qing'e formula, and Erzhi formula effectively improves the quality of life of perimenopausal women and relieves vasomotor symptoms. Therefore, we aimed to further assess the clinical efficacy of this traditional Chinese medicine on menopause-related VVA.

The MEG is a compound preparation based on our previous clinical and pharmacological studies and includes *Fructus Ligustri Lucidi*, *Eclipta*, *Herba Cistanches, Cynomorium Songaricum,* and *Cortex Phellodendri*. A randomized, double-blind, placebo parallel control trial was conducted to assess the efficacy and safety of MEG on menopause-related VVA by observing changes in patient's VHS, vaginitis score, VMI, FSFI, and modified KI.

2. Materials and Methods

2.1. Ethics and Registration. This study was approved by the ethics committee of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine and registered in the Chinese Clinical Trial Registry (Registration Number: ChiCTR-IOR-16009312). All the patients included provided signed informed consent before enrolment.

2.2. Principle of Study Design

2.2.1. Sample Content. Sample size for superiority trials for comparing two means was used to evaluate the formula for calculations.

It was expected that, after 12 weeks of treatment, the difference in VHS between test group and control group would be 4 points, for a total variance of 2.75. The optimal threshold value for statistical superiority was set at Δ=3. Class I and II errors were set at α=0.025 and β=0.10, respectively; therefore, at least 35 cases were required in each group.

2.2.2. Randomization. A completely randomized design was adopted; the random arrangement for treatments (therapeutic and control drugs) of subjects 01 to 88 was obtained with the SPSS software, which generated a random coding table.

2.2.3. Blinding Method. This was two level of blind design. The first level was processing a code corresponding to the groups (group A or B); the second level was the drug corresponding to the processing code. All the drug encoding process was written in the form of a document (blind code record) by a code-blinded person, which was kept as one of the clinical trial documents. The contents included preparation of drugs, drug packaging, prescription, storage requirements, drug delivery method, generation of random processing, drug packaging box for each subject, emergency letter, preservation of treatment code, and regulation of unblinding. The two levels of treatment codes were sealed separately and conserved by the Experimental Center and Research Department of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine. There was an emergency letter corresponding to each coded trial drug for unblinding in case of emergency.

2.3. Subjects. The subjects were eligible patients treated from November 2016 to April 2017 at the gynecologic outpatient clinic of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine.

Inclusion criteria were ① age from 45 to 65 years, with menopause time ≥1 year; ② at least one of VVA symptoms, such as vaginal dryness, vaginal or genital itching, painful sexual intercourse and so on; ③ percentage of superficial cells in the vaginal epithelium ≤10% and vaginal pH>5; ④ follicle stimulating hormone (FSH) ≥40 mIU/ml and estradiol (E_2) ≤20 pg/ml; ⑤ signed informed content. Exclusion criteria were: ① a history of malignant tumor of the reproductive system or breast cancer; ② unexplained uterine bleeding; ③ combined with other severe primary diseases or severely impaired hepatic or renal function; ④ abnormal cervical smear; ⑤ endometrium thickness by vaginal ultrasound >5 mm; ⑥ a history of antidepressant drugs, antipsychotic drugs or drugs containing other components in the past 3 months.

2.4. Methods

2.4.1. Medication and Administration. Patients in the treatment group were administered MEG (10 g preparation, 1 bag daily taken twice) for 12 consecutive weeks. The placebo group received placebo (starch, dextrin, bitterant, and so on, it is appearance is very close to that of MEG) as in the treatment group. All trial drugs, including treatment and placebo types, were provided by Huarun 39 medical Limited through Share Ltd. They were packaged according to the requirements of blinding and met quality requirements.

2.4.2. Observation Indexes

(1) Efficacy Indexes. The primary efficacy measure in this trial was the VHS [15], which includes vaginal elasticity, moisture, pH, mucosa and discharge. Scores for each item ranged from 1 to 4 points. The lower the score, the severer the local vaginal symptom. Scores were recorded by a doctor while performing gynecologic examination.

Secondary efficacy parameters included vaginitis score, VMI, FSFI, and modified KI.

Vaginitis score [8] was based on the patient's subjective feelings, including vaginal pain, painful sexual intercourse, vaginal itching, and burning and ranged from 0 to 3 points. The higher the score, the more serious the symptom.

For assessing the VMI [8], the vagina was dilated with a vaginal speculum. Aseptic cotton bubs were soaked in physiological saline, extended into the upper 1/3 vaginal wall and gently rolled, removed, transversely put on the slide, and rolled in one direction. After fixation with 95% ethanol for 30 minutes, Papanicolaou staining was performed, and the percentages of superficial and basal cells in the vaginal epithelium were determined, respectively.

The FSFI [16] included 19 items, with six domains of desire, arousal, lubrication, orgasm, satisfaction, and pain, which mainly reflect feelings and reactions about sexual life in the last 4 weeks.

Based on the original Kupperman scale, two items were added to the modified KI [17], including urinary system symptoms and vulvovaginal discomfort. The modified KI included 13 entries reflecting menopause symptoms. There were different basic and severity scores in each entry. Severity score ranged from 0 to 3 points; symptom score=Basic score×Severity score. The total score was derived as the sum of all symptom scores.

(2) Safety Parameters. Safety assessments included routine blood test, urine routine test, liver function test, renal function test, serum E_2 and FSH level assessment, electrocardiography, breast ultrasound and pelvic ultrasound. Meanwhile, adverse events were recorded.

2.4.3. Follow-Up. Efficacy indexes were evaluated at baseline, and at 4, 8, and 12 weeks during drug administration, as well as 4 weeks after drug withdrawal. Safety parameters, including laboratory examination and ultrasound, were obtained before group assignment and at 12 weeks during drug administration. Adverse events were observed during the whole follow-up period.

2.5. Statistical Analysis. The SPSS 23.0 software was used for statistical analysis. Normality and homogeneity of variance were assessed for VHS, vaginitis score, VMI, and modified KI. Normally distributed data were assessed by independent two-sample t-test, with paired sample t-test used for within-group comparison before and after treatment. For nonnormally distributed parameters, the nonparametric test was used for comparing groups before and after treatment.

3. Results

3.1. Baseline Patient Characteristics. A total of 88 patients were enrolled in this study, including 1 case of mistaken identity, 1 without medication record, 11 lost to follow up and 75 cases who completed treatment (39 and 36 cases in the treatment and placebo groups, respectively). All the enrolled patients met the set inclusion criteria. There was no statistical differences in baseline indexes, including demographic data (age, body mass index, menopause time and so on) and efficacy indexes (VHS, vaginitis score, VMI, FSFI, and modified KI). The baseline parameters of the two groups were comparable (Table 1).

3.2. Efficacy

3.2.1. Vaginal Health Score. All patients were followed up during the medication period and at 4 weeks after drug withdrawal. VHS in the treatment group were significantly increased in comparison with baseline values ($p<0.05$). VHS in the placebo group after treatment and during the follow-up period were not statistically different from baseline values ($p>0.05$). During the medication period and at 4 weeks after drug withdrawal, VHS in the treatment group were markedly increased in comparison with those of the placebo group ($p<0.05$).

During the medication period and at 4 weeks after drug withdrawal, vaginal moisture, pH, mucous, and discharge scores in the treatment group were significantly increased in comparison with baseline values ($p<0.05$). After 4 weeks of treatment, the vaginal elasticity score in the treatment group was increased in comparison with the baseline value, but the difference was not statistically significant ($p>0.05$). After 8 and 12 weeks of treatment as well as at 4 weeks after drug withdrawal, vaginal elasticity scores in the treatment group were significantly increased in comparison with baseline values ($p<0.05$). During the medication period and follow-up, vaginal elasticity, moisture, pH, mucous, and discharge scores in the placebo group were not statistically different with those at baseline ($p>0.05$).

During the medication period and at 4 weeks after drug withdrawal, vaginal moisture, mucous, and discharge scores in the treatment group were significantly increased in comparison with those of the placebo group ($p<0.05$). After 4 and 8 weeks of treatment, vaginal elasticity and vaginal pH in the treatment group were increased in comparison with those

Table 1: Baseline parameters in the two groups.

	treatment group	placebo group	p
demographic			
n	39	36	
Age, years	59.67±3.47	58.53±4.53	0.338
Body mass index, kg/m	24.23±3.45	24.59±3.51	0.726
age of menopause, years	49.43±3.93	49.44±4.25	0.061
Menopause time, years	10.24±5.37	9.09±5.96	0.241
Course of disease, years	1.56±0.88	1.21±1.21	0.979
vaginal health score	9.87±2.33	10.44±2.35	0.245
elasticity	2.18±0.56	2.25±0.55	0.585
moisture	2.00±0.76	2.08±0.73	0.700
pH	1.92±0.66	1.89±0.71	0.810
mucosa	1.95±0.89	2.14±0.72	0.330
discharge	1.82±0.68	2.08±0.73	0.127
Vaginitis score	4.50±1.57	4.58±1.57	0.925
pain	0.87±0.52	0.94±0.63	0.619
painful sexual intercourse	1.18±0.73	1.26±0.81	0.673
itching	1.38±0.75	1.19±0.58	0.276
burning	1.18±0.68	1.06±0.71	0.472
vaginal maturation index			
percentage of superficial cells	0.67±1.18	1.06±1.24	0.079
percentage of basal cells	55.23±10.32	53.53±9.95	0.494
female sexual function index	12.78±8.55	12.26±8.39	0.738
desire	2.38±0.76	2.40±0.98	0.841
arousal	1.65±1.66	1.48±1.55	0.710
lubrication	1.92±1.94	1.80±1.90	0.844
orgasm	1.81±1.82	1.58±1.62	0.527
satisfaction	2.79±0.89	3.01±1.01	0.212
pain	2.24±2.15	2.00±1.97	0.431
modified Kupperman Index	22.64±6.38	23.61±6.56	0.531
Sweating, hot flushes	1.08±0.81	1.14±0.80	0.796
Paresthesia	0.85±0.71	1.19±0.82	0.060
Insomnia	1.23±0.78	1.31±0.79	0.849
Nervousness	1.46±0.64	1.44±0.84	0.817
Melancholia	1.00±0.76	0.86±0.83	0.390
Vertigo	0.82±0.76	0.89±0.62	0.589
Fatigue	1.62±0.49	1.58±0.73	0.629
Arthralgia, myalgia	0.79±0.83	0.86±0.76	0.654
Headache	0.90±0.88	1.03±0.81	0.529
Heart palpitation	0.72±0.76	0.67±0.59	0.971
Formication	0.28±0.46	0.28±0.45	0.967
Sexual complaints	1.82±0.64	1.94±0.71	0.257
Urinary tract infection	0.74±0.85	0.56±0.65	0.410

of the placebo group, but the differences were not statistically significant ($p>0.05$). After 12 weeks of treatment and at 4 weeks after drug withdrawal, vaginal elasticity and pH in the treatment group were significantly higher than those of the placebo group ($p<0.05$) (Table 2)

3.2.2. Vaginitis Score. During the medication period and 4 weeks after drug withdrawal, vaginitis scores in the treatment group were significantly decreased in comparison with baseline values ($p<0.05$). Vaginitis scores in the placebo group after medication and during the follow-up period were not statistically different from baseline values ($p>0.05$). Meanwhile, vaginitis scores in the treatment group during the medication period and 4 weeks after drug withdrawal were starkly decreased in comparison with those of the placebo group ($p<0.05$).

Table 2: Vaginal health score.

	groups	baseline	4 weeks	8 weeks	12 weeks	4 weeks after drug withdrawal	p_1	p_2	p_3	p_4
VHS	treatment group	9.87±2.33	12.00±1.81	13.10±1.79	14.64±1.37	13.41±1.37	<0.001	<0.001	<0.001	<0.001
	placebo group	10.44±2.35	10.44±2.32	10.36±2.31	10.39±2.13	10.42±2.29	1.000	0.370	0.640	0.560
	p	0.245	0.002	<0.001	<0.001	<0.001				
elasticity	treatment group	2.18±0.56	2.21±0.57	2.31±0.57	2.82±0.39	2.77±0.43	0.32	0.03	<0.001	<0.001
	placebo group	2.25±0.55	2.25±0.55	2.22±0.54	2.22±0.54	2.22±0.54	1.000	0.320	0.320	0.320
	p	0.585	0.744	0.484	<0.001	<0.001				
moisture	treatment group	2.00±0.76	2.59±0.55	2.90±0.60	3.08±0.42	2.85±0.54	<0.001	<0.001	<0.001	<0.001
	placebo group	2.08±0.73	2.08±0.73	2.08±0.73	2.11±0.71	2.11±0.75	1.000	1.000	0.320	0.320
	p	0.700	0.001	<0.001	<0.001	<0.001				
pH	treatment group	1.92±0.66	2.10±0.60	2.23±0.67	2.59±0.59	2.54±0.60	<0.001	<0.001	<0.001	<0.001
	placebo group	1.89±0.71	1.94±0.71	1.94±0.67	1.92±0.69	1.92±0.73	0.160	0.160	0.320	0.320
	p	0.810	0.305	0.081	<0.001	<0.001				
mucosa	treatment group	1.95±0.89	2.54±0.60	2.77±0.48	2.97±0.28	2.85±0.43	<0.001	<0.001	<0.001	<0.001
	placebo group	2.14±0.72	2.08±0.69	2.03±0.61	2.08±0.55	2.06±0.58	0.160	0.160	0.480	0.260
	p	0.330	0.004	<0.001	<0.001	<0.001				
discharge	treatment group	1.82±0.68	2.56±0.55	2.90±0.45	3.18±0.39	2.41±0.50	<0.001	<0.001	<0.001	<0.001
	placebo group	2.08±0.73	2.08±0.73	2.08±0.73	2.06±0.67	2.11±0.75	1.000	1.000	0.560	0.320
	p	0.127	0.002	<0.001	<0.001	0.048				

P: significant difference between the treatment and placebo groups.
P_1: significant difference in score and baseline between the treatment and placebo groups after 4 weeks of medication.
P_2: significant difference in score and baseline between the treatment and placebo groups after 8 weeks of medication.
P_3: significant difference in score and baseline between the treatment and placebo groups after 12 weeks of medication.
P_4: significant difference in score and baseline between the treatment and placebo groups 4 weeks after drug withdrawal.

During the medication period and 4 weeks after drug withdrawal, vaginal pain, vaginal itching, and burning scores in the treatment group were significantly decreased in comparison with baseline values (all $p<0.05$). After 4 and 8 weeks of treatment, painful sexual intercourse scores in the treatment group were decreased in comparison with baseline values, but the differences were not statistically significant ($p>0.05$); after 12 weeks of treatment and 4 weeks after drug withdrawal, painful sexual intercourse scores in the treatment group were significantly decreased in comparison with baseline values ($p<0.05$). Vaginal pain, painful sexual intercourse, vaginal itching, and burning scores in the placebo group in the medication period and during follow-up were not statistically different from those at baseline ($p>0.05$). In the medication period and 4 weeks after medicine withdrawal, vaginal pain and burning scores in the treatment group were markedly decreased in comparison with those of the placebo group ($p>0.05$). After 4 weeks of treatment, vaginal itching scores in the treatment group were decreased in comparison with those of the placebo group, but the difference was not statistically significant ($p>0.05$). After 8 and 12 weeks of treatment as well as 4 weeks after drug withdrawal, vaginal itching scores in the treatment group were significantly lower than those of the placebo group ($p<0.05$). After 4 and 8 weeks of treatment, respectively, painful sexual intercourse scores in the treatment group were decreased in comparison with those of the placebo group, but the differences were not statistically significant ($p>0.05$). After 12 weeks of treatment, painful sexual intercourse scores in the treatment group were significantly lower than those of the placebo group ($p<0.05$). At 4 weeks after drug withdrawal, there were no statistical differences in painful sexual intercourse scores between the treatment and placebo groups ($p>0.05$) (Table 3)

3.2.3. Vaginal Maturation Index. During the medication period and 4 weeks after drug withdrawal, the percentage of superficial cells in the vaginal epithelium was markedly increased while that of basal cells was significantly reduced in the treatment group in comparison with values before treatment ($p<0.05$). In the medication period and during follow-up, the percentages of superficial and basal cells in the vaginal epithelium were not statistically different from baseline values in the placebo group ($p>0.05$). However, 4 weeks after drug withdrawal, the percentages of basal cells in the vaginal epithelium in the placebo group were significantly increased in comparison with the baseline value ($p<0.05$). In the medication period and 4 weeks after drug withdrawal, the percentages of superficial cells in the vaginal epithelium in the treatment group were starkly increased while those of basal cells were significantly decreased, in comparison with those of the placebo group ($p<0.05$) (Table 4).

3.2.4. Female Sexual Function Index. During the medication period and 4 weeks after drug withdrawal, the FSFI in the treatment group was significantly increased in comparison with the baseline value ($p<0.05$). FSFI in the placebo group in the medication period and during follow-up were not statistically different from those at baseline ($p>0.05$). In the medication period and 4 weeks after drug withdrawal, there was no significant difference in FSFI between the treatment and placebo groups ($p<0.05$).

During the medication period and 4 weeks after drug withdrawal, lubrication scores in the treatment group were significantly increased in comparison with baseline values ($p<0.05$). Satisfaction scores in the treatment group were significantly increased in the medication period compared with baseline values ($p<0.05$) but not at 4 weeks after drug withdrawal ($p>0.05$). After 12 weeks of treatment and 4 weeks after drug withdrawal, painful sexual intercourse scores in the treatment group were starkly increased in comparison with baseline values ($p<0.05$). In the medication and follow-up periods, desire, subjective arousal ability and orgasm scores in the treatment group were not statistically different from baseline values ($p>0.05$). The six domains of the FSFI in the placebo group in the medication and follow-up periods were not statistically different from baseline values ($p>0.05$). In the medication period and during follow-up,no significant differences in the 6 domains of the FSFI between the treatment and placebo groups ($p>0.05$) (Table 5).

3.2.5. Modified Kupperman Index. In the medication period and 4 weeks after drug withdrawal, modified KI in the treatment group were significantly decreased in comparison with baseline values ($p<0.05$). Modified KI in the placebo group in the medication and follow-up periods were not statistically significant from baseline values ($p>0.05$). After 4 weeks of treatment, modified KI in the treatment group were decreased in comparison with those of the placebo group, but the difference was not statistically significant ($p>0.05$). After 8 and 12 weeks of treatment, as well as 4 weeks after drug withdrawal, modified KI in the treatment group were significantly decreased in comparison with those of the placebo group ($p< 0.05$).

In the medication period and 4 weeks after drug withdrawal, scores for hot flashes, sweating, insomnia, fatigue and urinary tract infection in the treatment group were markedly decreased in comparison with baseline values ($p<0.05$). After 4 weeks of treatment, arthralgia and myalgia scores in the treatment group were decreased in comparison with baseline values, but the difference was not statistically significant ($p>0.05$). After 8 and 12 weeks of treatment, as well as 4 weeks after drug withdrawal, these scores were significantly decreased in the treatment group in comparison with baseline values ($p<0.05$). In the medication period, paresthesia, nervousness, melancholia, vertigo, headache, heart palpitation, sexual complaints scores in the treatment group were not statistically different from those at baseline ($p>0.05$). In the medication and follow-up period, modified KI in the placebo group were not statistically different from baseline values ($p>0.05$). In the medication period, scores for hot flashes, sweating, insomnia and fatigue in the treatment group were significantly decreased in comparison with those of the placebo group ($p<0.05$). At 4 weeks after drug withdrawal, fatigue scores in the treatment group remained significantly lower than those of the placebo group ($p<0.05$); however, no significant differences in scores for hot flashes, sweating

Table 3: Vaginitis scores.

	groups	baseline	4 weeks	8 weeks	12 weeks	4 weeks after drug withdrawal	p_1	p_2	p_3	p_4
Vaginitis score	treatment group	4.50±1.57	2.68±0.99	1.91±0.97	1.41±0.85	2.09±1.02	<0.001	<0.001	<0.001	<0.001
	placebo group	4.58±1.57	4.63±1.54	4.58±1.61	4.63±1.54	4.58±1.64	0.710	1.000	0.650	1.000
	p	0.925	<0.001	<0.001	<0.001	<0.001				
pain	treatment group	0.87±0.52	0.64±0.49	0.51±0.51	0.26±0.44	0.36±0.49	0.010	<0.001	<0.001	<0.001
	placebo group	0.94±0.63	0.97±0.65	0.97±0.65	0.94±0.63	1.00±0.68	0.320	0.320	1.000	0.480
	p	0.619	0.024	0.002	<0.001	<0.001				
painful sexual intercourse	treatment group	1.18±0.73	1.09±0.68	1.00±0.62	0.95±0.58	0.95±0.58	0.160	0.050	0.030	0.030
	placebo group	1.26±0.81	1.32±0.82	1.37±0.83	1.37±0.83	1.32±0.82	0.320	0.160	0.160	0.320
	p	0.673	0.272	0.076	0.046	0.080				
itching	treatment group	1.38±0.75	0.85±0.63	0.38±0.49	0.23±0.43	0.49±0.56	<0.001	<0.001	<0.001	<0.001
	placebo group	1.19±0.58	1.08±0.55	1.08±0.65	1.06±0.63	1.08±0.60	0.050	0.250	0.100	0.210
	p	0.276	0.086	<0.001	<0.001	<0.001				
burning	treatment group	1.18±0.68	0.28±0.51	0.08±0.27	0.03±0.16	0.44±0.50	<0.001	<0.001	<0.001	<0.001
	placebo group	1.06±0.71	1.08±0.69	1.11±0.71	1.08±0.73	1.03±0.81	0.560	0.320	0.560	0.650
	p	0.472	<0.001	<0.001	<0.001	<0.001				

Table 4: Vaginal maturation index.

	groups	baseline	4 weeks	8 weeks	12 weeks	4 weeks after drug withdrawal	p_1	p_2	p_3	p_4
percentage of superficial cells	treatment group	0.67±1.18	2.39±0.79	4.28±1.88	5.49±1.64	4.59±1.68	<0.001	<0.001	<0.001	<0.001
	placebo group	1.06±1.24	2.42±0.96	0.86±0.96	0.86±0.96	0.86±1.25	0.410	0.050	0.110	0.040
	p	0.079	<0.001	<0.001	<0.001	<0.001				
percentage of basal cells	treatment group	55.23±10.32	44.56±10.74	37.31±8.54	33.85±7.91	41.26±8.48	<0.001	<0.001	<0.001	<0.001
	placebo group	53.53±9.95	53.22±9.79	54.58±9.14	55.17±9.09	57.53±8.78	0.880	0.450	0.180	0.010
	p	0.494	0.001	<0.001	<0.001	<0.001				

Table 5: Female sexual function index.

	groups	baseline	4 weeks	8 weeks	12 weeks	4 weeks after drug withdrawal	p_1	p_2	p_3	p_4
FSFI	treatment group	12.79±8.55	13.09±8.83	13.45±9.05	13.76±9.36	13.46±9.16	<0.001	<0.001	<0.001	<0.001
	placebo group	12.26±8.39	12.47±8.33	12.42±8.48	12.38±8.52	12.27±8.43	0.549	0.905	0.411	0.190
	p	0.738	0.628	0.352	0.236	0.325				
desire	treatment group	2.39±0.76	2.77±1.90	2.40±0.76	2.43±0.78	2.42±0.79	0.527	0.317	0.059	0.102
	placebo group	2.40±0.98	1.11±1.35	2.42±0.97	2.43±0.99	2.42±0.98	0.317	0.564	1.000	0.564
	p	0.841	0.778	0.930	0.947	0.960				
arousal	treatment group	1.65±1.66	1.62±1.65	1.60±1.64	1.61±1.64	1.58±1.63	0.527	0.705	1.000	0.317
	placebo group	1.48±1.55	1.53±1.56	1.53±1.56	1.54±1.57	1.52±1.55	0.705	0.705	0.293	1.000
	p	0.710	0.818	0.924	0.951	0.906				
lubrication	treatment group	1.92±1.94	2.13±2.16	2.45±2.42	2.47±2.45	2.41±2.39	<0.001	<0.001	<0.001	<0.001
	placebo group	1.80±1.90	1.89±1.94	1.89±1.94	1.89±1.94	1.89±1.92	0.102	0.102	0.102	0.194
	p	0.844	0.587	0.246	0.226	0.260				
orgasm	treatment group	1.81±1.82	1.78±1.83	1.79±1.82	1.77±1.80	1.76±1.80	0.157	0.180	0.705	1.000
	placebo group	1.58±1.62	1.63±1.63	1.63±1.64	1.63±1.63	1.62±1.64	0.564	0.655	0.739	1.000
	p	0.527	0.644	0.597	0.669	0.698				
satisfaction	treatment group	2.79±0.89	2.92±0.87	2.93±0.88	3.02±0.94	2.89±0.86	0.028	0.044	0.006	0.144
	placebo group	3.01±1.01	3.05±0.96	3.01±1.03	2.98±1.01	2.94±1.03	0.380	0.739	0.448	0.234
	p	0.212	0.404	0.749	0.888	0.741				
pain	treatment group	2.24±2.15	2.25±2.23	2.29±2.26	2.47±2.42	2.40±2.36	0.102	0.067	0.005	0.020
	placebo group	2.00±1.97	1.95±1.98	1.95±2.00	1.90±1.95	1.89±1.92	0.317	0.414	0.323	0.283
	p	0.431	0.349	0.297	0.225	0.255				

and insomnia were found between the treatment and placebo groups ($p>0.05$). After 4 and 8 weeks of treatment, paresthesia scores in the treatment group were decreased in comparison with those of the placebo group, but the differences were not statistically significant ($p>0.05$). After 12 weeks of treatment and 4 weeks after drug withdrawal, paresthesia scores in the treatment group were significantly lower than those of the placebo group ($p<0.05$). After 4 weeks of treatment, bone and joint pain scores in the treatment group were decreased in comparison with those of the placebo group, but the difference was not statistically significant ($p>0.05$). After 8 and 12 weeks of treatment as well as 4 weeks after drug withdrawal, Arthralgia and myalgia scores in the treatment group were significantly lower than those of the placebo group ($p<0.05$). In the medication and follow-up periods, there was no statistical difference in modified KI between the treatment and placebo groups (Table 6).

3.3. Safety Parameters

3.3.1. Changes in Serum Hormone Levels, Endometrial Thickness, and Breast Ultrasound. At 12 weeks of treatment, serum E_2, and FSH levels, endometrial thickness and breast ultrasound findings for both groups were within the normal ranges after menopause.

3.3.2. Laboratory Indexes. At 12 weeks of treatment, no significant differences were found in blood routine, urine routine, hepatic and renal function parameters, as well as electrocardiograms between the two groups.

3.3.3. Adverse Events. During the medication period, adverse events occurred in 10 cases and were all mild or moderate. Of these, 2 cases were associated with medication, including 1 each in the treatment (diarrhea) and placebo (abdominal distention) groups, respectively; both cases were mild, and symptoms were relieved by taking medicine after a meal. No vaginal bleeding was found in this study. There was no case of withdrawal due to adverse drug reactions (Table 7).

4. Discussion

VVA is a common and frequently encountered disease in menopausal women. Unlike the symptoms of hot flashes, sweating, depression and anxiety, VVA is not relieved with time, and seriously affects the patients' quality of life.

From the Chinese medicine perspective, menopausal symptoms are thought to be associated with a decline in kidney Yin or Yang or both. Kidney is the congenital life basis and closely related to reproduction physiological activities. TCM believes that the menstruation, pregnancy, delivery, and lactation in woman's life are all supported by kidney Yin. Excessive consumption of kidney Yin such as irregular periods, repeated pregnancy, and long time lactation will lead to kidney Yin deficiency. It is typically characterized by syndrome such as vaginal dryness, hot flushes and night sweats, dizziness, and insomnia. According to TCM theory, Yin and Yang grow together and nourish each other. It will be accompanied by kidney Yang deficiency as kidney Yin is further exhausted with stages of menopause. Women may present with symptoms such as vaginal burning and itching, aversion to cold, urine incontinence, and loose motions.

MEG are composed of *Eclipta*, *Fructus Ligustri Lucidi*, Cynomorium Songaricum, *Herba Cistanches,* and *Cortex Phellodendri*. The efficacy of *Fructus Ligustri Lucidi* and *Eclipta* is nourishing kidney Yin, so as to moisten the vagina. *Herba Cistanches* and *Cynomorium Songaricum* are often used for warming kidney Yang. *Cortex Phellodendri* has the function of clearing heat, relieving toxicity and diminishing inflammations, and it has antipruritic properties in patients with vulvovaginal atrophy. Therefore, the combination of these herbs is very effective for vulvovaginal atrophy due to deficiency of kidney Yin and Yang which is accompanied with systemic symptoms.

The current study showed that MEG obviously improved the symptoms of reduced vaginal discharge, pale mucous membrane and reduced vaginal elasticity, relieved vaginal pain, painful sexual intercourse, and vaginal itching and burning, while increasing the percentage of epithelial cells and decreasing that of basal cells in the vaginal epithelium. At 12 weeks of medication with MEG, vaginal dryness and itching scores were decreased by 1.18 and 1.01 points, respectively; the percentages of superficial and basal cells in the vaginal epithelium increased by 4.82% and decreased by 21.38%, respectively. Previous studies showed that 12 weeks after treatment with vaginal E_2 soft capsule (10 μg), vaginal dryness and itching scores decrease by 1.5 and 0.8 points, respectively; meanwhile, the percentages of superficial and basal cells in the vaginal epithelium increased by 17% and decreased by 44%, respectively [18]. After 12 weeks of Ospemifene administration (60 mg), the vaginal dryness score decreased by 1.3 points; the percentages of superficial and basal cells in the vaginal epithelium increased by 7% and decreased by 31.7%, respectively [19]; at 12 weeks of dehydroepiandrosterone sulfate use (DHEA, 6.5 mg), the vaginal dryness score decreased by 1.44 points; the percentages of superficial and basal cells in the vaginal epithelium increased by 8.44% and decreased by 27.7%, respectively [20]. The above results indicated that the effects of MEG on vaginal dryness and vaginal itching were significant, and equivalent to those of local vaginal estrogens, Ospemifene, and vaginal DHEA, although VMI improvement was weaker compared with what found for the above drugs. In addition, MEG also improved female sexual function, mainly reflected in increased vaginal moisture, enhanced sexual life satisfaction, and relieved painful sexual intercourse, with significant differences compared with baseline values; however, there were no significant differences in comparison with the values of the placebo group, which might be related to the small sample size of this study. Therefore, larger sample studies are needed to draw a definite conclusion.

Cases with VVA are often accompanied by other menopausal symptoms. A study showed that vaginal dryness is significantly related to the occurrence of hot flashes (OR=1.52; 95%CI, 1.19-1.93) [21]. The duration of vasomotion is often over 5.5 years [22], and more than 50% of patients still have symptoms 4 years after menopause [21], which increases

Table 6: Modified Kupperman Index.

	groups	baseline	4 weeks	8 weeks	12 weeks	4 weeks after drug withdrawal	p_1	p_2	p_3	p_4
modified KI	treatment group	22.64±6.38	19.23±6.16	17.72±6.17	17.38±5.67	19.18±5.84	<0.001	<0.001	<0.001	<0.001
	placebo group	23.61±6.56	23.47±7.17	22.75±6.18	23.19±6.05	23.25±5.66	0.219	0.136	0.571	0.978
	p	0.531	0.054	0.002	<0.001	0.007				
sweating, hot flushes	treatment group	4.31±3.23	2.77±2.92	2.26±3.02	2.15±3.02	2.87±2.89	<0.001	<0.001	<0.001	0.001
	placebo group	4.56±3.19	4.56±3.19	4.44±3.28	4.44±3.28	4.56±3.19	1.000	0.317	0.317	1.000
	p	0.796	0.036	0.007	0.004	0.053				
paresthesia	treatment group	1.69±1.42	1.59±1.39	1.59±1.39	1.38±1.39	1.59±1.39	0.317	0.317	0.058	0.480
	placebo group	2.39±1.64	2.17±1.61	2.11±1.72	2.39±1.71	2.33±1.69	0.102	0.059	0.705	0.527
	p	0.060	0.058	0.110	0.004	0.026				
insomnia	treatment group	2.46±1.55	1.85±1.33	1.69±1.34	1.69±1.26	1.95±1.41	0.001	0.001	0.001	0.022
	placebo group	2.61±1.57	2.72±1.45	2.33±1.62	2.22±1.64	2.33±1.55	0.157	0.157	0.109	0.285
	p	0.849	0.012	0.042	0.047	0.126				
nervousness	treatment group	2.92±1.29	2.77±1.35	2.77±1.35	2.72±1.34	3.03±1.37	0.083	0.059	0.096	1.000
	placebo group	2.89±1.69	2.72±1.80	2.61±1.71	2.72±1.80	2.67±1.59	0.180	0.257	0.480	0.317
	p	0.817	0.409	0.542	0.554	0.322				
melancholia	treatment group	1.00±0.76	1.05±0.82	1.05±0.82	1.05±0.82	1.08±0.80	1.000	1.000	1.000	0.564
	placebo group	0.86±0.83	0.86±0.73	0.89±0.72	0.91±0.78	0.94±0.77	0.157	0.083	0.157	0.059
	p	0.390	0.347	0.440	0.539	0.551				
vertigo	treatment group	0.82±0.76	0.83±0.81	0.75±0.74	0.85±0.83	0.88±0.72	1.000	0.317	0.527	0.257
	placebo group	0.89±0.62	0.91±0.61	0.89±0.63	0.94±0.59	1.06±0.59	1.000	0.655	0.705	0.059
	p	0.589	0.419	0.329	0.406	0.227				
fatigue	treatment group	1.62±0.49	1.18±0.55	1.05±0.55	1.00±0.60	1.08±0.62	<0.001	<0.001	<0.001	<0.001
	placebo group	1.58±0.73	1.57±0.74	1.6±0.74	1.6±0.74	1.57±0.74	1.000	0.564	0.564	1.000
	p	0.629	0.017	0.001	<0.001	0.005				
arthralgia, myalgia	treatment group	0.79±0.83	0.73±0.75	0.53±0.64	0.50±0.60	0.53±0.64	0.317	0.004	0.002	0.004
	placebo group	0.86±0.76	0.86±0.73	0.91±0.70	0.91±0.70	0.91±0.74	0.317	0.564	0.655	0.655
	p	0.654	0.413	0.014	0.009	0.020				
headache	treatment group	0.90±0.88	0.90±0.81	0.90±0.78	0.95±0.78	1.00±0.82	1.000	1.000	0.564	0.356
	placebo group	1.03±0.81	1.03±0.82	1.03±0.75	1.03±0.75	0.97±0.79	1.000	1.000	1.000	0.480
	p	0.529	0.567	0.500	0.710	0.817				
heart palpitation	treatment group	0.72±0.76	0.80±0.82	0.65±0.74	0.65±0.70	0.73±0.68	0.257	0.257	0.366	1.000
	placebo group	0.67±0.59	0.66±0.59	0.60±0.60	0.63±0.60	0.60±0.55	1.000	0.527	0.739	0.527
	p	0.971	0.654	0.962	0.943	0.503				
formication	treatment group	0.28±0.46	0.35±0.62	0.25±0.44	0.28±0.45	0.28±0.45	0.317	0.317	1.000	1.000
	placebo group	0.28±0.45	0.29±0.46	0.29±0.46	0.26±0.44	0.20±0.41	1.000	1.000	0.317	0.18
	p	0.967	0.840	0.729	0.862	0.451				
sexual complaints	treatment group	3.64±1.29	3.69±1.17	3.54±1.17	3.54±1.17	3.54±1.17	0.564	0.317	0.414	0.527
	placebo group	3.89±1.43	3.83±1.46	3.94±1.39	4.00±1.35	4.00±1.35	0.317	0.564	0.157	0.157
	p	0.257	0.709	0.174	0.109	0.109				
urinary tract infection	treatment group	1.49±1.70	0.97±1.58	0.72±1.41	0.67±1.40	0.67±1.40	0.004	<0.001	<0.001	<0.001
	placebo group	1.11±1.30	1.06±1.31	1.11±1.30	1.11±1.30	1.11±1.30	0.317	1.000	1.000	0.317
	p	0.410	0.802	0.185	0.122	0.088				

Table 7: Adverse events.

adverse event	treatment group			placebo group			total		
	mild	moderate	severe	mild	moderate	severe	mild	moderate	severe
cough	1	0	0	0	0	0	1	0	0
pneumonia	1	0	0	0	0	0	1	0	0
diarrhea	1	0	0	1	0	0	2	0	0
knee arthritis	0	0	0	0	1	0	0	1	0
abdominal distention	0	0	0	1	0	0	1	0	0
zoster	0	0	0	0	1	0	0	1	0
hypertension	0	1	0	0	0	0	0	1	0
cold	0	0	0	1	0	0	1	0	0
somnolence	0	0	0	1	0	0	1	0	0

the risk of combined vulvovaginal symptoms. Karmakar N [23] found incidence rates of hot flashes, sweating, insomnia and fatigue among menopausal women in West Bengal (India) of 60%, 84%, and 93%, respectively. At 6 months after Ospemifene use (60 mg), changes in systemic symptoms such as insomnia, anorexia, dyspnea, nausea, and vomiting were shown not to differ from those of the placebo group [24]; the symptom of hot flashes was aggravated in 10% of patients after long-term Ospemifene administration, with 2% of subjects discontinuing the drug because of nontolerance [25]. In the present study, MEG significantly reduced the systemic symptoms of menopause syndrome, including vasomotor, and somatic symptoms and improved hot flashes, sweat, insomnia, fatigue, and bone and joint pain; the effects were obviously better than those of placebo. Li [26] found that Echinacetin (ECH), an extract from Herba Cistanche, has antiaging effect by increasing the antistress ability, increasing the levels of IL-2 in serum and NO and SOD in brain tissue, reducing the levels of IL-6 and MDA in brain tissue. Guo [27] found that intragastric administrate *Cynomorium Songaricum* in rats can alleviate the effect of overexercise on serum testosterone and corticosterone, promote the synthesis of protein, inhibit breakdown of amino acid and protein, and increase hemoglobin content and glycogen stores. These effects may help improve insomnia, fatigue, and joint pain in postmenopausal women. Moreover, MEG did not affect serum E_2 and FSH levels, and no damage to hepatic and renal function or serious adverse reactions occurred.

In the present study, recurrence in patients was followed up 4 weeks after drug withdrawal, and local manifestations in the vagina (vaginal elasticity, vaginal moisture, vaginal mucous and so on), atrophy symptoms (vaginal pain, itching, burning, and so on) and systemic symptoms (fatigue, bone and joint pain and so on) remained different from those of the placebo group. However, there are no reliable data about recurrence after withdrawal of other drugs such as Ospemifene, DHEA, and local estrogens.

Modern pharmacology found that the constituents of MEG, such as *Fructus Ligustri Lucidi*, *Eclipta*, *Herba Cistanches,* and *Cynomorium Songaricum*, have obvious estrogenic effects. There are terpenes, flavonoids, and coumaric esters in *Fructus Ligustri Lucidi* and *Eclipta*, with structures similar to that of estradiol [28–31]; they could induce the expression of the ERE-luciferase reporter gene and act as estrogens. Zheng [32] found that, compared with the blank control group, use of Er Zhi Wan (1.365 g/kg) results in overt keratinization of epithelial cells in the vaginal epithelium, epithelial thickening, increased ERα, and ERβ amounts in vaginal tissues, and selectively upregulated ERβ in uterine tissues; moreover, Er Zhi Wan does not significantly affect ERα levels and serum estrogen content in ovariectomized rats. Wang [33] found that the main active ingredients echinacoside and acteoside could bind the estrogen receptors ERα and ERβ, increase the luciferase activity of ERE and play estrogenic roles. In addition, according to the upregulation of both components in the luciferase activity of ERE, selectivity of acteoside for ERα and ERβ is not significant, and echinacoside has higher affinity to ERβ. The regulatory effects of these selective estrogen receptors might be the molecular mechanism by which MEG effectively improved the symptoms of VVA, increasing VMI, and relieve the symptoms of hot flashes and sweating without endometrial thickening and changing serum hormone levels [34].

Here, a randomized, double-blind, placebo-controlled clinical trial was firstly conducted for the traditional Chinese medicine treating VVA. VHS, vaginitis score, and VMI are all commonly used parameters for VVA evaluation worldwide. Vaginal atrophy was comprehensively evaluated from the objective and subjective aspects; the FSFI has been the most frequently used female function scale in the past few decades, with good evaluation validity, credibility, and reliability [16]. The modified KI is of high value for the evaluation of systemic symptoms in the menopause period [17]. By adopting the above scales, the clinical efficacy of the MEG was assessed comprehensively and systematically.

There were limitations in this study. First, only related symptoms were assessed in patients 4 weeks after drug withdrawal, and the duration of curative effect was not determined. Therefore, the follow-up time should be increased to further characterize MEG. Secondly, the sample size was relatively small, and subgroup analysis was not performed on age and symptom severity; in addition, efficacy indexes, such as sexual function dimensions in some females, were not consistent with those of the placebo group and before medication. Therefore, sample size should be further expanded. Finally, this study did not include a positive drug group. Therefore, similar trials are underway in our group to further compare the clinical efficacy of MEG with that of local estrogens in the treatment of VVA.

5. Conclusion

(1) Use of MEG for 4 weeks could obviously improve vaginal elasticity and vaginal moisture, increase vaginal discharge, decrease vaginal pH, obviously relieve vaginal pain, itching, and burning, and improve vaginal health in patients with menopause-related VVA. Moreover, the curative effects persisted 4 weeks after drug withdrawal.

(2) Use of MEG for 8 weeks could obviously relieve painful sexual intercourse, and the curative effects were maintained after drug withdrawal; however, there was no obvious effect on sexual desire and subjective sexual arousal after administration of MEG.

(3) With the prolongation of medication time, MEG could significantly increase the percentage of superficial cells in the vaginal epithelium, while decreasing that of basal cells, without significantly altering serum estrogen levels.

(4) MEG could obviously improve menopause-related systemic symptoms such as hot flashes, sweating, insomnia, fatigue, and bone and joint pain.

(5) No adverse effects on the endometrium, breast, liver, and kidney were observed with continuous use of MEG for 12 weeks.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Ranran Chen was responsible for data collection and manuscript writing; Dianrong Song was responsible for conception, design, interpretation, and critical revision and final approval of the manuscript; Wei Zhang and Xiumei Gao were responsible for conception and design, analysis, and critical revision; Guanwei Fan and Yingqiang Zhao were responsible for statistical design and data analysis. All authors read and approved the final manuscript before submission.

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Research Article

Association of Cold-Heat Patterns with Tongue Features, Body Composition, Anthropometric Indices, and Blood Parameters in Tae-Eum Type

Jihye Kim,[1] **Soo Jung Park**,[2] **Jiwon Yoon**,[1] **Bum Ju Lee**,[1] **and Keun Ho Kim**[1]

[1]*Future Medicine Division, Korea Institute of Oriental Medicine, 1672 Yuseong-daero, Yuseong-gu, Daejeon, Republic of Korea*
[2]*Department of Sasang Constitutional Medicine, College of Korean Medicine, Woosuk University, Republic of Korea*

Correspondence should be addressed to Keun Ho Kim; rkim70@kiom.re.kr

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Introduction. The purpose of this study was to elucidate the relationship between cold-heat patterns and body composition, anthropometric indices, blood parameters, and tongue features in Tae-Eum type subjects. We also sought to determine whether significant indicators could be used as risk factors for predicting cold-heat patterns in a clinic. *Methods.* This prospective, case-control pilot study was conducted at a single center. The subjects were males and females aged 19 years or older who had been analyzed as the Tae-Eum type. After screening, subject allocation was performed. The body composition, 11 anthropometric indices, blood parameters, and tongue features of the subjects were measured by well-trained practitioners. An independent t-test was conducted to compare the cold- and heat-pattern groups. Binary logistic regression was performed to determine significant differences between the two groups after adjusting for age, sex, and systolic blood pressure, with a focus on identifying significant indicators. *Results.* Eighty-nine participants were recruited, 39 of whom were excluded from the analysis. Ultimately, 20 cold-pattern and 30 heat-pattern subjects were included in the final analysis. We found that alanine aminotransferase and all anthropometric indices, except for neck and forehead circumference, were significant predictive factors in both groups according to the binary regression analysis. Additionally, the tongue body color in the cold-pattern group was significantly paler than that in the heat-pattern group. *Conclusions.* This study found that cold and heat patterns were significantly associated with body composition, anthropometric indices, blood parameters, and tongue features. We suggest that these factors could thus be used as objective indicators and predictors of cold-heat patterns. Our findings provide fundamental but also applicable data that will be useful for diagnosing and monitoring cold-heat patterns in Tae-Eum type patients.

1. Introduction

Sasang constitutional medicine is an exclusive and unique system of traditional Korean medicine (TKM) initiated in the late 19th century by Lee Je-Ma, which adopts a distinctive perspective viewpoint from the traditional Eastern medicine of China or Korea [1, 2]. Sasang constitutional types (SCTs) consist of four classifications: Tae-Yang, Tae-Eum, So-Yang, and So-Eum [3]. These types were diagnosed based on physical, physiological, and psychological characteristics such as susceptibility to particular external conditions or disease and types of weak or strong organ function [3, 4].

In Sasang constitutional medicine, patients diagnosed with one of the four SCTs are treated based on cold or heat patterns induced by conditions of physiological equilibrium among the internal organs, which also induce differences in body circumference [5]. For pattern identification, the most important and unique patterns are the cold-heat patterns, which reflect not only the temperature but also the metabolic activity of the subject [6]. Cold and heat patterns are determined by integrating four diagnostic methods. These patterns play an important role in deciding appropriate treatment regimens with modalities such as herbal prescriptions and acupuncture [7]. The cold pattern is related to low and slow metabolism, whereas the heat pattern is related to increase in the metabolic rate. Therefore, patients with a heat pattern show signs and symptoms such as red complexion, high fever, lots of sweat, thirst, yellow urine, and a rapid pulse.

On the other hand, patients with a cold pattern display symptoms including pale complexion, dislike of cold, cold limbs, little sweating and thirst, clear urine, and a slow pulse [8].

Although diagnosing cold-heat patterns is challenging, making an exact diagnosis of cold-heat patterns is very important. Pattern identification results are affected not only by external factors such as temperature, humidity, and luminance but also by subjective factors related to the knowledge, experience, and diagnostic skills of individual practitioners in assessing a patient's pulse, face, tongue, voice, body shape, stool, urine, sweating habits, and skin [9]. Several studies on cold-heat patterns have focused on specific diseases, such as rheumatoid arthritis [10] and the common cold [11], or on the development of cold-heat questionnaires [9, 12, 13]. However, existing studies on SCTs are insufficient, as there are currently no published reports evaluating the differences between cold-heat patterns in specific SCTs in terms of variables such as vital signs, body composition, anthropometric indices, blood parameters, and tongue features. In particular, it is very important to accurately diagnose the cold-heat patterns in Tae-Eum type because not only the cold-heat patterns of Tae-Eum type are very different from the manifestation symptoms but also the prescribed specific medical herbs are strictly different. Tae-Eum type diagnosed as cold pattern uses the following medical herbs: ephedrae herba, coicis semen, castaneae semen, liriopes radix, platycodi radix, and schisandrae fructus. Tae-Eum type diagnosed as heat pattern uses the following medical herbs: puerariae radix, scutellariae radix, cimicifugae rhizoma, angelicae dahuricae radix, rhei rhizoma, and ligustici tenuissimae radix.

Therefore, as a first step, we tried to determine the differences of variables such as vital signs, body composition, anthropometric indices, blood parameters, and tongue features between cold-heat patterns in Tae-Eum type.

The aim of this study was to evaluate the associations of cold-heat patterns in the Tae-Eum type with body composition, anthropometric indices, blood parameters, and tongue features. Additionally, we sought to determine whether significant indicators could be used as risk factors for predicting cold-heat patterns in a clinic.

2. Materials and Methods

2.1. Study Hypothesis. The hypothesis was that body composition, anthropometric indices, blood parameters, and tongue features would significantly differ between the cold- and heat-pattern groups in Tae-Eum type patients.

2.2. Subjects. This study was conducted at a single center as a prospective, exploratory pilot clinical study. The entire study was conducted at the Woosuk University Medical Center (WUMC) in Jeonju, Republic of Korea, between January and March 2017. This study was approved by the Institutional Review Board of WUMC (IRB number WSOH IRB M1709-01-01) and conducted according to the Declaration of Helsinki.

2.2.1. Inclusion and Exclusion Criteria. Subjects were recruited through posters that were displayed around the university and WUMC. The subjects were males and females aged 19 years or older who were diagnosed as the Tae-Eum type. Subjects with one or more of the following criteria were excluded: (1) subjects who had a hypersensitivity reaction after an examination that included a computerized tongue image analysis system; (2) pregnant or breastfeeding women; (3) subjects with a cognitive disorder; (4) subjects who did not sign the informed consent; (5) subjects who were involved in the administration of this research or who were deemed unfit for this research by the administrators; (6) subjects who were diagnosed as non-Tae-Eum types; and (7) subjects who had a fever over 38°C.

2.2.2. Sasang Constitutional Diagnosis. Sasang constitutional integrated diagnostic model developed by Do et al. [14, 15] was used to classify people into four SCTs based on probability values for each type. The model integrates quantitative data from facial images, body shape, voice features, and a questionnaire on personality and physiological symptoms into a single value. The integrated diagnostic model and measurement methods have previously been described in detail. Classification of subjects enrolled in this study was performed by using the final diagnostic model.

2.2.3. Cold-Heat-Pattern Diagnosis. Each subject was diagnosed as having a cold or heat pattern based on the 2015 Sasang constitutional diagnosis guidelines for the Tae-Eum and Tae-Yang types by Lee J et al. In this study, the diagnostic criteria for the cold-heat patterns were ordinary symptoms which means symptoms occurring in association with the individual constitution. The ordinary symptoms of the cold pattern include chilling, no perspiration, headache, backache, myalgia, arthralgia, palpitation, asthma, cough, diarrhea, dyspepsia/postprandial fullness, chest discomfort, and edema. On the other hand, the ordinary symptoms of heat pattern include pyrexia, perspiration, dry eye, dry nose, xerostomia, xeroderma, polydipsia, polyuria, and constipation. A more detailed diagnostic procedure is described in Lee's study [16]. A TKM expert at the hospital with more than 5 years of clinical experience then diagnosed individual cold-heat patterns. Subjects whose expert diagnosis differed from the result produced by the guidelines were excluded from the analysis. For accurate diagnoses, we adhered strictly to the defined qualifications of the expert and subject criteria.

2.3. Data Collection. After screening, the enrolled subjects were divided into cold- and heat-pattern groups. After completing the allocation process, the body composition, anthropometric indices, blood parameters, and tongue features of the subjects were obtained for use in a comparative analysis between cold- and heat-pattern groups of Tae-Eum type subjects.

2.3.1. Blood Parameters. A laboratory test was performed to obtain the blood parameters after more than 12 hours

Table 1: Definitions of anthropometric indices.

Anthropometric index	Definition
Height (cm)	Distance from the top of the subject to the bottom
Weight (kg)	Mass of the subject
Body mass index (kg/cm^2)	Body mass divided by body height squared
Forehead circumference (cm)	Levels of the glabella and opisthion
Neck circumference (cm)	Thyroid cartilage and cricoid cartilage
Axillary circumference (cm)	Left and right axilla
Chest circumference (cm)	Left and right nipple points
Rib circumference (cm)	Left and right 7th and 8th prominence of costochondral junction
Waist circumference (cm)	Umbilical cord
Pelvic circumference (cm)	Left and right anterior superior iliac spines
Hip circumference (cm)	Upper edge of the pubis

of fasting. Total cholesterol (TC), triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C), total protein (TP), albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), fasting blood glucose (FBG), blood urea nitrogen (BUN), serum creatinine (Crea), calcium (Ca), sodium (Na), potassium (K), chlorides (Cl), white blood cells (WBCs), red blood cells (RBCs), hemoglobin (Hb), platelets, lymphocytes, monocytes, eosinophils, basophils, erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP) were measured at a central laboratory at WUMC.

2.3.2. Body Composition and Anthropometric Indices. Body composition measurements obtained from a body composition analyzer (InBody 720, InBody, Seoul, South Korea) included the following: skeletal muscle mass (SMM), body fat mass (BFM), and body fat percentage (BFP). For the body shape analysis, height, weight, and the following eight circumference indices were collected: forehead circumference, neck circumference, axillary circumference, chest circumference, rib circumference, waist circumference, pelvic circumference, and hip circumference (Table 1). Body mass index (BMI) was simply calculated using a subject's height and weight. Anthropometric parameters were measured by well-trained staff with standard operating procedures (SOPs) developed for the Korea Constitutional Multicenter study based on Sasang constitutional medicine. Body measurement data were collected from each subject while wearing light clothing and maintaining stable breathing [17, 18]. The measurements were performed according to the SOP [17, 18].

2.3.3. Tongue Features. The following tongue features were obtained by using a computerized tongue image analysis system (CTIS; TAS-2000, Korea Institute of Oriental Medicine, Daejeon, South Korea): tongue body color and tongue coating color. The images were taken within 5-10 seconds and the captured images were automatically color-corrected by using a color chart (ColorChecker® Classic, X-Rite Pantone, USA). Afterward, the color-corrected images were semi-automatically segmented into the tongue region without background (Figure 1. (2)), tongue body area (Figure 1 (3)), and tongue coating area (Figure 1. (4)).

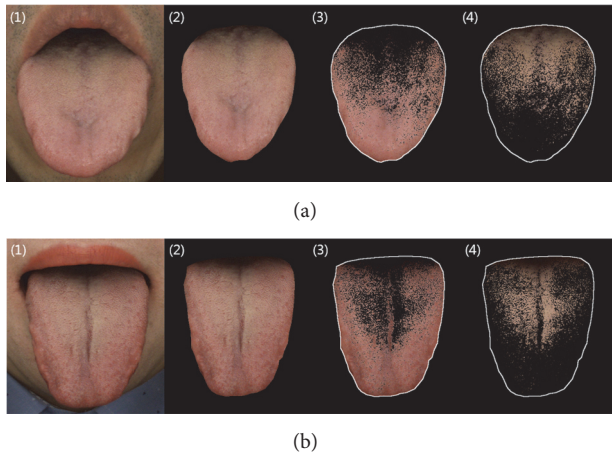

Figure 1: Row (a) presents tongue images from a cold-pattern subject. Row (b) presents tongue images from a heat-pattern subject. Illustrations show (1) the original tongue image, (2) segmented tongue region, (3) tongue substance area, and (4) tongue coating area.

Tongue body and coating color values were obtained from the International Commission on Illumination LAB which is usually abbreviated CIE LAB for its French name, Commission Internationale de l'éclairage LAB. The CIE LAB color space, developed in 1976, describes all the colors visible to human eyes and one of the most widely used today. The CTIS components and protocol for image analysis have previously been described in detail [19, 20].

2.4. Data Analysis. All analyses were conducted with SPSS version 23.0 (SPSS Inc., Chicago, Illinois, USA). The general characteristics of the participants were analyzed by using analyses of means and standard deviations. An independent t-test was conducted to compare the cold and heat groups. In the crude analysis and the analysis adjusted for age, sex, and systolic blood pressure (SBP), binary logistic regression was conducted to identify significant differences

Table 2: General characteristics of cold- and heat-pattern groups (n = 50).

Variables	Cold pattern (n = 20)	Heat pattern (n = 30)	p-value
Age (mean, SD)	43.7 (16.71)	47.93 (15.56)	0.365
Sex (no. of subjects, %)[†]			0.028
Male	7 (35.0)	20 (66.7)	
Female	13 (65.0)	10 (33.3)	
Vital signs			
Systolic blood pressure (mmHg)[‡]	121.4 (12.63)	131.7 (12.64)	0.007
Diastolic blood pressure (mmHg)	81.9 (9.94)	86.2 (10.47)	0.153
Pulse (beats/min)	77.55 (9.1)	78.47 (8.7)	0.722
Respiration rate (/min)	17.5 (2.24)	17.2 (2.76)	0.687
Temperature (°C)	36.66 (0.16)	36.64 (0.21)	0.676
Marital status (no. of subjects, %)			0.644
Single	9 (45.0)	10 (33.3)	
Married	10 (50.0)	19 (63.3)	
Other	1 (5.0)	1 (3.3)	
Occupation (no. of subjects, %)			0.183
White-collar worker	1 (5.0)	6 (20.0)	
Office worker	1 (5.0)	2 (6.7)	
Service	3 (15.0)	6 (20.0)	
Sales	3 (15.0)	0 (0.0)	
Blue-collar worker	0 (0.0)	1 (3.3)	
Other	12 (60.0)	15 (50.0)	
Education (no. of subjects, %)			0.599
Elementary school	0 (0.0)	3 (10.0)	
Middle school	2 (10.0)	2 (6.7)	
High school	9 (45.0)	12 (40.0)	
University	8 (40.0)	10 (33.3)	
Graduate school	1 (5.0)	3 (10.0)	

[†] $p < 0.05$; [‡] $p < 0.01$.

between the cold-pattern group (0) and the heat-pattern group (1). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. General Characteristics. A total of 89 subjects were recruited. Six subjects violated the protocol and were subsequently excluded from the data set. Another 33 subjects who were not diagnosed with cold or heat patterns (e.g., cold-heat complex or non-cold, non-heat) were excluded from the analysis. In total, 50 subjects were assigned to the cold- and heat-pattern groups.

The general characteristics of the participants are presented in Table 2 according to cold-heat-pattern group. The mean age and standard deviation of the cold- and heat-pattern groups were 43.7 ± 16.71 and 47.93 ± 15.56 years, respectively. There were no significant differences in mean age between the two groups. Additionally, there were no significant differences in marital status, occupation, or education. There was a slightly significant difference in sex and a strongly significant difference in SBP between the groups, but there were no significant differences in diastolic blood pressure, pulse, respiration rate or temperature.

3.2. Comparison of Body Composition and Anthropometric Indices between Cold- and Heat-Pattern Groups. The body composition data and anthropometric indices of the cold- and heat-pattern groups are presented in Table 3. All indices were significantly higher in the heat group than in the cold group, except for BFP and height. BFP and height did not differ between the two groups ($p = 0.342$ and $p = 0.105$, respectively).

3.3. Comparison of Blood Parameters between Cold- and Heat-Pattern Groups. Table 4 shows the comparison of blood parameters between the cold- and heat-pattern groups. There were no significant differences in the levels of TC, TP, albumin, AST, ALP, FBG, BUN, Crea, Ca, Na, K, Cl, WBCs, platelets, segmented neutrophils, lymphocytes, monocytes, eosinophils, basophils, ESR, or CRP between the two groups. However, there was a strongly significant difference in mean ALT and Hb between the groups ($p \leq 0.001$ and $p = 0.009$, respectively). There were also weak significant differences in

Table 3: Comparison of body composition and anthropometric indices between cold- and heat-pattern groups (n = 50).

Variables	Cold pattern (n = 20)	Heat pattern (n = 30)	p-value
Body composition			
Skeletal muscle mass‡	25.48 (4.89)	31.26 (6.31)	0.001
Body fat mass‡	18.78 (4.71)	24.97 (6.93)	0.001
Body fat percentage (%)	29.01 (6.89)	30.86 (6.55)	0.342
Anthropometric indices			
Height (cm)	163.34 (8.29)	167.25 (8.14)	0.105
Weight (kg)‡	65.16 (7.75)	80.77 (13.43)	0.001
Body mass index (kg/cm^2)‡	24.42 (2.32)	28.74 (3.16)	0.001
Forehead circumference (cm)†	56.22 (1.69)	57.18 (1.59)	0.046
Neck circumference (cm)†	35.20 (3.23)	37.39 (2.70)	0.013
Axillary circumference (cm)‡	91.85 (4.57)	100.70 (6.74)	0.001
Chest circumference (cm)‡	93.60 (5.04)	101.60 (7.33)	0.001
Rib circumference (cm)‡	81.49 (6.61)	93.02 (7.44)	0.001
Waist circumference (cm)‡	85.06 (6.59)	94.41 (8.55)	0.001
Pelvic circumference (cm)‡	89.94 (8.21)	97.57 (7.52)	0.001
Hip circumference (cm)‡	97.01 (4.00)	101.97 (6.37)	0.001

†$p < 0.05$; ‡$p < 0.01$.

Table 4: Comparison of blood parameters between cold- and heat-pattern groups (n = 50).

Variables	Cold pattern (n = 20)	Heat pattern (n = 30)	p-value
Total cholesterol (mg/dL)	189.05 (47.89)	205.53 (40.13)	0.194
Triglycerides (mg/dL)†	131.3 (84.36)	209.97 (136.71)	0.026
High-density lipoprotein (mg/dL)†	55.7 (12.19)	48.97 (8.64)	0.027
Total protein (g/dL)	7.46 (0.37)	7.46 (0.36)	0.975
Albumin (g/dL)	4.73 (0.26)	4.73 (0.3)	0.968
Aspartate aminotransferase (U/L)	24.75 (10.05)	27.4 (9.39)	0.346
Alanine aminotransferase (U/L)‡	20.45 (11.93)	37.2 (17.61)	0.001
Alkaline phosphatase (U/L)	27.9 (24.33)	38.17 (25.57)	0.163
Fasting blood glucose (mg/dL)	100.1 (26.54)	110.4 (43.99)	0.353
Blood urea nitrogen (mg/dL)	14.19 (3.89)	14.89 (3.67)	0.521
Creatinine (mg/dL)	0.87 (0.19)	0.93 (0.16)	0.198
Calcium (mg/dL)	8.97 (0.34)	8.98 (0.26)	0.829
Sodium (mmol/L)	138.65 (1.66)	138.23 (1.41)	0.345
Potassium (mmol/L)	4.21 (0.28)	4.35 (0.32)	0.116
Chloride (mmol/L)	102.65 (1.57)	102.57 (2.1)	0.880
White blood cells (K/uL)	6.36 (1.58)	7.17 (1.69)	0.096
Red blood cells (M/uL)†	4.6 (0.41)	4.92 (0.44)	0.011
Hemoglobin (g/dL)†	13.65 (1.88)	14.86 (1.25)	0.009
Platelets (K/dL)	249.8 (51.61)	250.23 (69.74)	0.981
Segmented neutrophils (%)	52.65 (6.81)	52.97 (8.24)	0.887
Lymphocytes (%)	35.95 (4.86)	36.13 (6.83)	0.918
Monocytes (%)	7.7 (2.18)	7.67 (1.97)	0.955
Eosinophils (%)	3.25 (3.37)	2.83 (2.07)	0.590
Basophils (%)	0.45 (0.51)	0.4 (0.5)	0.732
Erythrocyte sedimentation rate (mm/h)	6.45 (6.05)	5.17 (6.06)	0.466
C-reactive protein (mg/L)	1.44 (1.35)	1.73 (1.62)	0.510

†$p < 0.05$; ‡$p < 0.01$.

Table 5: Comparison of tongue features between cold- and heat-pattern groups (n = 50).

Variables	Cold pattern (n = 20)	Heat pattern (n = 30)	p-value
Tongue body area			
CIE L‡	48.23 (3.7)	45.76 (2.65)	0.008
CIE a	21.49 (1.56)	22.27 (1.03)	0.059
CIE b	15.81 (2.47)	14.78 (1.68)	0.114
Tongue coating area			
CIE L	39.9 (7.25)	36.94 (6.51)	0.139
CIE a†	13.48 (0.55)	13.02 (0.66)	0.012
CIE b†	14.66 (2.18)	13.45 (1.98)	0.047

† $p < 0.05$; ‡ $p < 0.01$.

Table 6: Associations between cold-heat pattern and body composition, anthropometric indices, blood parameters, and tongue features.

Cold pattern (reference)	Heat pattern			
	Crude		Adjusted	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Skeletal muscle mass	1.196 (1.059-1.351)	0.004	1.338 (1.045-1.713)	0.021
Body fat mass	1.044 (0.956-1.140)	0.337	1.248 (1.036-1.504)	0.020
Weight	1.171 (1.065-1.288)	0.001	1.184 (1.050-1.336)	0.006
Body mass index	1.840 (1.290-2.626)	0.001	2.100 (1.290-3.419)	0.003
Forehead circumference	1.458 (0.994-2.138)	0.054	1.265 (0.733-2.182)	0.399
Neck circumference	1.295 (1.042-1.610)	0.020	1.159 (0.860-1.562)	0.332
Axillary circumference	1.394 (1.147-1.693)	0.001	1.410 (1.113-1.785)	0.004
Chest circumference	1.266 (1.094-1.465)	0.002	1.316 (1.087-1.594)	0.005
Rib circumference	1.300 (1.116-1.514)	0.001	1.461 (1.143-1.868)	0.002
Waist circumference	1.194 (1.065-1.339)	0.002	1.180 (1.034-1.346)	0.014
Pelvic circumference	1.144 (1.041-1.257)	0.005	1.197 (1.060-1.353)	0.004
Hip circumference	1.210 (1.047-1.398)	0.010	1.197 (1.023-1.401)	0.025
Triglycerides	1.007 (1.000-1.015)	0.043	1.005 (0.998-1.012)	0.140
High-density lipoprotein	0.937 (0.882-0.995)	0.035	0.927 (0.856-1.002)	0.057
Alanine aminotransferase	1.086 (1.027-1.148)	0.004	1.066 (1.005-1.131)	0.035
Red blood cells	6.937 (1.382-34.81)	0.019	4.649 (0.523-41.29)	0.168
Hemoglobin	1.721 (1.098-2.697)	0.018	1.601 (0.792-3.233)	0.190
CIE L* value of tongue body	0.763 (0.611-0.951)	0.016	0.808 (0.633-1.032)	0.088
CIE a* value of tongue coating	0.274 (0.092-0.814)	0.020	0.232 (0.059-0.916)	0.037
CIE b* value of tongue coating	0.746 (0.554-1.005)	0.054	0.767 (0.531-1.106)	0.155

Results of binary logistic regression. OR: odds ratio, adjusted for age, sex, and systolic blood pressure.

the levels of TGs, HDL-C, and RBCs between the groups ($p = 0.026$, $p = 0.027$, and $p = 0.011$, respectively).

3.4. Comparison of Tongue Features between Cold- and Heat-Pattern Groups. The tongue features of the cold-pattern and heat-pattern groups are presented in Table 5. Based on the CIEL*a*b* model, the CIE L* value for tongue body area was higher in the cold-pattern group than in the heat-pattern group (48.23 ± 3.70 versus 45.76 ± 2.56, $p = 0.008$). No differences in the CIE a* and b* values for tongue body area were found between the two groups.

Additionally, there was no difference in the CIE L* value for tongue coating area between the groups. However, the CIE a* and b* values for tongue coating area were significantly higher in the cold-pattern group than in the heat-pattern group ($p = 0.012$ and $p = 0.047$, respectively).

3.5. Associations between Cold-Heat Pattern and Body Composition, Anthropometric Indices, Blood Parameters, and Tongue Features. The associations between the significant indicators and the cold-heat pattern are shown in Table 6. SMM was significantly associated with cold-heat pattern in the crude analysis ($p = 0.004$, OR = 1.196 [1.059-1.351]) and after adjusting for age, sex, and SBP (adjusted $p = 0.021$, adjusted OR = 1.338 [1.045-1.713]). BFM was not associated with cold-heat pattern ($p = 0.337$, OR = 1.044 [0.956-1.140]) in the crude analysis, but there was a significant association after adjusting for confounders (adjusted $p = 0.020$, adjusted OR = 1.248 [1.036-1.504]).

Of the anthropometric indices, weight and BMI were significantly associated with cold-heat pattern in both the crude ($p = 0.001$, OR = 1.171 [1.065-1.288] and 1.840 [1.290-2.626]), respectively) and adjusted analyses (adjusted $p = 0.006$, adjusted OR = 1.184 [1.050-1.336]; adjusted $p = 0.003$, adjusted OR = 2.100 [1.290-3.419], respectively). Cold-heat pattern was not related to forehead circumference in either the crude or adjusted analyses ($p = 0.054$, OR = 1.458 [0.994-2.138]; adjusted $p = 0.399$, adjusted OR = 1.265 [0.733-2.182]). Neck circumference was significantly associated with cold-heat pattern in the crude analysis ($p = 0.020$, OR = 1.295 [1.042-1.610]), but this association did not remain after adjusting for confounders. Axillary, chest, rib, waist, pelvic, and hip circumferences were associated with cold-heat pattern in both the crude and adjusted analyses.

Among the blood parameters, TG levels, RBCs, and Hb were associated with cold-heat pattern only in the crude analysis ($p = 0.043$, OR = 1.007 [1.000-1.015]); $p = 0.019$, OR = 6.937 [1.382-34.81]; $p = 0.018$, OR = 1.721 [1.098-2.697], respectively). HDL-C was significantly associated with cold-heat pattern in the crude analysis ($p = 0.035$, OR = 0.937 [0.882-0.995]), but this association did not remain significant after adjusting for confounders ($p = 0.057$, OR = 0.927 [0.856-1.002]). ALT was associated with cold-heat pattern in both the crude ($p = 0.004$, OR = 1.086 [1.027-1.148]) and adjusted analyses (adjusted $p = 0.035$, adjusted OR = 1.066 [1.005-1.131]).

Finally, for the tongue features, cold-heat pattern was associated with the CIE L^* value for tongue body ($p = 0.016$, OR = 0.763 [0.611-0.951]) and the CIE a^* value for tongue coating ($p = 0.020$, OR = 0.274 [0.092-0.814]) in the crude analysis, but the association between cold-heat pattern and CIE L^* was no longer significant after adjusting for confounders (adjusted $p = 0.088$, adjusted OR = 0.808 [0.633-1.032]). Additionally, the CIE b^* value for tongue coating was not related to cold-heat pattern in either the crude or adjusted analyses ($p = 0.054$, OR = 0.746 [0.554-1.005]; adjusted $p = 0.155$, adjusted OR = 0.767 [0.531-1.106]).

4. Discussion

In the present study, a comparative analysis was performed to examine differences in body composition, anthropometric indices, blood parameters, and tongue features between cold- and heat-pattern groups in Tae-Eum type patients.

The results showed that heat-pattern subjects presented a more reddish tongue body and were more obese than cold-pattern ones.

From the t-test and binary logistic regression analyses, we found that the body composition factors SMM and BFM as well as the anthropomorphic indices like weight, BMI, axillary, chest, rib, waist, pelvic, and hip circumferences were highly associated with the heat pattern in both the crude and adjusted analyses, although BFM was not significant in the adjusted analysis. There was a positive correlation between the heat pattern and the above anthropometric indices. Yoon J et al. observed that the indicators of body water balance (extracellular water/intracellular water ratio and extracellular water/total body water ratio) and Sasang personality questionnaire scores were significantly different between the So-Yang and non-So-Yang types [21]. Additionally, Jang E et al. evaluated whether SCTs could be a risk factor for abdominal obesity in Korean populations. The results of that study showed that, after adjustment, the Tae-Eum type was associated with increased abdominal obesity prevalence compared to the So-Eum and So-Yang types among both males and females [22]. Kim BS et al. compared differences in gut microbiota among three constitutions (So-Yang, So-Eum, and Tae-Eum) where Tae-Yang is excluded in the analysis due to the rare population. They also measured anthropometric and biochemical parameters. The results showed that height, weight, BMI, waist circumference, lean body mass, fat mass, and fat percentage differed significantly among the three types [23]. Park YJ et al. reported that there were no significant differences in age, height, weight, or BMI between cold- and heat-prescription groups in male and female Tae-Eum types [24]. According to Sasang constitutional medicine theory, lung hypofunction and liver hyperfunction are related to a large WC, and the Tae-Eum type is associated with hyperactive liver function and a trim waist area [25]. As mentioned above, various studies have investigated associations between Sasang constitutional medicine and BMI or the indices related to abdominal obesity. According to many previous studies, the prevalence and relative risk of obesity and metabolic syndrome are higher in the Tae-Eum type than in other types [26]. However, as there are currently no studies comparing body composition and anthropometric indices between cold- and heat-pattern patients of specific SCT types, the results of the present study are important.

In the present analysis of blood parameters, only ALT was significant in both the crude and adjusted analyses. Kim KY et al. found significant correlations between the Tae-Eum type and blood parameters such as hematocrit, TP, TGs, phospholipids, TC, low-density lipoprotein cholesterol, BUN, and cortisol ($p = 0.05$). Furthermore, Jang E et al. found that SCTs were significantly correlated with FBG, TG, and HDL-C ($p < 0.001$) [22]. Kim BS et al. compared blood parameters among the So-Yang, So-Eum, and Tae-Eum types and found that there were no significant differences in FBG, TGs, HDL-C, or TC among the three groups [23]. Park et al. compared 7 blood parameters, including ALT, TGs, WBCs, Hb, AST, and TC, between cold- and heat-pattern in Tae-Eum type and found that there were significant differences in ALT, TGs, WBCs, and Hb, but not AST and TC, between the two groups [27].

In the present study, we also found that the tongue body color of the cold-pattern subjects was significantly paler than that of the heat-pattern subjects. According to traditional medicine theory, changes in the tongue features indicate both the state of organ function and imbalances in essential components, allowing the doctor to determine whether the patient has a heat or cold pattern [28–30]. Patients with a typical cold pattern normally exhibit a pale tongue and thin tongue coating, whereas heat-pattern patients normally exhibit a reddish tongue and thick fur. Interestingly, the results of this study are consistent with TKM theory [30]. Although there are no previous studies examining tongue

color, a comparative study was conducted on the facial color of patients with cold and heat patterns in the Tae-Eum type by Park YJ et al. [31]. They observed that facial colors based on actual clinical data were similar among three types (Tae-Eum, So-Yang, and So-Eum). The results showed significant differences in complexion between the cold- and heat-prescription groups, which demonstrated that patterns differed according to Sasang constitution [29]. Since it is easier to observe the health condition in the tongue than in the face, it is possible to develop an objective diagnostic index that can distinguish the cold and heat patterns in Tae-Eum type patients with a tongue if sufficient in-depth research is conducted. Therefore, we suggest that the tongue body and coating colors should be used as a diagnostic and monitoring indicator for identifying cold and heat patterns in Tae-Eum type patients.

This study had two limitations. This study was designed as a single-center, prospective, cross-sectional pilot study. Therefore, it may be difficult to confirm the final results of the analysis. We aim to conduct a larger, multicenter trial after further considering the results of this study. Second, this study lacked observations regarding the relationships between other SCT types and body composition, anthropometric indices, blood parameters, and tongue features. This study was a preliminary study focused only on the Tae-Eum type. The results of this study show that it is possible to estimate cold and heat patterns in Tae-Eum type patients. Having confirmed this possibility, we plan to conduct a research on other SCT types in the future. Third, the difference between cold-heat patterns in the Tae-Eum type includes both the exterior and interior symptomatology of Tae-Eum type, as well as the nature and mind. This study lacks observations regarding the association of nature and mind between cold-heat patterns in Tae-Eum type. Further research should be conducted to overcome these limitations. Despite its limitations, this study was the first attempt to explore differences in body composition, anthropometric indices, blood parameters, and tongue features between cold- and heat-pattern patients of a specific SCT. Although this was a pilot study, the results represent valuable and important basic information. This analysis may provide fundamental but also applicable data that will be useful for diagnosing and monitoring cold-heat patterns in Tae-Eum type patients.

5. Conclusion

This study aimed to elucidate the relationship between cold-heat pattern and body composition, anthropometric indices, blood parameters, and tongue features among Tae-Eum type patients. It is very important to correctly diagnose cold and heat patterns after a patient's SCT type has been identified. We found significant differences in body composition, anthropometric indices, blood parameters, and tongue features between the cold- and heat-pattern groups in Tae-Eum type patients. These findings are consistent with previous studies and SCM theory. Thus it is believed that these indices may serve as elementary and supplementary means for the differentiation of syndromes in Tae-Eum type. We need to continue this research in a larger, multicenter trial after further consideration of the results of this study.

Data Availability

The clinical data collected at the Woosuk University Medical Center used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

All authors declare no conflicts of interest regarding the publication of this paper.

Authors' Contributions

Jihye Kim drafted the manuscript with Keun Ho Kim. Jihye Kim was responsible for the data analysis and interpretation. Jiwon Yoon and Keun Ho Kim reviewed the manuscript. Keun Ho Kim developed the computerized tongue image analysis system and tongue features. Soo Jung Park and Jiwon Yoon designed the study protocol. Soo Jung Park performed the clinical study. Keun Ho Kim and Bum Ju Lee supervised the study. All authors approved the final paper.

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Research Article

An Herbal Medicine, Yukgunja-Tang is more Effective in a Type of Functional Dyspepsia Categorized by Facial Shape Diagnosis: A Placebo-Controlled, Double-Blind, Randomized Trial

Seok-Jae Ko,[1] **Jae-Woo Park**,[1] **Jae-hyung Lee**,[1] **Jung-eun Lee**,[1] **Na-yeon Ha**,[1] **Seong-uk Nam**,[1] **Jae-hong Lee**,[1] **Soo-Hyung Jeon**,[2] **Jong-Won Kim**,[2] **Changwan Kang**,[3] **Inkwon Yeo**,[4] **and Jinsung Kim**[1]

[1]*Department of Gastroenterology, College of Korean Medicine, Kyung Hee University, Kyungheedae-ro 26, Dongdaemun-gu, Seoul 02447, Republic of Korea*
[2]*Department of Sasang Constitutional Medicine, College of Korean Medicine, Dong-Eui University, 62 Yangjeong-ro, Busanjin-gu, Busan 47227, Republic of Korea*
[3]*Production Information Technology Engineering Major, Dong-Eui University, 62 Yangjeong-ro, Busanjin-gu, Busan 47227, Republic of Korea*
[4]*Department of Statistics, Sookmyung Women's University, Cheongpa-ro 47-gil 100, Yongsan-gu, Seoul 140-742, Republic of Korea*

Correspondence should be addressed to Jinsung Kim; oridoc@khu.ac.kr

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Introduction. Functional dyspepsia (FD) is a functional gastrointestinal disorder characterized by persistent upper dyspeptic symptoms without organic lesions. There is no standard therapy for FD. Yukgunja-tang (YGJT) is an herbal medicine used for treating upper gastrointestinal symptoms in Asia. Studies on the effect of YGJT on FD have been conducted. However, the results were inconsistent. In *Hyungsang* medicine, traditional Korean medicine, FD patients are classified into bladder body (BB) or gallbladder body (GB) subtypes by the shape and angle of their faces. Each subtype may have different characteristics, physiology, and pathology of the same disease. YGJT is more effective for patients with BB subtype. The three-dimensional facial shape diagnostic system (3-FSDS) was shown to be effective in diagnosing BB or GB subtypes. This study aimed to investigate the effect of YGJT on FD patients classified using the 3-FSDS. *Materials and Methods*. The current study was a placebo-controlled, double-blinded, randomized, two-center trial. Eligible patients were diagnosed with either BB or GB FD subtype using the 3-FSDS. Ninety-six participants (48 BB and 48 GB subtypes) were randomly allocated to treatment or control groups in a 2:1 ratio. YGJT or placebo was administered for eight weeks. The primary outcome was assessed using the total dyspepsia symptom scale (TDS), while the secondary outcomes were assessed using the single dyspepsia symptom scale (SDS), proportion of responders, visual analog scale, Nepean dyspepsia index, functional dyspepsia-related quality of life, and spleen qi deficiency questionnaire. *Results and Discussion*. The result of TDS showed the superior effect of YGJT on BB over GB subtype. The subgroup analysis of TDS and SDS scores showed the superior effect of YGJT over placebo. Other outcome variables did not show any significant differences between groups. *Conclusion*. YGJT may be considered for FD patients diagnosed with BB subtype using 3-FSDS.

1. Introduction

Diagnosis by the shape and appearance of patients historically originated from ancient medical texts (*Huangdi Neijing,* Yellow Emperor's Internal Classic) in Asia and many traditional Korean medicine (TKM) doctors have been using it until now [1]. Based on this diagnostic method, Park et al. developed a unique medicine theory called *Hyungsang* medicine [2]. According to *Hyungsang* medicine, patients are classified into various subtypes such as bladder body (BB) or gallbladder

body (GB) based on the shapes of their face and body [3]. Depending on the subtype, the cause and treatment of the disease may vary. For example, patients with BB subtype are more vulnerable to dyspepsia, obesity, and lethargy [4]. They lack yang and qi and produce a lot of dampness and phlegm; therefore, TKM practitioners tend to prescribe drugs to invigorate qi and reduce dampness and phlegm [4, 5]. Considering that *Hyungsang* diagnosis focuses on morphological features of patients, the excellence of TKM in distinguishing individual characteristics is emphasized. However, though it plays an important role for TKM doctors, *Hyungsang* diagnosis mostly depends on individual experience and perspectives of practitioners; therefore, the diagnoses of doctors are often inconsistent, which makes communication difficult among TKM doctors and hinders the advancement of *Hyungsang* medicine. In order to overcome these limitations, research on standardization and objectification of diagnosis by the appearance of patients has been actively conducted [6–8]. As a result, questionnaires, three-dimensional (3D) body measuring machines, and 3D face automatic recognizers have been developed [9–12]. A 3D facial shape diagnostic system (3-FSDS) consists of a 3D diagnostic scanner combined with a face 3D scanner, data acquisition program, and facial shape measurement and diagnostic program. Three-dimensional stereoscopic images of the frontal and temporal faces are acquired, and 3D coordinates are obtained to calculate distance, angle, and area. A significant variable is selected from the results after calculation, and BB or GB subtype is diagnosed. The 3-FSDS proved to be effective in diagnosing BB/GB subtypes in a clinical trial for the approval of the Korea Food and Drug Administration (KFDA), and its diagnostic rate was over 70%. In 2011, the device was approved by the KFDA (No. 11-500 Medical Image Analysis Apparatus, May 4, 2011).

Functional dyspepsia (FD) is a common functional gastrointestinal disorder characterized by persistent or recurrent abdominal pain, discomfort, and other dyspeptic symptoms in the absence of organic diseases [13]. FD has a prevalence of approximately 11-25% in the total population, and about 50% of patients with FD complain of dyspepsia without any structural disease [14, 15]. Recently in the United Kingdom (UK), annual medical costs for FD have been reported to be more than one billion pounds, and the huge economic burden caused by FD has a considerable negative impact on the society [16, 17]. Several causative factors are involved in FD such as gastrointestinal motility disorder, sensory disturbance, and *H. pylori* infection, and they tend to interact with each other [18, 19]. Proton-Pump inhibitors, prokinetics, and dietary modifications are prescribed as western medical treatments [20]. However, due to unsatisfactory response to conventional treatments, patients are turning to alternative and complementary medicine such as herbal medicines [21, 22].

Yukgunja-tang (YGJT; *Rikkunshito* in Kampo Medicine; *Liu Jun Zi Tang* in Traditional Chinese Medicine) is an herbal medicine made up of eight crude herbs that have been used for treating upper gastrointestinal symptoms such as indigestion, abdominal pain, and epigastric discomfort in Asia [23, 24]. YGJT has been reported to maintain gastric storage capacity and facilitate stomach emptying leading to improved gastric accommodation [25, 26]. Several clinical studies also demonstrated the ameliorative effect of YGJT in FD, and a recent systematic review and meta-analysis reported that YGJT might be more effective compared to prokinetic drugs in the treatment of functional dyspepsia without any side effects [27, 28]. YGJT is known to be more effective for improving dyspeptic symptoms in patients with the BB subtype of FD [4, 5].

In this study, we classified FD patients, using 3-FSDS, into one of two subtypes based on the *Hyungsang* diagnosis: BB or GB subtype. After diagnosis, either YGJT or a placebo was administered for eight weeks and the effect of YGJT was compared. The aim of this study is to investigate the efficacy of YGJT on FD patients diagnosed with either BB or GB subtype by 3-FSDS. Through this study, the conventional *Hyungsang* theory that YGJT has superior effect on the BB subtype compared to the GB subtype of FD will be confirmed, and the usefulness of 3-FSDS will be verified.

2. Materials and Methods

2.1. Study Protocol. The current study was a placebo-controlled, double-blinded, randomized, two-center trial. After screening tests, eligible patients were diagnosed with either BB or GB subtypes of FD using the 3-FSDS. A total of 96 participants (48 BB and 48 GB subtypes) were recruited, and patients with each subtype were randomly allocated to treatment or control groups in a 2:1 ratio by an independent statistician. The number of patients in each group was similar to the number of patients who received placebo YGJT. The reason for the 2:1 ratio random allocation was that it would be unethical to assign an equal number of FD patients to the ineffective placebo treatment group [29]. The participants completed a one-week run-in (weeks −1 to 0), followed by eight weeks of either YGJT or placebo administration (weeks 0–8). The 8-week treatment duration was based on guideline for ROME III criteria [30]. During the administration period, participants were supposed to take one pack of YGJT or its placebo three times a day (1 h after each meal). The trial protocol was approved by the Institutional Review Board of the Kyung Hee University Korean Medicine Hospital (IRB number KOMCIRB-160115-HR-001) and the Dong-Eui University Korean Medicine Hospital (IRB number 2016-01). The protocol for this study was previously published [31] and there were no major changes to the design after initiation of the study.

2.2. Participants. Participants who met the criteria for FD based on Rome III [13] were recruited at two different sites in Korea: the Kyung Hee University Korean Medicine Hospital and the Dong-Eui University Korean Medicine Hospital. The inclusion and exclusion criteria for this study are shown in Table 1. Informed consent was obtained prior to the start of the trial, and participants were free to withdraw from the study at any time. This study was conducted between February 2016 and April 2018.

2.3. Randomization and Blinding. Randomization was performed with the PROC PLAN of SAS 9.4 (SAS Institute

Table 1: Inclusion and exclusion criteria of the study.

Inclusion criteria	(1) Subjects aged 19–75 years
	(2) Subjects who meet the Rome III criteria for functional dyspepsia
	(3) Subjects with more than 40 points on the visual analog scale (VAS; 0, no discomfort; 100, most severe discomfort) for the severity of dyspeptic symptoms
	(4) Subjects who agree to receive no other treatments during the study
	(5) Subjects who voluntarily agree with the study protocol and sign a written informed consent
Exclusion criteria	(1) Subjects with peptic ulcer or gastroesophageal reflux disease confirmed on esophagogastroduodenoscopy
	(2) Subjects with obvious signs of irritable bowel syndrome
	(3) Subjects with alarm symptoms, such as severe weight loss, melena, and dysphagia
	(4) Subjects with severe systemic organ diseases (cancer, diseases of heart, lung, liver, or kidney) or mental illness
	(5) Subjects who have had surgery related to the gastrointestinal tract, except for appendectomy more than six months ago
	(6) Subjects taking drugs that might affect the gastrointestinal tract; a minimum wash-out period of a week is required before participating in the study
	(7) Subjects who have had maxillofacial surgery or facial bone contouring surgery
	(8) Subjects who are pregnant or breastfeeding
	(9) Subjects who have malabsorption or maldigestion
	(10) Human immunodeficiency virus (HIV) positive subjects
	(11) Subjects with difficulties in taking part in the study (e.g., serious mental illness, dementia, drug addiction, time constraint, severe disorder in vision or hearing, illiteracy)
	(12) Subjects who have taken investigational drugs for other trials in the last three months

Inc., Cary, NC, USA) by the independent statistician. Eligible patients were assessed using the 3-FDSD and classified into either BB or GB subtypes. Each patient was assigned a randomization number according to a predetermined allocation list at each center. Patients were sequentially assigned to either treatment or placebo groups in a 2:1 ratio, and if any of the two groups containing either the BB or GB subtypes was full, patients in such groups were excluded from the study thereafter. To preserve allocation concealment, we used an opaque envelope containing randomization code. Only the independent statistician was not blinded to the randomization. Other researchers associated with the study (including subjects, investigators, clinical research coordinator, and clinical pharmacist) were blinded to the random allocation.

2.4. Three-Dimensional Facial Shape Diagnostic System (3-FSDS). The 3-FSDS is composed of a 3D facial scanner (Morpheus 3D), scanner driving and data generation program (Real Face), 3D facial shape measurement program (Renai MEF), and 3D facial shape diagnosis program (Renai FSD) (Figure 1(a)). The 3-FSDS photographs the front and side faces of subjects as a 3-dimensional image and obtains the 3D coordinates of 39 measurement points from the frontal face and 15 measurement points from the side face (Figures 1(b) and 1(c)). The distance and angular area between the points are calculated to generate 337 variables. After stepwise analysis among variables, significant variables are selected and either the BB or GB subtype is determined. For example, the length of the face can be calculated from the distance between the forehead (Figure 1(b) L1) and the jaw (Figure 1(b) L3), and the lateral width is calculated from the distance from the ear (Figure 1(c) S3) to the midpoint between both eyes (Figure 1(c) point 3.1). The width of the front side is calculated from the distance between the left and right eyebrow end points. Because the side face is more developed than the front one in the BB subtype, the area of the side face is larger than that of the front face. In the GB subtype, the area of the side face is smaller than that of the front face. A detailed explanation of the 3-FSDS was described in a previously published paper [31].

2.5. Interventions. Patients were supposed to take one pack (5 g) of YGJT or placebo three times a day for eight weeks (administration period). The YGJT used in the trial was a brown, bitter, herbal extract granule (Yukgunjatang granule®, Hankookshinyak Co., Ltd., Nonsan, Korea) and was produced according to the Korean Good Manufacturing Practice. Yukgunjatang granule®, a water-extracted YGJT combined with starch and lactose, was approved by the Korean Food & Drug Administration. One pack of YGJT is composed of the eight herbal plants listed in Table 2. Placebo YGJT consisted primarily of cornstarch powder with a similar color and tastes similarly to the real YGJT. The real YGJT and its placebo were identically packed and sealed in similar opaque aluminum bags with similar labeling. The patients were supposed to return the unused YGJT or placebo, and the treatment compliance was calculated by subtracting the remaining drugs from the planned drugs and dividing the resulting value by the planned drugs.

2.6. Outcome Assessments

2.6.1. Primary Outcome

Total Dyspepsia Symptom Scale (TDS Scale). The TDS scale is composed of eight items (postprandial fullness and bloating,

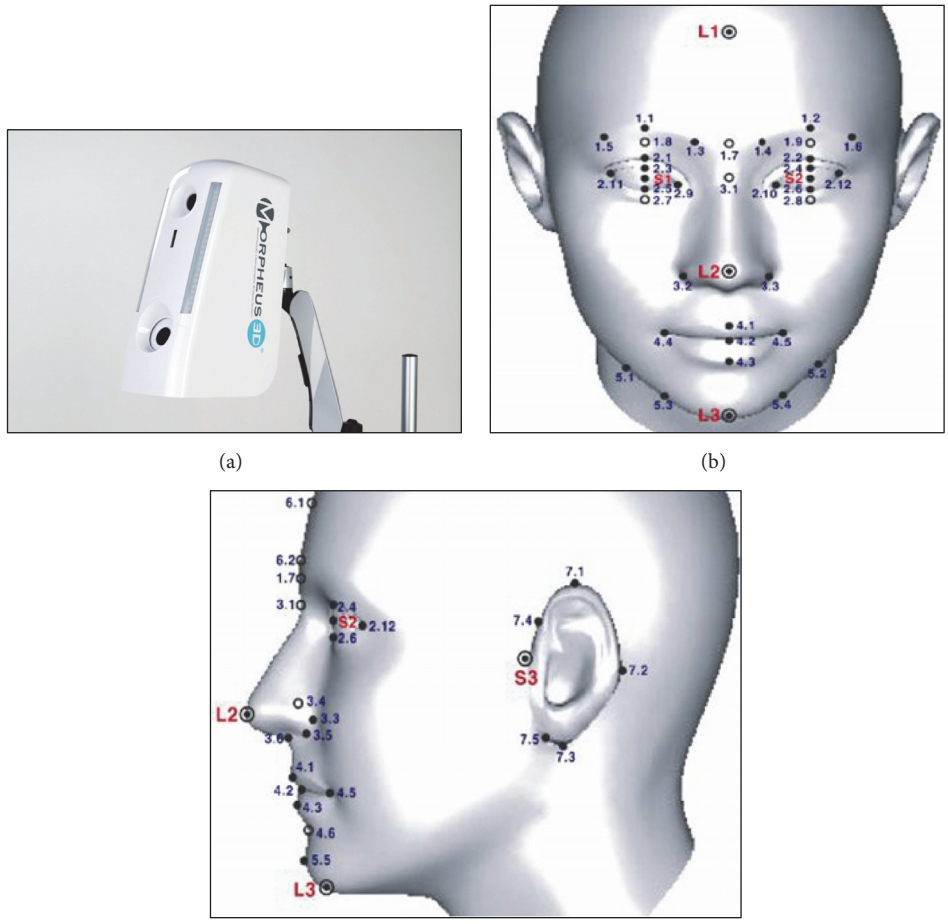

Figure 1: (a) 3-Dimensional facial shape diagnostic system (3-FSDS), (b) the 3D coordinates of frontal face used in 3-FSDS, (c) the 3D coordinates of side face used in 3-FSDS.

Table 2: Herbal plants contained in Yukgunja-tang granule.

Scientific name	Dosage per pack (gram)
Pinelliae tuber	1.33
Citriunshii pericarpium	1.33
Ginseng radix alba	1.33
Atractylodis rhizoma alba	1.33
Hoelen	1.33
Glycyrrhizae radix	0.50
Zingiberis rhizoma crudus	0.67
Zizyphi fructus	0.67

early satiety, epigastric pain, epigastric burning, nausea, vomiting, and belching), with a four-point Likert scale [29, 32]. The TDS score is obtained from the sum of the scores of the eight items. This scale was investigated at baseline and after four and eight weeks.

2.6.2. Secondary Outcomes

(1) Single Dyspepsia Symptom Scale (SDS Scale). The SDS scale comprises three properties (frequency, intensity, and level of discomfort) of four major symptoms (epigastric pain, epigastric burning, postprandial fullness and bloating, and early satiety) of FD with a 4-point Likert scale [29, 32]. The SDS score is the sum of the three properties of the four symptoms. The SDS scale was evaluated at baseline and after four and eight weeks.

(2) Proportion of Responders (PR) for FD Pain and Discomfort. PR was defined as the proportion of patients with at least 50% reduction in FD pain and discomfort from week 0 to week 8. PR was assessed using a weekly adequate relief (AR) question: "In the past 7 days, have you had adequate relief of your pain or discomfort related FD?" AR was assessed during visits or over the telephone. AR and PR have been effective for assessing improvement in abdominal pain and discomfort of functional gastrointestinal diseases [26, 33].

(3) Visual Analogue Scale (VAS). The VAS measures the severity of overall dyspeptic symptoms on a 100-mm visual analog scale during the entire trial (ranging from 0 mm, which signifies no symptom, to 100 mm, which signifies the most severe symptom ever experienced). The VAS was evaluated at baseline and after four and eight weeks.

(4) Nepean Dyspepsia Index-Korean Version (NDI-K). The Nepean dyspepsia index (NDI) developed by Talley et al. was reported to be a reliable questionnaire for measuring dyspeptic symptoms and health-related quality of life [34, 35]. The Korean version of the NDI (NDI-K) was validated by Lee et al. and has been used in clinical studies [9, 36]. We used symptom-based questions about the period, severity, and degree of distress of 15 symptoms with a 5- or 6-point Likert scale at baseline and after four and eight weeks.

(5) Functional Dyspepsia-Related Quality of Life (FD-QoL) Questionnaire. The FD-QoL questionnaire comprises four sections with a total of 21 questions regarding quality of life measured by a 5-point Likert scale (0: not at all or not applicable, 1: a little, 2: moderately, 3: quite a lot, and 4: extremely). The four sections comprise questions on diet (5 items), daily activity (4 items), emotion (6 items), and social functioning (6 items). The FD-QoL questionnaire is known to be reliable for assessing the overall quality of life of patients [37]. The FD-QoL questionnaire was evaluated at baseline and after four and eight weeks.

(6) Spleen Qi Deficiency Questionnaire (SQDQ). The SQDQ has been used to assess spleen qi deficiency syndrome, which is the most common symptom in FD patients [38, 39]. The questionnaire developed by Oh et al. [40] is composed of 11 items, and the total score is the sum of the score of each weighed item. The cut-off value of the SQDQ to determine spleen qi deficiency syndrome is 43.18 [41]. The SQDQ was measured at baseline and after four and eight weeks.

2.7. Safety and Adverse Events. To select eligible subjects and to confirm the safety of administering YGJT for eight weeks, blood biochemical tests (measurement of white blood cell count, red blood cell count, hemoglobin, hematocrit, platelet, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, blood urea nitrogen, creatinine, and erythrocyte sedimentation rate) were performed before randomization and after treatment was completed. Adverse events (AEs) were reported and documented in detail during the entire study period. Serious AEs were promptly reported to the institutional review board and the principal investigator within 24 hours.

2.8. Sample Size Calculation. The sample size was calculated according to the following formula:

$$N = n_t + n_c \left(n_t \text{ is the number of treatment groups; } n_c \text{ is the number of control groups}\right).$$
$$n_c = \frac{1}{2} n_t = \frac{\left\{\left(Z_{\alpha/2} + Z_{\beta}\right)^2 \sigma^2 (\lambda + 1)/\lambda\right\}}{\left(\mu_c - \mu_t\right)^2} \quad (1)$$

A previous study reported 1.57 points of improvement ($\mu_c - \mu_t = \delta$) in the TDS scale for FD after four weeks of YGJT administration compared to placebo [29]. A mean standard deviation (SD = σ) of 2.148 was obtained. The ratio of the treatment group to the control group in the present study was 2:1; therefore the ratio (λ) was 2. With a power ($1 - \beta$) of 80% and a significance level (α) of 5%, assuming $\delta = 1.57$ and $\sigma = 2.148$, a sample size of $n_t = 44$ and $n_c = 22$ subjects was calculated. Considering an assumed dropout rate of 30%, a total of 96 subjects were required.

2.9. Statistical Analysis. All data were collected and handled by an independent statistician. Other researchers associated with the study (including clinicians, clinical research coordinator, and clinical pharmacist) were rigorously isolated from data collection and analysis until the last participant completed the trial. Both the intention-to-treat (ITT) using the baseline observation carried forward approach for missing data and per-protocol (PP) populations were analyzed. All data are presented as mean ± standard deviation or number (%). Baseline characteristics between patients were analyzed using the chi-squared or Fisher's exact test for categorical variables and analysis of variance (ANOVA) for continuous variables. For the efficacy analysis, one-way ANOVA with Dunnett's test as a post hoc test was performed to compare changes in the scores of each outcome for eight weeks between the intervention groups or the facial shape types. Multiple comparisons of nonparametric data were performed using the Kruskal-Wallis H test followed by Dunnett's T3 post hoc test. For analysis between two subgroups, we used the independent two-sample t-test or Mann–Whitney U test as nonparametric statistical tests. Efficacy was analyzed based on the change rate variable which is defined as the value obtained by subtracting the value at the end of eight weeks from the value at baseline, and dividing it by the value at baseline. All statistical analyses of the data were performed using the SPSS software, version 20.0 (IBM SPSS Statistics, New York, USA), and a P value < 0.05 was regarded as statistically significant.

3. Results

3.1. Demographic Characteristics and Baseline Symptoms. A total of 137 patients were screened. Of these, 41 participants did not meet the screening criteria (Figure 2). Ninety-six patients were enrolled and randomly assigned to either the treatment or placebo group. Ninety participants completed the study and six patients were dropped from the study due to withdrawal of consent, violation of exclusion criteria, and adverse events (Figure 2). Baseline demographic characteristics, TDS scale, SDS scale, VAS for overall dyspeptic symptoms, NDI-K, FD-QoL questionnaire, and SQDQ were

Table 3: Characteristics of the patients and baseline TDS scale, SDS scale, VAS for overall dyspeptic symptoms, NDI-K, FD-QoL questionnaire, and SQDQ.

Variables/Group	Gallbladder body ($n = 32$)	Bladder body ($n = 32$)	Placebo ($n = 32$)	P value
Mean age	47.16 (11.83)	47.22 (11.60)	45.28 (12.27)	0.761
Mean weight (kg)	56.56 (7.93)	60.01 (9.39)	57.60 (10.28)	0.313
Mean BMI (kg/m^2)	21.98 (2.85)	23.28 (3.09)	22.21 (2.74)	0.165
Male (%)	15.6	15.6	15.6	1.000
TDS scale	8.81 (3.60)	8.41 (3.09)	8.66 (4.35)	0.907
SDS scale	15.28 (5.76)	14.78 (6.63)	14.72 (6.61)	0.927
VAS for overall dyspeptic symptoms	68.03 (12.09)	60.75 (18.24)	63.16 (9.06)	0.101
NDI-K	52.28 (27.74)	49.63 (30.48)	51.41 (30.56)	0.935
FD-QoL questionnaire	28.44 (20.98)	19.28 (11.77)	25.09 (17.80)	0.106
SQDQ	47.97 (19.22)	39.21 (15.50)	47.16 (16.15)	0.081

TDS: Total Dyspepsia Symptom; SDS: Single Dyspepsia Symptom; VAS: Visual Analogue Scale; NDI-K: Nepean Dyspepsia Index-Korean Version; FD-QoL: Functional Dyspepsia-Related Quality of Life; SQDQ: Spleen Qi Deficiency Questionnaire; BMI: Body Mass Index.
Both gallbladder and bladder body groups are treatment groups.
Baseline values were analyzed by Pearson's chi-squared test for categorical variables and one way-ANOVA for continuous variables.
Continuous variables are presented as mean (standard deviation).
P value < 0.05 is regarded as statistically significant.

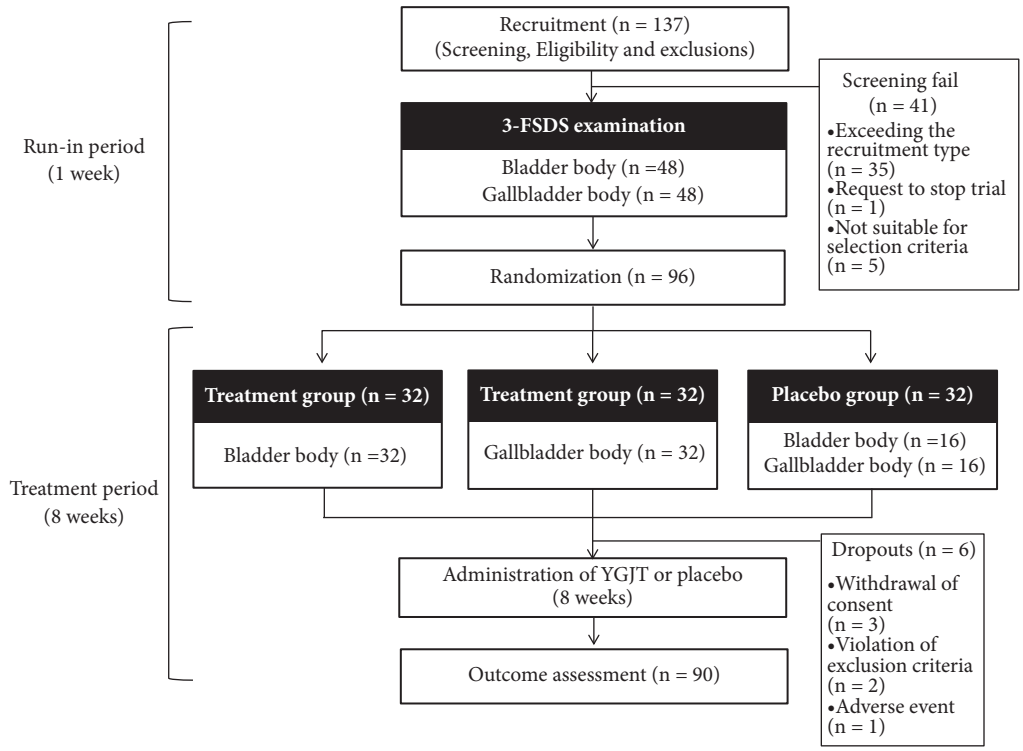

Figure 2: Flow chart of the trial. 3-FSDS, 3-dimensional facial shape diagnostic system; YGJT, Yukgunja-tang.

well balanced among groups at the beginning of the study (Table 3).

3.2. Primary Outcome. After the treatment period, the score on the TDS scale was significantly different among three groups (Table 4), and only the group comprising the BB subtype showed significant improvement compared to the placebo group according to the post hoc test (Figure 3(a)). After subgroup analysis between treatment and placebo groups, the TDS scale scores showed significant superior effect of real YGJT compared to placebo (Table 5 and Figure 3(b)).

3.3. Secondary Outcomes. There was no significant difference among the three groups based on the score on the SDS scale, VAS for overall dyspeptic symptoms, NDI-K, FD-QoL questionnaire, SQDQ, and PR (Table 4). Only the SDS scale score among the secondary outcomes displayed

Table 4: Change rate of TDS scale, SDS scale, VAS for overall dyspeptic symptoms, NDI-K, FD-QoL questionnaire, SQDQ, and proportion of responders.

Change rate of variables/Group	Gallbladder body ($n = 32$)	Bladder body ($n = 32$)	Placebo ($n = 32$)	P value
TDS scale	0.53 (0.38)	0.61 (0.31)	0.32 (0.54)	0.038∗
SDS scale	0.55 (0.36)	0.57 (0.43)	0.34 (0.72)	0.163
VAS for overall dyspeptic symptoms	0.43 (0.25)	0.48 (0.38)	0.41 (0.28)	0.692
NDI-K	0.57 (0.35)	0.61 (0.42)	0.44 (0.49)	0.274
FD-QoL questionnaire	0.58 (0.40)	0.64 (0.31)	0.19 (2.20)	0.338
SQDQ	0.36 (0.35)	0.44 (0.33)	0.34 (0.36)	0.457
Proportion of responders (%)	87.1	78.1	74.2	0.431

TDS: Total Dyspepsia Symptom; SDS: Single Dyspepsia Symptom; VAS: Visual Analogue Scale; NDI-K: Nepean Dyspepsia Index-Korean Version; FD-QoL: Functional Dyspepsia-Related Quality of Life; SQDQ: Spleen Qi Deficiency Questionnaire.
Both gallbladder and bladder body groups are treatment groups.
Change rate is defined as the value obtained by subtracting the value of 8 weeks from the value of baseline and dividing it by the value of baseline.
Values were analyzed by Pearson's chi-squared test for categorical variables and one way-ANOVA for continuous variables.
Continuous variables are presented as mean (standard deviation).
∗P value < 0.05 is regarded as statistically significant.

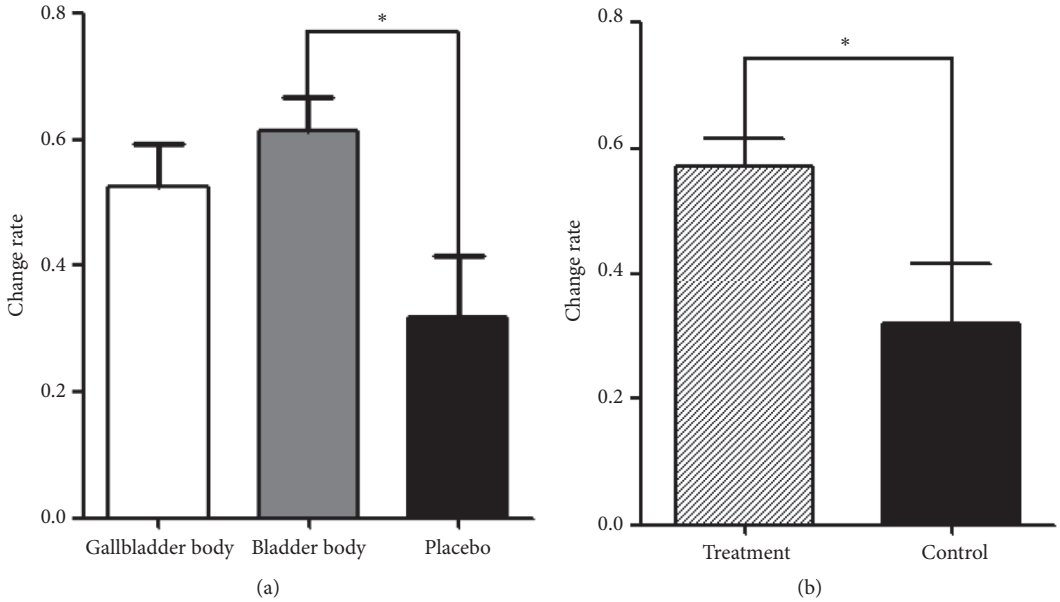

Figure 3: (a) Comparison of change rate of Total Dyspepsia Symptom (TDS) scale among 3 groups. Analysis was performed by one-way ANOVA with Dunnett T3 post hoc test. ∗$P < 0.05$. (b) Comparison of change rate of TDS scale between treatment and control group as subgroup analysis. Analysis was performed by Mann–Whitney U test. ∗$P < 0.05$.

significant improvement in the treatment group compared to the placebo group according to subgroup analysis (Table 5).

3.4. Adverse Events and Treatment Compliance. One major adverse event occurred during this study. During the treatment period, one patient underwent routine annual health check-ups and brain tumor was suspected through brain-MRI examination. During brain biopsy, for further evaluation, intracerebral hemorrhage occurred and the patient was admitted to the intensive care unit after an emergency operation. We reported an adverse event immediately to the institutional review board and the principal investigator within 24 hours and documented it in detail. We also carried out regular follow-up investigations on the patient, and the adverse event proved to be "definitely not related" to the study YGJT or placebo. The treatment compliance of treatment group was 89.16% and that of placebo group was 87.44%. Total treatment compliance was calculated as 88.58%.

4. Discussions

This study aimed to investigate the efficacy of YGJT on different types of FD patients classified by 3-FSDS. This study also aimed to verify the clinical usage of 3-FSDS as a diagnostic tool. The result of TDS as a primary outcome in this study showed the superior effect of YGJT on BB over GB subtype of FD. The subgroup analysis of the TDS and SDS scores also showed the superior effect of YGJT compared to

Table 5: Change rate of TDS scale, SDS scale, VAS for overall dyspeptic symptoms, NDI-K, FD-QoL questionnaire, SQDQ, and proportion of responder between treatment and placebo group as subgroup analysis.

Change rate of variables/Group	Treatment ($n = 64$)	Placebo ($n = 32$)	P value
TDS scale	0.57 (0.35)	0.32 (0.54)	0.031∗
SDS scale	0.56 (0.39)	0.34 (0.72)	0.046∗
VAS for overall dyspeptic symptoms	0.46 (0.32)	0.41 (0.28)	0.483
NDI-K	0.59 (0.39)	0.44 (0.49)	0.135
FD-QoL questionnaire	0.61 (0.35)	0.19 (2.20)	0.463
SQDQ	0.40 (0.34)	0.34 (0.36)	0.402
Proportion of responder (%)	82.5	74.2	0.415

TDS: Total Dyspepsia Symptom; SDS: Single Dyspepsia Symptom; VAS: Visual Analogue Scale; NDI-K: Nepean Dyspepsia Index-Korean Version; FD-QoL: Functional Dyspepsia-Related Quality of Life; SQDQ: Spleen Qi Deficiency Questionnaire.
Treatment group includes gallbladder and bladder body groups.
Change rate is defined as the value obtained by subtracting the value of 8 weeks from the value of baseline and dividing it by the value of baseline.
Values were analyzed by Pearson's chi-squared test for categorical variables and independent two-sample t-test as parametric statistical test or Mann–Whitney U test as nonparametric statistical test for continuous variables.
Continuous variables are presented as mean (standard deviation).
∗P value < 0.05 is regarded as statistically significant.

placebo regardless of subtypes of FD patients. Other outcome variables did not show any significant difference between the FD groups.

The significant effect of YGJT on FD has been demonstrated by a number of experimental and clinical studies [42, 43]. Basic studies showed that YGJT attenuated gastric dysmotility induced by a nitric oxide-synthesizing enzyme inhibitor and improved the delay of gastric emptying mediated by serotonin (5-HT) type 3 receptor [26, 44]. Conversely, YGJT increased plasma ghrelin level that has been known to have a strong orexigenic effect and enhancement of GI motility [45–48]. Clinically, YGJT may improve stress-induced gastric hypersensitivity and/or changes in gastric wall tone detected by gastric barostat method [49]. In addition, YGJT reversed the increase in plasma levels of neuropeptide Y, a representative neurotransmitter of the sympathetic nervous system, leading to improvement of the dyspeptic symptoms [50]. However, recent studies on YGJT did not show significant differences compared to the placebo in specific variables assessing upper dyspeptic symptoms such as epigastric burning, postprandial fullness, and early satiation [51, 52]. These inconsistent results might be due to reasons including small sample size, regression to the mean effect, or high placebo effect. From the results of this study, the differential effect of YGJT depending on subtypes of FD patients might be one of the major reasons for the insignificant effect of YGJT. YGJT, as an herbal medicine based on TKM theory, was prescribed after pattern identification in TKM diagnosis [29]. For example, a recent randomized controlled trial reported that YGJT appears to offer symptomatic improvement in FD patients with spleen-deficiency and qi-stagnation syndrome [29], and studies on *Hyungsang* medicine demonstrated that YGJT can improve FD symptoms for patients with BB subtype of FD [5]. Patients with BB and GB subtypes of FD have different characteristics, appearances, and pathological and physiological features for the same disease [3]. Patients with the BB subtype of FD tend to have deficient qi and excessive dampness. Therefore, they are susceptible to obesity, general weakness, joint disease, and narcolepsy [4]. On the other hand, patients with the GB subtype of FD are easily irritated, emotionally anxious, sleep deprived, and anemic, because they have deficient yin-blood and excessive heat [4]. YGJT is known to invigorate and eliminate dampness; therefore, it is a more appropriate prescription for patients with the BB subtype of FD [29].

We chose FD as the target disease to verify the usefulness of 3-FSDS for classifying either BB or GB subtypes. FD affects a large number of patients in the TKM field and the number of patients with FD is constantly increasing [53]. In *Hyungsang* medicine, which is part of TKM, functional gastrointestinal disease is the second most prevalent after metabolic disease [54]. There are currently no satisfactory standardized treatments for FD. TKM can play an important role in the treatment of diseases without organic causes such as FD. FD is a functional disease in which the efficacy of therapeutic medication varies according to the type of diagnosis. YGJT is a representative herbal prescription for FD in TKM.

There are several limitations in this study. First, 3-FSDS has a diagnosis rate of 70-80% and cannot distinguish between BB and GB subtypes completely. Therefore, there might be patients in the gray zone between BB and GB subtypes who affect the results of this study. Future studies with a large sample size may be a solution for this limitation. Second, other variables that affect classification into either BB or GB subtypes such as face area, face color, personality, and size of body could be considered in future studies. This would make it possible to improve diagnosis using 3-FSDS and obtain a higher diagnosis rate. Third, one reason for the insignificant results between groups of secondary outcomes might be that there are too many patients with mild levels of symptom severity. Functional diseases like FD are well known to be highly affected by placebos; therefore medications for FD might have more definite effect in patients with moderate

or severe dyspeptic symptoms which might be observed during long-term follow-up. Lastly, FD can be classified into subtypes including epigastric pain and postprandial distress syndromes according to the existing Rome criteria. Further studies on the relationship between the classification of FD using the Rome criteria and *Hyungsang* medicine are needed.

5. Conclusions

This study was the first to classify FD patients into different subtypes using *Hyungsang* medicine and to compare the effect of YGJT between subtypes. In the study, YGJT was more effective compared to the placebo, especially in FD patients diagnosed with BB subtype using 3-FSDS. In addition, 3-FSDS has been identified as a useful diagnostic tool for distinguishing between FD subtypes in *Hyungsang* medicine. In the present study, there were no significant differences in most secondary outcomes; therefore, further studies with a larger sample size are needed, and other variables affecting diagnosis rate should be considered in future studies.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare no conflicts of interest.

Authors' Contributions

Seok-Jae Ko, Jae-Woo Park, and Jinsung Kim were responsible for conception and design, manuscript writing and critical revision, and final approval of the manuscript. Jae-hyung Lee, Jung-eun Lee, Na-yeon Ha, Seong-uk Nam, Soo-Hyung Jeon, Jong-Won Kim, and Changwan Kang were responsible for data collection and design of the study. Jae-hong Lee and Inkwon Yeo were responsible for statistical design, data analysis, and interpretation. All authors read and approved the final manuscript before submission.

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Review Article

Acupuncture Treatment for Chronic Pelvic Pain in Women: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

Soo-Hyun Sung,[1] **Angela-Dong-Min Sung**,[2] **Hyun-Kyung Sung**,[3] **Tteul-E-Bom An**,[4] **Kyeong Han Kim**,[5] **and Jang-Kyung Park**[6]

[1]*Department of Pathology, College of Korean Medicine, Dae-gu Haany University, Daegu 38610, Republic of Korea*
[2]*Department of Preventive Medicine, College of Korean Medicine, Sangji University, Wonju 26339, Republic of Korea*
[3]*Department of Korean Pediatrics, College of Korean Medicine, Semyung University, Jechon 27136, Republic of Korea*
[4]*Department of Obstetrics and Gynecology, College of Korean Medicine, Dae-gu Haany University, Daegu 38610, Republic of Korea*
[5]*Department of Preventive Medicine, College of Korean Medicine, Woosuk University, Wanju 55338, Republic of Korea*
[6]*Department of Obstetrics and Gynecology, College of Korean Medicine, Sangji University, Wonju 26339, Republic of Korea*

Correspondence should be addressed to Jang-Kyung Park; vivat314@naver.com

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Aim of the Study. This systematic review and meta-analysis aims to evaluate the current evidence from randomized controlled trials (RCTs) related to the effectiveness and safety of acupuncture treatment (AT), including electroacupuncture or thread-embedding therapy in combination with modern technology, for chronic pelvic pain (CPP) in women. *Materials and Methods*. We searched 12 electronic databases up to December 2017. All randomized controlled trials evaluating the effect of AT for CPP were considered. *Results*. Four RCTs with 474 participants were included. The methodological quality of included studies was generally low. The results of meta-analysis of two studies showed that AT combined with conventional treatment (CT) was associated with significantly reduced CPP, based on the total effectiveness rate (n=277, mean difference = 1.29, confidence interval = 1.13 to 1.47, P=0.0001, I^2 = 0%). *Conclusions*. This review suggests the potential of AT combined with CT compared to CT alone for treating female CPP. However, there is insufficient evidence to conclude that AT can be recommended as a complementary and alternative (CAM) treatment for women with CPP. To draw a firm conclusion, future studies should require not only lager, more rigorously designed RCTs but also research on different AT types. *Protocol Registration Number*. This study is registered with PROSPERO 2018 (CRD42018088627).

1. Introduction

Chronic pelvic pain (CPP) is noncyclic pain of more than 6 months that localizes in the pelvis, the anterior abdominal wall at or below the umbilicus, the lumbosacral region of the spine, or the buttocks [1, 2]. A total of 14.7% of women aged 18-50 years in the United States experience CPP within the prior three months [3]. Severe CPP not only causes functional disability in patients, but also reduces quality of life [1, 4].

Although there is no clear understanding of the mechanism of CPP, the European Association of Urology (EAU) guidelines suggest that inflammation or infection of somatic or visceral tissue, central nervous system (CNS) activity, and emotional, cognitive, behavioural, and sexual components are involved [5]. The guidelines include a description of the diagnosis and treatment of CPP according to a predefined mechanism [5].

In traditional Korean medicine, the main causes of CPP are thought to be static blood or depression of seven emotions and are treated with acupuncture or herbal medicine [6, 7].

Acupuncture has long been used and is effective in relieving pain and is also minimally invasive, inexpensive, and safe [8, 9].

Acupuncture used in combination with modern technology, for example, electroacupuncture (EA), delivers electrical current through acupuncture, and acupoint thread-embedding therapy (TET) maximizes stimulation by inserting thread into meridian points [10].

Recently, comprehensive guidelines for the diagnosis and treatment of CPP have been developed by the EAU [5]. However, there is no evidence-based complementary and alternative medicine (CAM) treatment. Moreover, no published systematic review has determined whether acupuncture treatment (AT) (e.g., classic acupuncture, EA, and TET) for CPP is safe and effective.

This systematic review and meta-analysis aims to evaluate current evidence from randomized controlled trials (RCTs) to assess the effectiveness and safety of AT for CPP.

2. Methods

2.1. Protocol and Registration. This systematic review was registered in the PROSPERO 2018 (available from http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42018088627).

2.2. Data Sources and Searches. The following electronic databases were searched to identify relevant studies for inclusion in the review from inception to December 2017: the Cochrane Central Register of Controlled Trials (CENTRAL), MEDLINE, EMBASE, CINAHL Plus, two Chinese databases (the China National Knowledge Infrastructure (CNKI) and Wanfang), and six Korean databases (the National Digital Science Library (NDSL), the Korean Traditional Knowledge Portal, KoreaMed, the Oriental Medicine Advanced Searching Integrated System (OASIS), the Research Information Sharing Service (RISS), and The National Library of Korea).

The search terms used were ("chronic pelvic pain" OR "chronic pelycalgia" OR "chronic pain of pelvic" OR "chronic pelvic ache") AND ("acupuncture" OR "acupoint" OR "needling" OR "electroacupuncture" OR "electro-acupuncture" OR "electric acupuncture" OR "hand acupuncture" OR "scalp acupuncture" OR "auricular acupuncture" OR "ear acupuncture") AND ("Randomized controlled trial" OR "randomized clinical trial").

2.3. Study Selection. All RCTs evaluating the effect of AT of CPP were included. Nonrandomized trials, animal or cell studies, and reviews were excluded. Women participants diagnosed with CPP were considered. Any type of AT (e.g., classic acupuncture, electroacupuncture, scalp acupuncture, auricular acupuncture, and thread-embedding therapy) for treating CPP was included. AT that does not involve the insertion of needles into the skin (e.g., acupoint pressure, or acupressure) was not considered. We included RCTs comparing AT with no treatment, placebo/sham treatment, or conventional treatment (CT). RCTs that assessed the combined effects of AT plus CT were also included when the identical CT was applied to both groups. Our primary outcome measure was the patient-reported pain score (e.g., visual analogue scale, numeric rating scale for CPP, or total effectiveness rate for CPP). As secondary outcomes, we examined quality of life, activity score, and adverse events (AEs).

2.4. Data Extraction. Two of the authors (A. D. Sung and H. K. Sung) independently reviewed and screened the titles and abstracts of the retrieved studies based on the predefined eligibility criteria. Two independent reviewer (S. H. Sung and T. E. An) extracted the following data from the included studies: author information, sample size, types of diseases, intervention and control groups, outcome measures, main results, and any adverse events. Any disagreements arising between the reviewers during this process were resolved through discussion with a third author (J. K. Park).

2.5. Assessment of Risk of Bias (ROB). Two authors (S. H. Sung and K. H. Kim) independently evaluated the risk of bias of the included RCTs using the Cochrane Handbook V.5.1.0 [15]. This tool consists of seven domains, but we assessed the following six: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessments, incomplete outcome data, and selective reporting. For each domain, the risk of bias for each study was assessed according to three categories: low risk (L), high risk (H), or unclear (U). Disagreements encountered during the process were settled by a third author (J. K. Park) through discussion.

2.6. Data Analyses. For meta-analysis, we used RevMan software (Version 5.3.5 for windows; the Nordic Cochrane centre, Copenhagen, Denmark) [16]. Pooled dichotomous data were expressed as a risk ratio (RR) with 95% confidence interval (CI). In this case, we used a random-effects model for analysis and addressed heterogeneity among the included studies using the I^2 test. I^2 values above 50% or P values less than 0.10 showed considerable heterogeneity [11]. A summary of the findings was discussed in the results when a meta-analysis was not assessed.

3. Results

3.1. Study Selection and Description. The searches identified 117 potentially relevant studies, of which 4 RCTs (English databases: n = 1; Chinese databases: n=3) met our inclusion criteria (Figure 1). Details of the included RCTs are summarized in Table 1. Three [12–14] of the 4 RCTs were conducted in China and published in Chinese. The remaining study [11] was conducted in Egypt and published in English.

3.2. Participants. A total of 474 CPP patients were included in the review. The number of participants was 250 in the experimental group and 224 in the control group. Three of the included RCTs assessed clinical conditions: pelvic adhesion [12] and pelvic inflammatory disease [13, 14].

3.3. Interventions. The types of AT in the RCTs varied: warm acupuncture with moxibustion on the handle of the needle was used in two studies [12, 13]; and EA [11] and TET combined with auricular acupuncture [14] were utilized in one study each.

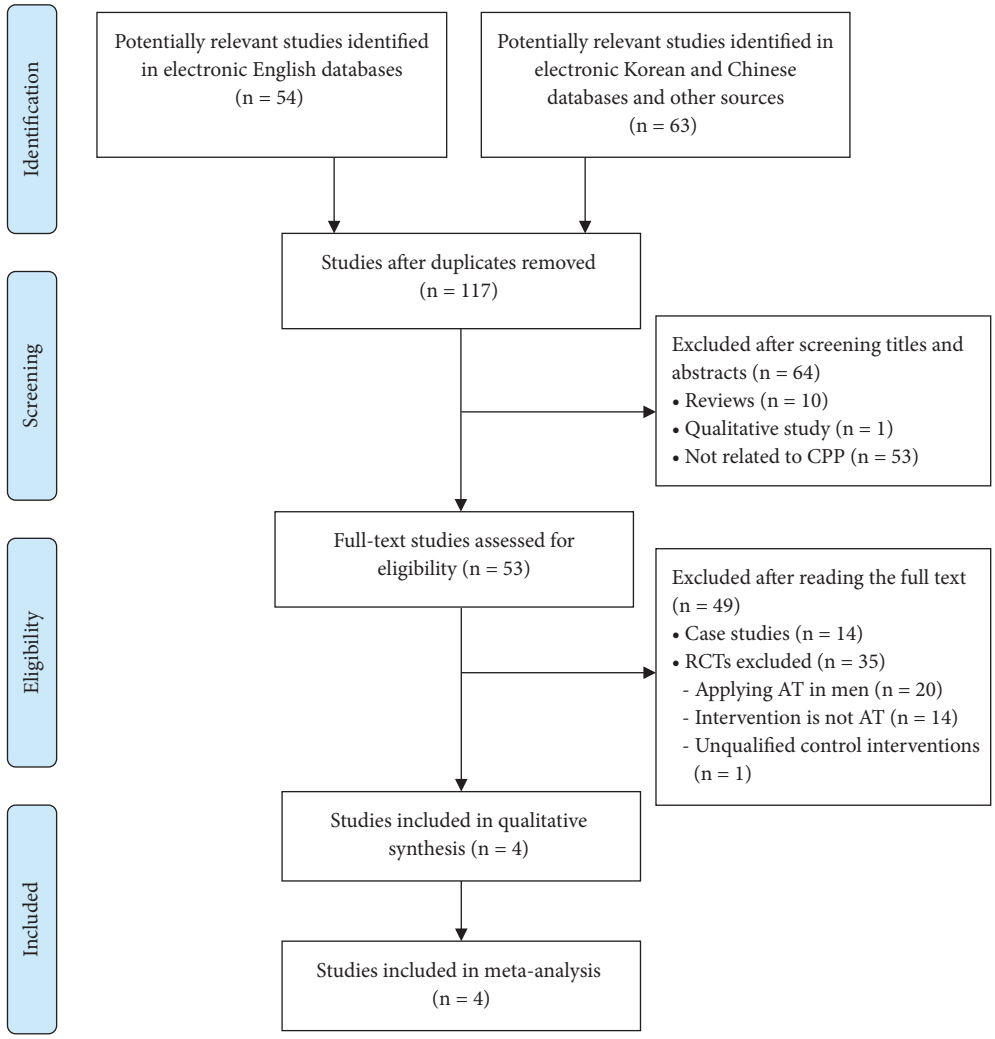


Figure 1: Flowchart of the RCT selection process. CPP: chronic pelvic pain; CCTs: controlled clinical trials; RCTs: randomized controlled trials; EAHM: external application of herbal medicine.

Two of the included RCTs compared AT, including EA and TET combined with auricular acupuncture, with CT [11, 14]. Two other trials compared warm acupuncture plus CT with CT alone [12, 13].

3.4. Outcomes

3.4.1. Acupuncture Treatment versus Conventional Treatment. Acupuncture treatment was compared with conventional treatment in two RCTs [11, 14], of which one contrasted EA with inferior hypogastric plexus blockade [11], while another compared TET plus auricular acupuncture with levofloxacin administration [14].

Two meta-analyses [11, 14] that compared the primary outcome of the total effectiveness rate (TER) for CPP between AT and conventional treatment showed no significant difference between the groups [Figure 2(a), mean difference (MD) = 1.00, confidence interval (CI) = 0.66 to 1.53, P = 0.99, I^2 = 92%]. Amin [11] reported significant pain relief, measured with the visual analogue scale (VAS), in the AT group (P<0.001) and conventional treatment group (P<0.001).

3.4.2. Acupuncture Treatment Plus Conventional Treatment versus Conventional Treatment. For the primary outcome of TER for CPP, data extracted from two RCTs [12, 13] showed significantly superior improvement in the experimental group compared to the control group [Figure 2(b), MD = 1.29, CI = 1.13 to 1.47, P=0.0001, I^2 = 0%]. Li [12] reported significant efficacy based on the numeric rating scale (NRS) (P<0.05).

3.4.3. Adverse Events. One RCT [11] reported that AEs did not occur.

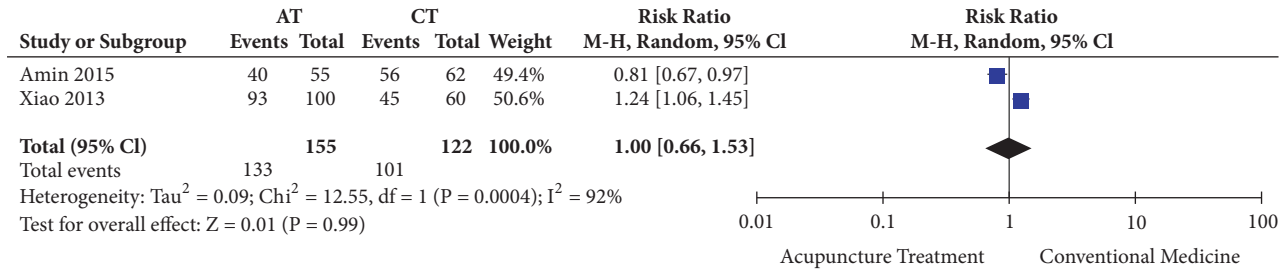
3.5. Cochrane Risk of Bias Assessment. The risk of bias of the included studies is presented in Figure 3. No included RCTs [8] mentioned the method of randomization or allocation concealment. Blinding of participants and practitioners was not performed in all of the included studies [8] due to

Table 1: Characteristics of the included RCTs for pelvic pain.

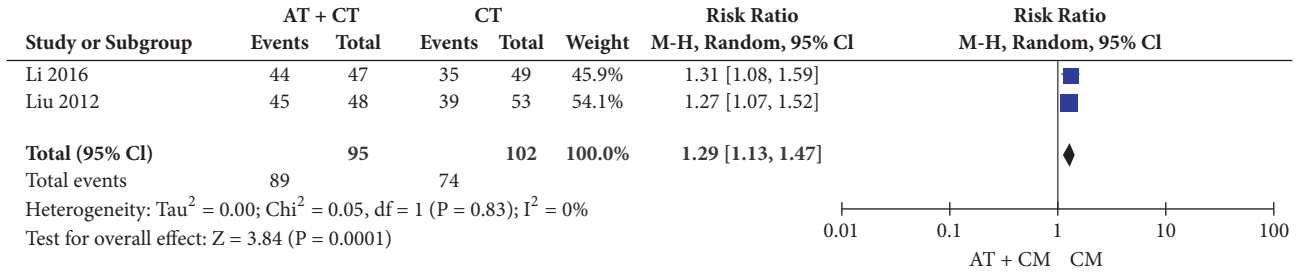
First author, year	Type of conditions related to pelvic pain	sample size (randomized//analysed)	Experimental group (intervention, regimen)	Control group (intervention, regimen)	Outcome measures	Main results	AEs
Amin, 2015 [11]	n.r.	127/117	(A) Electro-acupuncture, n=55, 12 sessions (2 times per week for 6 weeks)	(B) CT (inferior hypogastric plexus blockade), n=62, 1 sessions	(1) VAS (2) TER for chronic pelvic pain	(1) Significant difference in (A)[c] and (B)[c] (2) (B) better than (A)	None
Li, 2016[12]	Pelvic adhesion	96/96	(A) WA (using moxibustion) + CT (physical therapy), n=47, 10 sessions (1 times per day for 10 days)	(B) CT (physical therapy), n=49, 10 sessions (1 times per day for 10 days)	(1) NRS (2) TER for chronic pelvic pain	(1) Positive[a] (2) Positive[a]	n.r.
Liu, 2012 [13]	Pelvic inflammatory disease	101/101	(A) WA (using moxibustion) + CT (cefuroxime axetil), n=48, 7 sessions for WAT (1 times per day for 7 days) and 14 sessions for CM (2 times per day for 7 days)	(B) CT (cefuroxime axetil), n=53, 14 sessions (2 times per day for 7 days)	(1) TER for chronic pelvic pain	(1) Positive[b]	n.r.
Xiao, 2013[14]	Pelvic inflammatory disease	160/160	(A) TET + AA, n=100, 2 sessions for TEF (1 times per 4 weeks for 8 weeks) and 8 sessions for AA (1 times per week for 8 weeks)	(B) CT (levofloxacin capsule), n=60, 2 sessions (2 times for week)	(1) TER for chronic pelvic pain	(1) Positive[a]	n.r.

[a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.001$

AA: auricular acupuncture; AEs: adverse events; CT: conventional treatment; n.r.: not reported; NRS: Numerical Rating Scale; NS: no significant difference; QOL: quality of life; TET: thread embedding therapy; VAS: visual analogue scale; WA: warm acupuncture.



(a) Acupuncture treatment versus conventional treatment



(b) Acupuncture treatment plus conventional treatment versus conventional treatment

Figure 2: Meta-analysis of total effectiveness rate for chronic pelvic pain. AT: acupuncture treatment; CT: conventional treatment; CI: confidence intervals.

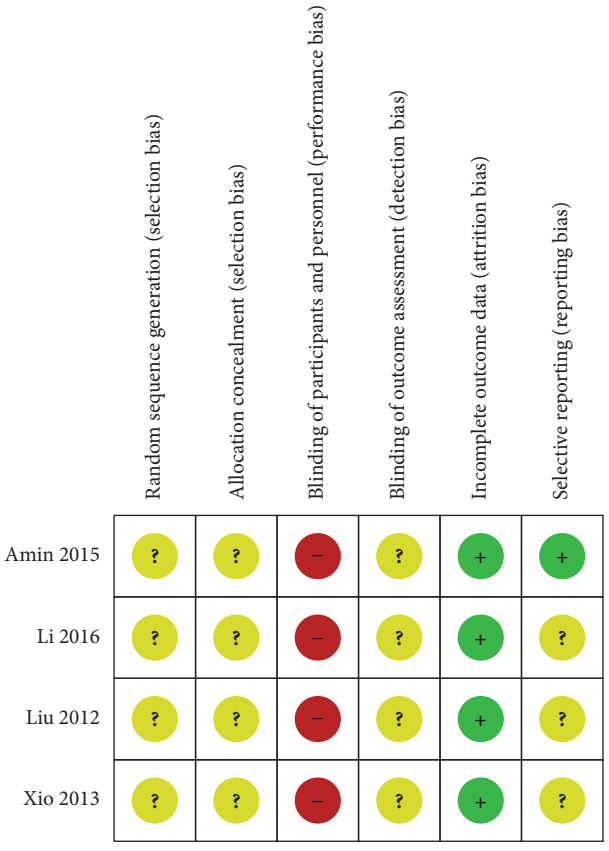


Figure 3: Summary risk of bias assessment.

differences in treatment type between groups. Moreover, these studies [8] did not report information about the blinding of the outcome assessors. All included RCTs [8] had a low risk of bias in addressing incomplete outcome data; three studies [8] had no missing outcome data; another trial [11] had missing outcome data, but the drop-out rate did not exceed 20% for short-term and 30% for long-term follow-up. In terms of selective reporting, only one [11] trial reported their protocol before conducting the studies.

4. Discussion

CPP in gynaecological practice is often associated with negative cognitive, behavioural, sexual, and emotional consequences and is often complex and difficult to treat [5]. Therapeutic options such as hormonal therapy or surgery are recommended in well-defined disease states and a multidisciplinary approach to pain management is used in persistent disease [5]. However in 30% of cases, no cause is ever determined and this presents a therapeutic challenge to the attending physician [5]. Thus, in the EAU guideline, the use of alternative therapies for chronic gynaecological pelvic pain is recommended [5].

Our systematic review provides suggestive evidence for the efficacy of AT, which is CAM therapy, in treating CPP. The meta-analysis that pooled data from two RCTs [12, 13] using the outcome measure of TER for CPP showed significant improvement with AT plus CT compared with CT alone (MD = 1.29, CI = 1.13 to 1.47, P=0.0001, I^2 = 0%). However, the meta-analysis in two studies [11, 14] indicated that AT showed no significant improvement on the outcome of TER for CPP compared to that with CT (MD = 1.00, CI = 0.66 to 1.53, P = 0.99, I^2 = 92%). Although our findings indicated that AT can be recommended as additional treatment when CPP patients are treated with CT, there is insufficient evidence

to recommend evidence-based treatment with AT for female CPP due to heterogeneity of control interventions and the small number of trials in the included studies.

The strength of our review is that we searched various databases without language restriction to avoid publication bias. Thus, three East Asian RCTs were included in the review; the researchers assess Chinese language articles.

Our review has several limitations. First, most of the included studies had low methodological quality in the Cochrane ROB assessment. None of the studies provided information on generation of random allocation and the method of allocation concealment. The blinding of participants, practitioners, and outcome assessors was not performed in all included RCTs. Low methodological quality RCTs led to overestimation of treatment effects [17]. Furthermore, three [12–14] of 4 studies did not provide a published protocol or register it prior to execution. Registration of clinical trial protocols is important to identify whether a trial is affected by selective or incomplete outcome reporting [18]. Future studies should be registered in an open-accessible registry such as ClinicalTrials.gov. or WHO.int/ICTRP [19, 20].

Second, in terms of safety, only one RCT [11] reported that AEs did not occur in 117 CPP patients. Safety is a fundamental principle in medical treatment. In general, there is a common impression that acupuncture is safer and is therefore recommended as alternative treatment. Recent research reported that minor and rare serious AEs can occur during acupuncture [21]. Therefore, AEs must be reported in RCTs of CPP in the future to draw firm conclusions on the severity and frequency of AEs due to AT.

Third, our findings were limited due to variation of AT types; three types of AT, including warm acupuncture, EA, and TET were included in the review. Traditionally, dry acupuncture needles have been used, and as new types of AT in combination with modern science and technology have been developed and utilized, the range of treatment tools is expanding. Thus, clinical studies according to different types of AT should be investigated in the future.

Lastly, the primary outcome of TER for CPP used in meta-analysis is not an internationally accepted tool for measuring pain. TER, an outcome measure generally used in China, was graded according to the following categories: clinical cure, markedly effective, effective, and ineffective [22]. The validity and reliability of TER have not yet been verified. In the future, internationally recognized measurements such as VAS or NRS should be used.

Although this review presented the applicability of AT for female CPP patients, standardization of AT intervention was not examined. Therefore, studies should consider the following factors: (1) AT type; (2) duration of treatment and number of treatment sessions based on each AT type; (3) size and depth of needle; (4) acupuncture points; and (5) appropriate placebo model for each AT type. Researchers need to investigate efficacy and safety of AT in CPP to establish CAM treatment guidelines that reflect our findings.

5. Conclusion

The results of our review and meta-analysis suggest the effectiveness of AT combined with CT for treating women with CPP compared to use of CT alone. However, current evidence is insufficient to verify the efficacy of AT for CPP because of the small number of RCTs and low methodological quality and heterogeneity of interventions. Therefore, larger, more rigorous and adequately powered multicentre RCTs are needed to provide clinical guidelines for AT in treating female CPP patients.

Conflicts of Interest

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

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Research Article

Evaluation of the Effectiveness of Protective Patches on Acupoints to Preserve the Bioenergetic Status against Magnetic Fields

Claudio Molinari,[1] Ian Stoppa,[1] Nicola Limardo,[2] and Francesca Uberti[1]

[1]*Laboratory of Physiology, Department of Translational Medicine, University of Piemonte Orientale, Via Solaroli 17, Novara 28100, Italy*
[2]*Health Sciences and Oral Hygiene, The Lifestyle Medicine, University "La Sapienza", Piazzale Aldo Moro 5, Roma 00185, Italy*

Correspondence should be addressed to Francesca Uberti; francesca.uberti@med.uniupo.it

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Guest Editor: Sang-Hoon Shin

The potentially harmful nature of electromagnetic fields (EMF) and static magnetic fields (SMF) has become a major problem in recent years. All these elements could be combined to produce cellular responses. For example, the orientation of molecules of water or other complex molecules, growth and cell viability, cell morphology, and intracellular metabolic pathways have demonstrated binding to magnetic fields. The effect of EMF and SMF on humans is a topic of great importance, especially because modern technology has introduced artificial magnetic fields such as those generated by power lines, mobile communications, and medical imaging equipment. A relevant problem is certainly that of professional exposure. The aim of this study was the evaluation of the effectiveness of a commercially available device, Skudo® patches (Edil Natura S.r.l., Novara, Italy), in protecting magnetic resonance operators from the influence of magnetic fields such as those present in the workplace. Skudo® patches are designed to protect microareas of the body from external electromagnetic disturbances. In this study, 10 male Italian volunteers aged between 50 and 60 were enrolled in the hospital. All participants were subjected to measurements at 4 specific time points to evaluate the effectiveness of Skudo® to counteract both EMF and SMF magnetic fields by evaluating the level of bioenergetic reactivity. To perform the measurements, a variant of the Ryodoraku method has been used, based upon the assessment of electropermeability. In particular, 12 acupoints were measured, one for each of the main meridians. This study shows that both SMF and EMF cause an alteration of the body's water system. The application of Skudo® patches determines a regularization of bioenergetic levels related to the water system. The application of Skudo® on the EMF source has suppressed the imbalance effect of the water system found in the subject without any protection.

1. Introduction

A growing body of evidence has shown that magnetic fields have the ability to interact with biological systems and to induce effects in the living matter. This topic has long been of interest in the scientific community both for its applicability in the therapeutic field and for determining whether they could be potentially harmful.

The potentially harmful nature of magnetic fields has become a serious problem in recent years due to the enormous increase in the number of electronic communication devices and also the increasing use of NMR in medicine [1]. During the evolution of life on Earth, living organisms have constantly been exposed to the geomagnetic field. On our planet it can vary from 20 to 70 μT. For this reason, the biological systems have developed specific mechanisms for the perception of the natural electric and magnetic fields involved, for example, in the orientation and migration of some animal species [2]. The mechanisms of detection and response to both electromagnetic fields (EMF) or static magnetic fields (SMF) can be found at different levels, for example, on the cell membrane or within a tissue. Sometimes

the sensitivity of a biological system to SMF is expressed through changes in the signal transduction cascade or nerve tissue activity [3, 4]. A recently studied aspect is the effect of low-intensity SMF on cell production of free radicals. Both reactive oxygen species (ROS) and nitrogen (RNS) were studied. ROS and RNS play significant roles in immunological defense [5], intracellular signaling [6], and intercellular communication [6]. It is assumed that EMF and SMF could change the duration of radical pairs. A radical pair consists of two radicals that have been created simultaneously, usually by a chemical reaction, and possesses magnetic properties. If an SMF affects cells through the radical pair mechanism, it can influence the rotation of electrons in free radicals, which can lead to changes in the kinetics of the chemical reaction and possible alterations of cellular function [7]. Most of the studies on the biological effects of SMF considered only low-intensity stimulations. Unfortunately, the effects of strong SMFs have not yet been evaluated sufficiently, although it is easy to think that even a strong SMF should have the ability to influence biological systems. Actually, the results of these studies are controversial. Sirmatel et al. [8] studied the effects of a high-intensity magnetic field produced by a magnetic resonance imaging (MRI) apparatus on oxidative stress. However, in this study, SMF does not seem to produce a negative effect; on the contrary, it has produced the positive effect of a decrease in oxidative stress in men after short-term exposure. On the other hand, a Nakagawa study in mice showed that [9] high-intensity SMF exposure induces increased peroxidation levels in the liver of mice and also enhances the effect of hepatotoxic substances such as carbon tetrachloride, CCl_4.

The cell is certainly a complex system formed by a set of components susceptible to the presence of EMF and SMF, such as electrical charges and molecules with their magnetic moment [10]. All these elements could be combined to produce cellular responses and, since the cellular environment includes a nonlinear system, magnetism-dependent phenomena could result from the combination of many conditions. For this reason, especially in the last decade, the behavior of cellular structures and characteristics has been studied following exposure to SMF. For example, cell and intracellular component's orientation, cell growth and viability, cell morphology, enzymatic activity, and biomolecules synthesis were investigated during exposure to SMF. For a review, see [10]. Based on numerous studies, a link between magnetic fields and observed cellular responses can be hypothesized. The mechanisms underlying these effects could be magnetosome presence, spin modulation of radicals, drifting of molecules in buoyancy following a magnetic field gradient, torques in molecules, linkage of ion to the enzyme active site, ion-protein attachment [11], calcium mobilization and diamagnetic anisotropy of lipids, mitochondria, DNA helix, and cytoskeleton.

Unlike SMF, EMF are waves that transport energy through space. Wavelength and frequency are the main features of EMF, and they are inversely correlated. Electromagnetic radiation is distributed in a spectrum ranging from radio waves to gamma rays, passing through visible light and microwaves. Starting from ultraviolet EMF are ionizing and produce damage to living organisms. EMF with frequency lower than the ultraviolet can induce thermal and nonthermal modifications in biological systems [12, 13].

The effect of EMF and SMF on humans is a topic of great importance, especially since the modern technology has introduced artificial magnetic fields such as those generated by power lines, mobile communications, and imaging equipment. In fact, the exposure to radio frequency and microwave electromagnetic fields, both in the work and in the general environment, has never experienced a growth like the one seen in the last 10 years. For this reason, it is of fundamental importance to address the problem of safety, using all the tools available to evaluate the potential risks of exposure. Moreover, a relevant problem is certainly that of occupational exposure. For example, individuals working in proximity of an MRI device are exposed to SMF coming from the scanner magnet. An important group of people regularly exposed to electromagnetic fields related to magnetic resonance imaging is the healthcare staff. Some publications describe subjective symptoms related to exposure to SMF, reported by people who have been exposed to MRI-related fields such as health personnel, patients, or healthy volunteers [14–17].

The evidence comes from both experimental and observational studies and includes general symptoms such as headaches and concentration problems, as well as specific sensory symptoms such as vertigo, balance problems, nausea, metallic taste, and flashes of light. Current literature suggests that these symptoms have an acute and transitory nature [17, 18], and many of these occur when people move through spatial gradients in the external static magnetic field outside the MRI scanner [19].

Although there is no clear evidence of a direct relationship between EMF or SMF and disease, some efforts have been made to try to develop protective methods or devices. Nowadays there are commercially available devices that claim to be able to screen the potential harmful effects of both static and electromagnetic magnetic fields. However, studies that evaluate the effectiveness of these devices in protecting the human body are still lacking. The purpose of this study was therefore the assessment of the effectiveness of one of these commercial devices in protecting MRI operators from the influence of magnetic fields present in the workplace.

2. Materials and Methods

2.1. Protective Device Tested. In order to carry on this study, we decided to test the effectiveness of a new and hi-tech device, in the form of patches, called Skudo® (Edil Natura S.r.l., Novara, Italy). The Skudo® patches are designed for the protection of microareas of the body from external electromagnetic disturbances, and each beneficial effect observed should be considered "indirect". They are composed with a base in Pe-eVA (transparent polyethylene foam) and with nonstick gauze. In addition, they have circular shape with a diameter of 25 mm and optimal weight and thickness (60 g / m2 of weight and 70 thick micrometers). The production process has been patented and certified at European level (European Patent Certificate No. 2073611). The effectiveness of each patch is about 12 hours and it can be used by

Figure 1: Experimental equipment. A schematic BFB-Z application and interpretation.

everyone regardless of age or gender. These patches are placed on the "energy points" of the meridian channels of body. If these points are effectively protected by environmental perturbations such as artificial electromagnetic fields and natural radioactivity, they can provide many beneficial effects to the body. The Skudo® patches protect these meridians by forming a physical barrier against environmental factors. These patches do not release substance, are not transdermal, have no side effect, and have no time limitations.

2.2. Subjects. In this study, 10 male Italian volunteers were enrolled within the hospital between 50 and 60 years of age, subject to written authorization at the Physiology Laboratory of the University of Piemonte Orientale (Novara, Italy). The approval for this study was conferred by the local Human Investigation Committee. To ensure the homogeneity of the study, the following exclusion criteria were applied: body mass index (BMI) <18.5 kg / m2 or> 30 kg / m2 [20]; autoimmune diseases; skin allergies; hypertension; surgery or critical medical history within the year prior to the study; metallic implants in the body; chronic diseases; contraindications for electrical stimulation; the inability to complete a form; and any other factors that the investigator judged to be inappropriate for the study. Then they were randomly assigned to either the control group or the Skudo® group. All participants did not receive any training or equipment to use.

2.3. Assessment of Body Energetic Status. The state of health corresponds not only to the biochemical balance but also to the electric equilibrium. In this study, the level of health was observed by evaluating the electropermeability of some points taken from the acupuncture meridians. The equipment used was the BFB-Zener (Zener S.r.l., Milan, Italy; BFB-Z), as reported in Figure 1. BFB-Z is an innovative tool that measures in an easy, precise, and fast way the electric balance (energetic homeostasis) that involves the vital capacity, the presence of functional and organic alterations, oxidative stress, and body functions. It is a computerized instrument based on the technique of evoked potentials in easily accessible peripheral electrodermal points placed on the hands and feet. BFB-Z can be considered a variant of the Ryodoraku method, which, as is known, was developed in 1951 Dr. Yoshio Nakatani. The Ryodoraku method is based on the presence of electropermeable points on the body surface. The electrical characteristics of these points, which largely coincide with the main points of classical acupuncture, vary not only with any pathological process but also with the detector probe voltage. Most traditional acupoints may be localized if a 21-volt circuit is used. However, if a 12-volt circuit is used, it is possible to find other electrically conductive points on the body, not associated with specific acupuncture points. Similarly, the BFB-Z analyzes the electropermeability characteristics of 12 skin points (6 from hands and 6 from feet), one for each of the main meridians. Once the electrical characteristics of the 12 points have been measured, the BFB-Z provides a diagram that represents the energetic status of the subject.

2.4. Study Design. All participants were measured at 4 specific time points to evaluate the effectiveness of Skudo® to counteract the electromagnetic field on different level of electromagnetic field using BFB-Z technique. In the first set of experiments 10 participants were measured: at basal level (after 2 days of consecutive work rest) without Scudo®, at basal level (after 2 days of consecutive work rest) with Scudo®, after front positioning to a WiFi antenna which was switch off without Scudo®, after front positioning to a WiFi antenna which was switch on and the electromagnetic waves affect the vertebral column longitudinally with Scudo®, and after front positioning to WiFi transmitter on which the patch is placed. In the second set of experiments, the same 10 participants were measured: at basal level (after 2 days of consecutive work rest) without Scudo®, after at least 10 hours of hospital work in radiology department without Scudo®, and after at least 10 hours of hospital work in radiology department with Scudo®. As illustrated in Figure 2, 3 patches were applied to each participant: 2 at the meridian position CV17 (Conception

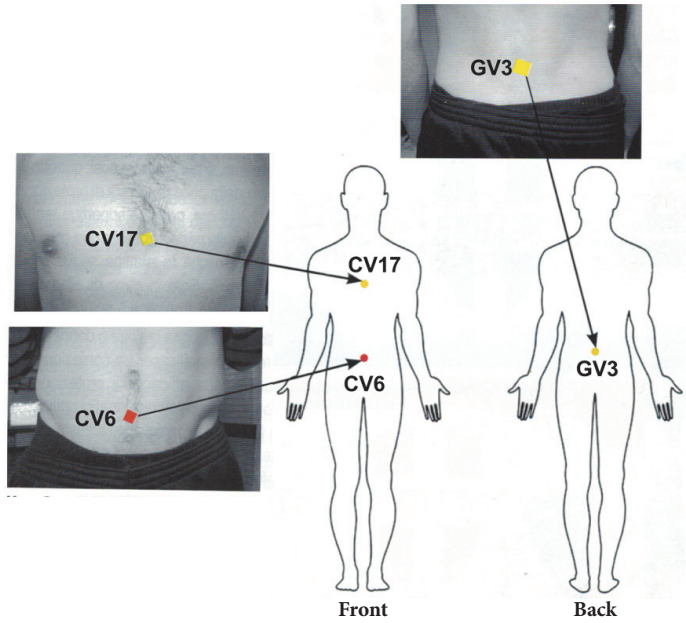

Figure 2: Experimental procedure. A schematic application of Skudo® patches.

Vessel 17) and at GV3 (Governing Vessel 3) and 1 at the meridian position CV6 (Conception Vessel 6), as shown in the picture.

2.5. Data Processing and Statistical Analysis. The raw data were processed using Prism GraphPad statistical software for normalization, peak picking, and comparison between groups. The images were produced directly by BFB-Z and ImageJ. One-way analysis for variance (ANOVA) with Tukey's post hoc tests was carried out for the comparison between groups, and all results were expressed as mean ± SD. Differences were considered to be statistically significant with $p < 0.05$.

3. Results and Discussion

3.1. Analysis of Standard Diagram. The term "biological reactivity" refers to the level of "vital energy of the organism", which corresponds with Qi of Traditional Chinese Medicine (TCM). An example of standard layout obtained by BFB-Z on healthy subject with good level of bioenergetic reactivity and vegetative balance is shown in Figure 3. In particular, the connection between H and F points of Ryodoraku system and the meridians of Traditional Chinese Medicine should be noted. In the standard diagram taken from a healthy subject, 3 steps of energy increase can be observed: at H3, F2, and F5 points, respectively. In addition, at F3 point, an important subsidence was observed. The meaning of this curve was explained by the correlation with meridians points. Indeed, H3 point corresponds to the fire, the energy of health which was represented by the heart; F2 and F5 points correspond to wood and the energy of blood; finally, F3 point corresponds to the water and the way of expulsion of impure substances and is connected to the metabolism of the body. Following

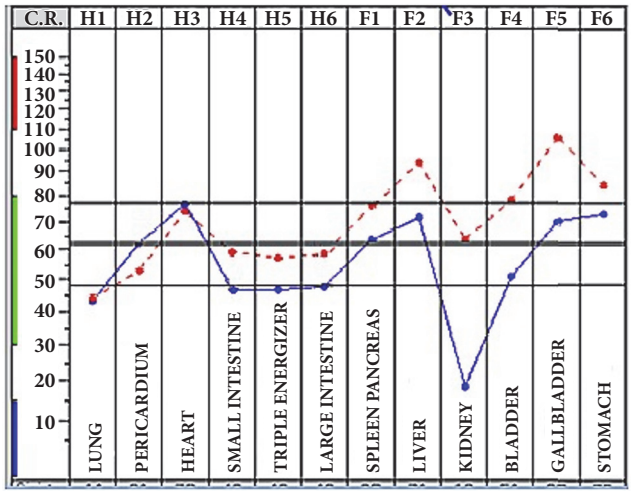

Figure 3: Standard layout obtained by BFB-Z on healthy subject.

the principles of TCM, the basic elements are five: water, fire, wood, metal, and earth; the relationships between these five elements represent a model of interaction between the internal organs and tissues and sense. The double horizontal line that crosses the whole layout indicates the level of bioenergetic reactivity. Finally, on the left side, there is a green line that corresponds to the normal energy level which is related to age and sex.

For this reason, the experiments were performed starting from analyzing the basal level without and with Skudo® patch. As reported in Figure 4(a), a significant ($p < 0.05$) reduction in the level of bioenergetic reactivity on all participants was observed in presence of Skudo® patches indicating that the

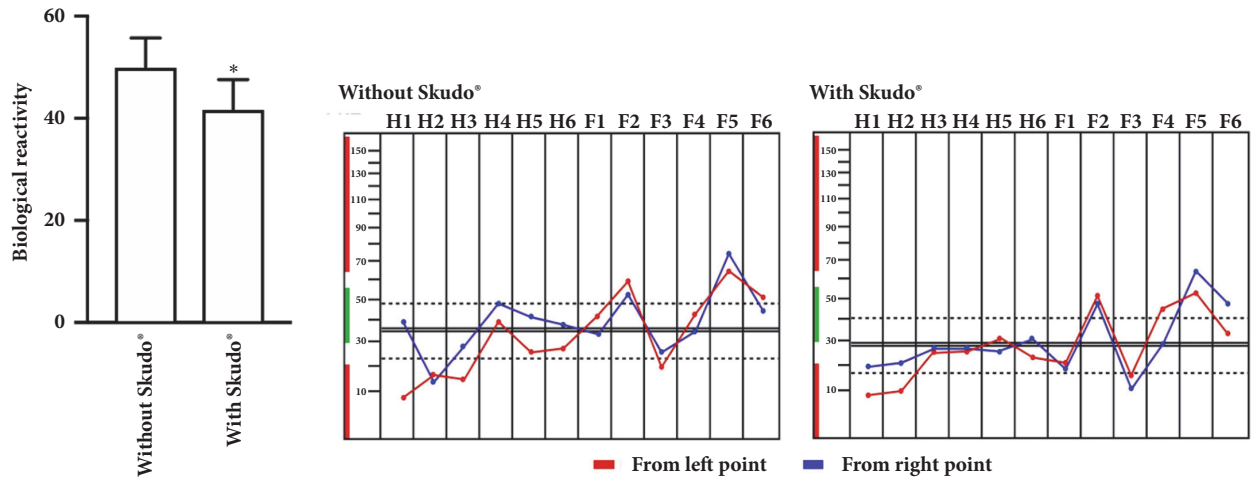

Figure 4: Analysis of the measurements at basal level with or without Skudo®. In (a) the biological reactivity measured by BFB-Z at 10 participants. In (b), an example of layout observed. Data reported are a means+/- SD. $* p < 0.05$ versus without Skudo®.

energy of individuals leads to stabilization, to achieve a more stable balance that is closely related to the neurovegetative activity, named as homeostasis. These are important data to support the hypothesis that the neurovegetative activity is a crucial regulator of stress. In addition, in presence of Skudo®, the shape of the layout (Figure 4(b)) demonstrated a better alignment between measurements from left and right points than in absence of Skudo®; these data demonstrated a lower perturbation of the biological impedance of the body and effectiveness of Skudo® into isolate the body from the external environment. Finally, a reduction of stress was also observed, as shown by the plateau phase of H2-H3-H4 points which represent the fire. On the contrary, wood and water elements remain unaffected, indicating absence of interfering effects on metabolism.

3.2. Skudo® Protection against EMF. The importance of the barrier from electromagnetic fields was confirmed by the successive experiments performed near a WiFi antenna, as reported in Figure 5 in which each measurements were described.

Analyzing the bioenergetic status of each measurements of participants, an evident unbalance was observed on meridians that regulate water. As reported in Figure 6, two water meridians related to kidney and bladder during the irradiation (phase 2) were unbalanced compared to the basal level (phase 1); the layouts of the left in red and of the right in blue were crossed. This phenomenon could be interpreted as an unbalance of the energy linked to water determined by the electromagnetic field of the Wi-Fi radiation source. Usually in physiological conditions, there are no crossing points of the left and right tracks. This phenomenon is relevant since, placing the Skudo® patches on the body (phase 3) and then on the source of EMF (phase 4), the traces corresponding to left and right side of the body return aligned and therefore are very similar to the basal layout.

3.3. Skudo® Protection against SMF. As has been observed on previous experiments in presence of WiFi antenna, also in these experiments performed during the working in radiological environment and in particular during magnetic resonance, the bioenergetic profiles measured on all participants in the different phases (phase 1, at basal rest; phase 2, working day without Skudo® patches; phase 3, baseline of working day; phase 4, day of work with Skudo® patches) show an imbalance of the meridians responsible for the control of water. As reported in Figure 7, the meridians corresponding to kidney and bladder during phase 2 (irradiation moment without Skudo® patches) have bioenergetic profiles of the left side (showed in red) and of the right side (reported in blue) that cross. This confirms the imbalance of water due to an electromagnetic field. The presence of the patches realigns the traces (phase 4), demonstrating the shielding and balancing capacity of the Skudo patches.

4. Discussion

In this study, the protective effects of specific patches on the effects of magnetic fields on the human body were evaluated. The studied devices, commercially available patches called Skudo®, have been placed on some important acupoints. In particular, the selected points were 6CV, 17CV, and 3GV. As known, the three points chosen belong to two of the extraordinary meridians: conception vessel and governor vessel, also known as Ren Mai and Du Mai. The choice of these meridians is motivated by the fact that Ren Mai represents a fundamental level of energetic functioning and has as main action the toning of the Kidney Yin and also nourishes and regulates the Blood. In addition, Ren Mai controls Sea of Yin meridians and circulates Yin Qi, including Blood, Essence, and Body Fluids. For what concerns the Du Mai meridian, its main functions are regulating the circulation of energy and blood in the Yang meridians (hence the name of "Yang meridian sea"), regulating the

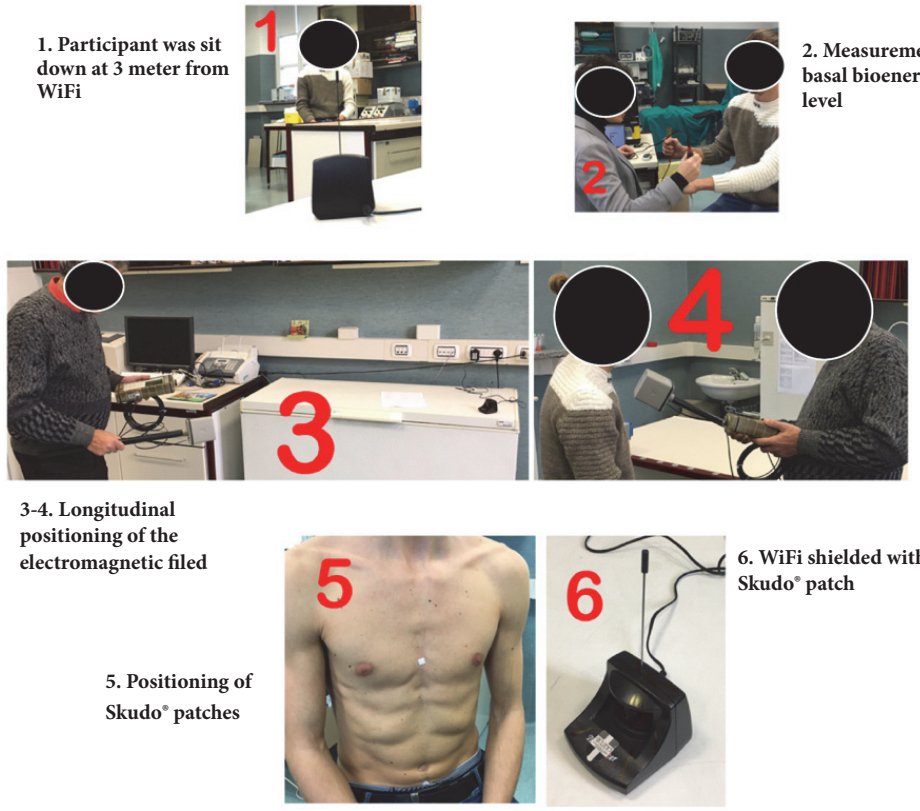

Figure 5: Description of EMF experiments with or without Skudo® patches.

functional activities of the brain and the marrow spinal, and regulating the function of urinary and reproductive systems. The Governing and Conception Vessels are the main rivers of the body's Yin and Yang energies. They are polar aspects of the body, perfectly complementary, like midnight and midday [21]. The two anterior points chosen for the positioning of the 6CV and 17CV patches correspond to the Lower Dantian and the Middle Dantian, respectively. The Lower Dantian is the major storage area for the various types of Kidney energies. The Kidney energies, in turn, are closely linked with the prenatal energies and provide the foundation for all other types of energy (like Jing, Qi, Yin, and Yang) in the body [21]. The Lower Dantian is connected to the first level of Wei Qi. This level of Protective Qi circulates outside the body, extending roughly two inches beyond the body's tissues. As the Lower Dantian fills with Qi, the Wei Qi field naturally becomes thicker. The Lower Dantian collects Earth energy and is associated with Jing and the energy of the physical body. The Earth energy that is transformed in the Lower Dantian is a dense, full, thick energy. In the above analogy of the transformations of water, the energy in the Lower Dantian relates to ice, the densest state of water. The Lower Dantian acts as a reservoir for heat and energy and is associated with the Kidneys. The Kidneys control the Water element in the body, and, in alchemical terms, Jing is said to be analogous to the water in the cauldron [21]. The third patch is placed on the midline of the lower back, in the depression below the spinous process of the second lumbar vertebra. This region corresponds to one of the most important point: called the Mingmen. It is the centre of vitality and is the point where the original life essence of the individual is based. The *Classic of Difficulties* said *"On the left is the Kidney, on the right is ming men"* whilst according to Zhang Jing-yue *"Ming men resides between the Kidneys"*. The exact location of ming men (Gate of Life) has been described differently at different times, but as its name makes clear, Mingmen is an important area to influence the ming men and the ministerial fire to which it is closely related. As well as influencing the ming men fire, this area located on the Governing vessel has a strong regulatory effect on the yang qi and the exterior portion of the body. For this reason, this area is particularly useful for the treatment of heat disorders, whether interior or exterior, excess or deficient. Treating this area it is possible to drain heat manifesting as 'heat in the body like fire' [21]. The diagram provided by the BFB-Z by measuring the electropermeability of the 12 sample points shows the bioreactivity status of the two halves of the body, right and left. Furthermore a double horizontal line indicates the level of biological reactivity. As described above, the protection provided by the patches mainly concerns the water (front points) and the focus (back point). For the TCM, water is one of the five fundamental elements of life. As already mentioned, water is managed by the Kidney and the Bladder: the Kidney both as an organ and as a meridian; the Bladder as a bowel and as a meridian. It should be noted that for the TCM the Kidney is not identified with the

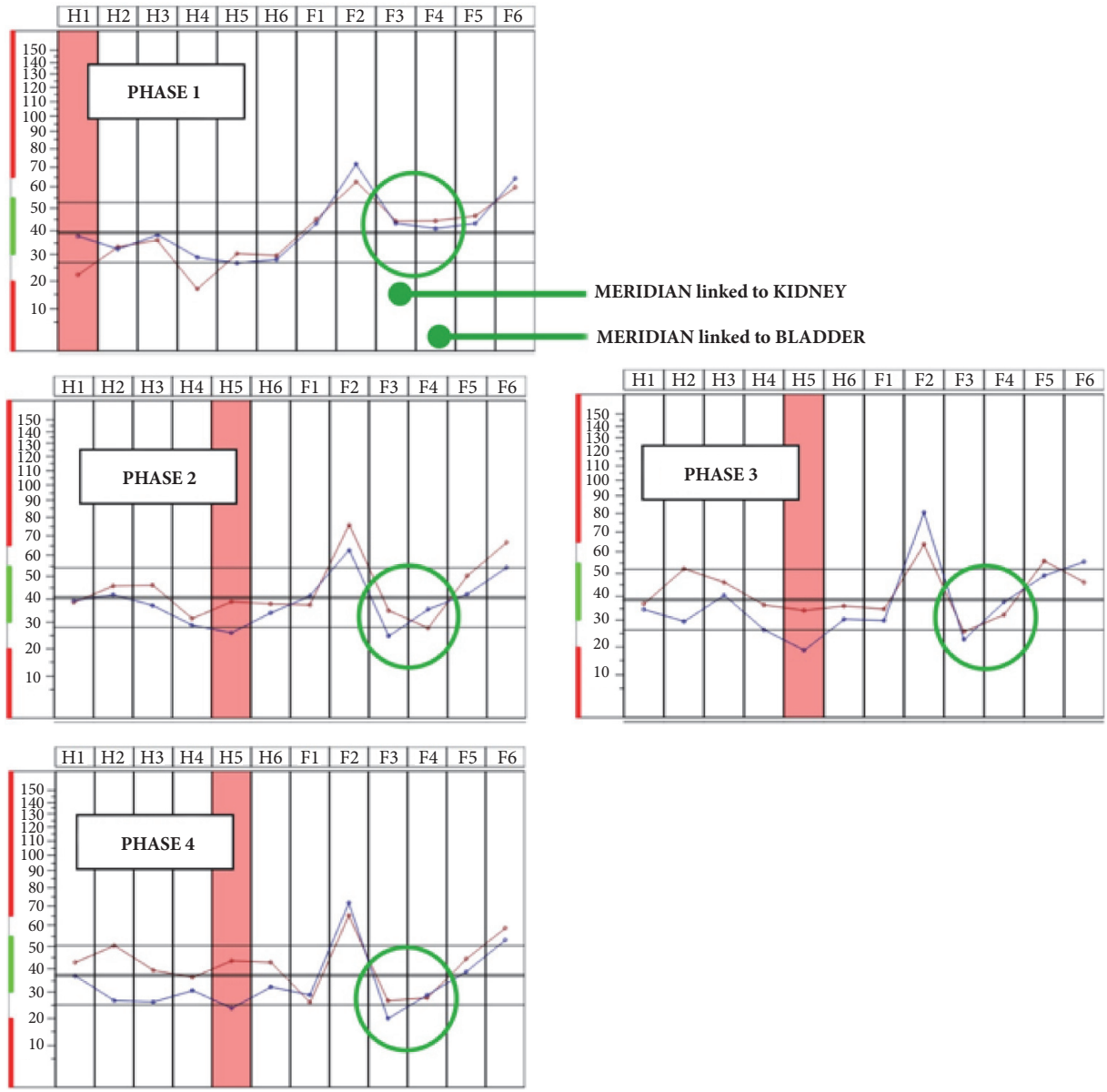

Figure 6: An example of layout obtained during the measurements by BFB-Z of 10 participants.

anatomical organ of modern medicine and its physiology, but with the set of energies expressed by the Water movement. The Kidney of TCM is, among all, perhaps the organ farthest from the kidney of Modern Medicine. Some Western doctors who support an integration between the two medicines have identified the Chinese kidney system with the immune system, endocrine and hormone, with important glands such as the thyroid, parathyroid, and adrenal glands. The Kidneys tesaurize the jing, the ancestral essence, that is the result not only of the union of the masculine with the feminine, but also of the transformation of nourishment and liquids. The jing presides over the development of the organism and represents the vital reserve of energy. The kidneys purify the liquids and reintroduce them into the life cycle. They are the "valves" which, opening and closing, favor the circulation of liquids.

As far as the bladder is concerned, its meridian crosses the whole body from head to toe, and, on its back, where the two branches of which it is composed run, its crosses the most important muscular bundles in its path. The bladder is responsible for the distribution of fluids throughout the body, but especially the muscles, but it deals with the elimination of toxins through the liquids. The bladder regulates the active liquids in the body, avoids both dryness and flooding, and guarantees a beneficial humidification to the muscles. The bladder transforms the liquids that come to it; on the one hand it makes their recoverable part rise; on the other it thickens the part with the waste and eliminates it through the urine. Life is based on water that represents its universal support. The human body contains more than 50% by volume, but if we consider the total number of molecules that

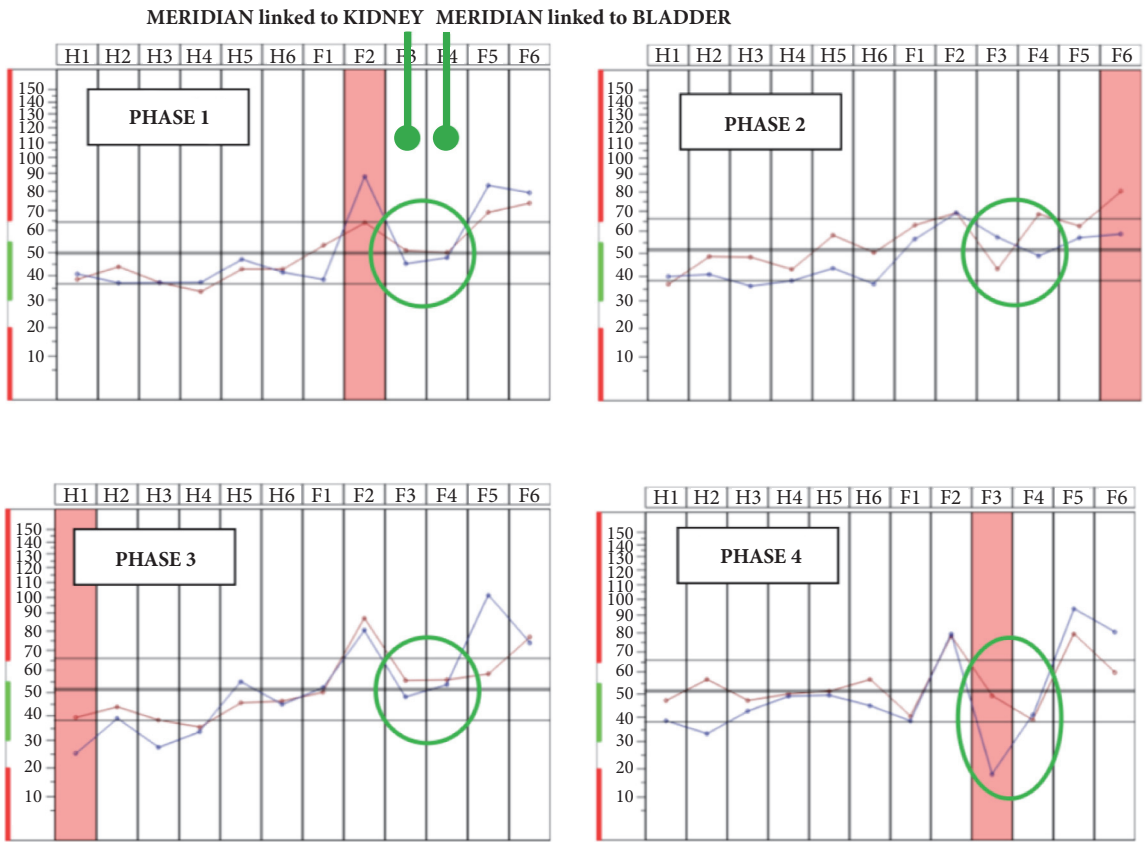

Figure 7: An example of layout obtained during the measurements by BFB-Z of 10 participants.

make up our body, water makes up 99% of the total [22]. Many studies have been carried out on the electromagnetic properties of water and on its behavior when it is exposed to electromagnetic fields [23, 24]. The water molecule, subjected to irradiation, absorbs the energy of the electromagnetic waves, if the latter have a frequency that approaches that of the microwaves, i.e., 2,450 GHz. This absorption results in a vibration of the water molecule that it could interfere with all the metabolic reactions of the cells, from enzymatic activity to protein synthesis, up to the processes of cell replication. Furthermore, a recent study [25] showed that a magnetic field of 1.2 micro-Tesla inhibits the action of melatonin. Another consideration that can be made by analyzing the results of the tests is that the patch on the back protects the body from an imbalance of heat, since the area corresponding to Mingmen. It is well known that magnetic fields, especially variable ones, generate thermal effects that can influence the metabolism [26]. The screen provided by these patches can therefore be effective on two important aspects of biological process control.

5. Conclusions

Maintaining a correct Yin/Yang balance through the use of acupoints is undoubtedly a method that allows the energies of the individual to be maintained longer and with a better quality. The true effectiveness of patches application is manifested in its rebalancing and preventive action on the disharmony that leads to the exhaustion of the energies of the individual.

This study shows that both SMF and EMF cause an alteration of the body's water system. The application of the Skudo® patches determines a regularization of the bioenergy levels correlated with the Water System.

The application of Skudo® on the EMF source suppressed the imbalance effect of the Water System found in the subject without any protection.

Data Availability

All data reported have been obtained from experiments carried out in author's laboratory.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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Research Article

Moxibustion-Simulating Bipolar Radiofrequency Suppresses Weight Gain and Induces Adipose Tissue Browning via Activation of UCP1 and FGF21 in a Mouse Model of Diet-Induced Obesity

Young Jun Koh, Ju-Hee Lee, and Sung Yun Park

College of Korean Medicine, Dongguk University, 32 Dongguk-ro, Ilsandong-gu, Goyang-si, Gyeonggi-do 10326, Republic of Korea

Correspondence should be addressed to Sung Yun Park; bmepark@dongguk.ac.kr

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Background. Obesity is a pathological condition associated with various diseases including diabetes, stroke, arthritis, infertility, and heart disease. Moxibustion is widely used to prevent and manage obesity in traditional Asian medicine. We tested our hypothesis that moxibustion-simulating bipolar radiofrequency (M-RF) can suppress total body and white adipose tissue (WAT) weight gain via induction of WAT browning in a mouse model of diet-induced obesity (DIO). *Methods*. We designed an M-RF device that could accurately adjust the depth and temperature at which heat stimulation was administered into the abdomen of DIO mice. High-fat-fed male C57BL/6 mice were treated with the M-RF device every two or three days for three weeks. We then harvested WAT and serum from the mice and measured total body and WAT weight, size of adipocytes, mitochondrial contents, features of the dead adipocyte environment, and levels of uncoupling protein 1 (UCP1) and fibroblast growth factor 21 (FGF21). *Results*. Heat stimulation by M-RF in DIO mice resulted in precise temperature adjustment in the mice abdomen, with variance less than 1°C. Additionally, M-RF stimulation inhibited body and WAT weight gain, resulting in increased formation of beige adipocytes, increased mitochondrial content, and decreased formation of dead adipocytes in WAT. Moreover, treatment of M-RF induced expression of UCP1 and FGF21 in serum and/or epididymal WATs in DIO mice. *Conclusion*. Heat stimulation by M-RF treatment induced upregulation of UCP1 and FGF21 expression in serum and/or WATs, which was correlated with reduced total body and WAT weight gain in DIO mice.

1. Introduction

Moxibustion based on natural materials such as herbs is widely used in traditional Asian medicine clinics to prevent and manage various diseases such as polycystic ovary syndrome, ulcerative colitis, heart disease, irritable bowel syndrome, diabetic limbs, and obesity [1–6]. Previous animal studies showed that preventive moxibustion can also be used to adjust the levels of fat accumulation, blood lipids, and female sex hormones in rats and mice [6, 7]. Some researcher has proved that moxibustion leads to significant decrease of 5-hydroxytryptamine and adrenocorticotrophic hormone which play a role in decreasing blood flow into fat tissue in mouse [8]. However, moxibustion is limited in that it directly stimulates the outer layer of the skin with relatively high temperatures that may be difficult to adjust, and it generates smoke during use [9–11]. Thus, development of an effective, easy-to-use, and safe moxibustion system is needed.

Application of radiofrequency (RF)-induced thermal effects based on electrical systems is a minimally invasive treatment method used in various medical fields. Radiofrequencies are commonly used to treat tumors in the liver, lung, pancreas, and kidney, as well as to induce fat reduction and cellulite improvement [12–16]. Because RF is used to increase the temperature of a target point from 42°C to 46°C for hyperthermia therapy [17, 18], or for ablation therapy (in which temperature changes ranging from 50°C to 102°C are applied), skin can be simulated using RF devices to increase the temperature [10, 19].

Obesity accompanied by WAT growth poses serious concerns because it is closely related to the onset of several metabolic diseases, including type 2 diabetes, fatty liver disease, and cardiovascular diseases [20–22]. Recent studies have spurred interest in the antiobesity effects of WAT browning, which induces the characteristics of brown adipose tissue (BAT) in WATs outside of typical BAT locations [23]. Browning of adipocytes in WAT, which has genetic differences because of its distinct developmental origin from BAT [24], can be induced by various stimulations such as small molecules and environmental cues [25]. WAT browning occurs via several transcriptional factors, coregulators, lipid droplet-associated proteins, microRNAs, and growth factors, as well as mitochondrial uncoupling proteins [25–27].

Obesity is associated with chronic inflammation, which leads to various diseases such as diabetes, hypertension, and cardiovascular diseases [28]. Chronic inflammation in obesity is mainly caused by recruitment of inflammatory macrophages into the WATs, as well as by conversion of resident macrophages in the WATs [29, 30]. In the adipose tissue of obese individuals, macrophages with phagocytic activities surround and remove dead adipocytes in the WATs [29]. Therefore, it is important to characterize the adipose tissue in terms of macrophage infiltration and conversion status.

Obese patients who try losing weight via lifestyle interventions such as adjusting food intake and increasing the amount of physical exercise often fail to see significant improvements in their condition; moreover, pharmacotherapeutics aimed at managing obesity are often accompanied by side effects such as metabolic and psychologic diseases [31]. Thus, a novel mode of intervention that safely manages obesity and its related conditions is needed. In light of this unmet need, several trials have used not only medications, but also noninvasive medical devices to treat obesity [32]. Here, we describe the effects of moxibustion-simulating bipolar radiofrequency (M-RF) treatment on body weight reduction and adipocyte browning induction in a mouse model of diet-induced obesity (DIO).

2. Materials and Methods

2.1. Equipment. To improve upon the method of classical moxibustion, we designed a bipolar RF device that is able to precisely control the depth and temperature at which stimulation is given, thereby allowing quantitative intervention. The main equipment consisted of two primary devices, an energy generator and a temperature measuring device (Figures 1(a) and 1(b)). A bipolar RF device mainly consisted of a power control board, display panel, and six bipolar probes. The power control board could generate a maximum power of 44.6 W with 50 Ω, 67.6 W with 200 Ω, and 50.8 W with 500 Ω, which was delivered to the subject through bipolar probes. The bipolar probes were circular (diameters of 5 mm, 1.8 mm, and 3 mm for the inner circle, outer circle, and width, respectively) and coated with Ag as a skin protectant (Figure 1(c)). The process was monitored and controlled by a display panel consisting of a 10.1″ thin film transistor-liquid crystal display that had high resolution (1280∗800), which is standard for 4-wire touch screens. K-type temperature detecting needles were used to measure the temperatures at the surface of the abdomen and approximately 1 mm below the surface. The temperature data were sampled at 20 Hz and transmitted to the LabVIEW system (National Instruments, Austin, TX USA). The heating performance of the M-RF Stimulator was measured with skin samples of Yucatan pig and mice as shown in Figures 1(d) and 1(e). Each experimental procedure was conducted over a course of three minutes. The subjects were contained in plastic cube boxes (350 mm × 450 mm × 350 mm) maintained at 23°C ambient temperature and 50–60% humidity.

2.2. Mouse and Treatment. All animal experiments were approved by the Institutional Animal Care and Use Committee of Dongguk University (approval No. IACUC-2017-017-1). Eight-week-old male C57BL/6 mice were purchased from Central Lab Animal Inc. (Seoul, Korea). The mice were provided with *ad libitum* access to water and high-fat diet (HFD). The HFD (60% Kcal fat; Research Diets, New Brunswick, NJ) was provided for four weeks to induce diet-induced obesity (DIO). Afterwards, the mice were randomly distributed into the following four treatment groups: (1) no heat application (control, $n = 10$); (2) low-temperature stimulation (RF-L, 36.7°C, $n = 10$); (3) middle-temperature stimulation (RF-M, 37.9°C, $n = 10$); and (4) high-temperature stimulation (RF-H, 38.8°C, $n = 10$). We applied M-RF onto the abdominal skin of DIO mice at the three different temperatures for one minute per day every two days over three weeks while maintaining the HFD. At the end of the three-week application of M-RF, mice were anesthetized by intramuscular injection of a combination of anesthetics (mixture of tiletamine, zolazepam, and xylazine, each 10 mg/kg), weighed, and sacrificed. Blood was collected by cardiac puncture and centrifuged at 848 g for 15 minutes at 4°C, after which the resulting serum was harvested. The WAT specimens were immediately weighed and fixed in neutral buffered 4% formaldehyde for histochemical studies. The serum and WAT were stored at -80°C until further use.

2.3. Anesthesia and Euthanasia. Mice were anesthetized and euthanized by intramuscular injection of the combination of anesthetics (tiletamine-zolazepam 50mg/kg and xylazine 12 mg/kg) before RF treatment and histologic analysis.

2.4. Histologic and Morphometric Analysis. Mice were anesthetized by intramuscular injection of anesthetics, and WATs were fixed by cardiac perfusion of 1% paraformaldehyde in PBS and whole-mounted. To visualize the adipocytes and mitochondrial content in WATs, the tissues were incubated for four hours at room temperature with the following chemicals: 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) for adipocytes (1 μg/ml in PBS; Invitrogen, Carlsbad, CA, USA) and MitoTracker Red CMXRos (MitoTracker) for mitochondrial contents (100 nM in PBS, Invitrogen). To verify the dead adipocytes, immunohistochemistry was performed as previously described [29, 33]. Briefly, the WATs were incubated for an hour at room temperature with blocking solution containing 5% whole donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) in

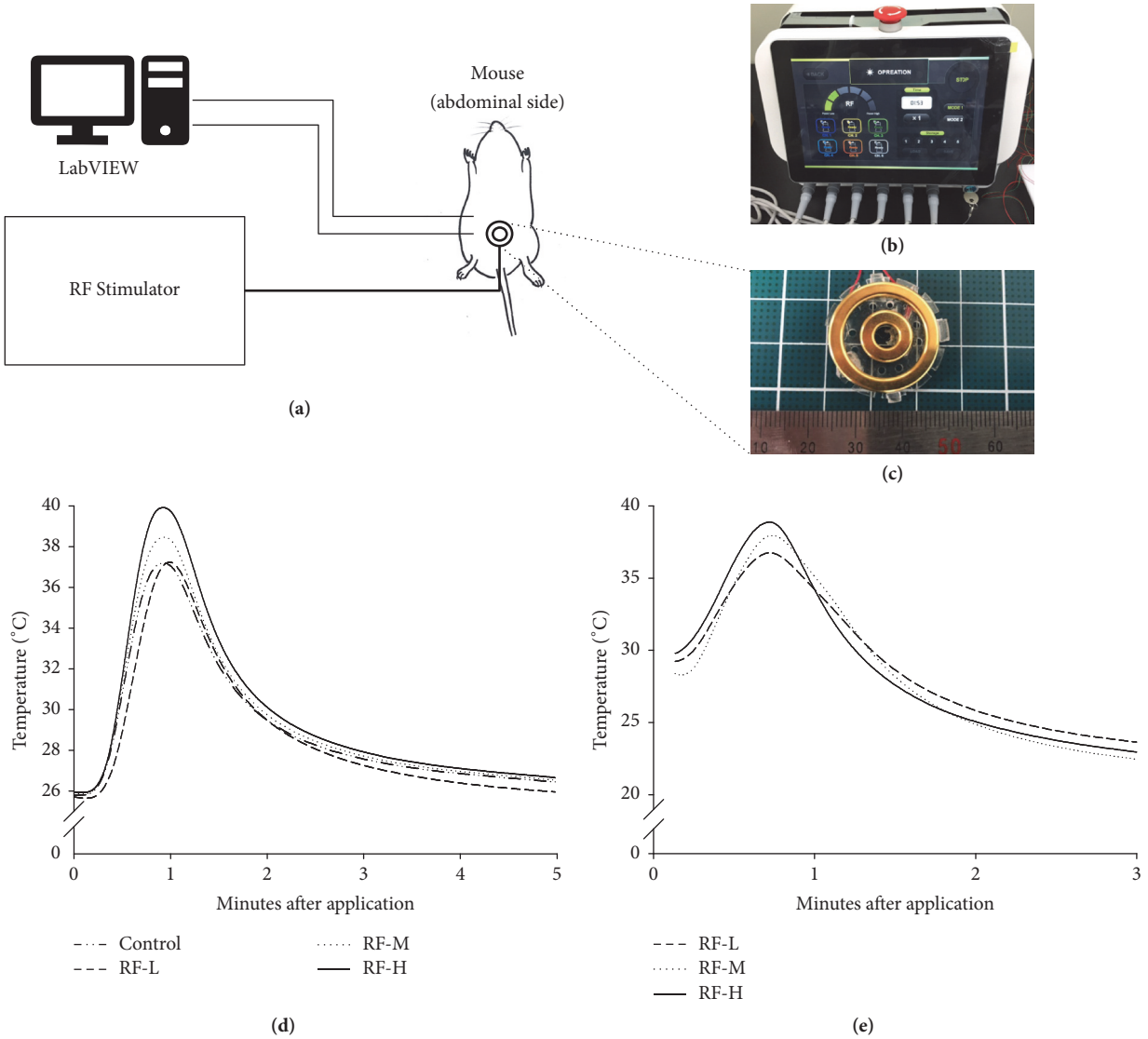

Figure 1: Development of the M-RF Stimulator. (a) Block diagram of the M-RF Stimulator and temperature measurement system. (b) Photo for front side of the M-RF Stimulator. (c) Bipolar electrode plate (5 mm, 1.8 mm, and 3 mm for diameters of the inner circle, outer circle, and width, respectively) in the M-RF Stimulator for use in mice. (d) Temperature change (maximum temperatures of 39.9°C, 38.4°C, 37.2°C, and 37.2°C for RF-H, RF-M, RF-L, and moxibustion (Moxibustion), respectively) in Yucatan pigs. (e) Temperature change (maximum temperatures of 38.8°C, 37.9°C, and 36.7°C for moxibustion, RF-H, RF-M, and RF-L, respectively) in mice.

PBS-T (0.3% Triton X-100 in PBS). After blocking, the tissues were incubated overnight at room temperature while shaking with rat anti-mouse F4/80 antibody (clone Cl:A3-1, diluted 1:1,000; Serotec) in blocking solution to visualize infiltration of phagocytic macrophages in WATs, which is a characteristic of apoptotic adipocytes [29]. After five washes in PBS-T, whole-mounted tissues were incubated for four hours at room temperature with Cy3-conjugated anti-rat antibody (diluted 1:500; Jackson ImmunoResearch Laboratories Inc.) in blocking solution. For negative control experiments, primary or secondary antibodies were omitted during the immunohistochemistry process. All images were captured using a Nikon Eclipse Ts2 inverted fluorescent microscope (Nikon, Japan) equipped with high-definition color camera (DS-Qi2, Nikon) and then analyzed with the NIS Elements Imaging Software (version 4.30; Nikon). To calculate the adipocyte size, mitochondrial content, and UCP1 expression, stained images for BODIPY, MitoTracker, and UCP1 were captured and measured. To determine the adipocyte size in WATs, the diameters of adipocytes were measured in five randomly selected regions (~100 adipocytes per each region) per WAT. The mitochondrial content and expression of UCP1 were measured by analyzing the densities of the MitoTracker- or UCP1-positive areas based on the pixels in the regions of interest. During analysis, only pixels with an intensity of more than 50 were chosen to exclude background fluorescence. The number of beige adipocytes in five randomly selected regions (~100 adipocytes per each region) per WAT was counted. Dead adipocytes were detected using double-stained color images for BODIPY and F4/80. We counted the number of

BODIPY$^-$/clustered macrophage$^+$ clustered regions for dead adipocytes in 10 randomly selected regions (~100 adipocytes per each region) per WAT in a blind manner.

2.5. Western Blotting. Total protein from WATs was extracted using a tissue homogenizer and lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% 4-nonylphenyl-polyethylene glycol, 5 mM ethylenediaminetetraacetic acid, 1 M *threo*-1,4-Dimercapto-2,3-butanediol) containing protease inhibitor cocktail (GenDEPOT, Barker, TX, USA). The homogenates were centrifuged for 15 min at 15,928 g and 4°C. The supernatants were collected and centrifuged again, and the second supernatants were used for Western blotting analysis. To detect mouse FGF21 protein, protein-loaded membranes were first reacted with anti-FGF21 antibody (diluted 1:1000, Abcam, Cambridge, MA, USA) and then incubated with HRP-conjugated secondary antibody (diluted 1:5000, Thermo Scientific, Rockford, IL, USA). Finally, the FGF21 protein was detected with HRP substrate (Amersham Bioscience, Buckinghamshire, UK) using a Fusion FX7 chemiluminescence imaging system (Vilber Lourmat, France).

2.6. ELISA for Serum FGF21. To quantify FGF21 in mouse serum, an enzyme-linked immunosorbent assay (ELISA) was performed using the mouse FGF21 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, mice were anesthetized by intramuscular injection of a combination of anesthetics, and 1 ml of whole blood was collected via cardiac puncture with 28-gauge syringe. The blood samples were then centrifuged for 15 minutes at 848 g and 4°C, after which the resulting serum samples were stored at -80°C until analysis.

2.7. Statistics. Values are presented as the means ± standard deviation (SD). Statistical analyses consisted of one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison or a Student's t-test. P values < 0.05 were considered statistically significant. All experiments were performed independently at least three times, and the data were analyzed using the Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Thermal Stimulation Using an M-RF Stimulator. To evaluate the performance of the M-RF Stimulator, we measured the temperature of Yucatan pigs and mice. The temperature curves of the M-RF Stimulator (39.9 ± 0.12°C, 38.4 ± 0.12°C, and 37.2 ± 0.13°C (mean ± SD) for maximum temperatures of RF-H, RF-M, and RF-L, respectively) showed patterns similar to those of moxibustion (37.2 ± 0.21°C maximum temperature) at 2 mm from the skin of Yucatan pigs (Figure 1(d)) and at 1 mm from the skin of mice (38.8°C, 37.9°C, and 36.7°C for RF-H, RF-M, and RF-L, respectively) (Figure 1(e)). The average times required for the temperature to increase from approximately 30°C to the maximum temperature for each temperature curve in the Yucatan pig experiment were 17.4 sec, 16.8 sec, 18.9 sec, and 13.3 sec for RF-H, RF-M, RF-L, and moxibustion, respectively (Figure 1(d)).

3.2. Thermal Stimulation by M-RF Suppresses Body and WAT Weight Gain in DIO Mice. To determine the effects of M-RF-induced heat stimulation on body weight gain in DIO mice (control), we applied M-RF on DIO mice at three levels of temperature, low (36.7°C; F-L), middle (37.9°C; RF-M), and high (38.8°C; RF-H), for three weeks (three min per day every two days). At three weeks after the start of M-RF treatment, total body weight measurements of the control group that received no heat stimulation had significantly increased (39.13 ± 3.736 g, $n = 10$), whereas those of RF-L (33.22 ± 2.371 g, $n = 10$, $P < 0.01$ versus control), RF-M (32.92 ± 0.8585 g, $n = 10$, $P < 0.01$ versus control), and RF-H (34.04 ± 3.505 g, $n = 10$, $P < 0.01$ versus control) had not increased significantly (Figure 2(a)). Moreover, in contrast with the control (at end of the experiment; 4.367 ± 1.268 g, $n = 10$), all three levels of M-RF stimulation groups showed changes in body weight relative to the day of first application (RF-L, -0.46 ± 0.826 g, $n = 10$, $P < 0.01$ versus control; RF-M, -1.08 ± 0.858 g, $n = 10$, $P < 0.01$ versus control; RF-H, -0.42 ± 1.85 g, $n = 10$, $P < 0.01$ versus control) (Figure 2(b)). Consistently, weights of epididymal and mesenteric WATs in M-RF-treated DIO mice did not significantly increase in all three levels (RF-L, 0.895 ± 0.0284 g, $n = 10$, $P < 0.01$ eWAT versus control and 0.538 ± 0.1039 g, $n = 10$, $P < 0.01$ mWAT versus control; RF-M, 0.8866 ± 0.0286 g, $n = 10$, $P < 0.01$ eWAT versus control and 0.565 ± 0.1373 g, $n = 10$, $P < 0.01$ mWAT versus control; RF-H, 1.359 ± 0.0427 g, $n = 10$, $P < 0.01$ eWAT versus control and 0.641 ± 0.1664 g, $n = 10$, $P < 0.01$ mWAT versus control) compared to the control (2.630 ± 0.0610 gram, $n = 10$ for eWAT and 1.194 ± 0.1673 g, $n = 10$ for mWAT) (Figures 2(c) and 2(e)). Moreover, ratios of WAT-to-body weight were lower in RF-L (2.69 ± 0.085%, $n = 10$, $P < 0.01$ eWAT versus control and 1.62 ± 0.313%, $n = 10$, $P < 0.01$ mWAT versus control), RF-M (2.69 ± 0.087%, $n = 10$, $P < 0.01$ eWAT versus control and 1.72 ± 0.417%, $n = 10$, $P < 0.01$ mWAT versus control), and RF-H groups (3.99 ± 0.125%, $n = 10$, $P < 0.01$ eWAT versus control and 1.88 ± 0.489%, $n = 10$, $P < 0.0101$ mWAT versus control) than in the control (6.72 ± 0.156%, $n = 10$ for eWAT and 3.05 ± 0.428%, $n = 10$ for mWAT) (Figures 2(d) and 2(f)).

3.3. Thermal Stimulation by M-RF Decreases the Size of Adipocytes and Increases Mitochondrial Contents in the WATs of DIO Mice. To describe the cellular changes in WATs induced by M-RF stimulation, we harvested the epididymal and mesenteric WATs from the M-RF-treated DIO mice and observed the size and mitochondrial contents of adipocytes in both WATs using whole-mounted immunostaining (Figures 3(a) and 3(d)). In the control group, the average diameter of the adipocytes was 124.3 ± 22.14 μm in epididymal ($n = 5{,}000$ cells) and 97.21 ± 17.89 μm in mesenteric WATs ($n = 5{,}000$ cells) (Figures 3(b) and 3(e)). In M-RF-treated groups, the sizes of adipocytes in both epididymal and mesenteric WATs were significantly smaller (73.19 ± 13.56 μm, $n = 5{,}000$ cells, $P < 0.01$ eWAT versus control and 58.60 ± 9.446 μm, $n = 5{,}000$ cells, $P < 0.01$ mWAT versus control), RF-M (75.03 ± 15.25 μm, $n = 5{,}000$ cells, $P < 0.01$ eWAT versus control and 50.57 ± 6.22 μm, $n = 5{,}000$ cells, $P < 0.01$ mWAT versus control), and RF-H (84.61 ± 18.48 μm, $n = 5{,}000$ cells, $P < 0.01$ eWAT versus control and 61.08 ± 15.78 μm, $n = 5{,}000$ cells, $P < 0.01$

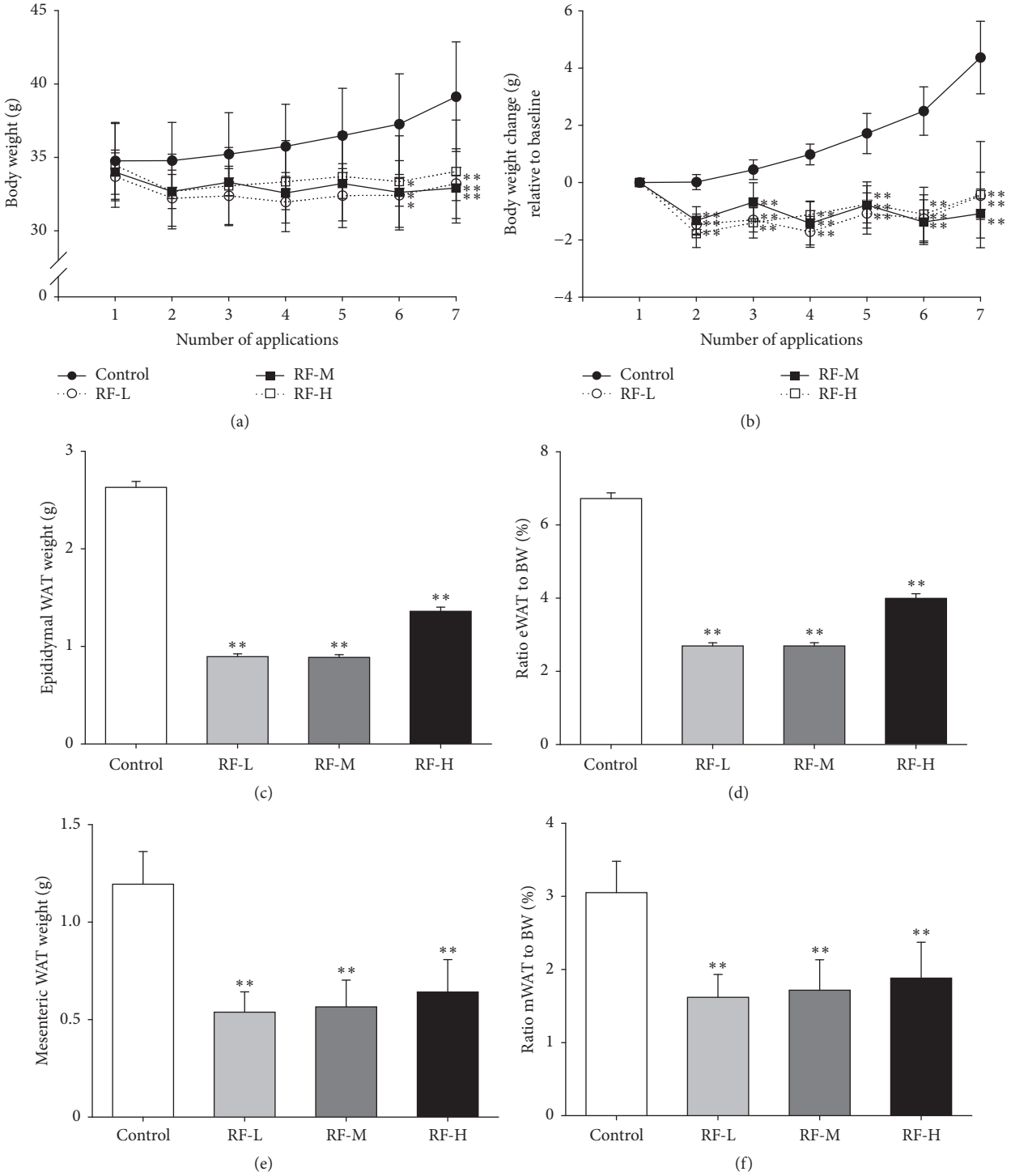

Figure 2: M-RF stimulation suppresses total body and WAT weight gain in DIO mice. (a) Body weights (g) of control ($n = 10$), RF-L ($n = 10$), RF-M ($n = 10$), or RF-H ($n = 10$) groups measured before M-RF stimulation. (b) Body weight changes (g) relative to the base level (the day of first application) in each group ($n = 10$). (c, e) Weights (g) of WATs in each group ($n = 10$). (d, f) Ratio of eWAT (epididymal WAT) weight (d) and mWAT (mesenteric WAT) (f) to body weight at the end-points of the experiment in each group ($n = 10$). Results are presented as the means ± SD. $*$, $P < 0.05$ versus control. $**$, $P < 0.01$ versus control.

mWAT versus control) (Figures 3(b) and 3(e)). Furthermore, contents of mitochondria in the adipocytes of epididymal and mesenteric WATs were increased by all three levels of M-RF stimulation (RF-L, 1.959 ± 0.1447 AU, $n = 5{,}000$ cells, $P < 0.01$ eWAT versus control and 2.275 ± 0.1275 AU, $n = 5{,}000$ cells, $P < 0.01$ mWAT versus control; RF-M, 2.077 ± 0.0999 AU, $n = 5{,}000$ cells, $P < 0.01$ eWAT versus control and 1.983 ± 0.2004 AU, $n = 5{,}000$ cells, $P < 0.01$ mWAT versus control;

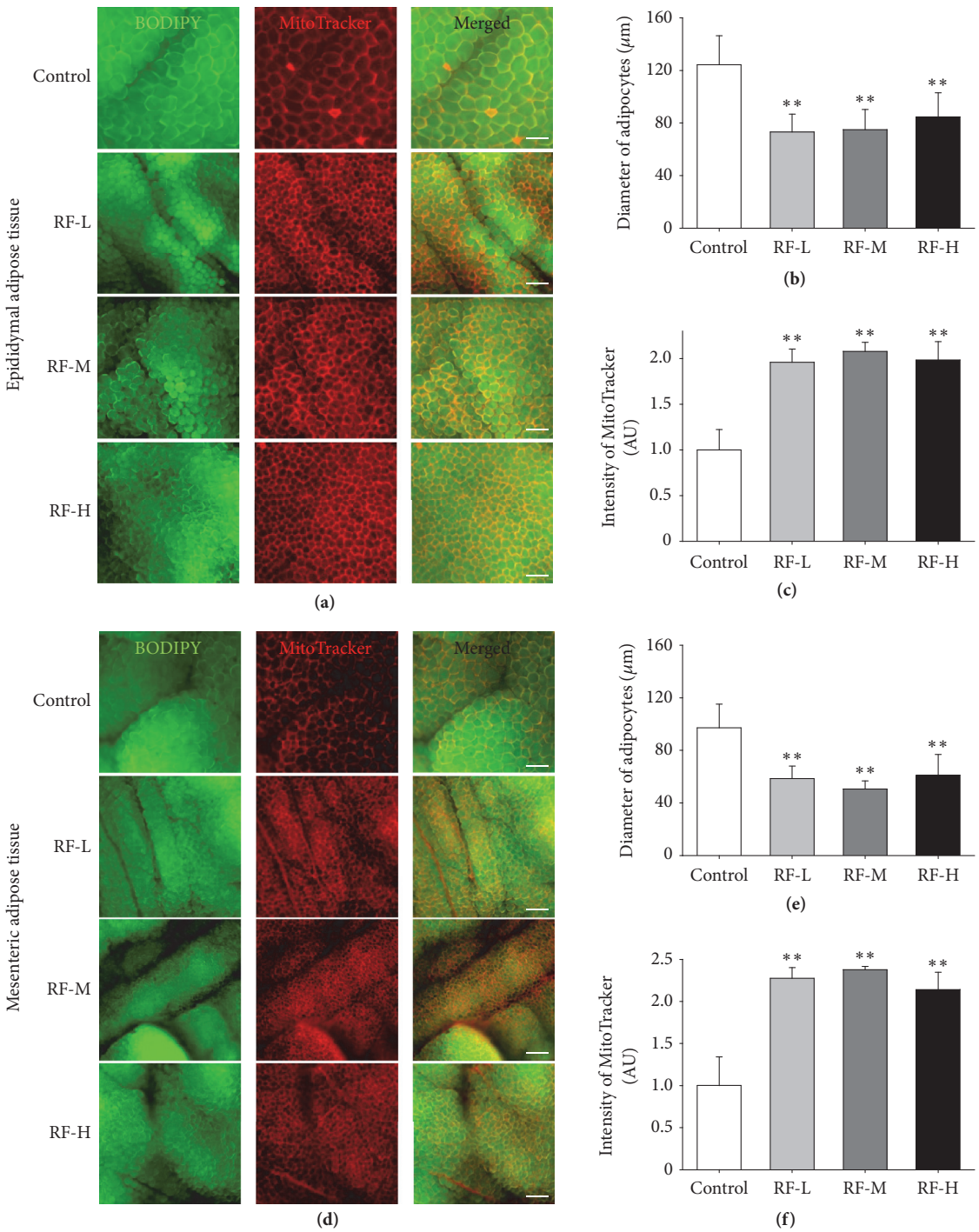

FIGURE 3: M-RF stimulation reduces the size of adipocytes and increases mitochondrial contents in the WATs of DIO mice. (a, d) Whole-mounted WATs stained with BODIPY (green) and MitoTracker (red). Note that all three levels of M-RF stimulation resulted in decreases in the size of adipocytes and increases in the mitochondrial contents compared to the control. Scale bar, 200 μm. (b, c, e, and f) Diameter of adipocytes and density of MitoTracker-positive area were measured in five randomly selected regions (~100 adipocytes per region) per WAT in control ($n = 10$), RF-L ($n = 10$), RF-M ($n = 10$), and RF-H ($n = 10$) and presented as micrometers for diameter of adipocytes and as an arbitrary unit (AU) for the ratio of the pixel densities compared to the control for intensity of MitoTracker. Results are presented as the means ± SD. $**$, $P < 0.01$ versus control.

RF-H, 1.983 ± 0.2004 AU, n = 5,000 cells, $P < 0.01$ eWAT versus control and 2.141 ± 0.2071 AU, n = 5,000 cells, $P < 0.01$ mWAT versus control) (Figures 3(c) and 3(f)).

3.4. Thermal Stimulation by M-RF Induces Expression of UCP1 and Formation of Beige Adipocytes in the WATs of DIO Mice. To identify the underlying mechanism of increased mitochondrial contents induced by M-RF treatment in DIO

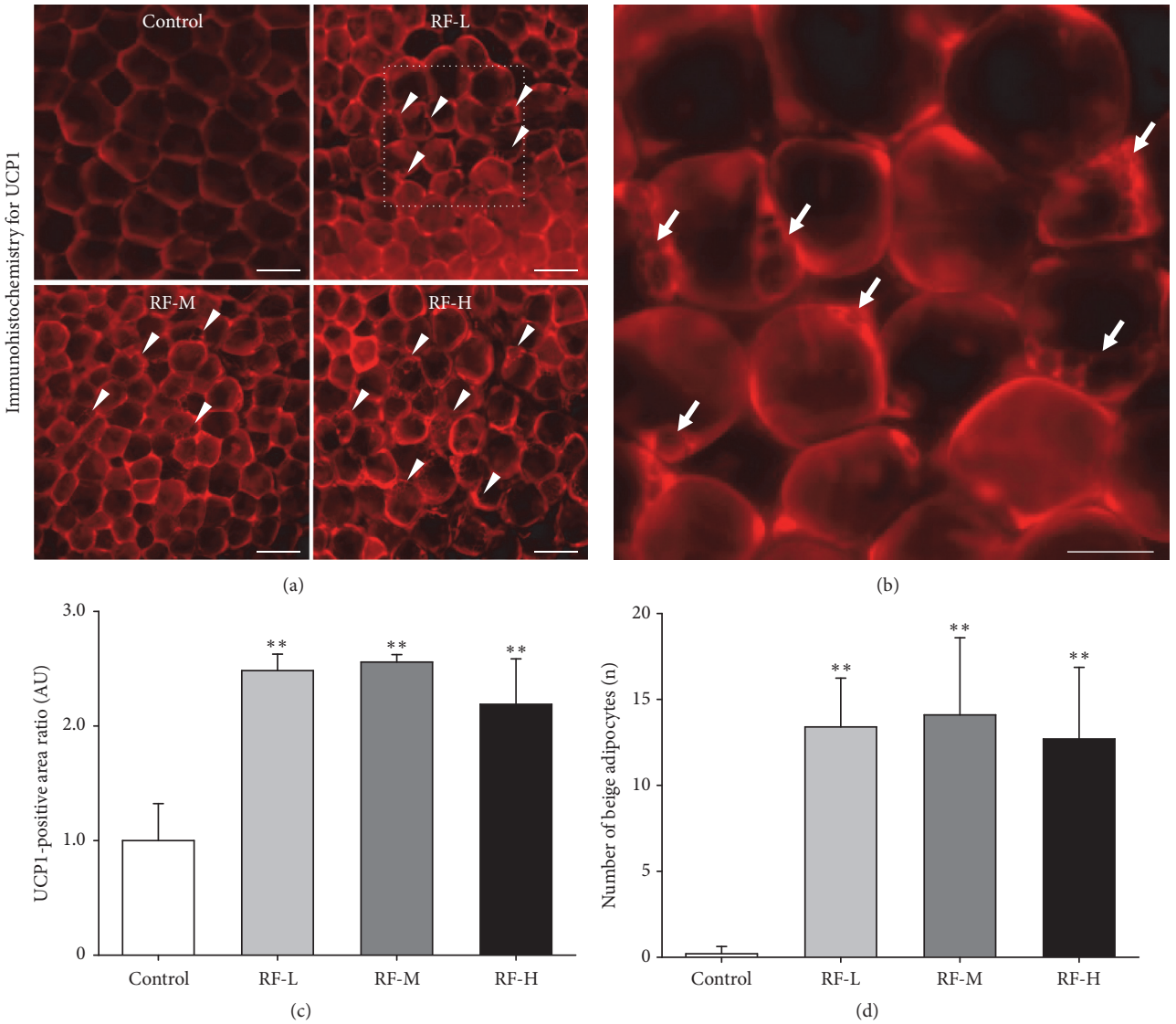

Figure 4: M-RF stimulation increases UCP1 expression and formation of beige adipocytes in the WATs of DIO mice. (a) Whole-mounted WATs immunostained for UCP1 (red). Scale bar, 100 μm. (b) Higher magnification image of RF-L in (a) showing characteristic beige adipocytes. Scale bar, 50 μm. Note that all three levels of M-RF stimulation increased UCP1 expression and formation of beige adipocytes compared to control. Scale bar, 50 μm. (c, d) Ratio of UCP1-positive areas compared to control (c) and numbers of beige adipocytes (d) calculated in five randomly selected regions (~100 adipocytes per each region) per WAT in control ($n = 10$), RF-L ($n = 10$), RF-M ($n = 10$), and RF-H ($n = 10$). Results are presented as means ± SD. **, $P < 0.01$ versus control.

mice, we analyzed UCP-1 expression in epididymal WATs because activation of UCP-1 is a component of mitochondrial activation [34] that is responsible for browning of WATs [25]. Similar to the changes in mitochondrial contents, UCP-1 was highly expressed in the epididymal WATs following RF-L (2.438 ± 0.1440 AU, $n = 5{,}000$ cells, $P < 0.01$ versus control), RF-M (2.558 ± 0.0646 AU, $n = 5{,}000$ cells, $P < 0.01$ versus control), and RF-H (2.189 ± 0.3965 AU, $n = 5{,}000$ cells, $P < 0.01$ versus control) (Figures 4(a) and 4(c)). Consistent with previous reports [33], while beige adipocytes were rarely observed in the WATs of control DIO mice (0.200 ± 0.421, $n = 5{,}000$ cells), large numbers of beige adipocytes with small droplets were observed in the eWATs of DIO mice that received RF-L (13.4 ± 2.84, $n = 5{,}000$ cells, $P < 0.01$ versus control), RF-M (14.1 ± 4.48, $n = 5{,}000$ cells, $P < 0.01$ versus control), and RF-H (12.7 ± 4.16, $n = 5{,}000$ cells, $P < 0.01$ versus control) stimulation (Figures 4(b) and 4(d)).

3.5. Thermal Stimulation by M-RF Inhibits Infiltration of Macrophages into Dead Adipocytes in the WATs of DIO Mice. To investigate the presence of dead adipocytes and macrophage infiltration into WATs, whole-mounted WATs were costained with BODIPY (neutral lipid binding chemical) and F4/80 (phagocytic macrophage marker). In control mice, we observed dead adipocytes with macrophage clumps ($BODIPY^{-}/F4/80^{+}$ cells) in their epididymal WATs (1.9 ± 0.88, $n = 5{,}000$ cells, $P < 0.01$), but these were not found in their mesenteric WATs (data not shown) (Figure 5(a)).

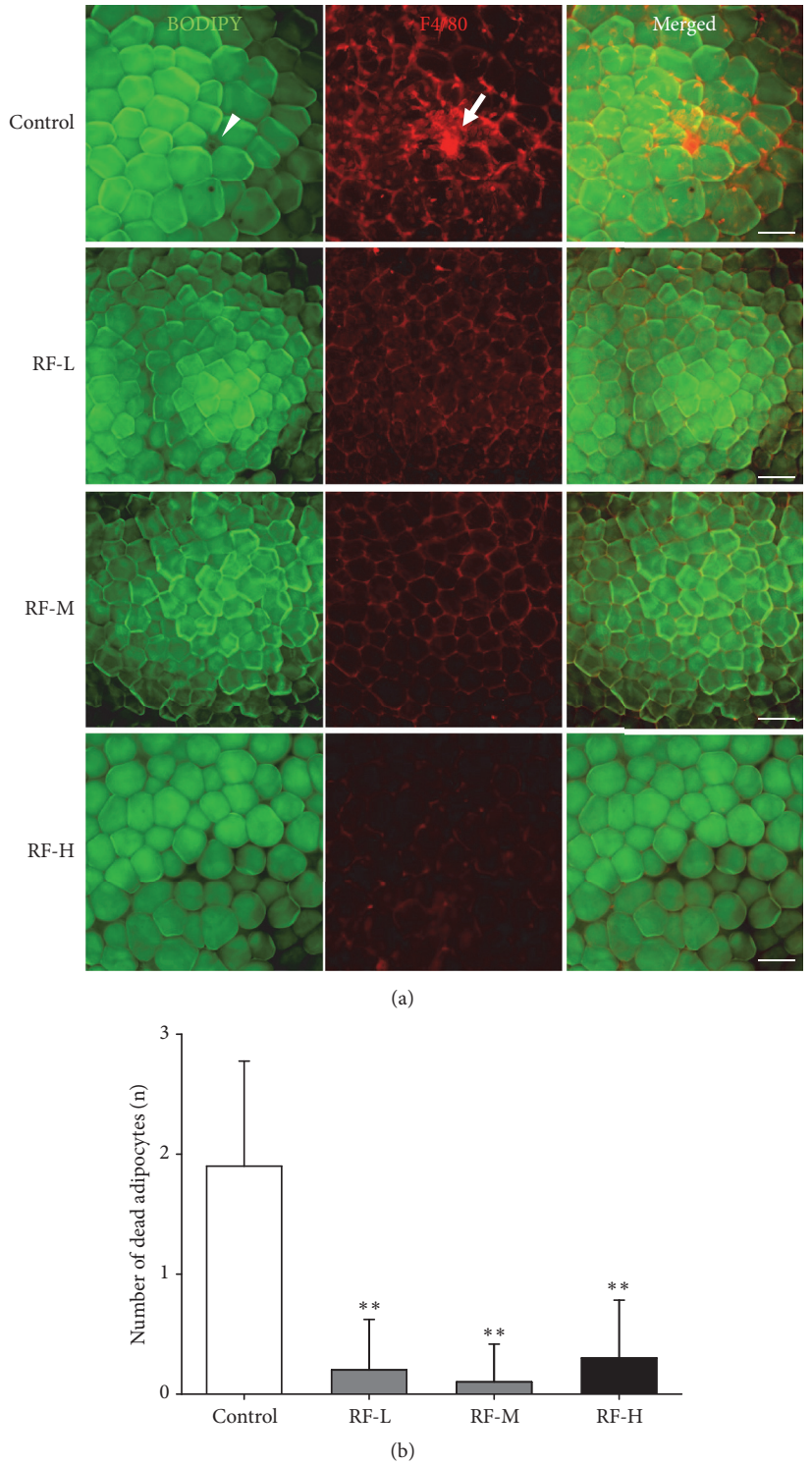

Figure 5: M-RF stimulation suppresses dead adipocyte formation in the WATs of DIO mice. (a) Whole-mounted WATs double-stained with BODIPY (green) and F4/80 (red). Note that all three levels of M-RF stimulation resulted in decreased dead adipocyte formation compared to control. Scale bar, 100 μm. (b) Number of BODIPY$^-$/clustered F4/80$^+$ dead adipocytes counted in 10 randomly selected regions (~100 adipocytes per region) per WAT in control ($n = 10$), RF-L ($n = 10$), RF-M ($n = 10$), and RF-H ($n = 10$). Results are presented as the means ± SD. **, $P < 0.01$ versus control.

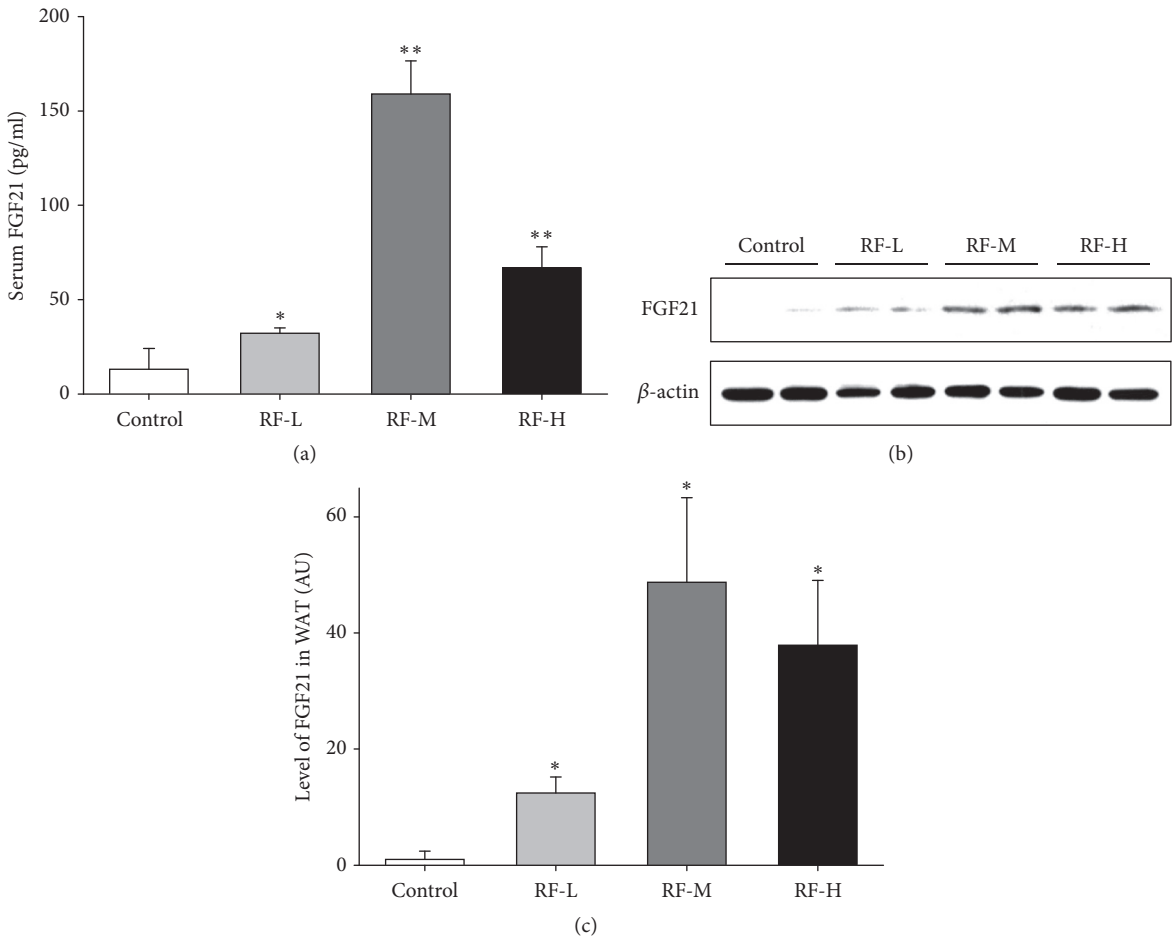

Figure 6: M-RF stimulation increases the level of FGF21 in serum and WAT of DIO mice. (a) ELISA for serum FGF21 in control ($n = 5$), RF-L ($n = 5$), RF-M ($n = 5$), and RF-H ($n = 5$). (b) Representative Western blotting for FGF21 in WATs of DIO mice in control ($n = 5$), RF-L ($n = 5$), RF-M ($n = 5$), and RF-H ($n = 5$). (c) Quantification of (b) and calculated in terms of ratio relative to control. Note that all three levels of M-RF stimulation significantly increased the level of FGF21 in serum and WAT compared to the control. Results are presented as the mean ± SD. ∗, $P < 0.05$ versus control. ∗∗, $P < 0.01$ versus control.

In contrast, we rarely detected $BODIPY^{-}/F4/80^{+}$ dead adipocytes in the epididymal WATs of RF-L (0.20 ± 0.42, $n = 5{,}000$ cells, $P < 0.01$ versus control), RF-M (0.10 ± 0.32, $n = 5{,}000$ cells, $P < 0.01$ versus control), and RF-H (0.30 ± 0.48, $n = 5{,}000$ cells, $P < 0.01$ versus control) groups (Figure 5(b)).

3.6. Thermal Stimulation by M-RF Increases the Expression of FGF21 in Both Serum and WATs of DIO Mice. FGF21 has favorable effects in several metabolic diseases including type 2 diabetes, dyslipidemia, and obesity. We analyzed the level of FGF21 from serum and epididymal WATs from DIO mice and found that M-RF stimulation increased serum FGF21 levels in RF-L (32.15 ± 2.839, $n = 5$, $P < 0.05$ versus control), RF-M (159.0 ± 17.43, $n = 5$, $P < 0.01$ versus control), and RF-H (66.81 ± 11.25, $n = 5$, $P < 0.01$ versus control) groups, whereas the control group showed a significantly lower amount of serum FGF21 (13.14 ± 10.92, $n = 5$) (Figure 6(a)). Similarly, FGF21 protein expression was increased in the epididymal WATs in response to all three levels of M-RF stimulation (RF-L, 12.45 ± 2.730 AU, $n = 5$, $P < 0.05$ versus control; RF-M, 48.78 ± 14.54 AU, $n = 5$, $P < 0.05$ versus control; RF-H, 37.94 ± 11.15 AU, $n = 5$, $P < 0.05$ versus control) (Figures 6(b) and 6(c)).

4. Discussion

We manufactured a moxibustion-simulating heat stimulation device using bipolar RF and showed that our bipolar RF was effective for precise adjustment of depth and temperature of thermal stimulation targeting abdominal WAT, which provides an advantage over monopolar RF devices. Our results demonstrated that thermal stimulation by M-RF treatment induced profound reductions in total body and WAT weight, which were accompanied by adipose tissue browning and elevation of UCP1 and FGF21 expression in DIO mice.

Tissue heating by RF stimulation occurs in response to the induction of ion currents into target tissue by applying an RF wave field between an electrode plate and a ground plate. While the two plates of monopolar RF are located on opposing sides, the two plates of bipolar RF are positioned closely on the same surface. Studies have shown that by

changing the power of an RF generator makes it easy to precisely increase the temperature by depth in bipolar RF compared with monopolar RF [35, 36]. Because of these advantages, bipolar RF is used in various medical fields for tumor treatment and fat reduction [12, 13, 37].

Our findings show that heat stimulation by RF treatment leads to beige adipocyte induction in DIO mice, which is interesting because beige adipocyte induction in WATs is a key feature of cold exposure [25]. Beige adipocyte induction can be promoted by several external stimuli, including β_3-adrenergic receptor agonist, A_{2A} agonist, thiazolidinedione, miRNA 155, and cold exposure [25, 38–40], which exert their effects by activating peroxisome proliferator-activated receptor gamma, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), β_3-adrenergic receptor, and adenosine receptor in WAT and other tissues [25, 38–40]. Specifically, cold exposure is a strong inducer of beige adipocyte induction that functions by increasing mitochondrial biogenesis [25]; however, the detailed mechanism responsible for beige adipocyte induction by heat stimulation has yet to be clearly identified. A previous study indicated that heat stimulation by RF may result in fat reduction and cellulite improvement and that heat stimulation may also cause minor inflammation and promote collagen formation [15]. Another report suggested a unique effect of bipolar RF in enhancing fat metabolism, which may contribute to treating cellulite. Because bipolar RF can penetrate to depths of more than 3 mm in depth, heat stimulation by bipolar RF can alter the surrounding adipose tissue by inducing thermal damage [41]. Thus, it is possible that heat stimulation by the M-RF device that we designed is strong enough to cause browning of WAT and lead to reduced total body weight.

The present findings also show that treatment with RF inhibits the infiltration of macrophages into dead adipocytes in WATs, which is a characteristic inflammatory phenotype in obesity. Obesity can induce chronic inflammation, thereby leading to onset of various diseases such as diabetes, hypertension, and cardiovascular diseases [28]. In obese individuals, phagocytic macrophages can be recruited into WATs from the bone marrow, or resident macrophages in WATs may be converted into phagocytic macrophages [29, 30]. Such changes in the macrophage environment lead to production of inflammatory cytokines such as interleukin-1β, monocyte chemoattractant protein-1, and tumor necrosis factor-α [28]. Therefore, it is possible that the decrease in the number of dead adipocytes and inhibition of macrophage infiltration in WATs are responsible for the weight reduction caused by RF stimulation.

Our results showed that heat stimulation by RF treatment induces profound mitochondrial biogenesis and expression of UCP1 in WATs of DIO mice. Browning of WATs is accompanied by generation of mitochondria and expression of related genes in the WATs [42]. During browning through mitochondrial biogenesis in WATs, activation of PGC-1α, peroxisome proliferator-activated receptor gamma, PR domain containing 16, and other coactivators induce the expression of mitochondria-related genes including UCP1 [23]. UCP1 is mitochondrial carrier protein expressed brown adipocytes that are responsible for generating heat through proton transport via ATP synthesis [43, 44]. Particularly, UCP1 plays crucial roles in the development of BAT and the production of beige adipocytes from white adipocytes, even outside typical BAT locations [23]. Expression of adipocyte-specific UCP1 leads to prevention of obesity by modulating mitochondrial membrane potential [45]. Conversely, deletion of UCP1 induces obesity by abolishing diet-induced thermogenesis in mice kept at thermoneutral temperature, regardless of the type of diet (normal chow, high-fat) [46]. Although the mechanism underlying RF stimulation-induced UCP1 expression and adipocyte browning in WAT remains unclear, our results demonstrated that browning can occur in the WATs of DIO mice upon RF stimulation.

A clinical study of twelve healthy males using a temperature-controlled water bath showed that levels of serum FGF21 and free fatty acids increased after immersing their lower legs in hot water at 39°C, 42°C, or 43°C [47]. Moreover, heat treatment and activation of heat shock proteins have been shown to improve insulin sensitivity of skeletal muscle, as well as to reduce plasma triglyceride and free fatty acids of genetically obese mice in conjunction with a decrease in WAT mass [48]. Traditionally, only cold exposure was associated with formation of beige adipocytes [24, 25, 42]; however, previous reports have shown that heat stimulation can also induce beige adipocyte formation [15, 47], which led us to test the effects of RF heat stimulation in WAT browning. Consistent with the results of a previous report [47], our results demonstrated that heat stimulation by RF treatment can induce elevation of FGF21 in the serum and WATs. FGF21 has diverse metabolic effects that are beneficial for managing obesity, increasing fatty acid oxidation in the liver, and improving insulin sensitivity in obese individuals [49, 50]. Recent studies have shown that elevation of adipose-derived and serum FGF21 levels lead to upregulation of UCP1 transcription in BAT and WAT [51–53]. Moreover, several *in vitro* and *in vivo* studies demonstrated that high concentrations of serum FGF21 cause browning of WATs and are positively correlated with nonshivering thermogenesis. Furthermore, FGF21 increases the expression of cell death activator CIDE-A, carnitine palmitoyltransferase-1 beta, and UCP1 in both brown and white adipocytes in a PGC-1α-dependent manner [51]. FGF21 also plays central roles in activation of the sympathetic nerve system and increases in energy expenditure, which are correlated with weight loss [54]. Moreover, FGF21 has been found to increase CCL11 expression to recruit eosinophils into WAT, where CCL11 leads to infiltration of macrophages and generation of beige adipocytes from adipocyte precursors [55]. Thus, we assume that M-RF treatment in DIO mice induced the expression of FGF21 and UCP1, which in turn influenced adipocyte browning by activation of the sympathetic nerve and changes in the commitment of adipocyte precursors.

5. Conclusions

Building upon the idea of moxibustion, we manufactured an M-RF heat stimulation device and demonstrated its antiobesity effects in DIO mice. Heat stimulation using M-RF effectively suppressed body and WAT weight gain while

increasing the formation of beige adipocytes, mitochondrial content, and higher expression of UCP1 and FGF21 in DIO mice.

Data Availability

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

Conflicts of Interest

The authors declared that no conflicts of interest exist.

Authors' Contributions

Young Jun Koh, Ju-Hee Lee, and Sung Yun Park participated in the study design, conducted the experiments and statistical analysis, and wrote the manuscript. All authors have read and approved the manuscript.

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Review Article

Cinobufacini Injection Improves the Efficacy of Chemotherapy on Advanced Stage Gastric Cancer: A Systemic Review and Meta-Analysis

Xing Zhang,[1,2] Yuan Yuan,[1,2] Yupeng Xi,[1,2] Xinyao Xu,[2] Qiujun Guo,[2] Honggang Zheng,[2] and Baojin Hua[2]

[1]*Graduate School, Beijing University of Chinese Medicine, Beijing 100010, China*
[2]*Department of Oncology, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, Beijing 100010, China*

Correspondence should be addressed to Qiujun Guo; drguoqiujun@126.com, Honggang Zheng; honggangzheng@126.com, and Baojin Hua; dr.huabaojin@hotmail.com

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Gastric cancer has a high morbidity and mortality. Chemotherapy regimens are routine advanced stage gastric cancer (AGC) treatment protocols, but most of these drugs have side-effects such as myelosuppression and gastrointestinal disorders. Cinobufacini, an extractive from TCM, could suppress cell proliferation and inhibit gastric cancer. In this study, we comprehensively reviewed the literature on the efficacy comparison between Cinobufacini injection combined with chemotherapy and chemotherapy solely used in AGC treatment. We extracted data for from six electronic databases to evaluate the efficacy of Cinobufacini injection on AGC patients. Twelve studies with a total of 853 patients were finally included in our study. The results indicated that Cinobufacini injection could increase response rate and disease control rate of chemotherapy on AGC, improve the life quality of AGC patients, increase leukocytes, improve anemia, improve hand-foot syndrome induced by chemotherapy, and relieve cancer pain. This study has its own limitations that prevented us from drawing a definite conclusion and more well-designed clinical trials of TCM are needed.

1. Introduction

Gastric cancer (GC) is one of the most common and lethal cancers worldwide and quite a number of GC patients are initially diagnosed with advanced stage gastric cancer (AGC) including local advanced GC (stage III and unresectable) and metastasis GC (stage IV). Chemotherapy regimens, such as FOLFOXs regimen (oxaliplatin, 5-fluorouracil, and leucovorin calcium), XELOX regimen (oxaliplatin and capecitabine), or other chemotherapeutic drugs, including paclitaxel, cisplatin, epirubicin, and etoposide [1, 2], are common AGC treatment protocols. But most of these drugs have side-effects such as myelosuppression (anemia, low count of leukocytes) and gastrointestinal tract disorders (nausea, vomiting, and diarrhea).

Traditional Chinese medicine (TCM) honors a long history in tumor treatment and it is accepted that TCM can inhibit tumor growth and metastasis, improve antitumor immunity, relieve tumor pains, and reduce side-effects of chemotherapy [3–5]. Combined treatment of TCM and modern medicine is widely used for AGC in China and studies showed TCM had an important potential value for improving the prognosis of patients with AGC [6, 7].

Cinobufacini (also called *Huachansu* in Chinese), extracted from the skins and parotid venom glands of the *Bufo bufo gargarizans Canto*, is a kind of traditional Chinese animal-derived drug used in the treatment of malignant neoplasms in ancient oriental countries. Recent studies showed that Cinobufacini could induce the apoptosis of tumor cells and downregulate protumor inflammatory signaling pathways in the tumor microenvironment [8–11]. Furthermore, researches also indicated that Cinobufacini can inhibit several kinds of human tumors in both clinical treatments and animal xenograft models [12–14].

While Cinobufacini antitumor activity has been proved, the gastrointestinal metabolic pathways of Cinobufacini remain unclear, so intravenous administration (e.g., Cinobufacini injection) is the most common route. Thus, Cinobufacini injection was increasingly used in clinical and basic studies. As there is no systemic review specifically for Cinobufacini injection on AGC treatment, this systematic review and meta-analysis comprehensively evaluated the effects of it according to the PRISMA statement for a high quality [15, 16].

2. Material and Methods

2.1. Literature Search. Studies were explored from databases including PubMed (from Jan. 1975 to Oct. 2017), Cochrane library (from Jan. 2010 to Oct. 2017), Excerpta Medica data BASE (Embase) (from Jan. 1990 to Oct. 2017), China National Knowledge Infrastructure (CNKI) (from Jan. 1979 to Oct. 2017), Weipu database (VIP) (from Jan. 1990 to Oct. 2017), and Wanfang database (WF) (from Jan. 1989 to Oct. 2017). All the studies were searched regardless of their publication types and without language restriction. The search terms were as follows: "Cinobufacini" OR "Cinobufotalin" OR "Huachansu" AND "gastric" OR "stomach". In addition to electronic databases, printed journals and relevant textbooks were manually searched from the libraries of Beijing University of Chinese Medicine, Peking Union Medical College and Guang'anmen Hospital. Specialized experts in particular fields were consulted for necessary supplements as well.

Inclusion criteria include the following: (1) types of studies: randomized clinical trials (RCTs); (2) participants: adult human populations (⩾18 years old) who were pathologically diagnosed as gastric cancer with clinical stages III (unresectable) and IV; (3) interventions: the control group was treated with chemotherapy while the experimental group was treated with the same chemotherapeutics plus Cinobufacini injection; and (4) outcomes: short/long-term chemotherapy response rate, Karnofsky's performance score, chemotherapeutic side-effects such as myelosuppression and gastrointestinal symptoms, and pain management. Exclusion criteria include the following: (1) studies such as reviews, animal researches, observational studies without control group, or other kinds of non-RCT studies; (2) trails about other types of gastrointestinal diseases; (3) participants who had nonpathological diagnosis, previously subjected to chemotherapy, radiotherapy or surgery, concurrent infection, or other malignancies or severe illnesses; and (4) participants in the control group who were treated with other antitumor TCM drugs.

2.2. Literature Selection and Data Extraction. Two independent reviewers (Yuan Y, Qiujun G) evaluated each title, abstract, citation, and selected relevant studies according to the inclusion criteria. Disagreements were discussed with and resolved by the third reviewer (Zizhen Y). Data from included studies were extracted separately by Yupeng X by using a specific form and checked by Xing Z. The characteristics of the data included name of first author, year of publication, gender and number of cases and controls, methods of randomization, interventions, treatment period, and outcomes. The hazard ratio (HR) was calculated from the Kaplan-Meier survival curve and survival outcome events as reported by Tierney [29].

2.3. Quality Assessment of Studies. The methodological quality of each randomized controlled trials (RCTs) was independently assessed by Yuan Y and Qiujun G via the Cochrane Risk of Bi as tool [30]. Disagreements were discussed with and resolved by Baojin H.

2.4. Data Synthesis and Analyses. The statistical analyses were performed using Review Manager (RevMan) 5.3.5 software (Cochrane Community, London, United Kingdom) and STATA 14 software. The total effectiveness rates of dichotomous data were pooled using risk ratios (RRs) with 95% confidence interval (CI). $P < 0.05$ was considered to indicate a statistically significant difference. The heterogeneity of the included studies was evaluated by the χ^2 and I^2 tests, and $P < 0.10$ or $I^2 > 50\%$ was defined as indicating heterogeneity. The fixed-effect models were used in merging homogeneity data and the random-effects models were applied to merge of heterogeneous data. The publication bias was evaluated by visual assessment of the asymmetry of funnel plots (RevMan 5.3.5) and Egger's test (STATA 14) with $p < 0.05$ indicating potential bias. The sensitive analysis was evaluated by reanalyzing the data using different statistical approaches or eliminating a variable which takes the largest proportion.

3. Results

3.1. Included Eligible Studies. 207 studies (including 22 additional records identified through other sources such as postgraduate dissertations and conference articles) were initially searched out by using the search strategy mentioned above, among which 88 duplicated studies were removed, and 75 studies were excluded because they were animal experiments, cell researches, or reviews. After reading the full text, 32 studies were excluded because they lacked control group, had insufficient outcomes conference abstracts, or were about Cinobufacini capsules. Eventually, 12 studies were included in the final research (Figure 1).

3.1.1. Characteristics of Included Studies. Twelve studies with a total of 853 patients were finally included (423 patients in the experiment group and 430 patients in the control group). Characteristics such as sample size, gender, age, interventions, and outcomes of each study were described in Table 1.

3.1.2. Quality Assessment of Included Studies. All of the included studies applied randomization methodology, but six of them did not describe the detailed random method. All of the included studies had complete data but none of them mentioned the details of allocation concealment and blinding of participants and personnel and outcome assessment. One study had high risk of reporting bias for its incompleteness of outcome, so it cannot be entered in the meta-analysis (Figures 2 and 3).

Table 1: Characteristics of the included studies.

Trials	Sample size (E/C)	Gender	Age (yr)	clinical stage	Experimental group (E)	Control group (C)	Period	Outcome measure
Zhu W [17]	32/32	M: 16, F: 16/M: 15, F: 17	32-74 (61.7)/34-72 (62.8)	III: 15, IV: 17/III: 16, IV: 16	Cinobufacini injection 30 ml iv. Qd + C	Xelox regimen	4 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO)
Zou H [18]	30/30	M: 13, F: 17/M: 21, F: 9	59.1/56.5	III, IV	Cinobufacini injection 20 ml iv. Qd + C	EOF regimen	6 weeks	tumor response (RECIST), Kamofsky Score, Side-effects of chemotherapy (WHO)
Zhang C [19]	35/32	M: 28, F: 7/M: 23, F: 9	46-82 (64)/42-79 (66)	III: 15, IV: 20/III: 13, IV: 19	Cinobufacini injection 20ml iv. Qd + C	ELF regimen	8 weeks	tumor response (UICC), Side-effects of chemotherapy (WHO)
Guo C [20]	43/43	M: 62, F: 24	43-74 (55)	IV	Cinobufacini injection 20 ml iv. Qd + C	Docetaxel	9 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO), analgesic effect
Zhang Y [21]	28/29	None	42-71 (57)/35-69 (54)	IV	Cinobufacini injection 50 ml iv. Qd + C	oxaliplatin + floxuridine	9 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO), analgesic effect, 1 year and 2 year survival time
Chen G [22]	62/86	M: 56, F: 30/M: 39, F: 23	65-87 (71.8 ± 18.6)/64-89 (73.1 ± 22.3)	IV	Cinobufacini injection 10 ml iv. Tid + C	Capecitabine	6 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO), overall survival time
Xu D [23]	30/30	M: 20, F: 10/M: 21, F: 9	66.3 ± 4.6/65.0 ± 3.9	IV	Cinobufacini injection 20 ml iv. Qd + C	Capecitabine	6 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO), analgesic effect
Zhang Z [24]	30/30	None	35-79 (53)/33-75 (56)	IV	Cinobufacini injection 20ml iv. Qd + C	Hydroxycamptothecin	6 weeks	tumor response (WHO), Kamofsky Score, Side-effects of chemotherapy (WHO), analgesic effect
Lu C [25]	31/31	M: 34 F: 28	37-71 (54 ± 17)	III	Cinobufacini injection 20 ml iv. Qd + C	FOLFOX4 regimen	9 weeks	tumor response (WHO), immune regulation
Wang Y [26]	36/32	M: 48 F: 20	40-72 (54)	IV	Cinobufacini injection 20 ml iv. Qd + C	FOLFOX4 regimen	8 weeks	tumor response (WHO), Side-effects of chemotherapy (WHO),

Table 1: Continued.

Trials	Sample size (E/C)	Gender	Age (yr)	clinical stage	Experimental group (E)	Control group (C)	Period	Outcome measure
Ren L [27]	32/22	Unclear	40-68 (53)	IV	Cinobufacini injection 20 ml iv. Qd + C	FOLFOX regimen	6 weeks	tumor response (WHO), Side-effects of chemotherapy (WHO), Kamofsky Score,
Chen H [28]	34/33	M: 20, F: 14/M:20, F: 13	50.6/40.9	III: 23, IV: 11/III: 24, IV: 9	Cinobufacini injection 30 ml iv. Qd + C	TPF regimen	6 weeks	tumor response (WHO), Side-effects of chemotherapy (WHO)

ELF regimen: oxaliplatin + epirubicin + floxuridine; ELF regimen: etoposide + cisplatin+ floxuridine; Xelox regimen: oxaliplatin + capecitabine; FOLFOX4 regimen: oxaliplatin + floxuridine + leucovorin; and TPF regimen: paclitaxel + cisplatin +floxuridine.

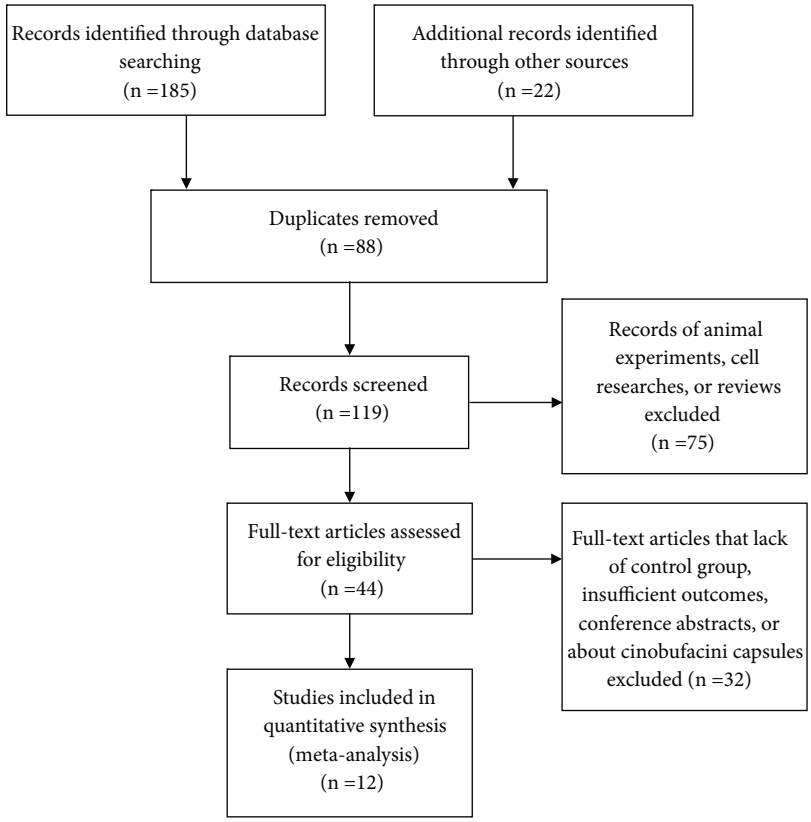

Figure 1: Flow diagram of the literature search process.

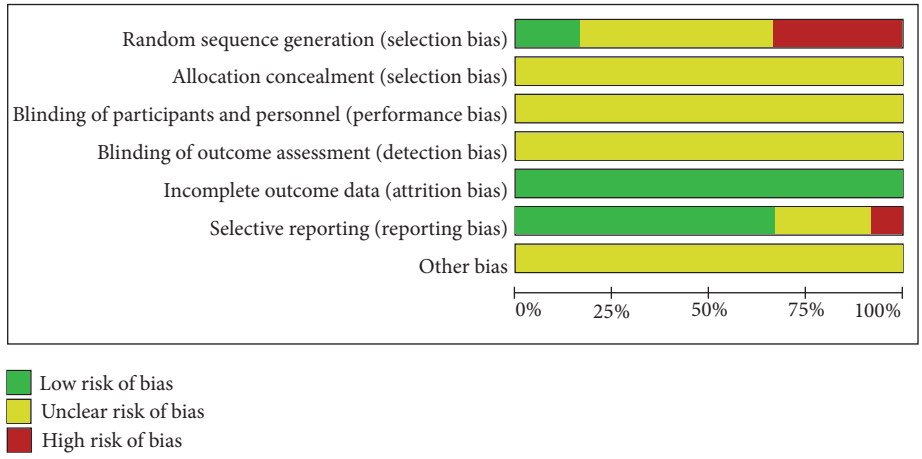

Figure 2: Risk of bias graph.

3.2. Meta-Analysis of Cinobufacini Injection on AGC Treatment

Cinobufacini Injection Could Enhance Response Rate (RR) of Chemotherapy on AGC. All of the twelve studies evaluated the RR. The RR in the experiment group (Cinobufacini injection combined with chemotherapy) was significantly higher than that in the control group (chemotherapy only), with the risk ratio = 1.28, 95% CI: 1.10-1.48, P = 0.001 in the Z test. The result did not indicate the heterogeneity with the $Chi^2 = 3.25$, df = 11, P = 0.99, $I^2 = 0\%$ (Figure 4).

Cinobufacini Injection Could Enhance Disease Control Rate (DCR) of Chemotherapy on AGC. Eleven studies evaluated the DCR which in the experiment group was significantly higher than that in the control group, with the risk ratio = 1.12, 95%

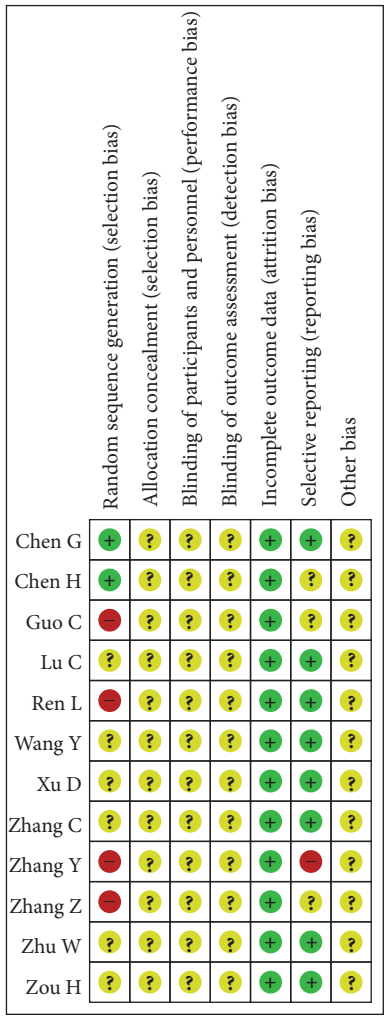

Figure 3: Risk of bias summary.

CI: 1.04-1.20, P = 0.003 in the Z test. The result did not indicate the heterogeneity with the Chi^2 = 11.02, df = 10, P = 0.36, I^2 = 9% (Figure 5).

Cinobufacini Injection Could Not Prolong the Overall Survival Time (OS) of AGC Patients. Two studies evaluated the OS of AGC patients. We pooled the hazard ratios (HRs) of OS and the result showed that pooled HR = 0.94, with 95% CI: 0.75-1.18, P = 0.59 in the Z test. The result did not indicate the heterogeneity with the Chi^2 = 0.20, df = 1, P = 0.65, I^2 = 0% (Figure 6).

Cinobufacini Injection Could Improve the Life Quality of AGC Patients. KPS is a recognized method for evaluating the quality of life, scoring integer 100 to 0 degressively with the decreased quality of life. Six studies included the KPS evaluation. Cinobufacini injection could improve KPS (KPS enhancement ≥ 10) when combined with chemotherapy, with the risk ratio = 1.83, 95% CI: 1.40-2.39, P < 0.00001 in the Z test. The result did not indicate the heterogeneity with the Chi^2 = 4.61, df = 5, P = 0.46, I^2 = 0% (Table 2).

Cinobufacini Injection Could Reduce the Declination of Leucocyte Count but Could Not Inhibit the Severe Declination (III-IV Degrees). Six studies evaluated the low count of leukocytes of AGC patients. As the result showed Chi^2 = 10.08, df = 5, P = 0.07, I^2 = 50% which indicated possible heterogeneity. The P values of Z test between experimental group and control group were 0.04 (random-effect model). These results indicated Cinobufacini injection could improve the situation of the low count of leukocytes due to the chemotherapy (Table 2). Four studies evaluated the severe situation of low count of leukocytes and the results showed that Cinobufacini injection could not inhibit the III-IV-degree declination of leukocytes count, with the risk ratio = 0.61, 95% CI: 0.33-1.14, P = 0.12 in the Z test. The result did not indicate the heterogeneity with the Chi^2 = 3.77, df = 3, P = 0.29, I^2 = 20% (Sup 2, Fig 3).

Cinobufacini Injection Could Reduce the Morbidity of (Severe) Nausea and Vomiting Caused by Chemotherapy. Five studies evaluated the incidence of nausea and vomiting between the two groups and the results showed a significant difference with the risk ratio = 0.68, 95% CI: 0.53-0.86, P = 0.001 in the Z test. The results did not indicate the heterogeneity with the Chi^2 = 7.52, df = 4, P = 0.11, I^2 = 47% (Table 2). The similar results were seen in four studies that involved Grades III-IV of nausea and vomiting, with the risk ratio = 0.34, 95% CI: 0.14-0.82, P = 0.02 in the Z test. The results did not indicate the heterogeneity with the Chi^2 = 3.11, df = 3, P = 0.37, I^2 = 4% (Sup 2, Fig 5).

Cinobufacini Injection Could Alleviate Hand-Foot Syndrome (HFS) Induced by Chemotherapy. Some chemotherapeutic drugs such as novel-fluorouracil derivatives could induce HFS sluggish feelings and red or black spots on hands and feet. Three studies evaluated number of HFS cases and the results showed Cinobufacini injection could reduce the morbidity of HFS. The result showed a significant difference with the risk ratio = 0.55, 95% CI: 0.33-0.91, P = 0.02 in the Z test. The results did not indicate heterogeneity with the Chi^2 = 1.48, df = 2, P = 0.48, I^2 = 0% (Table 2).

Cinobufacini Injection Could Relieve Tumor Pain. Two studies were conducted to evaluate the effectiveness of Cinobufacini injection in managing cancer pain. The result indicated that Cinobufacini injection significantly relieves pain with the risk ratio = 0.1.81, 95% CI: 1.30-2.54, P = 0.0.0005 in the Z test. The result did not indicate heterogeneity with the Chi^2 = 0.12, df = 1, P = 0.73, I^2 = 0% (Table 2).

Cinobufacini injection could not reduce the incidence of anemia, diarrhea, peripheral neurotoxicity, and oral mucositis caused by chemotherapy (Sup 2, Fig 8-11).

Three studies were conducted to compare the incidence of anemia between experimental and control groups. There were no significant differences in the incidence of anemia between two groups, with the risk ratio = 0.79, 95% CI: 0.58-1.08, P = 0.14 in the Z test. The results did not indicate heterogeneity with the Chi^2 = 0.37, df = 2, P = 0.83, I^2 = 0%.

Cinobufacini injection could not reduce the morbidity of diarrhea induced by chemotherapy. There was no significant

Table 2: Meta-analysis of KPS, side-effects and tumor-related pain.

Meta-analysis	No. of study	Risk Ratio [%95 CI]				heterogeneity	
		Fix-model	P value	Random-model	P value	I^2(%)	P value
KPS	6	1.83 [1.40, 2.39]	P < 0.00001	1.76 [1.35, 2.29]	P < 0.0001	0	0.46
leukocytopenia	6	0.78 [0.65, 0.93]	P = 0.007	0.76 [0.58, 0.99]	P = 0.04	50	0.07
Grades III-IV leukocytopenia	4	0.61 [0.33, 1.14]	P = 0.12	0.58 [0.23, 1.46]	P = 0.25	20	0.29
nausea and vomiting	5	0.68 [0.53, 0.86]	P = 0.001	0.68 [0.48, 0.96]	P = 0.03	47	0.11
Grades III-IV nausea and vomiting	4	0.34 [0.14, 0.82]	P = 0.02	0.44 [0.17, 1.13]	P = 0.09	4	0.37
hand-foot syndrome	3	0.55 [0.33, 0.91]	P = 0.02	0.54 [0.32, 0.91]	P = 0.02	0	0.48
tumor-related pain	2	1.81 [1.30, 2.54]	P = 0.0005	1.83 [1.31, 2.55]	P = 0.0004	0	0.73
anemia	3	0.79 [0.58, 1.08]	P = 0.14	0.80 [0.59, 1.09]	P = 0.15	0	0.83
diarrhea	5	0.77 [0.52, 1.15]	P = 0.21	0.76 [0.51, 1.14]	P = 0.19	0	0.8
peripheral neurotoxicity	3	0.64 [0.52, 0.80]	P < 0.0001	0.57 [0.23, 1.43]	P = 0.23	91	<0.00001
oral mucositis	2	0.46 [0.25, 0.83]	P = 0.01	0.37 [0.04, 3.47]	P = 0.39	88	0.004

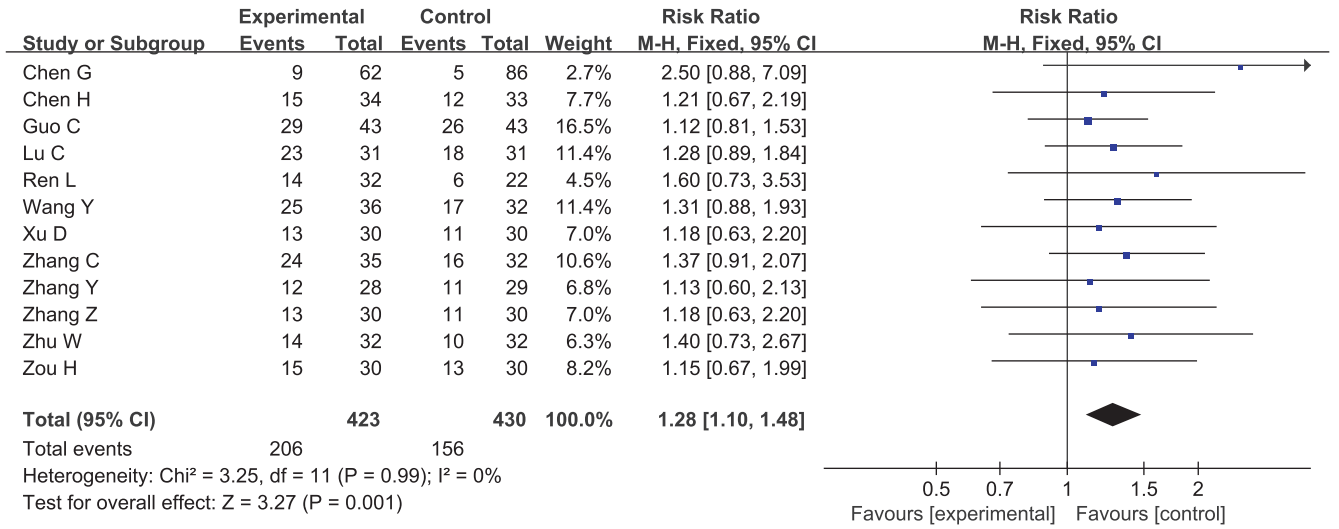

Figure 4: **Forest plot of RR (risk ratio) for evaluation of response rate in fixed-effect model.** The RR of chemotherapy response rate in Cinobufacini injection and chemotherapy group was compared with the chemotherapy group. Individual study is shown in the square with blue color, and the pooled datasets were shown in the diamond, representing the 95% confidence interval (CI) of each study. RR > 1 implied a better chemotherapy response rate of the experimental group. The size of each investigation represented the weighting factor (1/SE) assigned to the study.

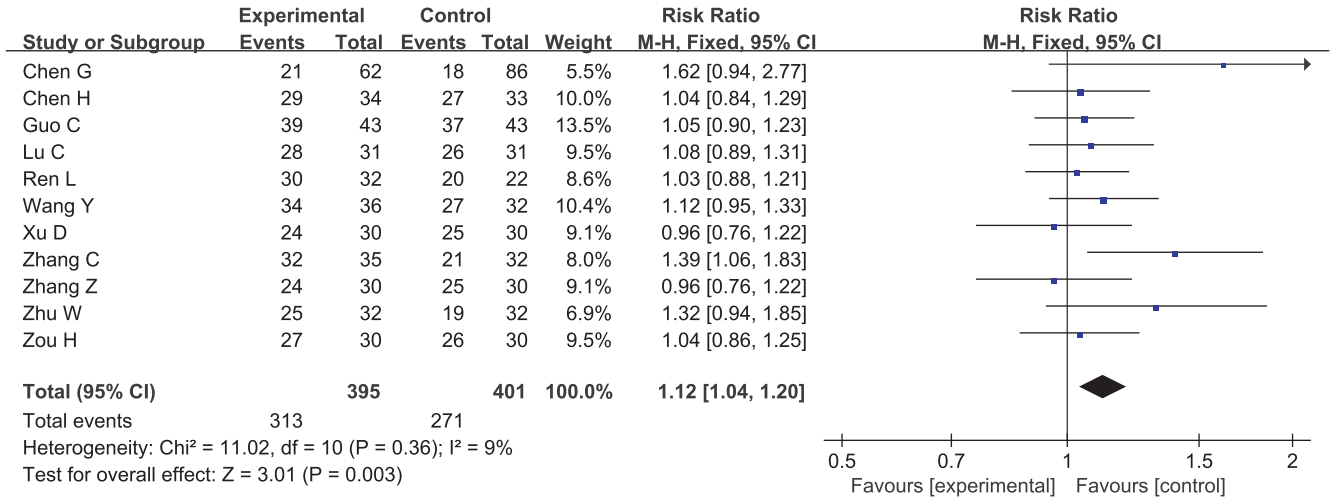

Figure 5: **Forest plot of RR for evaluation of disease control rate in fixed-effect model.** The RR of disease control rate in Cinobufacini injection and chemotherapy group was compared with the chemotherapy group. Individual study is shown in the square with blue color, and the pooled datasets were shown in the diamond, representing the 95% confidence interval (CI) of each study. RR > 1 implied a better disease control rate of the experimental group. The size of each investigation represented the weighting factor (1/SE) assigned to the study.

difference between the two groups, with the risk ratio = 0.77, 95% CI: 0.52-1.15, P = 0.21 in the Z test. The results did not indicate the heterogeneity with the $Chi^2 = 1.65$, df = 4, P = 0.80, $I^2 = 0\%$. The similar results were shown in four studies that involved the incidence of III-IV degree diarrhea with the risk ratio = 0.33, 95% CI: 0.08-1.38, P = 0.13 in the Z test. The results did not indicate heterogeneity with the $Chi^2 = 0.18$, df = 2, P = 0.91, $I^2 = 0\%$.

Cinobufacini injection could not reduce the incidence of peripheral neurotoxicity and oral mucositis. Three studies and two studies evaluated the recurrence of peripheral neurotoxicity and oral mucositis accordingly. There were no significant differences between experimental group and control group in the incidence of peripheral neurotoxicity and oral mucositis with the P = 0.23 and 0.39 accordingly. Significant heterogeneities were detected with P < 0.0.01 and $I^2 = 91\%$ and 88% accordingly.

3.3. Sensitivity Analysis. We conducted the sensitivity analysis to strengthen the reliability of the results of response rate

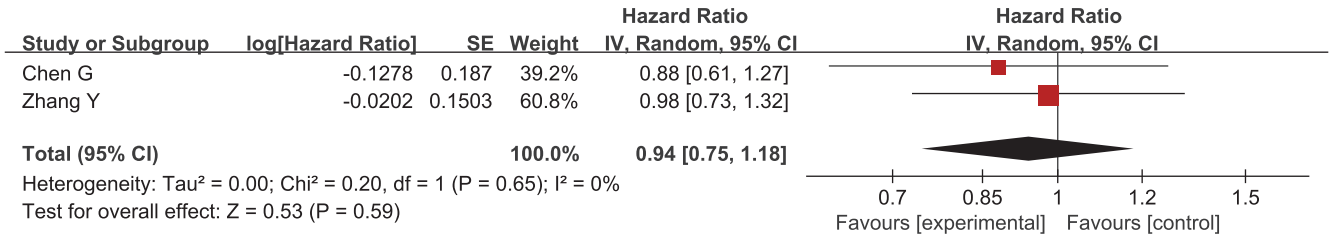

Figure 6: **Forest plot of HR (hazard ratio) for evaluation of overall survival in fixed-effect model.** The HR of overall survival in Cinobufacini injection and chemotherapy group was compared with the chemotherapy group. Individual studies are shown in the red-colored squares, and the pooled datasets are shown by the diamond, representing the 95% confidence interval (CI) of each study. HR < 1 implied improved overall survival in the experimental group. The size of each investigation represented the weighting factor (1/SE) assigned to the study.

Table 3: Egger's test.

Meta-analysis of publication bias	P value
response rate	0.114
disease control rate	0.004
KPS	0.250
leukocytopenia	0.224
nausea and vomiting	0.177
diarrhea	0.026

and disease control rate. The sensitivity analysis showed the same effect sizes among a fixed-effect model and a random-effect model of the response rate analysis, disease control rate analysis, and hazard ratio analysis. The same effects were shown in other outcome measures in the sensitive analysis except in the analysis of Grades III-IV nausea and vomiting, peripheral neuropathy, and oral mucositis (Table 2). By taking into consideration the heterogeneity, we adopted the corresponding result when there were inconsistent results in sensitivity analysis.

3.4. Publication Bias. The funnel plots (Figure 7) were not strictly symmetrical in the meta-analysis of response rate, disease control rate, KPS, and diarrhea. But Egger's test (Table 3) showed that there was no significant publication bias among the studies except the meta-analysis of disease control rate (P = 0.004) and diarrhea (P = 0.026).

4. Discussion

Gastric cancer has a high morbidity around the world. The comprehensive treatment including surgery, chemotherapy, radiotherapy, targeted therapy, support treatment, and treatment of TCM is the optimal treatment for gastric tumor. Chemotherapy is one of the most important treatments for advanced gastric cancer (AGC), but the response rate is far from satisfactory so far. The combination of TCM and modern medical treatments has been proved effective on AGC. For instance, a research showed that TCM herbal formula of invigorating spleen could prolong the median overall survival time and improve the prognosis of patients with AGC [7]. On fundamental research, *A. cucullata*, an extractive from TCM herb *Alocasia cucullata (Lour.) G. Don*, was reported to have a potent antigastric cancer activity both in vitro and in vivo via antiproliferation of G0/G1 arrest and cell proapoptosis, including PI3K/Akt pathway, ERK activity, stimulated cytochrome C release, and caspase 3/7 activity accompanied with an increase of Bax/Bcl-2 ratio [31]. As a kind of TCM extractive, Cinobufacini could suppress the cell proliferation of BGC-823 human gastric cancer cells via targeting BAG-1 (an antiapoptosis gene) and inhibit tumor growth and metastasis in xenograft models [8, 14, 32]. These may partially explain the mechanisms of how TCM and Cinobufacini injection inhibit gastric cancer. Some researchers started to work on the antitumor components of Cinobufacin injection, and Bufadienolides might be one of the antitumor agents in treating gastric cancer [33]. Further studies are needed to clarify how Cinobufacini injection could benefit cancer patients.

In this review, we comprehensively reviewed the literature on the efficacy comparison between Cinobufacini injection combined with chemotherapy and chemotherapy solely used in AGC treatment. Our results indicated that Cinobufacini injection could enhance the response rate and disease control rate of chemotherapy, which meant the experiment group had a better short-term efficacy than that in the control group. However, due to insufficient data, only two of our included studies included overall survival time, and our results showed that Cinobufacini injection could not prolong the overall survival time. High life quality is also important for tumor patients' living and recovery. Our study showed that Cinobufacini injection improved the life quality of AGC patients receiving chemotherapy by enhancing their KPS.

Side-effects such as myelosuppression and gastrointestinal toxicity constantly occur in tumor patients undergoing chemotherapy, which cause them great trouble. TCM plays an important role in alleviating side-effects when used in combination with chemotherapy. For instance, a double-blind clinical trial showed that the standardized ginger extract (the extract from a kind of traditional medicine in Asian countries to treat nausea and vomiting) acted as an antiemetic against chemotherapy-induced nausea and vomiting [34]. A meta-analysis based on eight trails indicated that Chinese herb medicine significantly protected peripheral blood WBCs from decreasing during the course of chemotherapy or

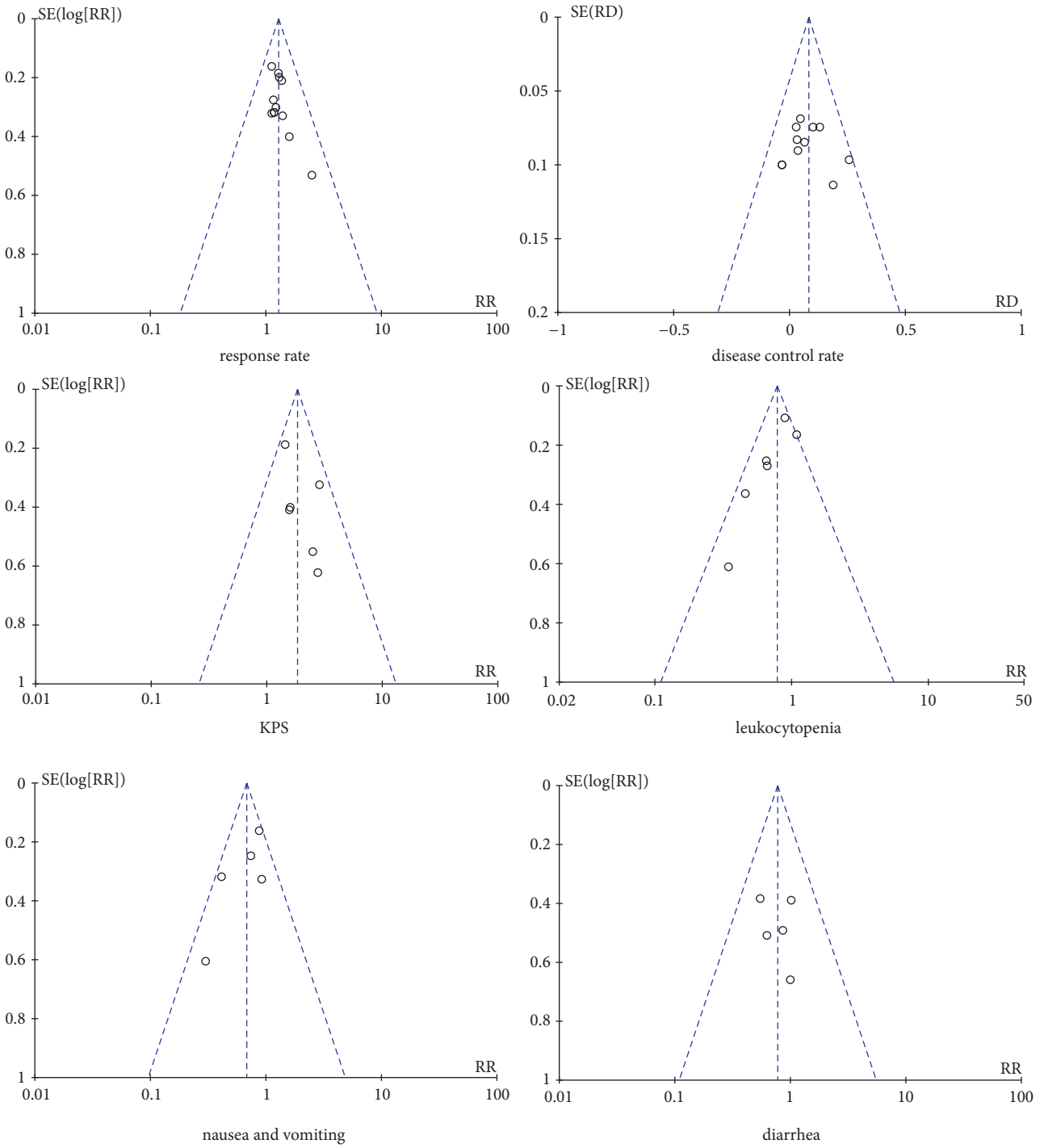

Figure 7: Funnel plots of response rate, disease control rate, KPS, leukocytopenia, and nausea, vomiting, and diarrhea.

radiotherapy [35]. Astragalus membranaceus was also proved to have a myelo-protective and myelo-therapeutic capacity against the chemotherapy-induced myelosuppression, evidenced at both laboratory and morphological levels in basic study [36]. Our results indicated that Cinobufacini injection could inhibit the declination of leukocytes in peripheral blood and allay nausea and vomiting caused by chemotherapy, but it could not prevent myelosuppression or gastrointestinal toxicity which commonly present as anemia or diarrhea. Most tumor patients suffer from cancer pain, which even painkillers cannot cure. Some Chinese herbal injectionsare proved to improve clinical efficacy and relieve adverse reactions when combined with the FOLFOX regimen in treating gastric cancer [37]. Chinese medicine such as Fufang Kushen injection could reduce cancer pain directly by blocking TRPV1 signaling pathway [38]. Cinobufacini

injection could help to relieve cancer pain as well based on our evaluation, but the exact mechanisms of these effects remain unclear.

However, this study has its own limitations. First, allocation concealment and blinding of all the included studies were unclear and there was publication bias in some evaluations since the included studies were all published in Chinese. Second, we failed to evaluate the long-term effects, because the treatment periods of included studies were generally short and they did not include long-term follow-ups. Thus, the long-term effects of Cinobufacini injection on AGC patients remain unclear. Third, the criteria for the evaluation of tumor response varied from one study to another, which might bring different results in subgroup analysis in RR and DCR evaluation. Taking into consideration all the above reasons, the evidence for this study might be insufficient. Although the above questions might exist that prevent us from drawing a definite conclusion about Cinobufacini injection, our study still provided helpful information for clinical practice that Cinobufacini injection could enhance the efficacy of other treatments in AGC patients, reduce the side-effects induced by chemotherapy, and help to relieve cancer pain, which might be helpful for clinical medication. However, in order to draw precise conclusion, more well-designed clinical trials with long-term follow-ups of Cinobufacini injection are needed for future study.

Data Availability

All the data are included in this article and its supplementary information files.

Conflicts of Interest

The authors disclose no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplementary File: forest plots of KPS, side-effects, and tumor-related pain (DOC). Supplement 1: forest plots of response rate, overall survival, and disease control rate (DOC). Supplement 2: sensitivity analysis (DOC). Supplement 3: publication bias of the meta-analysis (DOC). *(Supplementary Materials)*

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Research Article

Difference between Right and Left Facial Surface Electromyography in Healthy People

Bo-Hyun Kim,[1] Kyeong Han Kim,[2] Lak-Hyung Kim,[3] Jong-Uk Kim,[1] and Tae-Han Yook[1]

[1]*Department of Acupuncture & Moxibustion Medicine, College of Korean Medicine, Woosuk University, Republic of Korea*
[2]*Department of Preventive Medicine, College of Korean Medicine, Woosuk University, Republic of Korea*
[3]*Department of Neuropsychiatry, College of Korean Medicine, Woosuk University, Republic of Korea*

Correspondence should be addressed to Tae-Han Yook; nasiss@naver.com

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Introduction. The study was to see whether there were differences in values of facial surface electromyography in subjects of good heath by muscles, age, and sex. *Methods*. It draws ratio between lower value and higher value (R-LV/HV) and asymmetry index (AI), based on root mean square (RMS) from measurement of facial surface electromyography (sEMG) in 154 people of healthy people (male:female = 70:84) aging between more than 20 and less than 70. *Results*. For R-LV/HV, it averages 81.70±14.60% on frontalis muscle, 73.74±19.12% on zygomaticus muscle, and 79.72±14.77% on orbicularis oris muscle. With analysis of the AI average was 10.87±10.14% on frontalis muscle, 16.71±14.79% on zygomaticus muscle, and 12.10±10.05% on orbicularis oris muscle. Both values were statistically significant in three parts of muscles as shown. Both of R-LV/HV and AI show no statistically significant difference on age and sex ($p>0.05$). *Conclusions*. It could provide basic data for the future diagnosis of facial nerve palsy patients by measuring facial sEMG values for healthy people.

1. Introduction

Facial paralysis has a major symptom of atonia paralysis on the facial muscle and was a disease with accompanying symptoms, such as decreased taste, hearing impairment, saliva secretion, and tear reduction [1]. Various hypotheses for causes of facial paralysis include viral infection, ischemic vascular disease-causing paralysis, vascular disorders due to diabetes, multiple neuritis, autoimmune disease, and cold exposure, but no hypothesis yet provides a clear explanation as to what causes facial paralysis [2]. It has been reported that 20-30 persons every 100,000 in population annually experience facial paralysis [3]. As of 2015, there were 8,511 cases of facial paralysis reported in Korea [4].

Diagnostic techniques for patients with facial paralysis include House-Brackmann scale, Yanagihara grading system, and Sunnybrook facial grading system, which were performed on naked eyes [5, 6], and digital infrared thermal imaging, or DITI, electroneurography (ENoG), nerve excitability test (NET) or electromyography (EMG), and surface electromyography (sEMG) were utilized as diagnostic instruments [7].

Among other things sEMG was a kind of EMG diagnostic instrument that quantitatively measures electric signals for muscle movements. In general, EMG was measuring the electromyogram of a single muscle by inserting a needle, while sEMG was a noninvasive mechanism using surficial electrodes and was advantageous in conducting the overall assessment of facial movements, not just movements of a single muscle. Because of its strength, sEMG has been widely used as an assessing instrument of facial paralysis and was an index that has significance in determining facial states and degree of recovery with ratio between lower value and higher value(R-LV/HV), or asymmetry index (AI) [8].

Although there have been many studies on sEMG of patents with facial paralysis, less studies have been reported on whether the indexes were different by sex, age, or parts of the body. In some study [9], there was a difference in facial

sEMG values according to sex, but there was a limit of 40 small subjects. This study was to measure facial surface sEMG in subjects of good health aging between 20s and 60s to see any difference in the values depending on sex, age, or parts of the body.

2. Methods

2.1. Participants. The study mobilizes people aging older than 20 and less than 70 between September 13, 2016, and March 1, 2017. Prospective participants in the study were asked if they have the existing disease or have been administered with related medications through the basic examination and preliminary medical examination. Participants who were not subject to exclusion were selected as the final subjects. A total of 154 people wish to participate in the study and no subjects fall within exclusion. The final subjects include 154 people, 70 males and 84 females. Exclusion criteria are as follows.

(1) Persons with anamnesis of stroke

(2) Persons currently suffering from diseases associated with facial paralysis or with anamnesis of such diseases

(3) Persons with facial disease or body disease that could affect other facial electromyography

(4) Persons who have been given medications or experienced activity within one week that will affect measurement of sEMG

(5) Persons having discomfort with facial muscle movements due to plastic surgery or facial operation

(6) Persons who may have a displacement because they continuously use facial muscles in occupation (such as performer of brass instrument)

(7) Persons with facial asymmetry of Grade 2 on House-Brackmann scale through naked eye assessment

(8) Other cases of exclusion a researcher would determine inadequate

2.2. Study Implementation

2.2.1. Medical Device. For sEMG implementation, a four-channel adopting electromyography system QEMG-4 XL (manufactured by Laxtha Co. Ltd., Korea) was used, while QEMG-4 XL (version1.0 Neuromedi Inc.) was used for measurement. For electrode sensor, AM530 active electromyography system (manufactured by Laxtha Co. Ltd., Korea) was used.

2.2.2. R-LV/HV. A higher value from the left and right measurements in the total of 154 subjects was set on numerator and a lower value regarding as denominator. The formula is as follows.

$$\text{Ratio (\%)} = \frac{\text{EMG (low value side)}}{\text{EMG (High Value side)}} \times 100 \quad (1)$$

2.2.3. AI. AI is difference in the values divided by the sum of the values. In the study, the difference value obtained after deducting R-LV/HV, among root mean square (RMS) on the left and right measurements, was then divided by the sum of RMS values on the left and right parts to obtain AI. Higher AI means significant difference in RMS values on the left and right muscles. The formula is as follows.

$$\text{Asymmetry Index (\%)} = \frac{\text{EMG (high value side)} - \text{EMG (low value side)}}{\text{EMG (high value side)} - \text{EMG (low value side)}} \times 100 \quad (2)$$

2.3. Measurement. The placement of electrode was made in parallel to muscular fibers of frontalis muscle, zygomaticus muscle, and orbicularis oris muscle. A first electrode was placed on the left, while a second electrode was place on the right. To eliminate factors reducing any skin resistance to the sEMG measuring signals, the measurement site was cleaned with medical alcohol cotton and its surfaces were to be completely dried before the electrode was placed. The real measurement was conducted when a subject fully learned how to move after preliminary measurement. The measurement of sEMG uses signal processing of root mean square (RMS). A relaxation time for one-time electromyography signal measurement was set on five seconds and tension time on three seconds. Gain index was between the ranges of -1463 and 1463. Each test was repeated with three measurements and the average measurement of the three measurements was used as a measurement value. Subjects were required to take a 10-minute rest and then return to the measurement of sEMG by having a disposable electrode placed on acupuncture points of frontalis muscle, zygomaticus muscle, and orbicularis oris muscle after being introduced how to move muscles at each acupuncture point.

2.3.1. Frontalis Muscle (Yangbaek (GB14)). The acupuncture point of *Yangbaek* (GB14) is located directly above pupil by a finger joint from the eyebrow [8]. For movements of frontalis muscle and *Yangbaek* (GB14), a subject is required to move the eyebrows to form wrinkles on his forehead (Figure 1).

2.3.2. Zygomaticus Muscle (Gwonyo (SI18)). The acupuncture point of *Gwonyo* (SI18) is located sunken at ends of Yegol below Myeonpigol [8]. For movements of zygomaticus muscle and *Gwonyo* (SI18), a subject is required to pull angular upward and outside (Figure 1).

2.3.3. Orbicularis Oris Muscle (Seungjang (CV24)). The acupuncture point of *Seungjang* (CV24) is located sunken at edges of the lips [8]. Orbicularis oris muscle is located by a finger joint from both sides of Seungjang (CV24). For movements of orbicularis oris muscle, a subject is required to pucker lips forward to hold out (Figure 1).

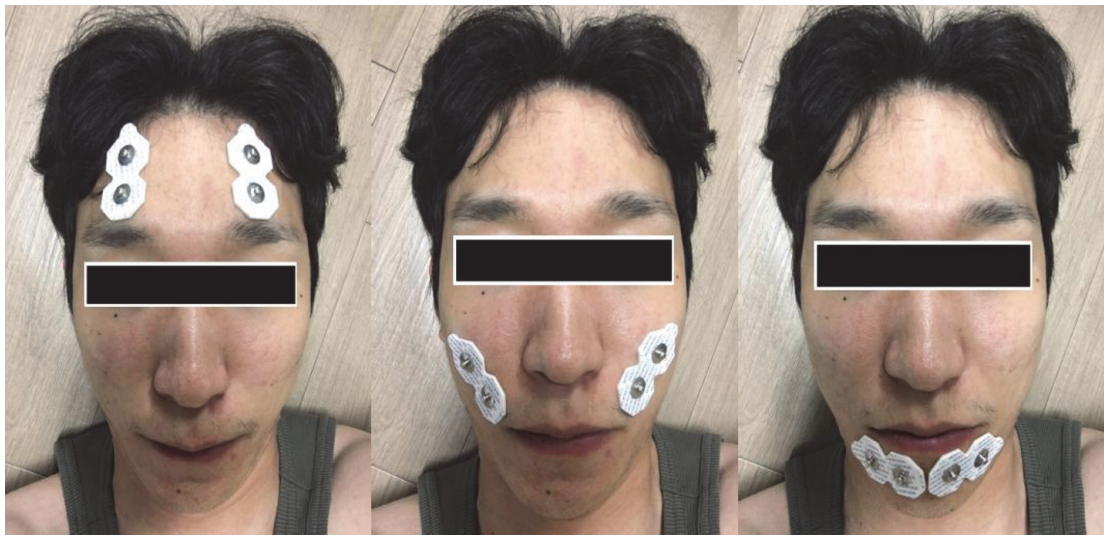

Figure 1: Attachment site of frontalis muscle, zygomaticus muscle, and orbicularis oris muscle.

2.4. Statistical Analysis. For statistics of research results, SPSS Statistics 22.0 version 64 bit edition (IBM, USA) was used and all the measurements were indicated in mean±SD. For sex comparison, paired t-test was conducted and one-way analysis of variance (ANOVA) followed by a post hoc Scheffe test was used to compare muscle and age. When p-value was less than 0.05, this was interpreted to have a statistically significance, and all the values were rounded up from the third decimal place.

2.5. Ethics. The study was approved by Institutional Review Board, IRB of Jeonju Oriental Hospital in affiliation with Woosuk University (No. WSOH IRB 1610-06).

3. Results

3.1. Sociodemographic Characteristics. The gathered group of males and female were in their 20s and 60s. For age distribution of the people gathered, 31 people were in the range of between older than 20 and younger than 30, 29 people were in the range of between older than 30 and younger than 40, 37 people were in the range of between older than 40 and younger than 50, 29 people were in the range of between older than 50 and younger than 60 and 28 people were in the range of between older than 60 and younger than 70. The average height of male was 171.56±5.29cm and average weight was 72.34±8.74kg. The average height of female was 160.25±5.22cm and average weight was 58.17±8.80 (Table 1).

3.2. Difference in R-LV/HV and AI between Muscles. The total of 154 subjects on the three parts have R-LV/HV and AI were measured (Table 2). For R-LV/HV, it averages on 81.70±14.60% on frontalis muscle, 73.74±19.12% on zygomaticus muscle, and 79.72±14.77% on orbicularis oris muscle. When they were compared using one-way ANOVA, R-LV/HV on each part shows statistically significant difference ($p<0.001$). Frontalis muscle value was higher than zygomaticus muscle value and orbicularis oris muscle value was higher than zygomaticus muscle value. However, it was no significant difference between frontalis muscle value and orbicularis oris muscle value.

For AI, it averages on 10.87±10.14% on frontalis muscle, 16.71±14.79% on zygomaticus muscle, and 12.10±10.05% on orbicularis oris muscle (Table 2). When they were compared using one-way ANOVA, the asymmetry index on each part shows statistically significant difference ($p<0.001$). Frontalis muscle value was lower than zygomaticus muscle value and orbicularis oris muscle value was lower than zygomaticus muscle value. However, there was no significant difference between frontalis muscle value and orbicularis oris muscle value.

3.3. Difference in R-LV/HV and AI between Ages. For R-LV/HV, it has its highest of 84.05±15.54% in 50s on frontalis muscle and its lowest of 78.75±19.01% in 30s. It has its highest of 79.76±17.87% in 30s on zygomaticus muscle and its lowest of 69.13±18.03% in 60s. It has its highest of 81.26±12.63% in 30s on orbicular oris muscle and its lowest of 76.52±16.33% in 20s. There was no significant difference between age in R-LV/HV ($p>0.05$) (Table 3).

For AI, it has its highest of 13.22±13.11% in 30s on frontalis muscle and its lowest of 9.17±7.64% in 20s. It has its highest of 19.88±16.14% in 60s on zygomaticus muscle and its lowest of 12.45±12.57% in 30s. It has its highest of 14.29±11.20% in 20s on orbicular oris muscle and its lowest of 10.88±8.19% in 30s. There was no significant difference between age in AI ($p>0.05$) (Table 3).

3.4. Difference in R-LV/HV and AI between Male and Female. For R-LV/HV, it averages on 82.37±15.29% in 70 males on frontalis muscle, 75.40±19.32% on zygomaticus muscle, and 80.00±15.50% on orbicularis oris muscle. It averages on 81.15±14.07% in 84 females on frontalis muscle, 72.35±18.96% on zygomaticus muscle, and 79.49±14.21% on orbicularis oris

Table 1: Demographic characteristics of 154 subjects.

Classification	Male	Female	Total
Age (person)			
Total	70	84	154
20s	14	17	31
30s	15	14	29
40s	14	23	37
50s	14	15	29
60s	13	15	28
height (cm)	171.56±5.29	160.25±5.22	165.39±7.70
weight (kg)	72.34±8.74	58.17±8.80	64.61±11.25

Table 2: Difference R-LV/HV and AI between muscles.

classification	FM Mean±SD (%)	ZM Mean±SD (%)	OM Mean±SD (%)	F	p	Post Hoc
R-LV/HV	81.70±14.60	73.74±19.12	79.72±14.77	9.965	<.001	FM>ZM ZM<OM FM=OM
AI	10.87±10.14	16.71±14.79	12.10±10.05	10.365	<.001	FM<ZM ZM>OM FM=OM

∗ FM: frontalis muscle; ZM: zygomaticus muscle; OM: orbicularis oris muscle.

muscle. When they were compared using paired t-test, the R-LV/HV by sex shows no statistically significant difference on all the three parts ($p>0.05$) (Table 4).

For AI, it averages on 10.56±10.89% in 70 males on frontalis muscle, 15.62±14.86% on zygomaticus muscle, and 12.03±10.84% on orbicularis oris muscle. It averages on 11.13±9.53% in 84 females on frontalis muscle, 17.62±14.77% on zygomaticus muscle, and 12.16±9.40% on orbicularis oris muscle. When they were compared using paired t-test, the R-LV/HV by sex shows no statistically significant difference on all the three parts ($p>0.05$) (Table 4).

4. Discussion

Most widely used methods for assessing facial paralysis include House-Brackmann scale, Yanagihara grading system, and Sunnybrook facial grading system which were methods of assessment with facial movements in patients as well as utilization of diagnosis devices such as DITI, NET, ENoG, EMG, and sEMG [10].

Among these methods, sEMG was a test that measures action potential by attaching electrodes on surfaces of the skin. Muscle forms a figure of the body by being attached to skins and the skeletal system and supervises exercise of the whole body that moves the skeleton system. When muscle retraction occurs, this simultaneously triggers motor impulse signal on motor cortex in the brain which was continuously transmitted into nerves of each motor unit through motor neurons of the spinal cord [11, 12]. When these nerve impulses were brought to neuromuscular junction, this would cause an electric transmission along muscular fibers to sarcolemma in both directions, which was called motor unit action potential, or MUAP [13]. Electrodiagnosis was defined as capturing, amplifying, and recording the electric action in muscles and was based on measurements of occurrence, mobilization, and propagation of these action potentials to be displayed on screen. The electrodes of the sEMG were divided into pole electrodes and surface electrodes based on measuring sites and convenience, and the surface electrode was used to alleviate pains of subjects when the measurement was conducted [14]. Unlike other methods, the sEMG was regarded as a relatively simple procedure not requiring artificial electric stimulus or noninvasive stimulus and thus has a potential for popularity, especially for the facial parts because a patient shows no resistance to its use on them. A study on surficial sEMG was emerging as the new paradigm in the field of rehabilitation for muscular and skeletal disease. There have been brisk study efforts in overseas going on the sEMG, including types of electrode and location of placement [15, 16].

For literature review on related studies on sEMG conducted in Asian countries such as Korea, Japan, and China, there were less than 10 studies on the sEMG in each country as of 2012 and there was the only clinical literature on patients with facial paralysis accompanying coordination movements [17]. Although there have been attempts to interpret results of the sEMG test on patients with facial paralysis by associating with the naked eye test, it was insufficient to represent the sEMG test with the smaller number of samples of 21 people. Another ongoing study on the sEMG measurements of 20 males and 40 females with good health when they move their facial parts was designed to draw biological electric features of the local specimen with good health but shows its limitation in that it fails to provide no classification other than

Table 3: Average of R-LV/HV and AI between ages.

classification	20s Mean±SD (%)	30s Mean±SD (%)	40s Mean±SD (%)	50s Mean±SD (%)	60s Mean±SD (%)	F	p
R-LV/HV							
FM	83.99±11.99	78.75±19.01	81.65±13.32	84.05±15.54	79.86±12.72	.781	.539
ZM	76.11±14.99	79.77±17.87	72.66±18.93	71.00±24.27	69.14±18.03	1.440	.224
OM	76.53±16.33	81.26±12.64	80.65±15.43	79.71±16.29	80.44±12.86	.487	.745
AI							
FM	9.18±7.65	13.23±13.12	10.76±9.31	9.61±11.71	11.76±8.35	.770	.546
ZM	14.41±10.34	12.46±12.57	17.32±14.05	19.58±19.34	19.89±16.15	1.413	.232
OM	14.30±11.20	10.89±8.19	11.60±10.75	12.28±11.38	11.40±8.17	.528	.715

∗ FM: frontalis muscle; ZM: zygomaticus muscle; OM: orbicularis oris muscle.

Table 4: Average of each muscles in R-LV/HV and AI between male and female.

classification	Male (N=70) Mean±SD (%)	Female (N=84) Mean±SD (%)	Total (N=154) Mean±SD (%)	t	p
R-LV/HV					
FM	82.37±15.29	81.15±14.07	81.70±14.60	.512	.610
ZM	75.40±19.32	72.35±18.96	73.74±19.12	.983	.327
OM	80.00±15.50	79.49±14.21	79.72±14.77	.213	.831
AI					
FM	10.56±10.89	11.13±9.53	10.87±10.14	-.348	.728
ZM	15.62±14.86	17.62±14.77	16.71±14.79	-.834	.406
OM	12.03±10.84	12.16±9.40	12.10±10.05	-.083	.934

∗ FM: frontalis muscle; ZM: zygomaticus muscle; OM: orbicularis oris muscle.

classification by males and females as well as with the smaller number of specimen [18, 19].

For sEMG analysis, the widely used statistical analysis was used. RMS was a method that analyzes amplitude of signal shown in sEMG. The analysis was available to measure the number of motor units activated and firing rate as it represents an increasing aspect of the signal amplitude when the muscle retracts and a decreasing aspect when muscle fatigue occurs [20]. It also has a significance in its utilization when R-LV/HV, Al determine conditions of the facial parts and degree of recovery. No report has been made as to whether the indexes of subjects with good health have a difference in sex, age, or parts of the body.

The authors in the study obtained R-LV/HV, Al from measurements of the facial surface sEMG in the subjects aged between 20s and 60s and with good health to clarify if the values have any difference in sex, age, or parts of the body. The study was based on a total of 154 males and females who aged between 20s and 60s and with good health gathered starting from September 13, 2016, to May 1, 2017. The selected subjects were required to take a 10-minute rest and introduced to learn how to move muscles of each acupuncture point to measure sEMG by placing a disposable electrode on acupuncture points of frontalis muscle, zygomaticus muscle, and orbicularis oris muscle. To measure orbicularis oris muscle, it selects a lower orbicularis oris muscle by a finger joint from both sides of Seungjanghyeol and this is based on results found in the study of Kim et al. [21] that orbicularis oris muscle has a higher measurement on the lower orbicularis oris muscle than on the higher orbicularis oris muscle.

The total of 154 subjects on the three parts have R-LV/HV and AI were measured. For R-LV/HV, it averages on 81.70±14.60% on frontalis muscle, 73.74±19.12% on zygomaticus muscle, and 79.72±14.77% on orbicularis oris muscle. When they were compared using one-way ANOVA, R-LV/HV on each part shows statistically significant difference. Frontalis muscle value was higher than zygomaticus muscle value and orbicularis oris muscle value was higher than zygomaticus muscle value. However, it was no significant difference between frontalis muscle value and orbicularis oris muscle value. For AI, it averages on 10.87±10.14% on frontalis muscle, 16.71±14.79% on zygomaticus muscle, and 12.10±10.05% on orbicularis oris muscle. When they were compared using one-way ANOVA, the asymmetry index on each part shows statistically significant difference. Frontalis muscle value was lower than zygomaticus muscle value and orbicularis oris muscle value was lower than zygomaticus muscle value. However, it was no significant difference between frontalis muscle value and orbicularis oris muscle value.

For R-LV/HV, it has its highest of 84.05±15.54% in 50s on frontalis muscle and its lowest of 78.75±19.01% in 30s. It has its highest of 79.76±17.87% in 30s on zygomaticus muscle and its lowest of 69.13±18.03% in 60s. It has its highest of 81.26±12.63% in 30s on orbicular oris muscle and its lowest of 76.52±16.33% in 20s. There was no significant

difference between age in R-LV/HV. For AI, it has its highest of 13.22±13.11% in 30s on frontalis muscle and its lowest of 9.17±7.64% in 20s. It has its highest of 19.88±16.14% in 60s on zygomaticus muscle and its lowest of 12.45±12.57% in 30s. It has its highest of 14.29±11.20% in 20s on orbicular oris muscle and its lowest of 10.88±8.19% in 30s. There was no significant difference between age in AI.

For R-LV/HV, it averages on 82.37±15.29% in 70 males on frontalis muscle, 75.40±19.32% on zygomaticus muscle, and 80.00±15.50% on orbicularis oris muscle. It averages on 81.15±14.07% in 84 females on frontalis muscle, 72.35±18.96% on zygomaticus muscle, and 79.49±14.21% on orbicularis oris muscle. When they were compared using paired t-test, the R-LV/HV by sex shows no statistically significant difference on all the three parts. For AI, it averages on 10.56±10.89% in 70 males on frontalis muscle, 15.62±14.86% on zygomaticus muscle, and 12.03±10.84% on orbicularis oris muscle. It averages on 11.13±9.53% in 84 females on frontalis muscle, 17.62±14.77% on zygomaticus muscle, and 12.16±9.40% on orbicularis oris muscle. When they were compared using paired t-test, the R-LV/HV by sex shows no statistically significant difference on all the three parts.

As found in the results stated above, individual differences in RMS values of the sEMG in subjects of good health were large and the same large differences were found in range of standard deviation, while R-LV/HV or AI in subjects of good health shows relatively certain range of values. This comes down to the conclusion as stated in Lee et al. [19] that RMS values were not suitable for determining conditions of subjects, rather comparisons using R-LV/HVs and AI values can be more reasonable method to determine whether a subject has a normal condition or not. Based on the conclusion, the results in this study can be utilized to assess abnormality of facial muscles and determine whether they fall within the normality.

5. Conclusion

The study analyzes R-LV/HV and AI with measurements of surface electromyography or sEMG on acupunctures points such as frontalis muscle, zygomaticus muscle, and orbicularis oris in a total of 154 subjects with good health.

(1) For R-LV/HV, it averages 81.70±14.60% on frontalis muscle, 73.74±19.12% on zygomaticus muscle, and 79.72±14.77% on orbicularis oris muscle. With analysis of the AI average was 10.87±10.14% on frontalis muscle, 16.71±14.79% on zygomaticus muscle, and 12.10±10.05% on orbicularis oris muscle.

(2) R-LV/HV was significance in three parts of muscles (FM>ZM, ZM<OM, FM=OM). And AI also was significance difference in muscles (FM<ZM, ZM>OM, FM=OM)

(3) Both of R-LV/HV and AI showed no statistically significant difference on age and sex.

In subjects of good health, no difference was found in terms of R-LV/HV and AI either by sex or by age. It was anticipated that the results in this study will be utilized to determine diagnosis, prognosis, and recovery in the future.

Data Availability

The data used to support the findings of this study may be released upon application to the IRB of Jeonju Oriental Hospital in affiliation with Woosuk University.

Conflicts of Interest

The authors have declared no conflicts of interest.

Authors' Contributions

Bo-Hyun Kim and Kyeong Han Kim equally contributed to this study.

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Research Article

Short-Term Efficacy of Pulsed Radiofrequency Thermal Stimulation on Acupoints for Chronic Low Back Pain: A Preliminary Study of a Randomized, Single-Blinded, Placebo-Controlled Trial

Boncho Ku,[1] **Minho Jun**,[1] **Jun-Hwan Lee**,[2,3] **Young-Ju Jeon**,[1] **Young-Min Kim**,[1] **Jaehui Kang**,[4] **Yu-Jung Lee**,[5] **Kahye Kim**,[1] **Hyun Heo**,[6,7] **and Jaeuk U. Kim**[1,3]

[1]*Future Medicine Division, Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea*
[2]*Clinical Medicine Division, Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea*
[3]*Korean Medicine Life Science, University of Science & Technology (UST), Campus of Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea*
[4]*Spinal and Joint Center, Cheonan Oriental Hospital of Daejeon University, Cheonan 31099, Republic of Korea*
[5]*Technology Licensing and Commercialization Team, Korea Institute of Oriental Medicine, Daejeon 34504, Republic of Korea*
[6]*Solco Biomedical Co., Ltd., Pyeongtaek, Gyeonggi 17704, Republic of Korea*
[7]*Department of Biomedical Engineering, Yonsei University, Wonju, Gangwon 26493, Republic of Korea*

Correspondence should be addressed to Jaeuk U. Kim; jaeukkim@kiom.re.kr

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Background. The objective of this study was to evaluate the pain-relief efficacy of thermal stimulation induced by a pulsed radiofrequency (PRF) thermal stimulation applied to acupoints (APs) in patients with low back pain (LBP). The study was designed as a randomized, single-blinded, placebo-controlled trial. *Methods.* Fifty-six LBP patients whose minimum pain intensity score on a visual analogue scale (VAS, 0-100 mm) was more than 30 mm were randomly allocated to either the placebo-controlled or the treatment group at a 1:1 ratio. The treatment and placebo-controlled groups received PRF thermal stimulation plus cupping therapy and cupping therapy only, respectively. Each patient was scheduled to receive a total of three treatment sessions over one week with allowing a window up to 4 days. Six of the 13 predefined APs were selected differently for each session depending on the change in patient's symptoms and intensity of pain. The primary outcome was the mean difference between the placebo-controlled and treatment group of VAS changes from the baseline to the end of the follow-up period. *Results.* The patients' reported VAS scores from baseline to the end of follow-up (average: 9.8 days) were significantly decreased by 8.036 points (two-sided 95% CI, -11.841 to -4.231) and 13.393 points (two-sided 95% CI: 17.198 to -9.588) in the treatment and the placebo-controlled groups, respectively. However, the change in VAS scores between the treatment group and the placebo-controlled group was not significantly different (2.015 mm, two-sided 95% CI: -5.288 to 9.317). *Conclusion.* The trial results indicated that treatment with either PRF thermal stimulation with cupping therapy or cupping therapy alone effectively relieved LBP. The efficacy of PRF thermal stimulation combined with cupping therapy was not superior to that of cupping therapy alone. **Trial registration number:** Clinical Research Information Service (KCT0002137). The trial was registered retrospectively on 10 November, 2016.

1. Introduction

Low back pain (LBP) has become a prevalent health problem in many economically developed countries [1]. More than 70% of the population in such countries has experienced LBP at some point in their lifetime, and the prevalence of chronic LBP is approximately 10 to 15% [2]. Due to the high prevalence of LBP, it is no longer considered a specific disorder limited to highly industrialized countries but is now considered a major health problem worldwide [2–4]. In

particular, according to a survey conducted in 2007 among the adult population in South Korea, the number of patients who suffered from LBP was estimated to be greater than 5 million, and approximately 55% of these patients developed chronic LBP [5]. LBP causes large burdens in terms of medical expenses, work absences, and disability [6]. For example, Kim et al. [7] reported that LBP was the most common disease for workers' compensation losses accounted for up to 40 % of cost.

Although conventional approaches for the management of LBP such as spinal manipulation, analgesics, nonsteroidal anti-inflammatory drugs, muscle relaxants, and many other treatments are available, no single therapeutic approach appears to be superior to other modalities [8]. Because conventional therapeutic interventions are often ineffective [9, 10] and are accompanied by adverse effects that lead to the dissatisfaction of patients [11], the use of complementary and alternative medicine (CAM) to manage LBP has been highlighted and has increased over the last two decades [12, 13]. Various CAM modalities, such as acupuncture, massage, and exercise, have been applied to alleviate LBP, although the precise mechanisms of action of each treatment remain ambiguous and their efficacies in reducing pain and disability are inconsistent or are based on low-quality evidence [14–20]. Nevertheless, numerous researchers and practitioners have sought to demonstrate the efficacy and mechanism of action of such treatments based on the perspectives of modern science [21]. Among these efforts, several systematic reviews and meta-analyses of randomized control trials (RCTs) have revealed some evidence for the efficacy and safety of CAM therapies for LBP [12, 21–26].

The most commonly applied therapeutic method among the various CAM modalities for the management of LBP is acupuncture. The efficacy of acupuncture in mitigating LBP has been consistently reported, and its safety is generally accepted [11, 21, 25, 27, 28]. Other types of CAM therapy, such as moxibustion and cupping, are also used alone or in combination with acupuncture to alleviate musculoskeletal pain [29–31]. Moxibustion is a therapeutic method that involves applying heat stimulation to APs by burning herbal powder primarily consisting of mugwort (moxa, *Artemisia argyi*) [29]. Cupping therapy is an ancient TCM modality that generates negative pressure, inducing hyperaemia or homeostasis, at acupoints using cups composed of various materials, such as bamboo or glass [30, 32, 33]. Both therapies have been generally accepted to be effective in improving blood circulation and alleviating pain [34, 35]. Experimentally, heat and negative pressure on the surface of the skin have been reported to induce similar physiological responses; both modalities induce the dilation of local blood vessels, increase local circulation and microcirculation, promote angiogenesis, and remove chemical substances that sensitize nociceptors [30, 36, 37]. Although moxibustion and cupping therapy are widely applied to alleviate LBP, their safety have not been well investigated. Especially, moxibustion may induce unexpected adverse effects, including air pollution, epidermal burning, blistering, suppuration, infection, and bruising, mainly due to the difficultly in controlling the magnitude of heat intensity [38].

Radiofrequency (RF) current has been used as a treatment modality to manage chronic pain syndromes such as chronic cervical pain, brachialgia, and cervicogenic headache, and cancer pain [39, 40]. In comparison to the conventional continuous RF (CRF) [41, 42], pulsed RF (PRF) does not generate thermal damage of nervous tissues by allowing time for heat dispersion [43]. Recently, PRF is regarded as a safe and less-destructive modality for the management of pain such as shoulder pain, lumbar facet joint pain, and various type of neuropathic pain [43–46]. A mechanism of PRF in pain relief is still unclear. Up to date, most studies related to biological effects of PRF postulate that a type of neuromodulatory effect induced by alternating electrical field inhibits synaptic transmission and neuron-specific gene expression [42, 43, 45, 47, 48]. Even though PRF and moxibustion are not directly linked in terms of clinical mechanism, both therapeutic modalities generate local thermal stimulation penetrating the subcutaneous skin layer [49]. In this respect, the PRF-based thermal stimulation system is often used as an alternative to the traditional moxibustion [50, 51].

Thus far, the available commercial products related to CAM therapeutic devices are primarily acupuncture-like devices that replace acupuncture needles, such as low-intensity lasers, electrical stimulators, or focused magnetic field generators [52]. In contrast, devices designed for use in moxibustion or cupping have rarely been reported. Recently, Myoung et al. [51] developed a temperature-controllable PRF probe generating heat distribution similar with moxibustion. Based on their research, a device that simultaneously applies thermal stimulation based on PRF electric fields and cupping therapy was developed for clinical use. In this study, we conducted a conventional RCT including LBP patients to assess the short-term pain-relieving effect of a newly developed PRF thermal stimulator.

2. Methods

2.1. Study Design. A randomized, single-blinded, and placebo-controlled trial was performed in 2013 at the Spinal and Joint Center, Cheonan Oriental Hospital of Daejeon University, Republic of Korea. This study was conducted in parallel with another study evaluating the effectiveness of a laser acupuncture device, previously reported by Shin et al. [53]. The design of this study was similar to the design of the study by Shin et al. except that patients were independently recruited.

2.2. Ethics Approval. This study was approved by the Institutional Review Board of Cheonan Oriental Hospital of Daejeon University, Korea (M2013-03-2, registered on 1 November, 2013). This study was regulated by the Ministry of Food and Drug Safety (No. 416, registered on 1 October, 2013), and a trial registration number was retrospectively obtained from the Clinical Research Information Service (KCT0002137, registered on 10 November, 2016), retrospectively.

2.3. Participants. Patients aged from 20 to 75 years with unspecified, uncomplicated, or chronic LBP were recruited

(a) (b)

Figure 1: (a) Appearance and accessories of the PRF thermal stimulation device. From the left to right in a clock-wise direction: main body cup-shaped probe equipped with PRF radiation tip; connection cable; and electrode pad. (b) Example of the operation of the PRF thermal stimulation device.

through advertisements and bulletin board postings at the hospital. LBP was diagnosed based on patient's history, symptom, previous radiographic records (e.g. X-ray, and CT), and independent physical examinations. The investigators or physicians provided full explanations of the purpose of study, interventions, and possible adverse events and complications, and written informed consent was obtained from all study candidates. LBP patients with a minimum pain intensity score greater than 30 mm on a visual analogue scale (VAS, 0 to 100 mm) were included. Enrolled patients were excluded if they met any of the following conditions: required the aid of medical devices or attached implantable equipment that could be affected by electromagnetic fields, such as pacemakers or hearing aids; had unendurable pain, bone fractures, severe disc herniation, or spinal tumours; were taking drugs such as corticosteroids, anticonvulsants, or anti-inflammatory agents; were pregnant; experienced any adverse effects due to the physical stimulation therapy; exhibited cognitive or mental dysfunction; or had participated in other clinical trials within the last month. Eligible patients were scheduled to visit three times over one week to receive treatment with allowing a window of 4 days, and follow-up investigations were performed within one week after the completion of treatment.

2.4. Randomization. Eligible patients were randomly allocated to the PRF thermal stimulation plus cupping therapy treatment group or the cupping therapy alone placebo-controlled group at a 1:1 ratio. Balanced block randomization was performed using the `blockrand()` function in the *blockrand* package [54] that is provided in the current version of the R statistical package (R Core Team, Austria). The block size was randomly selected with lengths of 2, 4, and 6 for group allocation.

2.5. Blinding. Access to the results of the randomization table was strictly prohibited with the exception of the independent statistician. The group assignment result was delivered to the hospital in the form of an opaque envelope labelled with consecutive numbers. All relevant investigators, including clinical coordinators and practitioners, were blinded to the type of treatment until the end of the study. The allocation was conducted by opening the envelope sequentially in front of the patient immediately before the first intervention. The patients received only partial information that corresponded to the masked group assignment (labelled as group A or B), and their actual treatment was concealed during the study. The devices used in both the treatment group and the placebo-controlled group were manufactured with identical appearances, but the PRF irradiation output of the device used for the placebo-controlled group was not operational. The practitioners were only able to identify each device according to the masked group labels. Acoustic sounds mimicking the application of PRF thermal stimulation and the pressure of cupping on peripheral APs were also delivered by the devices to preserve the group blinding of the practitioners.

2.6. Interventions. Patients received a combination of PRF thermal stimulation and cupping therapy or only cupping therapy three times over the course of one week. The PRF thermal stimulation and cupping device (Solco-HF100, Solco Biomedical, Co., Ltd., Republic of Korea) consisted of the following components: a main body to control the magnitude of PRF stimulation, cup-shaped probes equipped with a PRF stimulator at the tip, a connection cable for the probe, and an electrode pad for PRF induction. The detailed appearance of each component of the device is shown in Figure 1(a). PRF of 2 MHz was applied at intervals of 2000 ms through channel 1, and the actual stimulation duration of each application was configured to 400 ms. The output power was initially set at 20 W (10% of the maximum output power), and the negative pressure was 15 kPa.

In this study, an individualized acupuncture treatment for each LBP patient allowed flexible six APs within predefined APs depending on the patient's symptom progress and intensity of pain. Two licensed Korean Medicine doctors whose clinical experience was more than two years screened a total of 13 predefined APs based on their careful consensus and the literature [55]. The predefined APs include five bilateral

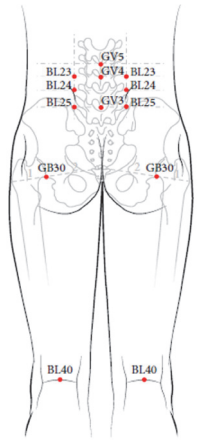

Figure 2: The location of the acupoints selected in the study. The figure was originated from the article of Shin and Jae-Young et al. "Short-Term Effect of Laser Acupuncture on Lower Back Pain: A Randomized, Placebo-Controlled, Double-Blind Trial," *Evidence-Based Complementary and Alternative Medicine* 2015 [53].

(BL23, BL24, BL25, GB30, and BL40) and three unilateral (GV3, GV4, and GV5) points (see Figure 2). APs including BL23, BL24, BL25, GV3, GV4, and GV5 were selected because these APs were located at low back and LBP patients usually feel pain at those points. The rest of predefined APs, GB30 and BL40, was one of frequently selected APs to LBP treatment not only in South Korea also worldwide [56, 57]. In addition, all these APs are located where the contact is possible, reflecting the structural feature of the probe mounted on the PRF thermal stimulation system.

For the treatment group, PRF thermal stimulation and cupping therapy were applied to each of the patient-specific APs for ten minutes. In the placebo-controlled group, an identical treatment procedure was performed except that PRF thermal stimulation was not actually applied. An example of the use of the probes is depicted in Figure 1(b).

2.7. Concomitant Medications. Patients in both the treatment group and the placebo-controlled group were suggested to voluntarily and independently perform daily exercises for LBP. Other treatments or therapies related to ameliorating LBP were prohibited during the study with the exception of the guided therapies. All significant medications and nonmedication treatments that were administered to patients after enrolment were reported in the "concomitant medications/significant non-drug therapies after the start of study" form.

2.8. Outcome Measures

2.8.1. Primary Outcome. The primary outcome measure was the change in LBP intensity measured before application of the intervention and one week after the end of the intervention. Patients were asked to score their subjective LBP intensity according to the 100 mm VAS. A decrease of VAS indicates the improvement of pain relief. The VAS score was evaluated on the first day of screening (visit 1), after each of the three interventions on visits 1 to 3, and at the follow-up visit. The difference of mean changes between placebo-controlled and treatment group is the primary concern in this study.

2.8.2. Secondary Outcomes. The secondary outcomes included the pressure pain threshold (PPT), the patient global impression of change (PGIC), and the European Quality of Life-5 Dimensions (EQ-5D).

The PPT represents a cut-off point at which a nonpainful pressure stimulation changes to a painful pressure [58]. A digital pressure algometer (AA129, JTECH Medical, USA) was used to quantify the PPT. The investigator placed an algometer on both sides of each patient's BL25 point and increased the pressure (kg/cm^2) applied perpendicular to the patient's skin. The pressure was increased until the patient noticed the first sensation of pain: each patient was instructed to raise a hand or announce when the pain was noticed. An increase of PPT indicates that the subject is more endurable to the pressure pain, so it can be interpreted that pain is alleviated. The PPT was evaluated on visits 1 to 3 and at the follow-up visit.

The PGIC is recommended tool to evaluate chronic pain in clinical trials [59]. The PGIC was adopted to assess the general improvement in the LBP of each patient after the intervention compared to before the intervention. The impression of this change was graded on a seven-point Likert scale (1: very much improved; 2: much improved; 3: moderately improved; 4: not improved; 5: slightly worse; 6: severely worse; or 7: very severely worse). The PGIC was reported on visit 1 and at the follow-up visit.

The EQ-5D is a representative instrument for measuring the patient's general health status and was developed by the EuroQol group [60]. The Korean translated version of the EQ-5D is composed of 5 dimensions: mobility, self-care, usual activities, pain/discomfort, and anxiety. Each dimension is graded on three levels and is scored using weights as estimated in a recent study of the EQ-5D for the Korean population [61]. The EQ-5D was assessed at visit 1 and at the follow-up visit.

2.9. Sample Size Calculation. Due to the limited existing evidence available to estimate the effect size of PRF thermal stimulation on LBP at the time of the study design, the sample size was determined based on previous results of a meta-analysis on the efficacy of acupuncture for LBP [27]. The pooled estimate of the effect size for the short-term effectiveness of acupuncture was 0.58 (95% CI: 0.36 – 0.80) based on four studies. It was necessary to conduct the preliminary study at minimal expense and, therefore, we intentionally overestimated the effect size and ultimately selected the value 0.80, which represented the upper bound of the 95% CI for the mean effect size. Using the above result, the sample size required to detect a significant difference in the primary outcome between groups on an independent two-sample t-test was 25 for each treatment group considering 80% power and a significance level of 5% (two-tailed). For a one-to-one group allocation ratio and allowing for a drop rate of 10%, a total of 56 patients were recruited.

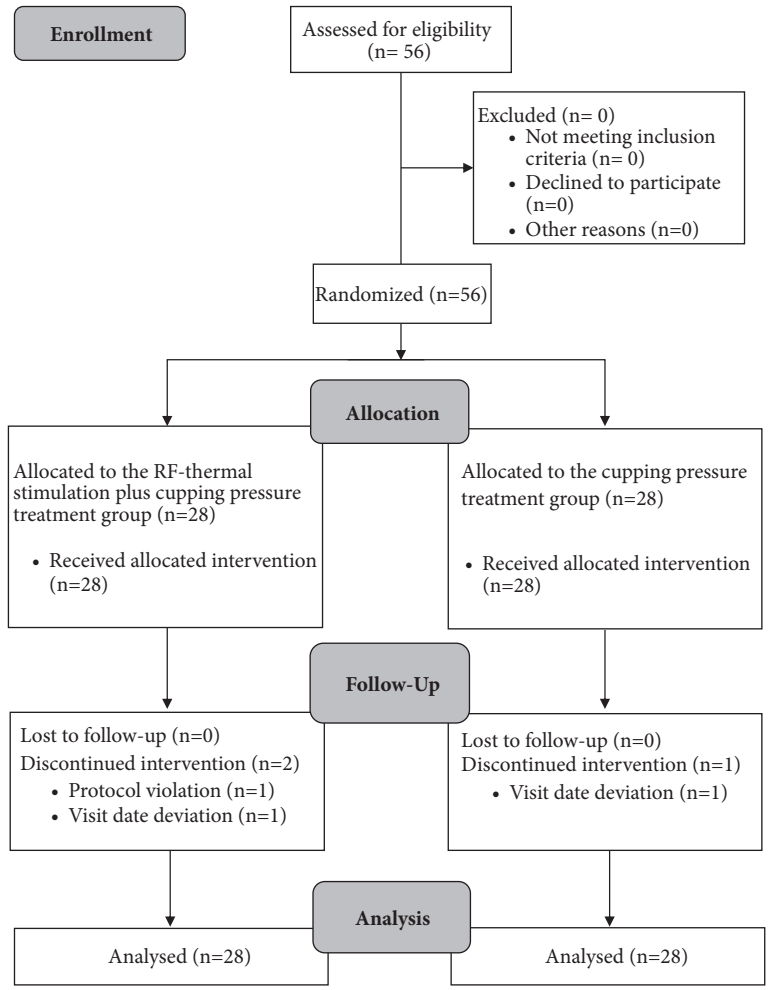

Figure 3: CONSORT flow diagram of the trial.

2.10. Statistical Analysis. Statistical analyses were performed using R statistical software, version 3.1.0. The level of significance was set to 0.05, and two-tailed comparisons were performed. The analyses of all measures, including the primary and secondary outcomes, were conducted on the full analysis set (FAS) based on the principle of intention-to-treat. Per protocol subsamples were also analysed for comparison with the results derived from the FAS analysis. There were no significantly discordant results between the two analyses and, therefore, the results of the PP analysis are not shown. Missing data were imputed using the last observation carried forward method. The differences in baseline characteristics between the active and placebo PRF stimulation groups were evaluated using independent two-sample t-tests or Wilcoxon's rank sum tests for continuous variables and Chi-squared tests or Fisher's exact tests for categorical variables.

An analysis of covariance (ANCOVA) was employed to evaluate the differences in the primary and secondary outcome measures between the treatment and placebo-controlled groups. The change in VAS scores between baseline and the endpoint was analysed using ANCOVA with adjustments for the baseline score and predetermined confounding factors of sex, age, the duration of back pain, and the number of exercise therapies performed during the study. A linear mixed effects model using the `lmer()` function in the lme4 package (R statistical package) was applied to investigate changes within each treatment group for each visit compared to baseline for the primary and secondary outcomes.

3. Results

A total of 56 patients recruited during November to December 2013 were assessed for eligibility and were equally randomized to the active (n=28) and control (n=28) PRF stimulation groups. Two patients in the treatment group and one patient in the placebo-controlled group dropped out. One patient in the treatment group violated the protocol, and two patients (one from the active group and one from the control group) did not appear on the scheduled visit dates. A flow diagram describing the study is presented in Figure 3.

3.1. Baseline Characteristics. The patients' demographics and baseline values for the main outcomes are summarized in Table 1. The samples of the two groups had equally balanced distributions: there were no significant differences between the treatment and placebo-controlled groups regarding sex

Table 1: Baseline characteristics of the treatment and placebo-controlled groups.

	Treatment (n=28)	Placebo-controlled (n=28)	p value
Baseline characteristics			
Sex			
Female	21 (75.0)	23 (82.1)	0.775
Male	7 (25.0)	5 (17.9)	
Age [years)	47.86 (9.58)	43.93 (11.58)	0.172
Duration of LBP [month)	7.38 (44.57)	3.53 (11.20)	0.204†
Exercise therapy during the study [count)	3.00 (0.00)	3.00 (0.00)	0.668†
BMI [kg/m^2)	23.81 (2.64)	24.77 (3.45)	0.245
Systolic BP [mmHg]	122.14 (14.18)	120.14 (14.16)	0.600
Diastolic BP [mmHg)	73.64 (11.04)	71.07 (9.03)	0.344
Pulse [bpm]	75.57 (10.64)	74.43 (10.81)	0.692
Body temperature [°C)	36.53 (0.53)	36.50 (0.41)	0.800
Outcomes at baseline			
VAS [mm]	41.96 (10.66)	46.25 (11.44)	0.153
PPT (at visit 1) [kg/cm^2]	6.72 (1.87)	6.62 (1.68)	0.847
EQ-5D (at visit 1)	0.77 (0.09)	0.78 (0.08)	0.844

Data are summarized as the mean (standard deviation: SD) for the continuous variables and N (%) for the categorical variables. The p values were derived based on the independent two-sample t-test or Wilcoxon's rank sum test for the continuous variables and chi-squared test for the categorical variables.
†Derived from Wilcoxon's rank sum test.
Treatment: PRF-thermal stimulation plus cupping therapy; Placebo-controlled: cupping therapy.
BP: blood pressure; BMI: body mass index; VAS: visual analogue scale; PPT: pressure pain threshold; EQ-5D: Euro Quality of Life-5 Dimensions.

ratio, age, duration of LBP, compliance with exercise guidance, body mass index, systolic/diastolic blood pressure, pulse and body temperature, or measured outcomes, including the VAS scores, the PPTs, and the EQol-5D results. The profile of the crude means and standard deviations of both primary and secondary outcomes measured in each session was illustrated in Figure 4.

3.2. VAS Change in LBP Intensity. The mean VAS scores were significantly reduced for both types of intervention (Table 2). After completion of the three intervention sessions over the course of one week, the changes in VAS scores at the time of follow-up were -8.036 (95% CI, -11.841 to -4.231; p<0.0001) for the PRF thermal stimulation plus cupping therapy group and -13.393 (95% CI, -17.198 to -9.588; p<0.0001) for the cupping therapy alone group. However, based on the between-group analysis, the adjusted mean difference in the change in VAS scores between the two groups at the time of follow-up was 2.015 (95% CI, -5.288 to 9.317; p=0.7090), and this difference remained insignificant throughout all intervention stages (Table 2).

3.3. Pressure Pain Threshold. Based on the within-group analysis, the mean changes in PPT at the time of follow-up were -1.421 (95% CI, -1.932 to -0.911; p<0.0001) and -0.935 (95% CI, -1.445 to -0.425; p=0.0012) in the treatment and placebo-controlled groups, respectively. Similar to the VAS score results, the adjusted mean differences in the change in PPT between the treatment and placebo-controlled groups were not significant during the intervention or at the follow-up visit (Table 2).

3.4. Patient Global Impression of Change. The mean change in the PGIC was significant at the time of follow-up in both groups: -0.786 (95% CI, -1.115 to -0.457; p<0.0001) in the treatment group and -0.929 (95% CI, -1.258 to -0.600; p<0.0001) in the placebo-controlled group. There was no significant difference in the adjusted mean difference in the change in PGIC between the two groups.

3.5. EQ-5D. The mean change in the EQ-5D result was 0.026 (95% CI, 0.000 to 0.052; p=0.0485) in the treatment group and 0.050 (95% CI, 0.024 to 0.076; p<0.0001) in the placebo-controlled group. There was no significant adjusted mean difference in the change in the EQol-5D result between the treatment and placebo-controlled groups (Table 2).

3.6. Safety Analysis. Although one patient in the placebo-controlled group complained of a mild sore throat during the study, the event was deemed irrelevant to the study. No adverse events directly related to the interventions were reported during the study.

4. Discussion and Conclusion

In the present randomized, double-blinded, placebo-controlled trial, we investigated the efficacy of PRF thermal stimulation using a newly developed device for modulating the functions of moxibustion and cupping therapy in patients with LBP. Combined treatment of PRF thermal stimulation and cupping therapy was not significantly different from treatment with cupping therapy alone based on the primary and secondary outcomes. Nevertheless, the results showed

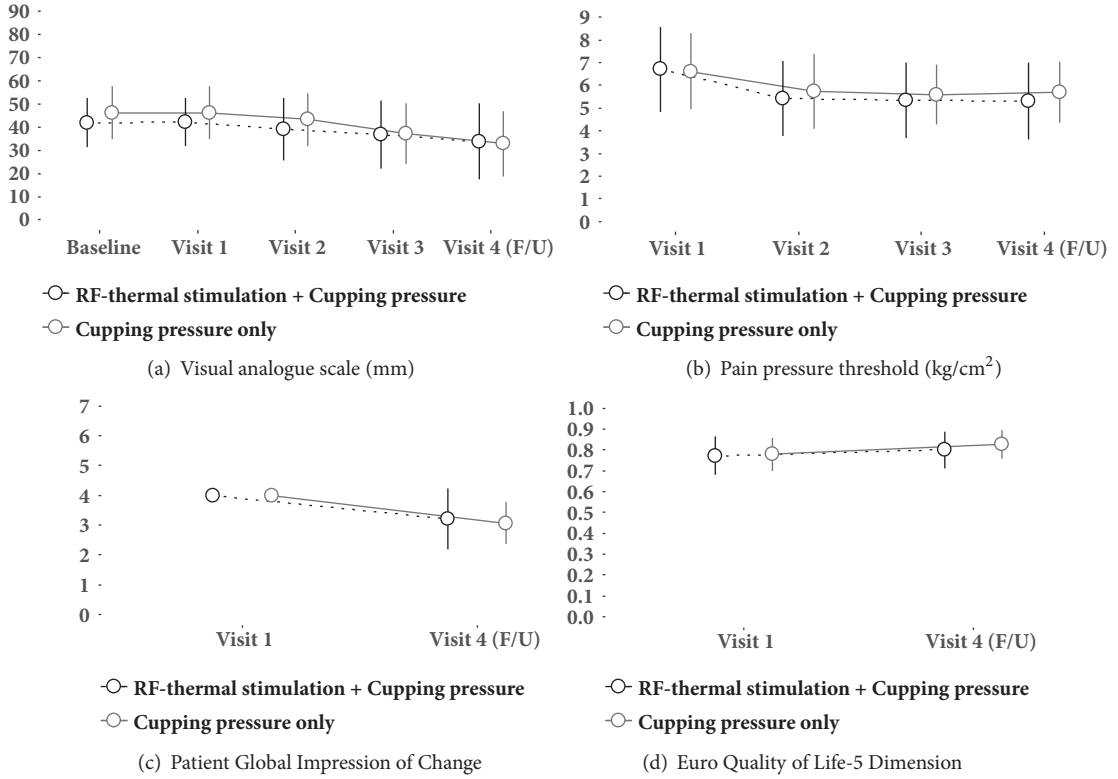

Figure 4: Crude mean profile of the primary and secondary outcomes (mean for each symbol and standard deviation for the error bars). Each panel represents (a) visual analogue scale (VAS), (b) pain pressure threshold (PPT), (c) patient global impression of change (PGIC), and (d) Euro Quality of Life-5 Dimension (EQol-5D).

that the PRF thermal stimulation and cupping therapy induced by the developed device were safe treatments for LBP patients and provided a clinical advantage in alleviating LBP. In both the treatment and control groups, LBP was significantly alleviated at the time of the follow-up visit after the end of all planned interventions. The VAS score revealed that the patients experienced a significant reduction in pain, and other secondary outcomes, including PGIC and EQol-5D, also showed improvements. In the case of PPT, unlike the results of other outcomes, LBP was increased in both placebo-controlled and treatment groups. This result is also opposite to the previous studies [62, 63] reporting PPT as an outcome. To clarify this discrepancy, a well-designed study that minimizes experimental biases is required.

The present study design was similar to that of the study conducted by Shin et al. [53], except for the different sources of stimulation and of enrolled LBP patients. Recent systematic reviews of LBP that have investigated the efficacy of CAM therapies generally defined the duration of 'short-term' follow-up as corresponding to an endpoint within two weeks to three months after treatment [11, 12, 27, 57]. The most common duration that patients used CAM therapies for treatment of a single disease in South Korea was reported to be approximately three to six days based on a survey conducted in 2009 [64]. In this respect, one-week duration selected for LBP treatment was of practical reason. The APs of BL23, BL25, BL40, GV3, GV4, and GB30 are globally used for the management of nonspecific and chronic LBP according to both textbook and clinical practice, as reviewed by Yuan et al. [57]. In addition, in a recent study, the result of a network analysis indicated that the set of APs applied in this study represented the most typically used points for treating LBP and that those 13 APs tended to be used together [65].

The results of the present study failed to reveal a difference in the efficacy of PRF thermal stimulation compared to cupping therapy alone. The strongest explanation for our result was that the initial temperature setting was insufficient to achieve the heat-sensitive de-qi sensation on the APs. The PRF stimulation module used in this study was introduced by Myoung et al. [51, 66]. They compared the temperature distribution between moxibustion and subcutaneous PRF thermal stimulation with a maximum power of 200 W applied to an anaesthetized rabbit. The study showed that the two subcutaneous temperature distributions were highly correlated and that the rate of epidermal heat loss during PRF stimulation was less than the heat loss rate during moxibustion. Thus, PRF thermal stimulation may be more effective in maintaining heat intensities than moxibustion. However, the PRF module used in this study was designed not to generate heat above 42°C. In addition, the thermal stimulation applied in this trial was generated at only 10% of the maximum power (20 W) due to safety considerations because there is no existing evidence regarding the optimal safe intensity of PRF stimulation for human subjects for the

Table 2: Mean change compared to the baseline and the mean difference in change between the treatment and placebo-controlled groups at each visit for the primary and secondary outcomes.

	Treatment		Placebo-controlled		Treatment–Placebo-controlled	
	Mean change from the baseline (95% CI)	p value†	Mean change from the baseline (95% CI)	p value†	Adjusted mean difference between the groups (95% CI)	p value‡
***VAS* [mm]**						
Visit 1 – Baseline	0.357 (-3.448, 4.162)	0.9935	0.000 (-3.805, 3.805)	1.0000	0.118 (-0.384, 0.620)	0.6811
Visit 2 – Baseline	-2.857 (-6.662, 0.948)	0.3837	-3.036 (-6.841, 0.769)	0.3324	-1.299 (-5.949, 3.351)	0.2886
Visit 3 – Baseline	-5.000 (-8.805, -1.195)	0.0366	-9.107 (-12.912, -5.302)	<0.0001	1.826 (-4.664, 8.316)	0.7127
Visit 4 (F/U) – Baseline§	-8.036 (-11.841, -4.231)	0.0002	-13.393 (-17.198, -9.588)	<0.0001	2.015 (-5.288, 9.317)	0.7090
***PPT* [kg/cm^2]**						
Visit 2 – Visit 1	-1.294 (-1.804, -0.784)	<0.0001	-0.878 (-1.388, -0.367)	0.0025	-0.581 (-1.308, 0.146)	0.0575
Visit 3 – Visit 1	-1.367 (-1.877, -0.857)	<0.0001	-1.020 (-1.530, -0.509)	0.0003	-0.417 (-1.087, 0.253)	0.1087
Visit 4 (F/U) – Visit 1§	-1.421 (-1.932, -0.911)	<0.0001	-0.935 (-1.445, -0.425)	0.0012	-0.507 (-1.217, 0.203)	0.0789
PGIC						
Visit 4 (F/U) – Visit 1§	-0.786 (-1.115, -0.457)	<0.0001	-0.929 (-1.258, -0.600)	<0.0001	0.053 (-0.419, 0.525)	0.5887
EQ-5D						
Visit 4 (F/U) – Visit 1§	0.026 (0.000, 0.052)	0.0485	0.050 (0.024, 0.076)	<0.0001	-0.022 (-0.056, 0.012)	0.9038

Data are summarized as the mean and 95% CI for the primary and secondary outcomes.
†Result of the within-groups analysis using a linear mixed effects model; p values were adjusted with Dunnett's test.
‡Result of the between-groups analysis using an ANCOVA with the following covariates: the baseline value, age, patient's duration of LBP, and compliance of daily exercise.
§Follow-up end point.
CI: confidence interval; VAS: visual analogue scale; PPT: pressure pain threshold; PGIC: patient global impression of change; EQ-5D: Euro Quality of Life-5 Dimensions.

treatment of LBP. Hence, use of the minimum intensity of PRF thermal stimulation was required in this trial, and the stimulation intensity chosen may be the main reason for the insignificant differences between the two different treatment groups. Similar results were found in other studies, and our study results are consistent with similar studies conducted by Lin et al. [67] and Shin et al. [53]. In those studies, there were no significant differences between the treatment group (laser acupuncture combined with cupping) and the placebo-controlled group (cupping), although significant differences in the VAS score within each treatment group were detected. The initial intensity of laser irradiation in both previous studies was a maximum power of 40-53 mW, wavelengths ranging from 660 to 808 nm, pulse frequencies of 20-200 Hz, a duty cycle of 50%, and 3 to 10 minutes of treatment. Regardless of the variation in laser dosage between the two studies, the length of infiltration was approximately 2-5 mm into the skin, which indicated that the intensity of stimulation was not sufficient to achieve the de-qi sensation: the optimal depth of acupuncture needle penetration has been suggested to be 10.3 to 90.3 mm [68]. Therefore, measurements of the de-qi sensation and further studies to identify the optimal intensity of electrically generated stimulation for humans are greatly needed for the development of convenient and safe medical devices to modulate CAM therapies for stimulating APs.

Another limitation of this study was that although the magnitude of negative pressure generated by the cupping instrument was not as strong as the pressure applied in conventional cupping therapy, its efficacy was not negligible. Recent studies reported that negative pressures generated from cupping therapy were ranged from 30 to 50 kPa [33, 69]. In contrast, low negative pressure (15 kPa) was applied in this study because the negative pressure was generated to adhere to the probes that were also used for PRF thermal stimulation in this stimulation system. Nonetheless, low-magnitude negative pressure is also widely used. In accordance with the survey of studies related to cupping therapy in South Korea performed in 2012, 50% of studies reported less than 100 mmHg (13.3 kPa) of cupping therapy, although the most frequently reported pressure was 600 mmHg (80 kPa) [70]. Moreover, the effectiveness of low negative pressure

cannot be ignored because the results of previous and present studies have indicated that low-pressure cupping therapy is significantly effective in reducing LBP [53, 67]. However, with the exception of those two previous studies, no study has reported the effectiveness of low negative pressure stimulation. Therefore, further studies on the efficacy of low negative pressure applied during cupping therapy are required to confirm our results.

Finally, patients were informed that the PRF thermal stimulation would not be performed in the placebo-controlled group. Despite the fact that we originally employed the double-blinded design, patient blinding was probably broken due to the nature of the heat stimulus. Therefore, the study was suspected to be a single-blinded clinical trial. This limitation may lead to bias in the results of the study. In addition, the fact that the trial was registered retrospectively is another limitation of this study.

Moxibustion and cupping are popular CAM modalities used to manage LBP. Both modalities are typical adjuvants of acupuncture. Traditional moxibustion and cupping can cause adverse effects because heat and negative pressure are difficult to tolerate. Because sensitivity to stimulation varies among individuals, the development of a medical device enabling control of the intensity of stimulation is required. In this respect, the assessment of the efficacy of the device used in this present study is meaningful. To our knowledge, this is the first study to investigate the efficacy of PRF thermal stimulation for LBP through RCT and, therefore, the present study provides a guideline for future studies and for device development.

In conclusion, the present study shows the effectiveness of both PRF thermal stimulation plus cupping therapy and cupping therapy alone in reducing LBP, although there was no evidence indicating the efficacy of the PRF thermal stimulation applied here. Future studies will be focused on identifying the optimal PRF intensity for generating heat stimulation equivalent to that applied in moxibustion.

List of Abbreviations

PRF:	Pulsed radiofrequency
AP:	Acupoints
LBP:	Low back pain
CAM:	Complementary and alternative medicine
TCM:	Traditional Chinese medicine
RCT:	Randomized control trial
VAS:	Visual analogue scale
PPT:	Pressure pain threshold
PGIC:	The patient global impression of change
EQ-5D:	European quality of life-5 dimensions
FAS:	Full analysis set
ANCOVA:	Analysis of covariance.

Data Availability

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

Ethical Approval

The Ethics Committee for Human Research at Cheonan Oriental Hospital of Daejeon University approved the trial procedures (M2013-03-2, registered on 1 November, 2013).

Consent

Written informed consent was obtained from all of the study candidates. Subject (Hyun Heo) in Figure 1 was one of the authors who participated in the establishment of the study protocol and consented to the publication.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Boncho Ku contributed to the design of the study, performed the statistical analyses, and drafted the manuscript. Minho Jun, Young-Ju Jeon, and Young-Min Kim participated in writing the manuscript and provided professional information on the medical devices. Jun-Hwan Lee and Jaeuk U. Kim conceived the study design. Jaehui Kang recruited the patients and conducted the clinical study. Yu-Jung Lee wrote the study protocol and managed the study. Kahye Kim carefully reviewed the manuscript. Hyun Heo contrived and provided the newly developed medical device and participated in the development of the study protocol. Jaeuk U. Kim supervised the study. All authors read and approved the final manuscript.

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Review Article

The Utilization of Medical Devices by Traditional Korean Medicine Doctors Investigated through Traditional Korean Medicine Clinical Studies

Soo-Hyun Sung,[1] **Hee-Ju Sim**,[2] **Eu-Gene Kim**,[3] **Angela Dongmin Sung**,[4] **Jung-Youn Park**,[5] **Byung-Cheul Shin**,[6] **Min-Jung Park**,[7] **Chang Hyun Han**,[8] **and Jang-Kyung Park**[9]

[1]*Department of Pathology, College of Korean Medicine, Dae-gu Haany University, Daegu 38610, Republic of Korea*
[2]*Department of Healthcare, Graduate School of Business, Ewha Womans University, Seoul 03760, Republic of Korea*
[3]*Department of Food and Nutrition, College of Natural Sciences, Inha University, Incheon 22212, Republic of Korea*
[4]*Department of Preventive Medicine, College of Korean Medicine, Sangji University, Wonju 26339, Republic of Korea*
[5]*Department of Social Welfare, Sungkyunkwan University, Seoul 03063, Republic of Korea*
[6]*Division of Clinical Medicine, School of Korean Medicine, Pusan National University, Yangsan 50612, Republic of Korea*
[7]*Graduate School of Public Health, Seoul National University, Seoul 08826, Republic of Korea*
[8]*Clinical Research Division, Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea*
[9]*Department of Obstetrics and Gynecology, College of Korean Medicine, Sangji University, Wonju 26339, Republic of Korea*

Correspondence should be addressed to Jang-Kyung Park; vivat314@naver.com

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Objective. The purpose of this study was to investigate the current status of modern medical devices utilized in diagnosis and treatment in traditional Korean medicine (TKM). *Methods*. We searched the following six Korean electronic databases to collect TKM clinical studies that were published in a five-year period (January 2012 to December 2016). Clinical studies of TKM when medical devices were used for diagnosis or treatment were investigated. *Results*. The search generated a total of 3,735 articles, and 1,328 of these were considered to be clinical studies. Of a total of 1,328 clinical studies of TKM, 774 articles (58.3%) used medical devices for diagnosis or treatment, and 554 articles (41.7%) did not use medical devices for diagnosis or treatment. The three most used diagnostic devices were as follows: MRI scanners, which were used in 194 (20.6%) studies; X-ray machines, which were used in 172 studies (18.3%); and CT scanners, which were used in 139 studies (14.8%). The three most used treatment devices were electroacupuncture equipment (20.3%), transcutaneous electrical nerve stimulation (TENS) equipment (18.4%), and interferential current therapy (ICT) equipment (16.4%). *Conclusions*. This study suggests that TKM doctors use diagnostic information derived from modern medical devices clinically. It is therefore necessary to institutionalize considering changes to the medical acts of traditional medicine (TM) doctors. Additionally, this information can be utilized as a reference for developing TM policy and education.

1. Introduction

A medical device is an instrument, machine, device, material, or any other similar product used alone or in combination in humans or animals, as specified in the following: a product used for the purpose of diagnosing, curing, alleviating, treating, or preventing a disease; a product utilized for the purpose of diagnosing, curing, alleviating, or correcting an injury or impairment; a product used for testing, replacing, or transforming a structure or function [1]. According to the World Medical Market Report, the global medical device market will grow at an average annual rate of 4.8% from 2010 to 2014, rising to an annual average of 6.1% from 2015 to 2020 due to an increasing trend in aging and awareness [2]. It is estimated that the market size will grow to approximately $ 435.8 billion by 2020 [2]. The size of the medical device

market in Korea also maintained a high annual growth rate of 10.4% from 2011 to 2015, and the production of medical devices reached $ 4.5 billion in 2015 [3].

In the past, doctors showed the medical behaviors of using stethoscope; recently, they presented the behaviors of utilizing modern medical devices (e.g., magnetic resonance imaging (MRI) scanners, computed tomography (CT) scanners, and medical laser) for diagnosing and treating patients [4, 5]. The reason why doctors have adopted modern medical devices is to gain a technical advantage and enhance self-determination and professional recognition [6].

Traditional tools such as acupuncture, herbal medicine, and cupping therapy have been used by traditional Korean medicine (TKM) doctors for a long time [7, 8]. Recently, TKM doctors have used medical devices such as electroacupuncture devices, electropulse graph, and distal arterial pulse wave analyzers, in combination with modern technology, for treatment [9, 10]. However, certain medical devices such as X-ray machines, MRI scanners, and CT scanners are not legalized for use by TKM doctors [11, 12].

China and Taiwan, similar to Korea, have a dual medical system that combines traditional medicine (TM) with conventional medicine (CM), and TM is included in the national system to provide medical service for nations [13]. In China, traditional Chinese medicine (TCM) doctors prescribe conventional medications, use modern medical devices, and perform surgeries, similar to licensed medical doctors [7, 14]. In Taiwan, only traditional treatments (e.g., acupuncture, moxibustion, and herbal medicine) were available to TM doctors, but recently complete blood counts, urinalysis, fecal analysis, and radiography have been allowed [7, 15]. Thus, the practice of TM doctors varies according to national systems and policies.

To our knowledge, the review on the use of medical devices by TKM doctors has not been published. The aim of this study was to investigate the current status of the medical behavior of TKM doctors by analyzing the data from clinical studies of TKM journals and to provide a reference for establishing TM policies and systems in countries that have a TM system

2. Methods

2.1. Data Sources and Searches. In April 2017, we searched the following six Korean electronic databases to collect TKM clinical studies that were published recent five years (January 2012 to December 2016); Korea Institute of Science and Technology Information, Korean traditional knowledge portal, KoreaMed, OASIS, RISS, and KISS. Clinical trials of TKM indexed in non-Korea databases such as PUBMED, EMBASE, or MEDLINE were not considered. The search keywords were as follows: "oriental medicine OR traditional medicine OR traditional Korean medicine OR complementary and alternative medicine" AND "clinical studies OR clinical trial OR case studies OR case report OR case series OR case controlled trial OR randomized controlled trial".

We did not limit study languages, but we excluded studies that did not have a paper format (e.g., abstract-only articles and conference presentations). The studies of master and doctoral degrees were not considered for inclusion.

2.2. Study Selection. We included only clinical studies of TKM. Reviews, surveys, qualitative studies, and experimental studies were excluded. Cohort studies or clinical studies in which participants were not provided any type of treatment were also excluded. Three authors (H. J. Sim, E. G. Kim, and A. D. Sung) independently screened and selected papers that met the study criteria. Disagreements were resolved by discussion with two authors (S. H. Sung and J. K. Park) to arrive at a consensus

2.3. Definition of Medical Device. This study classified medical devices into two categories: diagnostic devices and treatment devices. Diagnostic devices include medical imaging equipment (e.g., X-ray machines, MRI scanners, CT scanners, and ultrasound scanners), blood testing equipment, and infrared thermographic equipment used to examine the human body. Treatment devices include equipment for electric needle treatment, equipment for electric stimulus treatment, and laser treatment devices. However, equipment such as needles, lancets, and cups for cupping were excluded from the study as they were not considered to be machines.

2.4. Data Extraction. In TKM clinical studies where medical devices were used for treatment or diagnosis, we gathered information regarding the medical device. The collected information was classified into diagnostic devices and treatment devices. Furthermore, in order to investigate the current status of medical device use per year and the type of diseases, relevant information was gathered and further analyzed. Two independent reviewers (J. Y. Park and C. H. Han) extracted data using a form designed in the review. Discrepancies were resolved by discussions with two other reviewers (S. H. Sung and B. C. Shin).

When there were multiple diseases, the more serious disease was put first, and studies that focused on healthy people or case studies that treated more than 2 people with multiple diseases were excluded.

3. Results

3.1. Study Selection and Description. The search generated a total of 3,735 articles, of which 1,328 were considered to be TKM clinical studies. Four hundred and thirty-six studies were excluded for the following reasons: 117 studies were reviews, seven studies were surveys, 71 studies were experimental studies, 238 studies were clinical studies without interventions, and three studies were abstract-only articles or conference presentations (Figure 1).

3.2. Utilization of Medical Devices Classified by Type

3.2.1. Utilization of Medical Devices. From a total of 1,328 clinical studies of TKM, 774 articles (58.3%) used medical devices for diagnosis or treatment, and 554 articles (41.7%) did not use medical devices for diagnosis or treatment.

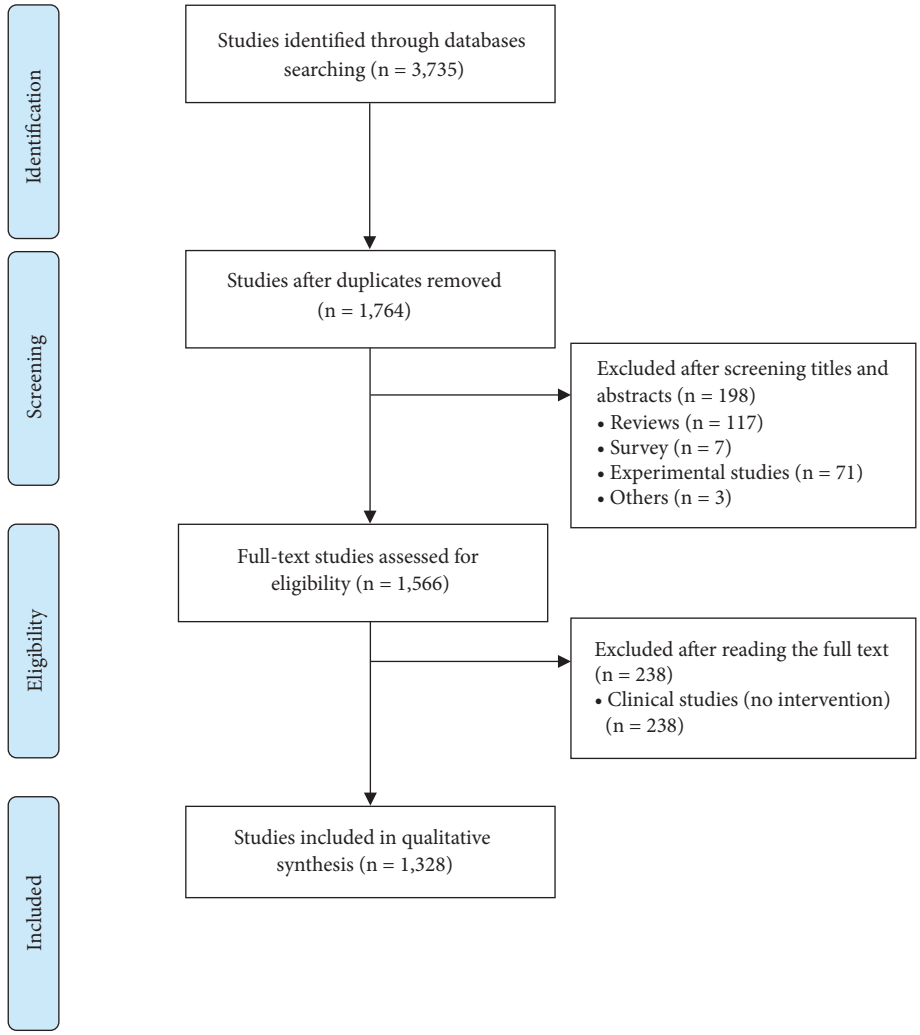

Figure 1: Flowchart of the study selection process.

Medical devices were used in TKM clinical studies a total of 1,345 times from 2012 to 2016; diagnostic devices were used 942 times (70%), while treatment devices were used 403 times (30%) (Table 1).

3.2.2. Utilization of Diagnostic Devices. Diagnostic devices were used 942 times in the TKM clinical studies. The 10 most used diagnostic devices are illustrated in Table 1. MRI scanners were used in 194 (20.6%) studies; X-ray machines were used in 172 studies (18.3%); CT scanners were used in 139 studies (14.8%); electrocardiography (EKG) equipment was used in 80 studies (8.5%); ultrasound scanners were used in 57 studies (6.1%); digital infrared thermal imaging (DITI) equipment was used in 43 studies (4.6%); heart rate variability (HRV) measurement equipment was used in 41 studies (4.4%); body composition analyzers were used in 28 studies (3.0%); electromyography (EMG) equipment was used in 21 studies (2.2%); and endoscopes in 19 studies (2.0%).

3.2.3. Utilization of Treatment Devices. In the 1,328 clinical studies investigated, medical devices were used for treatment 403 times. The 10 most used treatment devices are listed in Table 1. Electroacupuncture devices were used in 82 studies (20.3%); transcutaneous electrical nerve stimulation (TENS) equipment was used in 74 studies (18.4%); interferential current therapy (ICT) equipment was used in 66 studies (16.4%); ultrasound and infrared (IR) devices were used in 23 studies (5.7%); microwave therapy (M/W) equipment were used in 19 studies (4.7%); electrical stimulation treatment (EST) equipment was used in 12 studies (3.0%); lasers and silver spike points (SSPs) were used in 11 studies (2.7%); and electromagnetic stimulation therapy equipment was used in 6 studies (1.5%).

3.3. Utilization of Medical Devices per Year

3.3.1. Utilization of Medical Devices per Year. The use of medical devices decreased from 2012 to 2014 and increased by

Table 1: Medical devices reported in TKM clinical studies.

Types of Medical Device	Top 10 used Medical Device	Number of papers n (%)
Diagnostic device 942	MRI scanners	194 (20.6)
	X-ray machines	172 (18.3)
	CT scanners	139 (14.8)
	EKG equipment	80 (8.5)
	Ultrasound scanners	57 (6.1)
	DITI equipment	43 (4.6)
	HRV measurement equipment	41 (4.4)
	Body composition analyzer	28 (3.0)
	EMG equipment	21 (2.2)
	Endoscope	19 (2.0)
	Etc.	148
Treatment device 403	Electro acupuncture devices	82 (20.3)
	TENS equipment	74 (18.4)
	ICT equipment	66 (16.4)
	Ultrasound devices	23 (5.7)
	IR equipment	23 (5.7)
	M/W equipment	19 (4.7)
	EST equipment	12 (3.0)
	Laser devices	11 (2.7)
	SSP equipment	11 (2.7)
	Electromagnetic stimulation therapy equipment	6 (1.5)
	Etc.	76

MRI: magnetic resonance imaging; CT: computed tomography; EKG: electrocardiography; DITI: digital infrared thermal imaging; HRV: heart rate variability; EMG: electromyography; TENS: transcutaneous electrical nerve stimulation; ICT: interferential current therapy; IR: infrared rays; M/W: microwave therapy; EST: electrical stimulation therapy; SSP: silver spike point.

2016. Medical devices were used the most (312 times) in 2012. The utilization of diagnostic devices revealed a decreasing trend overall. The use of treatment devices declined from 2012 to 2015, and a steep increase occurred in 2016 (Figure 2).

3.3.2. Utilization of Diagnostic Devices per Year. Investigation of the 10 most used diagnostic medical devices per year in TKM clinical studies over the past 5 years revealed that MRI scanners, X-ray machines, and CT scanners were the most used medical devices from 2012 to 2016. MRI scanners were used a minimum of 32 and a maximum of 46 times every year. X-ray machines were used from 19 to 40 times, and CT scanners were used from 16 to 40 times. Electrocardiography use showed a decreasing trend from 2012. However, an increasing trend was demonstrated from 2014. Heart rate variability machines were not used much in 2015 and 2016, whereas they were used in 22 studies in 2012 (Figure 3).

3.3.3. Utilization of Treatment Devices per Year. Assessment of the 10 most used treatment devices that were utilized in more than 10 clinical studies per year revealed that electroacupuncture, TENS, and ICT devices were used on a regular basis every year, and IR equipment, laser equipment, and SSPs were used frequently in 2016 (Figure 4).

3.4. Diagnosis and Treatment of Diseases per Type of Medical Device

3.4.1. Diseases Diagnosed by Diagnostic Devices. The 5 most used diagnostic medical devices were used for various diseases. MRI was used to diagnose herniation of the lumbar disc in 16 studies (8.2%), stroke in 12 studies (6.2%), and rotator cuff tear in 5 studies (2.6%). X-ray machines were used for diagnosing scoliosis in 9 studies (5.2%) and pneumonia and herniation of the lumbar disc in 4 studies (2.3%). CT scanners were used to diagnose stroke in 8 studies (5.8%), lung cancer in 5 studies (3.6%), and herniation of the lumbar disc and gastric cancer in 4 studies (2.9%). For diagnosing amyotrophic lateral sclerosis and stroke, EKG was utilized in 4 studies (5.0%). Ultrasound devices were utilized in diagnosing infertility in 4 studies (7.0%), uterine myoma in 3 studies (5.3%), and hepatitis, frozen shoulders, gastrocnemius tear, drug-induced liver injuries, and uterine bleeding in 2 studies (3.5%) (Table 2).

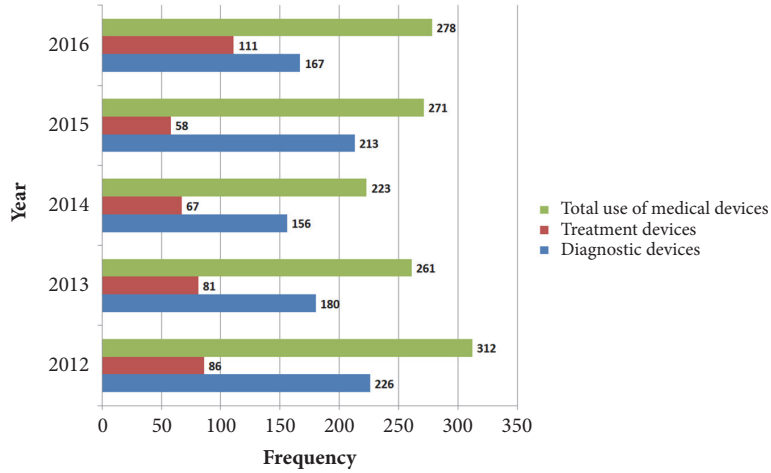

Figure 2: Utilization of medical devices in Korea from 2012 to 2016 by publication year.

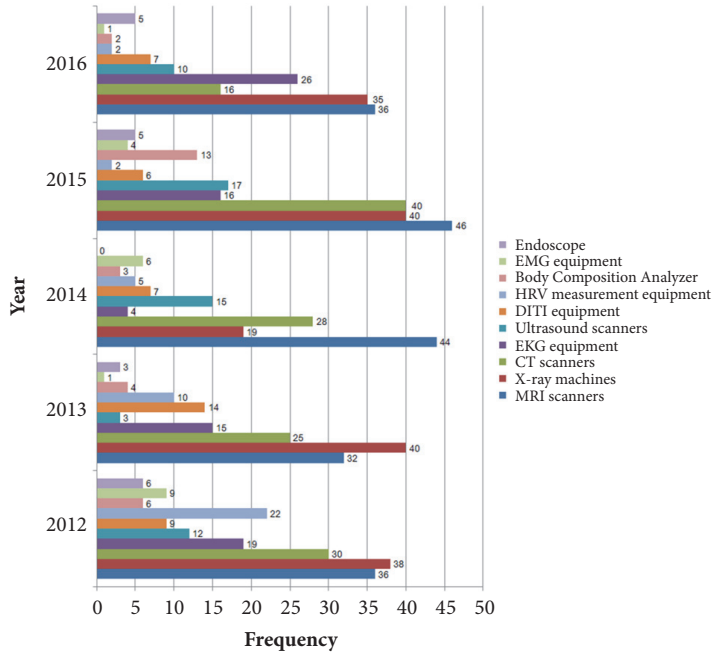

Figure 3: Study trend of diagnostic devices reported in clinical studies of TKM per year. MRI: magnetic resonance imaging; CT: computed tomography; EKG: electrocardiography; DITI: digital infrared thermal imaging; HRV: heart rate variability; EMG: electromyography.

3.4.2. Diseases Treated by Medical Treatment Devices. Investigation of the diseases treated by the 5 most used medical treatment devices revealed that electroacupuncture was used in treating facial palsy in 9 studies (11.0%), stroke in 5 studies (6.1%), and obesity and headaches in 3 studies (4.1%). Transcutaneous electrical nerve stimulation was utilized in treating herniation of the lumbar disc in 11 studies (14.9%), back pain in 4 studies (5.4%), and shoulder pain in 3 studies (4.1%). Interferential current therapy was used for treating herniation of the lumbar disc, in 7 studies (10.6%), while ultrasonography was used in 3 studies (13.0%). Infrared devices were used in 5 different studies (21.7%) to treat facial palsy (Table 3).

4. Discussion

The medical devices are important for quality of health service delivery [16]. It is an applied technique that combines various subject fields such as clinical medicine, electricity, electronics, mechanics, and optical science [17]. The industry is part of the health and medical treatment industry, with the aim of improving the quality of life of humans. The current medical device industry in Korea has gone beyond the simple research stage and is transforming itself to a customized industry where replaceable biomaterial and artificial organs are being developed with high technology [18].

When investigating previous research regarding TKM doctors use of medical devices, Kim et al. [19] conducted

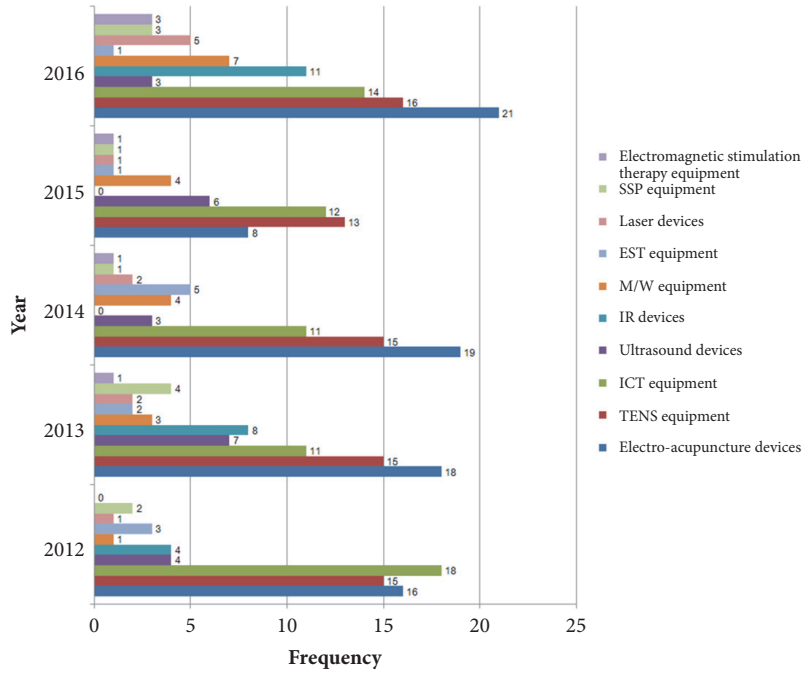

Figure 4: Study trend of treatment devices reported in clinical studies of TKM per year. TENS: transcutaneous electrical nerve stimulation; ICT: interferential current therapy; IR: infrared rays; M/W: microwave therapy; EST: electrical stimulation therapy; SSP: silver spike point.

Table 2: List of diseases diagnosed by diagnostic devices.

Diagnostic Devices	Diseases of Top 3 used	n (%)
MRI scanners	Herniation of lumbar disc	16 (8.2)
	Stroke	12 (6.2)
	Rotator cuff tear	5 (2.6)
X-ray machines	Scoliosis	9 (5.2)
	Pneumonia and herniation of lumbar disc	4 (2.3)
CT scanners	Stroke	8 (5.8)
	Lung cancer	5 (3.6)
	Herniation of lumbar disc and gastric cancer	4 (2.9)
EKG equipment	Amyotrophic lateral sclerosis and stroke	4 (5.0)
	Parkinson disease	2 (2.5)
Ultrasound scanners	Infertility	4 (7.0)
	Uterine myoma	3 (5.3)
	Hepatitis, frozen sholder, gastrocnemius tear, drug-induced liver injury and uterine bleeding	2 (3.5)

MRI: magnetic resonance imaging; CT: computed tomography; EKG: electrocardiography.

a survey where 900 TKM doctors were asked what types of medical devices are kept in TKM institutions. It was stated that although medical imaging equipment such as X-ray machines, CT scanners, and MRI scanners were not kept as much in these institutions, the frequency of their use was high. Sakong et al.'s [20] research provided critical views on restrictions imposed on TKM doctors in using medical imaging equipment by investigating cases where TKM doctors were restricted from using medical imaging equipment, how medical imaging equipment was brought into the medical industry, and the academic principles and study curriculum of TKM.

In Taiwan, there are no laws prohibiting the use of modern medical devices by TCM doctors, and some medical devices are available for the ministry of health [7, 15].

In China, there are no laws prohibiting the use of modern medical devices by TCM doctors, and the government does not restrict the use of modern medical devices and western medicine prescriptions by the Chinese government [7, 21]. For this reason, many studies have been conducted combining TCM with medical imaging devices [22–24].

This study shows that diagnostic devices have been used more than treatment devices in TKM clinical studies. When investigating the diagnostic medical devices and treatment

Table 3: List of diseases treated by treatment devices.

Treatment Devices	Diseases of Top 3 used	n (%)
Electro acupuncture	Facial paralysis	9 (11.0)
	Stroke	5 (6.1)
	Obesity and headache	3 (3.7)
TENS equipment	Herniation of lumbar disc	11 (14.9)
	Back pain	4 (5.4)
	Shoulder pain	3 (4.1)
ICT equipment	Herniation of lumbar disc	7 (10.6)
	Back pain	5 (7.6)
	Neck pain	4 (6.1)
Ultrasound devices	Herniation of lumbar disc	3 (13.0)
IR devices	Facial paralysis	5 (21.7)

TENS: transcutaneous nerve stimulation; ICT: interferential current therapy; IR: infrared rays.

devices used in the TKM clinical studies, the statistics of the usage per year show that both types of devices have been used steadily from 2012 to 2016. In the past, TKM doctors made diagnosis based on the traditional observing, smelling, asking, and touching methods and treated their patients with acupuncture, moxibustion, and herbal medicine. Currently, medical devices are used for diagnosis and treatment, indicating advancement in treatment. Initially, even doctors did not use medical devices for diagnosing and treating patients. Based on medical knowledge, objective information driven from utilizing modern medical devices allowed for this change, and it is thought that these types of medical acts will further transform themselves with the development of scientific technology.

The use of MRI scanners, X-ray machines, and CT scanners accounted for 53.7% of the total diagnostic device usage from 2012 to 2016 in TKM clinical studies. However, MRI scanners, CT scanners, X-ray machines, and diagnostic ultrasound machines are medical devices that TKM doctors are not allowed to use by law [25–27], and it is estimated that TKM institutions gather and utilize health information such as image materials taken from CM hospitals or image materials gathered through joint treatment of both TKM and CM doctors.

Clauses 37 and 38 under the Medical Service Act state each of the installment regulations and managing director decisions for MRI, radiography, and CT, and TKM doctors do not have the authority to direct [28]. Clause 1 of the Medical Technicians Act states that leadership over medical technicians can be given to dentists or medical doctors [29]. Furthermore, most of the preceding cases state that the use of modern diagnostic devices by TKM doctors is not permitted. However, there was an exceptional case where the use of 5 different medical devices (e.g., applanation tonometer, autorefractor and keratometer, slit lamp, pure tone audiometer, and automated perimeter) were allowed to a TKM doctor at the constitutional court [30]. The reasons for the denial are thought to be a dual medical system, differences in academic principles, insufficient legal grounds for TKM doctors using medical devices, and insufficient educational institutions. This study suggests that TKM doctors use diagnostic information derived from modern medical devices clinically. In order for the TKM study, which is heavily based on traditional knowledge and experience, to go through the process of digitization and produce evidence, collecting objective information by utilizing medical devices is needed. Although there can be differences in the methods of treatment in TKM and CM, the right to use medical devices utilized for treatment and diagnosing patients should be equally given to both types of doctors. Interferential current therapy and TENS are used for treatment every year. It is thought that they have the effect of relieving muscle tension, making the flow of qi smooth, and stimulating the meridian point to improve the pain. The use of the same treatment device may vary depending on the type of medical occupation. It is necessary to consider this aspect when developing medical devices and licensing items.

Diagnostic medical devices such as MRI scanners, CT scanners, and X-ray machines were widely used in herniation of the lumbar disc. In Korea, the Ministry of Health and Welfare operates a system to designate specialized hospitals that treat specific diseases when the medical institute meets the standards set by Medical Law [31]. A total of 109 specialized hospitals have been designated as of 2018; in the TKM field, 8 TKM hospitals specialized in the spine and 1 TKM hospital specialized in gynecology were designated [31]. It is thought that the results of this study reflect that MRI scanners, CT scanners, and X-ray machines are used for treatment in hospitals that specialize in the spine. Conventional medicine and TKM doctors in these hospitals cooperate in areas of treatment-related research [32].

There are some limitations in this review. First, TKM clinical studies indexed in English databases, including EMBASE and MEDLINE, were not considered in our review. Therefore, it is difficult to see that the clinical trials studies related to TKM included in this paper are representative of the use of medical devices in TKM doctors.

Second, it was impossible to fully explore how TKM doctors utilize medical devices as they have restrictions in using these devices in their practice. To understand how these medical devices are used in actual practice, studies such as surveys or interviews are needed and investigations of

medical behaviors of TM doctors in China and Taiwan are required.

Third, most of the clinical studies included in this review were conducted in TKM hospitals, and TKM hospitals make up only 1.9% of the TKM institutions [33]. It is therefore difficult to conclude that this review represents the majority of practices being carried out in TKM institutions.

Although TKM has been responsible for Korean people's health for thousands of years and holding sufficient historical evidence, there is no modern scientific evidence to support TKM. Therefore, this review is valuable because the medical behavior of TKM doctors was investigated for the first time. We cautiously propose that this review contributes to establishing TM policies in countries that practice TM and providing improved medical services of TM to patients.

5. Conclusion

We reviewed the use of medical devices by TKM doctors in Korea. It provided an understanding about the recent status and trends of the medical behaviors of TKM doctors related to medical devices. This study suggests that TKM doctors utilize medical devices for diagnosis and treatment of patients. It is necessary to institutionalize considering making changes to the medical acts of TM doctors. Additionally, this information can be utilized as a reference for making TM policy and education.

Conflicts of Interest

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

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Research Article

The Development and Application Evaluation of Meridian Energy Detection System in Traditional Oriental Medicine: A Preliminary Study

Yu-Chen Lee,[1,2] **Hui Ping Ng**,[1,3] **Yung-Hsien Chang**,[2] **and Wen-Chao Ho**[4]

[1]*Graduate Institute of Acupuncture Science, China Medical University, Taichung, Taiwan*
[2]*Acupuncture Department, China Medical University Hospital, Taichung, Taiwan*
[3]*International Master Program in Acupuncture, China Medical University, Taichung, Taiwan*
[4]*Department of Public Health, China Medical University, Taichung, Taiwan*

Correspondence should be addressed to Yu-Chen Lee; d5167@mail.cmuh.org.tw and Wen-Chao Ho; wcho@mail.cmu.edu.tw

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As technology advances, more modern medical devices are developed to help the physicians in performing objective assessment and diagnosis. In this study, our main objective was to evaluate the clinical application of the low voltage Meridian Energy Detection System in assessing the electrodermal activity (EDA) of the specific acupoints in a specific age group of healthy individuals and to assess the difference in the energy overview between the genders and specific time of assessment. 43 young healthy adults were recruited in a single group, nonrandomized, evaluation study. Written informed consent of each participant was obtained prior to the assessments. Results on energy overview between genders and specific time of assessment as well as factors influencing EDA were discussed. It was concluded that the study using Meridian Energy Detection System in healthy individuals provided an understanding of the difference in energy level of the meridians between the genders. Male healthy individuals had significantly higher values for Physical Status as well as Yin and Yang energy while female healthy individuals had significantly higher values for Mental Health and Autonomic Nervous System. There was no significant difference when comparing the assessments at the specific time of assessment. Hence there was no specific time in using the device. However, due to the limitation of the sample size and the healthy subjects, future research can be designed to investigate whether the time of assessment can affect the results in individuals with specific disease conditions in larger scale. It may merit further studies on the application of such device as preliminary diagnosis of the overall conditions and investigate the treatment efficacy by observing the change in the meridian energy level.

1. Introduction

As technology advances, more modern medical devices are developed to help the physicians in performing objective assessment and diagnosis. It was a step of advancement in clinical practice of Traditional Oriental Medicine since 1950 when devices for pulse diagnosis and meridian energy analysis were first developed. Experimental and translational studies are required for evaluating the clinical application of the modern tools in Traditional Oriental Medicine.

Based on the ancient publication "Yellow Emperor's Inner Canon" (Huang Di Nei Jing) compiled in the period of the Warring States (475-221 BC), the meridian system in the human body consists of twelve primary pathways (also known as "Primary Meridians" or "Channels and collaterals"). In the book of 'Magic Pivot, Chapter 33-Discourse on the Seas', the original text is translated as 'The twelve Primary Meridians in the body penetrate the interior visceral and bowels (also termed as '*zang-fu' or organs*) and connect the exterior limbs and joints'. In the book of 'Magic Pivot, Chapter 47-The Visceral', the original text is translated as 'The meridians are the pathways of the Qi (vital energy) and blood, nutrify the Yin and Yang, nourish the tendons and bones and lubricate the joint' [1, 2].

Based on the TCM theory, the meridians are longitudinally and laterally interconnected pathways that are

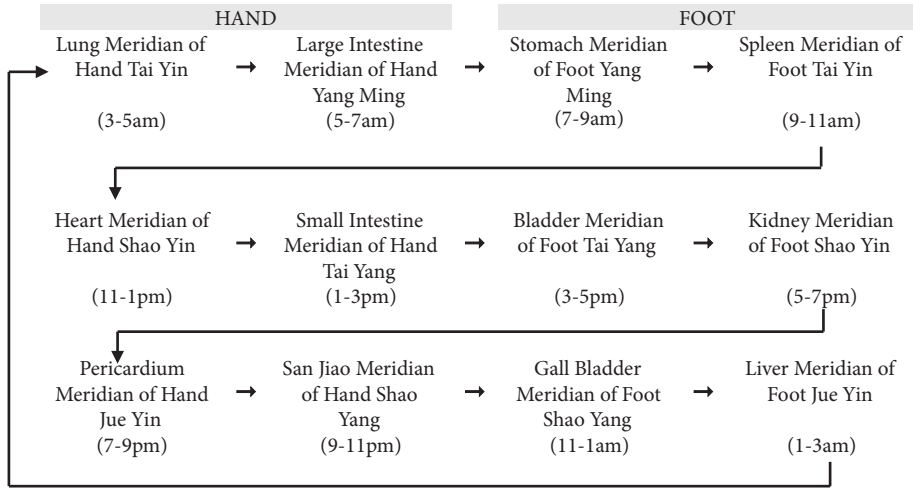


Figure 1: Meridian flow chart.

distributed throughout the body to provide nourishment to all the body tissues and organs. The twelve Primary Meridians and eight Extraordinary Meridians play important roles in the normal physiological functions of the body. The movement of Primary Meridian Qi is usually in a specific direction as illustrated in Figure 1 [2, 3].

The acupoints (or acupuncture points) are specific anatomical defined areas on the body along the meridians where Qi is gathered. These points are usually used for application of acupuncture in treatment of diseases.

Each of the twelve Primary Meridians has specific important group of acupoints on the upper and lower limbs, located distal to the elbows and knees. They are the 'Five Shu-points', also known as "Five Transport points". Table 1 describes the characteristics of the Qi flow in these acupoints [2, 3].

The Yuan-Source points were first discussed in the book of 'Magic Pivot, Chapter 1-The Nine Needles and Twelve Source'. The original text is translated as "The five *zang* (visceral) has six *fu* (bowels). Six *fu* have twelve Yuan-Source points which locate at the 4 joints of the extremities (wrists and ankles) that can treat the five *zang*. When five *zang* are diseased, the Yuan-Source points should be selected [1]." There are total 24 Yuan-Source points on both hands and feet.

In the 1950s, the electrical detection of acupoints was firstly introduced by various researchers, namely, Reinhard Voll (1953, Germany) [4], Yoshio Natakani (1956, Japan), and J.E.H. Niboyet (1957, France). Identifiable electrical characteristics of the skin points and the resembled traditional acupoints were independently concluded [5].

Dr. Yoshio Nakatani discovered a series of high electrical conductivity points that run longitudinally on the body which matched closely to the meridian acupoints [6]. He called these lines with high electrical conductivity points as Ryodoraku (Ryo=good; do=electrical conductivity; raku=line) while the points were called Ryodoten. Coincidentally, majority of these most energetically active points on the meridians corresponded to the Yuan-Source points that were located at the wrists and ankles. These were referred to as Representative Measuring Points (RMP) as illustrated in Table 2 and Figure 2. In his study, an amperometer (12V, 200 uA) was used to assess these points. Instead of absolute reading, a normalized scale of 0-200uA was reported due to the high variability in skin conductance measurements [4]. It was believed that an increase in conductance (i.e., decreased resistance) represents a surfeit of energy in the respective meridian while a decrease in conductance (i.e., increased resistance) represents a deficiency of energy in the respective meridian [7].

In fact, the Ryodoraku had incorporated the concept of electrodermal conductivity in the system. Electrodermal activity (EDA) was first introduced in 1966 as a common term to describe the electrical phenomena on the body skin. It is defined as all active and passive electrical characteristics in the skin and the appendages [8]. EDA is the current standardized preferred term for electrodermal response (EDR), psychogalvanic reflex (PGR), galvanic skin response (GSR), skin conductance, skin conductance level (SCL), skin conductance response (SCR), and sympathetic skin response (SSR) [9]. Overlying sweat glands and epidermis are involved in generating the EDA. It is mediated by dorsal thalamus, orbitofrontal cortex, posterior hypothalamus, and ventrolateral reticular formation. The spontaneous response is also known as peripheral autonomic surface potential or sympathetic skin response [10].

Colbert et al. (2008) reported that lower electrical skin resistance and higher capacitance could be found in acupoints compared to the tissues surrounding them; certain clinical diseases might be correlated with higher or lower resistance at specific acupoints; physiologic dysfunction that was experimentally induced and its subsequent recovery had correlated with the changes in electrical impedance at relevant acupoints. Hence electrical skin impedance of acupoints was a unique feature distinct from nonacupoints. Changes in skin impedance at the acupoints might have significant value in the areas of therapeutic, diagnostic, and research [11].

In this study, our main objective is to evaluate the application of low voltage Meridian Energy Detection System in assessing the EDA of the 24 RMP bilaterally on both wrists

Table 1: Five Shu-points & its characteristics.

Five Shu-points (Chinese, Pinyin-English Translation)	Characteristics	Each Yin Primary Meridian	Each Yang Primary Meridian	Total in 12 Primary Meridians
井 Jing-Well	The location where the Meridian Qi emanates	1	1	12
滎 Ying-Spring	The location where the Meridian Qi glides to form a small stream	1	1	12
俞 Shu-Stream	The location where the Original Qi infuses into the Meridian through the function of San Jiao (or Triple Energizer)	1	1	12
原 Yuan-Source	The location where the Original Qi resides and accumulates. In Yin Meridians, the Shu-Stream points are also the Yuan-Source points		1	6
經 Jing-River	The location where the Meridian Qi flows like river	1	1	12
合 He-Sea	The location where the Meridian Qi enters inward and return to the visceral & bowels (*zang-fu*). It is like the confluence of the water into the sea	1	1	12
			Total	66

and ankles in a specific age group of healthy individuals. The mean value of the meridian energy which is represented by the EDA at 2 specific times of measurement has been obtained. The secondary objective is to evaluate the clinical application of the result interpretation between the genders and the specific time of assessment.

To better understand the evaluation, we have streamlined and focused our study on a specific group of healthy individuals aged 20-30 in Taichung city, Taiwan.

2. Materials and Methods

2.1. Type of Study. A single group, nonrandomized, evaluation study was conducted. Method of randomization and blinding was not considered.

2.2. Location of Study. The study was conducted in China Medical University, Taichung, Taiwan. The study protocol was approved under ID: CMUH104-REC1-130 by China Medical University & Hospital Research Ethics Committee.

2.3. Subjects. A total of 43 young healthy adult participants were recruited. Written informed consent of each participant was obtained prior to the assessments.

Eligibility Criteria. Healthy participants aged 20-30 years who provided signed written informed consent were included.

The participants who met following criteria were excluded in the study:

(i) Pregnancy or lactation

(ii) Severe diseases such as carcinomas under chemotherapy, psychological/psychiatric disorders, and chronic heart failure

(iii) Have received pacemaker or coronary intravascular stent placement

(iv) Unable to undergo evaluation with the Meridian Energy Detection System

(v) Alcohol abuse or drug addiction

(vi) Communication disorder

(vii) Refusal to provide informed consent in the study

(viii) Exclusion at Project Investigator's discretion

(ix) Participation in other clinical trials within 3 months

Participants were advised to avoid coffee, tea, or caffeinated beverages before the procedure.

Table 2: Representative Measuring Points used in Ryodoraku.

RMP Name (Hand)	Meridians	Acupoints	RMP Name (Foot)	Meridians	Acupoints
H1	Lung	LU-9 Taiyuan∗	**F1**	Spleen	SP-3 Taibai∗
H2	Pericardium	PC-7 Daling∗	**F2**	Liver	LR-3 Taichong∗
H3	Heart	HT-7 Shenmen∗	**F3**	Kidney	KI-3 Taixi∗
H4	Small Intestine	SI-4 Wangu∗	**F4**	Bladder	BL-65 Shugu
H5	San Jiao (Triple Energizer)	TE-4 Yangchi∗	**F5**	Gall Bladder	GB-40 Qiuxu∗
H6	Large Intestine	LI-5 Yangxi	**F6**	Stomach	ST-42 Chongyang∗

∗ indicates Yuan-Source Points. The Acupoints code and name has followed WHO Standard Acupuncture Points Locations [12, 13].

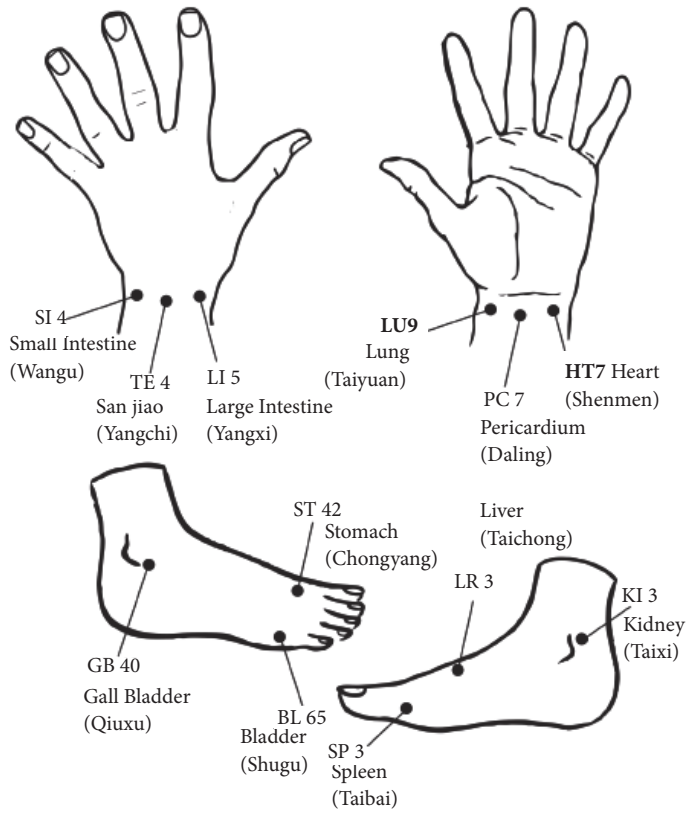


Figure 2: Representative measuring points.

2.4. Materials. Aetoscan Meridian Energy Detection System (Aeto Technology Co. Ltd., Taiwan) was used in this study. It was a simple, noninvasive Meridian Energy Detection System (MEDS) that use low voltage electrical current (3.7V, 200 uA) to detect the energy level (or the electrical conductivity) of the 24 RMPs on the skin of wrists and ankles. The device was connected to its mobile application with iOS system support through Bluetooth with connection encryption. The mobile application provided instant results and analysis as shown in Figure 3 [14].

The software mobile application version 1.0.0.0 was installed in iPad. The raw data of assessment was extracted into Microsoft Excel through the web application.

The result interpretation included the following 5 major indices illustrated in Table 3.

2.5. Procedure. The following procedure was carried out:

(i) The assessment was conducted at 2 specific times of the day: 9-11 am (Spleen meridian) and 1-3 pm (Small Intestine meridian).

(ii) Each participant was invited to sit in a room with controlled ambient temperature of 23.1°C.

(iii) For each session, the probe was sterilized with alcohol swab and the device was digitally calibrated to avoid any confounding factors.

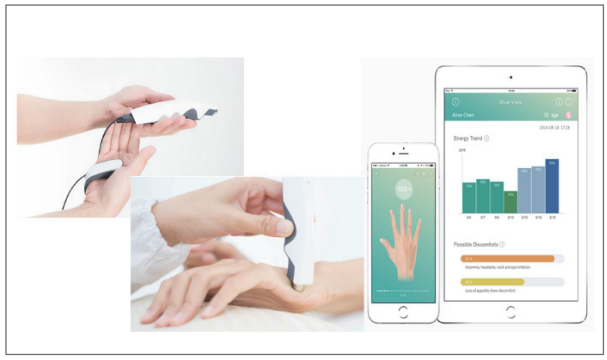


Figure 3: Meridian Energy Detection System.

Table 3: Major Indices used in the application.

Status	Function	Normal Range	Interpretation
Physical Status	It analyzes the average energy balance of all the meridians to evaluate the original Qi (energy) status of the individual.	25-55	Value < 25 indicates Qi deficient while > 55 indicates Qi excessive.
Metabolism Status	It analyzes the metabolism status through the assessment of Yin-Yang ratio in the individual.	0.8-1.2	Value < 0.8 indicates high metabolism due to excessive Yang and deficient Yin while > 1.2 indicates low metabolism due to excessive Yin and deficient Yang.
Musculoskeletal & Circulation Status	It analyzes the musculoskeletal & circulation status in the individual.	0.8-1.2	Value below or above normal range indicates possible musculoskeletal discomfort such as aches and pain.
Mental Health Status	It analyzes the mental health status in the individual.	0.8-1.2	Value below or above normal range indicates possible imbalance in the mental health which one may experience heavy-headed, lightheadedness, insomnia etc.
Autonomic Nervous System Status	It analyzes the autonomic nervous system status in the individual.	1.5-2.0	Value below or above normal range indicates underactive or overactive of the autonomic nervous system.

(iv) The following procedure was repeated in both sessions as illustrated in Figure 4.

(v) The participant was asked to grip the U-shape buckle (electrode) on one palm with constant pressure while the investigator placed the probe at each of the 24 RMP perpendicularly to the skin with even pressure without touching the participant's hands or feet.

(vi) During the procedure, the participant was advised to remove any metallic or electronic accessories including watches to avoid electrical disturbance. Electronic devices such as mobile phones were avoided to be used by the participants too.

2.6. Statistical Analysis. We calculated the mean and standard deviation for each variable to show the distribution and perform as the descriptive analysis. Further T-test was conducted for the comparison analysis. A two-tailed P-value of 0.05 was considered as significant. SAS statistical software (version 9.4; SAS Institute, Cary, NC) was used to conduct the analyses.

3. Results

A total of 43, including 13 males and 30 females, young healthy adult volunteers were participated in this study. The average age was 26.15±6.97 years and 23.27±2.94 years, respectively. There was no participant dropped out due to discomfort during the procedure or repeated assessments. Figure 5 illustrates the overview of the study.

3.1. Baseline Assessment. The baseline assessment such as body weight and height, body temperature (temporal), middle finger temperature, and body mass index (BMI) were

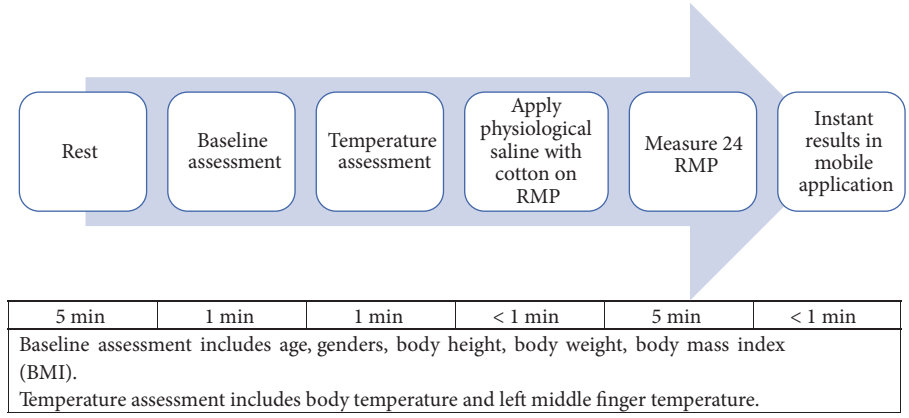


Figure 4: Procedure and duration of assessment.

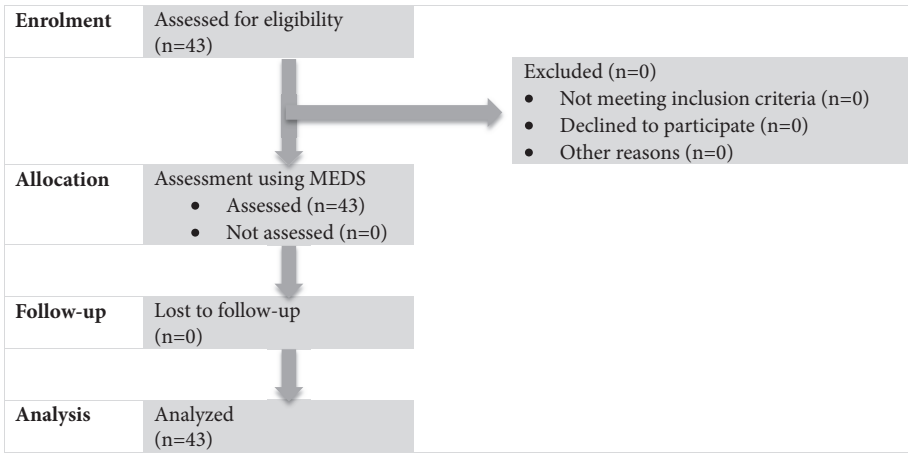


Figure 5: Overview of the study.

obtained. Table 4 provides the results of the baseline assessment.

Table 4 concluded that there was significant difference in the body height, body weight, and finger temperature between the genders within the participants.

3.2. Energy Overview Interpretation

3.2.1. Energy Overview between the Genders. Figure 6 provides the comparison of the 5 major indices between the genders.

It was concluded that there were significant differences in the Physical Status, Mental Health Status, and the Autonomic Nervous System status between the genders within the participants. Healthy male participants generally had higher Qi energy level than female. Healthy female participants had higher values for Mental Health and Autonomic Nervous System status than male. Both male and female had overactive Autonomic Nervous System status.

3.2.2. Energy Overview at Specific Time. Figure 7 illustrates the comparison of the 5 major indices at specific time within the genders.

It was concluded that there was no significant difference between the time of assessment for male participants but there was significant difference in the body temperature and mean Metabolism Status between the time of assessments for female participants.

3.2.3. Meridian Energy Balance of the Yin and Yang Meridians. The results were also categorized based on the Yin and Yang meridians of the left/right and hand/foot by summing the values of Physical Status obtained from genders and specific time as illustrated in Figure 8.

It was concluded that the Meridian Energy Balance of the Yin and Yang meridians of female participants was significantly lower than male participants.

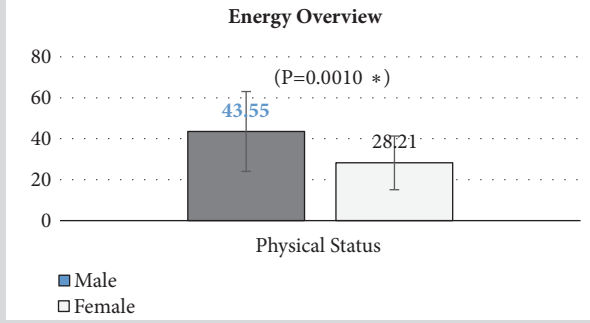
There was no significant difference in the Meridian Energy Balance of the Yin and Yang meridians at different time of assessment.

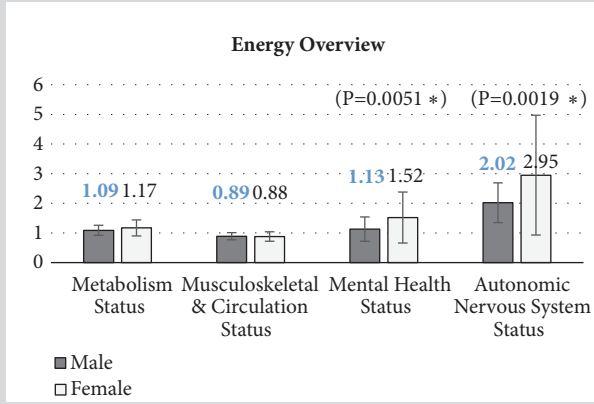
3.2.4. Meridian Energy Balance in Left and Right Meridians. We further categorized and compare the difference in Meridian Energy Balance in the left and right meridians as illustrated in Figure 9.

Table 4: Baseline assessment.

Parameters	Male		Female		P value
Sample Size	13		30		
	Mean	s.d.	Mean	s.d.	
Age (years)	26.15	6.97	23.27	2.94	0.1725
Body Height (m)	1.70	0.04	1.60	0.06	>0.0001∗
Body Weight (kg)	61.8	9.91	54.36	6.38	0.0100∗
BMI	21.42	3.10	21.21	2.17	0.8167
Body Temperature (°C)	35.55	0.71	35.38	0.68	0.3342
Left Middle Finger Temperature (°C)	35.81	2.46	33.93	4.43	0.0173∗

The values represent the mean difference between the genders.
The statistical significance is indicated with ∗ for $p < 0.05$.
s.d. = standard deviation.





The values represent the mean difference between the genders.
The statistical significance was indicated with ∗ for $p < 0.05$.

Figure 6: Energy overview between the genders.

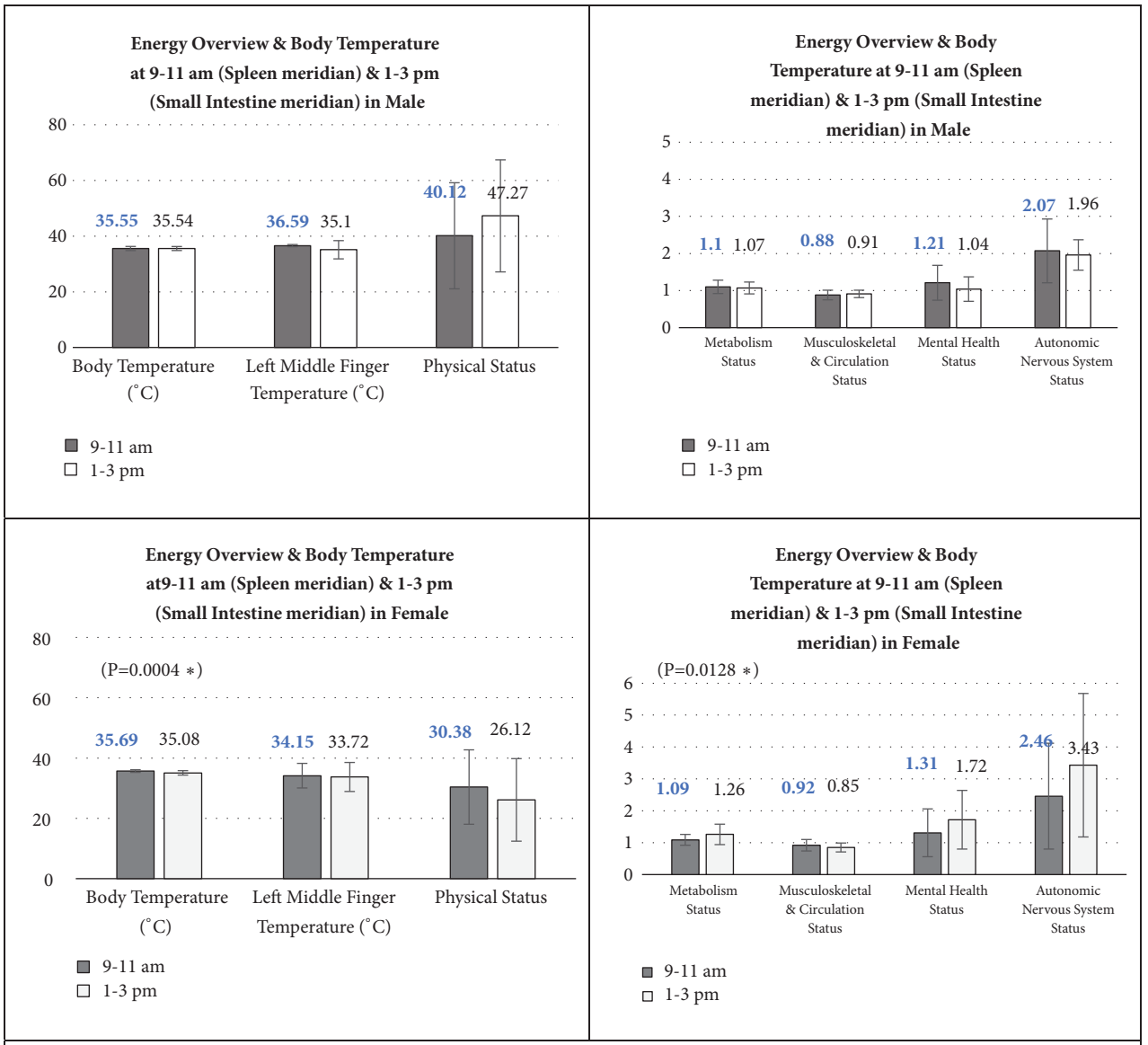
It was concluded that, in both genders, there was no significant difference in Meridian Energy Balance between the left and right meridians, though the right meridian acupoints have higher skin conductivity than the left with the exception of Lung and Pericardium Meridians in female.

3.2.5. Meridian Energy Balance in Left and Right Meridians at Specific Time. Figures 10 and 11 illustrated the comparison in the left and right meridians during the specific time of assessment.

It was concluded that there was no significant difference between the left and right meridians between the 2 specific times of assessment.

4. Discussion

Our specific goal of this clinical study is to evaluate the clinical application of the low voltage Meridian Energy Detection System in assessing the EDA of the 24 RMP bilaterally on both wrists and ankles in a specific age group



The values represent the mean difference between the specific time within the gender.

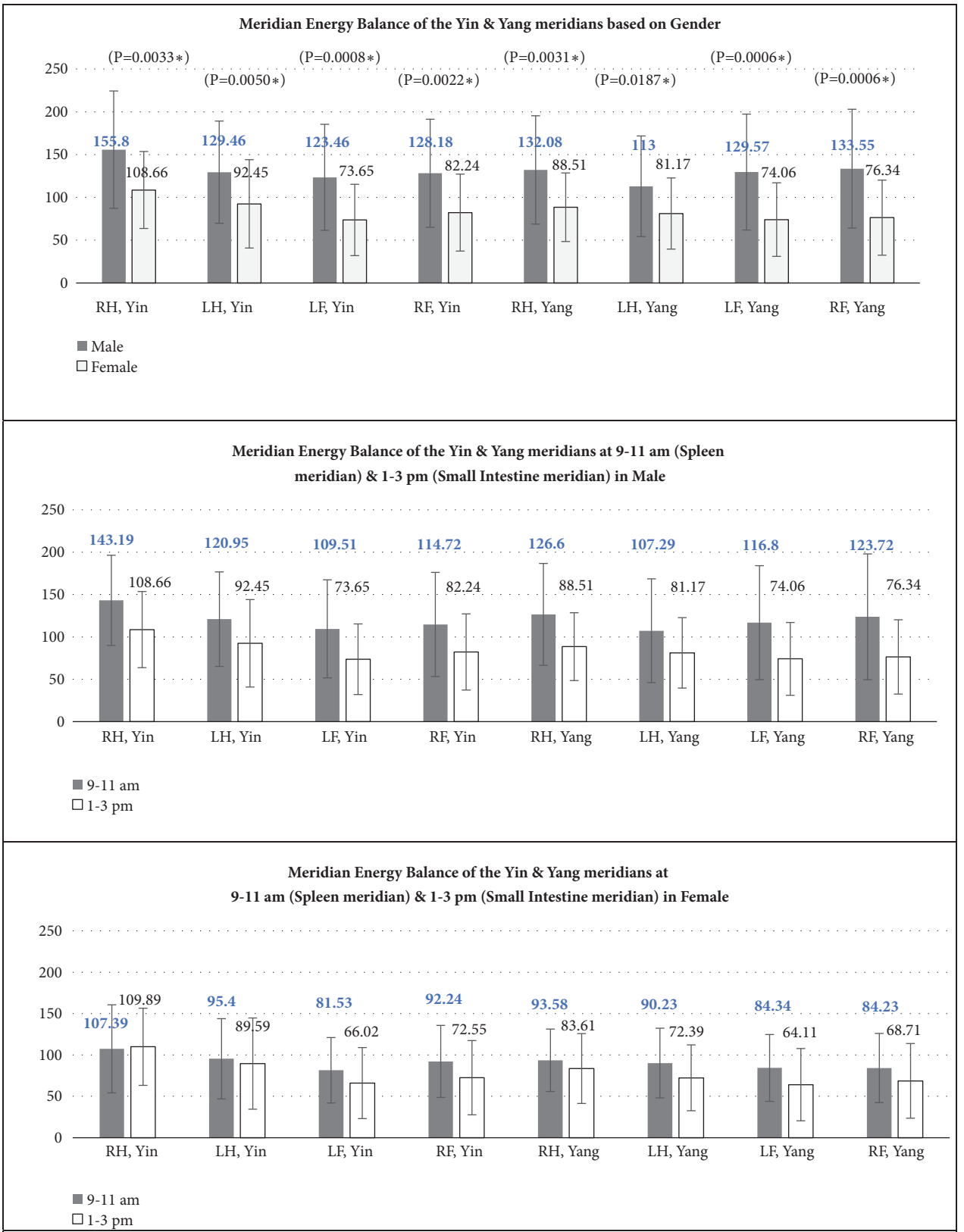
The statistical significance is indicated with ∗ for $p < 0.05$.

Figure 7: Energy overview at specific time within the genders.

of healthy individuals and to determine the difference in the energy overview between the genders and specific time of assessment.

4.1. Factors That Can Influence Electrodermal Activity. Various technical factors that could influence the electrodermal activity (EDA) on the skin has been reported by Andrew et al. (2007) [5]. These included the skin structures such as the integrity, hydration, and thickness; sweat gland density; electrode polarization such as the electrode material and size, current amplitude, and frequency as well as the contact medium used. Other influential factors discussed in other studies included the contact time on the skin, amount of pressure on the skin, precise location of acupoints, and control environment such as the room temperature as well as the degree of skin moisture. Variability in the measurement has resulted in doubt in the reliability of the measurement of EDA. Experienced operators also played an important role in avoiding any confounding factors and to ensure consistency by having sufficient knowledge regarding the use of the device [15–17].

As EDA can also be influenced by the emotion [18], a controlled ambient environment with constant temperature is maintained in the study. In the study, it was observed that generally, healthy female participants had significantly lower mean finger skin temperature than healthy male participants in the same controlled environment. This was similar to the findings by Kim et al. (1998) that the mean finger

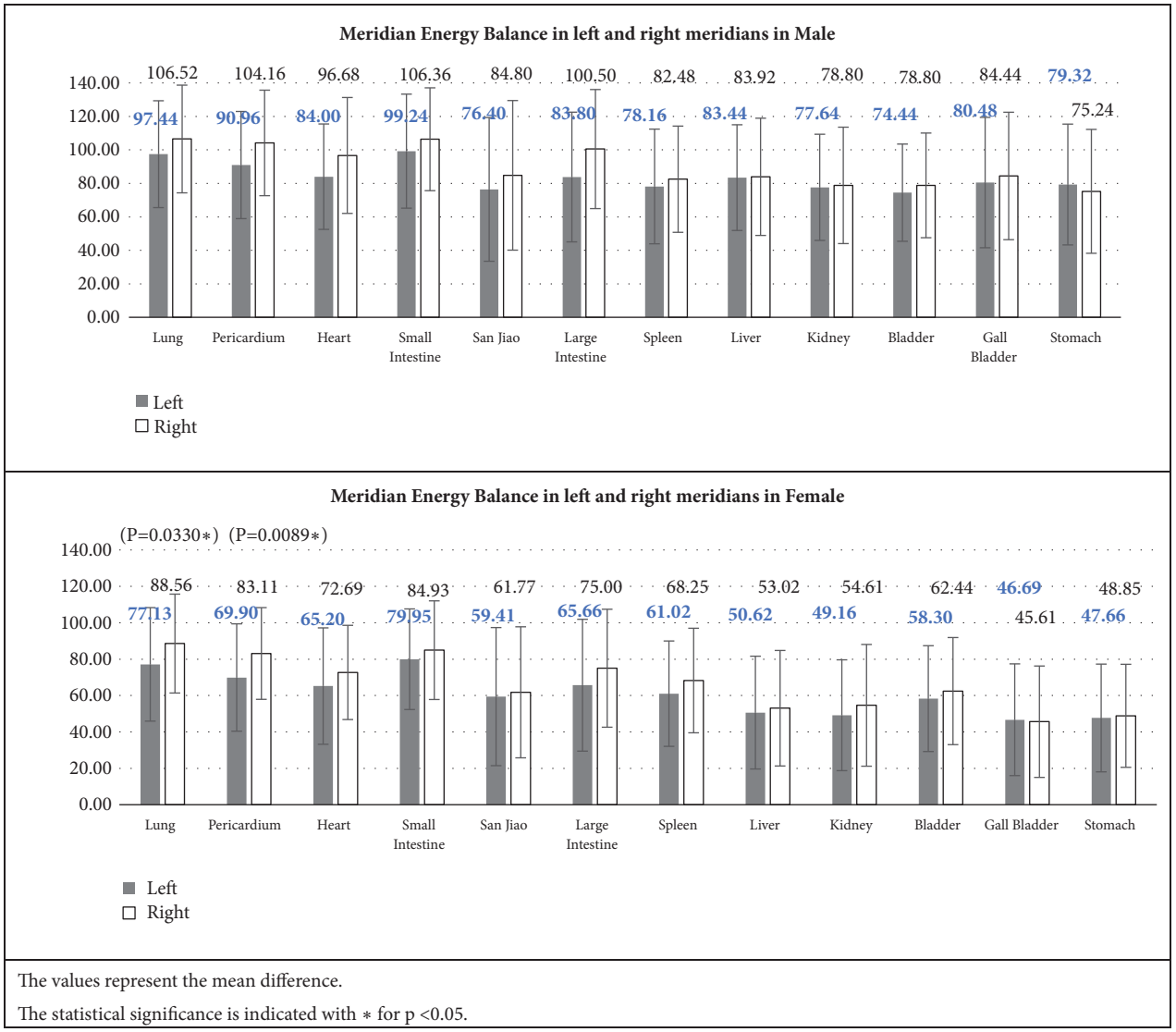


The values represent the mean difference.

The statistical significance is indicated with ∗ for $p < 0.05$.

RH = right hand; LH = left hand; RF = right foot; LF = left foot

Figure 8: Meridian Energy Balance of the Yin and Yang meridians.



The values represent the mean difference.

The statistical significance is indicated with ∗ for $p < 0.05$.

Figure 9: Meridian Energy Balance of the Yin and Yang meridians.

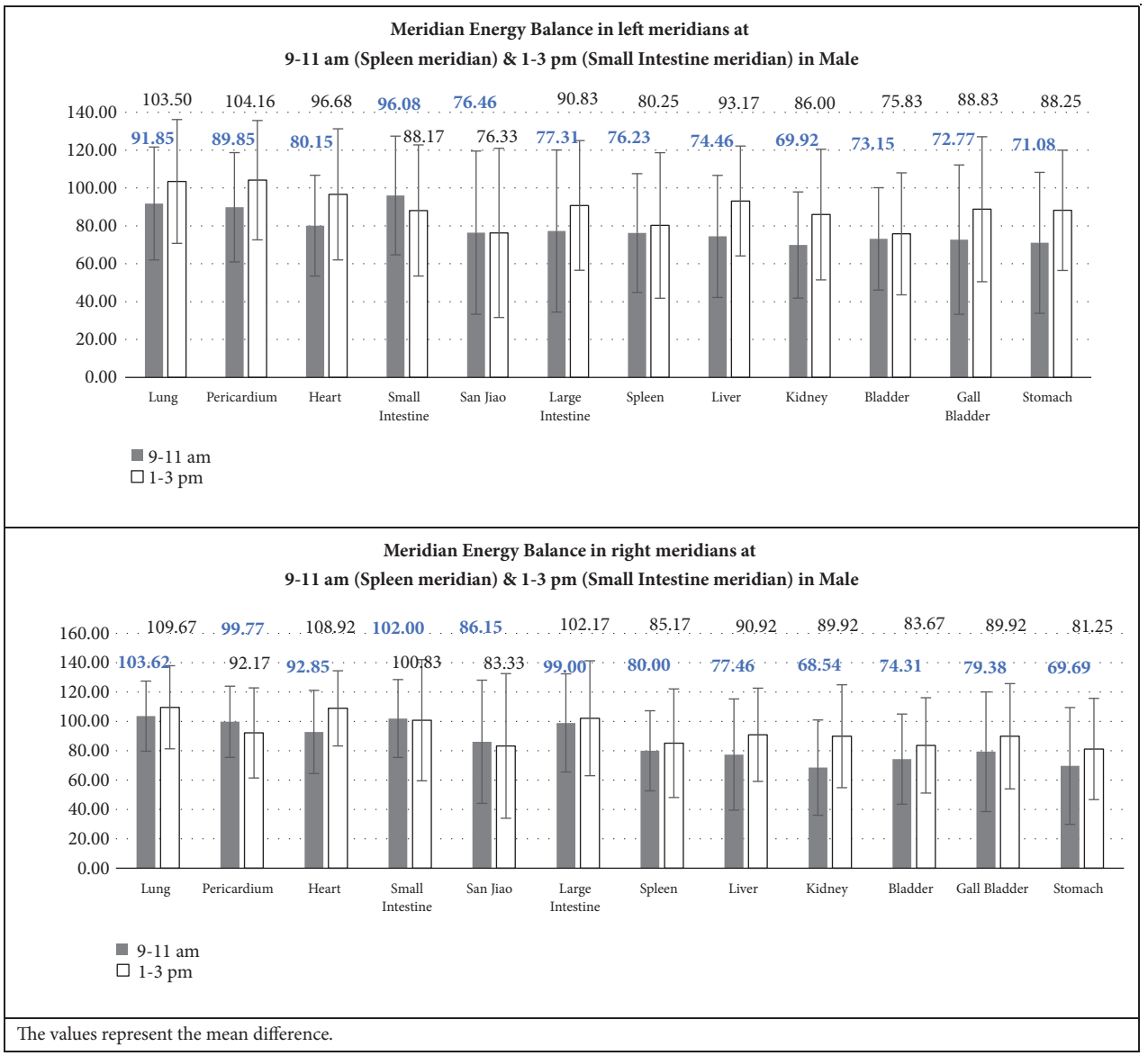
temperature of women is about 2.8 degrees lower than men [19]. However due to the small sample size, there was no significant difference in the mean core body temperature between the genders.

Beside the environmental factors, consumption of caffeinated beverages can also affect the electrical conductance. A study by Tsai et al. (2014) reported that the mean values of electrical conductance increased in most of the meridians 30 minutes after coffee consumption. Hence one should avoid caffeinated beverages before using the device [20].

4.2. Energy Overview between the Genders. Generally, male participants had significantly higher value in Physical Status than female participants. The Physical Status indicated the average energy balance of all the meridians. The higher the value is, the higher the Qi energy is. This results coincided with the study from S. Chamberlin *et al.* (2011) [21] in which a large scale clinical trial was conducted to determine the influence of age, genders, and time of the day on the skin conductance at the 24 Source points. It was reported that the mean skin conductance at acupoints was higher in male.

It was also observed that healthy female participants had higher values for Mental Health and Autonomic Nervous System status which could be possibly due to the fact that female usually experiences more stress and anxiety easier than male. Both male and female have overactive Autonomic Nervous System status. In studies related to stress and depression among university students, it was reported that female students had higher level of stress, depression, frustration, and anxiety than male students [22, 23].

There was no significant difference between the Metabolism Status and Musculoskeletal and Circulation Status in the genders. This could be due to the fact that the subjects recruited were healthy individuals. Future research may be merited to investigate these indices in unhealthy individuals.



The values represent the mean difference.

FIGURE 10: Meridian Energy Balance in left and right meridians at 9-11 am (Spleen meridian) and 1-3 pm (Small Intestine meridian) in Male.

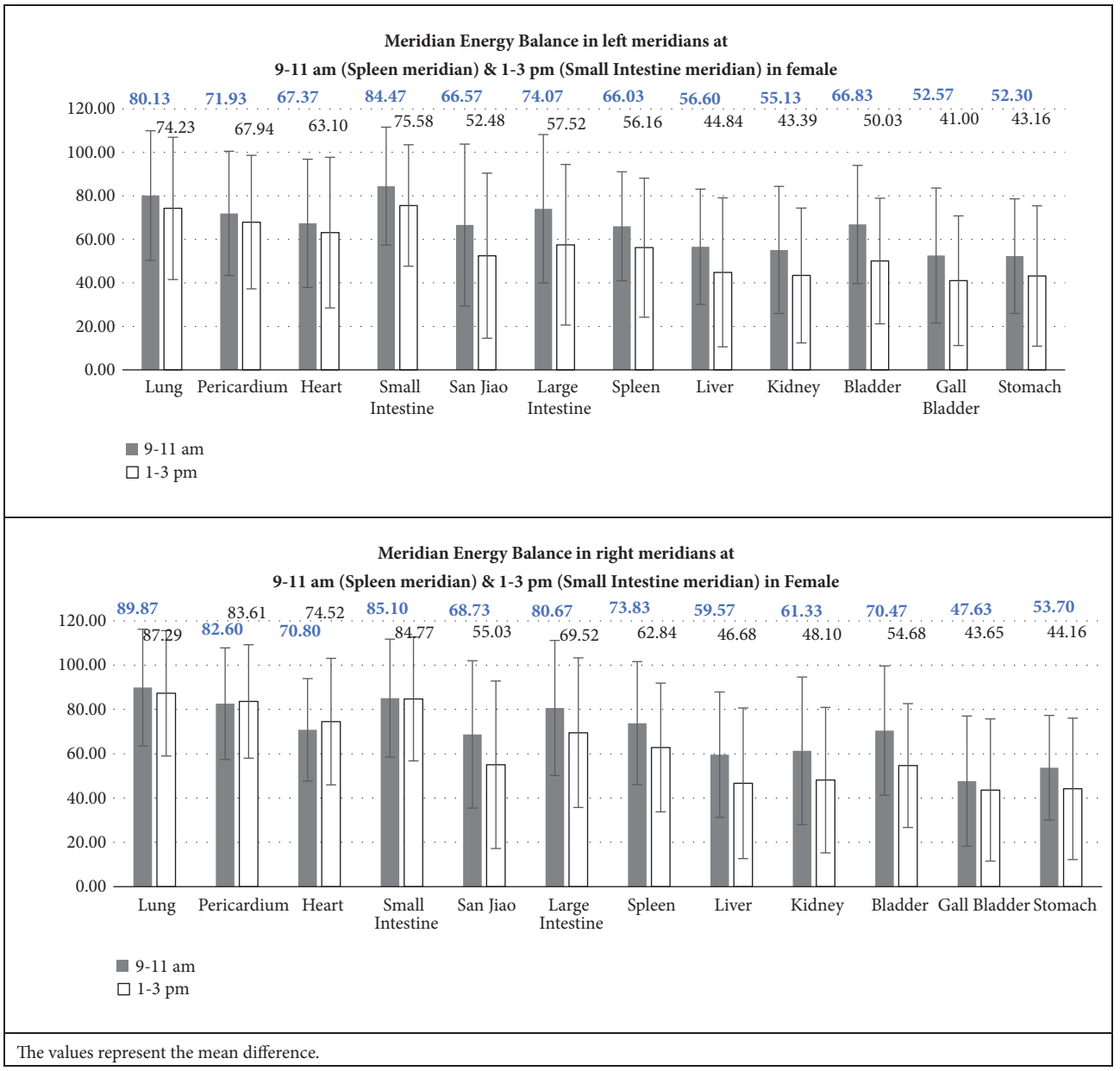
When comparing the Yin and Yang meridian energy, female participants had significantly lower energy level compared to male participants. This affirmed the observation on the Physical Status between the genders that female Qi is more deficient than male.

4.3. Energy Overview at Specific Time of Assessment. There was no significant difference in using the device at the specific time of assessment, i.e., 9-11 am and 1-3 pm for Physical Status, Metabolism Status, Musculoskeletal and Circulation Status, and Mental Health and Autonomic Nervous System status except the Metabolism Status in female. This could be due to some exception that required further investigation.

There was no significant difference when comparing the Yin and Yang or left and right meridians at the specific time of assessment.

Hence the device could be used at these two specific periods of the day without causing variation in the results. However, future research can be designed to investigate whether the time of assessment can affect the results in unhealthy individuals.

4.4. Limitation in This Study. The limitation in this study is the low sample size. Further study with larger sample size, different age groups, and individuals with various disease conditions to be more conclusive in the clinical application of the device is recommended. Studies on the effect of meridian energy level in specific disease conditions and treatments using Meridian Energy Analysis Device, e.g., in abnormal gastroscopy [24], Low Back Pain [25], and Endometriosis-Related Chronic Pelvic Pain [26], have been reported. Similar studies on other disease conditions can be designed to



The values represent the mean difference.

Figure 11: Meridian Energy Balance in left and right meridians at 9-11 am (Spleen meridian) and 1-3 pm (Small Intestine meridian) in Female.

investigate if such device can be used as preliminary diagnosis as well as for treatment evaluation.

We have excluded the study of other functions in the application that can assist in preliminary diagnosis such as Possible Discomfort, Meridian Summary, and Body System Report. Further research could be done to conclude the accuracy.

5. Conclusions

In conclusion, the study using Meridian Energy Detection System in healthy individuals provided an understanding of the difference in energy level of the meridians between the genders. Male healthy individuals had significantly higher values for Physical Status as well as Yin and Yang energy compared to female healthy individuals. Female healthy individuals had significantly higher values for Mental Health and Autonomic Nervous System due to the fact that female experienced stress, depression, and anxiety easier than male. There was no significant difference when comparing the Yin and Yang or left and right meridians at the specific time of assessment. Hence there was no specific time in using the device. However, due to the limitation of the sample size and the healthy subjects, future research can be designed to investigate whether the time of assessment can affect the results in individuals with specific disease conditions in larger scale.

As people are getting more health conscious, the healthcare professionals play an important role in counselling the

patients in their health maintenance. Such Meridian Energy Detection System may be used as a tool for preliminary diagnosis of the overall conditions of the individual. It may be used as a tool to investigate the treatment efficacy by observing the change in the meridian energy level. It merited further studies to be more conclusive.

Data Availability

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

Conflicts of Interest

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

Authors' Contributions

Yu-Chen Lee and Hui Ping Ng equally contributed to this study as co-first authors. Yu-Chen Lee and Wen-Chao Ho are the corresponding authors. Yu-Chen Lee was the principle investigator and Wen-Chao Ho participated in the statistical analysis of the study. Yung-Hsien Chang provided supervision on the study. All authors reviewed and approved the final version of the manuscript.

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Research Article

Safety Assessment of the Auto Manipulation Device for Acupuncture in Sprague-Dawley Rats: Preclinical Evaluation of the Prototype

Geng-Hao Liu,[1,2,3,4] Meng-Yen Tsai,[1] Gwo-Jyh Chang,[2] Chao-Min Wu,[5] Sheng-Kai Lin,[6] Yu-Sheng Chen,[1,2] and Tzung-Yan Lee[7,8]

[1]*Division of Acupuncture and Moxibustion, Department of Traditional Chinese Medicine, Chang Gung Memorial Hospital, Linkou, Taiwan*
[2]*Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[3]*School of Traditional Chinese Medicine, Chang Gung University, Taoyuan, Taiwan*
[4]*Sleep Center, Chang Gung Memorial Hospital, Taoyuan, Taiwan*
[5]*Department of Electrical Engineering, National Central University, Jhongli, Taiwan*
[6]*Delta Electronics, Inc., Taoyuan, Taiwan*
[7]*Graduate Institute of Traditional Chinese Medicine, College of Medicine, Chang Gung University, Taoyuan, Taiwan*
[8]*Department of Traditional Chinese Medicine, Chang Gung Memorial Hospital, Keelung, Taiwan*

Correspondence should be addressed to Yu-Sheng Chen; cuspat@yahoo.com.tw
and Tzung-Yan Lee; joyamen@mail.cgu.edu.tw

Geng-Hao Liu and Meng-Yen Tsai contributed equally to this work.

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Background. The Auto Manipulation Device for Acupuncture (AMDA) is designed for providing stable, quantified effects and higher frequency when doing lifting and thrusting manipulation. The purpose of this study is to investigate the safety of manipulation by AMDA in different frequency and duration in healthy rats. *Methods.* The study was divided into two parts: single intervention and once a day for a week. 12 rats and 15 rats were randomly allocated to different groups: Control (needle insertion only), AMDA (2Hz/10Mins), AMDA (2Hz/20Mins), AMDA (20Hz/10Mins), and AMDA (20Hz/20Mins) for single and repeated interventions. Real-time physiological functions, laboratory data, and the bilateral muscle tissue of acupoint (ST 36) were obtained after the intervention. *Results.* We found neither real-time physiological functions nor laboratory data differences between control group and AMDA groups in both parts. In the muscle tissue samples, the slight damage had been observed in the AMDA group with a frequency of 2 Hz for 20 minutes after once intervention, and the repeated session groups noted more obvious tissue damage with fibrotic change. Although the period was shorter, higher frequency manipulation caused more damage that fibroblast nuclei became more slender and obvious. However, no significant adverse effect was noted such as crippled and molting in the whole process. *Conclusion.* Our study suggested that the safety issue of AMDA operation in rats is feasible because there was no difference between control group and AMDA groups among real-time physiological functions and laboratory data. However, manipulation with higher frequency should be more preserved.

1. Introduction

Acupuncture is one of the most frequently requested complementary therapies [1]. Various disorders can effectively be cured by inserting long, fine needles into specific "acupuncture points" (acupoints) on the skin of the patient's body. Besides China, acupuncture has spread to over 160 countries and regions. The World Health Organization recommends

the use of acupuncture treatment for 43 diseases [2]. However, acupuncture needle manipulation is one of the most fundamental yet widely variable components of acupuncture treatments [3].

Because of the variability forms of manipulation and individual difference of acupuncturists, there exist artificial errors that make scientific studies more difficult to quantize. For providing stable and quantified effects, many researchers provide devices to monitor the frequency and amplitude of manipulation [4]. The Auto Manipulation Device for Acupuncture (AMDA) is designed for providing stable, quantified effects, and higher frequency when doing lifting and thrusting manipulation. The preliminary results have demonstrated the developed AMDA and its plausibility in the clinical application of acupuncture in simulated tissues [5].

The purpose of this study is to investigate the new method of manual manipulation (MA) by AMDA and the safety of different frequency and duration after acupuncture intervention in healthy rats.

2. Material and Methods

The study was divided into two parts: single intervention and once a day for a week (the detailed protocol was shown in Figure 1). Manual lifting-thrusting acupuncture was given with Auto Manipulation Device for Acupuncture (AMDA, prototype).

2.1. Animal Preparation and Recording Procedures. 27 healthy male Sprague-Dawley rats weighing 280±50 g and aged 6-8 weeks were provided by the Bio LASCO animal centre. The animals were maintained in a controlled environment (22±2°C and 50±5% humidity) and under a 12 h/12h light/dark cycle with free access to food and water. This study was performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals of the Animals in Science Regulation Unit of UK. All experimental procedures were approved by the Chang Gung University Institutional Animal Care and Use Committee (IACUC Approval no. CGU15-088) and were conducted in a manner that minimized the number of animals used and the number of procedures per animal.

In experiment 1 (once intervention), 12 rats were randomly allocated to different groups: Control (needle insertion only, AMDA 0 Hz/20Mins, n=4), $AMDA^{ls}$ (2Hz/10Mins), $AMDA^{ll}$ (2Hz/20Mins), $AMDA^{hs}$ (20Hz/10Mins), and $AMDA^{hl}$ (20Hz/20Mins) (n=2 each AMDA group). Real-time physiological functions, including heart rate, systolic blood pressure, mean arterial pressure, diastolic blood pressure, are measured using the tail-cuff method, recorded before the manual acupuncture with AMDA intervention and every five minutes during the course. Then, blood samples, including hepatic, renal function (AST/ALT, BUN/Cr), electrolytes (Na/K), and hemogram (CBC/DC), were also collected after sacrifice. Third, the bilateral muscle of ST36 acupoint was harvested and the histological sections were stained with hematoxylin and eosin (H&E) and were observed under a light microscope (40X, 100X) after the intervention.

In experiment 2 (repeated sessions), all 15 rats received daily manual ST36 acupuncture with AMDA intervention for 7 days and were randomly allocated to different groups: Control (0Hz/20Mins, n=3), $AMDA^{LS}$ (2Hz/10Mins), $AMDA^{LL}$ (2Hz/20Mins), $AMDA^{HS}$ (20Hz/10Mins), and $AMDA^{HL}$ (20Hz/20Mins) (n=3 each AMDA group). Real-time physiological functions, body weight change, the muscle histological sections, and blood samples were also collected in the seventh days.

2.2. Experimental Procedures. The rats were kept in supine position under anesthesia with 4% isoflurane inhalation and maintain the depth of anesthesia as stage III, which was assessed by pedal reflex, preserve normal body temperature using warm thermal pads. The ST36 point in the rat is located at a point 5 mm lateral and inferior to the tibial tubercle. Based on the comparative anatomical localization in rats as compared with that in human, selected points (the location of acupoints is shown in Figure 2) [6, 7].

The region of acupoint was shaved and disinfected; and then a sterilized single-use stainless steel needle measuring 0.27 mm ×13 mm (0.27 mm in diameter and 13 mm in length; Ching-Ming Medical Co., Ltd., Taiwan) was placed on the left side ST36 by a single experienced acupuncturist. The insertion depth was about 6 mm. After the *de qi* sensation, the AMDA was connected to the handle of the acupuncture needle. Afterward, adjust the device to the specific frequency and start the lifting-thrusting manipulation for each group.

In experiment 2, acupuncture was repeated for 7 days. All rats were sacrificed by decapitation, and tissue samples were collected and analyzed [8].

The procedure was carried out in accordance with the IACUC Guidelines.

2.3. Statistical Analysis. Statistical analysis was conducted using IBM SPSS Statistics 21.0 Software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). All data were analyzed using Kruskal-Wallis ANOVA test and presented as mean±SD of the mean (SEM). Significance was considered when $p<0.05$.

3. Results

3.1. Experiment 1 (Once Intervention). In experiment 1 (once intervention), real-time physiological functions before the intervention were collected and showed no significant difference in each group (all $P>0.05$). After the intervention, we found neither real-time physiological functions nor blood samples differences between control group and AMDA groups (Table 1).

In the muscle tissue samples, the slight damage had been observed in the AMDA group with a frequency of 2 Hz for 20 minutes (Figure 3(a)). As long as the period prolonged, the damage increased in an order: control (AMDA 0 Hz/20Mins) < $AMDA^{ls}$ (2Hz/20Mins) < $AMDA^{hs}$ (20Hz/10Mins) < $AMDA^{hl}$ (20Hz/20Mins) (Figure 3(a)).

3.2. Experiment 2 (Repeated Sessions). After 7 days of repeated acupuncture intervention, the data presented in

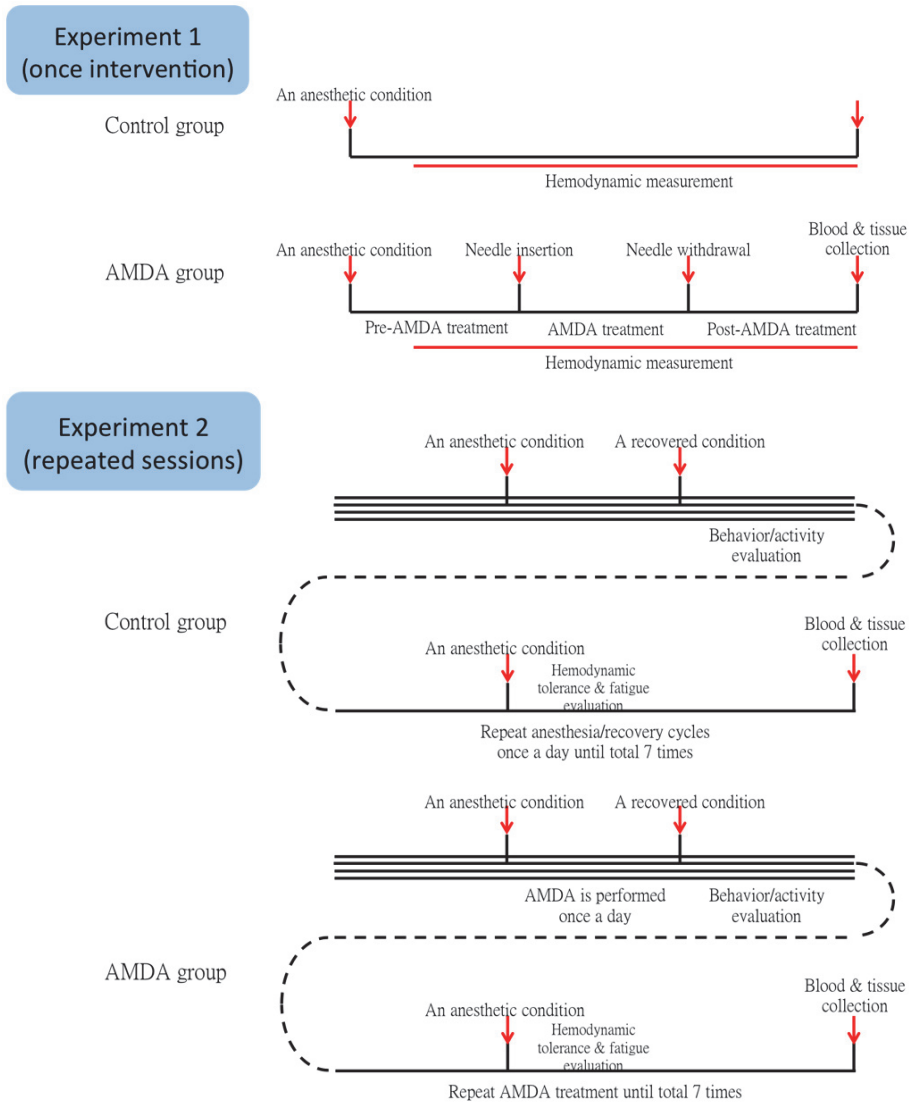


Figure 1: Protocol of experiment 1 (once intervention) and experiment 2 (repeated sessions).

Table 2 showed that it still had no significant change in the real-time physiological function, including heart rate, systolic blood pressure, mean arterial pressure, and diastolic blood pressure in the rats. Also, it did not affect hepatic or renal function (AST/ALT, BUN/Cr), electrolyte (Na/K), or hemogram (CBC/DC).

In addition, there was no significant body weight change between those groups (P=0.220). No significant adverse effect was noted such as crippled and molting in the whole process.

In the muscle tissue samples, the damage had been observed in all AMDA groups. In comparison to once intervention group with a frequency of 2Hz for 10 minutes, the repeated session groups noted more obvious tissue damage with fibrotic change (Figure 3(b)). Our study found that although the period was shorter, higher frequency manipulation causes more damage that fibroblast nuclei became more slender and obvious (Figure 3(b)). In conclusion, the damage increased in an order: control (AMDA 0 Hz/20Mins) < $AMDA^{LS}$ (2Hz/10Mins) < $AMDA^{LL}$ (2Hz/20Mins) < $AMDA^{HS}$ (20Hz/10Mins) < $AMDA^{HL}$ (20Hz/20Mins).

4. Discussion

The main purpose addressed by this study was whether AMDA was available for providing a stable and safe new method acupuncture manipulation with different frequency and duration in rats. As most patients receive more than one acupuncture therapy in the treatment course, this study was conducted in two phases for investigating short-term and repeated effects. We hypothesized that MA manipulation by AMDA may affect the rats in three ways: real-time physiological functions, laboratory finding, and tissue damage. It follows that neither real-time physiological functions nor

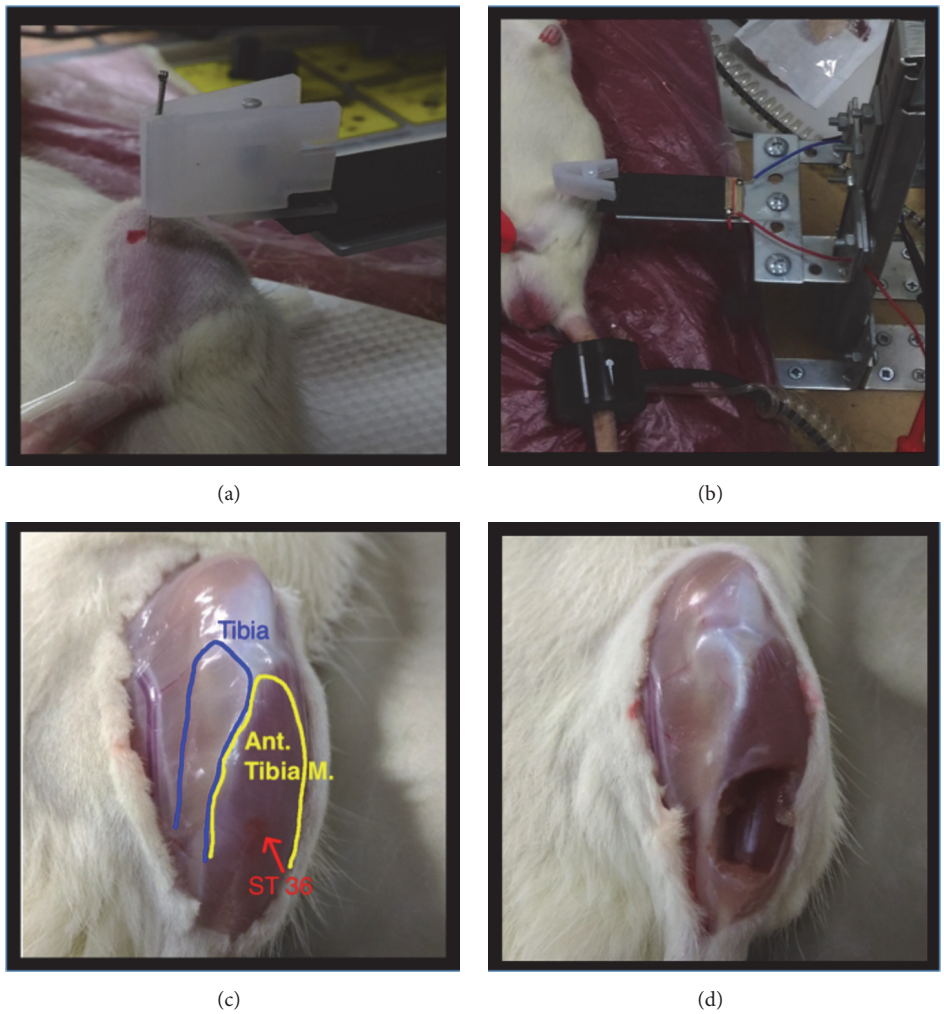


Figure 2: **Manual acupuncture at ST36 with AMDA and tissue sampling from ST 36. (a)** ST 36 was located 4 mm bellowed and 1-2 mm lateral to the midpoint of the knee. Manual acupuncture with AMDA **(b)** was performed after well anesthesia. **(c)** After rechecking the margin of muscle (yellow) and bone (blue), the muscle tissues of ST 36 (red) were obtained **(d).**

laboratory blood test differences between control group and AMDA groups in both time courses. Moreover, there was no significant adverse effect noted during the course. The results presented here reveal the safety issue of AMDA operation in rats is feasible.

However, according to the histologic review, a single AMDA operation with 20 Hz will have a slight damage to muscle tissue. After daily manipulation with AMDA, regardless of the needle frequency, muscle tissue over acupoint (ST36) became fibrosis. Moreover, AMDA with higher frequency (20Hz) make clearly visible tissue tear. As the needle retention time was extended to 20 minutes, the fibroblast nuclei are also more slender and obvious.

For fibroblasts and endothelial cells, focal adhesions form mechanical links between extracellular collagen matrix and intracellular cytoskeleton. It was evident that the needle movement was an effective mechanical stimulus leading to tissue displacement [9]. Tissue tension is likely sensed by fibroblast by their adhesion to collagen fibers [10, 11]. Langevin observed that mechanical coupling between the needle and connective tissue with a winding of tissue around the needle during needle manipulation transmits a mechanical signal to connective tissue cells via mechanotransduction [9, 12].

We knew that the therapeutic effectiveness of acupuncture could be influenced by multiple factors. Basic science experiments, mostly in animals and healthy human subjects, show that acupuncture needling has demonstrable physiological effects that are dependent on needling parameters, including needle insertion depth, type, amplitude, and frequency of needle stimulation [13]. Between therapeutic effectiveness of acupuncture and connective tissue damage, the control of parameters of manipulation was quite important. In this study, we can deduce that the most important factor cause tissue damage is the frequency of manipulation, and

Table 1: Baseline vital signs, hemogram, and biochemistry laboratory data in single intervention.

Baseline vital signs	Control group (n=4)	AMDA group (n=8)	P
HR (bpm)	377.8±15.9	355.9±16.8	.056
SBP (mmHg)	89.0±4.5	85.6±6.5	.379
MBP (mmHg)	70.3±4.6	63.1±7.3	.109
DBP (mmHg)	61.0±5.0	52.0±9.5	.110
Vital signs	Control group (n=4)	AMDA group (n=8)	P
HR (bpm)	431.3±43.4	423.4±40.1	.734
SBP (mmHg)	103.5±20.6	101.4±18.5	.932
MBP (mmHg)	80.5±9.7	80.1±12.9	.865
DBP (mmHg)	69±4.8	69.8±10.3	1.000
Hemogram			
WBC (1000/uL)	5.25±3.78	5.79±3.32	.610
RBC (million/uL)	5.013±1.513	5.28±1.496	.610
Hb (g/dL)	10.63±3.43	11.19±3.25	.552
Hct (%)	33.65±11.01	35.83±10.34	.396
MCV (fL)	66.7±2.28	67.81±2.68	.497
MCH (pg/Cell)	21.08±0.67	21.18±0.64	.495
MCHC (gHb/dL)	31.6±0.22	31.23±0.73	.200
Plt (1000/uL)	436.5±494.4	470.3±420.6	.552
Seg (%)	21.98±12.26	15.13±6.04	.308
Lym (%)	74.3±14.29	82.56±6.1	.396
Mono (%)	3.45±2.34	1.75±0.99	.234
Eosin (%)	0.1±0.2	0.38±0.43	.096
Baso (%)	0.18±0.21	0.19±0.15	.930
Biochemistry laboratory data			
BUN (mg/dL)	11.95±3.72	11.24±2.33	.609
Cr (mg/dL)	0.27±0.062	0.244±0.041	.670
Na (mEq/L)	146±0.8	146.1±1.8	1.000
K (mEq/L)	5.93±0.57	6.59±0.29	.061
AST (U/L)	112.3±5.3	111.3±7.8	.932
ALT (U/L)	44.5±4.2	49.6±7.5	.147

HR, heart rate; SBP, systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure. WBC, white blood cell; RBC, red blood cell; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelet; Seg, segment; Lym, lymphocyte; Mono, monocyte; Eosin, eosinophil; Baso, basophil. BUN, blood urea nitrogen; Cr, Creatinine; Na, sodium; K, potassium; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

Mean ± standard deviation was presented for vital signs (HR, SBP, MBP, and DBP), hemogram (complete blood count and differential count), and biochemistry laboratory data (BUN/Cr, Na/K, and AST/ALT). No significant difference between the 2 groups was found at baseline and once intervention study.

then it is the time of needle retention and repeated times of manipulation.

Another study that evaluates the variable frequencies of manual acupuncture at ST36 in rats with atropine-induced inhibition of gastric motility also found that twirling manipulations with frequencies of 1, 2, and 3 Hz had better therapeutic effects than a frequency of 4 Hz on the recovery of the gastric motility amplitude [14]. Therefore, the frequency of manual acupuncture influences not only the therapeutic effects but the safety of acupuncture intervention.

On the other hand, electroacupuncture at ST36 increases the concentration and reorganization of collagen in the rat model of tendon healing [15]. The subtle differences in acupuncture needle manipulation techniques can affect cellular responses in mouse subcutaneous connective tissue [16]. Further studies will be needed to determine whether those manipulations are related to therapeutic responses.

Now, modern imaging and cell biology techniques have been employed to study the nature of acupuncture and many researchers build many models to explain acupuncture such as mechanistic function, neural response, or electrical response. For example, earlier studies have shown that rotation of an inserted acupuncture needle stretches nearby connective tissue by pulling collagen fibers from the periphery toward the needle [11, 17]. On the other hand, current study found that the acoustic shear wave, being a mechanical energy, is capable of mechanotransduction, stimulating cytosolic Ca2+ rise in both excitable and nonexcitable cells, producing Ca2+ oscillations and memory, and giving rise to in vivo calcium fluorescence and endorphin release into

Table 2: Vital signs, hemogram, and biochemistry laboratory data in repeated sessions study.

	Control group (n=3)	AMDA group (n=12)	P
Rat body weight gain (%)	12.85±2.25	12.69±2.65	.942
Vital signs			
HR (bpm)	411.3±17.4	382.7±29.7	.149
SBP (mmHg)	84.7±11.2	87.1±9.8	.771
MBP (mmHg)	68.3±13	67.3±10.6	1.000
DBP (mmHg)	59.3±14.2	57.6±11.8	.828
Hemogram			
WBC (1000/uL)	7.4±2.96	8.43±4.09	.613
RBC (million/uL)	6.357±1.384	6.801±0.698	.773
Hb (g/dL)	13.47±2.8	14.21±1.3	.772
Hct (%)	41.83±8.82	44.48±3.89	.773
MCV (fL)	65.87±0.81	65.58±3.11	.563
MCH (pg/Cell)	21.2±0.26	20.93±0.89	.347
MCHC (gHb/dL)	32.23±0.21	31.94±0.36	.128
Plt (1000/uL)	516.7±373.2	523.7±322.2	.885
Seg (%)	16.33±2.25	16±7.41	.248
Lym (%)	81.5±2.43	81.45±7.45	.278
Mono (%)	1.4±0.1	1.53±0.93	.884
Eosin (%)	0.5±0.2	0.76±0.6	.563
Baso (%)	0.27±0.06	0.26±0.12	.939
Biochemistry laboratory data			
BUN (mg/dL)	15.2±3.87	14.3±3.43	.773
Cr (mg/dL)	0.213±0.015	0.23±0.045	.716
Na (mEq/L)	145.3±1.5	146.8±2.2	.210
K (mEq/L)	6.57±0.12	6.93±0.61	.346
AST (U/L)	121.7±16.3	125.8±23.7	.885
ALT (U/L)	50.7±2.3	54.1±8.1	.469

Mean ± standard deviation was presented for the percentage of rat body weight gain, vital signs (HR, SBP, MBP, and DBP), hemogram (complete blood count and differential count), and biochemistry laboratory data (BUN/Cr, Na/K, and AST/ALT). No significant difference between the 2 groups was found at chronic AMDA study.

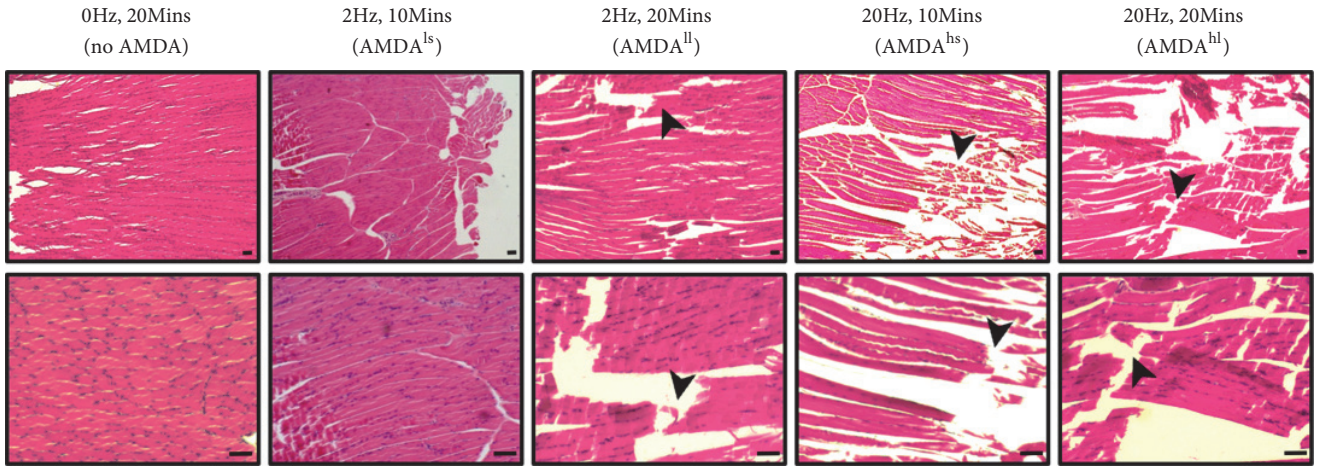
blood plasma in mice [18]. However, there were some current studies which suggest that the initial action of acupuncture appears to be mechanical and not neural or electrical. The mechanism of acupuncture still needs further evidence to be proved.

As for the manipulation effect for fibroblasts, previous studies have shown that both acupuncture needle rotation and simple tissue stretching cause fibroblasts to increase their cross-sectional area, as their cell bodies expand and spread out [11, 19]. The fibroblast responsiveness along a plane of connective tissue could be the source of purines that led to adenosine-mediated acupuncture analgesia some distance away from the needle [20]. The response of fibroblasts to acupuncture still needs further study to distinguish the benefit and the adverse effect of a high frequency intervention.

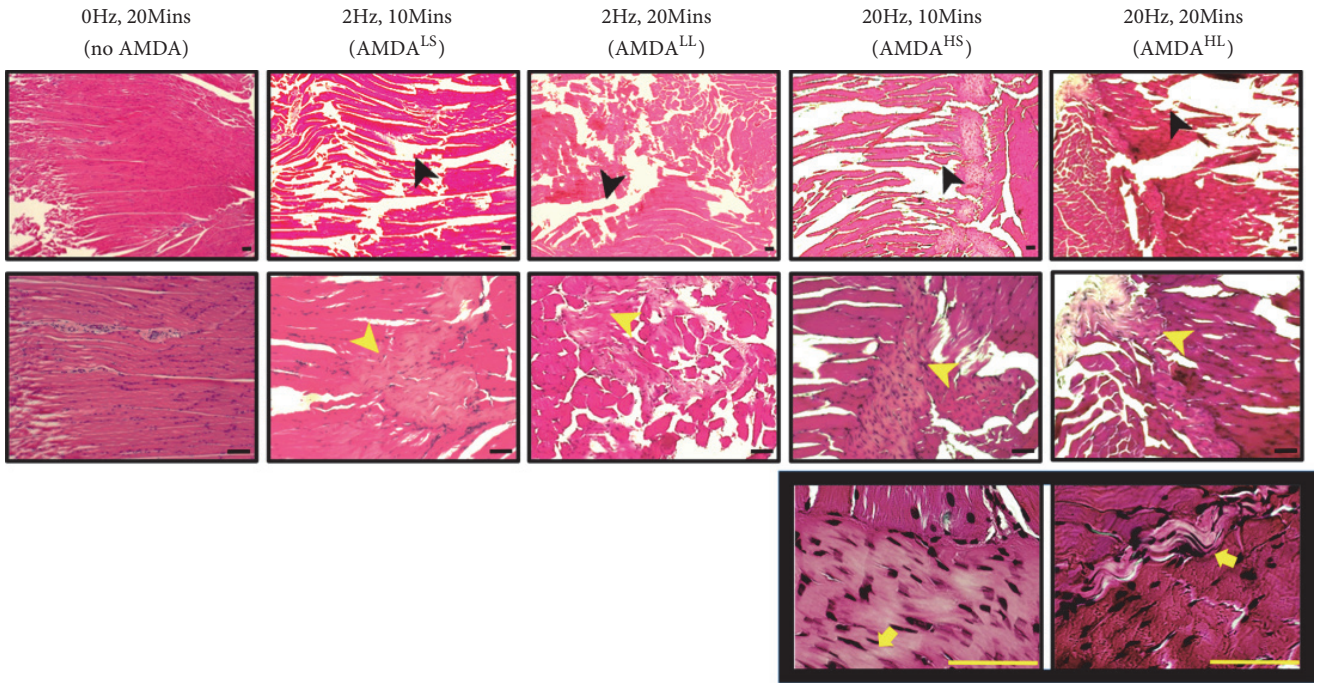
However, the size proportion of the needle to the body of a rat was different from that of a human. Thus, the muscle tissue damage may be overrated in the animal model. Even though our study only focuses on rats, the damage of repeated needle intervention still was an important issue. Skin changes such as localized lipoatrophy and hypertrophic scar have been reported in some review articles, especially during a relatively long treatment period [21]. In some case reports, epithelioid granuloma, pseudolymphoma, and scars at needling sites were also mentioned [22].

Guidelines of World Health Organization on basic training and safety in acupuncture have proposed the safety in acupuncture, including prevention of infection, contraindications, management of accidents, and untoward reactions [23]. We suggested avoiding high frequency and repeated manipulation, based on our result of muscle pathology and clinic observation of patients with acupoint-fibrosis. At the same time, try to avoid the same acupoint in the treatment course for decreasing the stimulation of same muscle tissue and the incidence of muscle tissue fibrosis.

One advantage of the study is that we try to provide a new and safe method for acupuncture study to solve the variability forms of manipulation and individual difference of acupuncturists, which is another way to reduce artificial errors and quantified the effects. The limit of our study includes the related small amount sample and may need further study for evaluation the fibrosis tissue and the



(a)



(b)

Figure 3: Once intervention (a) and repeated sessions (b) of AMDA effect on muscle tissue at acupoint ST36 in different needle frequency and retention time. The bar of proportional scale is 100 um. AMDA with higher frequency (20Hz) make clearly visible tissue tear (black arrowhead). Regardless of the needle frequency in repeated sessions, fibrotic change (yellow arrowhead) was noted. As the needle retention time was extended to 20 minutes, the fibroblast nuclei (yellow arrow) are also more slender and obvious. (a) Once intervention. ls, low frequency (2Hz) and short duration (10mins); ll, low frequency (2Hz) and long duration (20mins); hs, high frequency (2Hz) and short duration (10mins); hl, high frequency (2Hz) and long duration (20mins). (b) Repeated sessions. LS, low frequency (2Hz) and short duration (10mins); LL, low frequency (2Hz) and long duration (20mins); HS, high frequency (2Hz) and short duration (10mins); HL, high frequency (2Hz) and long duration (20mins).

differences between manipulations. On the other hand, even though the experienced acupuncturist performed the needle manipulation to the *de qi* sensation first, the followed AMDA could not sense and maintain it in the whole process. Another question that may be asked is whether the finding from rats is applicable to humans.

5. Conclusions

Our study suggested that the safety issue of AMDA operation in rats is feasible because there was no difference between control group and AMDA groups among real-time physiological functions and laboratory sample test in both intervention courses. However, lifting-thrusting manipulation with

higher frequency should be more preserved, especially in patients that need more often acupuncture intervention, such as chronic arthritis, sciatica, and cerebrovascular disease. Further studies will be needed to investigate the potential of AMDA.

Data Availability

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

Conflicts of Interest

The authors declared no conflicts of interest.

Authors' Contributions

Details of the each author with his/her contribution in this paper are as follows: Geng-Hao Liu contributed to acupuncture, sample collection, statistics, and writing of manuscript; Meng-Yen Tsai contributed to writing of manuscript; Gwo-Jyh Chang contributed to tail-cuff equipment lending; Chao-Min Wu contributed to AMDA prototype supervisor; Sheng-Kai Lin contributed to AMDA prototype maker; Tzung-Yan Lee is supervisor; Yu-Sheng Chen contributed to origin idea of AMDA and is supervisor. Geng-Hao Liu and Meng-Yen Tsai contributed equally to this study.

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Supplementary Materials

The low and high frequency (2/20 Hz) manipulation of the AMDA prototype have been recorded as a video file and as a supplementary document. *(Supplementary Materials)*

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Research Article

Effectiveness and Safety of Acupotomy for Lumbar Disc Herniation: A Randomized, Assessor-Blinded, Controlled Pilot Study

So Yun Kim,[1] Eunseok Kim,[2] Ojin Kwon,[3] Chang-Hyun Han,[3] and Young-Il Kim[1]

[1]*Department of Acupuncture and Moxibustion Medicine, Daejeon University Dunsan Korean Medicine Hospital, Daejeon 35235, Republic of Korea*
[2]*Department of Acupuncture & Moxibustion Medicine, College of Korean Medicine, Daejeon University, 62 Daehak-ro, Dong-gu, Daejeon 34520, Republic of Korea*
[3]*Clinical Research Division, Korea Institute of Oriental Medicine, Daejeon 34054, Republic of Korea*

Correspondence should be addressed to Chang-Hyun Han; chhan@kiom.re.kr and Young-Il Kim; omdkim01@dju.kr

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Objective. Patients with lumbar disc herniation (LDH) suffer from pain, physical disabilities, and low quality of life. This study was designed to evaluate the effectiveness and safety of acupotomy in patients with LDH. *Method*. Fifty participants with LDH were recruited to this randomized, assessor-blinded, controlled study and randomly assigned to the acupotomy (n = 25) or manual acupuncture (n = 25) group. The acupotomy group received acupotomy four times in 2 weeks, while the manual acupuncture group received manual acupuncture six times in 2 weeks. The follow-up visit was planned in the 4th week (i.e., 2 weeks after the final intervention). The primary outcome was the change in the Visual Analogue Scale (VAS) at follow-up. The changes in the Oswestry Disability Index (ODI), Modified-Modified Schober Test (MMST), and EuroQol Five Dimensions (EQ-5D) questionnaire were also evaluated. An intention-to-treat analysis was applied and adverse events were recorded. *Results*. The acupotomy group showed significant changes in VAS, ODI, and EQ-5D after intervention. VAS and ODI in the 4th week were lower in the acupotomy than in the manual acupuncture group. The acupotomy group showed consistent changes in VAS and ODI in the 1st, 2nd, and 4th week. No serious adverse event was reported in the acupotomy group. *Conclusion*. This study suggests greater therapeutic effects of acupotomy on relieving pain and improving the functional disability associated with LDH than those observed with manual acupuncture.

1. Introduction

Lumbar disc herniation (LDH) is a state of displaced intervertebral disc material, including the nucleus pulposus or annulus fibrosis, which can cause low-back pain and/or radicular pain, paresthesia, or weakness in the lower limbs [1, 2]. Current therapies include nonsurgical and surgical methods. The nonsurgical treatments include conservative management and invasive treatments [3, 4]. For the conservative management, nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, muscle relaxants, and steroidal drugs are commonly used among others. Nonpharmacological strategies such as acupuncture, physical therapy, and exercise are also recommended. If the conservative therapy fails to relieve the symptoms, more invasive treatments such as epidural steroid injection can be applied. When these more invasive procedures fail to control the LDH symptoms, subsequent surgeries are considered.

Before considering surgery, it is important to discuss other therapeutic options in patients who received nonsurgical treatments with no success. Epidural injections are effective in pain relief for a short time but may require additional injections because of recurring symptoms [5, 6]. There have been attempts to find effective and safe treatments before surgical procedures for symptomatic LDH [7–9].

As a valuable option in LDH, acupotomy has the characteristics of both acupuncture and surgical procedures [10, 11]. Acupotomy uses a bladed needle composed of a

thick, flat-head, and cylindrical body, which is optimized for alleviating the adhesion of a lesion. Acupotomy is used for musculoskeletal pain such as low-back, postneck, knee, and shoulder pain and even in metabolic diseases such as diabetes and lymphatic edema [12–15].

Although the potential benefit of acupotomy in patients with LDH has been previously suggested, more evidence is needed regarding its effectiveness and safety [11, 16, 17]. Therefore, we designed this controlled, pilot study on the effectiveness and safety of acupotomy in patients with LDH compared with manual acupuncture.

2. Method

2.1. Design and Participants. The study protocol has been previously published [18]. In summary, 50 participants with symptomatic LDH were recruited to this randomized, two parallel-armed, controlled, assessor-blinded, single center, pilot clinical trial. The participants were recruited from the outpatients at Daejeon University Dunsan Korean Medicine Hospital (DUDKMH) from July 11, 2016, to January 20, 2017. The study was approved by the Institutional Review Board of DUDKMH (DJDSKH-16-BM-05) and registered in the Clinical Research Information Service (CRIS) (KCT0002188) in South Korea.

To test the effectiveness of acupotomy in 40 participants (20 per group), a total of 50 participants (25 per group) were recruited after considering the 20% drop-out rate. Patients aged between 20 and 80 years who were diagnosed with LDH and showed symptoms such as low-back pain, radicular pain, and paresthesia in the lower limbs were recruited. The LDH diagnosis was based on medical imaging findings. Patients who were at the bulging stage based on medical images were excluded, and those who were diagnosed with LDH at the protrusion and extrusion stages or above were recruited.

2.2. Randomization and Blinding. Participants were randomly assigned to either the acupotomy group (n = 25) or the manual acupuncture group (n = 25). The random number list was generated by an independent statistician. Participants were informed of the possibility of random allocation and of each intervention procedure. Different researchers were in charge of the intervention procedure, outcome assessment data management, and statistical analysis. As the intervention procedure and needle sizes vary distinctively between acupotomy and acupuncture, it was difficult to blind the participants and intervention practitioners. Outcome assessors and data managers were blinded to the allocation status of each participant. Each intervention practitioner performed only one intervention, either acupotomy or acupuncture, and was not involved in the outcome measurements.

2.3. Interventions. In acupotomy group, four acupotomy treatments were administered twice per week for 2 weeks using flat-head-screw-driver-shaped stainless steel disposable sterilized acupotomy needles (1.2 mm in diameter and 75 mm in length; Hansung Precision Manufacture, Seoul, South Korea). The insertion points were set at the corresponding disc level based on the medical imaging finding, 20–30 mm away from the spinous process, to the depth of 50–60 mm. They could be inserted on one side or both sides, according to the symptom and appearance of LDH on the medical image, and no more than three acupotomy sites were chosen. After local sterilization with 10% betadine solution and anesthetization with lidocaine, the needle was inserted, manipulated, and removed immediately. Disposable sterilized wet-cuppings were applied on the acupotomy site for 5 minutes to prevent local hematoma [19].

In the manual acupuncture group, six manual acupuncture treatments were administered three times per week for 2 weeks using 0.25 mm × 40 mm, single-use, sterile, stainless steel needles (Dongbang Acupuncture Inc., Chungnam, Republic of Korea). The acupuncture treatment was performed at GV3 and bilateral BL23, BL24, BL25, BL26, GB3, BL40, and BL60 [20]. The depth of insertion was 20 mm for BL40 and BL60 and 30 mm for the other acupuncture points. The needles were removed after 15 minutes.

Acetaminophen was administered to the acupotomy group for rescue medication. Details of the intervention and cointervention have also been published [18].

2.4. Outcome Measurements. The primary outcome was the change in the Visual Analogue Scale (VAS) score for low-back pain and/or leg pain between baseline and follow-up visit on the 4^{th} week (2 weeks after the last intervention). The secondary outcome measures included the Korean version of Oswestry Disability Index (ODI) [21], Modified-Modified Schober Test (MMST) [22], and EuroQol Five Dimensions (EQ-5D) questionnaire [23]. All the above outcomes were measured at the screening visit and 1^{st}, 2^{nd}, and 4^{th} week after randomization.

The adverse events were assessed during each visit based on the vital signs, medical examinations, and other test results. The causal relationships between adverse events and acupotomy or acupuncture intervention and severity of the adverse events were assessed, such as pain, bleeding, hematoma, or bruise.

All the outcome values were registered on the eCRF, which was designed by Korea Institute of Oriental Medicine and only accessible to nonblinded researchers.

2.5. Statistical Analysis. Statistical analyses were performed using SAS® software (version 9.1.4, SAS institute, Inc., Cary, NC) by a statistician blinded to the participant allocation. If a participant received the intervention and provided the outcome measurement more than once, the data would be analyzed. The independent sample t-test or Wilcoxon signed-rank test for continuous variables and the chi-squared or Fisher's exact test for categorical variables were used to examine potential differences in baseline demographics and medical history variables between the acupotomy and manual acupuncture groups. The analysis of covariance (ANCOVA) was used to compare the mean changes in VAS, ODI, MMST, and EQ-5D from the baseline to 4^{th} week. The paired t-test or Wilcoxon signed-rank test was used to compare the outcomes before and after treatment within each group. The repeated measures analysis of variance (ANOVA) was used to assess the differences between the two groups

Table 1: Baseline VAS, MMST, ODI, and EQ-5D between two groups.

	Acupotomy group (n = 25)	Manual acupuncture group (n = 25)	p-value
VAS	63.88 ± 16.599	60.60 ± 17.260	.497
MMST	4.57 ± 1.774	4.66 ± 1.566	.853
ODI	38.40 ± 12.657	32.37 ± 14.190	.120
EQ-5D	0.78 ± 0.117	0.72 ± 0.118	.115

M, mean; SD, standard deviation; VAS, Visual Analogue Scale, MMST, Modified-Modified Schober Test; OD, Oswestry Disability Index; EQ-5D questionnaire, EuroQol Five Dimensions questionnaire.

Table 2: Observed outcomes and p-value of adjusted group differences in the analysis of covariance.

Variable	Week	Acupotomy group (n=25)	Manual acupuncture group (n=25)	Adjusted mean difference	p-value
VAS	Baseline	62.74 (16.666)	58.83 (16.846)	18.56	.002
	4	37.52 (24.714)	52.48 (23.216)		
MMST	Baseline	4.69 (1.786)	4.84 (1.348)	0.55	.129
	4	5.48 (1.581)	5.01 (1.304)		
ODI	Baseline	36.81 (11.678)	30.84 (13.641)	8.56	<.001
	4	27.24 (12.302)	30.76 (14.078)		
EQ-5D	Baseline	0.779 (0.121)	0.737 (0.112)	.005	.458
	4	0.792 (0.114)	0.757 (0.111)		

Observed outcomes are presented as mean (SD).
VAS: Visual Analogue Scale; MMST: Modified-Modified Schober Test; ODI: Oswestry Disability Index; EQ-5D questionnaire: EuroQol Five Dimensions questionnaire.

over time. A value of P <.05 was considered statistically significant.

3. Results

3.1. Recruitment and Baseline Data. Of the 56 participants with LDH who were interviewed during the recruitment period, 50 met the inclusion criteria and were randomly assigned to the acupotomy group (n = 25) and the manual acupuncture control group (n = 25) (Figure 1). Of the 50 participants, 46 completed this study, while the remaining four dropped out. Of the four participants who dropped out, two participants in the acupotomy group withdrew their consent during their participation in the research, and one was withdrawn due to the side effects of lidocaine. One participant in the control group was dropped because of receiving additional acupuncture treatment for back pain at another clinic before completing this trial.

Demographic and medical data like sex, age, weight, height, smoking, drinking, medical history of hypertension, diabetes mellitus, or others and medication use were collected and analyzed. No significant differences in age, sex, weight, height, smoking status, alcohol consumption, medical history, and medication use were found between the two groups (p >.05).

Table 1 shows the mean baseline VAS, MMST, ODI, and EQ-5D for the acupotomy and manual acupuncture group.

3.2. Primary Outcome. We compared the change of mean VAS scores between two groups by ANCOVA. The covariate value of the mean VAS score was more significantly reduced in the acupotomy group at the 4th week from the baseline compared with the manual acupuncture group (P <.01) (Table 2).

We also compared the difference in VAS score before and after the intervention within each group by independent sample t-test. In the acupotomy group, the mean VAS score significantly decreased after acupotomy (from 62.74±16.666 to 37.52±24.714, P <.001). In the manual acupuncture group, although the mean VAS score decreased after manual acupuncture, there was no significant difference (from 58.83±16.846 to 52.48±23.216, P >.05) (Table 2).

3.3. Secondary Analysis. After the paired t-test of the baseline and 4th week outcomes, other secondary outcomes showed the following results: The mean MMST score significantly increased after acupotomy (from 4.69±1.786 to 5.48±1.581, P <.05), whereas it did not increase significantly after manual acupuncture (from 4.84±1.348 to 5.01±1.304, P >.05). The mean ODI score significantly decreased after acupotomy (from 36.81±11.678 to 27.24±12.302, P <.001), whereas it did not decrease significantly after manual acupuncture (from 30.84±13.641 to 30.76±14.078, P >.05). The mean EQ-5D score significantly increased after both acupotomy (from 0.779±0.121 to 0.792±0.114, P <.05) and manual acupuncture (from 0.737±0.112 to 0.757±0.111, P <.001) (Table 2).

We performed ANCOVA to compare the change in the secondary outcomes between the two groups from the baseline to 4th week. The covariate value of the mean ODI score was more significantly reduced in the acupotomy group

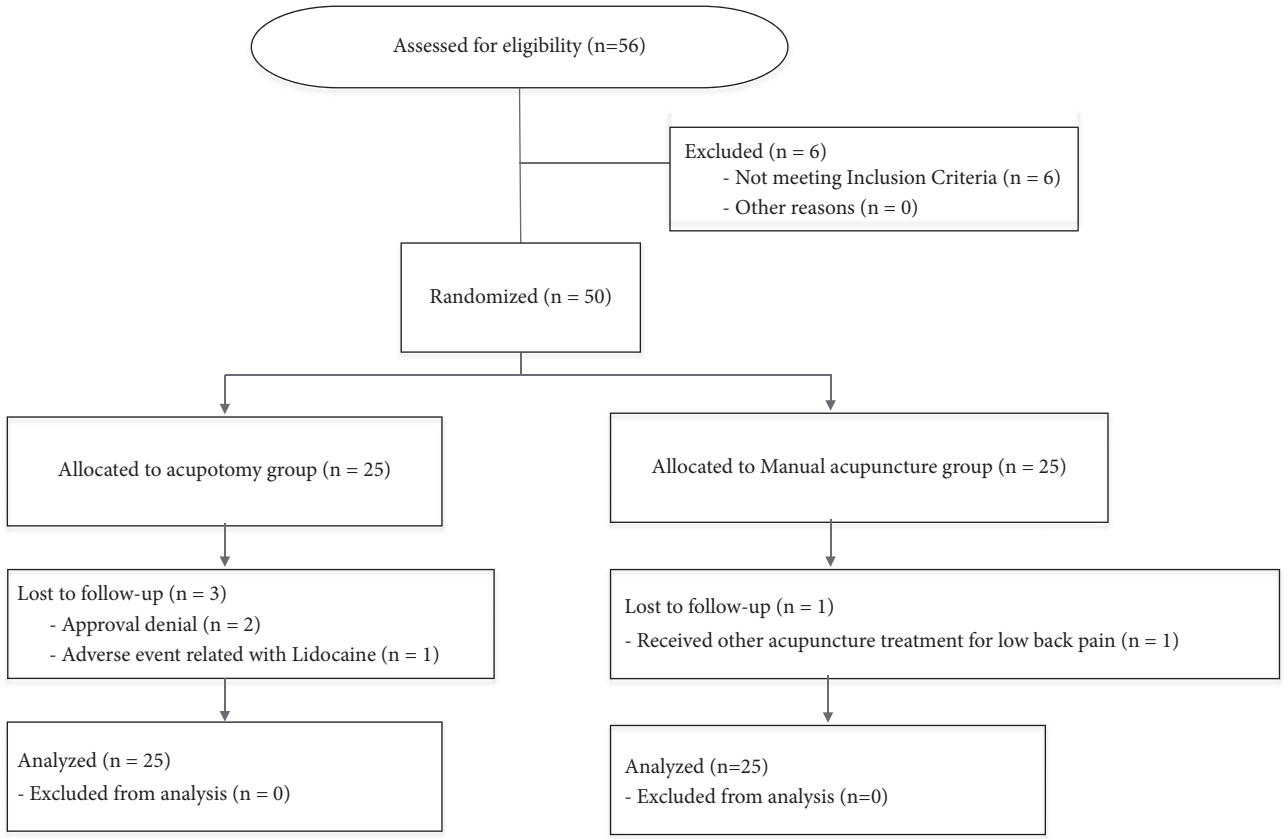

Figure 1: Flow diagram.

at the 4th week from baseline compared with the manual acupuncture group (P <.001). No significant differences in the changes in the mean MMST and EQ-5D scores were found between the two groups (P >.05) (Table 2).

To investigate if the time × group interaction effect on outcome measures is significant, a repeated measures ANOVA was performed using the measurements obtained at the 1st, 2nd, and 4th weeks. For accurate results, we first investigated whether the variables satisfied the assumption of sphericity before performing the repeated measures ANOVA. The VAS and ODI scores satisfied the assumption of sphericity, and MMST and EQ-5D scores did not satisfy the assumption of sphericity. The statistical significance of the trends in the VAS, ODI, MMST, and EQ-5D scores at the 1st, 2nd, and 4th weeks was presented in Figure 2. The change was significant in VAS score (P <.01) and ODI score (P <.001) but not in MMST and EQ-5D score.

3.4. Safety of Acupuncture. Seven participants reported a total of nine adverse events. There were two adverse events in two participants, which were thought to be related to this clinical trial. Two cases were observed in the acupotomy group. In one case, a participant became dizzy after undergoing the first acupotomy session and recovered the day after. The participant underwent the subsequent acupotomy sessions as scheduled, not feeling dizzy. The second case included delirium, dizziness, nausea, and vomiting, which was considered to have resulted from lidocaine use [24]. The participant was withdrawn from this trial, although recovering without any sequela. The other seven cases included premenstrual disorder, common cold, and stomach ache, which were not related to this trial.

4. Discussion

This study was designed to investigate the clinical effectiveness of acupotomy compared with manual acupuncture and the safety of both interventional methods. The VAS score decreased more significantly in the acupotomy group, in which participants received four acupotomy sessions for 2 weeks, than in manual acupuncture group, in which participants received six manual acupuncture sessions for 2 weeks, suggesting a better therapeutic effect on relieving the pain caused by LDH. After acupotomy, MMST, ODI, and EQ-5D were also improved significantly, and more significant changes were shown in the ODI compared with the manual acupuncture. The VAS and ODI decreased constantly as the acupotomy sessions went on, which can be interpreted as the fact that the improvement of pain and disability is not temporary but consistent.

The concept of acupotomy existed in ancient times, but the modern form of acupotomy was first introduced by

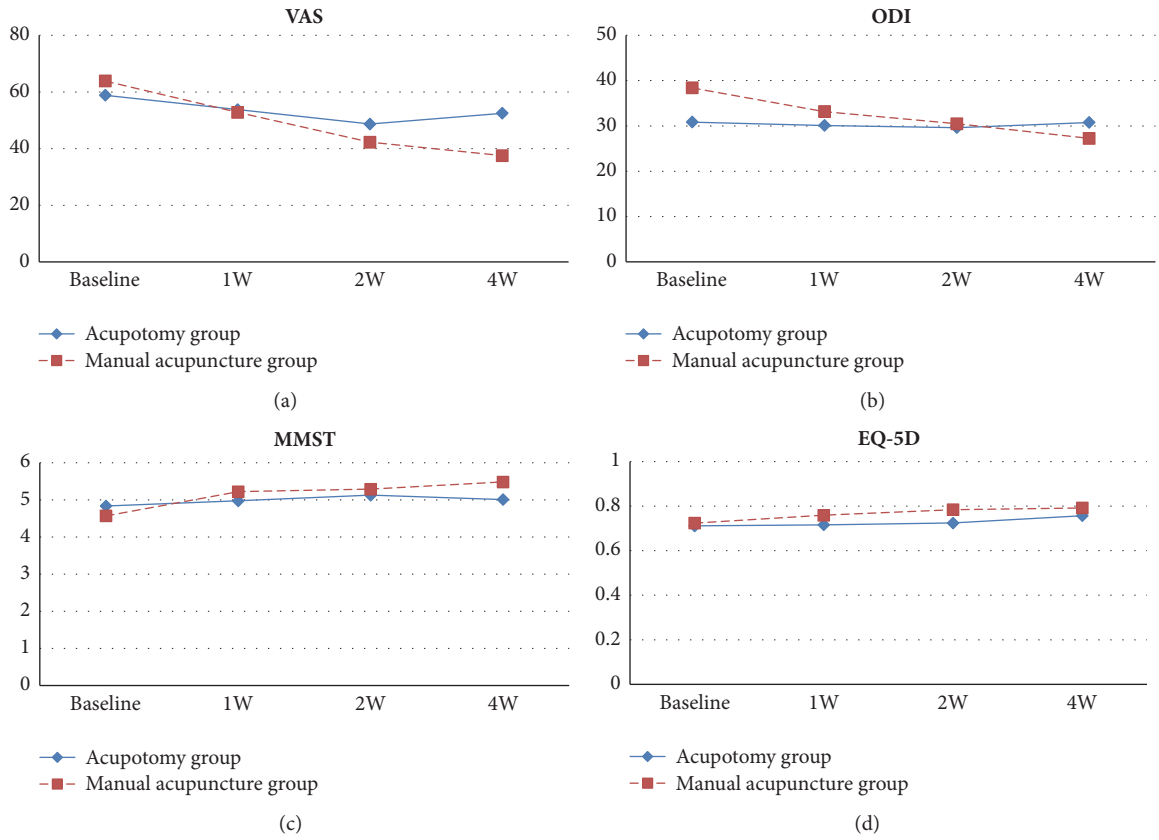

Figure 2: The time × group interaction effect on VAS, ODI, MMST, and EQ-5D.

a Chinese professor named Hanzhang Zhu in 1975 [25]. Acupotomy needle possesses greater diameters than a manual acupuncture needle and can be applied not only on traditional acupoints but also on musculotendinous junctions in contact with muscles, tendons, and bones [26]. Acupotomy can recover the dynamic function of soft tissues, relieve abnormal pressure applied on nerves, and promote Qi-blood circulation to relieve pain [27]. These acupotomy mechanisms have been utilized in the treatment of various disorders [11–17]. There have been extensive researches on appropriate methods for LDH, which can induce chronic pain and reduce the quality of life (QOL) [28], and acupotomy is thought to be another option. In clinical practice, we have applied acupotomy on patients with lumbar spinal disorders including herniated disc and stenosis and observed satisfactory therapeutic effect.

In this study, acupotomy produced superior results compared to manual acupuncture, possibly because of the following reasons. First, the needles used in acupotomy could restore the biodynamic balance at the lumbar spine by releasing unnecessary tension in deep muscles, recovering muscle strength, and reducing muscle fatigue to mitigate the lumbar extensor muscle imbalances [16]. Second, acupotomy may be more effective than manual acupuncture in that acupotomy can ameliorate the nerve entrapment physically, as well as promote the circulation of a lesion. When a lumbar nerve root gets compressed by fibrous tissues, the adhesion can cause low-back pain and/or radiating pain [29]. Furthermore, local adhesion disrupts the blood flow, and when it comes to the circulation to nerves, the symptoms above can be worse [30]. In another point, because LDH is associated with the atrophy of the paraspinal muscles [31], acupotomy may help to recover the atrophy by relieving the nerve root entrapment indirectly. Third, we can explain the therapeutic effects of acupotomy with a stronger stimulus than manual acupuncture procedure. By directly applying a strong stimulus at the painful spot with an acupotomy needle, the pain threshold can be lowered and the signals that affect pain perception can be reduced [32].

The present study has several limitations. First, the population of recruited participants was small. Second, the follow-up period was short. Lastly, the effects of manual acupuncture were not very evident. This result might be due to short intervention period and weak stimulation made by thin needle compared with acupotomy. It would be useful for future studies to consider monitoring more participants for a longer term, increasing the number of treatment sessions, and using larger acupuncture needles.

5. Conclusion

The present results indicated a significant difference in the intensity of pain and disability in patients with LDH after 4 times of acupotomy compared with 6 times of manual acupuncture for 2 weeks. The ability to reduce the pain and disability continued significantly as the acupotomy treatment

was repeated suggesting that multiple acupotomy treatments can relieve pain and disability caused by LDH effectively.

Data Availability

All the outcome values were registered on the eCRF, which was designed by Korea Institute of Oriental Medicine.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Analysis of Facial Features according to Sasang Types between Native Japanese and Native Korean Populations

Lin Ang,[1,2] Jong Yeol Kim,[1,2] and Jeongyun Lee[3]

[1]*Korea Institute of Oriental Medicine (KIOM), 1672 Yuseong-daero, Yuseong-gu, 34054 Daejeon, Republic of Korea*
[2]*Korean Medicine Life Science, University of Science and Technology, 217 Gajeong-ro, Yuseong-gu, 34113 Daejeon, Republic of Korea*
[3]*Division of Clinical Medicine, School of Korean Medicine, Pusan National University, 49 Busandaehak-ro, Mulgeum-eup, Yangsan-si, 50612 Gyeongsangnam-do, Republic of Korea*

Correspondence should be addressed to Jeongyun Lee; prajnamoon@gmail.com

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Background. Facial diagnosis is a common practice and essential diagnostic method used in the Sasang Constitution Medicine (SCM). SCM is a kind of personalized medicine in Traditional Korean Medicine which categorizes people into four types, namely, Tae-Yang (TY) type, Tae-Eum type (TE), So-Yang (SY) type, and So-Eum (SE) type. This study was conducted to compare and analyze the differences in the facial feature across Sasang types among native Japanese and native Koreans. *Methods.* A total of 843 subjects were recruited for this study, 127 native Japanese and 716 native Koreans, respectively. Facial feature points and the measurements of facial features were assigned and calculated automatically using a facial analysis program. Data of each Sasang type for both genders were also extracted and analyzed. Analysis of covariance was then used to examine the differences in facial feature variables among native Japanese and native Koreans and Sasang types. *Results.* Significant differences were seen in the facial feature variables related to lower face area and eye shape. In males, TE types had wider mid-face and lower face as compared to other constitutions. Male TE types were also seen to have narrower eyes whereas male SY types had rounder eyes. In females, TE types had wider lower face width and area compared to SY types and SE types. Female SY types also had rounder eyes. *Conclusions.* This study presented distinctive feature in the lower face area and eye shape among the Sasang types in both native Japanese and native Koreans. This proposed that facial feature variables can also be used as an objective tool in distinguishing the Sasang types in native Japanese. Further studies are needed in the future to generalize these results.

1. Background

Sasang Constitutional Medicine (SCM) is a kind of typological personalized medicine in Traditional Korean Medicine which categorized people into four types, Tae-Yang (TY) type, Tae-Eum (TE) type, So-Yang (SY) type, and So-Eum (SE) type. This theory was established and written in the classic Longevity and Life Preservation in Eastern Medicine by Lee Jema around the year 1894 [1]. According to this classic, the determination of Sasang type comprised several diagnosis elements such as facial appearance, type of body shape, biopsychological traits, pathophysiological symptom diagnosis, and type-specific clinical response [2–4].

In the midst of those diagnosis elements, facial appearance can be regarded as one of the most important aspects [5]. Although SCM classics had descriptions of the facial characteristics of the four Sasang types, those statements are relatively subjective and less quantitative [1, 6–13]. Therefore, many efforts had been made to standardize and objectify the diagnosis of Sasang types using facial features [14–22].

In addition, SCM specialists had strongly accounted for facial features as one of the most reliable elements in distinguishing Sasang types [23]. Many studies had been conducted and facial features were shown to be highly employed in the determination of Sasang types [6, 16, 24–26]. Another study had also mentioned that face shape was found to be more utilized among all the facial elements. Recently, a standardization approach on the facial diagnosis of Sasang types according to quantitative analysis of facial features was established and the representative facial images

of each Sasang type among native Koreans were made public in the year 2012 [5, 27].

The purpose of this study was to expand the usage of facial features in the determination of Sasang type towards other ethnicities. We investigated and compared the facial features among native Japanese and native Koreans across Sasang types by collecting their facial photographs and analyzing them quantitatively.

2. Methods

2.1. Subjects

2.1.1. Native Japanese (Sample A). This study was conducted at Tohoku University, Sendai, Japan, from 2010 to 2011 after receiving approval from the Institutional Review Board of Tohoku University where a total of 127 native Japanese were recruited. All the subjects who were eligible met the following inclusion criteria: (1) age ranging from 18 to 40 years and (2) being healthy and not suffering from chronic diseases. Subjects who have undergone plastic surgery or facial reconstruction surgery due to trauma were excluded. Male subjects with excessive facial hair were also excluded. A written consent form was signed by all the subjects before participating in this study.

2.1.2. Native Korean Subjects (Sample B). Data collected by Korea Constitutional Multicenter Bank (KCMB) between 2007 and 2010 were used in this study. Data of 716 native Koreans whom their age ranged from 18 to 40 years were extracted and analyzed. This procedure was done with the approval of the Korean Institute of Oriental Medicine Institutional Review Board (I-0910/02-001).

2.2. Classification of Sasang Types. The determination of Sasang types for both native Japanese and native Korean was performed by trained experts using Sasang Constitutional Analysis Tool (SCAT) under standard operating procedures, where the SCAT system analyzed the combination of information on facial images, body shape, voice, and questionnaires [20, 22]. In terms of questionnaires, the Korean language questionnaires developed for the use of SCAT were translated into Japanese language and the reliability assessment of the questionnaire was performed [28].

In order to further confirm the Sasang constitutions of the subjects, the Sasang constitutions of native Japanese subjects were confirmed by a certified Sasang medicine specialist (JY Kim) who has more than 8 years of clinical experience accompanied by a professional translator whereas Sasang constitutions of native Korean subjects were then further determined based on the response of subjects towards Sasang type-specific herbal medicine. Sasang constitution of the native Korean subjects was confirmed by a certified Sasang medicine specialist when they showed improvements in ordinary symptoms and did not suffer from adverse effects after taking the prescription for 50 or more days.

2.3. Facial Photography. Frontal full face and profile pictures are essential and should be taken with a neutral facial expression for all subjects. Pictures were taken at a fixed subject-camera distance of 1.6 m using a Nikon D700/D5100 digital camera with 85 mm lens under bilateral illumination. The camera was maintained at the same height as each subject. Images were taken at a resolution of 3184 × 2120 pixels in JPEG format using 24-bit RGB encoding. As for the subjects, ears and hairline of each subjects have to be revealed using a hair tie or hair band during the photography shoot. A ruler used for converting pixels into millimeters was placed approximately 1 cm below the chin. The first shot is the full face frontal view. Subjects were instructed to look at the lens of the camera with their heads positioned in a way that the central point of the two pupils and the upper auricular points were horizontal. The next shot is the profile view where the subject's face is turned approximately 90 degrees from the front. Only one side of their face and not the eye on the far side should be seen. The central point of the pupil from the side and point of upper auricular should also be on the same horizontal line.

2.4. Measurement of Facial Feature. The facial feature points were automatically allocated by uploading the facial images into the Sasang Constitutional Analysis Tool (SCAT) as shown in Figure 1. Facial feature variables were also automatically calculated using length, length to length ratio, angle, and area between facial feature points (Table 1).

2.5. Statistical Analysis. Data analysis of facial features was conducted independently for each sample according to gender. The differences in the general characteristics (age, height, weight, and BMI) of both samples were tested using Student's t-test. The differences in general characteristics (age, height, weight, and BMI) of Sasang types for native Koreans were tested using one-way ANOVA with Bonferroni or Dunnett's T3 as post hoc analysis, depending on the result of Levene's test. For native Japanese, general characteristics (age, height, weight, and BMI) of Sasang types were tested with Kruskal-Wallis test with Mann–Whitney as post hoc analysis. Statistical results were presented as mean (standard deviation).

The facial features of the samples were analyzed according to the Sasang types using one-way ANCOVA, with age as covariates and sample and Sasang types as factors. Post hoc analysis was performed with a Bonferroni adjustment. Statistical results were presented as adjusted mean (standard error).

Statistical analyses were performed using IBM SPSS Statistics 23.0 for Windows (IBM, Armonk, New York) at the significant level of 0.05 as the p value.

3. Results

3.1. General Characteristics of the Subjects. The general characteristics of the subjects by samples were shown in Table 2. The mean age of native Japanese (Sample A) was 23.8 ± 4.4 years (ranging from 20 to 40 years old) for males and 24.2 ± 5.4 years (ranging from 19 to 40 years old) for females. The mean age of native Koreans (Sample B) was 31.2 ± 6.1 years (ranging from 18 to 40 years old) for males and 31.4 ± 5.9

Table 1: Description of facial feature variables.

Variables	Description
FD_*n1_n2* [or PD_*n*,_*n2*]	The length between two points in the frontal (side) picture
FDH_*n*,_*n2* [or PDH_*n1_n2*]	The horizontal length between two points in the frontal (side) picture
FDV_*n1_n2* [or PDV_*n1_n2*]	The vertical length between two points in the frontal (side) picture
FDL_*n1_n2_n3* [or PDL_*n1_n2_n3*]	The length between the point *n1* and segments *n2*, *n3*
FHD_*n1_n2_n3_n4* [or PHD_*n1_n2_n3_n4*]	FDH_*n1_n2* / FD_*n3_n4* [or PDH_*n1_n2* / PD_*n3_n4*]
FDH_*n1_n2_n3_n4* [or PDH_*n1_n2_n3_n4*]	FD_*n1_n2* / FDH_*n3_n4* [or PD_*n1_n2* / PDH_*n3_n4*]
FDD_*n1_n2_n3_n4* [or PDD_*n1_n2_n3_n4*]	FD_*n1_n2* / FD_*n3_n4* [or PD_*n1_n2* / PD_*n3_n4*]
FVD_*n1_n2_n3_n4* [or PVD_*n1_n2_n3_n4*]	FDV_*n1_n2* / FD_*n3_n4* [or PDV_*n1_n2* / PD_*n3_n4*]
FVV_*n1_n2_n3_n4* [or PVV_*n1_n2_n3_n4*]	FDV_*n1_n2* / FDV_*n3_n4* [or PDV_*n1_n2* / PDV_*n3_n4*]
FVH_*n1_n2_n3_n4* [or PVH_*n1_n2_n3_n4*]	FDV_*n1_n2* / FDH_*n3_n4* [or PDV_*n1_n2* / PDH_*n3_n4*]
FA_*n1_n2* [or PA_*n1_n2*]	The angle that the straight-line vector (n1, n2) makes with the horizontal line in the frontal (side) image
FAs_*n1_n2* [or PA_*n1_n2*]	180—The angle that the straight-line vector (n1, n2) makes with the horizontal line in the frontal (side) image
FAi_*n1_n2* [or PAi_*n1_n2*]	The angle that the straight-line vector (n1, n2) makes with the horizontal line in the frontal (side) image ∗ (-1)
FAis_ *n1_n2* [or PAis_*n1_n2*]	180—The angle that the straight-line vector (n1, n2) makes with the horizontal line in the frontal (side) image
FA_*n1_n2_n3* [or PA_*n1_n2_n3*]	The angle formed by the three points n1, n2, and n3 in the frontal (side) image
FArea02	The area of the face defined using points 53, 94, 194, and 153
FArea03	The area of the face defined using points 94, 43, 143, and 194

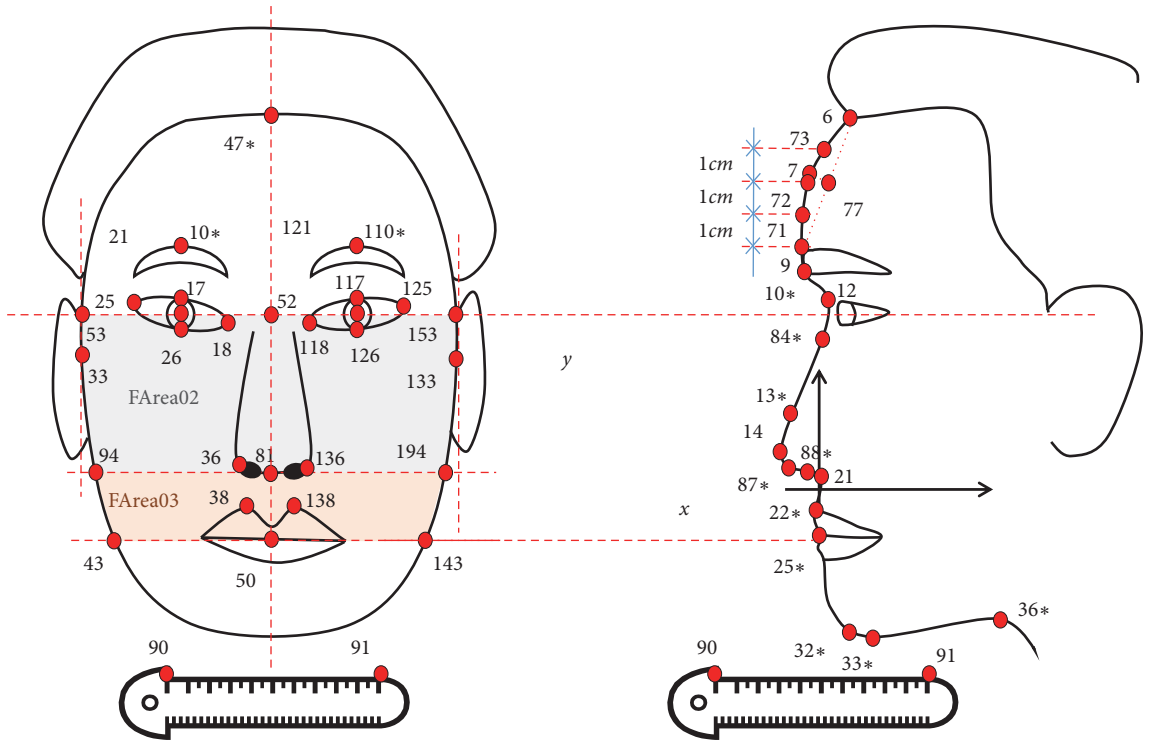

Figure 1: Automatically allocated facial feature points using facial analysis program [29].

Table 2: General characteristics of the subjects by samples.

	Males			Females		
	Sample A (n = 61)	Sample B (n = 252)	*P* value	Sample A (n = 66)	Sample B (n = 464)	*P* value
Age (yrs)	23.83 (4.39)	31.18 (6.08)	<0.001	24.19 (5.36)	31.43 (5.93)	<0.001
Height (cm)	170.80 (6.06)	173.50 (5.67)	0.001	158.67 (5.28)	160.92 (5.18)	0.001
Weight (kg)	64.93 (9.13)	72.54 (11.23)	<0.001	52.27 (6.37)	55.81 (8.89)	<0.001
BMI (kg/m^2)	22.20 (2.48)	24.36 (3.14)	<0.001	20.75 (2.22)	21.55 (3.24)	0.012

Sample A: native Japanese, Sample B: native Koreans, and BMI: Body Mass Index.
Data were presented as the mean (standard deviation). *P* values were calculated using *t*-test.

Table 3: General characteristics of the subjects by Sasang types.

(a) Sample A

Sample A (n = 127)	Male (n = 61)				Female (n = 66)			
	TE (n = 17)	SE (n = 35)	SY (n = 9)	*P* value	TE (n = 9)	SE (n = 19)	SY (n = 38)	*P* value
AGE (yrs)	22.34 (1.72)	24.03 (4.66)	25.84 (6.08)	0.320	22.82 (4.29)	22.71 (4.78)	25.26 (5.70)	0.127
Height (cm)	174.07 (5.47)^a	169.42 (6.42)^b	169.99 (3.01)	0.022	158.27 (7.23)	158.82 (4.58)	158.70 (5.23)	0.855
Weight (kg)	72.30 (9.27)^a	60.60 (6.72)^b	67.82 (7.34)^a	<0.001	60.58 (6.66)^a	48.32 (4.56)^c	52.28 (5.18)^b	<0.001
BMI (kg/m^2)	23.85 (2.76)^a	21.08 (1.72)^b	23.46 (2.30)^a	<0.001	24.14 (1.56)^a	19.15 (1.55)^c	20.75 (1.69)^b	<0.001

(b) Sample B

Sample B (n = 716)	Male (n = 252)				Female (n = 464)			
	TE (n = 101)	SE (n = 79)	SY (n = 72)	*P* value	TE (n = 145)	SE (n = 156)	SY (n = 163)	*P* value
AGE (y)	31.63 (6.12)	30.85 (5.89)	30.94 (6.27)	0.670	30.83 (6.23)	31.86 (5.77)	31.53 (5.81)	0.326
Height (cm)	174.26 (5.72)	172.70 (5.92)	173.28 (5.30)	0.212	161.41 (4.98)	161.03 (5.07)	160.41 (5.44)	0.239
Weight (kg)	79.37 (10.73)^a	66.52 (9.76)^b	69.66 (8.22)^b	<0.001	63.00 (9.42)^a	51.03 (5.21)^c	54.19 (7.20)^b	<0.001
BMI (kg/m^2)	26.09 (2.83)^a	22.26 (2.70)^b	23.18 (2.36)^b	<0.001	24.20 (3.59)^a	19.67 (1.74)^c	21.05 (2.43)^b	<0.001

Sample A: native Japanese, Sample B: native Koreans; TE: Tae-Eum, SE: So-Eum, SY: So-Yang.
Data were presented as the mean (standard deviation).
Sample A: *P* values were calculated using Kruskal-Wallis test. Post hoc comparisons using Mann–Whitney.
Sample B: *P* values were calculated using one-way ANOVA. Post hoc comparisons using Bonferroni or Dunnett T3.
a, b, c: Significant difference between the groups, in which the value descends by a and b followed by c.

years (ranging from 18 to 40 years old) for females. In both males and females, age, height, body weight, and BMI were significantly higher in Sample B than in Sample A.

The general characteristics of the samples' Sasang types were shown in Table 3. There were no significant differences in age and height among the Sasang types for both genders in Sample B and females only in Sample A. For males in Sample A, there was no significant difference in age but there were significant differences in height. However, there were significant differences in weight and BMI among the Sasang types for males and females in both samples. Weight and BMI for both samples were the highest in TE types and lowest in SE types according to post hoc analysis.

3.2. Differences in Facial Features among the Samples and Sasang Types. After adjustment for age, there were several statistically significant differences in the facial features variables among samples and Sasang constitution in both genders.

3.2.1. Difference in Facial Features in Males. In males, lower face angle and area variables such as FA_18_17_43, FA_118_117_143, and FArea03_aD showed significant differences between the samples and also among the Sasang constitutions. Lower face area was larger in Sample B and also in the TE types as compared to SE types and SY types. Mid-face area variable, FArea02_aD, was also seen significantly different where the mid-face of Sample B is larger than Sample A. The value of this variable was also highest in TE types compared to SE types and SY types (Table 4).

There was also a significant difference in the eye horizontal distance variable, FDH_25_125, and also in the variables related to eye slanting angle, FA_18_17_25 and FA_118_117_125. This showed that the outer eye horizontal distance and eye slanting angle were larger in Sample B and also in TE types. Moreover, inner eye slanting angle, FAis_18_17 and FAi_118_117, was larger in Sample A and also in SY types as compared to other Sasang types (Table 4).

Forehead related variable, PD_7_77, was also significantly different across samples and Sasang types. The forehead of Sample A and SY types was more protruding than Sample B and other Sasang types. Furthermore, there is also a significant difference in the nose related variable such as PDH_12_14 where Sample A had shorter nose than Sample B.

Table 4: Male facial feature variables with differences among the samples and Sasang types.

	Samples			Sasang types				Sample ∗ Sasang types
Facial variables	Sample A	Sample B	*P* value	TE	SE	SY	*P* value	*P* value
FA_18_17_43	77.55 (1.16)	83.12 (0.50)	<0.001	82.74 (0.98)[a]	80.68 (0.77)[ab]	77.58 (1.29)[b]	0.006	0.160
FA_118_117_143	78.39 (1.08)	82.81 (0.47)	<0.001	82.96 (0.92)[a]	80.48 (0.72)[ab]	78.37 (1.21)[b]	0.008	0.079
FArea02_aD	6787.01 (114.31)	7266.28 (49.14)	<0.001	7245.54 (96.90)[a]	7064.10 (76.28)[ab]	6770.29 (127.62)[b]	0.012	0.074
FArea03_aD	3831.44 (72.95)	4089.65 (31.36)	0.002	4119.47 (61.84)[a]	3948.55 (48.68)[ab]	3813.62 (81.45)[b]	0.008	0.288
FDH_25_125	97.86 (0.92)	104.46 (0.39)	<0.001	102.63 (0.78)[a]	101.65 (0.61)[ab]	99.20 (1.02)[b]	0.027	0.123
FA_18_17_25	128.72 (1.21)	133.30 (0.52)	0.001	133.14 (1.02)[a]	131.89 (0.81)[a]	127.99 (1.35)[b]	0.009	0.162
FA_118_117_125	127.94 (1.16)	132.30 (0.50)	0.001	132.27 (0.98)[a]	130.84 (0.77)[ab]	127.26 (1.29)[b]	0.008	0.132
FAis_18_17	32.03 (0.80)	29.47 (0.34)	0.005	28.89 (0.68)[b]	30.52 (0.53)[ab]	32.85 (0.89)[a]	0.002	0.265
FAi_118_117	31.71 (0.82)	29.48 (0.35)	0.015	29.01 (0.69)[b]	30.42 (0.55)[ab]	32.35 (0.91)[a]	0.014	0.230
PD_7_77	3.15 (0.28)	2.36 (0.12)	0.011	2.60 (0.24)[ab]	2.39 (0.18)[b]	3.28 (0.31)[a]	0.048	0.224
PDH_12_14	19.48 (0.40)	20.66 (0.17)	0.009	20.58 (0.34)[a]	20.53 (0.27)[a]	19.11 (0.45)[b]	0.015	0.099

Sample A: native Japanese, Sample B: native Koreans; TE: Tae-Eum, SE: So-Eum, SY: So-Yang.
Data were presented as adjusted mean (standard error)
P values were calculated using one-way ANCOVA. Post hoc comparisons using Bonferroni.
a, b, c: Significant difference between the groups, in which the value descends by a and b followed by c.
ab: no significant difference among the groups.

Table 5: Female facial feature variables with differences among the samples and Sasang types.

	Samples			Sasang types				Sample ∗ Sasang types
Facial variables	Sample A	Sample B	*P* value	TE	SE	SY	*P* value	*P* value
FDV_52_50	75.28 (0.65)	71.83 (0.20)	<0.001	73.82 (0.74)[ab]	74.36 (0.53)[a]	72.49 (0.39)[b]	0.011	0.105
FDV_81_50	28.45 (0.38)	26.07 (0.12)	<0.001	27.76 (0.43)	27.42 (0.30)	26.62 (0.22)	0.018	0.103
FA_18_17_43	77.98 (0.96)	75.52 (0.30)	0.016	78.53 (1.08)[a]	75.10 (0.77)[b]	76.63 (0.57)[ab]	0.029	0.816
FArea03_aD	3788.77 (64.90)	3525.75 (20.10)	<0.001	3794.18 (73.24)[a]	3631.84 (52.16)[ab]	3545.76 (38.50)[b]	0.009	0.073
FD_17_26	8.89 (0.18)	10.02 (0.06)	<0.001	9.03 (0.21)[b]	9.80 (0.15)[a]	9.53 (0.11)[ab]	0.010	0.090
FAis_18_17	31.57 (0.69)	35.51 (0.22)	<0.001	32.41 (0.78)[b]	34.70 (0.56)[a]	33.52 (0.41)[ab]	0.044	0.620

Sample A: native Japanese, Sample B: native Koreans; TE: Tae-Eum, SE: So-Eum, SY: So-Yang.
Data were presented as adjusted mean (standard error)
P values were calculated using one-way ANCOVA. Post hoc comparisons using Bonferroni.
a, b, c: Significant difference between the groups, in which the value descends by a and b followed by c.
ab: no significant difference among the groups.

For this variable, the noses of SY types were shorter than SE types (Table 4).

3.2.2. Difference in Facial Features in Females. In females, the facial variable related to the distance between the root of nose and lips, FDV_52_50, and philtrum related variable, FDV_81_50, showed significant differences among the samples. The values of these variables were larger in Sample A than Sample B which suggested that Sample A had longer nose and philtrum (Table 5).

Lower face angle and area variables, FA_18_17_43 and FArea03_aD, also showed significant differences among the samples and Sasang constitutions. This indicates that lower face width and area was overall wider in Sample A and in the TE types compared to the SY and SE types. There was also significant difference seen in variable FD_17_26, which is the distance between upper and lower eyelid in vertical alignment. Furthermore, variable related to the angle of eye slanting, FAis_18_17, also showed significant differences where Sample B and SE types had a wider angle than Sample A and other Sasang types (Table 5).

4. Discussion

As the diagnosis of facial features is one of the fundamental aspects of SCM, understanding the differences in facial features among Sasang types is essential. Currently, the standard approach to Sasang types facial diagnosis among native Koreans has already been established using quantitative analysis. However, the use of Sasang types facial diagnosis in other populations is still limited. This study was performed to extend the use of Sasang facial diagnosis in native Japanese.

In this study, we analyzed the differences in facial feature variables among the samples and Sasang types. In terms of demographic characteristics, we found that the age, height, weight, and BMI were significantly higher in Sample B than in Sample A for both genders (Table 2). The average height and weight of our samples correspond with the average height and

weight of both nationalities in general, which could suggest that the results in our study could speak for native Japanese and native Koreans in general [30–34]. Besides, TE types also had the highest weight and BMI followed by SE types as the lowest in both samples (Table 3). This shows that the results of Sample A are consistent with the previous study of Sample B where TE type was reported to have highest BMI score and it was lowest in SE type [4, 17, 35].

After adjustment for age, facial variables such as FA_18_17_43, FArea03_aD, and FAis_18_17 were the common variables significantly different in both males and females. Therefore, lower face variables and eye-related variables were considered the main indicators that distinguish the Sasang types in both genders among both samples. The value of lower face angle and area variables, FA_18_17_43 and FArea03_aD, was higher in male and lower in the female of Sample B indicating that lower face of Sample B was larger than Sample A in male and vice versa in the female. However, the values of these variables were higher in TE type of both males and females (Tables 4 and 5). These results are similar to those previous studies that analyzed facial feature according to Sasang types among native Koreans. It was mentioned that the width over length was larger in TE type with the width of jaw serving as standard and generally TE type had a wider jaw than other Sasang types [16, 17, 25].

On the other hand, the value of the variable related to the area of mid-face, FArea02aD, was also higher in the male of Sample B and TE types (Table 4). This indicates that male TE types have larger mid-face. Looking at the results of both mid-face and lower face area variables for males, we can suggest that males of TE types have wider face area compared to other Sasang types. These results could be considered consistent with the previous studies where the face of TE types was mentioned to be larger than other types [16].

Furthermore, the value of eye slanting angle variables, FA_18_17_25 and FA_118_117_125, was higher in the males of Sample B compared to the males of Sample A and vice versa for the inner eye slanting angle variables, FAis_18_17 and FAi_118_117. This indicates that Sample A males have rounder eyes whereas Sample B males have narrower eyes. However, in females, the value of inner eye slanting angle variable FAis_18_17 and the value of variable FD_17_26, which is the vertical distance of upper and lower eyelid, were higher in Sample B (Tables 4 and 5). This shows that Sample A females have narrower eyes and Sample B females have rounder eyes. Our findings showed that the eye shape of both genders in Sample A and Sample B is opposite to each other.

In terms of Sasang types, the value of eye slanting angle variables, FA_18_17_25 and FA_118_117_125, is the highest in TE types compared to other types in males. This shows that TE types of males have narrower eyes. In addition, males of SY types and females of SE types have the highest value in the inner eye slanting angle variable, FAis_18_17, indicating that SY types males and SE types females have rounder eyes (Tables 4 and 5). A previous study had stated that female TE types had narrower eyes and female SE type had rounder eyelids [25]. Another study also suggested that the eye of SE type is round in shape and the eye of SY type was relatively rounder than TE types [16]. Our results showed similar consistency with previous studies. Moreover, the value of forehead related variable, PD_7_77, is highest in SY types according to our post hoc analysis. This finding also matched the descriptions of previous studies which mentioned that the SY types had the most protruding forehead and bulging head [23, 25].

We also found that there are a few significant results when we look only at the differences among the Sasang types without regard to the differences in samples. In males, there were significant differences in the variable PDV_14_21 among the Sasang types. This showed that TE types have longer vertical distance between the apex of nose and subnasale. This results indicated that, regardless of sample types, the nose of TE types males was more turn-up compared to other types.

In addition, the consistency between our study and previous studies implies that the algorithm used in Sasang Constitutional Analysis Tool for native Koreans can also be utilized in the analysis of Sasang types of native Japanese provided that the standardized values compensating for the difference of sample size are assigned to the algorithm [22]. Hence, further studies are needed to validate our findings.

There are several limitations in this study. Firstly, the determination of the subjects' Sasang types is inconsistent between the samples. Sasang types of the subjects in Sample B were decided based on their response towards Sasang type-specific herbal medicine while Sasang types of the subjects in Sample A were determined by only one certified clinical specialist and they were not prescribed Sasang type-specific herbal medicine. Therefore, there might be a question on the generality of data. Secondly, subjects in Sample B were chosen from data bank based on the predefined inclusion criteria whereas subjects in Sample A were recruited from one university, resulting in unequal sample size which may affect the ability to generalize our findings. Although we did necessary statistical adjustment for age, conclusion involving these data should be drawn with caution.

Hence, future studies should include a larger sample size from various places of Japan and Sasang types of subjects should be determined by at least two or more certified clinical specialists with their interrater reliability tested. The improvement in the reliability of data collection will greatly improve the accuracy of our research. Such follow-up studies will greatly generalize our findings and may be able to yield new discoveries.

5. Conclusion

This study is the first study which attempts to analyze the facial features of native Japanese individuals according to Sasang types. Although there were differences in facial features among Sample A and Sample B, the facial features of both samples across Sasang types showed a similar tendency. If the distinctive variables are applied after compensating for the differences between samples, the Sasang Constitutional Analysis Tool may be valid and usable in distinguishing the Sasang types of native Japanese.

Data Availability

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

Disclosure

The authors would also like to mention that this study was presented in part as conference abstract in the 2018 International Congress on Integrative Medicine and Health, Baltimore, Maryland, USA, May 2018 (Abstract 4039).

Conflicts of Interest

There are no conflicts of interest in this study.

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Review Article

Acupuncture and Related Therapies for Treatment of Postoperative Ileus in Colorectal Cancer: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

Yihong Liu[1], **Brian H. May**[2], **Anthony Lin Zhang**[2], **Xinfeng Guo**[1], **Chuanjian Lu**[1], **Charlie Changli Xue**[1,2] and **Haibo Zhang**[1]

[1]*Guangdong Provincial Academy of Chinese Medical Sciences, Guangdong Provincial Hospital of Chinese Medicine and The Second Clinical College, Guangzhou University of Chinese Medicine, Guangzhou, China*
[2]*China-Australia International Research Centre for Chinese Medicine, RMIT University, Bundoora, VIC 3083, Australia*

Correspondence should be addressed to Charlie Changli Xue; charlie.xue@rmit.edu.au
and Haibo Zhang; haibozh@aliyun.com

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Delays in recovery of intestinal function following abdominal surgery are associated with longer hospital stays, increased postoperative complications, and higher costs to the health care system. Studies of acupuncture for postoperative ileus and other postoperative issues have reported improvements. This systematic review and meta-analysis aimed to assess whether acupuncture assisted recovery following surgery for colorectal cancer (CRC). Randomized controlled trials (RCTs) were identified from major English and Chinese language biomedical databases. Participants (aged 18 years plus) had received surgical resection for CRC. 22 studies (1,628 participants) were included. Five were sham-controlled. Outcomes included gastrointestinal function recovery (21 studies), recovery of urinary function (1 study), postoperative abdominal distension (3 studies), and quality of life (1 study). Meta-analyses found significant reductions in time to first bowel sounds, first flatus, and first defecation in both the sham-controlled and nonblinded studies. These results suggested that the addition of acupuncture following CRC surgery improved recovery of gastrointestinal function based on four blinded good quality RCTs (281 participants) and 17 nonblinded lower quality RCTs (1,265 participants). The best available evidence was for interventions that included electroacupuncture at the point ST36 *Zusanli* and there is supporting evidence for other types of acupuncture therapies that involve stimulation of this point. This review is registered with the following: systematic review registration in PROSPERO: CRD42017079590.

1. Introduction

Delay in resumption of intestinal function following the surgery occurs in most patients after abdominal surgery including surgery for CRC [1]. Known as postoperative ileus (POI), this condition typically resolves by day five following open abdominal surgery and by day three following laparoscopic surgery but it may be prolonged or recur and may be accompanied by abdominal distension, pain, and/or nausea and vomiting [2]. POI is associated with longer hospital stays and increased postoperative complications and results in higher costs to the health care system [1, 3].

Integrative medicine is utilised by a substantial proportion of people with cancers [4, 5] with acupuncture being used in both out-patient [6] and in-patient settings [7, 8]. A review of acupuncture for symptom management in cancer found evidence of benefits [9] and an update found support for improvement in POI [10]. A meta-analysis of acupuncture for POI found that acupuncture might be effective in improving POI [11] and a systematic review of acupuncture for recovery after CRC surgery concluded that there was low-to-moderate quality evidence for the efficacy and safety of acupuncture for postoperative outcomes including POI [12]. Since these reviews were published, additional studies have become available.

The objective of this systematic review was to assess whether acupuncture and related therapies were effective in

assisting in recovery following surgery for colorectal cancer. If so, we aimed to determine the best available evidence in order to inform clinical practice.

2. Method

Searches were conducted of (1) major English language biomedical databases: PubMed, Embase, CINAHL, AHMED, and Cochrane Library; (2) major Chinese language biomedical databases: Chinese BioMedical Literature Database (CBM), VIP Database for Chinese Technical Periodicals (CQVIP), China National Knowledge Infrastructure (CNKI), and Wanfang Data from their respective inceptions to October 2017; and (3) reference lists in studies and reviews (see Supp. 1 for PubMed search strategy). Only prospective randomized controlled trials (RCTs) were included.

Included participants had been diagnosed with colorectal, colon, or rectal cancer by pathology and had received surgical resection for this cancer and were aged 18 years or older. Studies that included participants with other cancers or other diseases were excluded.

The test interventions were acupuncture and related therapies including electroacupuncture, manual acupuncture, acupressure, moxibustion, point application and laser acupuncture, or any combination of these therapies. Studies of acupoint-injection, suture embedding (also called "catgut" embedding), transcutaneous electrical nerve stimulation (TENS), acupuncture combined with oral herbal medicine, or other nonacupuncture-related therapy were excluded. Studies in which the details of the acupuncture or related therapy were unclear were excluded.

The control interventions were sham/placebo acupuncture or related therapy, or no additional intervention. The cointerventions were any conventional surgery for CRC, including open or laparoscopic surgery, plus conventional postoperative or perioperative care. The conventional care was required to be the same in all groups. The study setting could be a hospital or similar clinic suitable for postsurgical recovery.

Studies that reported numerical data for an outcome directly related to recovery from CRC surgery were included. Outcomes included recovery of physical functions, quality of life, and postoperative adverse events. Studies of acupuncture for anaesthesia or pain relief were excluded.

Search results were screened by two reviewers and full-text papers were obtained for any paper considered a potential inclusion. These were assessed against the inclusion and exclusion criteria. Data were extracted to a predesigned spread-sheet for: citation details, year, country; study design, duration, and setting; methodological aspects; participant characteristics (number, age, gender, and cancer type); details of the acupuncture or related intervention, type of surgery, type of conventional care; details of outcome measures; data for included outcome measures; safety, dropouts, and adverse events for each group. If there were any disagreements between reviewers, a third reviewer was consulted. In the case of discrepancies in the published data it was planned to contact authors but this was not required. Risk of bias was assessed using the Cochrane tool [13] by two reviewers independently with a third reviewer available or consultation to resolve any issues.

Assessments of effect sizes were based on published data and conducted in Stata 12. Meta-analyses were conducted when studies were comparable and used the same outcome measures. Random-effects models with 95% confidence interval (CI) were applied. Heterogeneity was quantified as I-square. Publication bias was assessed using a funnel plot and Egger's test for asymmetry when ten or more studies were available. Subgroup analyses were planned based on participant characteristics such as the cancer type (colorectal, rectal, and colon); the type of acupuncture or related intervention; the type of surgery (open, laparoscopic); the type of conventional care; and methodological quality. Sensitivity analyses were planned to explore sources of heterogeneity.

3. Results

Twenty-two RCTs of acupuncture and related therapies for recovery following surgery for colorectal cancer were identified (Figure 1). One study was conducted in the USA [14], 19 in various locations in mainland China [15–33], one in Hong Kong [34, 35], and one in Taiwan [36].

The studies enrolled 1,628 participants ranging in age from 22 to 87 years (Table 1, study IDs 1-22). The mean ages ranged from 45 to 73 years. In the twenty-one studies that reported data on gender, there were 843 males and 625 females. All participants were diagnosed with CRC in 15 studies, with colon cancer in three studies, and rectal cancer in four studies. Eleven reported that acupuncture sensation (*deqi*) was produced but none mentioned Chinese medicine syndrome differentiation [37].

In two studies laparoscopic surgery was used [27, 34]. One used Dixon surgery for rectal cancer [15] and one used Miles surgery for rectal cancer [27]. The other studies used conventional open surgery, excluded Miles surgery, or did not specify the type of surgery. Two studies used the Fast Track Program (FTP) of perioperative care [22, 24] and the other 20 studies used conventional postoperative care. Two studies involved three intervention groups [25, 34]. One of these had two test groups [25], so whenever both groups were included in the same pool the number in the control group was halved to avoid double counting.

Test interventions included manual acupuncture (3 studies), electroacupuncture (7 studies), manual acupuncture plus electroacupuncture (1 study), acupressure (2 studies), manual acupuncture plus moxibustion (1 study), warm needling (1 study), ear acupressure (3 studies), acupuncture plus ear acupressure (1 study), moxibustion plus ear acupressure (1 study), acupuncture plus electroacupuncture plus ear acupuncture (1 study), and point application (1 study).

Outcome data were available for gastrointestinal function recovery (21 studies), recovery of urinary function (1 study) [27], postoperative abdominal distension (3 studies) [22, 24, 25], and quality of life (1 study) [18].

3.1. Risk of Bias. All studies were described as "randomized" but only 13 described an appropriate method for sequence generation and were judged "low risk" while three studies were judged "high risk" since patient order was used (Supp. Table S1). Four studies described the method of allocation

Table 1: Characteristics of included studies of acupuncture and related therapies for postoperative recovery for colorectal cancer.

Study ID No. [ref]	Author, year [location]	N participants (baseline); mean (SD) age at baseline in years; (M/F);	N groups; Duration and frequency of treatment	Conventional treatment, cancer type	Acupuncture therapy	Control intervention	Acupuncture points
1 [36]	Chao HL *et al.*, 2013 [3]	66; T: 61.9(13.3), C: 62.8(15.7); (31/35)	2; from postoperative days 1-5, three-mins, three times per day	Open surgery, CRC	Acupressure∗	Sham acupressure	ST36 *Zusanli* 足三里, NS
2 [14]	Deng G *et al.*, 2013 [2]	90; T: 56, C: 59; (52/38)	2; from postoperative days 1-3, 30 min, twice daily	Open surgery, CRC	Acupuncture + electro-acupuncture + ear acupuncture	Sham acupuncture + sham electro-acupuncture + sham ear acupuncture	ST36 *Zusanli* 足三里, PC6 *Neiguan* 内关, LI4 *Hegu* 合谷, SP6 *Sanyinjiao* 三阴交, SP9 *Yinglingquan* 阴陵泉, ST25 *Tianshu* 天枢 and ear point TF4 *Shenmen* 神门, bilateral
3 [15]	Hong X 2017 [1]	80; T: 61.2(7.2), C: 58.8(9.4); (47/33)	2; ear acupressure: press Vaccaria seeds 5 times per day for 7 days, change to other ear every 2 days; moxibustion: once a day for 7 days	Dixon surgery, rectal cancer	Moxibustion + ear acupressure	no acupuncture therapy	Moxibustion points: ST36 *Zusanli* 足三里 and CV12 *Zhongwan* 中脘; ear points: CO4 *Wei* 胃, TF4 *Shenmen* 神门, CO6 *Xiaochang* 小肠, CO3 *Benmen* 贲门, unilateral
4 [16]	Li JM *et al.*, 2016 [1]	160; T: 51.3(3.16), C: 52.17(3.34)	2; press Vaccaria seeds 30-60s per point, three-five times per day, change to other ear every 3-7 days	Open radical surgery, rectal cancer	Ear acupressure	no acupuncture therapy	Ear points: CO4 *Wei* 胃, CO5 *Shierzhichang* 十二指肠, CO6 *Xiaochang* 小肠,CO7 *Dachang* 大肠, CO13 *Pi* 脾, CO3 *Benmen* 贲门, unilateral
5 [17]	Lu JY & Jin HM 2011 [1]	78; T: 62.25(9.38), C: 61.21(10.16); (43/35)	2; Vaccaria seeds attached immediately after surgery, point pressure for 30-60s per point every 2-3 hours (more than 5 min/cycle); until recovery	Radical surgery excluding Miles surgery, CRC	Ear acupressure∗	no acupuncture therapy	Ear points: CO13 *Pi* 脾, CO4 *Wei* 胃, CO7 *Dachang* 大肠, CO6 *Xiaochang* 小肠, AH6a *Jiaogan* 交感, CO18 *Neifenmi* 内分泌, AT4 *Pizhixia* 皮质下, bilateral
6 [18]	Meng ZQ *et al.*, 2010 [1]	85; T: 54.3, C: 53.1; (47/38)	2; from postoperative days 1-6, 20 min, once a day, until first bowel movement or day 6, whichever came first	Intraperitoneal surgery, epidural anaesthesia, colon cancer	Electro-acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, TE6 *Zhigou* 支沟, GB34 *Yanglingquan* 阳陵泉, bilateral
7 [34, 35]	Ng SSM *et al.*, 2013 [4] †	165; T: 67.4(9.7), C1: 67.4(10.7), C2:68.5(10.6); (99/66)	3; T & C1: from postoperative days 1-4, 20 min, once a day, until defaecation occurred or day 4, whichever was earlier	Laparoscopic surgery, CRC	Electro-acupuncture∗	C1: sham electro-acupuncture, C2: no acupuncture therapy	ST36 *Zusanli* 足三里, SP6 *Sanyinjiao* 三阴交, LI4 *Hegu* 合谷, TE6 *Zhigou* 支沟, NS

Table 1: Continued.

Study ID No. [ref]	Author, year [location]	N participants (baseline); mean (SD) age at baseline in years; (M/F);	N groups; Duration and frequency of treatment	Conventional treatment, cancer type	Acupuncture therapy	Control intervention	Acupuncture points
8 [19]	Niu CF *et al.*, 2008 [1]	32; T & C: 52; (21/11)	2; from postoperative day 1, 15 min. twice daily	Radical surgery excluding Miles surgery, CRC	Electro-acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, PC6 *Neiguan* 内关, NS
9 [20]	Si JG & Ding RS 2015 [1]	40; T: 45, C: 46; (23/17)	2; from 24 hours after surgery, 20 min once a day, till bowel sounds and passing flatus occurred	Radical surgery, CRC	Electro-acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, SP6 *Sanyinjiao* 三阴交, ST25 *Tianshu* 天枢, LI4 *Hegu* 合谷, bilateral
10 [21]	Tan S & Zheng CM 2015 [1]	76; T: 63.8(11.2), C: 63.2(10.5); (43/33)	2; ear acupressure: Vaccaria seeds attached after surgery, pressure for 5 min every 2-3 hours; acupuncture: after surgery, 20 min once a day; until recovery	Radical surgery, CRC	Acupuncture∗ + ear acupressure∗	no acupuncture therapy	ST36 *Zusanli* 足三里 and ear sensitive points 敏感点, NS
11 [22]	Tong WY *et al.*, 2014 [1]	84; T: 58.6(15.1), C: 59.2(14.7); (50/34)	2; from 2 hours after surgery, 30 min, once a day	Open surgery, FTP, rectal cancer	Acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, PC6 *Neiguan* 内关, ST37 *Shangjuxu* 上巨虚, SP4 *Gongsun* 公孙, NS
12 [23]	Wang EM 2012 [1]	60; T: 57.4(13.6), C: 56.6(12.3); (34/26)	2; Vaccaria seeds attached 4 hours after surgery, pressure for 1 min on each point, every 6 hours, until recovery	Usual surgery, colon cancer	Ear acupressure∗	no acupuncture therapy	Ear points: TF4 *Shenmen* 神门, AH6a *Jiaogan* 交感, AT4 *Pizhixia* 皮质下,CO7 *Dachang* 大肠, CO4 *Wei* 胃, *Ashixue* 阿是穴 (sensitive point); unilateral, change side every 4 days
13 [24]	Wang HM 2011 [1]	30; T: 60.4(11.01), C: 58(10.24); (20/10)	2; from 24 hours after surgery, 30 min, once a day, for 5 days	Open surgery, FTP, CRC	Acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, PC6 *Neiguan* 内关, ST37 *Shangjuxu* 上巨虚, SP4 *Gongsun* 公孙, NS
14 [25]	Xiao C 2014 [1]	90; T1: 55.87(10.49), T2: 55.33(10.83), C: 54.63(10.25); (51/39)	3; from 24 hours after surgery, 20 min, once a day, for 5 days	Open radical surgery excluding Miles surgery, CRC	T1:acupuncture;∗ T2: electro-acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, bilateral
15 [26]	Xiao L *et al.*, 2016 [1]	60; T: 67.43(16.35), C: 68.52(17.16); (33/27)	2; from postoperative days 1-14, 30 min twice daily	Radical surgery, colon cancer in elderly patients	Acupuncture∗ + electro-acupuncture	no acupuncture therapy	ST36 *Zusanli* 足三里, LI4 *Hegu* 合谷, SP6 *Sanyinjiao* 三阴交, LU5 Chize 尺泽 (all electro); TE6 *Zhigou* 支沟, LU7 *Lieque* 列缺 (both manual); all bilateral

Table 1: Continued.

Study ID No. [ref]	Author, year [location]	N participants (baseline); mean (SD) age at baseline in years; (M/F);	N groups; Duration and frequency of treatment	Conventional treatment, cancer type	Acupuncture therapy	Control intervention	Acupuncture points
16 [27]	Yan YB 2011 [1]	30; T: 50.73(6.076), C: 50.33(5.802); (9/21)	2; from postoperative days 3-7, 30 min, once a day	Laparoscopic Miles surgery, rectal cancer Dukes A-C1, recovery of urinary function	Acupuncture + moxibustion	no acupuncture therapy	ST36 *Zusanli* 足三里, LI4 *Hegu* 合谷, SP10 *Xuehai* 血海, SP9 *Yinglingquan* 阴陵泉, SP6 *Sanyinjiao* 三阴交, LR3 *Taichong* 太冲, CV4 *Guanyuan* 关元 (moxa), CV8 *Shenque* 神阙(moxa), NS
17 [28]	Yang JJ *et al.*, 2011 [1]	60; T: 60.9(6.63), C: 62(6.98); (39/21)	2; from postoperative day 1, 30 min, once a day, until 3 days after defecation occurred	Radical surgery, CRC	Electro-acupuncture∗	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, ST39 *Xiajuxu* 下巨虚, bilateral
18 [29]	Yang JF *et al.*, 2016 [1]	72; T: 72(5.4), C: 73.4(5.6); (44/28)	2; performed three times at 24 hours, 48 hours and 72 hours after surgery, application for 6 hours each time	Open surgery, CRC in elderly patients	Point application with warming cataplasm	Point application with non-warming cataplasm	ST36 *Zusanli* 足三里, bilateral
19 [30]	Zhang SY & Du YQ 2011 [1]	70; T: 57.1(11.7), C: 59.1(8.5); (52/18)	2; from postoperative days 1-10, 45 min, once a day	Radical surgery, CRC	Warm needling	no acupuncture therapy	ST36 *Zusanli* 足三里, ST37 *Shangjuxu* 上巨虚, ST39 *Xiajuxu* 下巨虚, SP6 *Sanyinjiao* 三阴交, SP9 *Yinglingquan* 阴陵泉, NS
20 [31]	Zhang XY & Lu JB 2017 [1]	80; T: 64.8(5.6), C: 67.5(6.0); (42/38)	2; from 6 hours after surgery, 1 min pressure for each point, three times per day, 1 hour after meals, until postoperative day 5	Radical surgery excluding Miles surgery, CRC	Acupressure∗	no acupuncture therapy	ST36 *Zusanli* 足三里, LI4 *Hegu* 合谷, ST37 *Shangjuxu* 上巨虚, NS
21 [32]	Zhang ZD *et al.*, 2014 [1]	40; T: 63(9), C: 60(10); (22/18)	2; at 30 min after surgery, then postoperative days 1–4, 30 min, once a day	Open surgery, CRC	Electro-acupuncture∗	Sham electro-acupuncture	ST36 Zusanli 足三里, bilateral
22 [33]	Zhang XY *et al.*, 2016 [1]	80; T: 64.8(6.8), C: 67.5(4.2); (41/39)	2; from 6 hours after surgery, 1 min pressure for each point, three times per day, 1 hour after meals, until postoperative day 5, change ear every 2 days	Usual surgery, CRC	Acupressure∗ + ear acupressure∗	no acupuncture therapy	Acupressure points: ST36 *Zusanli* 足三里, LI4 *Hegu* 合谷, ST37 *Shangjuxu* 上巨虚, NS; ear points: CO4 *Wei* 胃, CO6 *Xiaochang* 小肠, CO7 *Dachang* 大肠, unilateral

Location in which the study was conducted: 1: Mainland China; 2: USA; 3: Taiwan; 4: Hong Kong. NS: side not specified
∗ means mentioned acupuncture sensation (*deqi*) was obtained. † means reported in two journal articles, reference Nos. [34, 35].

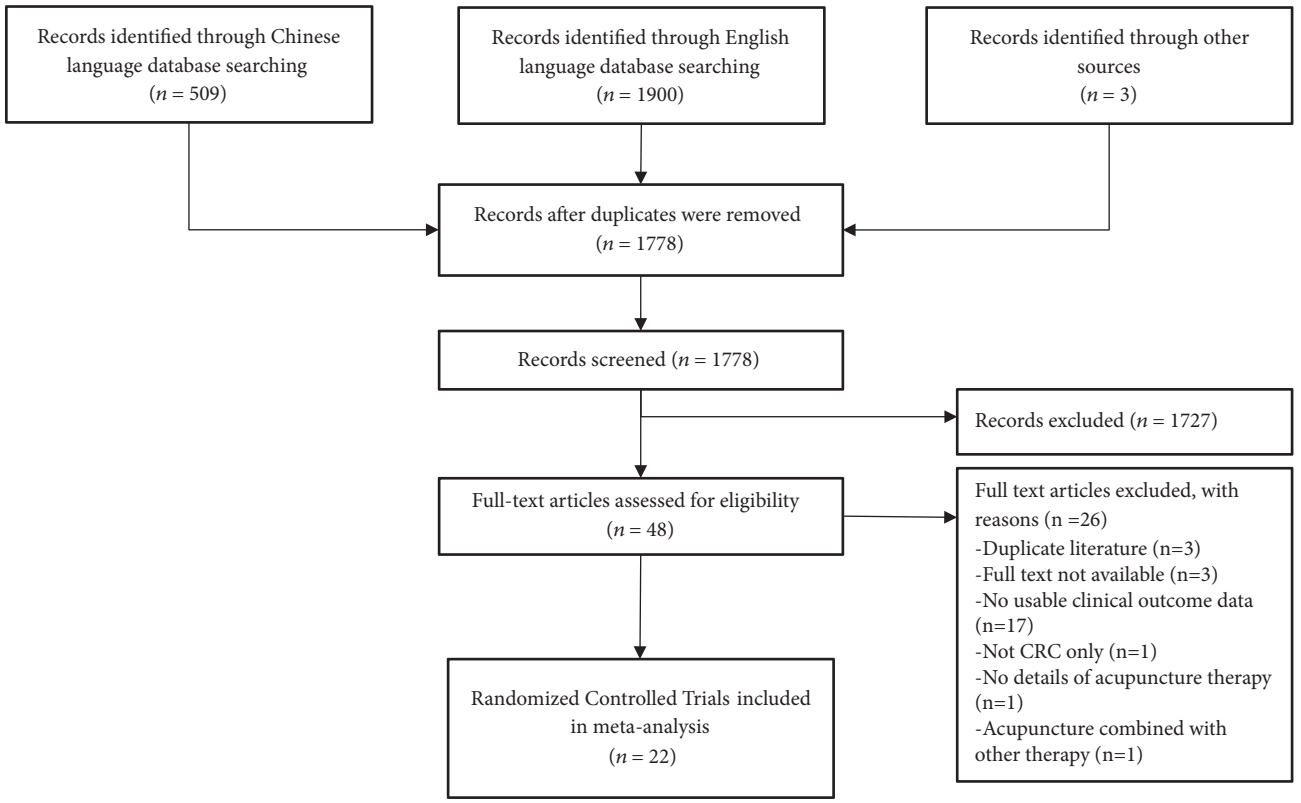

Figure 1: Flow diagram of the search, screening, and inclusion process.

concealment and were judged "low risk". The others were judged "unclear risk" since there was no description.

Five studies used a sham intervention. Four of these were judged "low risk" for blinding of participants and one was judged "unclear risk" since the method was not well described. All studies were judged "high risk" for blinding of study personnel since the acupuncturists could not be blinded. Five studies were judged "low risk" for blinding of outcome assessors and the other studies were "high risk". In each study there were no dropouts or few dropouts so all were assessed as "low risk" of bias for incomplete outcome data. Study protocols were located for two studies [14, 34]. Since all outcomes were reported, both these studies were judged "low risk" for selective outcome reporting. The other studies were judged "unclear risk".

Funnel plots for time to first bowel sounds, time to first flatus, and time to first defecation were generated for acupuncture and related therapies versus conventional care alone. These showed no apparent asymmetry and the Eggers tests were not significant (Supp. Figures S2, S4, and S6). This indicated that the risk of publication bias was not high.

3.2. Recovery of Gastrointestinal Function. Of the 21 studies that reported results for recovery of gastrointestinal function, five included comparisons with a sham acupuncture therapy and 17 studies compared the acupuncture therapy with a no acupuncture group. All groups used a form of usual postoperative or perioperative care.

3.2.1. Acupuncture Therapy versus Sham Acupuncture Therapy. All five studies were of CRC. One study used laparoscopic surgery [34] and four used open surgery. All used usual postoperative care in both groups. In two studies the test intervention was electroacupuncture [32, 34]. One study used manual acupuncture plus electroacupuncture plus ear acupuncture [14], one study used acupressure on traditional points [36], and one used point application with warming cataplasm [29]. The most commonly used traditional acupuncture points were ST36 *Zusanli* 足三里 (n=5), SP6 *Sanyinjiao* 三阴交 (n=2), and LI4 *Hegu* 合谷 (n=2). The single ear point was TF4 *Shenmen* 神门.

The sham interventions included

(i) disabled electrostimulator [14, 32, 34];

(ii) needles taped on the same points with no insertion [14];

(iii) needles inserted subcutaneously at a sham point superior and lateral to the verum point [32];

(iv) shallow insertion using short needles, 15mm away from the verum acupoints, with avoidance of acupuncture sensation "*deqi*" [34];

(v) acupressure using the same method on a nonpoint [36];

(vi) point application with nonwarming cataplasm [29].

All studies reported data for recovery of gastrointestinal function but one study used composite measures which are

reported separately [14]. Data could be pooled for time to first bowel sounds (2 studies), first flatus (4 studies), and first defecation (4 studies) (Figure 2). The other outcomes were reported by single studies only.

There was a significant reduction in time to first bowel sounds in the pooled result for two studies (MD -11.41 [-20.96, -1.85] hours, I^2=81.8%) but the heterogeneity was considerable (Table 2). For time to first flatus, there were significant reductions in the pool of two studies of electroacupuncture (MD -8.00 [-14.72, -1.28] hours, I^2=0%) without heterogeneity and in the total pool of four studies (MD -15.79 [-26.10, -5.49] hours, I^2=79.9%) with considerable heterogeneity. For time to first defecation, the pooled results showed significant reductions for electroacupuncture (MD -18.04 [-31.90, -4.19] hours, I^2=0.1%) and all acupuncture therapies (MD -22.42 [-39.14, -5.70] hours, I^2=75.4%). The source of heterogeneity in the previous two total pools was the study of point application therapy [29]. When removed, the meta-analysis results were significant for time to first flatus (MD -10.96 [-17.98, -3.94] hours, I^2=26.7%) and time to first defecation (MD -16.03 [-27.34, -4.73] hours, I^2=0%) without important heterogeneity.

In the study of acupuncture plus electroacupuncture plus ear acupuncture [14], the two composite measures were GI-3 (the later of the following two events: time that the patient first tolerated solid food, AND time that the patient first passed flatus OR a bowel movement) and GI-2 (the later of the following two events: time patient first tolerated solid food AND time patient first passed a bowel movement). There were no significant differences between groups for GI3 (MD 3.00 [-26.12, 32.12] hours, n=81) or GI2 (MD -3.00 [-31.74, 25.74] hours, n=81) but the CI were very wide.

3.2.2. Acupuncture Therapy versus Postoperative Care Alone. Seventeen RCTs compared acupuncture plus postoperative care to postoperative care without acupuncture. In two of these studies [22, 24] the Fast Track Program (FTP) of perioperative care was used in both groups.

The most commonly used traditional acupuncture points were ST36 *Zusanli* 足三里 (n=14), ST37 *Shangjuxu* 上巨虚 (n=10), LI4 *Hegu* 合谷 (n=5), SP6 *Sanyinjiao* 三阴交 (n=4), PC6 *Neiguan* 内关 (n= 3), and TE6 *Zhigou* 支沟 (n=3). Notably, ST36 *Zusanli* 足三里 was included in all studies except the three that used ear acupressure alone. The most frequently used ear points were CO4 *Wei* 胃 (n=5), CO7 *Dachang* 大肠 (n=4), and CO6 *Xiaochang* 小肠 (n=4). In one study, sensitive points on the ear were chosen on an individual basis [21].

Meta-analysis results are presented separately for time to first bowel sounds, first flatus, and first defecation (Figures 3, 4, and 5 and Tables 3, 4, and 5). Studies of a commonly used acupuncture therapy on traditional points were grouped together, with separate results presented for manual acupuncture, electroacupuncture, manual plus electroacupuncture, acupressure, and warm needling. Studies that combined traditional points with ear points (3 studies) were included as a subgroup within this traditional points group, since this approach is typical of modern acupuncture practice [38]. Ear acupressure alone was treated as a separate group since no traditional points were used.

Time to First Bowel Sounds. There were significant reductions in time to first bowel sounds (hours) in the pooled results for studies of manual acupuncture, electroacupuncture, and acupuncture/moxibustion plus ear acupressure and in the single studies of acupressure and warm needling (Figure 3).

The pool of 10 studies of acupuncture or acupressure on traditional points showed a mean reduction of 8.61 hours in the test groups (MD -8.61 [-10.60, -6.61] I^2=84.8%) but the heterogeneity was considerable (Table 3). Therefore the following sensitivity analyses were conducted. Since FTP has been found to improve recovery [39, 40], it was excluded from the pool. The result for the remaining eight RCTs was significant with reduced heterogeneity (MD -9.73 [-12.21, -7.25] I^2=80.8%, n=606). Six RCTs in this group were judged low RoB for SG. These also showed a similar result to the total pool (MD -6.95 [-9.90, -4.00] I^2=82.2%, n=410). The pool of 5 RCTs with low RoB for SG, excluding the remaining study that used FPT, also showed a similar result (MD -8.02 [-10.56, -5.47] I^2=73.2%, n=380) with reduced heterogeneity. The group for ear acupressure (3 RCTs) showed significant reductions in time to first bowel sounds with considerable heterogeneity. Due to differences between studies there were no reasonable approaches to sensitivity analyses for these groups.

The total pool of 13 RCTs showed a significant reduction with considerable heterogeneity (89.0%). In total, 7 RCTs were judged low RoB for SG. These showed a similar result to the total pool (MD -6.69 [-9.34, -4.04] I^2=80.5%, n=470). When the remaining study that used FPT also was excluded, the pool of 6 RCTs with low RoB for SG showed a similar result to the total pool with reduced heterogeneity (MD -7.57 [-9.92, -5.21] I^2=73%, n=440).

Time to First Flatus. Seventeen RCTs reported data on time to first flatus (hours) (Figure 4). There were significant reductions in the studies of manual acupuncture and electroacupuncture and in the pooled result for 14 studies that used an acupuncture therapy on traditional points (MD -15.93 [-21.44, -10.41] I^2=95.5%, n=967) but the heterogeneity was considerable (Table 4).

In the sensitivity analysis of 12 RCTs, after excluding the two studies that used FTP, the result was similar (MD -14.47 [-20.06, -8.88] I^2=95.3%, n=853). In the pool of nine RCTs judged low RoB SG the result remained significant (MD -9.28 [-13.12, -5.44]) I^2=80.8%, n=655) with reduced heterogeneity. When the remaining study that used FTP was also excluded, the result for the remaining eight RCTs was similar (MD -8.90 [-12.72, -5.09] I^2=81.9%, n=625). In the three RCTs of ear acupressure without any traditional points, the pooled result showed a significant reduction. In the total pool of all 17 RCTs, the result were similar to that for the traditional points group with considerable heterogeneity (I^2=98.4%).

In the sensitivity analyses for the total pool, removing the two studies that used FTP produced a similar result (MD -13.49 [-18.70, -8.29] I^2=98.6%, n=1151). In the 10 RCTs judged low RoB SG, there was reduced heterogeneity (MD -8.97 [-12.40, -5.55] I^2=78.9%, n=715) and when the study that also used FTP was excluded, the pooled result for the remaining 9 RCTs was similar (MD -8.67 [-12.06, -5.27] I^2=79.9%, n=685).

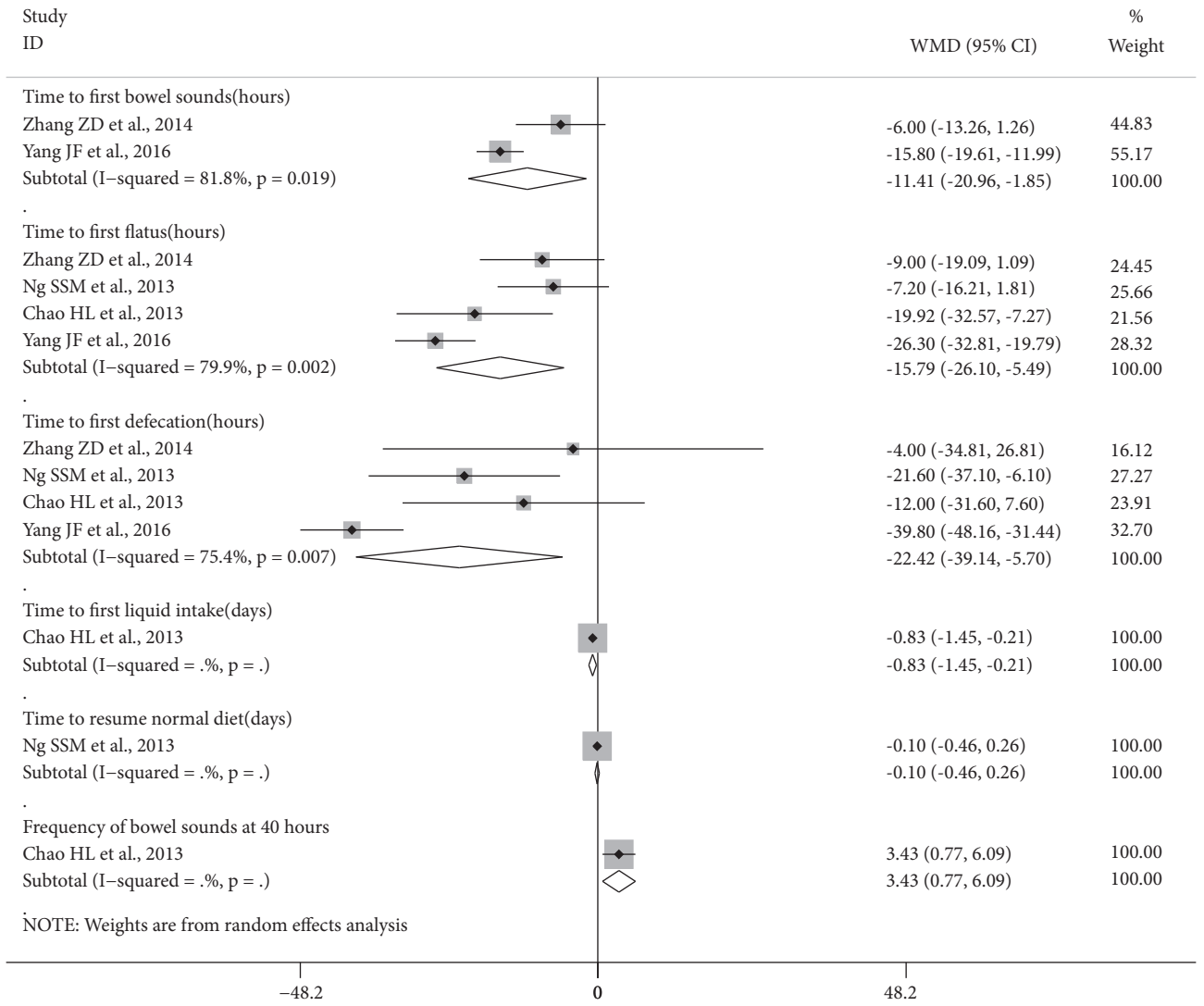

Figure 2: **Forest plot of acupuncture therapy versus sham acupuncture therapy for recovery of gastrointestinal function**. Note: frequency of bowel sounds at 40 hours: frequency per minute assessed during a three-minute interval.

Time to First Defecation. There were significant reductions in time to first defecation in the studies of manual acupuncture and electroacupuncture (Figure 5) and in the pooled result of 11 RCTs of acupuncture therapies that used traditional points (MD -13.53 [-18.38, -8.67] I^2=94.1%, n=850) but the heterogeneity was substantial (Table 5).

In the sensitivity analyses, removal of the two studies that used FTP reduced the heterogeneity (MD -11.31 [-14.58, -8.04] I^2=79.2%, n=736) and heterogeneity was further reduced in the pool of nine studies judged low RoB SG (MD -10.07 [-12.99, -7.15] I^2=71%, n=656).

In the total pool of all 13 studies of an acupuncture therapy, the result was significant with substantial heterogeneity (MD -12.34 [-16.84, -7.84] I^2=94.9%). This heterogeneity was reduced when the two FTP studies were removed (MD -10.29 [-13.31, -7.27] I^2=83.8%, n=926) and was further reduced in the pool of 10 RCTs with low RoB SG (MD -9.97 [-12.69, -7.25] I^2=67.7%, n=716).

Other Measures of Gastrointestinal Recovery. In addition to the above outcomes, four studies reported on other measures of gastrointestinal recovery (Table 6). In the pool of two studies of CRC that reported time to first liquid intake [17, 21], there was significant improvement in the acupuncture therapy group (MD -19.72 [-20.22, -19.22] I^2=0%). For time to first semifluid food intake, a single study of colon cancer in elderly patients found no difference between groups [26]. For time to resume normal diet, a single study of laparoscopic surgery for CRC found a significant improvement in the electroacupuncture group [34].

3.3. Other Postoperative Recovery Outcomes. One study of manual acupuncture plus moxibustion [27] that reported the incidence of postoperative urinary retention found one case in the acupuncture group and two cases in the usual care group. There was no significant difference between groups (RR 0.50 [0.05, 4.94] n=30).

Table 2: Acupuncture therapy versus sham acupuncture therapy for recovery of gastrointestinal function.

Outcome	Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD [95% CI] I^2	Study ID No.†
Time to first bowel sounds (hours)	Open surgery, CRC (39)	Electro-acup.	-6.00 [-13.26, 1.26]	21
	Open surgery, elderly, CRC (72)	Point application	-15.8 [-19.61, -11.99]∗	18
	Pooled result (111) 2 RCTs	All acupuncture therapies	-11.41 [-20.96, -1.85]∗ 81.8%	18, 21
Time to first flatus (hours)	Laparoscopic surgery, CRC (110)	Electro-acup.	-7.20 [-16.21, 1.81]	7
	Open surgery, CRC (39)	Electro-acup.	-9.00 [-19.09, 1.09]	21
	Pooled result (149)	Electro-acup.	-8.00 [-14.72, -1.28]∗ 0%	7, 21
	Open surgery, CRC (60)	Acupressure	-19.92 [-32.57, -7.27]∗	1
	Open surgery, elderly, CRC (72)	Point application	-26.30 [-32.81, -19.79]∗	18
	Pooled result (281) 4 RCTs	All acupuncture therapies	-15.79 [-26.10, -5.49]∗ 79.9%	1, 7, 18, 21
Time to first defecation (hours)	Laparoscopic surgery, CRC (110)	Electro-acup.	-21.60 [-37.10, -6.11]∗	7
	Open surgery, CRC (39)	Electro-acup.	-4.00 [-34.81, 26.81]	21
	Pooled result (149)	Electro-acup.	-18.04 [-31.90, -4.19]∗ 0.1%	7, 21
	Open surgery, CRC (60)	Acupressure	-12.00 [-31.60, 7.60]	1
	Open surgery, elderly, CRC (72)	Point application	-39.80 [-48.16, -31.44]∗	18
	Pooled result (281)4 RCTs	All acupuncture therapies	-22.42 [-39.14, -5.70]∗ 75.4%	1, 7, 18, 21
Time to first liquid intake (days)	Open surgery, CRC (60)	Acupressure	-0.83 [-1.45, -0.21]∗	1
Time to resume normal diet (days)	Laparoscopic surgery, CRC (110)	Electro-acup.	-0.10 [-0.46, 0.26]	7
Frequency of bowel sounds at 40 hours[1]	Open surgery, CRC (60)	Acupressure	3.43 [0.77, 6.09]∗	1

∗ means statistically significant; † see Table 1; 1: frequency per minute assessed during a three minute interval.
CI: confidence interval; MD: mean difference.

Three studies reported on abdominal distension at five days after surgery. Since each study used a different approach to reporting data, data were not pooled. One study included two test groups [25] (Table 7). One of the studies that used FTP showed no difference between groups while the others found significant reductions in the groups that received acupuncture.

One study of electroacupuncture (n=76) reported on quality of life using a modified Edmonton Symptom Assessment System (ESAS) which consists of five items (pain, nausea, insomnia, abdominal distension, and general sense of well-being), which are each rated using a 0-10 numeric rating scale. The authors reported no differences between groups for any outcome [18].

3.4. Safety. One RCT [18] mentioned that there were no AEs greater than CTCAE grade I [41] for electroacupuncture. Another RCT stated there was no AE for manual acupuncture [27]. The other studies did not mention AEs. Therefore it was not possible to make an assessment of the safety of the acupuncture therapies.

3.5. Post Hoc Analyses. It is plausible that acupuncture may show a dose-response effect which is influenced by the type of acupuncture, the number of points needled, the patient response in terms experiencing typical acupuncture sensations (*deqi*), needle retention time, stimulation method, number and frequency of treatments, and other factors [42, 43]. In order to explore this issue we conducted post hoc analyses of the acupuncture therapy versus usual postoperative care group based on available data for the number of acupuncture points used and whether the study mentioned the experience of *deqi*.

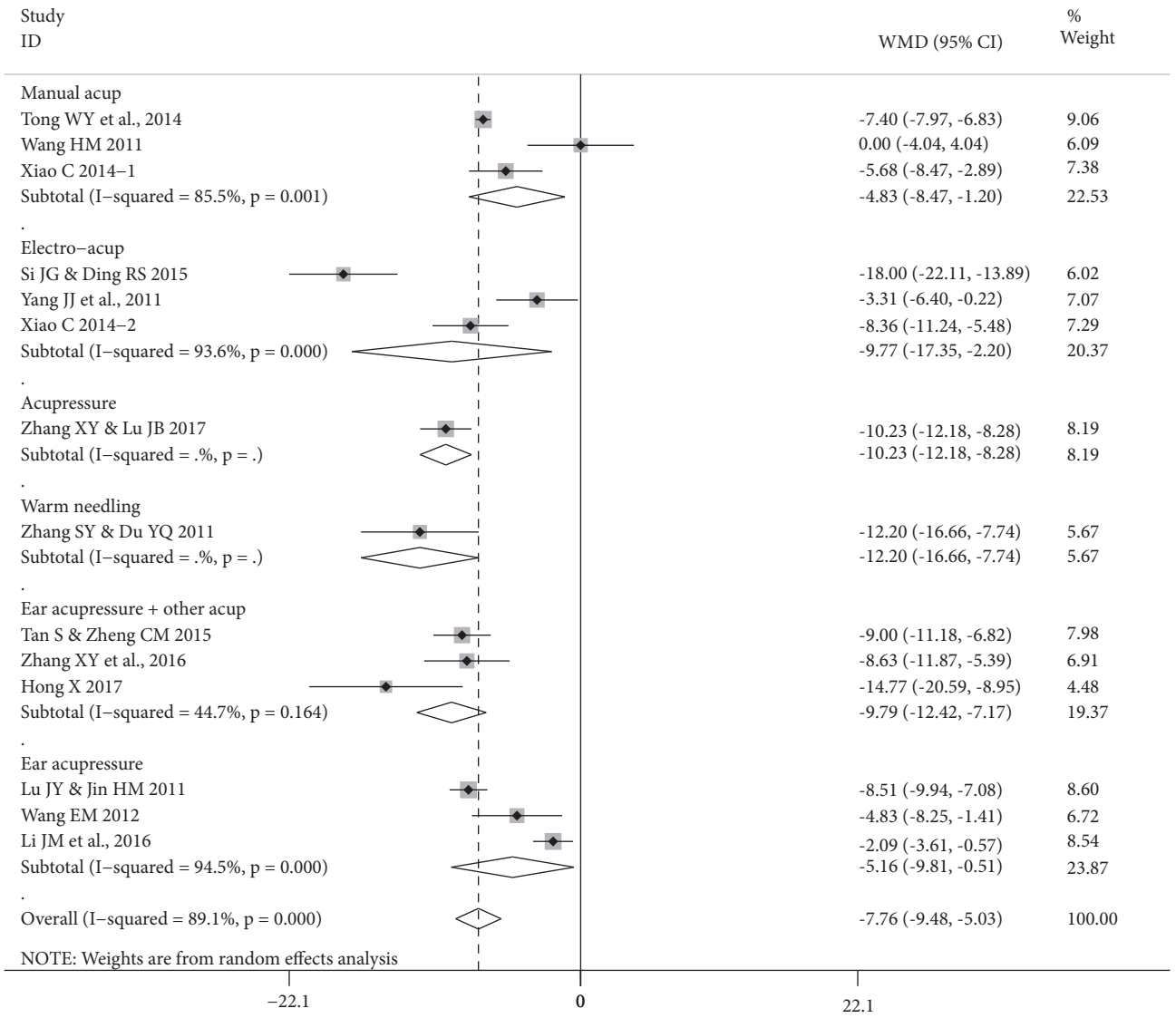

Figure 3: **Forest plot of acupuncture therapy versus postoperative care for time to first bowel sounds (13 RCTs, 14 groups)**. Note: this forest plot focuses on the effect sizes for each study. Since Xiao C 2014 is a three-group study with two test groups of n=30 and one control group n=30, in the meta-analysis in Table 3 the number of participants was halved n=15 in each comparison. In this figure, the control group remains n=30, so this has a small effect on the result for the total pool. The pooled result in Table 3 is the more accurate estimate of the pooled effect size, while the effects for Xiao C 2014-1 and Xiao C 2014-2 are accurate in this figure.

There was variation in the number of studies that reported on the three main outcomes with time to first flatus providing the most complete data. For analysis of number of points we selected the subgroup of studies that used acupuncture (manual or electro-), acupressure, and/or moxa on traditional points excluding studies that also used ear points. In this group of 11 studies, which all reported time to first flatus, the number of traditional acupuncture points used ranged from four to 12 (one point used bilaterally was counted as two points), so studies were divided into two groups: 1. 4-6 points; and 2. 8-12 points, which was the most equal division. For each of the three main POI outcomes, both groups showed significant reductions in time to outcome, and the effect sizes were larger for the 8-12 points groups (Supp. Table S2). However the confidence intervals were overlapping and heterogeneity was substantial to considerable. So while the results suggest a dose-response trend, this could not be confirmed.

For experience of *deqi,* all 11 studies that mentioned that the patient experienced acupuncture sensation(s) were treated as a subgroup irrespective of the points or acupuncture therapy used (Supp. Table S2). For each of the three main POI outcomes there were significant improvements in the acupuncture therapy groups. The effect sizes were comparable with the results for the total pools and the confidence intervals overlapped. Heterogeneity was reduced but remained substantial to considerable.

Table 3: Acupuncture therapy versus postoperative care for time to first bowel sounds.

Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD [95% CI] hours, I^2	Study ID No.†
Open surgery, FTP, rectal cancer (84)	Manual acup.	-7.40 [-7.97, -6.83]∗	11
Open surgery, FTP,[1] CRC (30)	Manual acup.	0.00 [-4.04, 4.04]	13
Open radical surgery,[2] CRC (60)	Manual acup.	-5.68 [-8.47, -2.89]∗	14.1 (T1)
Pooled result (174) 3 RCTs	Manual acup.	-4.83 [-8.47, -1.20]∗ 85.5%	11, 13, 14
Radical surgery, CRC (40)	Electro-acup.	-18.00 [-22.11, -13.89]∗	9
Radical surgery, CRC (60)	Electro-acup.	-3.31 [-6.40, -0.22]∗	17
Open radical surgery,[2] CRC (60)	Electro-acup.	-8.36 [-11.24, -5.49]∗	14.2 (T2)
Pooled result (160) 3 RCTs	Electro-acup.	-9.77 [-17.35, -2.20]∗ 93.6%	9, 14, 17
Radical surgery,[2] CRC (80)	Acupressure	-10.23 [-12.18, -8.28]∗	20
Radical surgery, CRC (70)	Warm needling	-12.20 [-16.66, -7.74]∗	19
Pooled result (454) 7 RCTs	Manual, electro-, acupressure, warm needling	-8.06 [-10.65, -5.47]∗ 88%	9, 11, 13, 14, 17, 19, 20
Radical surgery, CRC (76)	Manual acup. plus ear acupressure [3]	-9.00 [-11.18, -6.82]∗	10
Usual surgery, CRC (80)	Acupressure plus ear acupressure	-8.63 [-11.87, -5.39]∗	22
Dixon surgery, rectal cancer (80)	Moxa plus ear acupressure	-14.77 [-20.59, -8.95]∗	3
Pooled result (236) 3 RCTs	Manual acup./ moxa plus ear acupressure	-9.79 [-12.42, -7.17]∗ 44.7%	3, 10, 22
Pooled result (690) 10 RCTs	All acup., acupressure, moxa on trad. points	-8.61 [-10.60, -6.61]∗ 84.8%	3, 9-11, 13, 14, 17, 19, 20, 22
Radical surgery,[2] CRC (78)	Ear acupressure	-8.51 [-9.94, -7.08]∗	5
Usual surgery, colon cancer (60)	Ear acupressure	-4.83 [-8.25, -1.41]∗	12
Open radical surgery, rectal cancer (160)	Ear acupressure	-2.09 [-3.61, -0.57]∗	4
Pooled result (298) 3 RCTs	Ear acupressure	-5.16 [-9.81, -0.51]∗ 94.5%	4, 5, 12
Total pool (988) 13 RCTs	All acupuncture therapies	-7.78 [-9.55, -6.01]∗ 89.0%	All above

∗ means statistically significant; † see Table 1; 1: acupuncture began 24 hours after surgery; 2: excluding Miles surgery; 3: using sensitive ear points.
CI, Confidence Interval; MD, mean difference; moxa, moxibustion; trad., traditional.

4. Discussion

Of the 21 studies that reported results for recovery of gastrointestinal function, all but the three studies of ear acupressure alone used the point ST36 *Zusanli* 足三里, usually in combination with other points. In the case of the ear acupressure alone studies, these all used CO4 *Wei* 胃 and CO7 *Dachang* 大肠 plus other points. Each of these points is commonly used for gastrointestinal disorders [38, 44]. Of the stimulation methods, manual acupuncture with or without electrostimulation was the most frequently used. Other commonly used methods included acupressure, moxibustion, and ear acupuncture or acupressure [38, 44]. Overall, this group of studies was reflective of the scope of acupuncture practice internationally.

For the five blinded sham-controlled studies, data pooling was feasible for four studies and for the three main POI outcomes. All these outcomes showed significant reductions in the time to these events. The heterogeneity in the total pools was attenuated by removal of the single study of point application therapy without affecting the overall result. There was no apparent effect on the result for the type of sham used. This was consistent with earlier reviews [45, 46].

In the remaining 17 studies, there were significant differences in the pooled data in favour of the acupuncture and related therapy groups compared to the conventional care alone groups for all three main measures of gastrointestinal function recovery but the heterogeneity was considerable in the pooled results. Heterogeneity was still evident when

Table 4: Acupuncture therapy versus postoperative care for time to first flatus.

Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD [95% CI] hours I^2	Study ID No.†
Open surgery, FTP, rectal cancer (84)	Manual acup.	-28.56 [-32.45, -24.67]∗	11
Open surgery, FTP, CRC (30)	Manual acup.	-31.20 [-58.91, -3.49]∗	13
Open radical surgery[2], CRC (60)	Manual acup.	-6.49 [-11.88, -1.10]∗	14.1 (T1)
Pooled result (174) 3 RCTs	Manual acup.	-20.51 [-39.19, -1.84]∗ 95.3%	11, 13, 14
Radical surgery[2], CRC (32)	Electro-acup.	-28.13 [-34.65, -21.61]∗	8
Radical surgery, CRC (40)	Electro-acup.	-37.90 [-42.34, -33.46]∗	9
Radical surgery, CRC (60)	Electro-acup.	-2.93 [-5.51, -0.35]∗	17
Open radical surgery[2], CRC (60)	Electro-acup.	-9.95 [-14.89, -5.01]∗	14.2 (T2)
Intraperitoneal surgery, colon cancer (75)	Electro-acup.	3.02 [-6.44, 12.48]∗	6
Laparoscopic surgery, CRC, (110)	Electro-acup.	-14.40 [-23.41, -5.39]∗	7
Pooled result (377) 6 RCTs	Electro-acup.	-15.17 [-28.81, -1.54]∗ 97.6%	6-9, 14, 17
Radical surgery, colon cancer, elderly patients (60)	Manual plus electro-acup.	-8.00 [-17.12, 1.12]	15
Radical surgery[2], CRC (80)	Acupressure	-11.05 [-13.44, -8.66]∗	20
Radical surgery, CRC (70)	Warm needling	-18.55 [-23.86, -13.24]∗	19
Pooled result (731) 11 RCTs	Manual, electro-, acupressure, warm needling	-15.68 [-23.03, -8.33]∗ 96.1%	6-9, 11, 13-15, 17, 19, 20
Radical surgery, CRC (76)	Manual acup. plus ear acupressure [3]	-18.70 [-21.01, -16.39]∗	10
Usual surgery, CRC (80)	Acupressure plus ear acupressure	-9.67 [-13.58, -5.76]∗	22
Dixon surgery, rectal cancer (80)	Moxa plus ear acupressure	-22.90 [-30.10, -15.70]∗	3
Pooled result (236) 3 RCTs	Manual acup./ moxa plus ear acupressure	-16.72 [-23.79, -9.65]∗ 89%	3,10, 22
Pooled result (967) 14 RCTs	All acup., acupressure, moxa on trad. points	-15.93 [-21.44, -10.41]∗ 95.5%	3, 6-11, 13-15, 17, 19, 20, 22
Radical surgery[2], CRC (78)	Ear acupressure	-19.85 [20.35, -19.35]∗	5
Usual surgery, colon cancer (60)	Ear acupressure	-6.73 [-11.16, -2.30]∗	12
Open radical surgery, rectal cancer (160)	Ear acupressure	-2.09 [-3.26, -0.92]∗	4
Pooled result (298) 3 RCTs	Ear acupressure	-9.59 [-23.58, 4.39] 99.7%	4, 5, 12
Total pool (1265) 17 RCTs	All acupuncture therapies	-14.77 [-19.75, -9.79]∗ 98.4%	All above

∗ means statistically significant; † see Table 1; 1: acupuncture began 24 hours after surgery; 2: excluding Miles surgery; 3: using sensitive ear points.
CI, Confidence Interval; MD, mean difference; moxa, moxibustion; trad., traditional.

data were grouped according to the type of acupuncture intervention, while the significant differences were maintained. Removal of the studies that used FTP, which is likely to have provided an independent contribution to recovery, reduced heterogeneity somewhat but there were only two such studies so the reductions were small. The sensitivity analyses of studies that were judged low RoB SG showed lower heterogeneity and some reductions in effect sizes but

Table 5: Acupuncture therapy versus postoperative care for time to first defecation.

Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD [95% CI] I^2	Study ID No.†
Open surgery, FTP, rectal cancer (84)	Manual acup.	-22.56 [-24.36, -20.76]∗	11
Open surgery, FTP[1], CRC (30)	Manual acup.	-28.80 [-62.11, 4.51]	13
Open radical surgery[2], CRC (60)	Manual acup.	-5.71 [-10.25, -1.17]∗	14.1 (T1)
Pooled result (174) 3 RCTs	Manual acup.	-16.30 [-31.35, -1.25]∗ 95.7%	11, 13, 14
Radical surgery, CRC (60)	Electro-acup.	-5.16 [-7.98, -2.34]∗	17
Open radical surgery[2], CRC (60)	Electro-acup.	-12.96 [-16.67, -9.25]∗	14.2 (T2)
Intraperitoneal surgery, colon cancer (76)	Electro-acup.	-0.34 [-24.71, 24.03]	6
Laparoscopic surgery, CRC (110)	Electro-acup.	-36.20 [-53.26, -19.14]∗	7
Pooled result (306) 4 RCTs	Electro-acup.	-12.39 [-20.97, -3.81]∗ 86%	6, 7, 14, 17
Radical surgery, colon cancer, elderly patients (60)	Manual plus electro-acup.	-3.00 [-13.32, 7.32]	15
Radical surgery[2], CRC (80)	Acupressure	-9.65 [-11.23, -8.07]∗	20
Radical surgery, CRC (70)	Warm needling	-16.30 [-23.13, -9.47]∗	19
Pooled result (660) 9 RCTs	Manual, electro-, acupressure, warm needling	-12.68 [-18.60, -6.77]∗ 94.8%	6, 7, 11, 13-15, 17, 19, 20
Usual surgery, CRC (80)	Acupressure plus ear acupressure	-11.16 [-13.66, -8.66]∗	22
Dixon surgery, rectal cancer (80)	Moxa plus ear acupressure	-25.47 [-33.91, -17.03]∗	3
Pooled result (160) 2 RCTs	Manual acup./ moxa plus ear acupressure	-17.72 [-31.70, -3.75]∗ 90.2%	3, 22
Pooled result (820) 11 RCTs	All acup., acupressure, moxa on trad. points	-13.53 [-18.38, -8.67]∗ 94.1%	3, 6, 7, 11, 13-15, 17, 19, 20, 22
Usual surgery, colon cancer (60)	Ear acupressure	-9.28 [-16.73, -1.83]∗	12
Open radical surgery, rectal cancer (160)	Ear acupressure	-4.08 [-6.02, -2.14]∗	4
Pooled result (220) 2 RCTs	Ear acupressure	-5.38 [-9.80, -0.97]∗ 42.9%	4, 12
Total pool (1040) 13 RCTs	All acupuncture therapies	-12.34 [-16.84, -7.84]∗ 94.9%	All above

∗ means statistically significant; † see Table 1; 1: acup. began 24 hours after surgery; 2: excluding Miles surgery.
CI, Confidence Interval; MD, mean difference; moxa, moxibustion; trad., traditional.

Table 6: Acupuncture therapy versus postoperative care for other measures of gastrointestinal recovery.

Outcome	Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD[1] [95% CI] I^2	Study ID No.†
Time to first liquid intake (hours)	Radical surgery, CRC (76)	Manual acup. plus ear acupressure[2]	-18.90 [-21.20, -16.60]∗	10
	Radical surgery[1], CRC (78)	Ear acupressure	-19.76 [-20.27, -19.25]∗	5
Pooled result (hours)	2 RCTs (154)	Manual acup., ear acupressure	-19.72 [-20.22, -19.22]∗ 0%	5, 10
Time to first semifluid food intake (hours)	Radical surgery, colon cancer, elderly patients (60)	Manual plus electro-acup.	-4.00 [-37.46, 29.46]	15
Time to resume normal diet (days)	Laparoscopic surgery, CRC (110)	Electro-acup.	-0.80 [-1.40, -0.20]∗	7

∗ means statistically significant; † see Table 1; 1: excluding Miles surgery; 2: on sensitive ear points.
CI: confidence interval; MD: mean difference.

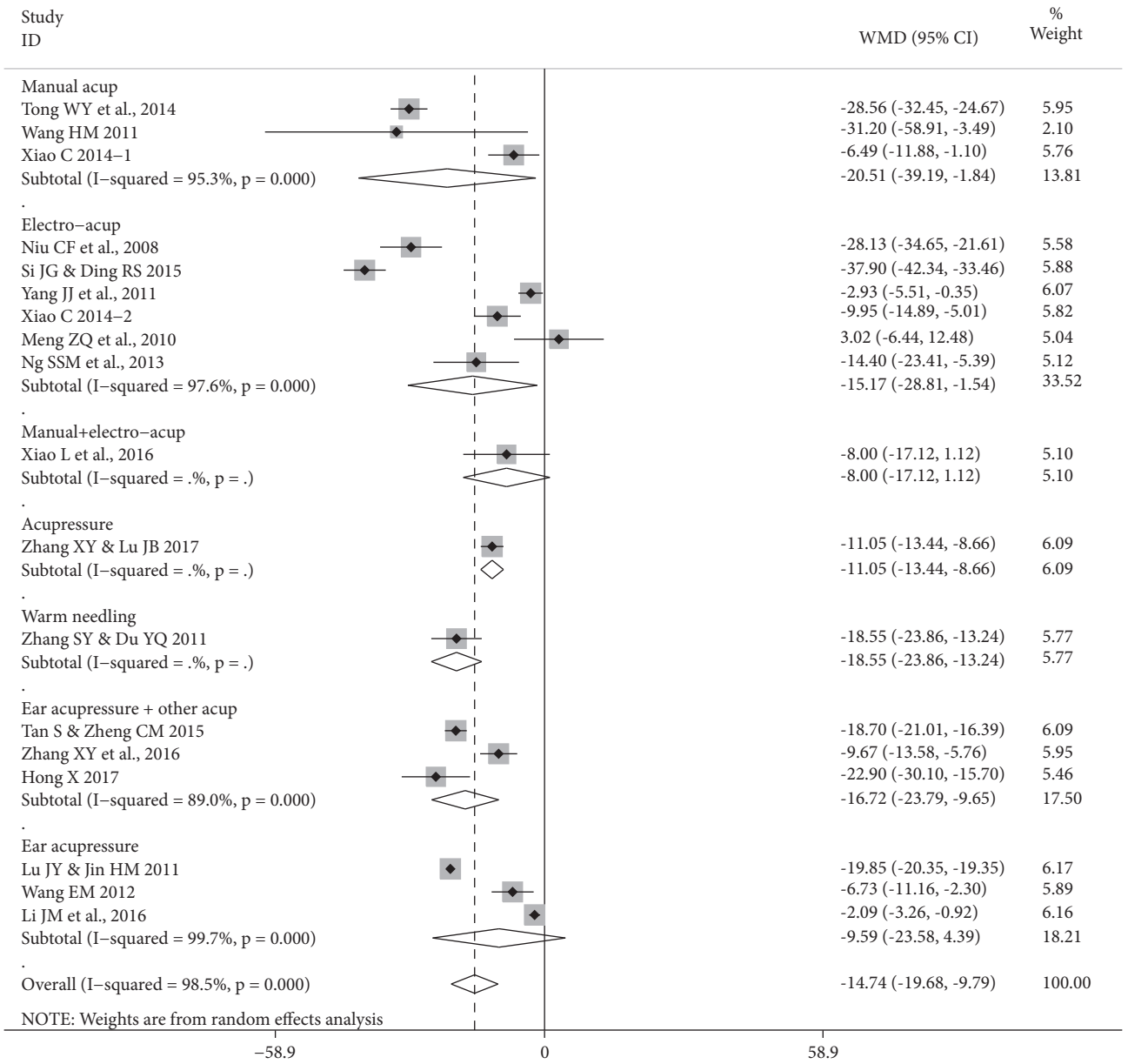

Figure 4: **Forest plot of acupuncture therapy versus postoperative care for time to first flatus (17 RCTs, 18 groups)**. Note: this forest plot focuses on the effect sizes for each study. Since Xiao C 2014 is a three-group study with two test groups of n=30 and one control group n=30, in the main meta-analysis (Table 4) the number of participants was halved n=15 in each comparison. In this figure, the control group remains n=30, so this has a small effect on the result for the total pool. The pooled result in Table 4 is the more accurate estimate of the pooled effect size, while the effects for Xiao C 2014-1 and Xiao C 2014-2 are accurate in this figure.

Table 7: Acupuncture therapy versus postoperative care for postoperative abdominal distension.

Treatment type, cancer, participants (number)	Acupuncture therapy	Effect Size MD/RR [95% CI] I^2	Study ID No.†
Open surgery, FTP[1], CRC (30)	Manual acup.	RR 0.67 [0.13, 3.44][3]	13
Open surgery, FTP, rectal cancer (84)	Manual acup.	MD -0.77 [-0.80, -0.74]∗	11
Open radical surgery[2], CRC (60)	Manual acup.	MD -0.27 [-0.51, -0.03]∗	14.1 (T1)
Open radical surgery[2], CRC (60)	Electro-acup.	MD -0.53 [-0.75, -0.32]∗	14.2 (T2)

∗ means statistically significant; † see Table 1; 1: acupuncture began 24 hours after surgery; 2: excluding Miles surgery; 3: incidence of medium to severe (grades II-III) abdominal distension.
CI: confidence interval; MD: mean difference.

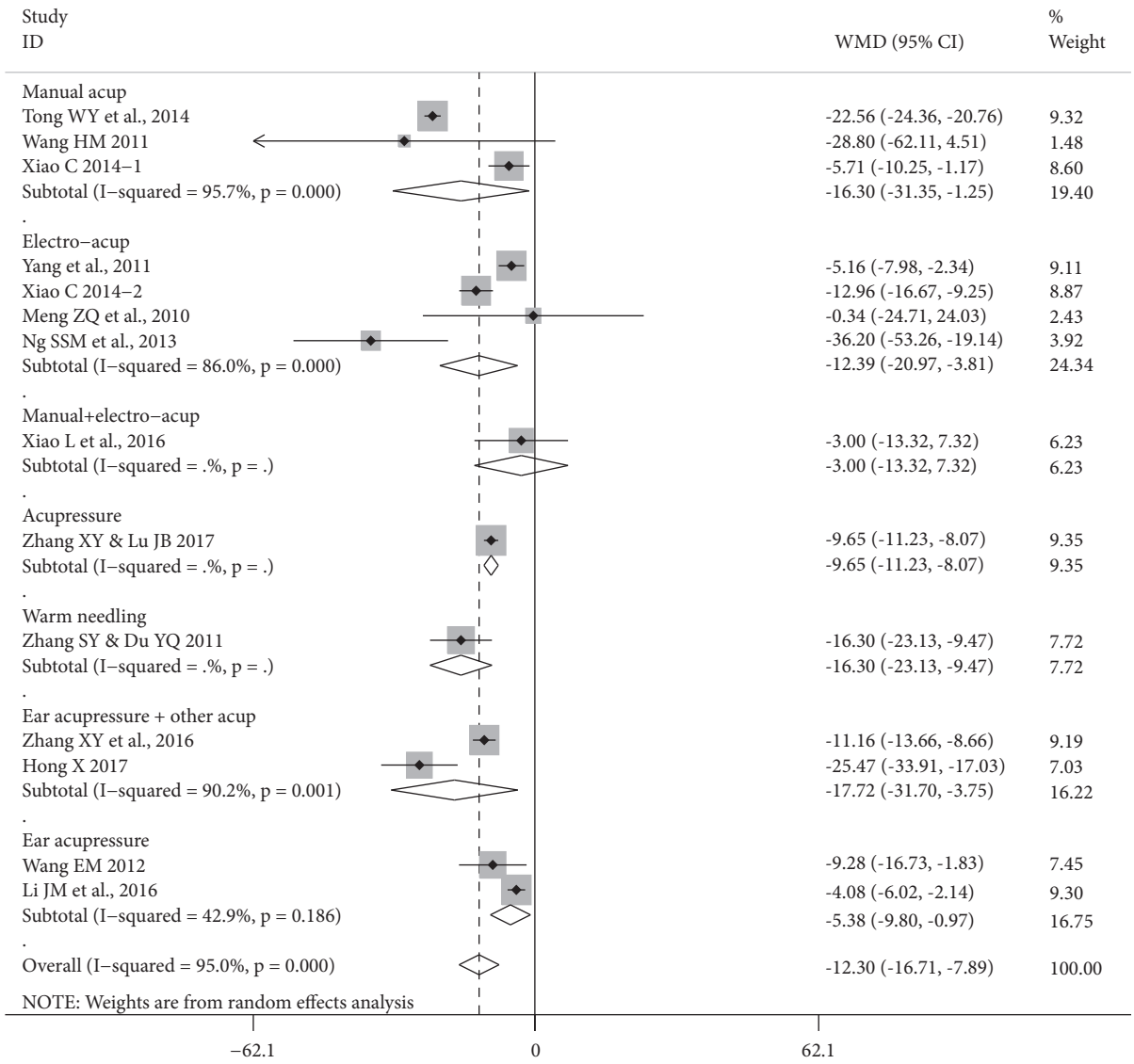

Figure 5: **Forest plot of acupuncture therapy versus postoperative care for time to first defecation (13 RCTs, 14 groups)**. Note: this forest plot focuses on the effect sizes for each study. Since Xiao C 2014 is a three-group study with two test groups of n=30 and one control group n=30, in the meta-analysis in Table 5 the number of participants was halved n=15 in each comparison. In this figure, the control group remains n=30, so this has a small effect on the result for the total pool. The pooled result in Table 5 is the more accurate estimate of the pooled effect size, while the effects for Xiao C 2014-1 and Xiao C 2014-2 are accurate in this figure.

the heterogeneity remained moderate to substantial. Further attempts to group studies by combinations of surgery type, cancer type, and acupuncture type were not productive since any resultant pools comprised too few studies. It is notable that the direction of the effect in the majority of studies was in favour of the acupuncture interventions. So it appears likely that the statistical heterogeneity reflected the clinical diversity amongst the studies and variation in effect sizes. It was not an indicator of an unclear direction in the results. In addition to measures of POI, acupuncture appeared to reduce postoperative abdominal distension but there were too few studies for any strong conclusions.

Overall, the results of the 21 RCTs indicated that acupuncture therapies reduced time to recovery of gastrointestinal function following CRC surgery. In studies of other abdominal surgeries, acupuncture has been reported to reduce POI following gastrectomy [47, 48] and caesarean section [49], so the effects found in this meta-analysis are not limited to surgery for CRC. With regard to type of surgery, most data were for open surgery but significant reductions in time to recovery were also evident in studies of laparoscopic surgery for CRC and surgery for rectal cancer.

With regard to type of acupuncture therapy, the sham-controlled studies which constitute the better quality evidence support electroacupuncture on the point ST36 *Zusanli* 足三里, with or without other points, as effective for recovery of POI. In the nonblinded studies, the heterogeneity in meta-analysis pools precluded detailed assessment of which type of

acupuncture was more effective. The results appear to support various types of acupuncture therapy on ST36 *Zusanli* 足三里 combined with other points. In general, the categories of acupuncture were not obviously different from each other in terms of effect sizes. Notably, electroacupuncture did not appear to be any better than manual acupuncture. Also, the pooled effect size results for the sham-controlled studies were comparable with those for the nonblinded studies. This suggests that lack of blinding did not lead to inflation of effect sizes. It is notable that all the studies were conducted in a hospital setting, so it is likely that there was little opportunity for participants to interact with each other over the short durations of the studies, and the outcome data were usually collected by nursing staff. These features of the setting appear conducive to the collection of more objective data than may be the case in longer studies in out-patient settings.

A previous meta-analysis of acupuncture for headache suggested that electroacupuncture was more effective than manual acupuncture, longer needle retention was better, and twice-a-week treatment was better than once-a-week treatment [50], a review of acupuncture for menstrual pain found effects for needle location, number of needles used, and frequency of treatment [51], but a clinical study found no significant effects of needle retention duration on outcomes in oncology [52]. In attempting to determine whether there was any dose-response effect for the acupuncture on POI outcomes, the only feasible parameters were overall number of points used and patient experience of *deqi*. It was not possible to determine estimates of the total number of treatments or total duration of treatment since treatment typically ceased once the outcome had been achieved. The results suggested that more points may be better but the wide confidence intervals and statistical heterogeneity precluded any strong conclusions. There were too few studies for any effects of number of ear points to be examined. Future studies could consider designs that directly test potential dose-related factors.

Patient experience of *deqi* did not appear to affect results. Since *deqi* is a typical aspect of acupuncture practice, it is likely that mention was omitted in a number of study reports. Therefore, the meaningfulness of this result is unclear. Notably, the sham-controlled studies of acupuncture, which were reported in more detail, did mention *deqi*.

One limitation with these meta-analyses was the methodological quality and associated risk of bias in the included studies. The majority of the studies were not blind to participants and the acupuncturists were not blinded in any of the studies. Nevertheless, the results of the multiple sham-controlled studies tended to agree with those of the nonblinded studies. Many studies conducted in China do not report according to the CONSORT or STRICTA guidelines, resulting in omission of important aspects of trial methodology [37, 53]. From the point of view of meta-analysis, inadequacies in study reporting substantially limit the opportunities for exploration of clinically relevant variables [54], so it is vital that journals endorse these guidelines.

Although most of the studies were conducted in China, integrative cancer therapy employing acupuncture is used in hospitals outside China and has proven acceptable to patients in Europe, America, and Australia [52, 55]. Based on the results of this meta-analysis, the extension of acupuncture use in postoperative care should be considered.

5. Conclusions

The addition of an acupuncture intervention following surgery for CRC improved outcomes for recovery of gastrointestinal function based on pooled data from four blinded good quality RCTs (281 participants) and 17 nonblinded lower quality RCTs (1,265 participants). The best available evidence was for interventions that included electroacupuncture at the point ST36 *Zusanli*. There is supporting evidence for other types of acupuncture therapies that involve stimulation of this point plus other points. Further well-designed blinded studies are needed to confirm these findings and determine optimal acupuncture interventions for POI.

Disclosure

Yihong Liu and Brian H. May are co-first authors

Conflicts of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplementary Materials

Supp. 1: Search strategy used to identify studies of acupuncture and related therapies for CRC: PubMed. Figure S1: Funnel plot of acupuncture therapy versus postoperative care for time to first bowel sounds. Figure S2: Funnel plot of acupuncture therapy versus postoperative care for time to first flatus. Figure S3: Funnel plot of acupuncture therapy versus postoperative care for time to first defecation. Table S1: Risk of bias judgements for included studies. Table S2: Post hoc analyses of potential dose-response parameters for POI outcomes. *(Supplementary Materials)*

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Research Article

A New Strategy to Uncover the Anticancer Mechanism of Chinese Compound Formula by Integrating Systems Pharmacology and Bioinformatics

Yifei Dai,[1] Liang Sun,[2] and Weijie Qiang[1]

[1]*Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing, China*
[2]*The China Institute for History of Medicine and Medical Literature, Beijing, China*

Correspondence should be addressed to Weijie Qiang; qiangwj92@163.com

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Currently, cancer has become one of the major refractory diseases threatening human health. Complementary and alternative medicine (CAM) has gradually become an alternative choice for patients, which can be attributed to the high cost of leading cancer treatments (including surgery, radiotherapy, and chemotherapy) and the severe related adverse effects. As a critical component of CAM, traditional Chinese medicine (TCM) has increasing application in preventing and treating cancer over the past few decades. Huanglian Jiedu Decoction (HJD), a classical Chinese compound formula, has been recognized to exert a beneficial effect on cancer treatment, with few adverse effects reported. Nevertheless, the precise molecular mechanism remains unclear yet. In this study, we had integrated systems pharmacology and bioinformatics to explore the major active ingredients against cancer, targets for cancer treatment, and the related mechanisms of action. These targets were scrutinized using web-based Gene SeT Analysis Toolkit (WebGestalt), and 10 KEGG pathways were identified by enrichment analysis. Refined analysis of the KEGG pathways indicated that the anticancer effect of HJD showed a functional correlation with the p53 signaling pathway; moreover, HJD had potential therapeutic effect on prostate cancer (PCa) and small cell lung cancer (SCLC). Afterwards, genetic alterations and survival analysis of key targets for cancer treatment were examined in both PCa and SCLC. Our results suggested that such integrated research strategy might serve as a new paradigm to guide future research on Chinese compound formula. Importantly, such strategy contributes to studying the anticancer effect and the mechanisms of action of Chinese compound formula, which has also laid down the foundation for clinical application.

1. Introduction

According to a WHO report, cancer has become the leading killer of human health, which is associated with high recurrence rate and high mortality. Typically, the year 2012 has witnessed about 14 million new cancer cases and 8.2 million cancer-related deaths. It is estimated that the annual new cases will increase from 14 million to 22 million over the coming 20 years [1]. The existing anticancer treatments mainly include surgery, radiotherapy, and chemotherapy. However, the patients would eventually choose to discontinue the treatment due to the high cost of radiotherapy and chemotherapy, as well as the serious related adverse effects [2]. With the development of medicine, cancer is treated based on a comprehensive and diversified treatment, and complementary and alternative medicine (CAM) has become an alternative option for patients under such circumstances. Traditional Chinese medicine (TCM), a critical component of CAM, has been increasingly applied in preventing and treating cancer over the past few decades [3, 4]. As an adjuvant therapy, Chinese medicine shows beneficial effect on cancer treatment with few adverse effects reported [5].

Huanglian Jiedu Decoction (HJD), first recorded in the *Prescriptions for Emergent Reference (Zhouhou Beiji Fang)* written by Ge Hong, consists of four herbs, including *Coptidis Rhizoma* (Huanglian), *Scutellariae Radix* (Huangqin), *Phellodendri Chinrnsis Cortex* (Huangbo), and *Gardeniae Fructus* (Zhizi). HJD is a representative formula for cancer

treatment, which is frequently employed to treat pancreatic cancer, breast cancer, liver cancer, and colorectal cancer (CRC) in clinical practice [6]. For instance, some results of pharmacological experiment suggest that HJD has anticancer effect on human liver cancer cells both in vitro and in vivo, which can also markedly extend the survival time of liver cancer bearing mice [7, 8]. However, the precise mechanism of its anticancer effect remains unclear so far.

Chinese compound formula is characterized by the synergistic effects of multicomponent and multitarget. On this account, a method suitable for its characteristics is needed to reveal the underlying mechanism of action. Systems pharmacology is a new discipline studying the regularity and mechanism of drug-organism interaction at the system level [9]. It can study the changes in body function mechanisms caused by drug treatment for diseases from molecules, cells, tissues, to organs. Moreover, it would establish the interrelationships between drug efficacy and the organism at both microscopic levels (molecular and biochemical network levels) and macroscopic levels (tissue, organ, and overall levels). Besides, extremely abundant cancer data have been produced in recent years, with the rapid development of bioinformatics technology, including microarray, proteomics, and other high-throughput screening assays. By integrating systems pharmacology and bioinformatics, this study aimed to explore the relationships of HJD with its cancer-related targets and interactive genes and to reveal the underlying molecular mechanisms of action. Such strategy would be helpful for investigating the anticancer effect and the mechanism of action of Chinese compound formula, which could also provide the basis for clinical application. A flowchart of the research approach was presented in Figure 1. In addition, The Chinese herbal compound can be considered as a weak inhibitor with multicomponent and multitarget, and there are synergistic effects among multiple components. We hope to explore how this compound can actually work in the treatment of cancer, but it must be taken into account that the components of the compound are complex and not every component can play a role. Therefore, we screen out the main active components through multiple parameters and predict the targets of the active ingredients, so as to infer the therapeutic effect.

2. Materials and Methods

2.1. Construction of Cancerous Target Network and Chemical Component Database. All targets for cancer treatment could be accessed in DrugBank database (http://www.drugbank.ca/), and the cancerous target network was thereby constructed through Cytoscape [12]. In addition, HJD was comprised of four herbs, including *Coptidis Rhizoma* (Huanglian), *Scutellariae Radix* (Huangqin), *Phellodendri Chinrnsis Cortex* (Huangbo), and *Gardeniae Fructus* (Zhizi). All chemical components of these Chinese herbs had been collected into TcmSP [13], TcmID [14], TCM Database@Taiwan [15], and NCBI Pubchem databases and had been standardized to a constituent data supplemented in the TcmSP database. Finally, the number of chemical compounds in HJD was obtained, as shown in the Appendix.

2.2. Screening the Active Ingredients by OB Prediction. Oral bioavailability (OB) in vivo (%F), the unchanged fraction of the orally administered dose achieving systemic circulation, is one of the most commonly used pharmacokinetic parameters in drug screening cascades. In this study, a robust calculative system OBioavail 1.1 [16] was employed to predict the OB of the compounds, since it was difficult to assess the bioavailability of the complex TCM by "wet" experiments. It has combined the metabolism (cytochrome P450 3A4) and transporter (P-glycoproteins) information. Using this system, compounds with lower OB could be discarded, so that the amount of the original compounds could be distinctly reduced to a smaller set suitable for Chinese compound formulas. Compounds with the OB of ≥30% were selected as the active ingredients in this study. Such a threshold was selected based on (1) the use of a minimum number of components to maximally extract HJD information and (2) the fact that the obtained model could be reasonably explained by the reported pharmacological data.

2.3. Screening the Active Ingredients by Drug-Likeness Prediction. Before target prediction, some compounds considered chemically unsuitable for use were removed by drugs similarity index, which could be deduced as a delicate balance among the molecular properties affecting pharmacodynamics and pharmacokinetics, ultimately influencing its absorption, distribution, metabolism, and excretion (ADME) in human body like a drug. In this study, the drug-likeness (DL) index of a new compound was calculated according to the Tanimoto similarity [17].

$$f(A, B) = \frac{A \cdot B}{|A|^2 + |B|^2 - A \cdot B} \quad (1)$$

where A represented the new compound and B stood for the average DL index of all the 6511 molecules in the DrugBank database based on the Dragon soft descriptors. Accordingly, molecules with the drug-likeness of <0.18 were also removed. Finally, compounds with both the OB of ≥30% and DL of ≥0.18 were considered as the active ingredients.

2.4. Prediction of the Targets of Active Ingredients. SysDT [18], the drug-target prediction model, was adopted to predict the targets of active ingredients. Briefly, SysDT was based on the 6511 drugs and 3987 targets of DrugBank database as well as the mutual correlation degree. Moreover, it was established using the stochastic forest algorithm and the support vector machine (SVM) algorithm, respectively. It turned out that the prediction model constructed by SVM was superior, with the consistency of 82.83%, sensitivity of 81.33%, and specificity of 93.62%. Using such model, targets with the SVM of > 0.7 were predicted as the putative targets of active ingredients. In addition, target information was integrated from SEA [19], STITCH [20], TTD [21], and HIT [22] databases to supplement this predictive model. Moreover, information regarding the physiological functions of all targets was obtained from the TTD and UniProt databases.

2.5. Construction of the Network and Topological Analysis. Associations between active ingredients and putative targets were constructed into the compound-target network of HJD

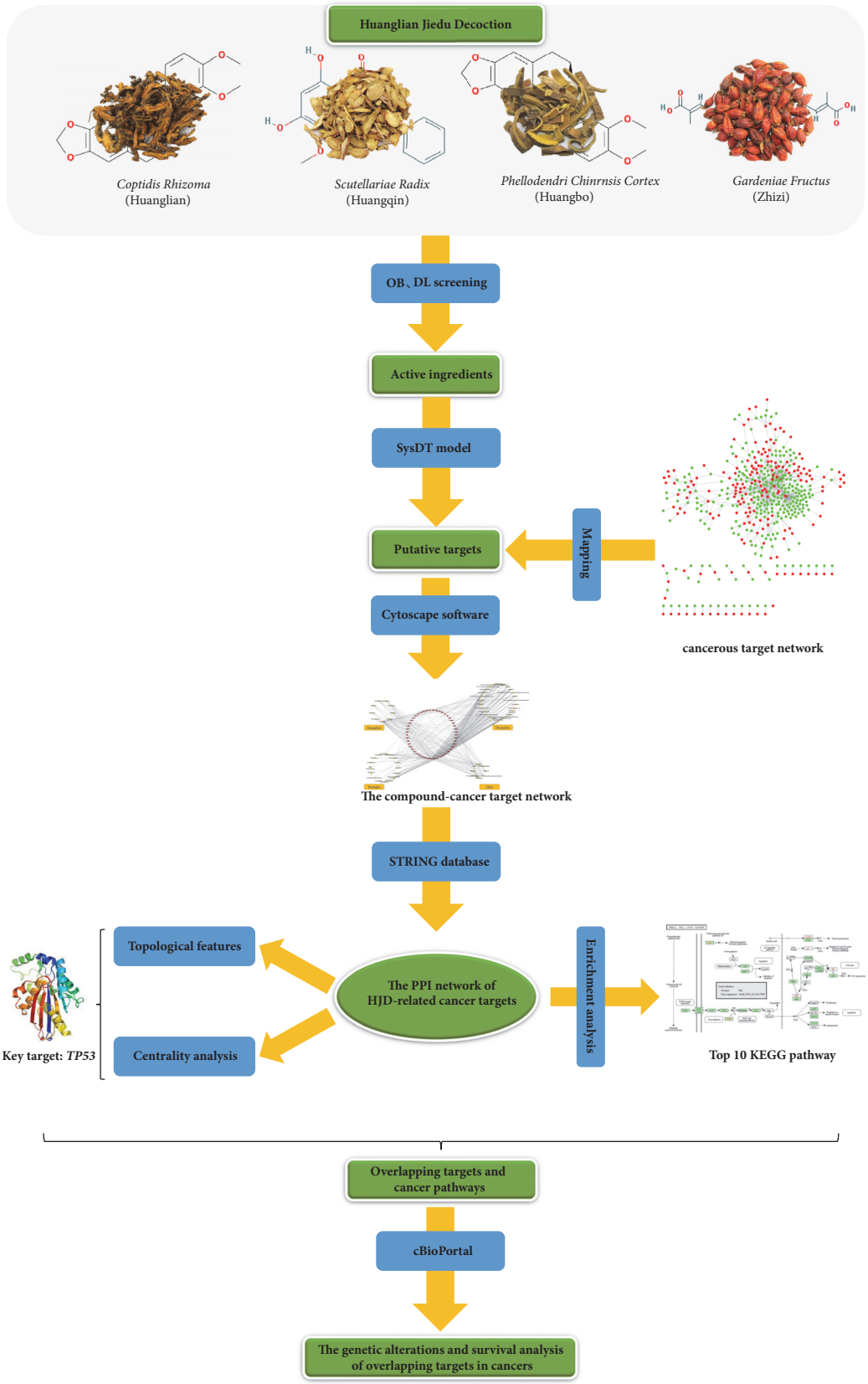


Figure 1: Integrated systems pharmacology and bioinformatics approach.

Table 1: The topological features of the PPI network.

Parameters	numerical value	Parameters	numerical value
Clustering coefficient	0.667	Number of nodes	98
Connected components	1	Number of edges	1027
Network diameter	4	Network density	0.216
Network radius	2	Network heterogeneity	0.676
Network centralization	0.474	Isolated nodes	0
Shortest paths	9506(100%)	Number of self-loops	0
Characteristic path length	2.006	Multiedge node pairs	0
Avg. number of neighbors	20.959	-	-

using Cytoscape v3.4.0 software [12], which was then mapped with the cancerous target network to obtain the compound-cancer target network of HJD, including all HJD-related targets for cancer treatment. Afterwards, the protein-protein interaction (PPI) network of HJD-related targets for cancer treatment was constructed by STRING [23]. Subsequently, topology analysis was performed using the Network Analyzer plug-in to output the main topological parameters of this network [24].

2.6. Screening Key Targets and KEGG Pathway Enrichment Analysis. The centrality algorithm is a key method to measure the importance degree of nodes in the whole network, with a larger value indicating a higher importance degree of node in the whole network and greater influence on the structure and function of the whole network. In this study, the degree centrality algorithm was adopted as the major algorithm, supplemented by the closeness centrality and the betweenness centrality algorithm, so as to select and evaluate the key anticancer targets of HJD. Additionally, the biological information and attribution embedded in the anticancer targets were then analyzed using a web-based integrated data mining system, WebGestalt [25]. Biochemical pathways and functions linked to the anticancer targets of HJD were specifically queried and navigated by the KEGG pathway enrichment analysis tool in WebGestalt. Eventually, the top 10 pathways with an adjusted P value of <0.01 were selected.

2.7. Exploration of the Cancer Genomics Data Linked to HJD by cBio Cancer Genomics Portal. The cBio Cancer Genomics Portal (http://cbioportal.org), an open platform to explore the multidimensional cancer genomics data, can encapsulate the molecular profiling data obtained from cancer tissues and cell lines into the readily understandable genetic, epigenetic, gene expression, and proteomic events [26]. Specifically, the complex cancer genomics profiles can be easily accessed using the query interface of the Portal, which enables the researchers to explore and compare the genetic alterations across samples. Furthermore, the obtained underlying data can thereby be linked to clinical outcomes, which has facilitated the novel discovery in biological systems.

In this study, the cBio Portal was utilized to examine the connectivity of HJD-related targets for cancer treatment across all studies on PCa and SCLC available in the databases. These targets in all sample studies on PCa and SCLC were classified as altered or nonaltered using the Portal search function. The genomics datasets were then presented using OncoPrint as the heatmap, a visually appealing display of alterations in microarrays across cancer samples [27]. Another feature of the Portal was that, it could generate multiple visualization platforms through grouping PCa abd SCLC-associated alterations using the input from key HJD-related targets for cancer treatment [27–31]. In the meantime, the survival of these targets in PCa and SCLC was analyzed using survival option embedded in the Portal, a tool integrating the survival Kaplan-Meier estimate and the survival data in the TCGA database.

3. Results

3.1. Screening the Active Ingredients and Visualization of the Compound-Cancer Target Network. Compounds contained in all 4 herbs constituting HJD were collected through several databases, including Huanglian (48), Huangqin (143), Huangbo (140), and Zhizi (98). A total of 85 compounds with OB of $\geq 30\%$ and DL of ≥ 0.18 were identified, among which only 59 active ingredients targeting the anticancer targets were screened (the Appendix). Correlations of the active ingredients with their anticancer targets were visualized through Cytoscape, and the compound-cancer target network was also obtained for subsequent analysis (Figure 2).

3.2. Construction of the PPI Network of HJD-Related Targets for Cancer Treatment as well as Topological Analysis. The HJD-related targets for cancer treatment could be obtained through the compound-cancer target network. In addition, the "protein-protein interaction (PPI) option" embedded in STRING was also adopted for further analysis, and a PPI network containing 98 interactive targets was also identified (Figure 3). Later, the topological features of this network were calculated with the Network Analyzer plug-in (Table 1), which consisted of an entire portion of the interaction between the anticancer targets, with an average number of direct neighbors of 20.959. Besides, the degree of some nodes was much higher than the average number of direct neighbors. In the degree centrality algorithm, a higher degree of a node indicated greater impact on the whole network. In this network, the degree distribution between nodes was uneven. These nodes, which were twice the average number of direct neighbors, were then define as Hub nodes in this study, indicating their importance in the network for subsequent investigation.

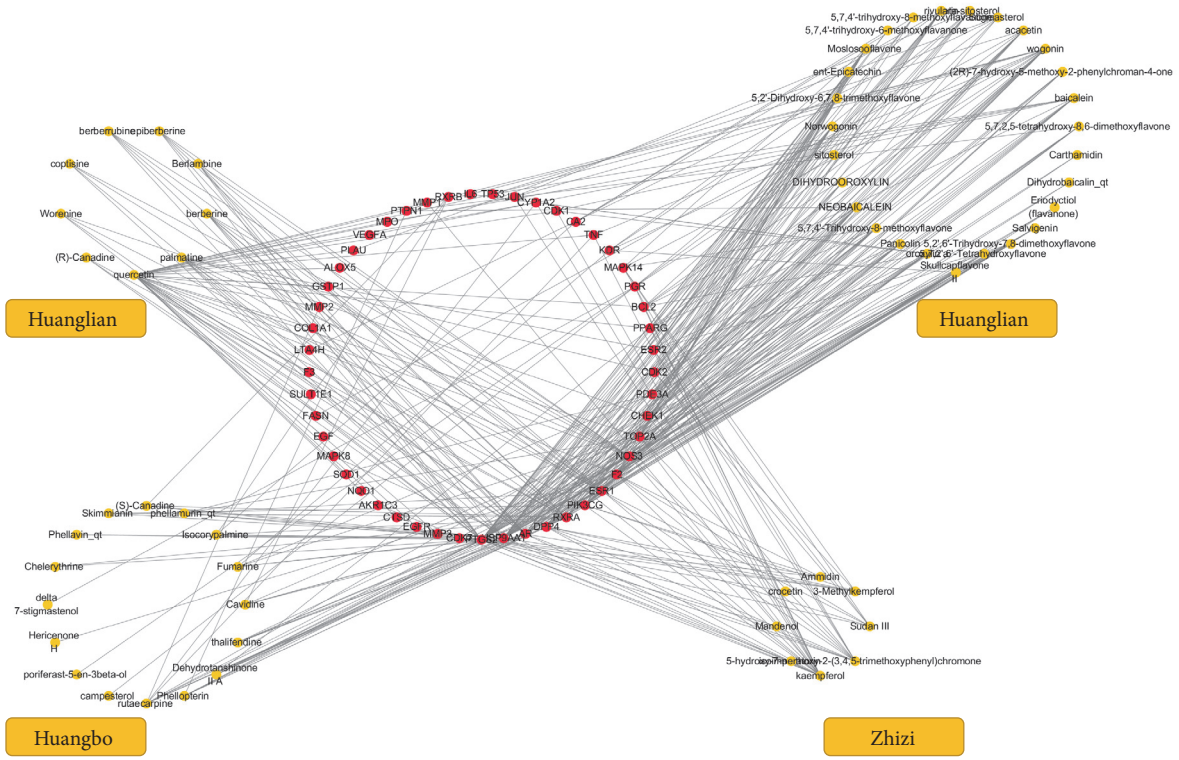


Figure 2: The compound-cancer target network of HJD. The yellow nodes represented active ingredients, while the red ones stood for anticancer targets.

3.3. Searching and Analysis of the Key Targets. Three centrality algorithms were employed for key target screening, including degree centrality, closeness centrality, and betweenness centrality. Of them, the closeness centrality algorithm has emphasized the average shortest path length between nodes and other nodes. In contrast, the betweenness centrality algorithm measures the number of nodes on the shortest path of other nodes, which suggests the frequency that the shortest path between the other nodes passes through one node. In other words, if the shortest path of the other nodes often passes through this node, then this node shows a high importance or ability, which can modulate information transmission of other nodes as a link between the other nodes.

These 3 algorithms were used to calculate the whole network, and the top 30 targets were summarized based on the algorithm results, as shown in Table 2. Consistently, nodes that were twice the average number of direct neighbors were defined as Hub nodes, including *TP53*, *AKT1*, *EGF*, *PCNA*, *JUN*, *VEGFA*, *ESR1*, and *IL6*. It should be noted that *TP53* ranked the top among the three centrality algorithms, indicating that the primary target pathway under control or mediated by HJD was associated with *TP53*. In addition, *AKT1* took up the second place, which was only second to *TP53*. As a critical component in the PI3K-AKT signaling pathway, *AKT1* was closely correlated with the occurrence and development of human cancers. Baicalin and baicalein, the main active ingredients of Huangqin, had been reported to show a definite relationship with the downregulation of the PI3K-AKT pathway in anticancer effect [23, 24]. Consequently, the *AKT1*-related signaling pathway might also have an important link with the anticancer effect of HJD.

3.4. Analysis of the KEGG Pathway. To explore the biological mechanism underlying the anticancer effect of HJD, the KEGG pathway enrichment analysis embedded in WebGestalt was performed. Typically, the top 10 KEGG pathways linked to all targets in the PPI network were obtained, including cell cycle (24), pathways in cancer (31), the p53 signaling pathway (15), the AGE-RAGE signaling pathway in diabetic complications (17), prostate cancer (16), endocrine resistance (16), hepatitis B (18), the PI3K-Akt signaling pathway (25), small cell lung cancer (14), and the FoxO signaling pathway (15) (Table 3). Broad grouping of the KEGG pathway analysis suggested that the anticancer effect of HJD was closely correlated with the following cancer-related signaling pathways with potential mechanisms, including (1) control of cancer cell proliferation and survival by p53-mediated cell cycle control, (2) the PI3K-Akt signaling pathway regulating the growth, proliferation, and invasion and metastasis of cancer cells by mediating the FoxO signaling pathway, and (3) the potential treatment of breast cancer achieved through regulating endocrine resistance. *TP53* ranked the top among the 3 centrality algorithms (Table 2); as a result, emphasis was directed to the p53 signaling pathway. The KEGG analysis results probably indicated that the anticancer effect of HJD

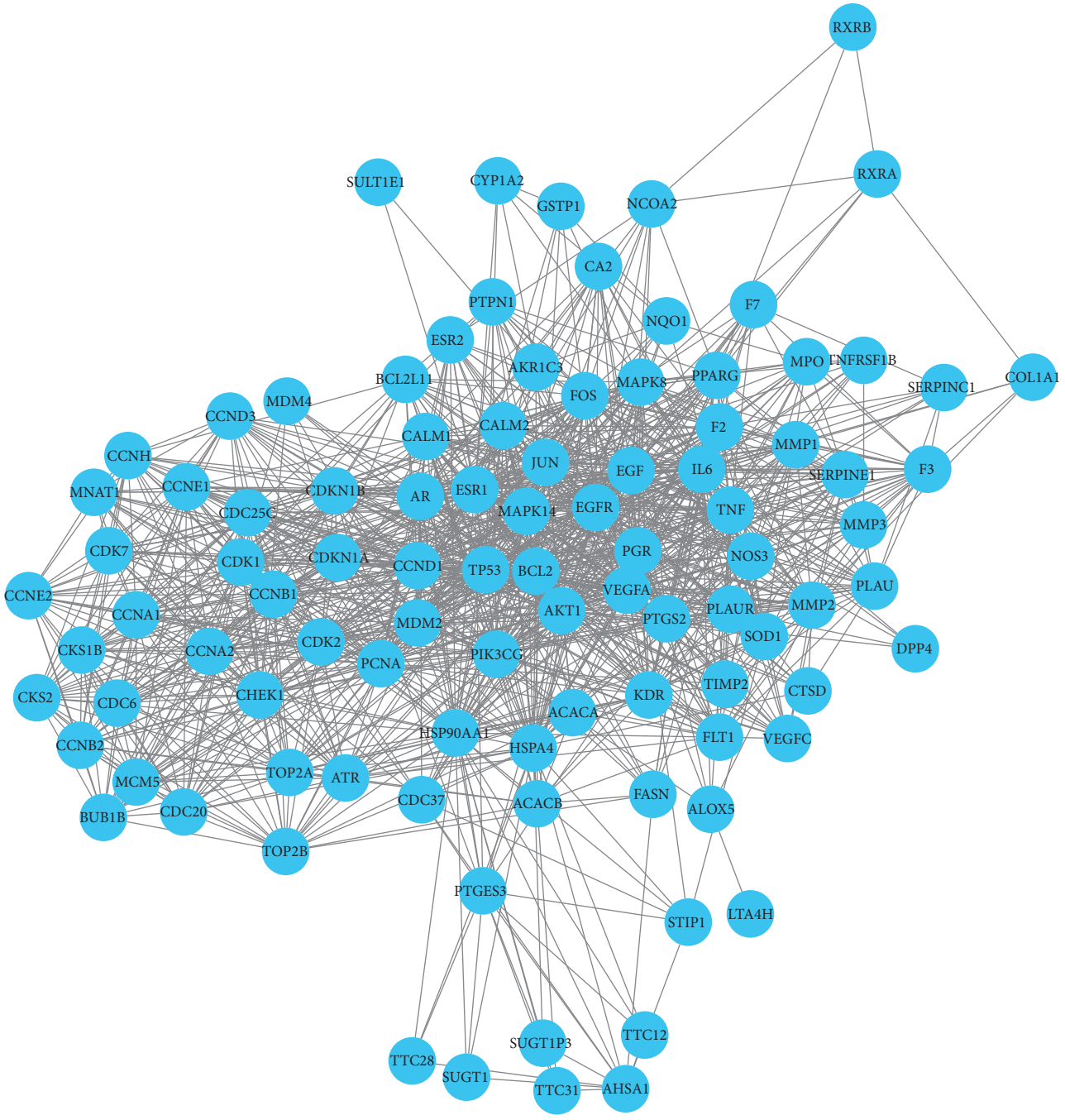


Figure 3: The PPI network of HJD-related targets for cancer treatment. The blue nodes represented HJD-related targets, while the edges represented the interaction between targets.

showed a functional correlation with *TP53*. In addition, the enrichment KEGG pathway analysis also suggested that 16 and 14 targets were associated with PCa and SCLC, respectively (Table 3).

3.5. Mining the Genetic Alterations and Survival Analysis. It had been proved that HJD displayed therapeutic effects on different cancers; however, its specific biological mechanisms remained unclear so far. KEGG enrichment analysis revealed that HJD was correlated with the cancer-related pathways (Table 3). To further explore the validity of such correlation, cBio Portal, a web-based integrated data mining system, was adopted to examine the genetic alterations and survival analysis associated with HJD-related targets in PCa and SCLC. The p53 signaling pathway was the main target of HJD; consequently, the overlapping targets of the p53 signaling pathway with PCa and SCLC were studied. The results discovered that 8 overlapping targets were associated with the KEGG assay embedded in WebGestalt, including 7 in PCA (*CDK2, CDKN1A, MDM2, CCND1, TP53, CCNE1,* and

Table 2: The centrality analysis of PPI network of HJD-related cancer targets.

Name	Degree	Name	Betweenness Centrality	Name	Closeness Centrality
TP53	66	*TP53*	0.12965202	*TP53*	0.75193798
AKT1	49	*HSP90AA1*	0.07551998	*AKT1*	0.65986395
EGF	47	*HSPA4*	0.06902023	*EGF*	0.65100671
PCNA	47	*IL6*	0.06776382	*VEGFA*	0.64666667
JUN	46	*AKT1*	0.04867524	*PCNA*	0.64666667
VEGFA	44	*VEGFA*	0.04389668	*JUN*	0.64666667
ESR1	42	*PCNA*	0.03662677	*IL6*	0.63815789
IL6	42	*EGF*	0.034913	*ESR1*	0.62987013
CDK1	41	*ESR1*	0.02917485	*BCL2*	0.62580645
BCL2	41	*JUN*	0.02822688	*EGFR*	0.62179487
HSP90AA1	40	*TNF*	0.02701286	*HSP90AA1*	0.61783439
CDK2	40	*CDK1*	0.02175952	*TNF*	0.61783439
CCND1	40	*PTGS2*	0.02156667	*CDKN1A*	0.61006289
EGFR	40	*PPARG*	0.02083383	*FOS*	0.61006289
TNF	39	*AKR1C3*	0.02075601	*PIK3CG*	0.61006289
CDKN1A	38	*ALOX5*	0.02061856	*HSPA4*	0.60625
PIK3CG	37	*AR*	0.01863776	*CDK2*	0.60625
FOS	37	*CDK2*	0.01810019	*AR*	0.60248447
HSPA4	37	*NOS3*	0.017597	*MAPK8*	0.60248447
MAPK8	35	*EGFR*	0.01749873	*PTGS2*	0.59876543
AR	34	*CCND1*	0.01665403	*CDK1*	0.59509202
CCNB1	33	*MAPK8*	0.01587777	*CCND1*	0.59509202
NOS3	33	*CDKN1A*	0.01556023	*NOS3*	0.59146341
PTGS2	33	*BCL2*	0.0151564	*MMP2*	0.58083832
CHEK1	30	*FOS*	0.01323593	*MAPK14*	0.58083832
CDKN1B	30	*MMP2*	1.25E-02	*CALM2*	0.57058824
MMP2	30	*PGR*	9.72E-03	*CALM1*	0.57058824
MAPK14	30	*CDKN1B*	9.65E-03	*CDKN1B*	0.56725146
CCNA2	29	*PIK3CG*	9.52E-03	*MDM2*	0.56725146
MDM2	29	*CALM2*	9.18E-03	*KDR*	0.55747126

CCNE2) and 5 in SCLC (*CDK2, CCND1, TP53, CCNE1,* and *CCNE2*). Therefore, the genomic and clinical characteristics of these targets in PCa and SCLC were examined, respectively (Table 2).

13 studies on PCa were analyzed [10, 32–40], the results of which indicated 1.9% to 63.9% alterations in the gene sets/pathways submitted for analysis (Figure 4(a)). Multiple genetic alterations observed across each set of cancer samples from the Michigan study [10] with the most significant genomic changes were summarized and presented using OncoPrint. The results indicated that 37 cases (63%) had an alteration in at least one of the 7 targets, and the alteration frequency in each of the selected targets was presented in Figure 4(b). *CDK2, CDKN1A,* and *CCNE1* were not associated with genetic alterations. For *MDM2, CCND1,* and *CCNE2*, most alterations were classified as amplification. *TP53*-associated genetic alterations included deep deletions and missense/truncating mutations. The alterations in these targets showed a cooccurrence trend across samples. However, mutual exclusivity analysis revealed no statistical significance (p=0.183) (data not shown). More interestingly, cases with genetic alterations were linked with a poorer survival compared with those without alterations (P=0.443, Figure 4(c)).

Among the 3 SCLC studies analyzed [11, 41, 42], 78.6% to 93.6% alterations were found in the gene sets/pathways submitted for analysis (Figure 5(a)). Multiple genetic alterations observed across each set of cancer samples from the U Cologne study with the most significant genomic changes were summarized and presented using OncoPrint [11]. The results indicated that 103 cases (94%) had an alteration in at least one of the 5 targets, and the alteration frequency in each of the selected targets was shown in Figure 5(b). Different from results of PCa study, these results indicated that almost all genetic alterations occurred in TP53, whereas no genetic alterations were seen in CDK2 or CCND1. CCNE1-associated genetic alterations were classified as missense mutations, while CCNE2-associated ones were classified as truncating mutations. In comparison, TP53-associated

Table 3: KEGG pathway analysis.

Pathway Name	#Gen	Uniprot name (corresponding gene set)	Statistics
Cell cycle	24	CDK2 CDK7 CDKN1A CDKN1B CHEK1 MCM5 MDM2 PCNA ATY CCND1 BUB1B TP53 CCNA2 CCNA1 CCNB1 CCND3 CCNE1 CCNH CCNB2 CCNE2 CDK1 CDC6 CDC20 CDC25C	C=124; O=24; E=1.59; R=15.09; rawP=0e+00; adjP=0e+00
Pathways in cancer	31	CDK2 CDKN1A CDKN1B CKS1B CKS2 EGF EGFR AKT1 FOS GSTP1 HSP90AA1 IL6 AR JUN MDM2 MMP1 MMP2 PIK3CG PPARG MAPK8 PTGS2 CCND1 BCL2 RXRA RXRB TP53 VEGFA VEGFC CCNA1 CCNE1 CCNE2	C=397; O=31; E=5.09; R=6.09; rawP=0e+00; adjP=0e+00
p53 signaling pathway	15	CDK2 CDKN1A CHEK1 MDM2 MDM4 SERPINE1 ATR CCND1 TP53 CCNB1 CCND3 CCNE1 CCNB2 CCNE2 CDK1	C=69; O=15; E=0.88; R=16.95; rawP=4.22e-15; adjP=3.78e-13
AGE-RAGE signaling pathway in diabetic complications	17	CDKN1B COL1A1 MAPK14 AKT1 F3 IL6 JUN MMP2 NOS3 SERPINE1 PIK3CG MAPK8 CCND1 BCL2 TNF VEGFA VEGFC	C=101; O=17; E=1.3; R=13.13; rawP=5e-15; adjP=3.78e-13
Prostate cancer	16	CDK2 CDKN1A CDKN1B EGF EGFR AKT1 GSTP1 HSP90AA1 AR MDM2 PIK3CG CCND1 BCL2 TP53 CCNE1 CCNE2	C=89; O=16; E=1.14; R=14.02; rawP=1.15e-14; adjP=7e-13
Endocrine resistance	16	CDKN1A CDKN1B MAPK14 EGFR AKT1 ESR1 ESR2 FOS JUN MDM2 MMP2 PIK3CG MAPK8 CCND1 BCL2 TP53	C=98; O=16; E=1.26; R=12.73; rawP=5.64e-14; adjP=2.85e-12
Hepatitis B	18	CDK2 CDKN1A CDKN1B AKT1 FOS IL6 JUN PCNA PIK3CG MAPK8 CCND1 BCL2 TNF TP53 CCNA2 CCNA1 CCNE1 CCNE2	C=146; O=18; E=1.87; R=9.62; rawP=2.03e-13; adjP=8.77e-12
PI3K-Akt signaling pathway	25	BCL2L11 CDK2 CDKN1A CDKN1B CDC37 COL1A1 EGF EGFR AKT1 FLT1 HSP90AA1 IL6 KDR MDM2 NOS3 PIK3CG CCND1 BCL2 RXRA TP53 VEGFA VEGFC CCND3 CCNE1 CCNE2	C=341; O=25; E=4.37; R=5.72; rawP=4.23e-13; adjP=1.6e-11
Small cell lung cancer	14	CDK2 CDKN1B CKS1B CKS2 AKT1 PIK3CG PTGS2 CCND1 BCL2 RXRA RXRB TP53 CCNE1 CCNE2	C=86; O=14; E=1.1; R=12.7; rawP=2.52e-12; adjP=8.48e-11
FoxO signaling pathway	15	BCL2L11 CDK2 CDKN1A CDKN1B MAPK14 EGF EGFR AKT1 IL6 MDM2 PIK3CG MAPK8 CCND1 CCNB1 CCNB2	C=134; O=15; E=1.72; R=8.73; rawP=1.03e-10; adjP=3.12e-09

The following statistics were listed in the row: C: the number of reference targets in the category; O: the number of targets in both the gene set and the category; E: the expected number in the category; R: ratio of enrichment; rawP: p value upon hypergeometric test; and adjP: p value adjusted by the multiple test adjustment.

genetic alterations included both missense mutations and truncating mutations. The mutual exclusivity analysis still displayed no statistical significance (p = 0.876) (data not shown). More interestingly, cases with genetic alterations also had a poorer survival relative to those without (P=0.166, Figure 5(c)).

4. Discussion

HJD serves as the object of study in this work. To elucidate the anticancer molecular mechanism of HJD, we have integrated systems pharmacology and bioinformatics. As a result, a number of public databases as the research basis and a set of tools are available to elucidate the molecular mechanisms and the relationship with the clinical outcomes of cancers. 3 steps are carried out in our workflow. (i) The cancerous target network is constructed through the DrugBank database, and all chemical components contained in the 4 medicines are obtained by databases, such as TcmSP, TcmID, TCM Database@Taiwan, and NCBI Pubchem. Subsequently, the active ingredients are screened based on the criteria of OB of ≥30% and DL of ≥0.18, and the targets of these active ingredients were then predicted using the SysDT model. Ultimately, 59 anticancer active ingredients and their anticancer targets were identified by mapping with the cancerous target network (the Appendix). (ii) Based on these anticancer targets, a PPI network containing 98 targets is constructed by STRING (Figure 2), and topological analysis is therefore performed. Eight key anticancer targets (including TP53, AKT1, EGF, PCNA, JUN, VEGFA, ESR1, and IL6) are screened through the topological parameters (Table 1) and 3 centrality algorithms (Table 2). Afterwards, the top 10 KEGG pathways are identified by enrichment analysis of the 98 targets (Table 3). (iii) Taking TP53 as the main object of study, we have compared the p53 signaling pathway between PCa and SCLC, and 8 overlapping targets are obtained. Then, the genetic alterations and survival analysis of the overlapping targets in PCa and SCLC are performed, so as to evaluate the relevance of the p53 signaling pathway with HJD in treating cancer.

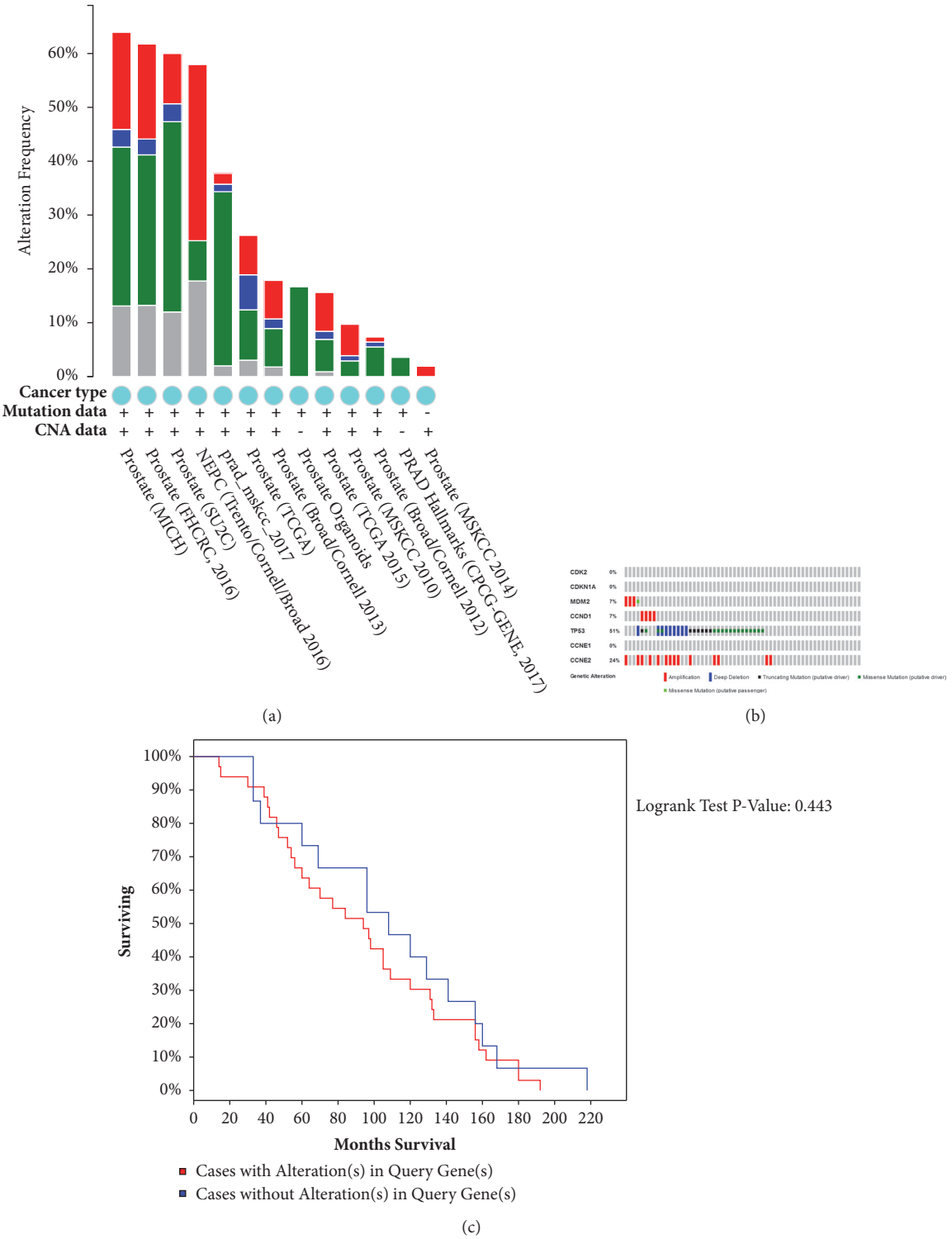


Figure 4: The genetic alterations and survival analysis related to 7 overlapping targets (including CDK2, CDKN1A, MDM2, CCND1, TP53, CCNE1, and CCNE2) in PCa studies embedded in cBio cancer genomics Portal. (a) Overview of changes in 7 overlapping targets in genomics datasets available in 13 different PCa studies. (b) OncoPrint: a visual summary of alterations across a set of prostate samples (data taken from the Michigan studies, Nature 2012) [10] based on a query of the 7 overlapping targets. Distinct genomic alterations including mutations and copy number alterations (CNAs, exemplified by gene amplifications and homozygous deletions) were summarized, and the color codes represented % changes) in particular targets in individual cancer samples. Each row stood for a gene, and each column represented a cancer sample. Red bars stood for gene amplifications, blue bars represented homozygous deletions, and green squares indicated nonsynonymous mutations. (c) K-M curve between groups with alterations and without alterations. Red line represented cases with alterations, and the blue one indicated cases without. The X-axis was overall survival (OS, months), and the Y-axis stood for the survival rate. Kaplan-Meier test was performed.

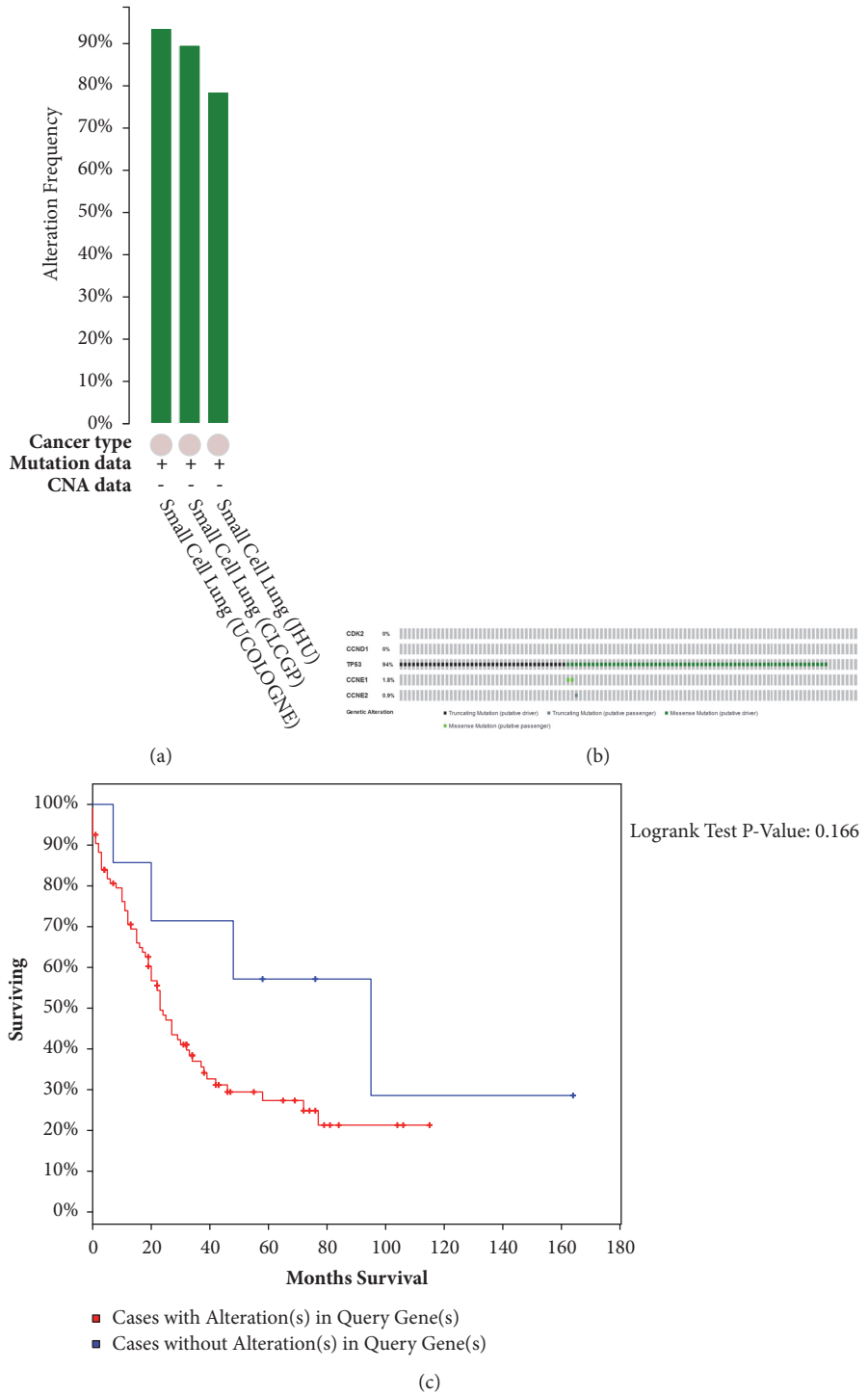


Figure 5: The genetic alterations and survival analysis related to the 5 overlapping targets (including CDK2, CCND1, TP53, CCNE1, and CCNE2) in SCLC studies embedded in cBio cancer genomics Portal. (The annotations were consistent with those in Figure 4.) (a) Overview of changes in 5 overlapping targets in genomics datasets available in 3 different SCLC studies. (b) OncoPrint (data taken from the U Cologne studies, Nature 2015) [11] based on a query of the 5 overlapping targets). (c) K-M curve between groups with alterations and without alterations.

Table 4: Anticancer active ingredients, oral bioavailability (OB), and drug-likeness (DL) of HJD.

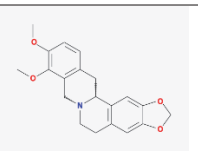
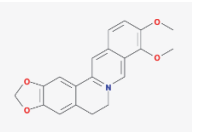
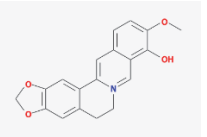
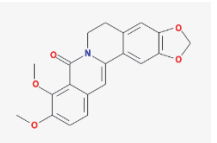
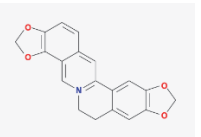
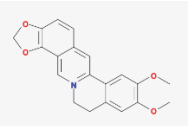
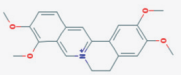
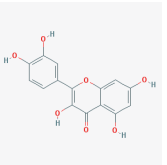
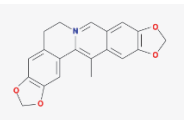
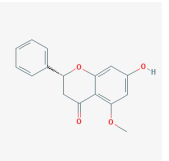
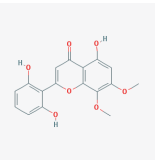
Name	Active Ingredient	Chemical Structure	OB/%	DL
Huanglian	(R)-Canadine		55.37	0.77
	berberine		36.86	0.78
	berberrubine		35.74	0.73
	Berlambine		36.68	0.82
	coptisine		30.67	0.86
	epiberberine		43.09	0.78
	palmatine		64.6	0.65
	quercetin		46.43	0.28
	Worenine		45.83	0.87
Huangqin	(2R)-7-hydroxy-5-methoxy-2-phenylchroman-4-one		55.23	0.2
	5,2′,6′-Trihydroxy-7,8-dimethoxyflavone		45.05	0.33

Table 4: Continued.

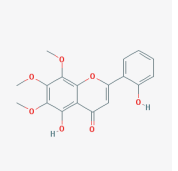
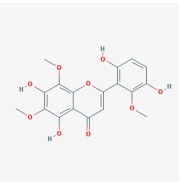
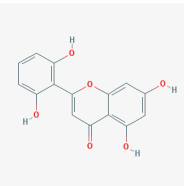
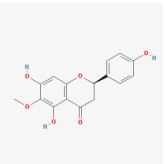
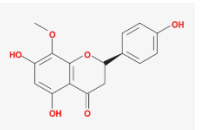
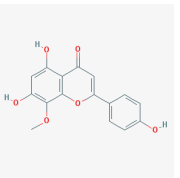
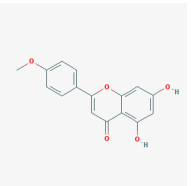
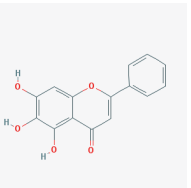
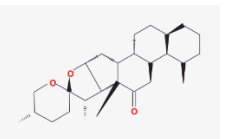
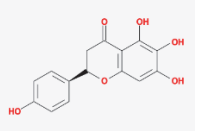
Name	Active Ingredient	Chemical Structure	OB/%	DL
	5,2′-Dihydroxy-6,7,8-trimethoxyflavone		31.71	0.35
	5,7,2,5-tetrahydroxy-8,6-dimethoxyflavone		33.82	0.45
	5,7,2′,6′-Tetrahydroxyflavone		37.01	0.24
	5,7,4′-trihydroxy-6-methoxyflavanone		36.63	0.27
	5,7,4′-trihydroxy-8-methoxyflavanone		74.24	0.26
	5,7,4′-Trihydroxy-8-methoxyflavone		36.56	0.27
	acacetin		34.97	0.24
	baicalein		33.52	0.21
	beta-sitosterol		36.91	0.75
	Carthamidin		41.15	0.24

Table 4: Continued.

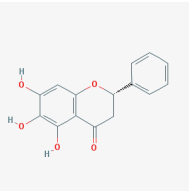
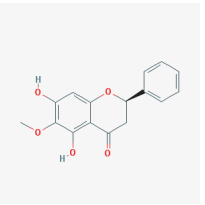
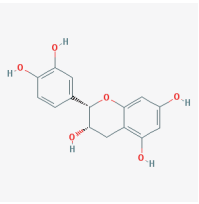
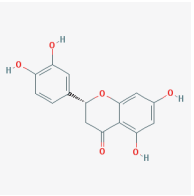
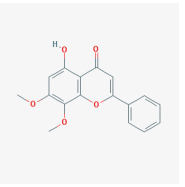
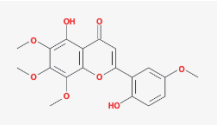
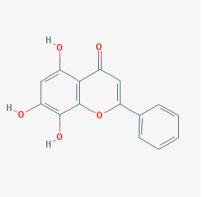
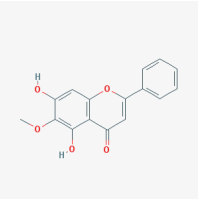
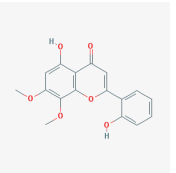
Name	Active Ingredient	Chemical Structure	OB/%	DL
	Dihydrobaicalin_qt		40.04	0.21
	Dihydrooroxylin		66.06	0.23
	ent-Epicatechin		48.96	0.24
	Eriodyctiol (flavanone)		41.35	0.24
	Moslosooflavone		44.09	0.25
	NEOBAICALEIN		104.34	0.44
	Norwogonin		39.4	0.21
	oroxylin a		41.37	0.23
	Panicolin		76.26	0.29

Table 4: Continued.

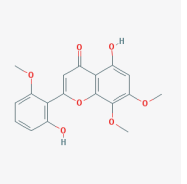
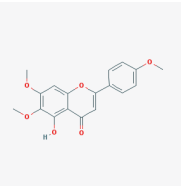
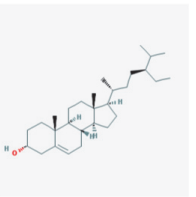
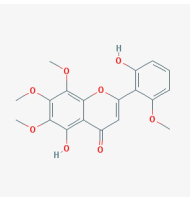
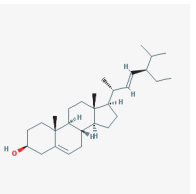
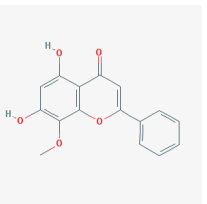
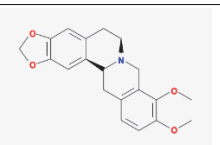
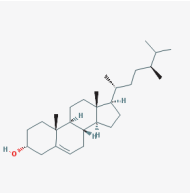
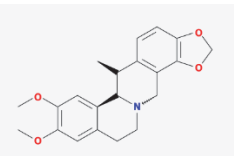
Name	Active Ingredient	Chemical Structure	OB/%	DL
	rivularin		37.94	0.37
	Salvigenin		49.07	0.33
	sitosterol		36.91	0.75
	Skullcapflavone II		69.51	0.44
	Stigmasterol		43.83	0.76
	wogonin		30.68	0.23
Huangbo	(S)-Canadine		53.83	0.77
	campesterol		37.58	0.71
	Cavidine		35.64	0.81

Table 4: Continued.

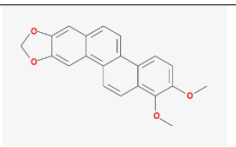
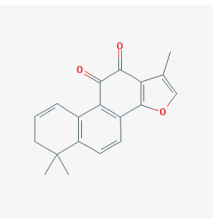
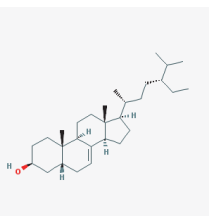
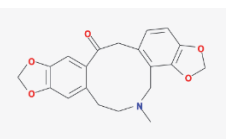
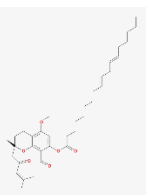
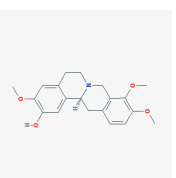
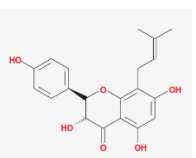
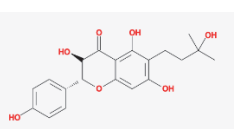
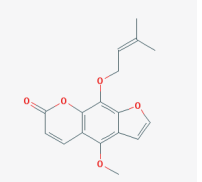
Name	Active Ingredient	Chemical Structure	OB/%	DL
	Chelerythrine		34.18	0.78
	Dehydrotanshinone II A		43.76	0.4
	delta 7-stigmastenol		37.42	0.75
	Fumarine		59.26	0.83
	Hericenone H		39	0.63
	Isocorypalmine		35.77	0.59
	phellamurin_qt		56.6	0.39
	Phellavin_qt		35.86	0.44
	Phellopterin		40.19	0.28

Table 4: Continued.

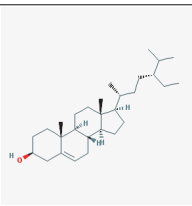
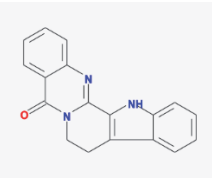
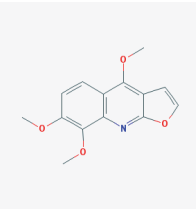
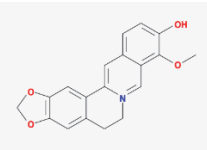
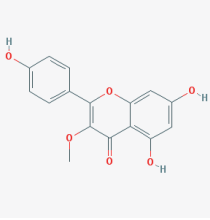
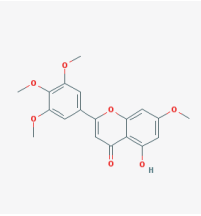
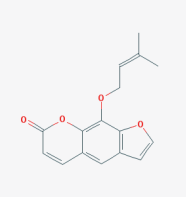
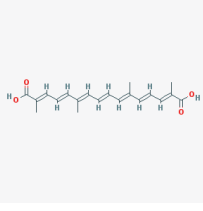
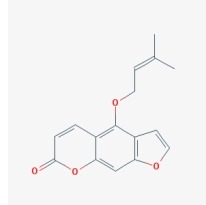
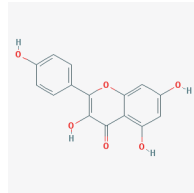
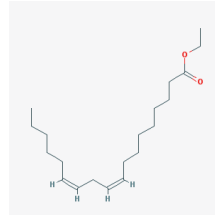
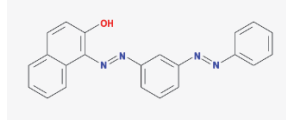
Name	Active Ingredient	Chemical Structure	OB/%	DL
	poriferast-5-en-3beta-ol		36.91	0.75
	rutaecarpine		40.3	0.6
	Skimmianin		40.14	0.2
	thalifendine		44.41	0.73
Zhizi	3-Methylkempferol		32.03	0.76
	5-hydroxy-7-methoxy-2-(3,4,5-trimethoxyphenyl) chromone		34.55	0.22
	Ammidin		84.07	0.59
	crocetin		36.91	0.75

Table 4: Continued.

Name	Active Ingredient	Chemical Structure	OB/%	DL
	isoimperatorin		42	0.19
	kaempferol		33.55	0.42
	Mandenol		45.46	0.23
	Sudan III		60.16	0.26

HJD has been suggested in a report to inhibit angiogenesis through suppressing the expression of VEGFA and MMP-9, thus further restraining cancer growth [43]. Similarly, we also discover that VEGFA is a key target in the anticancer activity of HJD using network analysis (Table 2). In addition, a study shows that HJD can obviously inhibit the proliferation of human SCLC NCI-H446 cells [44]. Coincidently, our findings also support that HJD has certain therapeutic effect on SCLC, which is probably achieved through regulating the p53 signaling pathway. However, no other related literature reports that HJD has therapeutic effect on PCa, which may account for a future research direction pending further validation of the experiment. Interestingly, we find through KEGG enrichment analysis that the AGE-RAGE signaling pathway is also present in diabetic complications. The therapeutic effect of HJD on diabetes and its complications has been approved in lots of literature; nonetheless, no existing study indicates HJD works through this pathway. Therefore, it remains to be further studied whether the AGE-RAGE signaling pathway may be a potential mechanism of HJD in treating diabetes and its complications.

Compared with studies integrating systems pharmacology and network pharmacology, the current study has a certain biological rationality, since it has bridged HJD to its target genes and linked it with biological effects. Moreover, this study has also illustrated the relationship between the molecular mechanism of HJD and the clinical outcome of cancer through a set of network-based tools. This approach is greatly different from the use of experimental techniques to prove a few relationships at a time; instead, it can reduce redundant experiments from different laboratories. The use of such a new research strategy may remarkably contribute to (i) understanding the molecular biological mechanisms of Chinese compound formula, (ii) revealing the primary effects and targets of HJD on cancers, and (iii) promoting the clinical use of Chinese compound formula and laying down the clinical foundation. This method can be used not only in the study on HJD, but also on other Chinese compound formulas and on medicine combination therapy.

However, there are some shortcomings deserving our attention. The compounds contained in the herbal medicines are obtained based on databases; therefore, the quality of databases would directly affect the final compounds obtained. Moreover, the selection of screening parameters and the setting of threshold can also affect the number of active ingredients obtained. All of these may influence the final analysis.

In conclusion, the targets of HJD will undoubtedly be confirmed thanks to a growing number of studies on HJD carried out using traditional experimental techniques and methods. However, the relationship with the biological effects of HJD remains unclear yet. We believe that the use of this method can help to offset some uncertainties of HJD related to its target and its subsequent phenotypic expression. Furthermore, this approach contributes to determining the feasibility of future experiments. In the future, molecular biology experiments about the key targets and pathways of HJD can be carried out on the basis of the current study. Apart from PCa and SCLC, many studies have also reported

the antitumor effect of HJD on other tumors, such as lung cancer, liver cancer, breast cancer, and colon cancer. These findings reveal that it remains to be further studied whether the connectivity between HJD and PCa as well as SCLC can be extended to other cancers.

Appendix

See Table 4.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Systems Pharmacological Approach to Investigate the Mechanism of *Acori Tatarinowii Rhizoma* for Alzheimer's Disease

Zhenyan Song, Fang Yin, Biao Xiang, Bin Lan, and Shaowu Cheng

The Key Laboratory of Hunan Province for Integrated Traditional Chinese and Western Medicine on Prevention and Treatment of Cardio-Cerebral Diseases, Hunan University of Chinese Medicine, Changsha, Hunan 410208, China

Correspondence should be addressed to Shaowu Cheng; 702058195@qq.com

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In traditional Chinese medicine (TCM), *Acori Tatarinowii Rhizoma* (*ATR*) is widely used to treat memory and cognition dysfunction. This study aimed to confirm evidence regarding the potential therapeutic effect of *ATR* on Alzheimer's disease (AD) using a system network level based *in silico* approach. Study results showed that the compounds in *ATR* are highly connected to AD-related signaling pathways, biological processes, and organs. These findings were confirmed by compound-target network, target-organ location network, gene ontology analysis, and KEGG pathway enrichment analysis. Most compounds in *ATR* have been reported to have antifibrillar amyloid plaques, anti-tau phosphorylation, and anti-inflammatory effects. Our results indicated that compounds in *ATR* interact with multiple targets in a synergetic way. Furthermore, the mRNA expressions of genes targeted by *ATR* are elevated significantly in heart, brain, and liver. Our results suggest that the anti-inflammatory and immune system enhancing effects of *ATR* might contribute to its major therapeutic effects on Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is one of the most common age-related serious fatal neurodegenerative diseases, and its prominent feature is progressive cognitive, learning, and memory dysfunction. Gradually, bodily functions are lost, eventually leading to death [1]. According to a statistical report issued by Alzheimer's disease association in 2016, one new case of AD is expected to develop every 33 seconds, resulting in nearly 1 million new cases per year, and the estimated prevalence is expected to range from 11 million to 16 million by 2050 [2]. Therefore, prevention and treatment of AD are particularly important. Although AD is a global health problem, its pathology remains poorly understood [3]. About 70% of the risk is thought to be genetic, and many genes are usually involved. The disease process is associated with high density of senile plaques and neurofibrillary tangle in brain [4]. Currently, no drugs or supplements can reduce the processes [2].

Recent study shows that AD might result from a series of complex reasons, such as aging, immunity dysfunction, and metabolic disturbances [5, 6]. In order to understand the pathogenesis of complex diseases a new "multitarget, multidrug" model strategy of drug discovery was presented [7]. Interestingly, as a multidisciplinary system of multicomponent therapeutics, Traditional Chinese Medicine (TCM) Systems revealed a probability to explain the relationship between multicomponents and drug synergistic effects [8]. *Acori Tatarinowii Rhizoma* (ShiChangPu, *ATR*) is a well-known herb from Chinese traditional medicine (TCM) being used for neurodegenerative diseases in China at least 2,000 years. *ATR* was recorded in the Chinese pharmacopoeia (2015) as the official botanical source. It contains several phytochemicals, and the main ingredients include β-asarone, α-asarone, estragole, *cis*-methylisoeugenol, isoshyobunone, δ-cadinene, and methyleugenol. Some experimental researches by cellular and animal models support that *ATR* had anti-AD effects. Volatile oils of *ATR* were being used in AD

for promoting neural progenitor proliferation, protecting β-amyloid induced neurotoxicity, and improving memory and cognitive function [9–11]. β-Asarone, a major essential oil component from *ATR,* has anti-AD effects by nerve growth factor signaling pathways, ameliorating oxidative stress, autophagy, and neuronal apoptosis *in vivo* [12–14]. In addition, many studies had confirmed that *ATR* has therapeutic effects on nervous disorders, depression, epilepsy, and dementia [15–19].

In recent years, systems pharmacology has emerged as a new field that integrates pharmacology, biochemistry, genomics, and bioinformatics techniques, to establishe a model based on computer network analysis and therapeutic targets prediction, and it highlights the holistic thinking of TCM [20]. It provides a new powerful tool to analyze and visualize the complex interaction data in herb, compound, target, and disease. To introduce the TCM application on AD by using systems pharmacology approach, Cai's group used a systems pharmacology approach to reveal the underlying molecular mechanisms of BSYZ in treating AD [21]. Luo's group established multiple herbs-compounds-targets-pathway-cooperation networks model to analyze the effects of Danggui-Shaoyao-San in AD [22]. Fang et al. performed literature mining of PubMed for top 10 anti-AD herbs (include *ATR*), based on systematic pharmacological analysis, and they interpreted the multiscale mechanisms of action of herbs in AD management [23]. Although these researches provided valuable support for the mechanisms of TCM in the treatment of AD, there is no analysis on the mechanism of the single herb, such as *ATR*, because they were based on the combined action of multiple herbs.

In this study, we discussed how system pharmacology demonstrated the relationship of *ATR* to treat AD from the system level. First, ingredients of ATR were collected from traditional Chinese medicine systems pharmacology (TCMSP, http://lsp.nwu.edu.cn/) database [24]. Oral bioavailability (OB), drug-likeness (DL), and blood-brain barrier (BBB) analysis were carried out to filter the compounds for subsequent analysis. Next, system pharmacology approach was used to explore the compounds-targets interactions and to establish the compounds-targets network and the compounds-targets-AD network. Finally, based on bioinformatics analysis, we illuminated the multiscale mechanisms of action of ATR on AD. A workflow of the systems pharmacological study was summarized as shown in Figure 1.

2. Material and Methods

2.1. Establishment of Database. All the compounds of *ATR* are collected by TCMSP database (a free phytochemical database of herbal medicine). Evaluating the absorption, distribution, metabolism, and excretion (ADME) parameters: oral bioavailability (OB), drug-likeness (DL), and blood-brain barrier (BBB) for all the natural compounds, the active compounds were collected to illustrate the proposed model.

2.2. OB Evaluation. OB shows the percentage of an active compound that reaches the systemic circulation, which defines one of the most important pharmacokinetic parameters to the convergence of the ADME process [25]. OB is essential to determine whether the chemical components of TCM have pharmacodynamic activity. The OB values are predicted by using the OBioavail 1.1 [26], which is a tool that contains 805 different structures of drugs and drug-like molecules and metabolism information. In this study, the OB threshold was set as ≥15%.

2.3. DL Prediction. DL helps filter out "drug-like" compounds in the traditional Chinese herbs, as DL is a qualitative concept used in drug design for an estimate on how "drug-like" a prospective compound is [27]. Compounds with high DL values are more likely to have certain biological properties that can increase the possibility for drug treatment. The calculation of DL value is based on Tanimoto coefficient formula in TCMSP database [28].

2.4. BBB Screening. The blood-brain barrier (BBB) is anatomically characterized by the presence of intercellular tight junctions between continuous nonfenestrated endothelial cells, whose normal function is to limit the passage of protein, potentially diagnostic and therapeutic agents into the brain parenchyma [29]. Understanding and evaluating the capacity of compounds of entering into the central nervous system, the compounds with BBB <-0.3 were considered as nonpenetrating (BBB-), those from -0.3 to +0.3 moderate penetrating (BBB±), and those >0.3 strong penetrating (BBB+) [8].

2.5. Target Fishing. To obtain active compounds of *ATR* target information, a comprehensive drug targeting protocol includes text mining database search and chemometric analysis was applied. First, the information on molecular targets was found from the TCMSP. Second, based on the set-wise chemical similarity among their ligands of active compounds, the targets were adopted by Similarity Ensemble Approach (SEA, http://sea.bkslab.org) [30] and the Binding Database (http://www.bindingdb.org) [31]. Third, the targets obtained from different databases were input to UniProt (http://www.uniprot.org/) [32] to make the targets names uniformly standardized. Finally, noise and error information was eliminated by PharGKB (https://www.pharmgkb.org/) [33], Therapeutic Target Database (DTT, http://bidd.nus.edu.sg/group/cjttd/) [34], and the Comparative Toxicogenomics Database (CTD, http://ctdbase.org/) [35].

2.6. Drug-Target Network. In order to understand the complex relationship between *ATR* active compounds and potential targets, a visual network is established by CytoScape_v3.4.0 [36]. This network is composed of node and edge. Nodes represent to molecules (compounds and targets), and edges indicate intermolecular interactions (compounds and targets interactions), namely, the connections between nodes.

2.7. Drug-Target-AD Network. DisGeNET database (http://www.disgenet.org/) [37] is a discovery platform that serves as one of the largest publicly available databases, containing

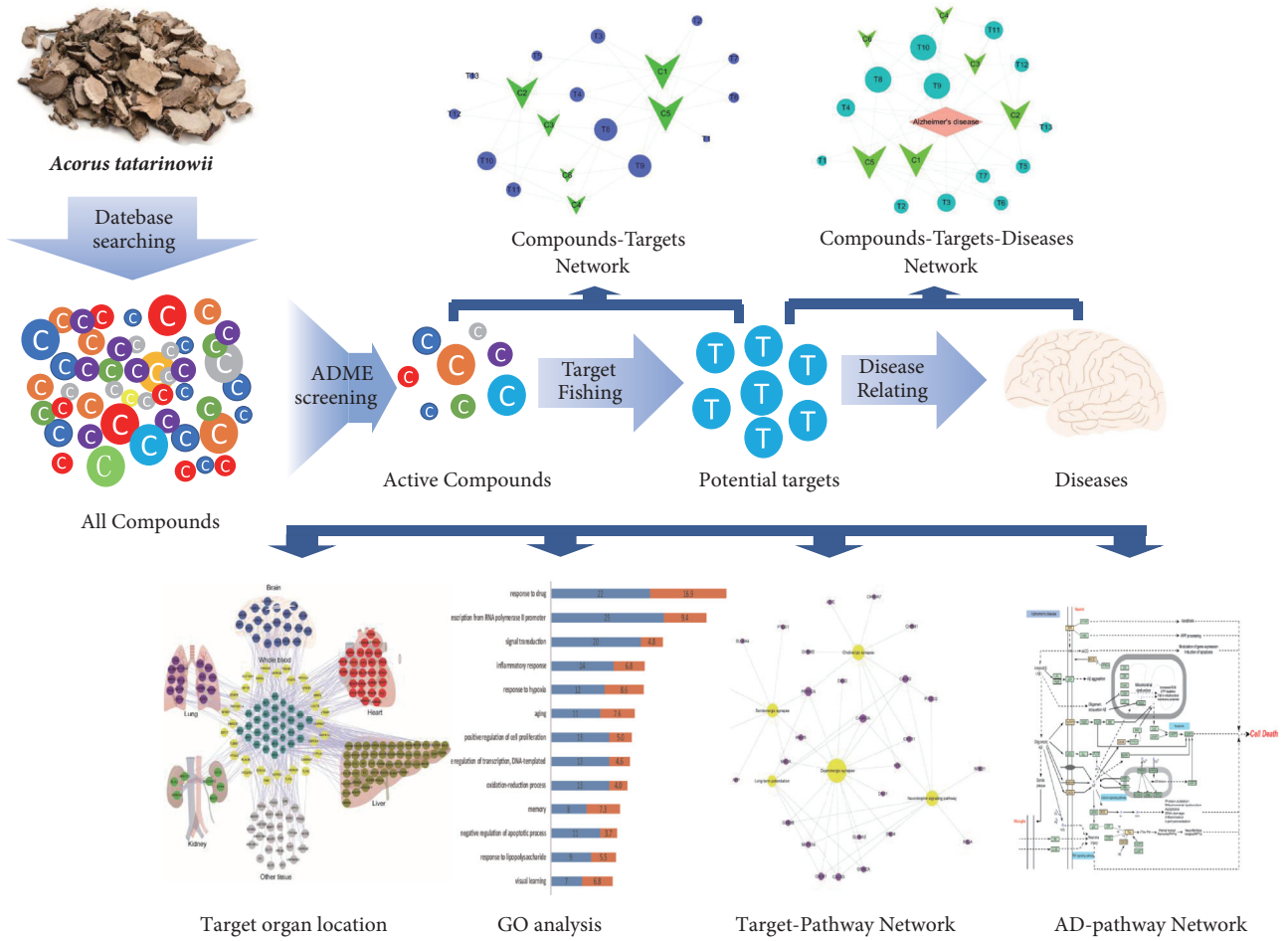


Figure 1: Workflow of system pharmacology by *Acori Tatarinowii Rhizoma* (*ATR*): screening and target fishing, network analysis, relevant organ location network analysis, gene ontology, and KEGG pathway analysis.

a mass of collections of genes and variants associated with human diseases. It contains 561,119 gene-disease associations and 135,588 variant-disease associations. Based on expert curated repositories, GWAS catalogues, animal models, and the scientific literature, it can be used for different research purposes, including the generation of hypothesis on drug therapeutic action and drug adverse effects. In order to elucidate the action relations of *ATR* in Alzheimer's disease, the integration is performed by means of gene and AD vocabulary mapping and using the DisGeNET association type ontology to select the targets related to AD. The active compounds-targets-AD network is established by CytoScape_v3.4.0.

2.8. Target-Organ Location Network. BioGPS is a database for querying and organizing genetic annotation resources [38]. It provides gene expression abundance data in cells or tissues by microarrays analysis [39]. The target-organ location network is constructed with the use of the dataset: GeneAtlas U133A, gcrma (http://biogps.org). First, the mRNA expression patterns of each target gene are obtained in 84 parts of organ tissues. Second, average values for each gene are applied to computer. Third, genes will locate in the relevant organ tissues where the mRNA expression level is higher than the mean. Finally, a target-organ location network is constructed using Cytoscape 3.4.0 and organ-specific, Alzheimer's disease related gene overexpression data.

2.9. Gene Ontology (GO) Analysis. Gene Ontology (GO) is a framework for the model of biology. The GO defines gene classes that describe gene functions and relationships between these concepts. It covers three domains: Cellular Component (CC) explains where gene products are active; Molecular Function (MF) means molecular activities of gene products; Biological Process (BP) interprets the pathways and larger processes made up of the activities of multiple gene products [40]. In this study, GO terms with P values < 0.01 and Benjamini < 0.05 were employed and the data were collected by the DAVID 6.8 Gene Functional Classification Tool [41] (http://david.abcc.ncifcrf.gov/).

2.10. Network Pathway Analysis. KEGG pathway enrichment analysis provides not only pathway functional annotations of given gene set but also pathway enrichment analysis [42]. First, gene numbers of each pathway in the given gene set are calculated. Then, significantly enriched pathways in the given

gene set compared to the genome background are defined by hypergeometric test. The calculating formula of P value is

$$P = 1 - \sum_{i=0}^{m-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}}. \quad (1)$$

Here N is the number of all genes with KEGG pathway annotation (http://www.genome.jp/kegg/) [43] which represents the gene number of given genes set in N; M is the number of all genes that are annotated to the certain pathway; m is the gene number in the given gene set that is annotated to the certain pathways. Taking P values < 0.01 and FDR ≤ 0.05 as threshold, pathways that meet this condition were defined as significantly enriched pathways in the given genes set. In this research, pathway enrichment analysis was performed by using the OmicShare tools, a free online platform for data analysis (http://www.omicshare.com/tools/). In order to illuminate the action mechanisms of *ATR* in Alzheimer's disease, filtered target proteins were entered into the pathway map of Alzheimer's disease acquired from the KEGG.

3. Results

3.1. Identification of Active Compounds. As 105 compounds were identified belonging to *ATR* in TCMSP (as shown in Table S1), 30 compounds were selected with the conditions of OB≥15%, BBB≥-0.3, and DL≥0.1. Removing 2 compounds (4-aromadendrene, isocembrol) with no target information, 28 compounds with known target information were chosen for the following analysis. Another 4 compounds (β-asarone, γ-asarone, cis-methylisoeugenol, and methyleugenol) reported to have anti-inflammation, antioxidation, and antimicrobial effects were added additionally [13, 44–46], and finally 32 compounds were analyzed as shown in Table 1.

Most of these 32 compounds showed various biological activities experimentally. For example, eudesmin (M23; DL=0.62, BBB=0.05, and OB=52.35) was experimentally identified which has anti-inflammation [47] and anticonvulsion effects [48]. A angiogenesis inhibitor of marmesin (M28; DL=0.18, BBB=0.07, and OB=50.28) was reported to have regulatory effect on endothelial cell fate and angiogenesis [49]. Antioxidation and anti-inflammation effect of patchoulene (M31; DL=0.11, BBB=2.17, and OB=49.06) was proved through inhibition of nuclear factor κB (NF-κB) and downregulation of COX-2/iNOS [50]. Bergapten (M12; DL=0.13, BBB=0.69, and OB=42.21) was reported to have anti-inflammatory properties [51] and the ability of ameliorating the insulin resistance [52]. Aminacrine (M9; DL=0.12, BBB=0.33, and OB=35.00) and its derivatives were experimentally identified to be reversible inhibitors of acetyl cholinesterase (AChE) [53]. α-Cedrene (M2; DL=0.10, BBB=2.16, and OB=55.56) was reported to exhibit antimicrobial activity [54]. Veraguensin (M7; DL=0.39, BBB=0.72, and OB=25.53) was reported to have analgesic and anti-inflammatory activities [55] and neuroprotective effects[56]. β-Asarone (M16; DL=0.06, BBB=1.24, and OB=35.61) increased striatal level of dopamine by enhancing dopa decarboxylase activity which improved behavioral competence in Parkinson's rat model [57]. γ-Asarone (M24; DL=0.06, BBB=1.33, and OB=22.76) prevented oxidative stress-induced cell injury in cultured astrocytes through Akt signaling activation [46]. Aristolone (M11; DL=0.13, BBB=1.54, and OB=45.31) was reported to have regulatory effects on lipid peroxidation and proliferation in human cancer cells [58]. α-Cubebene (M10; DL=0.11, BBB=2.1, and OB=16.73) was experimentally identified with significant antifungal activity inhibition of *A. flavus* growth [59]. β-Gurjunene (M15; DL=0.10, BBB=2.07, and OB=51.36) was experimentally identified with antioxidation activity and antimicrobial activity [60]. Isopimpinellin (M26; DL=0.17, BBB=0.5, and OB=25.93) was reported to exhibit antiallergic inflammation [61]. α-Longipinene (M3; DL=0.12, BBB=2.05, and OB=57.47) was reported to inhibit biofilm formation and hyphal growth in *Candida albicans* [62]. Anti-inflammation effect of aristolene (M11; DL=0.11, BBB=2.08, and OB=52.2) was proved through measuring secretion of the proinflammatory cytokines IL-6 and TNF-α by using a human-derived macrophage cell line [63]. Calarene (M20; DL=0.11, BBB=2.04, and OB=52.16) and caryophyllene oxide (M4; DL=0.13, BBB=1.76, and OB=32.67) from the essential oil of *Patrinia scabiosaefolia* were both found to have antineuroinflammation, anticancer, and antioxidation effects[64]. As mentioned above, many compounds of *ATR*, which are universal in herbs, plants, and fruits, contribute to neuroprotective, anti-inflammation, antimicrobial, and antioxidation effects.

3.2. Target Fishing. These 32 identified active compounds interact with 181 target proteins (as shown in Table S2) based on a target fishing technique [65]; that is, on average, they interact with 5.7 target genes, which did fully explain the multiple-target effects of pharmacology by *ATR* [66]. Among the 181 obtained targets entered to DisGeNET database for screening AD-related targets, only 97 potential targets (as shown in Table S3) were associated with AD, and they were reserved for further analysis.

3.3. Network Construction. To facilitate the visualization of multiple-target effects of *ATR* and AD interrelation, network analysis was performed based on the context of the whole human genome [67]. Two networks (A) C-T and (B) C-T-AD were constructed that might show multicompound and multitarget effects and they might explain why *ATR* can be used for the treatment of AD.

3.3.1. Compound-Target Network (C-T Network). C-T interactions data between 181 targets and 32 compounds of *ATR* were collected by the method in Section 2.5. The network comprised 213 nodes and 509 edges. Circular nodes represented targets and triangle nodes represented compounds. Node size was regulated by degree centrality, resulting in an average degree of 4.70 nodes per target and 15.91 edges per compound, respectively (as shown in Figure 2). Among the 32 active compounds, 10 compounds with high-degree distributions were identified, and each of these compounds hit more than 21 potential targets. Usually, higher degree meant that the compounds had more important pharmacological effects on the potential treatment of AD [68]. For example, veraguensin

Table 1: 32 potential active compounds of *Acori Tatarinowii Rhizoma* (*ATR*).

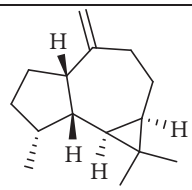
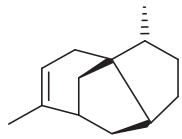
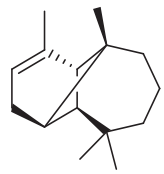
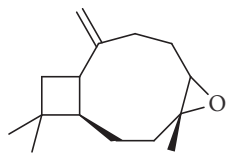
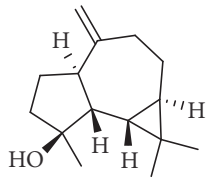
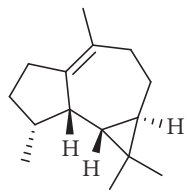
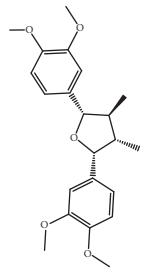
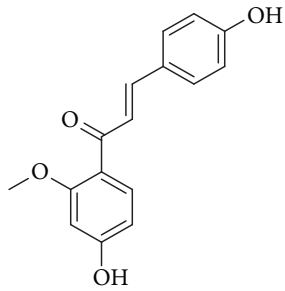
ID	Active compounds	OB	BBB	DL	Structure
M1	(-)-Alloaromadendrene	54.04	2.07	0.1	
M2	(-)-α-Cedrene	55.56	2.16	0.1	
M3	(+)-α-Longipinene	57.47	2.05	0.12	
M4	(-)-Caryophyllene oxide	32.67	1.76	0.13	
M5	(+)-11-Epispathulenol	81.61	1.55	0.12	
M6	(+)-Ledene	51.84	2.16	0.1	
M7	(+)-Veraguensin	25.53	0.72	0.39	
M8	2′-O-Methylisoliquiritigenin	75.86	-0.16	0.17	

Table 1: Continued.

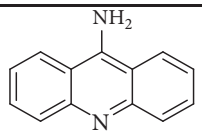
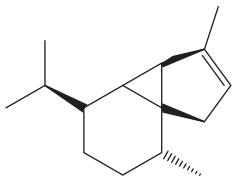
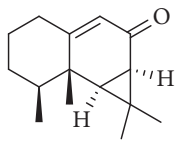
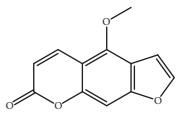
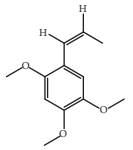
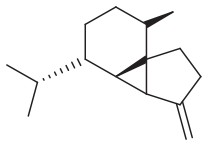
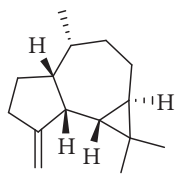
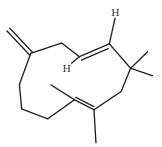
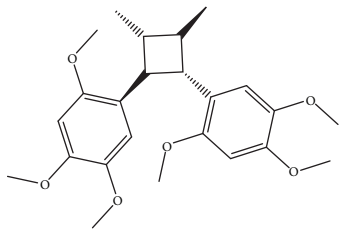
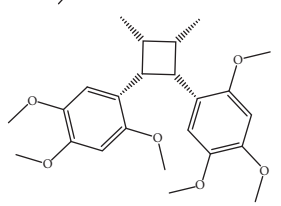
ID	Active compounds	OB	BBB	DL	Structure
M9	Aminacrine	35	0.33	0.12	
M10	α-Cubebene	16.73	2.1	0.11	
M11	Aristolone	45.31	1.54	0.13	
M12	Bergapten	42.21	0.69	0.13	
M13	β-Asarone	35.61	1.24	0.06	
M14	β-Cubebene	32.81	2.02	0.11	
M15	β-Gurjunene	51.36	2.07	0.1	
M16	β-Humulene	26.87	2.01	0.06	
M17	Bisasarcin	18.55	0.54	0.5	
M18	Bisasaricin	28.94	0.65	0.5	

Table 1: Continued.

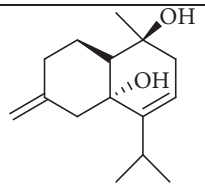
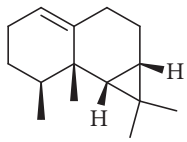
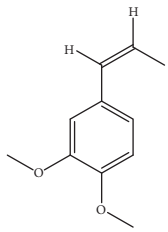
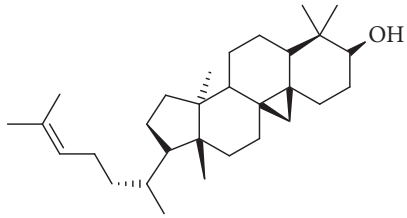
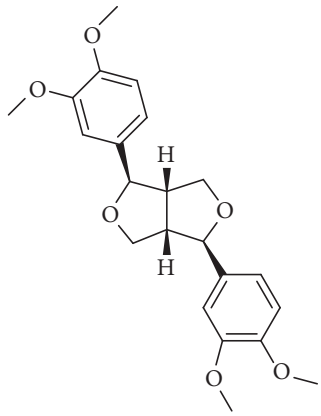
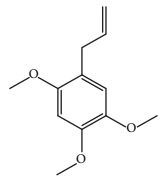
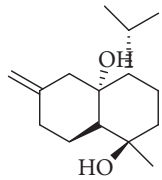
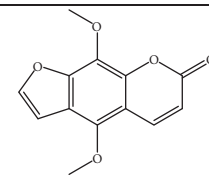
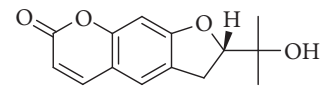
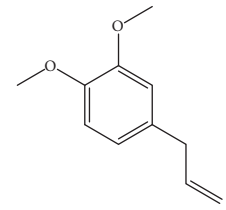
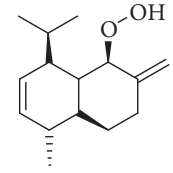
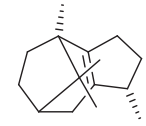
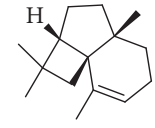
ID	Active compounds	OB	BBB	DL	Structure
M19	Calamendiol	61.13	0.67	0.11	
M20	Calarene	52.16	2.04	0.11	
M21	cis-Methylisoeugenol	74.21	1.48	0.04	
M22	Cycloartenol	38.69	1.33	0.78	
M23	Eudesmin	52.35	0.05	0.62	
M24	γ-Asarone	22.76	1.33	0.06	
M25	Isocalamendiol	57.63	0.74	0.11	

Table 1: Continued.

ID	Active compounds	OB	BBB	DL	Structure
M26	Isopimpinellin	25.93	0.5	0.17	
M27	Longicyclene	46.07	2.16	0.15	
M28	Marmesin	50.28	0.07	0.18	
M29	Methyleugenol	73.36	1.41	0.04	
M30	Murolan-3, 9(11)-diene-10-peroxy	36.72	1.04	0.11	
M31	Patchoulene	49.06	2.17	0.11	
M32	α-Panasinsene	56.77	2.11	0.12	

(M7, degree =82) had the highest number of interactions with targets, followed by methyleugenol (M29, degree =47) and cis-methylisoeugenol (M21, degree = 43), indicating that those compounds might have more pharmacological effects than other compounds in this C-T network. Meanwhile, muscarinic acetylcholine receptor M3 (CHRM3, degree =20) showed the most intimate connections with compounds, followed by muscarinic acetylcholine receptor M1 (CHRM1, degree = 19) and prostaglandin G/H synthase 2 (PTGS2, degree=18). The C-T network results demonstrated the multitarget effects of active compounds by *ATR*.

3.3.2. Compound-Target-AD Network (C-T-AD Network). Network pharmacological methods provided a visual approach to understanding the complex relationship between disease and therapeutic spots [69, 70]. In the present study, 97 potential targets of AD were selected form DisGeNET database and 30 involved compounds (M27 and M30 have no target) were used to construct the C-T-AD network for further cluster analysis. The C-T-AD network was built in the same way as C-T network, as shown in Figure 3. Veraguensin (M7, degree=42), cis-methylisoeugenol (M21, degree=28), methyleugenol (M29, degree =24), and β-asarone (M13, degree=20) might play an important role in the treatment of AD. CHRM1 (degree = 20), PTGS2 (degree = 19), CHRM2 (degree = 19), and ADRB2 (degree = 9) might be the important drug-targets. 26 active compounds connected with more than two targets and all of the 97 targets interact with more than one compound, indicating that many proteins associated with AD might display similar binding patterns with ligands.

3.4. Multiple-Target/Organ Cooperation for AD Treatment. Traditional Chinese medical workers consider the human body as an organic whole, and none of the organs in this whole organization are independent. Theses organs are structurally inseparable, functionally coordinated, and complementary to each other. They interact with each other

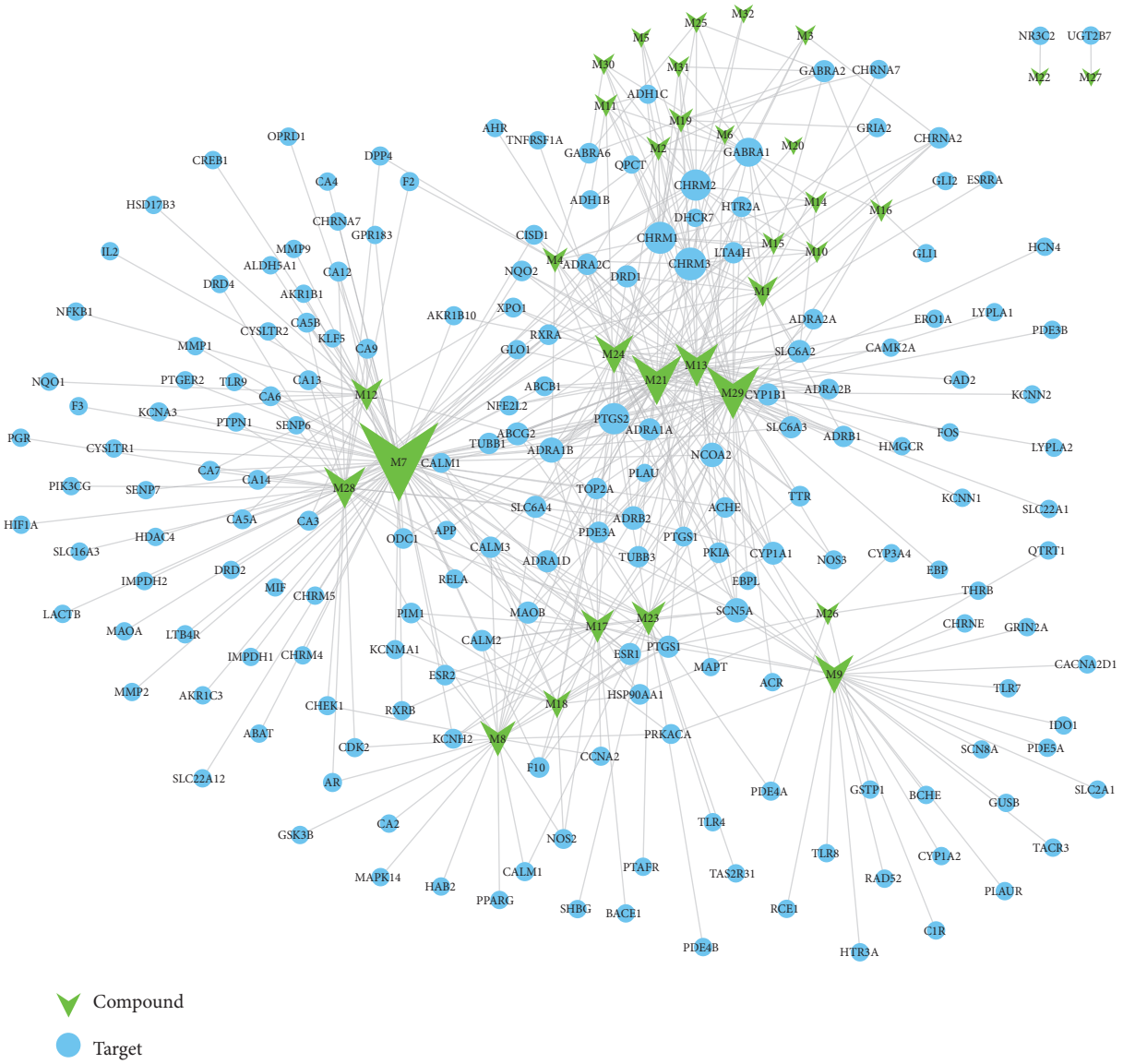


Figure 2: C-T network: a compound-target (C-T) network and nodes represent compounds and targets.

in pathology. Therefore, in this study, the complex process of exploring *ATR* for AD was used by multiorgan cooperation and multitarget action.

3.4.1. GO Analysis. GOBP describes a series of events accomplished by one or more organized assemblies of molecular functions [40]. GOBP showed that these targets were enriched to 20 biological process terms, and the 181 genes were highly related to response to drug, signal transduction, inflammatory response, response to hypoxia, aging, memory, and so on (as shown in Figure 4). In detail, phosphatidylinositol-4, 5-bisphosphate 3-kinase catalytic subunit, gamma isoform (PIK3CG), toll-like receptor 4 (TLR4), tumor necrosis factor receptor superfamily member 1A (TNFRSF1A), protooncogene c-fos (FOS), nuclear factor erythroid 2-related factor 2 (NFE2L2), macrophage migration inhibitory factor (MIF), and nuclear factor NF-kappa-B p105 subunit (NFKB1) were involved. 14 genes were related to "inflammatory response," including sodium-dependent serotonin transporter (SLC6A4), matrix metalloproteinase-2 (MMP-2), hypoxia-inducible factor 1-alpha (HIF1A), dopamine D2 receptor (DRD2), inducible nitric oxide synthase (NOS2), and cyclic AMP-responsive element-binding protein 1 (CREB1). 12 genes were related to "response to hypoxia," such as NFE2L2, Alpha-1A adrenergic receptor (ADRA1A), neuromedin-K receptor (TACR3), 5-hydroxytryptamine 2A receptor (HTR2A), transcription factor p65 (RELA), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), sodium-dependent dopamine transporter (SLC6A3), FOS, and CREB1. 11 genes were related to "aging" including SLC6A4, HTR2A, PTGS2, DRD1, CREB1, neuronal acetylcholine receptor subunit alpha-7 (CHRNA7), glutamate receptor ionotropic, and NMDA 2A (GRIN2A). 11 genes were related to "memory."

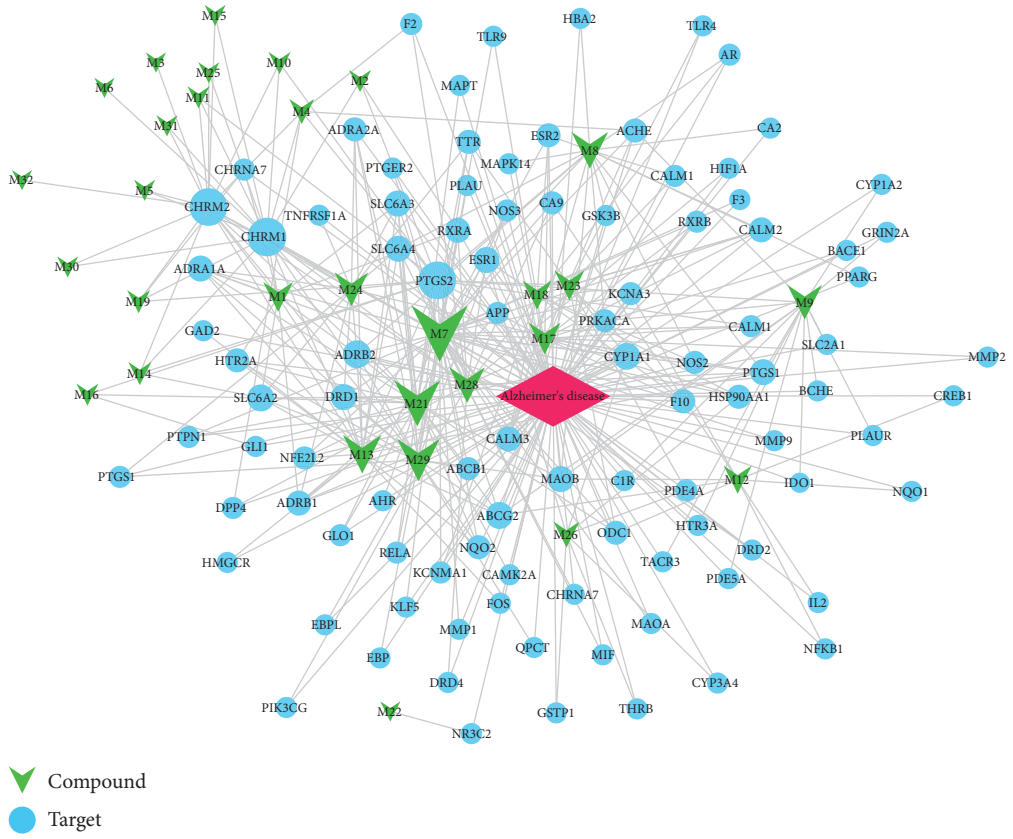


Figure 3: C-T-AD network: a compound-target-Alzheimer's disease (C-T-AD) network and nodes represent compounds and targets.

In conclusion, *ATR* is entirely possible as a therapeutical herb on AD, which is attributed to affect certain biological processes, such as aging, memory, anti-inflammation, and response to hypoxia.

3.4.2. Target Tissue Location. The mRNA specific expression patterns in tissues are important clues to the gene function [71]. Thus, they are of great significance to determine the tissue mRNA expression profiles of various genes at the organ level which is to explore the beneficial effects of *ATR* on AD [72]. In this paper, the results of mRNA expression were demonstrated by using BioGPS microarray analysis. 175 of the 181 targets had their expression profiles in 84 normal tissues (as shown in Table S3).

The 175 targets were generally found in human tissues but showed different levels of mRNA expression. We focused on some of these organizations, including brain, heart, kidney, liver, lung, and whole blood. A target was positioned to the organization where it had the highest mRNA expression level. For example, we compared the expression patterns of different tissues and observed that the 21 targets contained had higher mRNA expression levels in the brain than the remaining 84 normal tissues, 27 targets were positioned in heart, and, meanwhile, 32 targets located in whole blood were linked with tissues in any forms. The organization distribution network of 175 targets (as shown in Figure 5) is based on their expression patterns.

Results from our research suggested that the 21 high-abundant targets in brain were regarded as therapeutic targets for AD, and the other targets were also thought to be associated with AD. Our results implied that drug targets worked on these tissues rather than focusing on a specific one, and the whole blood serves as bridge between these tissues, so as to promote the coordination of organs to achieve the positive effect of AD. The effects of AD are not just in the brain but also in other organs. For example, AD can cause a series of bodily functions damage incidents such as cardiac autonomic dysfunction, urinary incontinence, and metabolic disorders [2, 73, 74]. Meanwhile, other organ lesions may also affect the progression of AD. For example, insulin resistance, hyperglycemia, hyperinsulinemia, and type 2 diabetes mellitus could provoke AD [75]. Coronary heart disease and heart surgery correlate with the development of AD cognitive dysfunction [76, 77]. Therefore, the prevention of AD requires not only attention to the brain but also the coordination of other organs. An interpretation of these findings is that the holistic view of TCMs diagnosis and treatment theory is correct.

3.4.3. Alzheimer's Disease-Relating Pathway. Through the C-T-AD network, a macroscopic visual of the relationship between *ATR*, targets, and AD was obtained, but the detailed mechanism of these remains unclear. Therefore, for determining the interactions among ATR and AD multiple targets,

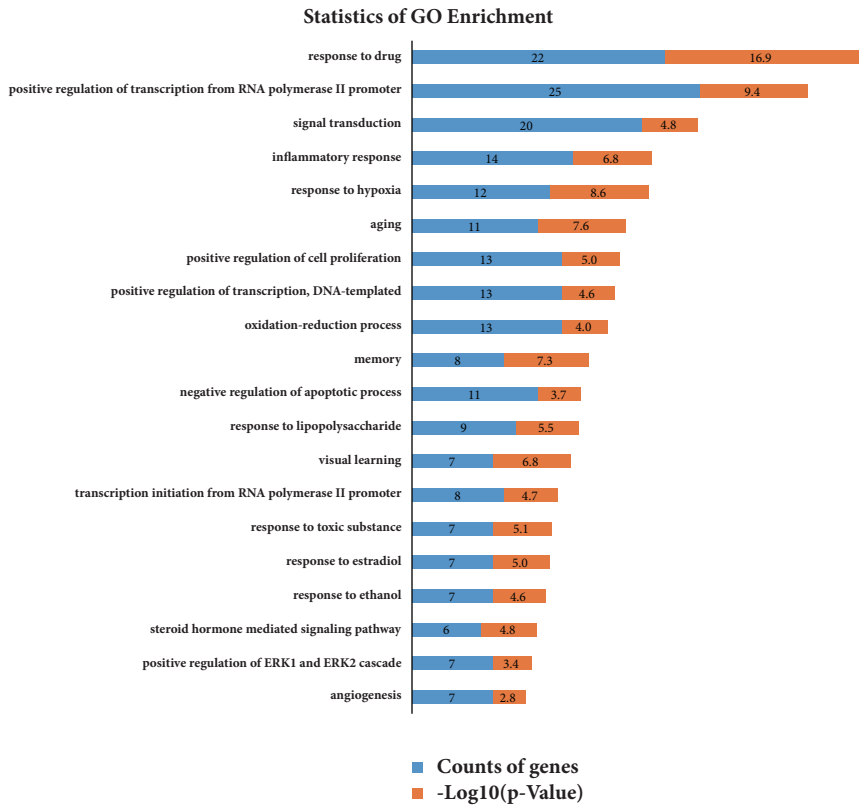


Figure 4: GO analysis: 20 biological processes (BP) of gene ontology (GO) terms sorted by P value < 0.01. Counts of genes and P value related to each BP terms are shown. The x-axis represents BP terms for the target genes, and the y-axis shows counts of genes and −log10 (P value).

we did the following things. First, 97 potential targets of AD underwent pathway enrichment analysis, and a holistic picture of KEGG pathway annotation constructed by the use of the OmicShare tools was provided (as shown in Figure 6). Second, an "AD-pathway" was established based on Alzheimer's disease pathway which was provided by the KEGG pathway database (as shown in Figure 9).

(1) KEGG Pathway Annotation. KEGG pathway annotation can be divided into several modules, such as "Environmental Information Processing", "organismal systems", and "metabolism." Two representative modules, "environmental information processing" and "organismal systems," were described in detail to clarify the underlying mechanism.

Signal transduction in "environmental information processing" module was studied, and a target-pathway network (T-P) was displayed (as shown in Figure 7) whose node size was regulated by degree centrality. 12 signal transduction pathways were concentrated.

There were 4 inflammation-related pathways shown in signal transduction module, including TNF signaling pathway (degree=9), NF-kappa B signaling pathway (degree=6), HIF-1 signaling pathway (degree=9), and VEGF signaling pathway (degree=4), and these pathways indicated that the anti-inflammatory action is important for the treatment of AD. The synthesis of inflammatory critical cytokines and receptors could induce a wide range of intracellular biological processes including apoptosis and cell survival as well as inflammation and immunity [78, 79]. We found that γ-asarone (M24) targets TNF receptor superfamily member 1A (TNFRSF1A), cis-methylisoeugenol (M21) and veraguensin (M7) target NF-kappa B p65 subunit (RELA), bergapten (M12) targets NF-kappa B p105 subunit (NFKB1), and eudesmin (M23) targets toll-like receptor 4 (TLR4), and so on.

The G-protein-coupled receptor-triggered signaling cascade related pathways about cAMP signaling pathway (degree=20) had the maximum interaction targets, suggesting that it was closely related to AD. Research shows that activation of cAMP-dependent pathway may lead to phosphorylate alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) [80], tau hyperphosphorylation, and memory impairment [81].

Calcium signaling pathway also plays an important role (degree=17). Calcium can act in signal transduction resulting from activation of ion channels or as a second messenger caused by indirect signal transduction pathways such as G-protein-coupled receptors [82]. In neurons, calcium is important for the synchronization of neuronal electrical activity with mitochondrial energy metabolism when it increases in cytosolic and mitochondrial system [83], and the increasing of Ca^{2+} signals due to Ca^{2+} release has been implicated to play roles in synaptic plasticity and memory, neurotransmitter release, and neuronal excitability[84].

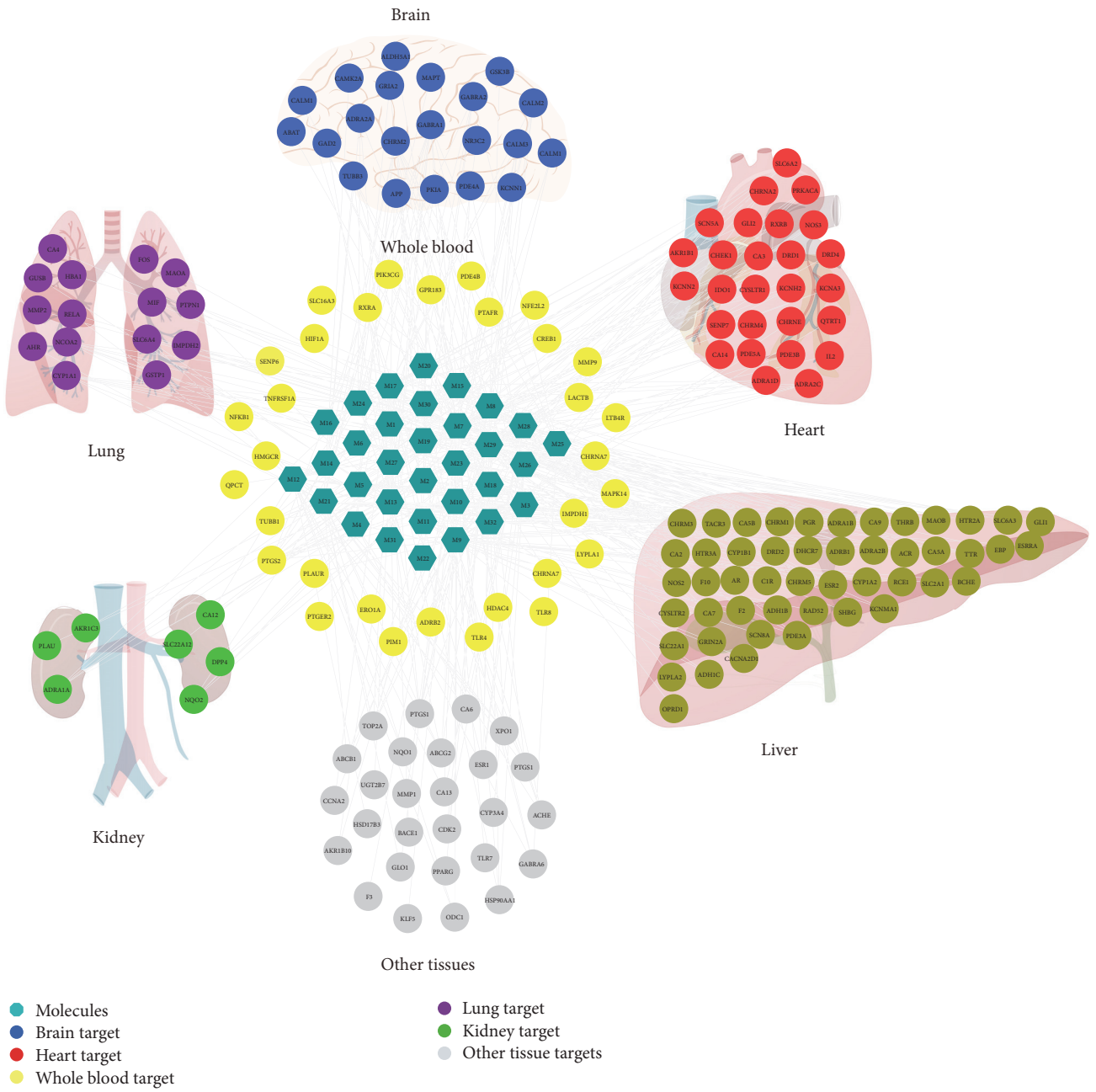


Figure 5: Target-organ location map. The node pie chart represents the organs in which each target is located.

The signaling pathway mediated by small GTPase affects the structure and function of synapses, such as Ras signaling pathway (degree=8) and Rap1 signaling pathway (degree=7). For example, Ras protein family (Ras, Rap1, and Rap2), which belong to small GTPase, regulate the secretion of presynaptic neurotransmitters and the transshipment of the postsynaptic glutamate receptors in the cell membrane [85].

PI3K-Akt signaling pathway (degree=12) is important to regulate the cell cycle and promote growth and proliferation neural stem cells (NSCs) differentiate into motor neurons in adult [86] and promote neuroprotection from NSCs injury [87]. 2′-O-Methylisoliquiritigenin (M8) targets glycogen synthase kinase-3 beta (GSK3β). GSK3β plays an important role in the ultraphosphorylation of tau, which is one of the pathological features of AD. Increased GSK3β activity causes PI3K/Akt dysfunction, which regulates glucose metabolism in the brain and leads to tau hyperphosphorylation in the brain of AD patients [88]. Moreover, in long-term potentiation (LTP), the PI3K binds to an AMPA receptor in a conservative region to determine the receptor in the membrane, especially in the GluR subunit, which causes AMPA receptors to be inserted postsynaptically [89].

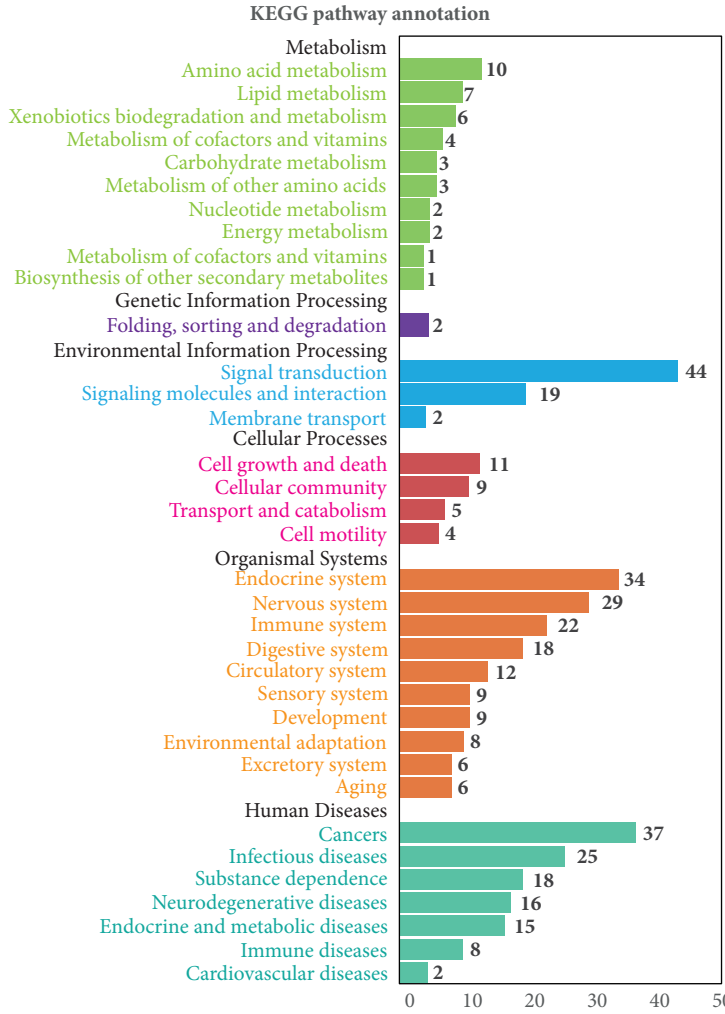


FIGURE 6: KEGG pathway annotation. *x*-axis represents B level classification of pathways, and the *y*-axis shows the gene number in the given gene set that are annotated to the certain pathways.

In addition, cGMP-PKG signaling pathway (degree=12), sphingolipid signaling pathway (degree=6), and AMPK signaling pathway (degree=5) are also thought to be related to AD.

Nervous system in organismal systems module was also studied, and a target-pathway network (T-P) was established (as shown in Figure 8). Node size was regulated by degree centrality. They display extremely significantly close functional correlation to the AD, including dopaminergic synapse, cholinergic synapse, neurotrophin signaling pathway, serotonergic synapse, and long-term potentiation (LTP). Some researches showed that AD was also recognized as a disease of synaptic failure. Beta-secretase 1 (BACE1) cleaved amyloid precursor protein (APP) products impact learning and memory through proteins localized on glutamatergic, GABAergic, and dopaminergic synapses [90]. Acetylcholine is combined with both muscarinic acetylcholine receptor M1 (CHRM1) and muscarinic acetylcholine receptor M2 (CHRM2). To protect the memory and attention deficit caused by the loss of cholinergic neurons [91], acetylcholinesterase (ACHE) inhibitor works by partially blocking the degradation of acetylcholine in the synapse and enabling more of the neurotransmitter to reach and activate cholinergic receptors [92]. Soluble oligomers of the amyloid-β peptide (AβO) decreased brain serotonin (5-HT) levels in mice, whereas treatment with 5-HT prevented AβO-induced microglial activation and increased TNF-α levels [93]. We found that bisasarcin (M17) targets BACE1 and ACHE. (-)-Caryophyllene oxide (M4), veraguensin (M7), and aminacrine (M9) target ACHE. Among the 32 identified active compounds, 19 compounds target CHRM1 and 18 compounds target CHRM2, respectively. (-)-Alloaromadendrene (M1), β-asarone (M13), cis-methylisoeugenol (M21), and methyleugenol (M29) target 5-hydroxytryptamine 2A receptor (HTR2A).

(2) AD-Pathway Network. Nine protein targets were mapped to the AD-pathway network, indicating that these proteins play an important role in AD (as shown in Figure 9). AD is associated with senile plaques and neurofibrillary tangles (NFTs). Amyloid-beta (Aβ), a major component of senile plaques, has various pathological effects on cell and organelle function. Intracellular Aβ may contribute to pathology

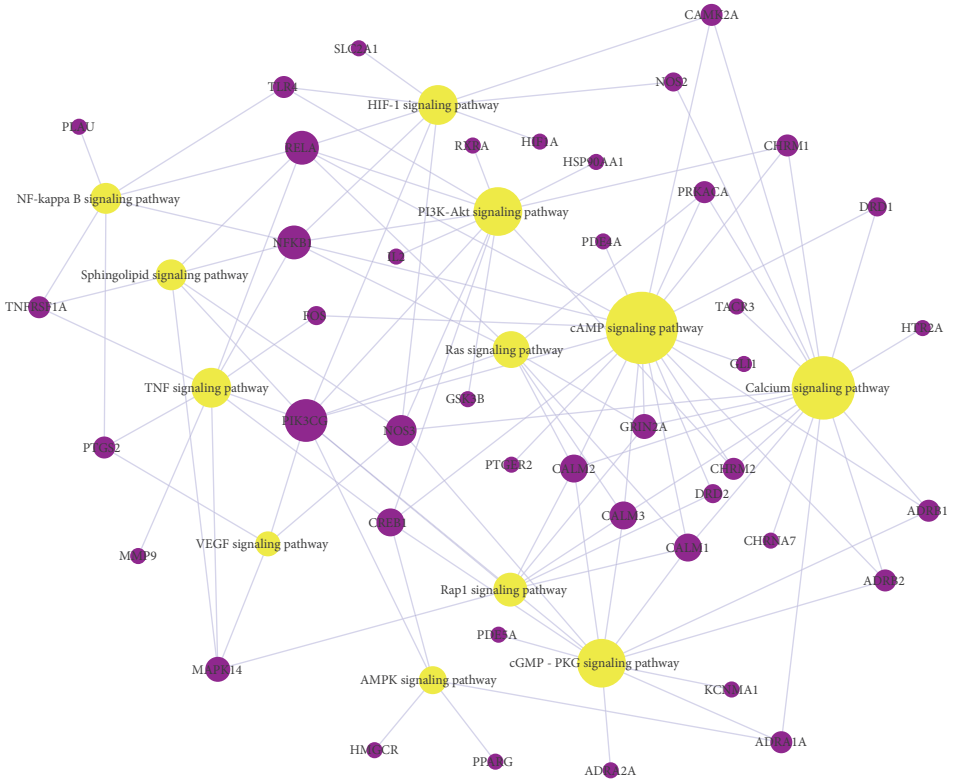


Figure 7: A target-pathway network (T-P) was displayed, signal transduction in environmental information processing.

by facilitating tau hyperphosphorylation, disrupting mitochondria function, and triggering calcium dysfunction. As displayed in Figure 9. Some target receptors involved in Aβ aggregation and tau hyperphosphorylation were affected by *ATR* components to produce neuroprotective functions.

Recent studies have shown that the soluble oligomers of Aβ polypeptides may be a pathogenic factor for the development of Alzheimer's disease [94]. The amyloidogenic pathway triggered from two sequential cleavages of APP by BACE1 resulted in the formation of Aβ peptides [95]. Based on the above findings, blocking the enzyme (BACE inhibitor) in theory would prevent the accumulation of Aβ and (per the amyloid hypothesis) may help slow or stop Alzheimer's disease. For example, Albany Molecular Research Inc. reported on their development of an experimental drug for the treatment of Alzheimer's disease called verubecestat (MK-8931), which is an inhibitor of BACE1[96]. AstraZeneca and Eli Lilly and Company announced an agreement to codevelop lanabecestat (AZD3293), which is an oral beta-secretase 1 cleaving enzyme (BACE) inhibitor [97], and the drug has been developed with the FDA's fast-track designation. Our research data indicated that veraguensin (M7) and cis-methylisoeugenol (M21) target APP and bisasarcin (M17) can target BACE1 being used to treat AD through decreasing production of amyloid.

Excessive or abnormal tau phosphorylation destroys the cytoplasm function and interferes with axonal transport, resulting in cell death [98]. GSK3β has a great influence on activated overphosphorylation factor. 2′-O-Methylisoliquiritigenin (M8) targets GSK3β with AD treatment by preventing tau overphosphorylation.

As far as we know, glutamate-mediated excitotoxicity is one of the major reasons for chronic neurodegeneration. Aminacrine (M9) targets GRIN2A (NMDAR), and calamendiol (M19) and γ-asarone (M24) have shown connections with GRIA2 (GLuR). GRIA2 is a mediated glutamate receptor, which can mediate excitatory synaptic transmission in synaptic hyperactivity [99]. NMDA subtypes of glutamate receptors are agents that rely on the death of cells dependent on Ca^{2+} [100], which can lead to degenerative disorders due to excessive or inappropriate activity [101]. Therefore, GRIN2A and GRIA2 are the intrinsic neural protection targets for regulating Ca^{2+} concentration. Meanwhile, aminacrine (M9) targets voltage-dependent calcium channel subunit alpha-2/delta-1 (CACNA2D1), a protein in a voltage-dependent calcium channel mediates calcium ions into the membrane polarization. GRIN2B and GRM5 are associated with calcified neuropathy. Therefore, neuronal Ca^{2+} signaling is partially targeted through *ATR* for the treatment of AD.

In addition, TNFRSF1A (TNFR) participated in the MAPK signaling pathway and has been associated with inflammation, which is a risk factor for AD.

4. Discussion and Conclusion

TCM is a complex system composed of multicomponent and multitarget, and the function of each component is

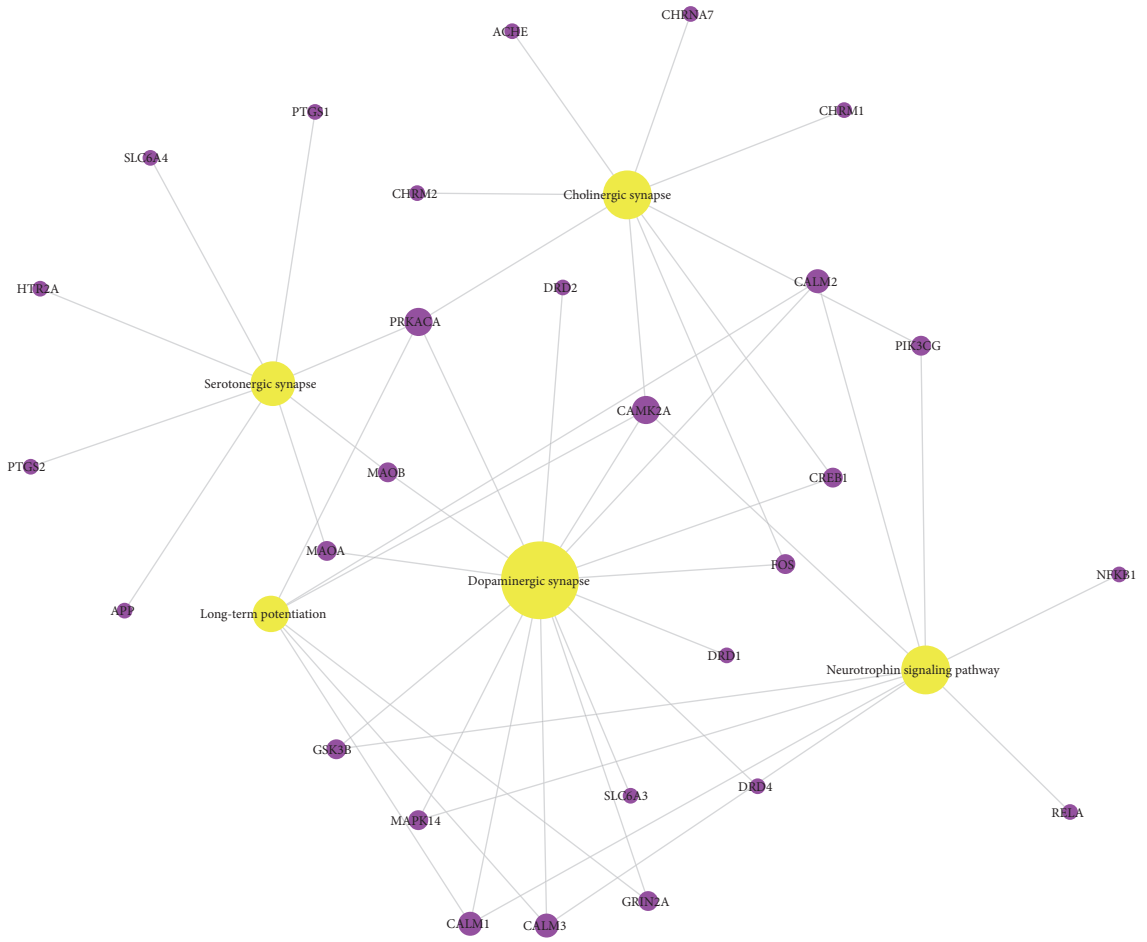


Figure 8: A target-pathway network (T-P) was displayed, nervous system in organismal systems module.

synergistic. The composition of TCM is so complex that it is difficult to study its effects from the mixture and to elaborate the mechanism of action from the perspective of modern medicine. The systematic pharmacological method provides new ideas and perspectives for the research of complicated Chinese medicine system. The pharmacology of TCM system has established a model of interaction of elements, such as drug molecules, protein targets, tissues, and organs, to clarify and predict the efficacy and toxicity of TCM [102]. In this study, a systematic pharmacological method was used to study the active ingredients of *ATR*. The potential targets of pharmacodynamic components, gene ontology, network analysis, organ location network, and pathway enrichment analysis were employed to explore the relationship between active ingredients and targets of Alzheimer's disease. Our result showed that *ATR* and AD are highly correlated in biological processes, organs, and signaling pathways. Our main findings are as follows.

(1) Through defining components in this system, we have studied systematic pharmacological methods to integrate multiple technologies, including ADME-system assessment, drug targeting, and target tissue distribution. 32 active compounds were detected. These compounds interact with 181 different targets by drug targeting. The resulting C-T network indicated that compounds veraguensin (M7), methyleugenol (M29), and cis-methylisoeugenol (M21) and targets CHRM3, CHRM1, and PTGS2 are the key factors to play an important role in the drug-target interaction network.

(2) The identified 97 targets associated with Alzheimer's disease are essential to understanding the pharmacological mechanisms of *ATR*. The C-T-AD network has clearly elucidated the pharmacological action and mechanism of *ATR* on Alzheimer's disease. For example, aristolone (M11) mainly targets at acetylcholine receptors CHRM1 and CHRM2 to treat AD through the cholinergic synapse pathway. The resulting network builds a more comprehensive visualization of the C-T-AD interaction pattern.

(3) TCM is the embodiment of holistic medicine, which is characterized as multicomponent treatment, multiobjective/approach regulation, and multiorgan cooperation. The response to inflammatory, hypoxia, aging, and memory mechanism of *ATR* is also illustrated by GO analysis. For instance, the 14 potential targets from the 7 compounds were enriched to the biological process of inflammatory response, which indicated that anti-inflammation may be the key to *ATR* treatment of AD. The target tissue location results had shown that 27 and 48 of 185 targets of *ATR* were highly expressed in heart and liver, respectively. These organs were

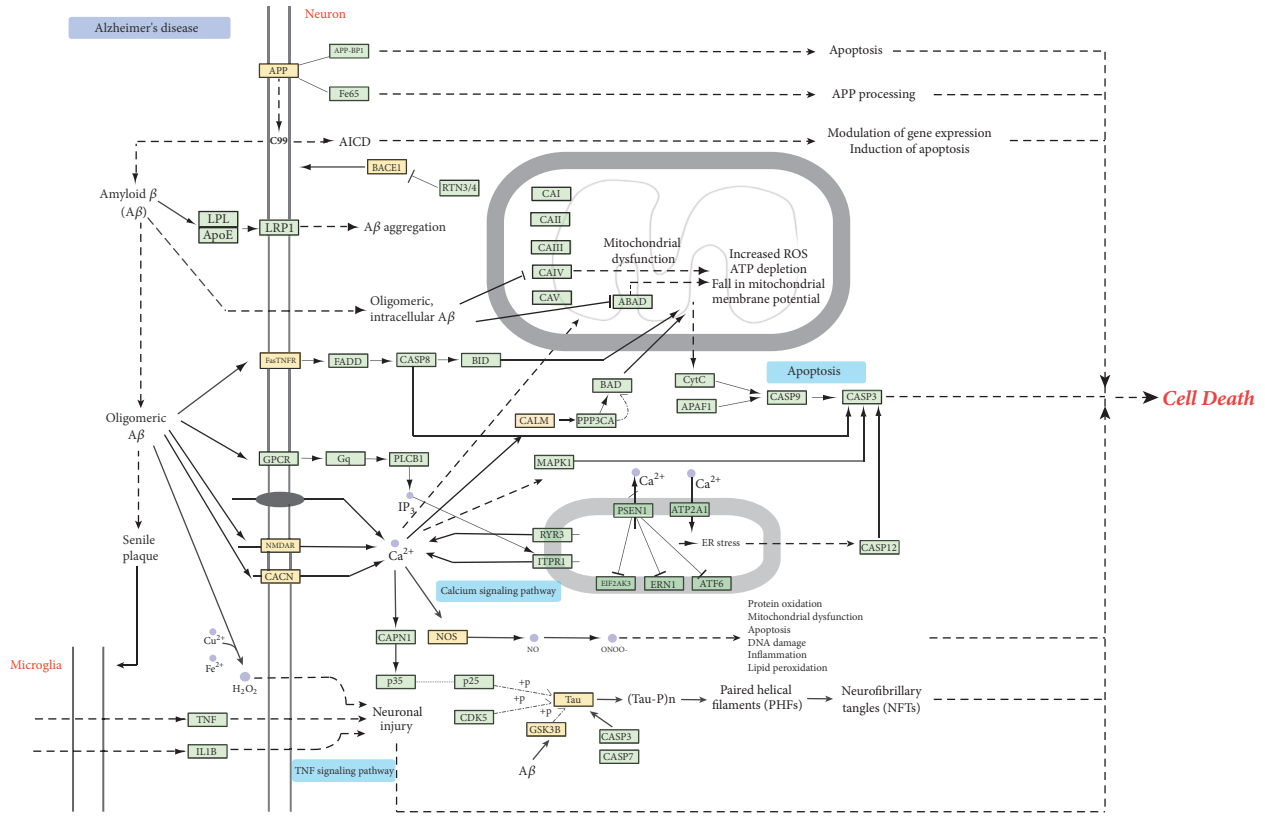


Figure 9: Network pathway: pathway enrichment analysis was performed. 97 filtered target genes were mapped into Alzheimer's disease pathway from the Kyoto Encyclopedia of Genes and Genomes (KEGG) to confirm the possible effect pathway of *Acori Tatarinowii Rhizoma* (*ATR*) on Alzheimer's disease.

closely related to AD, and *ATR* was closely related to these organs in the meridian theory.

(4) KEGG pathway annotation analysis showed that the potential targets of the compounds were clustered in 12 signaling pathways, mainly related to inflammation, calcium signaling pathway, G-protein mediated signaling pathway, and energy metabolism, and effects on the nervous system are mainly manifested in the dopaminergic synapse, cholinergic synapse, neurotrophin signaling pathway, serotonergic synapse, and LTP. Finally, pathway-mapping result showed that 9 protein targets were closely related to the *ATR* treatment AD.

In summary, this study not only conducts a comprehensive analysis of relevant AD through systematic pharmacological methods and discovers potential active compounds but also explains the treating mechanism of *ATR* in Alzheimer's disease, widely applied to clarify the effectiveness and mystery of the medicinal plant *ATR*; what is more, it provides an example for the treatment of complex diseases in the near future. Although the results are interesting, further research requires that the support of experimental data includes the confirmation of drug dose relationships. In addition, the present study needs to test the molecular mechanisms of the active compounds *in vivo* to support further assessment of potential clinical applications.

Data Availability

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

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Table S1 shows the structure of 105 compounds in *Acori Tatarinowii Rhizoma* (ShiChangPu, *ATR*). Compounds information was found in the TCMSP (http://lsp.nwu.edu.cn/) and was input to Pubchem (https://www.ncbi.nlm.nih.gov/pccompound/) for compounds names to be uniformly standardized. Table S2 shows the targets prediction of 32 identified

active compounds of *ATR*. Molecular targets information was found in the TCMSP (http://lsp.nwu.edu.cn/), SEA (http://sea.bkslab.org) and the Binding Database (http://www.bindingdb.org). The targets were obtained from different databases and input to UniProt (http://www.uniprot.org/) to make the targets names uniformly standardized. Noise and error information was eliminated by PharGKB (https://www.pharmgkb.org/), Therapeutic Target Database (DTT, http://bidd.nus.edu.sg/group/cjttd/), and the Comparative Toxicogenomics Database (CTD, http://ctdbase.org/). Table S3 shows the potential targets of 32 identified active compounds of *ATR* were associated with AD. In order to elucidate the action relations of *ATR* in Alzheimer's disease, the integration was performed by means of gene and AD vocabulary mapping by using the DisGeNET association type ontology to select the targets related to AD. Table S4 shows the 175 targets mRNA expression profiles in 84 normal tissues. The targets organ location network was used with the dataset: GeneAtlas U133A, gcrma (http://biogps.org). *(Supplementary Materials)*

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Review Article

An Insight into Ginsenoside Metabolite Compound K as a Potential Tool for Skin Disorder

En Hyung Kim[1] **and Wonnam Kim**[2]

[1]*Department of Dermatology, Cheil General Hospital and Women's Healthcare Center, Dankook University College of Medicine, Cheonan, Republic of Korea*
[2]*Division of Pharmacology, College of Korean Medicine, Semyung University, Jecheon, Republic of Korea*

Correspondence should be addressed to Wonnam Kim; wonnam_kim@semyung.ac.kr

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Ginsenosides are the major bioactive natural compounds derived from *Panax ginseng*. Several studies report the pharmaceutical benefits of several ginsenosides, including antidementia, antitumor, and anti-inflammatory activity. Biotransformations by gut microbiome contribute to the biological function of these ginsenosides. After ingestion ginsenosides are hydrolyzed to Rg2, Rg3, compound K, and others by human gut flora. Compound K is considered the representative active metabolite after oral administration of ginseng or ginsenosides. Various studies report the diverse biological functions of compound K, such as antitumor, antidiabetic, antiallergic, and anti-inflammatory activity. Recent clinical trial and *in vitro* studies demonstrate the antiaging activities of ginsenosides in human skin. Ginsenosides have been considered as an important natural dermatological agent. In this review, we will cover the modern tools and techniques to understand biotransformation and delivery of compound K. Also the biological function of compound K on skin disorder and its potential dermatological application will be discussed.

1. Introduction

Ginseng, referring to the root and rhizome of *Panax ginseng*, is a representative medicinal herb commonly used thousands of years in Asia. Its active constituents are ginsenosides, a class of triterpenoid saponins, and are exclusively contained in *Panax* species and more than 150 ginsenosides are currently identified from ginseng roots, fruits, flower heads, leaves, and stems. [1]. Ginsenosides are divided into two main types by their chemical structures: protopanaxadiols (PPDs) and protopanaxatriols (PPTs) [2, 3]. PPD-type includes ginsenoside Rc, Rd, Rb1, and Rb2, while PPT-type includes ginsenosides Re, Rf, Rg1, and Rg2. There have been many reports describing the biological actions of several ginsenosides including antidementia, antitumor, and anti-inflammatory activities [4–6]. After ginseng or ginsenosides are orally consumed, compound K is considered the major functional component determined by plasma or organ [7]. The biotransformation by gut microbiome is closely linked to the diverse biological activities of these ginsenosides [8]. The deglycosylation of ginsenosides Rc, Rb1, and Rb2 by human gut bacteria produce compound K (20-*O*-*β*-(D-glucopyranosyl)-20(S)-protopanaxadiol) is an active metabolite. [9]. Numerous experimental studies of compound K have shown the antitumor, antidiabetic, antiallergic, and anti-inflammatory effects [10, 11].

For thousands of years, the benefits of ginseng are well known to treat a wide variety of diseases. It also has been used to improve the overall condition of skin [12]. Chinese traditional medicine textbooks describe its ability as a topical treatment for wounds, atopic dermatitis, and other inflammatory skin symptoms [13]. Recently, there have been a few studies to clarify the efficacy of ginseng in skin [12]. A number of human and animal studies have demonstrated that dermatological formulations comprising crude extracts of ginseng show positive benefits on the skin [14–16]. Other reports indicate that ginsenoside Rb1 [17] and Rb2 [15] stimulates the recovery of burn injury, and topical treatment of compound K may help to avoid or ways to improve skin deteriorations with age caused by loss of hyaluronan

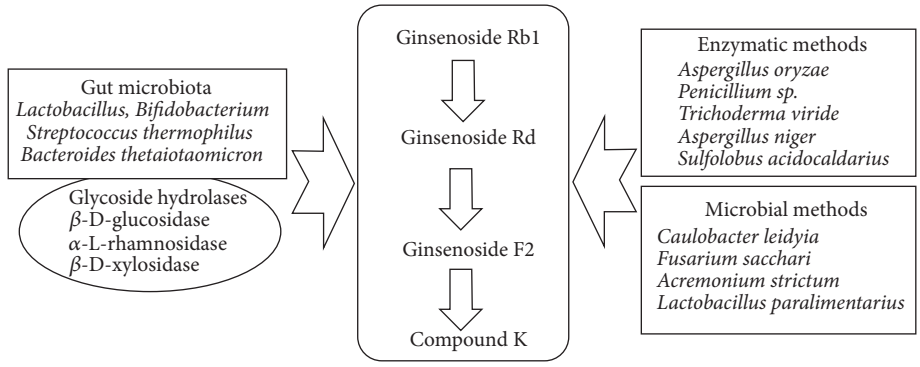

Figure 1: Biotransformation to compound K.

in human skin [18]. This review summarizes the current understanding of compound K, and its dermatological and cosmeceutical benefits.

2. Overview of Compound K

2.1. Role of Intestinal Microbiota. The human intestine is populated with a large community of microorganisms and is a site where they affect human health as well as drugs' fate [19]. The intestinal microbiota, represented as a "microbial organ," can contribute important roles in the metabolic function of drugs and affect the stability and oral bioavailability of drugs [20–22]. Gut is an emerging therapeutic target, especially for herbal products and dietary supplements [21, 23]. As herbal products are mostly consumed orally, it can inevitably affect the gut microbiota in different ways [24].

First, herbs may change the population of the gut microbiota to maintain a homeostatic balance [25]. Green tea has been reported to exert anti-inflammatory and antiobesity effects and also have been found to change composition of the gut microbiota [26, 27]. Seo's group reported that fermented green tea extract restored the ratio changes in *Firmicutes/Bacteroidetes* and *Bacteroides/Prevotella* induced by high-fat diet, which may explain the underlying mechanism that improves obesity and its related disorders [28]. Axling's group added one strain of *Lactobacillus plantarum* with green tea powder and found reduction in inflammatory markers affected from high-fat diet and expansion in gut microbial diversity which may act as a positive health factor [29]. Several studies have reported the potent anticancer activities of *Gynostemma pentaphyllum* (Gp) [30]. Chen's group first demonstrated that Gp saponins (GpS) elicit anticancer responses on tumor xenograft models [31]. They also showed that tumor implants significantly altered the gut microbiota compositions assessed with ERIC-PCR and 16S pyrosequencing procedures [31]. Interestingly, GpS treatment augmented the relative abundance of probiotics such as *Clostridium cocleatum* and *Bacteroides acidifaciens* modulated by tumor implantation [31].

Second, herbs may undergo gut microbiota-mediated bioconversion process influencing the drug metabolism [24]. *Coptis chinensis* contains alkaloids such as berberine, which has been widely studied due to its potent antimicrobial, antioxidant, anti-inflammatory, anticancer, antidiabetic, neuroprotective, nephroprotective, and hepatoprotective activity [32]. However, berberine exhibits poor water solubility partly contributing to its low bioavailability and poor intestinal absorption [33]. A recent study by Feng's group suggests that interaction between the gut microbiota and berberine enhances its absorption [34]. In fact, the gut microbiota transforms berberine to dihydroberberine, a 5-fold higher absorbable form, and if treated with antibiotics the level of gut flora was lowered and as a result the plasma concentrations of berberine were lowered, reducing its therapeutic efficacy [34]. Ginsenosides from *Panax ginseng* are involved in modulating numerous physiological functions [35]. Ginsenoside Rb1, a 20(S)-protopanaxadiol (PPD) type ginsenoside, one of the important components in ginseng total saponins, possesses various beneficial effects [36]. However, biotransformation may be required for ginsenoside Rb1 due to its poor membrane permeability and higher susceptibility to degradation [35]. Increased biological effects of ginsenoside Rb1 is mediated by metabolites metabolized by human intestinal microbes [9].

2.2. Biotransformation to Compound K. After ginsenosides are consumed orally, ginsenosides are metabolized by deglycosylation reactions [37, 38]. Gut microbiota including *Lactobacillus*, *Bifidobacterium*, *Streptococcus thermophilus*, and *Bacteroides thetaiotaomicron* possess different types of glycosidases, such as β-D-glucosidase, α-L-rhamnosidase, and β-D-xylosidase [37]. Ginsenoside Rb1 undergoes stepwise hydrolysis of the sugar moieties to secondary ginsenosides or aglycone by β-D-glucosidase [37]. Ginsenoside Rb1 is rapidly hydrolyzed to ginsenoside Rd and then in a rate-limiting step deglycosylated to ginsenoside F2 and further converts to the compound K through hydrolysis [39] (Figure 1). Due to its diverse biological activities, compound K has attracted growing interests in methods on how to increase its quantity. Conventional chemical approaches, such as heating, hydrolysis with weak acid, and cleavage by alkali, have been studied; however, microbial or enzymatic conversion methods are considered more favorable due to their prominent selectivity, moderate reaction conditions, and environmental compatibility [40–45]. Enzymatic methods to produce compound K use lactase, cellulose, and β-D-glycosidase, which are

purified from *Aspergillus oryzae*, *Penicillium sp*., *Trichoderma viride*, *Aspergillus niger*, and *Sulfolobus acidocaldarius* [46–48] (Figure 1). Microbial methods using crude enzymes from *Caulobacter leidyia*, *Fusarium sacchari*, *Acremonium strictum*, and *Lactobacillus paralimentarius* were reported to achieve compound K [49–52] (Figure 1).

To understand the pharmacokinetics of compound K, *in vitro* and *in vivo* studies have been processed by dose-dependent oral administration [53]. An open trial study on single oral dose of red ginseng product shows that absorption of compound K is not affected by its parent compound, ginsenoside Rb1, except the fact that the delay to reach the maximum serum concentration explains the required transformation process [54]. Moreover, a human pharmacokinetic study comparing the pharmacokinetic parameters of compound K between fermented and nonfermented red ginseng indicates that fermented group absorbed higher and faster in greater amounts than nonfermented group [55]. Recent human pharmacokinetic data from single and multiple dose studies of compound K suggest the influence of sex and food related factors [56, 57].

2.3. Advances in Delivery of Compound K. The therapeutic use of compound K may be restricted because of poor aqueous solubility, low membrane permeability, and P-glycoprotein mediated efflux [58]. To improve the solubility and stability of active constituents several approaches were developed, including polymeric nanoparticles, solid lipid nanoparticles, liquid crystal systems, precursors systems for liquid crystals, liposomes, and microemulsions [59]. Polyethylene glycol (PEG) is a widely used hydrophilic, nonionic, and nontoxic polymeric carrier in drug delivery systems [60]. Surface modification using PEG increases water solubility protects from proteolytic degradation, prolongs circulation half-life in blood, reduces systemic toxicity, and improved therapeutic indices [61]. Mathiyalagan's group generated a pH-sensitive PEG-compound K conjugate through an acid-labile ester-linkage that enhanced water solubility of compound K [62]. They also covalently conjugated hydrophobic compound K with hydrophilic glycol chitosan backbone by an acid-labile linkage to improve aqueous solubility and targeted delivery [63]. The nanoparticles were stable under physiological pH, whereas they degraded easily under acidic pH that mimics the intracellular pH levels [63].

D-α-Tocopheryl polyethylene glycol 1000 succinate monoester (vitamin E TPGS or simply TPGS) possesses the benefits of both promoting solubility and suppressing P-glycoprotein [64]. TPGS based formulation could increase solubility, permeability, and stability, prolong the half-life, and improve the cellular uptake of the drug [65, 66]. Yang's group prepared ginsenoside compound K-loaded TPGS-modified liposomes (GCKT-liposomes) to increase the solubility and targeting capability of compound K [67]. The GCKT-liposomes significantly increased the cellular uptake and its cytotoxicity *in vitro* and also showed higher antitumor efficacy by grafting A549 cells into nude mice *in vivo* [67]. Zhang's group used a novel ascorbyl palmitate (AP)/TPGS mixed micellar system with compound K and reported an increased antitumor effect *in vitro* [68]. The compound K-loaded AP/TPGS mixed micelles significantly enhanced cellular uptake and tumor targeting resulting in decreased tumor volumes in the A549 xenograft models [68]. Furthermore, Yang's group used TPGS/PEG-poly(ε-caprolactone) (PCL) mixed micelles with compound K to increase the water solubility and the cellular uptake in tumor tissue [69]. This carrier system enhanced the antitumor effect of compound K by promoting apoptosis and inhibiting cell invasion and migration in A549 and PC-9 cells [69].

3. Biological Activity of Skin

3.1. Dermatological Activity. Pruritus or itching is an unpleasant skin sensation that frequently provokes scratching and is generally relevant with primary skin lesions such as urticaria, atopic dermatitis, or systemic diseases such as cholestasis and uraemia [70]. A number of chemical agents, like proteases, cytokines, prostaglandins, histamine, neuropeptide substance P, and bile salts, can act as pruritogens [71]. Shin's group investigated the antipruritic effects of ginsenoside Rb1 and compound K in response to compound 48/80, substance P, and histamine using behavioral mouse model for itch [70]. Compound K treatment reduced scratching behaviors and skin vascular permeability activated by compound 48/80, substance P, and histamine [70].

The anticancer effects of compound K have been investigated by focusing on skin related cancer. Lee's group studied the effects of compound K on tumor progression and mediated molecular changes [72]. Tumor progression is regulated by elevation of ornithine decarboxylase (ODC), free radicals, reactive oxygen species (ROS), COX-2, and NF-κB activity [73, 74].

To induce mouse ear edema, prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was applied [72]. Compound K pretreatment inhibited the TPA induced activity of COX-2 and ODC by interfering with extracellular signal regulated kinase (ERK) and nuclear factor-κB (NF-kB) pathway [72]. Melanoma is notoriously resistant to most approaches to treat the aggressive and lethal skin cancer. A series of functional, biochemical, and gene sequencing indicated that melanoma cells frequently acquire chemoresistance by exploiting their intrinsic apoptosis resistance and by reprogramming pathways associated with cell proliferation and survival during melanoma progression [75]. Development of highly potent and specific compounds is urgently needed to block signaling networks critical for melanoma [76]. Kang's group reported the mechanism of action responsible for the antitumor effect of compound K in melanoma progression [77]. Compound K appears to inhibit melanoma cell proliferation and growth in anchorage independent conditions [77]. Also, compound K treatment activated AMPK/JNK signaling and induced cell death mediated by autophagy and apoptosis [77].

Studies have reported the effect of compound K on inflammatory skin conditions. Atopic dermatitis (AD), or atopic eczema, is a common chronic, relapsing, and often intensely pruritic inflammatory disorder of the skin [78]. Kim's group demonstrated that compound K treatment in NC/Nga mice attenuates *Dermatophagoides farinae* body

extract (DFE) antigen-induced AD-like symptoms, including increased dermatitis severity score, ear thickness, and infiltration of inflammatory cells in the skin lesions [79]. These effects were regulated by decrease in serum levels of macrophage derived chemokine and production of T cell-derived proinflammatory cytokines in cultured *ex vivo* splenocytes, including IFN-γ, GM-CSF, TNF-α, IL-4, IL-5, IL-10, and IL-12 [79].

As described above, compound K is a promising therapeutic approach for inflammatory related skin disorders; however there are limited experimental studies and clinical trials to fully understand and evaluate the pharmacological activities.

3.2. Cosmeceutical Activity. The nutritional benefits of ginseng on skin health are characterized by activating skin metabolism due to enhanced blood flow and cell proliferation which may be related to the antiaging capabilities [80]. Many studies support that ginsenosides elicit antiaging effects by free radical scavenging and suppressing lipid peroxidation [81].

Hyaluronic acid (HA) also called hyaluronan is an, evolutionarily conserved, abundant linear polysaccharides [82]. Since its discovery in 1934, HA has been widely applicable in the field of cutaneous wound repair, neurosurgery, and cosmetic practice [83]. The HA synthesis and turnover have been shown to decline with age [84]. This decline is important for decreased turgidity, wrinkling, reduced elasticity, and weakened support for microvessels in aged skin [85]. HA is synthesized by three different plasma membrane bound hyaluronan synthase (HAS) enzymes, namely, HAS1, HAS2, and HAS3 [86]. Kim's group treated immortalized keratinocyte, HaCaT cells, with compound K, and examined the gene expressions of 100 transcripts using cDNA microarray technology [18]. HAS2 gene expression was upregulated significantly by compound K and enhanced HA content in aged skin by HA synthesis [18]. A later study by Lim's group reported the underlying mechanism for augmented HA production by compound K [87]. The study provides evidence that the production of HA induced by compound K is mediated by Src-dependent Akt and ERK activation, but not EGFR or Ca2+ changes [87].

Exposure to ultraviolet (UV) radiation on human skin is highly correlated with skin diseases [88]. Prolonged UV exposure affects many different biological alterations that are directly or indirectly associated with skin aging and cancer incidents [89]. UVA comprises most of the UV radiation that reaches the earth's surface; chronic exposure to UVA penetrates deeply through into the human skin and damages the underlying support by the dermis causing premature photoaging and forms wrinkles and sagging skin [88, 90, 91]. UV radiation activates particular matrix metalloproteinase (MMP) family members that mediate collagen degradation that is observed in photoaged skin [92]. Dermal fibroblasts express matrix metalloproteinase-1 (MMP-1) by exposure to both UVA and B [93]. He's group treated UVA-irradiated fibroblasts with compound K and showed that type I collagen production increased while, under the same experimental conditions, MMP-1 activity decreased [94]. UVB irradiation stimulates MMP expression by regulating transcription factors, such as activator protein-1 (AP-1) and NF-κB in the epidermis [95]. The mitogen-activated protein kinase (MAPK) signaling pathway results in expression of AP-1 activation; depending on the cell type IκB kinase (IKK), phosphoinositide 3 kinase- (PI3K-) Akt and p38 MAPK have been associated with NF-κB activation [96, 97]. Thus, investigation of compounds targeting UVB-induced MMP levels and/or its upstream regulators may offer advantages to prevent and treat skin aging [98]. Shin's group reported the inhibitory effect of compound K on MMP-1 levels in human dermal fibroblasts (HDFs) by UV, which is due to the effect of adenosine monophosphate-activated protein kinase (AMPK) as a downstream of the cAMP-dependent protein kinase- (PKA-) liver kinase B1 (LKB1) pathway [99]. Damaged DNA by UVB causes cyclobutane pyrimidine dimers (CPDs), while UVA exposure mostly damages indirectly through ROS generation [93]. Most of UVB-induced DNA damage in humans is removed by the response of nucleotide excision repair (NER) pathway [100]. Cai's group reported that compound K augment UVB induced cell death in HaCaT cells [101]. Compound K, by DNA repair induction, caused a notable reduction against CPD in later stages after UVB irradiation [101]. Compound K augmented the decrease in specific components of the NER complex, such as XPC and ERCC1 by UVB [101]. Hong's group used BIOGF1K, a fraction rich in compound K, to study the antiphotoaging effect induced by UVB irradiation on NIH3T3 and B16F10 cells [102]. BIOGF1K inhibited the UVB-induced apoptosis, morphological changes, and melanin secretion [102]. Skin inflammation is closely linked to skin aging because inflammation induced inflammatory cytokines and halogenated tyrosine increases protein denaturation resulting in skin aging [103]. Lee's group showed that compound K inhibits TNF-α induced MMP-1 secretion, a characteristic feature of skin aging in human [104]. The ability of compound K to inhibit the degradation of collagen in human fibroblasts by TNF-α stimulated MMP-1 secretion is regulated by inactivation of c-Src/EGFR-dependent ERK/AP-1 signal pathways [104].

As a cosmeceutical, skin (percutaneous, dermal) absorption of compound K is an important factor when applied topically. However, hydrophilic properties of glycosides due to the glycosyl group limit skin permeability which is disadvantageous for cosmetics purposes [105]. The aglycones are more hydrophobic and can effectively permeate the skin [106]. Therefore, enhancing biological activity of extracts by glycosides hydrolyzed into aglycones has attracted much attention [105]. Previous study has mentioned the antiallergic effects of compound K through mast cell via a membrane stabilizing activity [107]. Thus skin problems such as irritation and sensitization may be lower in compound K. A significant amount of research has been conducted to evaluate the pharmacological effects of compound K, to expand the scope of its potential applications further clinical studies will be required.

Summary. Medicinal use and safety of ginseng have been recognized for thousands of years with evidence suggesting the antiaging activities, such as wrinkle reduction and sun

protection of ginseng extract and ginsenosides. To express the pharmacological actions of ginsenosides, orally administered ginseng is biotransformed by intestinal microbiota into compound K. However as a dermatological agent, compound K is primarily used topically and due to the omission of intestinal absorption and biotransformation, strategies to enhance skin absorption are an important step. Moreover, the dermatological effect of compound K at the molecular level is poorly understood. Therefore, a better mechanistic understanding of compound K can lead to more effective delivery method. Also the safety of compound K when applied frequently onto skin still remains unclear. Further study to improve skin penetration and clinical tests for efficacy and safety of compound K is needed for its commercial use.

Conflicts of Interest

There are no conflicts of interest to declare by the authors.

Authors' Contributions

En Hyung Kim and Wonnam Kim designed and prepared the paper. All authors revised and approved this manuscript.

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Research Article

MUC1 and MUC5AC Acting on *Helicobacter pylori*-Related Deficiency and Solid Syndrome of Spleen and Stomach

Ling Hu,[1] **Wanqun Chen**,[2] **Ming Cheng**,[1] **Ting Zhang**,[1] **Shaoyang Lan**,[3] **Peiwu Li**,[3] **and Weijing Chen**[1]

[1]*Institute of Gastroenterology, Guangzhou University of Chinese Medicine, Guangzhou 510405, China*
[2]*Chongqing Hospital of Traditional Chinese Medicine, Chongqing 400037, China*
[3]*The First Affiliated Hospital, Guangzhou University of Chinese Medicine, Guangzhou 510405, China*

Correspondence should be addressed to Ling Hu; hl.cn@139.com

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To investigate the relationship of MUC1, MUC5AC, and the syndrome of spleen and stomach, 109 subjects (34 peptic ulcer (PU), 62 chronic gastritis (CG), and 13 healthy volunteers (CON)) were included. All the subjects included were surveyed with questionnaire to classify them into damp-heat syndrome of spleen and stomach (DHSS), spleen-qi deficiency syndrome (SQD), and CON, examined by gastric endoscope, and biopsied. Rapid urease and methylene blue staining (MBS) were performed on every subject to diagnose for *Helicobacter pylori* (Hp) infection, and both were defined as Hp-positive. Hematoxylin and eosin (HE) staining was performed on every specimen to explore the histomorphology, inflammatory degree, and inflammatory activity of different groups; then Elivision™ plus kit was used to test the expression of MUC1 and MUC5AC. All the results of digital images were reviewed by two experts blindly. The inflammatory degree with Hp infection was higher than those uninfected or CON, but no significant difference was found between DHSS and SQD. And the expressions of MUC5AC with positive Hp was higher than those with negative Hp or CON regardless of the deficiency and solid syndrome of spleen-stomach but not for MUC1. We speculate that the deficiency and solid syndrome of spleen-stomach is a condition like Tai Ji symbol of dynamic equilibrium, showing the higher expression of MUC5AC but no change of MUC1 in the circumstance of Hp infection.

1. Introduction

It is well established that *Helicobacter pylori* (Hp) is the main etiologic factor in a range of pathologies including chronic gastritis (CG), peptic ulcer (PU), and even gastric cancer (GC) [1, 2]. The theory that intestine GC is a multistep process starting with CG and progressing through atrophy, intestinal metaplasia (IM), and dysplasia triggered by Hp is well known [3]. Therefore, the multiple and complicated disruptions of organism caused by Hp have long been the research highlights.

Previous studies have shown that infection with Hp is able to induce a cascade of innate and adaptive immune response for the gastric mucosa [4, 5]; furthermore, triggered by Hp infection, the alteration of mucins (MUCs) in the gastric epithelium and their functions have been widely investigated [6–9]. In particular, MUC1 and MUC5AC are believed to be the most critical proteins for protection from Hp or in the process of carcinogenesis, which have previously been assumed. On one hand, based on the in vitro and mouse model research, studies have elucidated that Hp dwelling exerts the reduction of MUC1 expression due to the mucosal barrier injury [9–11], whereas, on the other hand, it was suggested that glycan-rich niche produced by mucins provides a preferential binding point for Hp [9]; notably, the abnormal expression of MUC1 was recognized as oncogene in the development of gastric carcinomas [12, 13]. In contrast, the expression of MUC5AC was proven to be reduced in the gastric endoscopic biopsy specimens with Hp infection [14], and the significant decrease was demonstrated to represent a marker of worse survival probability in GC [15]. In conclusion, as the critical mucin and the major receptor for Hp, the dual role of MUC1 and MUC5AC can be considered as powerful two-edged sword.

Table 1: Clinical parameters for all included individuals.

Group	Number	Gender		Diseases		Age (Y)
		M	FEM	PU	CG	$\overline{\chi} \pm S$
DHSS	68	40	28	32	36	39.84 ± 10.44
Hp(+)	39	19	20	16	23	40.62 ± 10.76
Hp(−)	29	21	8	6	23	38.79 ± 10.10
SQD	28	15	13	2	26	41.75 ± 9.24
Hp(+)	13	1	12	1	12	41.84 ± 8.60
Hp(−)	15	2	13	1	14	41.66 ± 10.06
CON	14	8	6	—	—	38.75 ± 8.64

Notes. There is no statistical difference for the gender and age among the groups of DHSS, SQD, and CON ($P > 0.05$). Hp(+): *Helicobacter pylori* (Hp) positive; Hp(−): Hp negative; SQD: spleen-qi deficiency syndrome; CON: control group; M: male; FEM; female; PU: peptic ulcer; CG: chronic gastritis; —: none.

On the basis of holism concept and syndrome differentiation, Chinese medicine (CM) has been paid more attention recently [16, 17]. And syndrome or Zheng differentiation is the critical step in clinic; thus our team has long been contributing to the research of solid and deficiency syndrome of spleen and stomach. The establishment of the animal model of damp-heat syndrome of spleen and stomach (DHSS) and the diagnostic standards of spleen-qi deficiency syndrome (SQD) laid a solid foundation for the research on the relationship of inflammatory cytokines and syndromes triggered by Hp [18–21]. Therefore, based on the previous studies, we hypothesize that, in the circumstance of Hp infection, MUC1 and MUC5AC may be involved in solid or deficiency syndrome of spleen and stomach.

2. Materials and Methods

The present study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou University of Chinese Medicine, and each individual gave signed informed consent.

2.1. Materials and Chemical Regents. The gastric endoscope was obtained from Olympus (Nagano, Japan), and the rapid urease was obtained from Kedi (Guangzhou, China). Monoclonal antibody of MUC1 and MUC5AC was purchased from ZSGB-BIO (Beijing, China), and the cytokine assay of Elivision plus kit was obtained from Maxin (Fujian, China). The microscope used in this study was Olympus (Nagano, Japan).

2.2. Subjects Selection. From March 2010 to March 2011, 109 pairs of gastric endoscopic biopsy specimens, including 34 PU, 62 CG, and 13 healthy volunteers, were collected from the First Affiliated Hospital of Guangzhou University of Chinese Medicine. The diagnostic of CG and PU was reference to the consensus of CG in China and diagnostic criteria of Lancet, respectively [22, 23]. The diagnostic standards of Hp infection were followed by the associated detection technique [24–26]. Moreover, by reference to the previous study of our team and the state administration of CM in 2002, the diagnostics of DHSS and SQD were established [21, 27]. Similarly, the inclusion and exclusion criteria were the same as our previous research [21, 28]. And the detailed information of all subjects included in this study is described in Table 1.

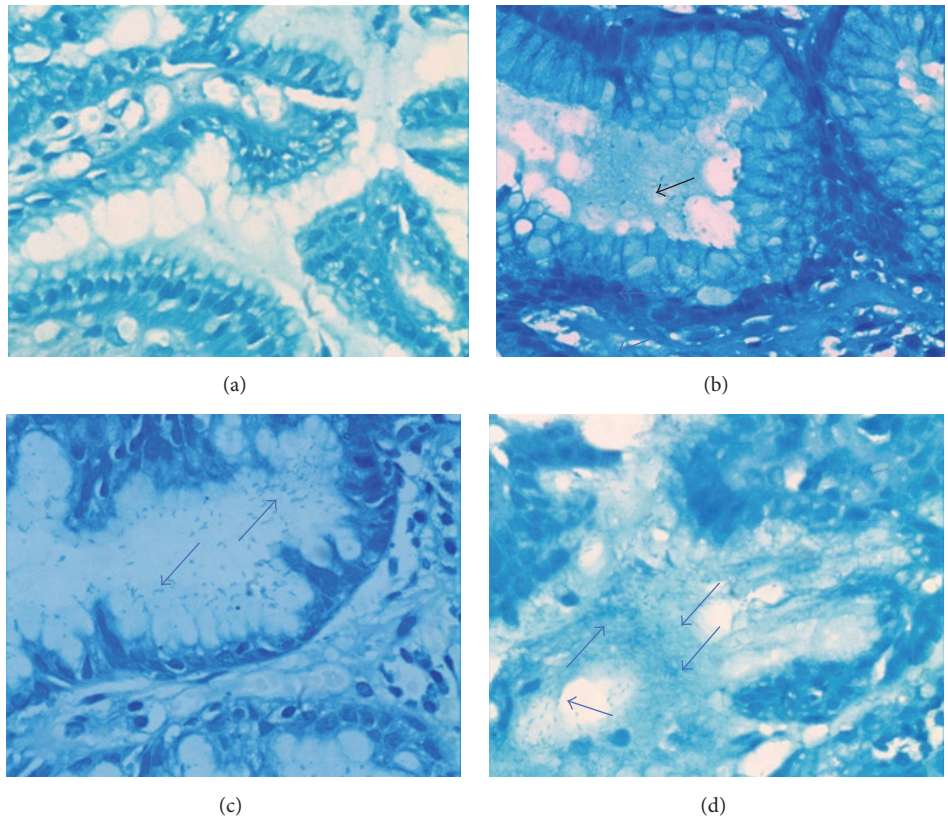
In order to evaluate the symptoms and sighs, all the subjects included in the present research were surveyed by two experts of our team in a scientific, objective, and professional way.

2.3. Sample Preparation. All the subjects included were asked to be examined by the gastric endoscopy, and two samples (a pair) were collected from each stomach antrum, namely, from the greater curvature and the opposite position, respectively. In order to make the initial diagnosis of Hp infection, one of the samples was tested with a rapid urease immediately; simultaneously, the other was fixed with formalin. Aiming to test Hp infection in a histological way, the specimens were paraffin-embedded and sectioned, and methylene blue staining (MBS) was performed. Only those with double positive results of a rapid urease and MBS were defined as Hp positive.

In order to observe the morphological characteristic of each specimen, hematoxylin and eosin (HE) staining was prepared routinely. In accordance with the consensus of 2006 [23, 29], two professional experts examine the degree of inflammation, inflammatory activity, and Hp infection condition as none, mild, moderate, and severe independently and blindly.

2.4. Mucins Protein Assay. Following the Elivision plus kit manufacturer's instruction, the expression of MUC1 and MUC5AC was detected by immunohistochemistry (IHC). As the same method as the HE staining observation, additionally referring to the diagnostic standards of IHC [30, 31], the expression of mucins protein was evaluated.

2.5. Statistical Analysis. By using SPSS software version 22.0 for Windows, a two-tailed $P < 0.05$ was defined as statistical significance in this study. And related data was expressed as the mean ± standard deviation; one-way ANOVA or t-test was applied. For the clinical parameters, Pearson's χ^2 test was conducted, and with quantitative variables, Chi-square test with 95% confidence intervals was screened.



(a) (b) (c) (d)

Figure 1: Methylene blue staining (MBS) of gastric mucosa (×400): (a) no *Helicobacter pylori* (Hp) infection; (b) mild infection: spiral-shaped or small blue rods (arrows) Hp are visible in the gastric pit or mucus layer (arrow); (c) moderate infection; (d) severe infection. The arrow refers to Hp infection in the gastric mucosa.

3. Results

3.1. The Clinical Parameters of the Subjects Included. As shown in Table 1, no statistical significance of different groups exists among gender, diseases, or age, which is the reasonable prerequisite of the following experiment.

3.2. Hp Infection of Gastric Mucosa with MBS. Aiming to increase the sensitivity and specificity for Hp infection test, the specimen was detected with rapid urease; additionally, MBS was performed routinely, and both were defined as positive Hp infection. As shown in Figure 1, under microscope, Hp is shown like curved or spiral bacillus in the epithelial surface, mucus layer, or gastric pits. According to the different amount of Hp dwelling, none, mild, moderate, and severe infections were screened.

3.3. Inflammatory Condition of Gastric Mucosa in Different Groups. With HE staining of specimens, histomorphology appearance of different groups is shown in Figures 2(b)–2(f). In conclusion, compared with CON and those with negative Hp, the subjects with Hp infection had more inflammatory cells infiltrating ($P < 0.05$), even with intestinal metaplasia (IM) or dysplasia, regardless of DHSS or SQD. However, no statistical difference was found between DHSS and SQD. By reference to the criteria of IHC [30], the inflammatory degree and activity were present in Figures 2(a) and 2(g).

3.4. Expression of MUC1 and MUC5AC. As shown in Figures 3 and 4, marked by Elivision plus kit, positive expression of MUC1 and MUC5AC was stained with yellow-brown color. And MUC1 was predominantly screened in gastric epithelium mucosa and gland cells, while MUC5AC was mainly detected in the crypt of gastric epithelial gland cells. There was statistical significance for the expression of MUC5AC between the group of DHSS with Hp infection and CON and also between the group of SQD Hp positive and CON ($P < 0.05$). In contrast, no significance existed for the expression of MUC1.

4. Discussion

Nowadays, based on the acknowledgement and communication between CM and Western medicine, some theory has been suggested. For example, Western medicine has been paid more attention to recognize the individual spiritual fulfillment and proposed diagnostic strategies as system-based diagnosis [17, 32]. On the other hand, for exploration of the spirit of CM, microcosmic point of view of CM has been investigated [33, 34]. Our team has long been contributing to the research on the essence of deficiency and solid syndrome

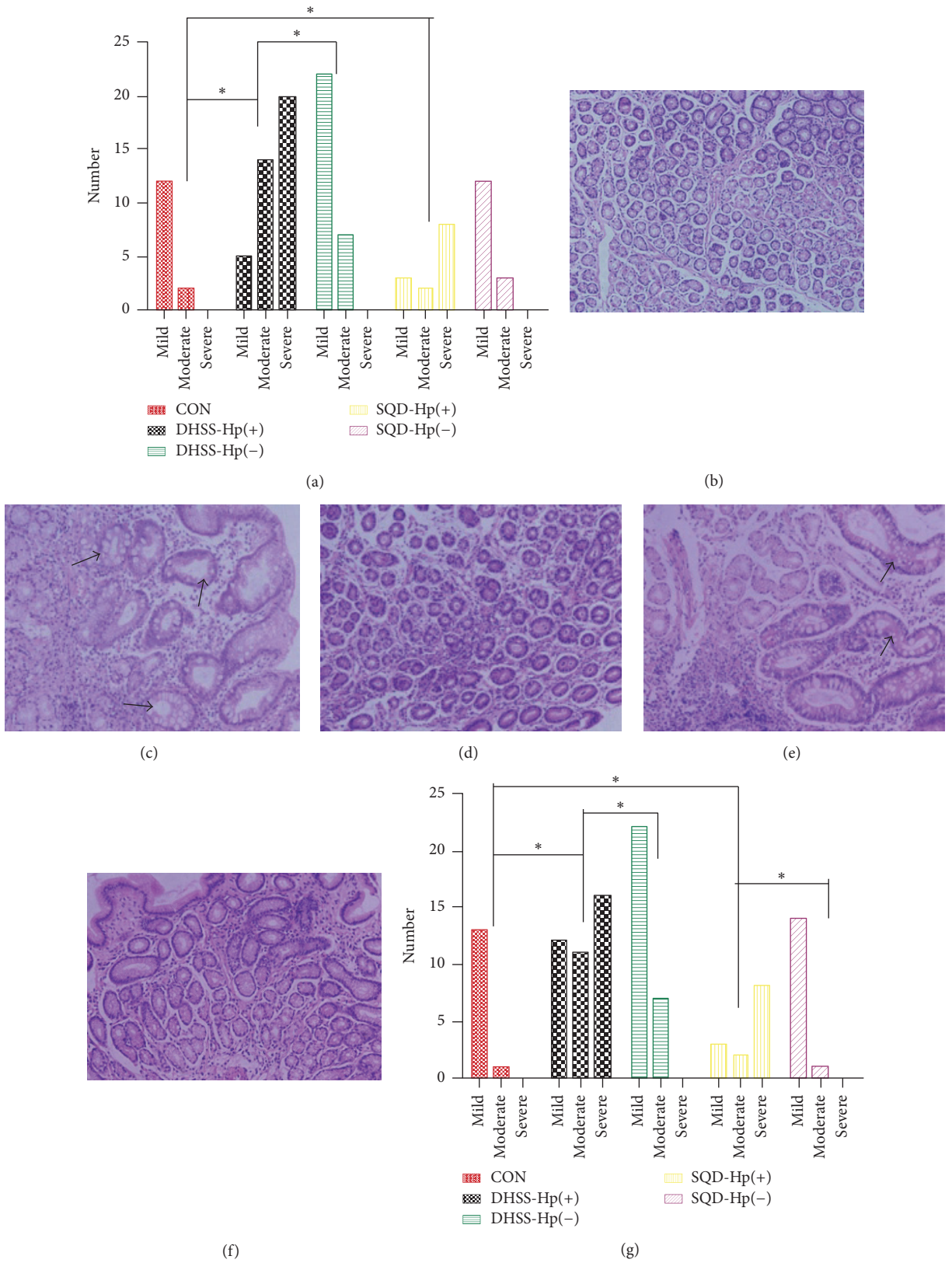
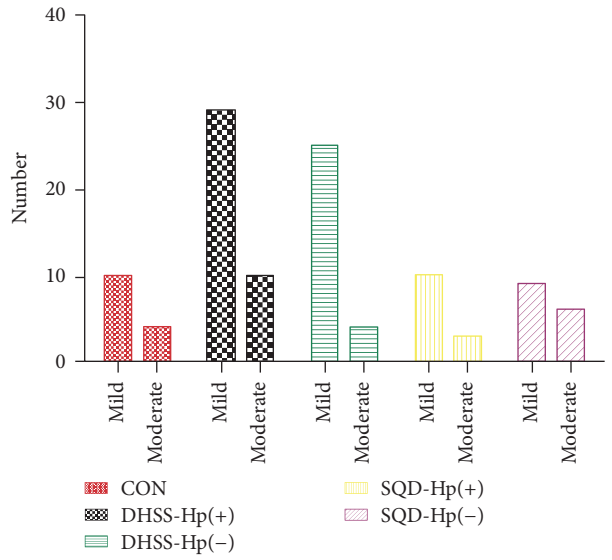
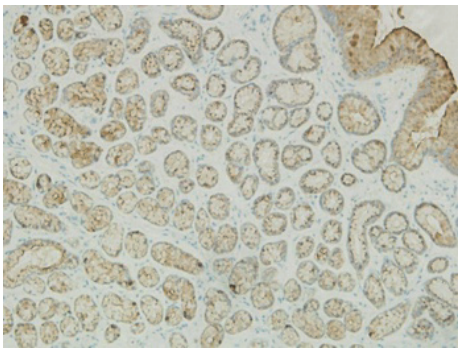


Figure 2: Histomorphology and inflammatory condition of gastric mucosa samples: (a) inflammatory degree of different groups, $^{*}P < 0.05$; (b) normal gastric mucosa of control group (CON): inflammatory cells are rare and gastric glands arrange in order (×100); (c) histomorphology of damp-heat syndrome of spleen and stomach (DHSS) with Hp infection: a few number of inflammatory cells infiltrate the gastric mucosa, with intestinal metaplasia visible (IM) (arrow) (×100); (d) histomorphology of DHSS without Hp infection: several inflammatory cells and IM or dysplasia present infrequently (×100); (e) histomorphology of spleen-qi deficiency syndrome (SQD) with Hp infection (×100); (f) histomorphology of SQD without Hp infection (×100); (g) the inflammatory activity of different groups, $^{*}P < 0.05$. The arrow refers to Hp infection in the gastric mucosa.



(a)



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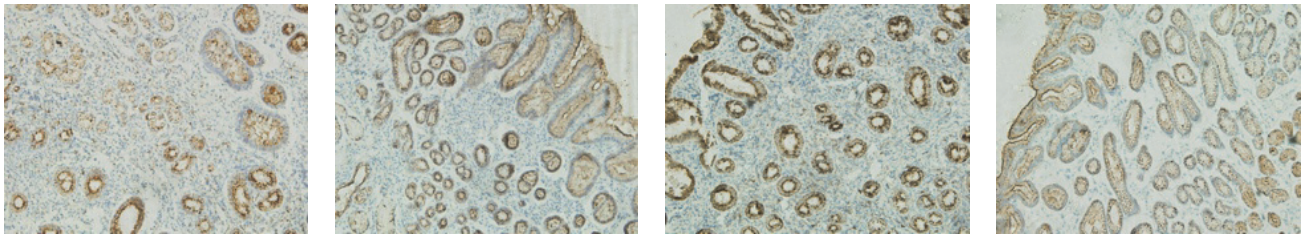


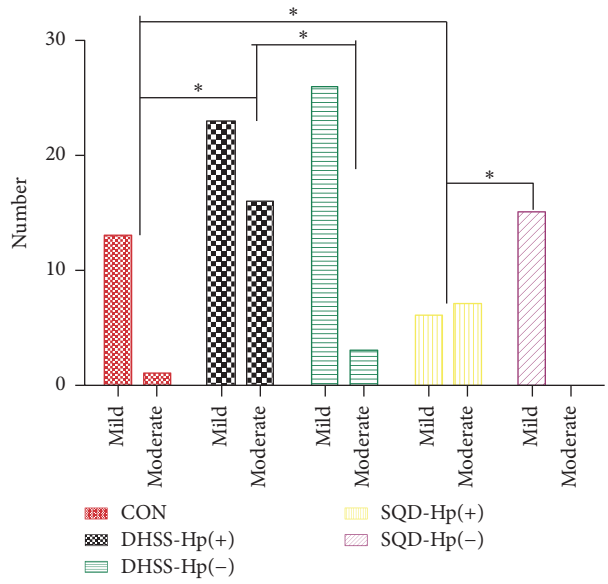
(c) (d) (e) (f)

Figure 3: Expression of MUC1: (a) expression of MUC1 in different groups: no statistical difference was observed; (b) expression of MUC1 in control group (CON) (×100); (c) expression of MUC1 in damp-heat syndrome of spleen and stomach (DHSS) with Hp infection (×100); (d) expression of MUC1 in DHSS with Hp negative (×100); (e) expression of MUC1 in spleen-qi deficiency syndrome (SQD) with Hp infection (×100); (f) expression of MUC1 in SQD with Hp negative (×100).

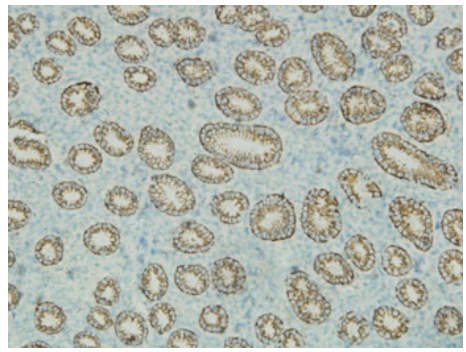
of spleen and stomach; previous studies on the elucidation of the theory of DHSS and SQD have laid solid foundation for this research [28, 35].

As we all know, Hp has been considered as I carcinogen for human beings [36], and continuous infection can cause CG, PU, IM, dysplasia, and even gastric malignancy [3]. In the point view of CM, Hp invasion and colonization in stomach are the representative of evil-qi, while the protection barrier for Hp (gastric epithelium) is the delegate of healthy-qi, whereas mucins (MUCs) are the critical polyprotein components of the gastric epithelium. Consequently, we investigated the relationship of MUCs and the deficiency and solid syndrome of spleen and stomach triggered by Hp infection in order to imply the syndrome spirit of CM.

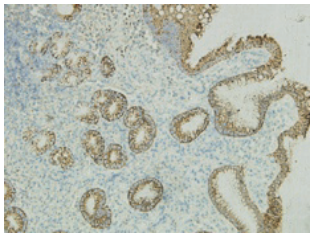
The human MUC consists of secreted mucins and transmembrane ones, in which MUC1 is transmembrane glycoprotein involved in the signal of epithelial-mesenchymal transition (EMT), while MUC5AC is secreted protein [13], recognized as the major receptor for Hp in the human stomach [37]. There is evidence that MUC1 is a physical barrier to protect gastric mucosa from Hp dwelling in murine infection model and in vitro experiment; in turn, by injuring the



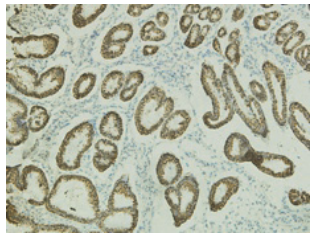
(a)



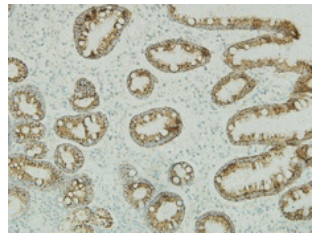
(b)



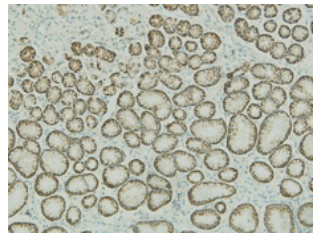
(c)



(d)



(e)



(f)

Figure 4: Expression of MUC5AC: (a) expression of MUC5AC in different groups, $^{*}P < 0.05$; (b) expression of MUC5AC in control group (CON) (×100); (c) expression of MUC5AC in damp-heat syndrome of spleen and stomach (DHSS) with Hp infection (×100); (d) expression of MUC5AC in DHSS with Hp negative (×100); (e) expression of MUC5AC in spleen-qi deficiency syndrome (SQD) with Hp infection (×100); (f) expression of MUC5AC in SQD with Hp negative (×100).

physical barrier, Hp infection would decrease the expression of MUC1 [9–11]. However, for human gastric tissues, studies have shown that no significant association exists between the expression of MUC1 and Hp infection, which is in accordance with our results in this study, whereas it was overexpressed in the dysplasia and adenocarcinoma tissue, showing its carcinogen characteristics [12, 38].

The deficiency and solid syndrome of spleen and stomach is the dynamic procession of transportation and transformation for spleen with the whole body. And the deficiency and solid syndrome is just another form of Yin and Yang like Tai Ji symbol, both of which grasp each other but are also present within each other. Namely, the two sides alter as ecologic succession to sustain dynamic equilibrium, and change of some critical element may disturb the homeostasis. Although, compared with CON, the expression of MUC1 was not different, we still cannot conclude that MUC1 has no association with SQD and DHSS, because Hp inhabitant in gastric epithelium is like wind invading the skin barrier; of course, there is no change for the skin in a modern point of

view, but it is considered to be due to insecurity of the interstices in CM.

In addition, previous study has shown that only small portion of subjects infected with Hp will develop into malignancy, and the abnormal expression of MUC1 acts like oncogene [12, 39]. Therefore, we cannot deny that MUC1 sustaining may be the related element of dynamic equilibrium regardless of the deficiency and solid syndrome.

On the other hand, for the expression of MUC5AC, results show some difference and even contradiction in different teams. By detection of the expression of endoscopic biopsy with IHC, Kocer et al. [14], have demonstrated that MUC5AC was decreased in patients with Hp positive, with its localization in the superficial epithelium, upper parts of gastric glands, and dysplastic areas but not in IM. In contrast, with the same method, Park et al. [40], elucidated that MUC5AC was overexpressed in IM of young adults compared to normal and Hp-infected gastric mucosa of children. In consistence with our study, as Figure 4 shows, the extent of MUC5AC expression with Hp infection was more than that with no infection. We speculated that it might result from the fact that the specimens with Hp positive included in our study showed more IM and dysplasia compared with CON and those with no Hp infection (Figure 2).

In the circumstance of Hp long-time inhabitant in gastric epithelium, to protect the gastric epithelium from deeper damage, MUCs (mainly MUC5AC and MUC1) serve as healthy-qi resistant to Hp. From the holism perspective, the individual syndrome can demonstrate the two sides: deficiency (SQD) and solid (DHSS), but it is another relative homeostasis for gastric epithelium to get dynamic equilibrium showing higher expression of MUC5AC and no change of MUC1. We speculate that it may be the evidence that stomach-qi was still strong even though subjects showed SQD until the equilibrium was broken when showing abnormal expression of MUC1. Consequently, the deficiency and solid syndrome is just relative condition like Tai Ji symbol.

However, with Hp positive, no statistical significance was found between the groups of DHSS and SQD ($P > 0.05$); we still cannot conclude that MUC5AC gets no correlation with the difference of deficiency and solid syndrome of spleen and stomach because of the limited subjects. Thus, in the following, the extension of included samples is the critical approach. Furthermore, investigation of the single nucleotide polymorphisms (SNPs) for the genotype of MUC1 and MUC5AC may be another method to imply the essence of deficiency and solid syndrome of spleen and stomach.

Disclosure

Ling Hu and Wanqun Chen are co-first authors.

Conflicts of Interest

The authors declare that there are no conflicts of interest with respect to the authorship and/or publication of this paper.

Authors' Contributions

Ling Hu and Wanqun Chen contributed equally to this work. Ling Hu conceived and supervised the study; Ming Cheng and Ting Zhang performed experiments; Shaoyang Lan, Peiwu Li, and Weijing Chen cooperated the experiments; Wanqun Chen analyzed data; Ling Hu and Wanqun Chen wrote and revised the manuscript.

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