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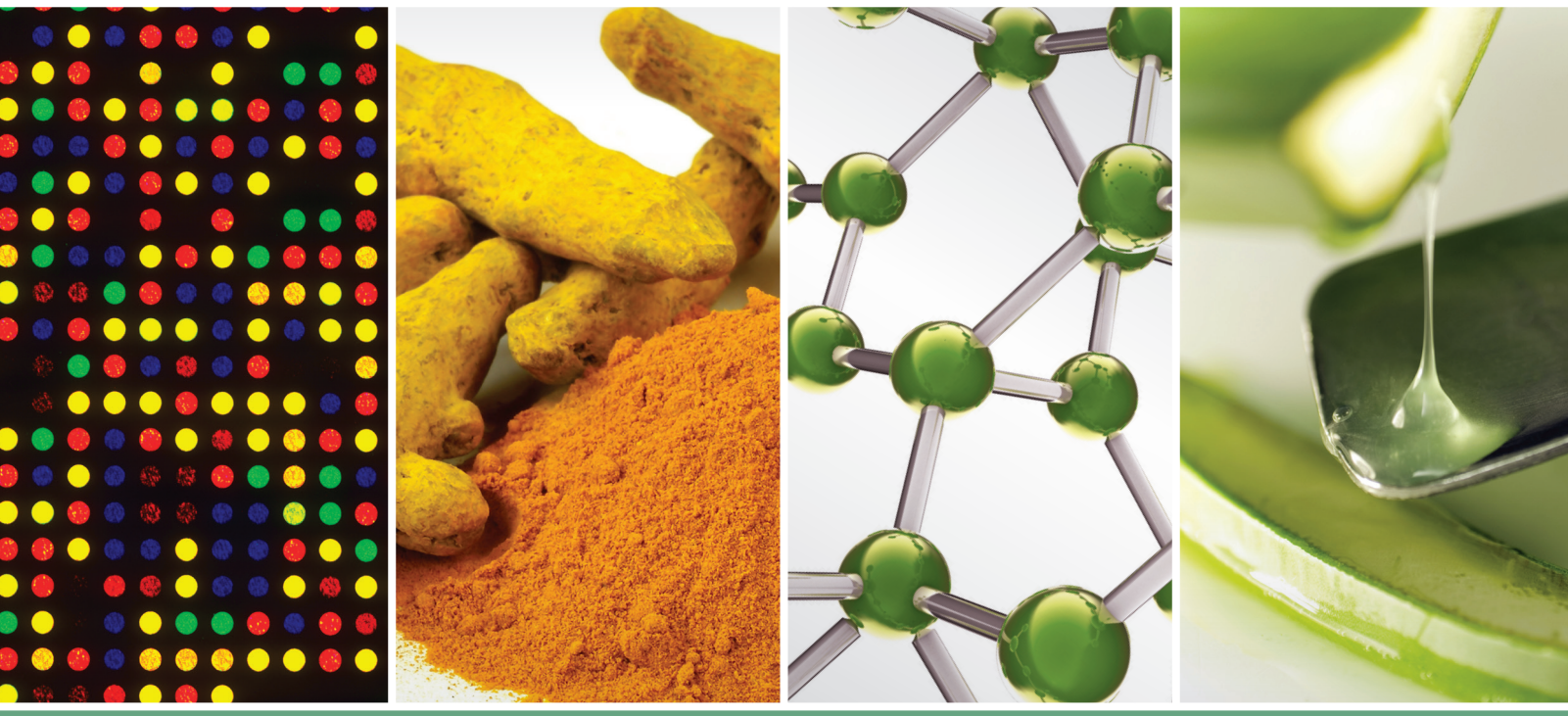
Efficacy of Herbal Drugs in Human Diseases and Disorders

Guest Editors: Suaib Luqman, Syed Ibrahim Rizvi, André-Michael Beer, Sunil Kumar Khare, and Pinar Atukeren



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Editorial Board

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Editorial

Efficacy of Herbal Drugs in Human Diseases and Disorders

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According to a ballpark figure by World Health Organization, more than three-quarters of population of the developing nations exploit herbal medicine for their primary health care. Herbal drugs and their constituents have advantageous effects on long-term fitness and can be used to efficiently treat human diseases or disorders. In veracity, herbs frequently include various active ingredients that imaginably have drug allying commotion in the body. Bringing into play, herbs are often more affordable than procuring expensive contemporary pharmaceuticals to take care of ailments. A large number of herbs have proved their usefulness in managing various illnesses. Latest advances in biology and medicine have introduced new technologies to study the biological significance of herbal drugs in various human diseases and disorders. Hence it is important to understand the mechanism(s) of herbal drug action for the knowledge and development of successful therapies.

The current special issue brings together several exciting papers defining the efficacy of herbal drugs in an assortment of human diseases and disorders.

J. Li et al. have tested the effects and mechanism of Bufei Yishen formula (BYF) in a rat chronic obstructive pulmonary disease (COPD) model and provided the basis for mechanisms of BYF on COPD and new therapeutic drug targets.

X. Yu et al. have reported the treatment of Si-Jun-Zi decoction, a famous Chinese medicine that promotes the restoration of intestinal function after obstruction by regulating intestinal homeostasis.

Tong xinluo (TXL), a Chinese herbal compound, has been used in China with traditional therapeutic efficacy in patients with diabetic nephropathy (DN). N. Zhang et al. demonstrated that TXL successfully inhibits TGF-β1-induced epithelial-to-mesenchymal transition in DN, which may account for the therapeutic efficacy in TXL-mediated renal protection.

W. Zhang et al. explored the effects of Jian-Pi-Zhi-Dong decoction (JPZDD) on Tourette syndrome (TS) by investigating the expression of gamma-aminobutyric acid (GABA) and its type A receptor (GABAAR) in the striatum of a TS mice model induced by 3,3′-iminodipropionitrile (IDPN) treatment. It was revealed that JPZDD alleviates TS symptoms which may be associated with GABAAR expression downregulation in striatum thereby controlling GABA metabolism.

TZQ-F has been traditionally used as a traditional Chinese medicine formula for the treatment of diabetes. H. Yuhong et al. compared the pharmacologic effects and gastrointestinal adverse events between TZQ-F and acarbose in Chinese healthy volunteers.

T. Numata et al. reported the treatment of posttraumatic stress disorder (PTSD) using the traditional Japanese herbal medicine Saikokeishikankyoto in survivors of the great east Japan earthquake and tsunami. The traditional medicine may

be a valid choice for the treatment of psychological and physical symptoms in PTSD patients.

Y. Ewnetu et al. evaluated the synergetic antimicrobial effect of Ethiopian honey and ginger powder extract mixtures on standard and resistant clinical isolates of *Staphylococcus aureus*, *Escherichia coli,* and *Klebsiella pneumoniae*. The combination has the potential to serve as cheap source of antibacterial agents especially for the drug resistant strains.

Herbs are now gaining much attention as the main source of effective drugs for lowering serum lipids and lipid peroxidation. M. Rafieian-kopaei et al. assessed the impact of *Ferulago angulata* on serum lipid profiles and on levels of lipid peroxidation in male Wistar rats. Administration of a hydroalcoholic extract reduces the serum levels of total cholesterol, triglycerides, and LDL comparable to atorvastatin (a standard lipid lowering drug).

A-J. Lee et al. investigated the absolute counts and percentages of peripheral blood (PB) lymphocyte subtypes in end-stage cancer patients before and after localized radiotherapy (RT) and after oral administration of Bojungikki-tang water extract (BJITE) and to evaluate the changes mediated by RT and BJITE. It was observed that immune deterioration occurs after RT and administration of BJITE was found effective in its restoration.

T. Shimizu provides a good overview of efficacy of Kampo medicine (KM) in treating atopic dermatitis (AD). KM is a traditional herbal medicine in Japan and has a long history and plays an important role in the prevention and treatment of various diseases including AD.

Y. Ma et al. explored the efficacy of herb-partitioned moxibustion in patients with diarrhoea-predominant irritable bowel syndrome (DPIBS) and suggest it as a promising, efficacious, and well-tolerated treatment for patients.

Melinjo (*Gnetum gnemon* L.) seed extract containing transresveratrol (3,5,4′-trihydroxy-trans-stilbene) and other derivatives exerts diverse beneficial effects. H. Konno et al. reported melinjo seed extract decreases serum uric acid levels in nonobese Japanese males regardless of insulin resistance and may improve lipid metabolism by increasing HDL cholesterol.

We anticipate that readers and scientific fraternity working in the area of *herbal drugs* will find not only the updated review on the subject but also a compilation of precise data on the efficacy of herbal drugs in some lifestyle diseases and disorders with their suggested mechanism of action.

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Research Article

Effects and Mechanism of Bufei Yishen Formula in a Rat Chronic Obstructive Pulmonary Disease Model

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Bufei Yishen Formula (BYF) has been used for centuries in Asia to effectively treat patients with chronic obstructive pulmonary disease (COPD). This study established a COPD animal model in rats, wherein three groups (control, COPD, and BYF) were used to evaluate the mechanism(s) and curative effect of BYF. Pulmonary function and histomorphology demonstrated that BYF had an evident effect on COPD. Gene microarray was then exploited to analyze the effects of BYF on COPD. ClueGO analysis of differentially expressed genes indicated that BYF improved COPD by regulating expression of interleukins, myosin filament assembly components, and mitochondrial electron transport-related molecules. Moreover, ELISA revealed that expression of several interleukins (*IL1β*, *IL6*, *IL8*, and*IL10*) was reduced in peripheral blood and bronchoalveolar lavage fluid by BYF treatment. It was concluded that BYF has therapeutic effects on COPD in rats through its effects on interleukin expression and/or secretion. Furthermore, pharmacological or targeted expression of two differentially expressed genes, *F2R* and *Sprik1*, might be useful in novel COPD therapies. This study provides the basis for mechanisms of BYF on COPD and new therapeutic drug targets.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a slowly progressive, poorly reversible disease characterized by an abnormal inflammatory response in the lung [1]. The greatest risk factor in COPD development is cigarette smoking [1]. Other risk factors include air pollutants, dust, and inherent susceptibility [1]. The incidence of COPD in general populations is increasing, along with its great burden on public health [2, 3]. Although some glucocorticoids and bronchodilators can alleviate acute onset of COPD, significant side effects exist [4]. For example, β2 agonists can induce muscle tremor, tachycardia, sweats, and agitation [5]; theophylline can induce headache, nausea, vomiting, arrhythmias, and seizures [6]; and glucocorticoids can induce osteoporosis [7]. Therefore, the development of new longer lasting, targeted therapeutic strategies is a matter of great urgency.

At present, clinical trials and experimental studies have shown that certain Chinese medicines can effectively treat COPD, by improving pulmonary function, respiratory muscle fatigue, immunity, and lung blood flow [8–10]. Deng [11] found one Chinese herb formula that could improve COPD pathological presentation in a rat model, including inflammatory reactions and airway and pulmonary vasculature plasticity. Moreover, compared to western medicine, many Chinese herbs have few side effects [12]. However, the complexity and various actions of herbal components have limited their application and hindered study of their underlying mechanisms. With the development of high-throughput molecular techniques such as gene microarray, it has become possible to observe the effects of Chinese herbs on genomic expression and seek for their molecular targets.

As a traditional Chinese medicine formula, Bufei Yishen Formula (BYF) has been used for centuries in East Asia

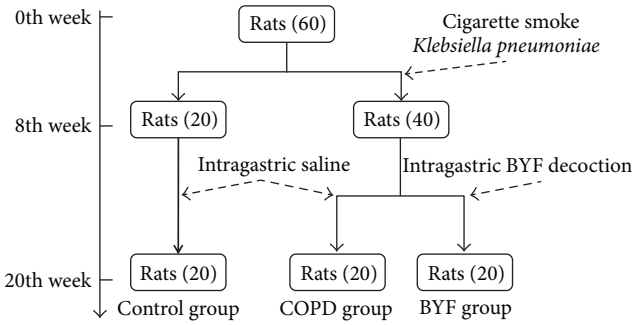

Figure 1: Establishment of COPD animal model.

to effectively treat patients with COPD. BYF principally employs the use of the herb Huang Qi (*Radix Astragali*) in its herbal formula. Multicentered clinical studies suggest that herbal formulae with Huang Qi were effective for stable COPD [13–18]. However, the quality of these studies has not been evaluated systematically, and no studies have yet explored the molecular mechanism of this particular herbal formula. Our previous studies have shown that BYF has more obvious beneficial effects on clinical symptoms of stable COPD than western medicine. Moreover, apparent positive long-term effects were observed for BYF treatment [19–21]. In this study, a modified COPD rat model was established as described previously by Chen et al. [22] for evaluating the effect(s) of BYF. Three experimental groups (COPD, BYF, and controls) were used to compare pulmonary function and pathology. To investigate the molecular mechanistic effect of BYF on COPD, differentially expressed genes were screened by microarray, and gene function enrichment analyses were conducted.

2. Methods

2.1. BYF Preparation and Animal Model Establishment. BYF was prepared at the First Affiliated Hospital of Henan University of Traditional Chinese Medicine (Zhengzhou, Henan, China). BYF components were comprised of 15 g Huang Qi (*Radix Astragali*), 15 g Ren Shen (*Radix Ginseng*), 15 g Shanzhuyu (*Fructus Corni*), and 9 g Wuweizi (*Fructus Schisandrae*). All herbs were decocted with water, steam sterilized, and brought to a final concentration of 0.6 g/mL. The BYF was supported by [9] and Chinese patent (number 2011101175781).

Experimental protocols were approved by the Experimental Animal Care and Ethics Committees of the First Affiliated Hospital, Henan University of Traditional Chinese Medicine. Two-month-old Sprague-Dawley rats were purchased from Henan Experimental Animal Center (Henan XK2005-0001) with a body weight of 200 ± 20 g. They were maintained on a 12-hour dark/light cycle with ambient temperature of 25 ± 1°C and relative humidity 50 ± 10%, as well as sufficient food (sterile rat chow) and water (sterile). All rats were anesthetized and sacrificed under the experimental protocols mentioned above and all efforts were made to minimize suffering.

Sixty Sprague-Dawley rats were randomized and divided into three experimental groups (control, COPD, and BYF) (Figure 1) with equal numbers of males and females in each group. Forty rats underwent intranasal instillation with *Klebsiella pneumoniae* once every 5 days lasting for 8 weeks. Rats were placed in a 300 L smoke box for 30 min with 3 h intervals between smoke treatments, wherein eight cigarettes were burned twice daily in the first two weeks and 15 cigarettes were burned three times daily during weeks 3–8. After 8 weeks of treatment, the 40 COPD-induced rats were randomly divided into two groups: COPD and BYF. The COPD group was administered 2 mL intragastric saline vehicle (0.9%) twice daily during weeks 9–20, and the BYF group was administered 4.44 g/kg/d BYF twice daily during weeks 9–20. The remaining 20 rats were used as a control group with no treatment for the first 8 weeks followed by 2 mL intragastric saline vehicle (0.9%) during weeks 9–20. All rats were weighed weekly to determine dosing and underwent pathologic examination by lung tissue excision (6 samples/group) during the 21st week.

2.2. Preparation and Determination of Pulmonary Function and Pathology. Tidal volume (TV), peak expiratory flow (PEF), and 50% tidal volume expiratory flow (EF50) were detected by unrestrained pulmonary function testing plethysmographs (Buxco Inc., Wilmington, NC, USA) conducted every fourth week from weeks 0 to 20. Paraffin-embedded sections of lung tissue were stained with hematoxylin and eosin and images were taken by light microscope (Olympus, Tokyo, Japan).

2.3. Interleukin Detection in BALF and Peripheral Blood. Following repeated saline bronchoalveolar lavage (3 mL/lavage), collected BALF underwent 10 min centrifugation (2000 r/min) and the supernatants were used to detect *IL8* and *10* by ELISA (BOSTER Inc., Wuhan, China). All processes were kept at 4°C. Peripheral blood was collected from rat *aorta abdominalis,* and serum was used to detect *IL1β, IL6, IL8*, and *IL10* by ELISA (BOSTER Inc.).

2.4. RNA Extraction. Six lung tissue samples were excised from each of the three experimental groups for microarray analysis. Total RNA was isolated from skeletal muscle by Trizol reagent (Invitrogen, Breda, Netherlands) and purified using a Qiagen RNeasy Micro kit (Qiagen, Venlo, Netherlands). RNA quality was verified by Agilent 2100 bioanalyzer (Agilent Technologies, Amsterdam, Netherlands).

2.5. Microarray Processing. Purified RNA samples (2 μg ea) were PCR amplified and labeled using an Agilent Quick Amp kit (Agilent Technologies, Santa Clara, CA, USA) and hybridized with Agilent Whole Rat Genome Oligo Microarray (4 × 44 K) in Agilent's SureHyb Hybridization Chambers. After hybridization and washing, processed slides were scanned by an Agilent DNA microarray scanner (part number G2505B) using manufacturer recommended settings.

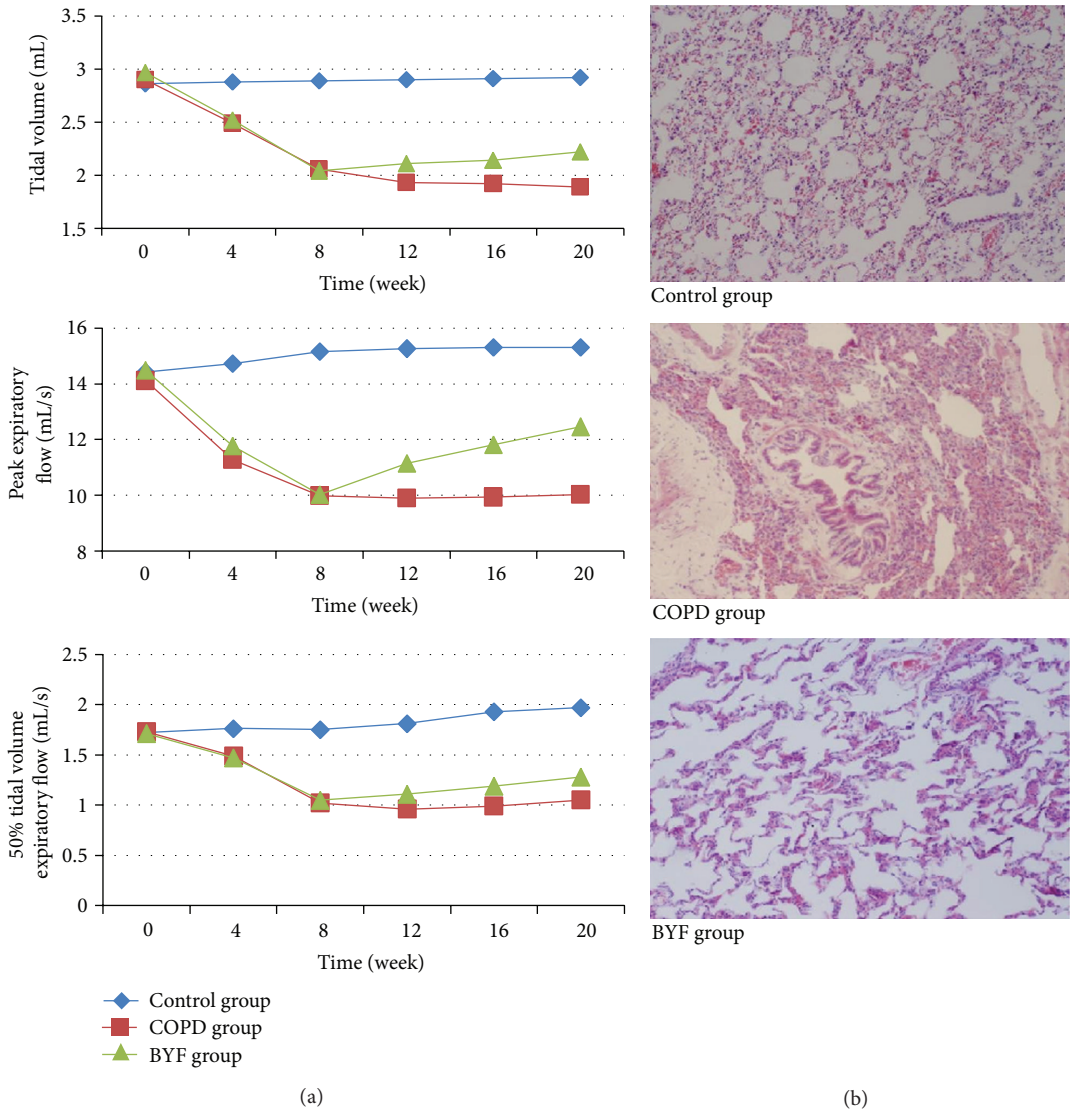

Figure 2: Pulmonary function and pathology in each experimental rat group. (a) TV, PEF, and EF50. (b) Lung histomorphology observations by light microscope. Control group (magnification ×100): pulmonary tissue was smooth and there was no inflammation and cell infiltration, no hyperemia, or no swelling. COPD group (magnification ×100): pulmonary tissue showed upregulation of a severe inflammatory response with visible increases in lymphocytes, monocytes, and neutrophils. Bronchial and pulmonary wall thickness, degree of bronchial stenosis, and alveolar diameter were significantly higher. BYF group (magnification ×100): inflammation around trachea lessened after BYF therapy. The number of inflammatory cells decreased dramatically.

Table 1: TV, PEF, and EF50 in three experimental rat groups ($\overline{x} \pm S$).

Index	Group	0 weeks	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks
TV (mL)	Control	2.86 ± 0.46	2.88 ± 0.33	2.89 ± 0.34	2.90 ± 0.32	2.91 ± 0.36	2.92 ± 0.24
	COPD	2.90 ± 0.38	2.49 ± 0.24	2.06 ± 0.22	1.93 ± 0.22	1.92 ± 0.30	1.89 ± 0.36
	BYF	2.97 ± 0.46	2.52 ± 0.21	2.04 ± 0.33	2.11 ± 0.18	2.14 ± 0.25	2.22 ± 0.36
PEF (mL/s)	Control	14.43 ± 2.69	14.72 ± 2.48	15.16 ± 1.99	15.25 ± 2.02	15.31 ± 1.75	15.30 ± 2.24
	COPD	14.11 ± 2.47	11.28 ± 2.00	9.99 ± 2.33	9.90 ± 1.96	9.93 ± 2.40	10.02 ± 2.18
	BYF	14.49 ± 2.53	11.78 ± 1.96	10.03 ± 2.48	11.15 ± 2.51	11.81 ± 2.61	12.35 ± 2.84
EF50 (mL/s)	Control	1.72 ± 0.16	1.76 ± 0.12	1.75 ± 0.15	1.81 ± 0.17	1.93 ± 0.28	1.97 ± 0.21
	COPD	1.73 ± 0.11	1.49 ± 0.13	1.02 ± 0.17	0.96 ± 0.20	0.99 ± 0.17	1.05 ± 0.17
	BYF	1.71 ± 0.12	1.47 ± 0.20	1.05 ± 0.20	1.11 ± 0.22	1.17 ± 0.28	1.24 ± 0.28

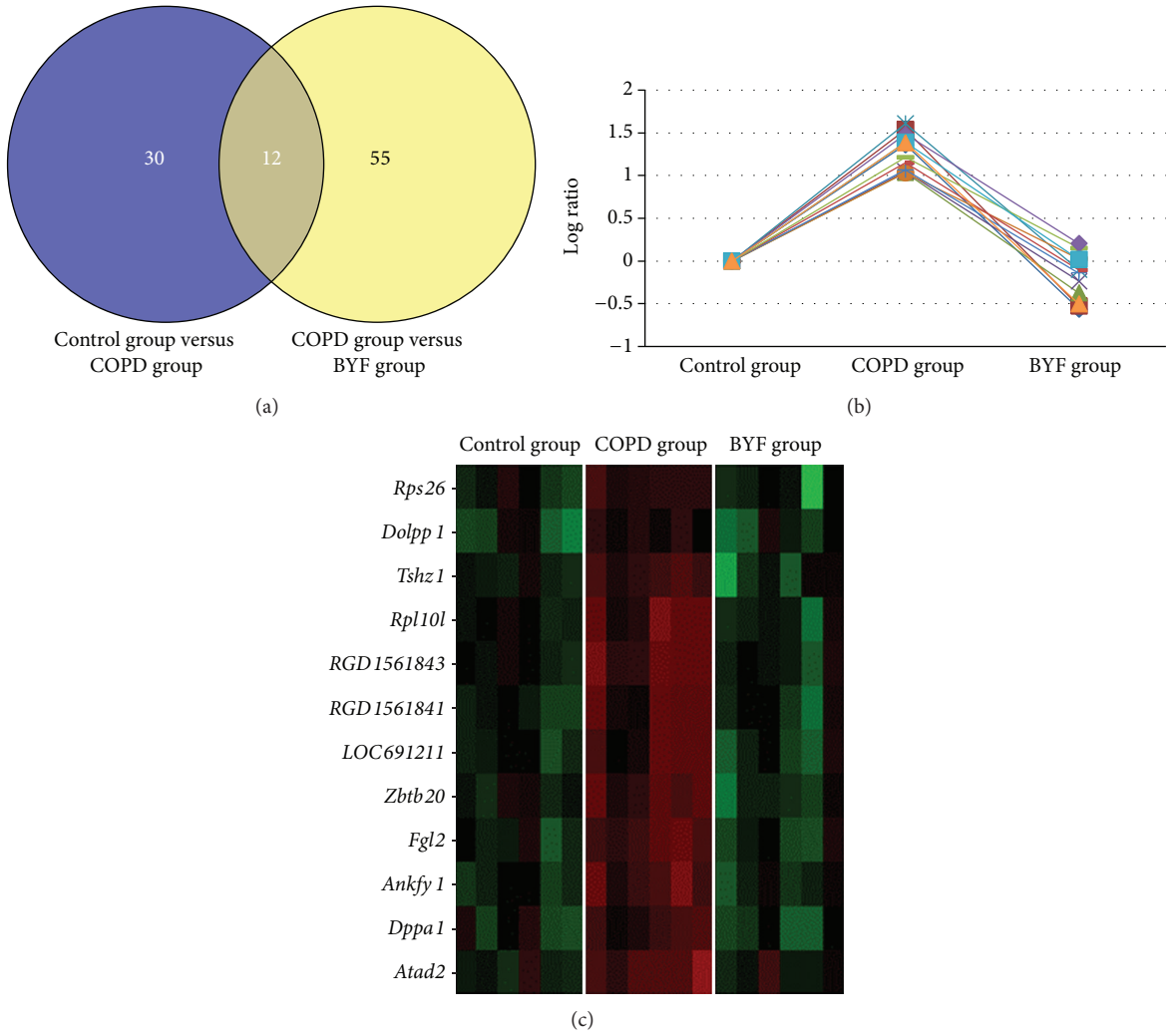

Figure 3: Venny plot of DEGs between each experimental group and expression of 12 overlapping genes. (a) Venny plot of two DEG lists (COPD versus controls and COPD versus BYF). (b) Expression of 12 overlapping genes from all three rat groups. (b) Gene expression heat map of 12 overlapping genes.

2.6. Data Preprocessing. Raw image data were converted to CEL and pivot files using Agilent Feature Extraction Software version 10.5.1.1. All downstream microarray analyses were performed using Agilent GeneSpring GX software version 11.0. Microarray datasets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization); log 2-transformed data were normalized by quantile normalization and used for comparisons. Differentially expressed genes were identified through fold change ($|\log \text{ratio}| > 1$) and Student's t-test screening ($P < 0.05$).

ClueGO software [24] was used for gene function enrichment analysis. We incorporated both gene ontology (GO) and the KEGG pathway in ClueGO analysis; GO term fusion and restriction with $P < 0.05$ were chosen, which integrates GO categories and creates a functionally organized GO category network based on overlap between different GO categories and significance. Interleukin expression differences between groups were compared by Student's t-test ($P < 0.01$).

3. Results

3.1. Pulmonary Function and Pathology Improvements in COPD Rats with BYF Treatment. After 20 weeks, control group rats were active and restless, with smooth and burnished fur. Their body mass increased gradually and respiration remained stable. Before BYF treatment, rats in COPD and BYF groups exhibited "spiritual malaise," which is characterized by appetite suppression and wriggling with gathered fur in the first 8 weeks. The body mass of these rats slowly increased with short respiration accompanied by frequent cough. These symptoms in BYF group rats were significantly alleviated following BYF treatment from weeks 9 to 20.

Pulmonary function for all three experimental groups was detected via TV, PEF, and EF50 every four weeks for 20 weeks. TV, PEF, and EF50 were found to be stable in the control group but were dramatically declined in the first eight

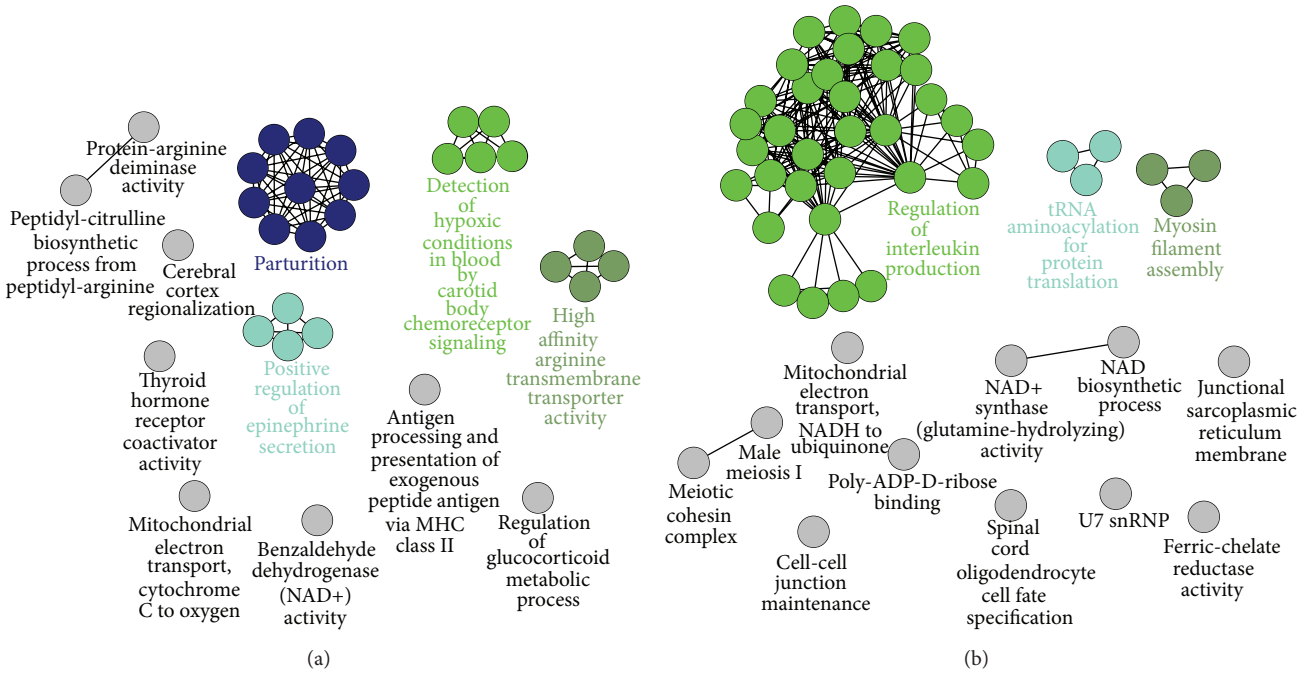

Figure 4: ClueGO analysis for DEGs. (a) ClueGO analysis for DEGs between COPD and control groups. (b) ClueGO analysis for DEGs between COPD and BYF groups.

weeks in the COPD and BYF groups ($P < 0.05$). Following BYF treatment, TV, PEF, and EF50 in the BYF group were significantly improved (all $P < 0.05$; Table 1; Figure 2(a)) compared to rats of COPD group.

Lung histomorphology images from each of the three experimental groups are shown in Figure 2(b). Compared to controls, COPD rats showed upregulation of a severe inflammatory response with visible increases in lymphocytes, monocytes, and neutrophils. Bronchial and pulmonary wall thickness, degree of bronchial stenosis, and alveolar diameter were significantly higher in the COPD group, while the alveolar count per unit area was significantly lower compared to control rats. These COPD-related phenomena were dramatically relieved with BYF treatment. Furthermore, BYF treatment significantly alleviated the inflammatory response, as shown by a significant decrease in the number of inflammatory cells present in lung tissues.

3.2. Gene Microarray Data Analysis. To investigate the molecular mechanism of BYF on COPD pathogenesis, we randomly chose eighteen samples from the three experimental groups (six samples from each experimental group) for gene expression experiments. Using $|\log \text{ratio}| > 1$ and $P < 0.05$, 42, and 67, differentially expressed genes (DEGs) were detected between control and COPD groups as well as COPD and BYF groups (Figure 3(a); see Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/381976), respectively. Twelve genes were overlapped between the two DEG lists (control versus COPD and COPD versus BYF). As shown in Figures 3(b) and 3(c), all 12 overlapped genes were increased in COPD rats and then decreased in the BYF group. GO enrichment analysis by Bingo software [23] showed that these 12 overlapping genes were involved in ribosomal structure/function, gene expression, translation, and negative regulation of RNA splicing (Table 2). In particular, four overlapped genes (*RPL10L, RPS26, RGD1561843,* and *RGD1561841*) are located in the ribosome. This indicates that BYF treatment may reduce mRNA translation and translational editing in COPD.

3.3. ClueGO Analysis of Differentially Expressed Genes. To facilitate understanding of the biological implications of DEGs, functional enrichments were performed by ClueGO [24], which incorporates gene ontology and KEGG pathway annotation. ClueGO integrates GO categories and creates functionally organized GO category networks based on overlap between different GO categories and statistical significance. In line with previous studies of COPD and anoxic and oxidative stress [25], DEGs detected between our COPD and control groups were largely implicated in detection of hypoxic conditions in blood by carotid body chemoreceptor signaling, mitochondrial electron transport, cytochrome C to oxygen, and benzaldehyde dehydrogenase (NAD+) activity (Figure 4(a)). An especially interesting finding of this analysis was the significant enrichment for terms involved in parturition.

Significant GO and DEG pathway terms between COPD and BYF groups are presented in Figure 4(b). These DEGs were mainly enriched in terms involved in regulation of interleukin production, mitochondrial electron transport, NADH to ubiquinone, NAD+ synthase activity, NAD biosynthesis, poly-ADP-D-ribose binding, and myosin filament assembly.

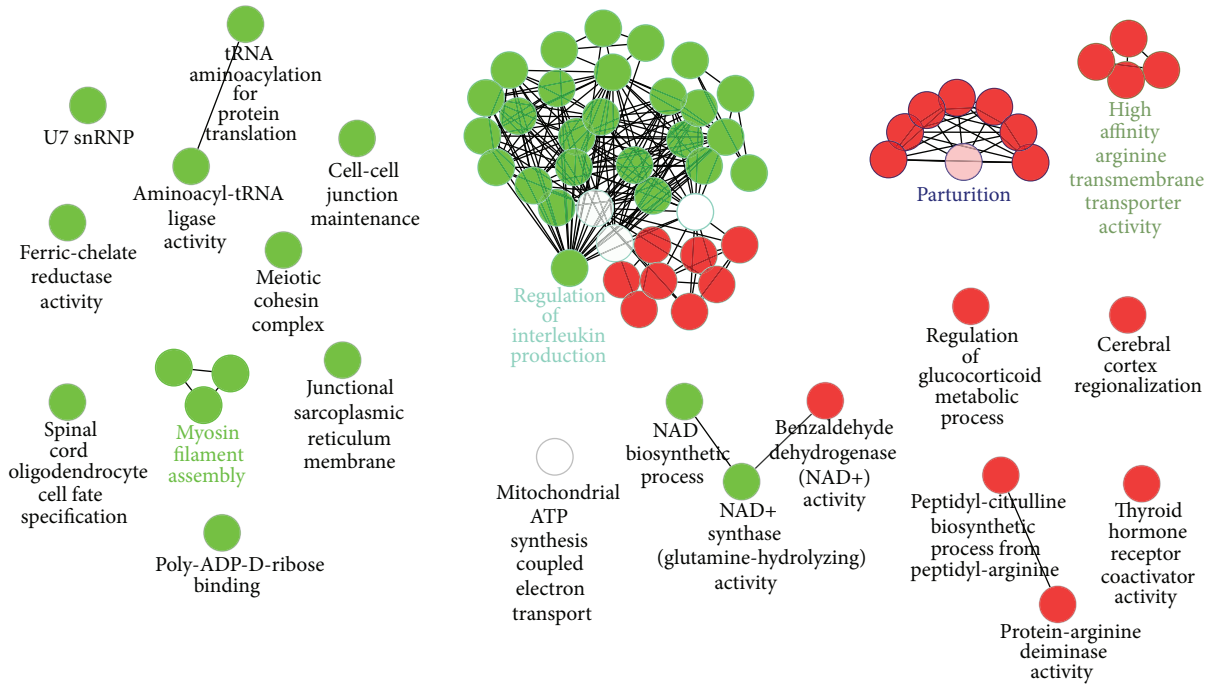

Figure 5: Comparative ClueGO analysis for two DEG lists (COPD versus controls and COPD versus BYF). Red node: functions only enriched by DEGs between control and COPD groups; green node: functions only enriched by DEGs between COPD and BYF groups; white node: functions enriched by both DEG lists.

Table 2: Bingo analysis of 12 overlapping genes [23].

GO term	*P* value	FDR	Gene
Structural constituent of ribosome	$1.31E-04$	$1.16E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Ribosome	$1.76E-04$	$1.16E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Translation	$2.65E-04$	$1.17E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Ribonucleoprotein complex	$5.64E-04$	$1.86E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Structural molecule activity	$7.05E-04$	$1.86E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Soft palate development	$2.16E-03$	$3.55E-02$	*TSHZ1*
Negative regulation of RNA splicing	$2.16E-03$	$3.55E-02$	*RPS26*
Macromolecular biosynthesis	$2.42E-03$	$3.55E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*
Gene expression	$3.36E-03$	$4.44E-02$	*RPL10L/RPS26/RGD1561843/RGD1561841*

Because COPD is known to be accompanied by pulmonary inflammation, oxidative stress, and muscle fiber dysfunction [25–27], BYF treatment may improve these COPD symptoms by regulating the above GO term functions.

Comparative ClueGO analysis between the two DEG lists (COPD versus control and COPD versus BYF) demonstrated that both DEG lists shared at least three relatively enriched GO terms in common including interleukin production, NAD/NADH, and mitochondrial electron transport-related function (Figure 5).

3.4. Interleukin Expression Level in Serum and BALF. Interleukin production and inflammatory functions were evaluated by ClueGO analysis [24], which demonstrated increases in the levels of several common serum (*IL1β, IL6, IL8,* and *IL10*) and BALF (*IL8* and *IL10*) interleukins by ELISA. At the end of 20 weeks, serum levels of *IL1β, IL6, IL8,* and *IL10* in the COPD group were significantly higher than in the control group ($P < 0.01$), while those in the BYF group were significantly lower than the COPD group ($P < 0.01$). Similar to serum, *IL8* and *IL10* levels were also decreased with BYF treatment in BALF ($P < 0.01$; Table 3; Figure 6(b)). These findings indicate that BYF can alleviate COPD inflammation by reducing interleukin levels.

4. Discussion

Although technological advances have been made, there are still considerable complications associated with human tissue sampling. Animal models that imitate COPD pathogenesis

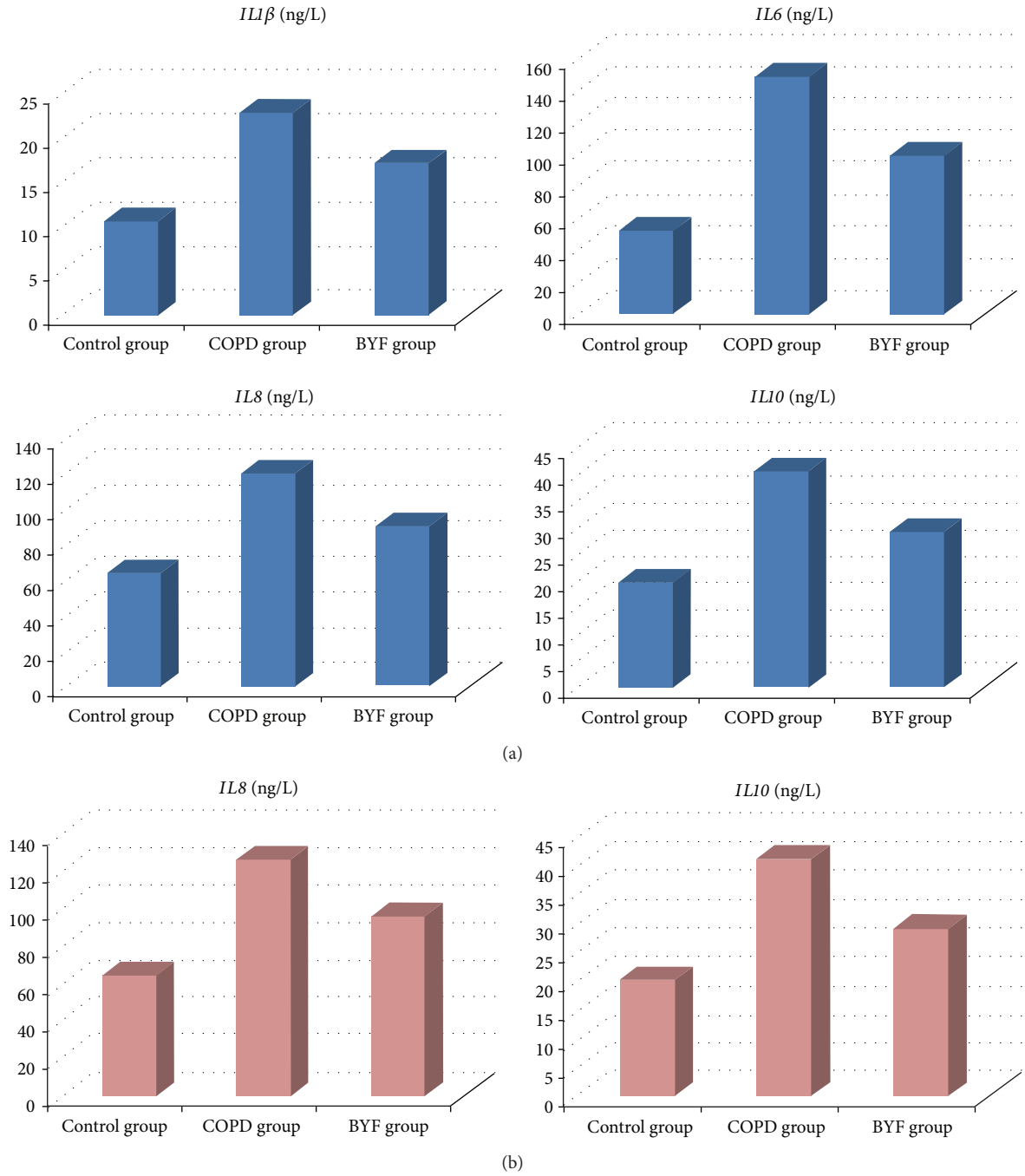

Figure 6: Interleukin expression levels in BALF and serum. (a) Expression level of *IL1β*, *IL6*, *IL8*, and *IL10* in serum. (b) Expression level of *IL8* and *IL10* in BALF.

avoid the risks of human experimentation [28–30] and provide a basis for evaluating the effectiveness of new therapeutic strategies. At present, COPD animal models that are consistent with human COPD are available [9, 10]. In this study, rat COPD was induced by cigarette smoking in combination with repeated instillation of *Klebsiella pneumonia*. COPD lung tissue pathology by light microscope demonstrated that lesions in the rat model closely resemble those occurring in human COPD.

The complexity and varying action of components of Chinese herbs have limited their extensive utilization and hindered study of their underlying molecular mechanism(s). With development of high-throughput molecular techniques such as gene microarray, it is possible to examine the effects of Chinese herbs on genomic expression. To evaluate the effect of BYF on COPD in the present study, we established three groups of rats (COPD, BYF, and controls) and compared their pulmonary function and pathology. Results showed

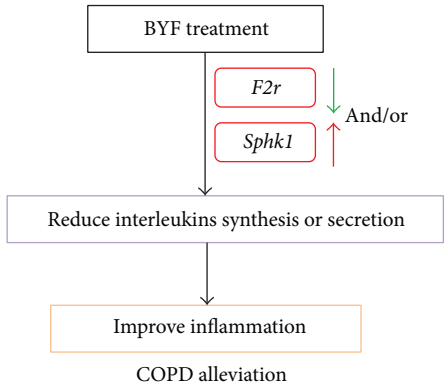

Figure 7: Mechanism of BYF treatment for COPD.

Table 3: Interleukin expression levels in serum and BALF in the 20th week.

Interleukin	Group	Serum (ng/L)	BALF (ng/L)
IL1 β	Control	10.62 ± 1.68	
	COPD	22.81 ± 1.34	
	BYF	17.13 ± 0.71	
IL6	Control	52.48 ± 3.42	
	COPD	148.71 ± 4.72	
	BYF	99.32 ± 4.45	
IL8	Control	64.06 ± 8.17	64.60 ± 8.77
	COPD	120.33 ± 7.15	126.60 ± 6.89
	BYF	90.33 ± 4.37	96.52 ± 6.90
IL10	Control	19.72 ± 2.93	20.18 ± 2.43
	COPD	40.50 ± 2.13	41.16 ± 2.58
	BYF	29.18 ± 1.55	29.12 ± 1.54

that BYF treatment can significantly improve pulmonary function and lung tissue pathology of COPD rats. Gene expression profiles were used to explore the multitarget characteristics of BYF treatment. Gene function enrichment analysis indicated that the BYF can improve COPD through mitochondrial electron transport-related molecules (NAD/NADH/ADP), regulation of interleukin expression, and myosin filament assembly components, relating to muscle dysfunction. Many studies suggested that COPD was related to respiratory, diaphragmatic, and skeletal muscle dysfunction [27, 31–33]. Mitochondrial electron transport-related molecules (NAD/NADH/ADP) are also in relation with redox reactions and oxidative stress, which are known to be the key factors in COPD [33, 34]. In addition, regulation of interleukin expression, involved in inflammation, is also a symptom of COPD [35–37].

Chronic airway inflammation is a key aspect in the pathogenesis of COPD, associated with almost all structural and functional damage of airway and lung tissue [38]. Previous studies showed that members of inflammatory cells such as neutrophils, lymphocytes, and alveolar macrophages were aggregated in blood, sputum, BALF, and bronchial mucosa in stable COPD patients [39]. Inflammatory cells also release various cytokines, including *IL1β, IL6, IL8,* and *IL10,* which play an important role in inflammatory response. In this study, we detected expression of *IL1β, IL6, IL8,* and *IL10* in peripheral blood and BALF. Results show that all four interleukins were reduced by BYF treatment in COPD rats, which was consistent with the gene function enrichment analysis.

In gene expression profiles, no difference was found for interleukin expression among treatment groups. However, expression changes of several interleukins were detected in BALF and serum, suggesting that BYF treatments cannot downregulate interleukin transcription but reduce translation and/or protein secretion. Gene function enrichment analysis of 12 overlapping DEGs (between control versus COPD and COPD versus BYF groups) indicates that BYF treatment can reduce translation and RNA splicing in COPD. As confirmation, we focused on two DEGs (*F2R* and *Sphk1*) that are related to the regulation of interleukin production. Interestingly, studies by Gigante et al. [40, 41] showed that *F2R* haplotypes influence serum *IL6* levels in humans and regulate *IL6* synthesis and production in endothelial cells. In other words, *F2R* haplotypes may influence *IL6* synthesis and secretion. Besides, the human protein-protein interactions predictions (PIPs) database predicts that *F2R* may physically interact with *IL8* [42], while Li et al. [43] found that *Sphk1* expression and activity could reduce *IL1β* and *IL6* concentrations in the serum. Therefore, we speculate that *F2R* and *Sphk1* can regulate the synthesis and/or secretion of certain interleukins in our COPD rat models, while BYF treatment does not influence transcription but reduces interleukin translation or secretion by regulating expression of *F2R* and *Sphk1*, thereby improving COPD-related inflammation (Figure 7).

Conflict of Interests

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

Authors' Contribution

Jiansheng Li and Liping Yang contributed equally to the work.

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Research Article

Si-Jun-Zi Decoction Treatment Promotes the Restoration of Intestinal Function after Obstruction by Regulating Intestinal Homeostasis

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Intestinal obstruction is a common disease requiring abdominal surgery with significant morbidity and mortality. Currently, an effective medical treatment for obstruction, other than surgical resection or decompression, does not exist. Si-Jun-Zi Decoction is a famous Chinese medicine used to replenish qi and invigorate the functions of the spleen. Modern pharmacological studies show that this prescription can improve gastrointestinal function and strengthen immune function. In this study, we investigated the effects of a famous Chinese herbal formula, Si-Jun-Zi Decoction, on the restoration of intestinal function after the relief of obstruction in a rabbit model. We found that Si-Jun-Zi Decoction could reduce intestinal mucosal injury while promoting the recovery of the small intestine. Further, Si-Jun-Zi Decoction could regulate the intestinal immune system. Our results suggest that Si-Jun-Zi Decoction promotes the restoration of intestinal function after obstruction by regulating intestinal homeostasis. Our observations indicate that Si-Jun-Zi Decoction is potentially a therapeutic drug for intestinal obstruction.

1. Introduction

Intestinal obstruction is a common disease requiring abdominal surgery with significant morbidity and mortality. Intestinal obstruction often occurs in the small or large intestines [1]. Regardless of the initial cause of the obstruction, a series of pathophysiological changes occur in the obstructed segments. These changes are responsible for symptoms such as bloating, vomiting, abdominal cramps, and constipation and may lead to intestinal failure [2].

The function of the intestine rests on the normal and balanced homeostasis of the intestine, while intestinal homeostasis depends upon complex interactions between the intestinal epithelium and the intestinal immune system [3]. On a cellular level, the dynamic crosstalk between intestinal epithelial cells (IECs) and local immune cells represents one of the fundamental features of intestinal homeostasis. These interactions are not only important for the pathogenesis of intestinal disorders such as IBD, Crohn's disease, and intestinal obstruction, but also essential for maintaining normal intestinal homeostasis [4]. Our previous study has shown that intestinal obstruction can induce severe dysfunction of intestinal homeostasis. The intestinal epithelial cells and the intestinal immune system are significantly compromised as the obstruction progresses. Therefore, restoration of intestinal homeostasis may be an attractive strategy for treatment of human intestinal obstruction.

The Chinese medicine Si-Jun-Zi Decoction is a famous herbal formula composed of four Chinese herbs: Ginseng Root, *Atractylodes macrocephala*, licorice root, and Poria root.

This formula is considered mild in nature. It balances qi and invigorates the spleen. Modern pharmacological studies show that this prescription affects several other physiological functions such as improving gastrointestinal function [5], strengthening the immune system [6, 7], improving bone marrow hematopoietic function, and speeding up the production of red blood cells. This prescription can also be used for the treatment of malignant tumors of the digestive tract [8]. Xiao and Yang found that Si-Jun-Zi Decoction could improve the immune function and quality of life and reduce the side effects of chemotherapy in patients with colorectal cancer undergoing chemotherapy [7]. Liu et al. found that Si-Jun-Zi Decoction could promote the recovery of glucose uptake in the small intestine of reserpine induced Pi-qi deficiency syndrome rats [9]. Modified Si-Jun-Zi Decoction could slow down the formation of prednisone-induced osteoporosis through promoting osteoblast differentiation and inhibiting osteoclastogenesis [10]. A clinical observation by Guo et al. found that Chenxia Si-Jun-Zi Decoction could promote severe patient's gastrointestinal function recovery and reduce hospitalization days [5]. However, the therapeutic effects of Si-Jun-Zi Decoction on cases of intestinal obstruction are unknown.

In the present study, we investigate the effects of Si-Jun-Zi Decoction on the restoration of intestinal function after the relief of obstruction in a rabbit model. Our results indicate that Si-Jun-Zi Decoction promotes the restoration of intestinal function by regulating intestinal homeostasis. Our observations indicate that Si-Jun-Zi Decoction may have potential therapeutic effects on patients suffering from intestinal obstruction.

2. Materials and Methods

2.1. Animals and Reagents. Healthy New Zealand rabbits with a body weight of 2.5–3 kg were used in this study. The animals were purchased from Mingle Laboratory Animal Center (Tianjin, China) and maintained in a temperature-controlled room with a 12 h light/dark cycle and with access to regular chow and water. The experimental procedures were approved by the Laboratory Animal Care Committee at Tianjin Medical University. All animals received care according to the Guide for the Care and Use of Laboratory Animals (NIH, Bethesda, MD). The Si-Jun-Zi Decoction was supplied by the pharmaceutical preparation section of Nankai Hospital (Tianjin, China). The formula was composed of tuckahoe root (*Scierotium poriae cocos*), ginseng root (*Radix ginseng*), white *Atractylodes* rhizome root (*Rhizoma Atractylodes macrocephalae*), and licorice root (*Radix glycyrrhizae*) in the ratio of 3 : 3 : 2 : 2 (tuckahoe root 60 g, ginseng root 60 g, rhizome root 60 g, and licorice root 40 g), which were submerged in 2.2 L distilled water for 30 min and then decocted twice (2 h per time), then filtered and concentrated to 1 g/mL using a routine method, and stored in a refrigerator at 4°C until use.

2.2. Description of Experimental Groups and the Rabbit Intestinal Obstruction Model. Thirty-six New Zealand rabbits were randomly divided into six groups: sham operation control group (Sham), 48 h after obstruction group ($O_{48\,h}$), natural recovery at 48 h or 96 h after relief of the obstruction groups ($S_{48\,h}$, $S_{96\,h}$), and Si-Jun-Zi Decoction treatment for 48 h or 96 h after the relief of the obstruction groups ($T_{48\,h}$, $T_{96\,h}$), ($n = 6$ per group). To establish a rabbit model of intestinal obstruction that was controllable, we transformed the parts of infusion sets that are widely used in clinical applications into an *in vitro* pulled-type lock. After being anesthetized with an i.v. injection of urethane (1 g/kg), a laparotomy was performed on sedated rabbits and a uniform controllable loop obstruction was created in the mesenteric nonavascular zone by placing a clamp eight cm from the distal end of the ileum. Sham-operated rabbits received mock manipulation of the gut without placement of the lock. The animals were allowed to recover postoperatively for 3 days to remove any influence of the anesthesia on our test parameters. Three days after the operation, the clamp was locked according to the color label, resulting in the obstruction of the intestine. The obstruction lasted for 48 h after which the intestinal obstruction was relieved by cutting off the lock. The animals were then treated with or without drug (5 g/kg, twice a day) for 48 h or 96 h. At the end of the experiment, the animals were sacrificed for further analysis.

2.3. Quantification of Intestinal Damage and Determination of Intraepithelial Lymphocytes (IEL) and Lamina Propria Lymphocytes (LPL) Cell Numbers. Two-centimeter or longer intestinal segments from the ileum (5 cm from the distal ileum with obstruction) were excised, fixed in 4% paraformaldehyde, and embedded in paraffin blocks. Four-micron thick sections were stained with H&E and PAS using standard protocols [11]. The sections were examined using a Champion-500w graphic report management system. Intestinal mucous membrane damage was evaluated and Chiu's [12] histopathological scores were determined. Briefly, the tissue damage was graded from 0 to 5 according to the following criteria: grade 0, normal structure of villi; grade 1, development of small subepithelial space at the villous apex; grade 2, enlarged subepithelial space but without change in villous length and width; grade 3, few shortened villi and presence of cells in the lumen; grade 4, the majority of villi are shortened and widened with crypt hyperplasia and cells in the lumen; and grade 5, blunting of all villi with elongated crypts and a large number of cells in the lumen. The percentage of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were quantified in photomicrographs of the intestines. The percentage of IEL cells was calculated using the following formula: (the number of total IEL cells in 20 villi per section/the total cell number in 25 villi per section) × 100. The percentage of LPL cells was calculated using the following formula: (the number of total LPL cells in 20 villi per section/the total lamina propria cell number in the central axis of 25 villi per section) × 100.

2.4. High-Performance Liquid Chromatography (HPLC) Analysis of Ornithine Decarboxylase Activity and Citrulline Levels. Theornithine decarboxylase (ODC) activity of intestinal tissues and the level of citrulline in blood serum were analyzed by HPLC as described elsewhere [13, 14].

2.5. RNA Extraction and Real-Time PCR. RNA was extracted from intestinal segments (100 mg) from the ileum (5 cm from the distal ileum with obstruction) using a Total RNA Kit (Qiagen) by following the manufacturer's instructions. First-strand cDNA was synthesized from 1 μg mRNA using reverse transcriptase (Fermentas, Glen Burnie, MD) and oligo (dT) primers. The primer sequences used were as follows: Claudin 1 forward: 5′-GTGCCTTGATGGTGATTG-3′, reverse: 5′-AAAGTAGCCAGACCTGAAAT-3′. β-actin forward: 5′-TGATGGTGGGCATGGGTC-3′, reverse: 5′-CGATGGGGTACTTCAGGGTG-3′. Real-time PCR was performed using an Applied Biosystems PRISM7300 (Applied Biosystems) and SYBR Green PCR master mix (Applied Biosystems). Reagents concentration were 2× Realtime Mix 10 μL, forward primer 0.3 μL, reverse primer 0.3 μL, ddH_2O 7.4 μL, and cDNA templates 2 μL. PCR cycling conditions were 95°C for 30 sec followed by 40 cycles of 95°C for 5 sec and 50°C for 30 sec. The mRNA expression was normalized to the expression of the β-actin housekeeping gene.

2.6. Flow Cytometry Analysis of Peyer's Patch (PP) Lymphocytes. The PP lymphocytes were isolated as described previously [15]. Briefly, the excised PP lymphocytes were incubated under sterile conditions with RPMI medium containing 1 mM DTT (5 min, 37°C). Thereafter, the PP lymphocytes were washed with RPMI medium and passed through a steel mesh. The resulting cell suspension was washed and resuspended in RPMI containing 10% FBS. The PP lymphocytes were stained with FITC-anti-rabbit CD4, PE-anti-rabbit CD8, and Percp-Cy5.5-anti-rabbit CD3. A negative control was stained with isotype-matched mAb. Cells were stained at 4°C for 30 min. The cells were then washed and resuspended in PBS for FACS analysis. Data were acquired using a FACSCalibur (BD Bioscience) flow cytometer and analyzed using the CellQuest software.

2.7. ELISA. A fresh 8 cm intestinal segment from the ileum (5 cm from the distal ileum with obstruction) was obtained, the intestinal solid contents were removed, and casing slime was collected by douching with 3 mL of sterile PBS. The obtained extracts were centrifuged and the supernatants were collected. The levels of s-IgA were measured using an ELISA kit according to the manufacturer's instructions. The serum level of D-lactate was measured by ELISA kit (ADL co.), according to the manufacturer's instructions.

2.8. Statistical Analysis. Results were expressed as mean ± SD. The data were analyzed using a two-tailed Student's t-test (GraphPad Prism 5) and a P value of <0.05 was regarded as statistically significant.

3. Results

3.1. The Si-Jun-Zi Decoction Reduces Intestinal Mucosal Injury. The intestinal mucosal barrier is the first line of host defense against intestinal pathogens, and intestinal obstruction induces sever intestinal mucosal injury [16]. Intestinal mucosal injury is also a common clinical complication that may lead to dysfunction of the intestinal barrier. Here, by using a modified and controllable rabbit intestinal obstruction model, we evaluated the ability of the Si-Jun-Zi Decoction to reduce the obstruction-induced intestinal mucosal damage after relief of the intestinal obstruction. As shown in Figures 1(a) and 1(b), the intestinal obstruction induced severe intestinal mucosal injury, including villous blunting and epithelial sloughing. The overall histopathological damage score of the intestines of the sham and $O_{48\,h}$ groups was statistically different. After relieving the obstruction, the damage to the intestinal mucosa was gradually repaired, while the Si-Jun-Zi Decoction treatment groups showed significantly increased levels of repair compared to treatment groups without Si-Jun-Zi Decoction treatment. The overall histopathological damage score of the $S_{48\,h}$ versus $T_{48\,h}$ groups and the $S_{96\,h}$ versus $T_{96\,h}$ groups was statistically different ($P < 0.05$), suggesting that the Si-Jun-Zi Decoction treatment had a profound protective effect against intestinal mucosal injury. This protection provided by the Si-Jun-Zi Decoction was confirmed by changes in the level of blood D-lactate, which is a measure of intestinal mucosa permeability [17], and the intestinal mucins, which are key components of the intestinal mucosal barrier [18]. The data showed that D-lactate levels significantly declined after Si-Jun-Zi Decoction treatment ($S_{48\,h}$ versus $T_{48\,h}$ and $S_{96\,h}$ versus $T_{96\,h}$) (Figure 1(c)), while levels of the intestinal mucins significantly increased relative to the control groups ($S_{48\,h}$ versus $T_{48\,h}$ and $S_{96\,h}$ versus $T_{96\,h}$) (Figure 1(d)). These results indicate that the Si-Jun-Zi Decoction could reduce the permeability of intestinal mucosa and enhance the secretion of intestinal mucins. Taken together, our results showed that the Si-Jun-Zi Decoction was beneficial for the reduction of intestinal mucosal injury after relieving the intestinal obstruction.

3.2. The Si-Jun-Zi Decoction Promotes the Recovery of the Small Intestine. We next evaluated the effects of the Si-Jun-Zi Decoction on the function and integrity of the small intestine. We first examined the change in ornithine decarboxylase (ODC) levels, which indicate intestinal epithelial cells proliferation [19]. Our previous study showed that the level of ODC increased after obstruction, reaching a peak at 12 h after obstruction, and then rapidly decreased. This observation suggests that, during the early stages of obstruction, the intestinal epithelial cells renewed quickly, promoting the recovery of the intestinal epithelium, and that the damage and recovery were balanced. However, at later stages, this balance was upset due to the severity of intestinal epithelial damage.

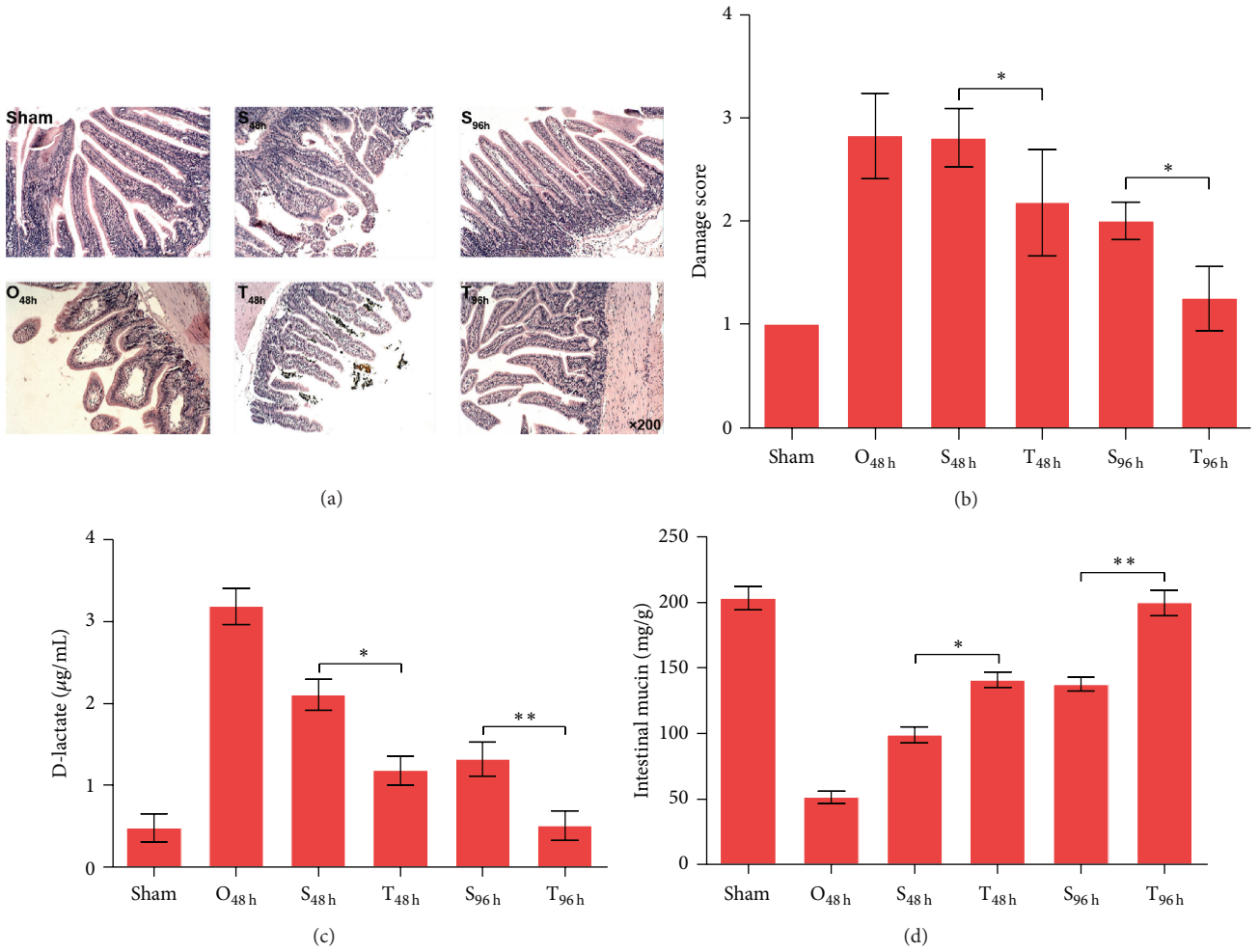

FIGURE 1: Si-Jun-Zi Decoction treatment reduces intestinal mucosal injury. (a) and (b): intestinal segments from the ileum were excised and fixed in 4% paraformaldehyde and embedded in paraffin blocks. We stained 4 μm thick sections with H&E. Intestinal mucous membrane damage and Chiu's histopathological score were evaluated. The H&E staining (a) and the damage score (b) are shown. In panel (c), the serum level of D-lactate is shown. In panel (d), the level of intestinal mucins, examined by Coomassie brilliant blue G250 staining, is shown. The data are representative of three independent experiments, with each using 6 mice per group. Data are shown as mean ± SD. ($^{*}P < 0.05$, $^{**}P < 0.01$).

As shown in Figure 2(a), the ODC level of the $O_{48\,h}$ group was equal to the sham control group and gradually increased after the obstruction was relieved. The level of ODC was significantly elevated after 48 h of Si-Jun-Zi Decoction compared with the $S_{48\,h}$ group. At 96 h after relieving the obstruction, the ODC level returned to normal and thus maintained intestinal homeostasis. This result indicates that the Si-Jun-Zi Decoction could promote the recovery of the intestinal epithelium. We next quantified the level of blood citrulline, which serves as an indicator of the absorptive function of small intestine [20]. Our data showed that serum levels of citrulline in the $T_{48\,h}$ or $T_{96\,h}$ groups were significantly higher than those in the $S_{48\,h}$ or $S_{96\,h}$ groups (Figure 2(b)), suggesting that the Si-Jun-Zi Decoction treatment promoted the recovery of the absorptive function of the small intestine. Because the integrity of the intestinal mucosa, which is maintained by tight junction proteins (TJPs) and adherens junction proteins, is essential for the function of the intestinal barrier [21], we examined Claudin 1 gene expression by real-time PCR analysis. Claudin 1 is the functional component of tight junction transmembrane protein (TJPs) in intestinal epithelial cells [22]. We found almost undetectable levels of Claudin 1 gene expression at 48 h after obstruction, indicating that the intestinal epithelial integrity at later stages of obstruction was completely lost (Figure 2(c)). Once the obstruction was removed, Claudin 1 gene expression increased ($S_{48\,h}$ group). In animals treated with the Si-Jun-Zi Decoction for 48 h, expression of the Claudin 1 gene was significantly increased compared with the $S_{48\,h}$ group, and this enhancement was more obvious in $T_{96\,h}$ group versus $T_{48\,h}$ group. This result

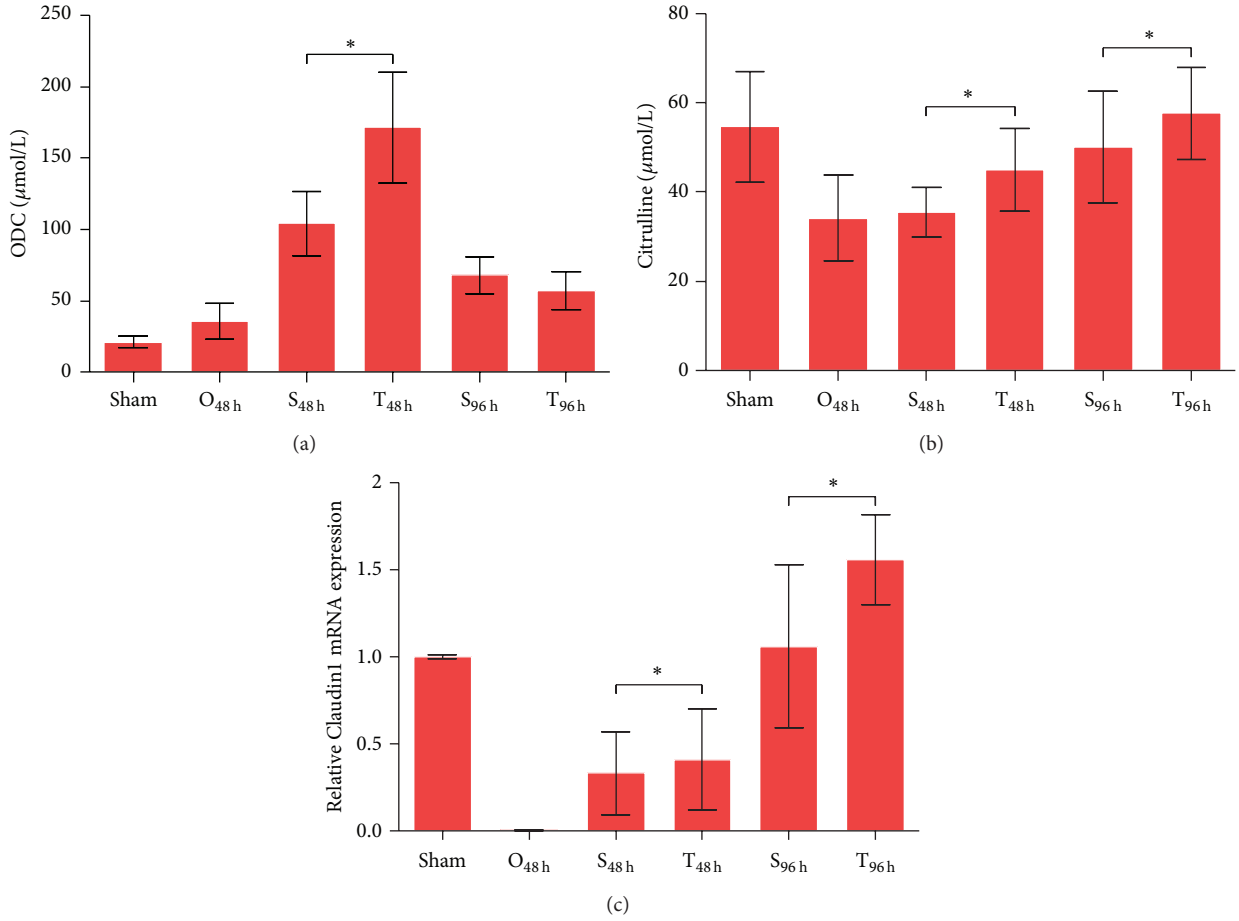

FIGURE 2: Si-Jun-Zi Decoction treatment promotes recovery of the small intestine. (a) and (b): the ODC activity (a) in intestinal tissues and the level of citrulline (b) in blood serum were analyzed by HPLC. The data are shown as mean ± SD ($n = 6$). (c) The gene expression level of Claudin 1 was examined by qRT-PCR. The data are shown as the mean ± SD ($n = 6$). The data are representative of at least three independent experiments. $^{*}P < 0.05$.

indicates that the Si-Jun-Zi Decoction could promote the recovery of the integrity of the small intestine. Taken together, our results suggest that the Si-Jun-Zi Decoction can promote the recovery of the small intestine after relieving intestinal obstruction.

3.3. The Si-Jun-Zi Decoction Regulates the Intestinal Immune System. Intestinal epithelial cells are well known for their role as the boundary between the external environment and the intestinal tract. Apart from being a physical barrier, intestinal epithelial cells play a pivotal role in regulating immune responses in order to maintain intestinal homeostasis [23]. However, once intestinal epithelial cell damage occurs, the balance between intestinal epithelial cell barrier and intestinal immune system cannot be maintained. Our previous study found that intestinal obstruction induces intestinal immune system dysfunction and therefore we investigated whether the Si-Jun-Zi Decoction could regulate the intestinal immune system after intestinal obstruction was relieved. We first assessed the number of intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) and intestinal epithelial cells (IEC) using PAS stained tissue sections. The data showed that significant differences in the numbers of IELs after Si-Jun-Zi Decoction treatment (data not shown) were not found, while the number of LPLs decreased with the Si-Jun-Zi Decoction treatment ($S_{48\,h}$ versus $T_{48\,h}$, $P < 0.05$) (Figure 3(a)). In addition, we analyzed the level of s-IgA in the intestinal lumen at various times, with or without drug treatment, after relieving the obstruction. We found that the s-IgA level was not significantly changed, suggesting that the Si-Jun-Zi Decoction does not affect the innate immunity of the intestine. We next examined alterations in T cells subtype percentages in PP lymphocytes by flow cytometry. As shown in Figures 3(c)–3(f), Si-Jun-Zi Decoction treatment could reduce the percentage of $CD3^{+}T$ cells 48 h after relieving the intestinal obstruction compared with the no treatment group (Figure 3(c)). Si-Jun-Zi Decoction treatment could increase the percentage of $CD4^{+}T$ cells and reduce the percentage of $CD8^{+}T$ cells at

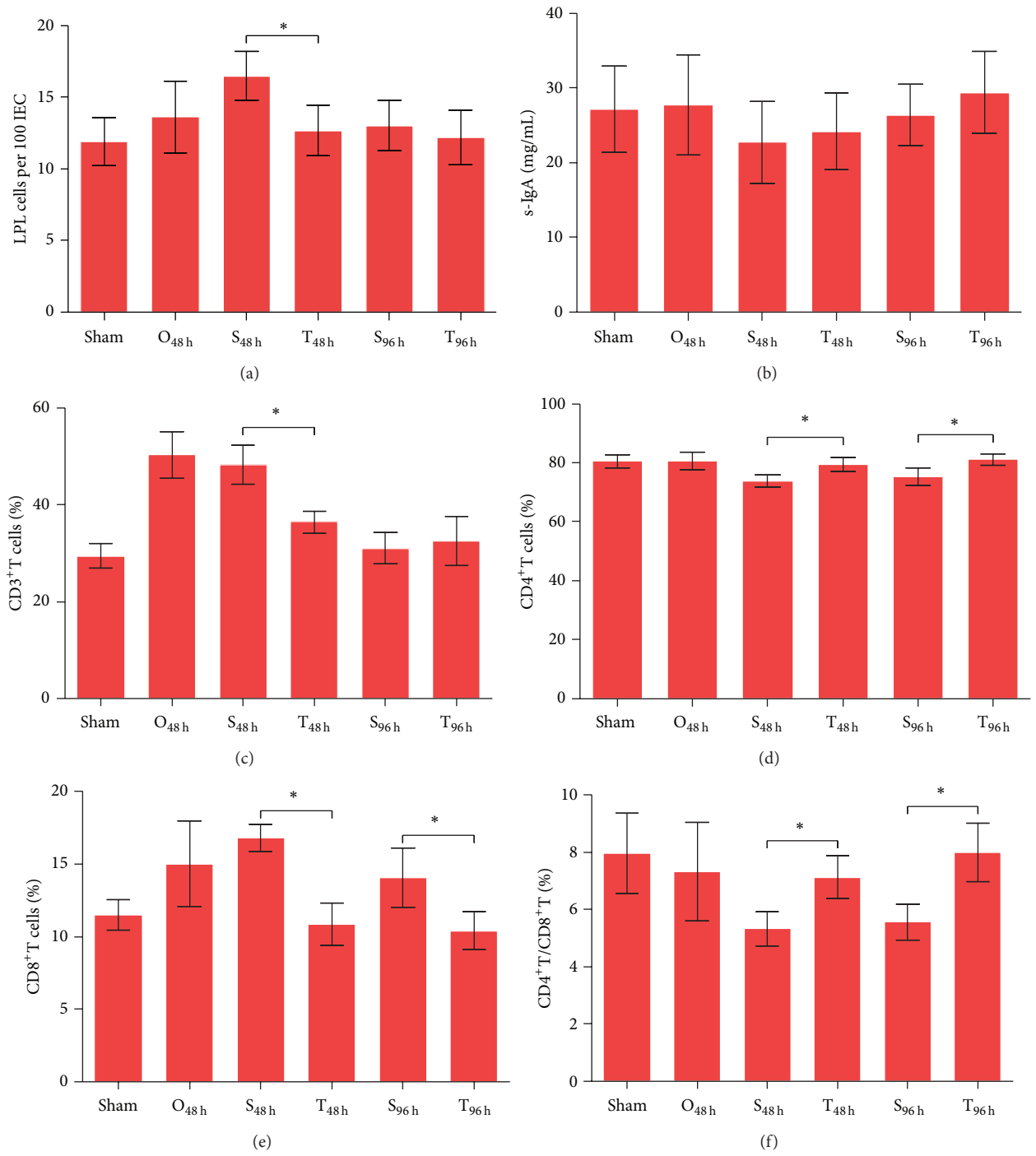

FIGURE 3: Si-Jun-Zi Decoction treatment regulates the intestinal immune system. (a) Intestinal segments from the ileum were excised and fixed in 4% paraformaldehyde and embedded in paraffin blocks. We stained 4 μm thick sections with PAS, and the percentage of LPLs was quantified in photomicrographs of the intestines. (b) The level of s-IgA in the intestinal lumen was examined by ELISA. (c)–(f) The PP lymphocytes were isolated and the percentage of T cells subtypes was analyzed by flow cytometry. The percentage of $CD3^+T$ cells (c), the percentage of $CD4^+T$ cells (d), the percentage of $CD8^+T$ cells (e), and the $CD4^+/CD8^+T$ cells ratio (f) are shown. The data are representative of three independent experiments, with each using 4-5 mice per group. Data are shown as mean ± SD ($^*P < 0.05$).

different times with or without drug treatment after the relief of obstruction (Figures 3(d) and 3(e)). The $CD4^+/CD8^+T$ cells ratio was also sharply elevated by Si-Jun-Zi Decoction treatment (Figure 3(f)). These data suggest that the Si-Jun-Zi Decoction could regulate the adaptive immune response by reducing the number of $CD3^+T$ cells and $CD8^+T$ cells while increasing the number of $CD4^+T$ cells. However, the precise mechanisms involved still require further investigation.

Based on the above data, we suggest that the Si-Jun-Zi Decoction can promote the restoration of intestinal function by regulating the intestinal immune system.

4. Discussion

Intestinal obstruction is a common disease requiring abdominal surgery with significant morbidity and mortality [1]. The major morbidity associated with intestinal obstruction is related to excessive distension that leads to strangulation and bowel necrosis. Until now, effective medical treatment for obstruction, other than surgical resection or decompression, is lacking. However, even if the obstruction is surgically removed, many patients continue to have disturbed motility function in the bowel proximal to the site of resection for many years to come [24, 25]. There are very few drugs for the treatment of intestinal obstructions. Recently Alvimopan, a drug that behaves as a peripherally acting μ-opioid receptor antagonist, was approved by the FDA for the treatment of patients with postoperative ileus (POI) following partial large or small bowel resection surgery with primary anastomosis [26]. However, there are several side effects and limitations for the use of this drug [27, 28].

Si-Jun-Zi Decoction is a famous Chinese medicine used to replenish qi and invigorate the functions of the spleen. It is used when there is a deficiency of qi of the spleen and stomach, which is marked by anorexia and loose bowels. Modern pharmacological studies show that this prescription can improve gastrointestinal function [5] and strengthen immune function [6, 7]. Therefore, we hypothesized that this prescription may be beneficial for the restoration of intestinal function after relieving the obstruction.

In the current study, we used a controllable rabbit intestinal obstruction model to investigate the effects of a Si-Jun-Zi Decoction on the restoration of intestinal function after relieving the obstruction. We found that the Si-Jun-Zi Decoction can reduce the intestinal mucosal injury. Si-Jun-Zi Decoction treatment significantly alleviated intestinal mucosal damage, reduced intestinal mucosal permeability, and enhanced the secretion of intestinal mucins. Furthermore, the Si-Jun-Zi Decoction promotes the recovery of the small intestines. Si-Jun-Zi Decoction treatment could promote the recovery of both the intestinal epithelium and the absorptive function of the small intestine by increasing ODC and citrulline levels. In addition, the Si-Jun-Zi Decoction promotes the recovery of small intestine integrity by upregulating the Claudin 1 gene expression. Finally, we found that the Si-Jun-Zi Decoction regulates the intestinal immune system. Although the Si-Jun-Zi Decoction does not affect the innate immunity of the intestine, it may regulate the adaptive immune response by reducing $CD3^+$T cell and $CD8^+$T cell numbers while increasing the number of $CD4^+$T cells in PP lymphocytes.

Although we found that the famous Chinese herbal formula Si-Jun-Zi Decoction has the ability to promote the restoration of intestinal function by regulating the intestinal homeostasis, the precise mechanisms involved are still to be elucidated. Si-Jun-Zi Decoction as a potential therapeutic drug for intestinal obstruction treatment needs to be investigated in the future.

5. Conclusion

The present study clearly demonstrates that Si-Jun-Zi Decoction promotes the restoration of intestinal function after obstruction by regulating intestinal homeostasis. Our results indicate that the Si-Jun-Zi Decoction may be a potential therapeutic drug for intestinal obstruction. Further studies are needed to assess the side effects and limitations of this drug in the treatment of intestinal obstruction.

Conflict of Interests

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

Authors' Contribution

Xiangyang Yu and Zhigang Cui contributed equally to this work.

Acknowledgments

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Research Article

Effects of Chinese Medicine Tong xinluo on Diabetic Nephropathy via Inhibiting TGF-β1-Induced Epithelial-to-Mesenchymal Transition

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Diabetic nephropathy (DN) is a major cause of chronic kidney failure and characterized by interstitial and glomeruli fibrosis. Epithelial-to-mesenchymal transition (EMT) plays an important role in the pathogenesis of DN. Tong xinluo (TXL), a Chinese herbal compound, has been used in China with established therapeutic efficacy in patients with DN. To investigate the molecular mechanism of TXL improving DN, KK-Ay mice were selected as models for the evaluation of pathogenesis and treatment in DN. In vitro, TGF-β1 was used to induce EMT. Western blot (WB), immunofluorescence staining, and real-time polymerase chain reaction (RT-PCR) were applied to detect the changes of EMT markers in vivo and in vitro, respectively. Results showed the expressions of TGF-β1 and its downstream proteins smad3/p-smad3 were greatly reduced in TXL group; meantime, TXL restored the expression of smad7. As a result, the expressions of collagen IV (Col IV) and fibronectin (FN) were significantly decreased in TXL group. In vivo, 24 h-UAER (24-hour urine albumin excretion ratio) and BUN (blood urea nitrogen) were decreased and Ccr (creatinine clearance ratio) was increased in TXL group compared with DN group. In summary, the present study demonstrates that TXL successfully inhibits TGF-β1-induced epithelial-to-mesenchymal transition in DN, which may account for the therapeutic efficacy in TXL-mediated renoprotection.

1. Introduction

Diabetic nephropathy (DN) and other chronic kidney diseases are characterized by glomeruli and interstitial fibrosis. Traditionally, resident fibroblasts are considered to be the key mediators of renal fibrosis; now, convincing evidences suggest that the appearance of interstitial myofibroblasts also contributes to fibrosis. Central to this process is epithelial-to-mesenchymal transition (EMT) [1]. Researches show that about 30% fibroblasts are derived from the tubular epithelial cells via EMT in kidney [2].

EMT is regulated by different signaling molecules and transforming growth factor β1 (TGF-β1) is proved to be the principle mediator in EMT [3]. In diabetes, high glucose and other stimuli increased the production of TGF-β1 [4]. TGF-β1 has the ability to increase its own expression leading to the accumulation of ECM and fibrosis [5]. Combined with TβRII, TGF-β1 initiates the expressions of several downstream signal proteins such as small mothers against decapentaplegic (smad) and mitogen-activated protein kinases (MAPKs). Loss of E-cadherin (E-CA) and gain in α-smooth muscle actin (α-SMA) expression are hallmarks of EMT [6]. As a result, extracellular matrix (ECM) such as collagen IV (Col IV) and fibronectin (FN) excessively accumulated. With the development of renal fibrosis, many patients may undergo end-stage renal disease (ESRD) and DN is the leading cause of ESRD, accounting for millions of deaths worldwide [7]. In treating DN and other chronic renal

diseases, most therapies aim at the heteropathy such as the control of blood pressure and blood glucose.

With the development of modern technologies, preparation of Chinese medicine has also been improved. Tong xinluo (TXL), a Chinese herbal compound developed two decades ago, includes a group of medicines such as *Panax ginseng* C. A. Mey. extract and *Paeonia lactiflora* Pall. extract [8, 9]. It was registered in the State Food and Drug Administration of China. TXL has already been proven to have a cohort of potentially therapeutic value such as preventing apoptosis, improving endothelial cell function, reducing inflammation, and lowering lipids [10]. It has already been used in treating angina pectoris diseases in China for many years [8]. According to these positive effects, many studies have investigated the mechanism of it, that is, TXL modulates vascular endothelial function by inducing eNOS expression via the PI-3K/Akt/HIF-dependent signaling pathway [11]. TXL dose-dependently enhanced stability of vulnerable plaques compared with a high-dose simvastatin [12]. TXL showed protective effects on free fatty acid induced endothelial injury by boosting intracellular antioxidant capacity through AMPK pathway [8]. In treating DN, TXL also shows positive effect. Meta-analysis showed that TXL significantly decreased 24-hour urine albumin excretion ratio (24 h-UAER) and blood urea nitrogen (BUN) [13]. In the treatment of early DN, TXL could improve renal microcirculation, reduce Cys-C and UAER, and delay the progress of renal damage [14]. But the mechanism of TXL improving DN remains unclear. Fibrosis is a key pathology in DN and excessive extracellular matrix (ECM) synthesis and accumulation, resulting in glomerular and tubular pathology and ultimately death in diabetic patients [15]. Studies have proven the positive effect of TXL and some components of it in suppressing renal fibrosis or EMT. TXL attenuated renal fibrosis and decreased the expressions of TGF-β1 and α-SMA in unilateral ureteral occlusion mice [16]. Ginsan, a polysaccharide extracted from *Panax ginseng*, significantly suppressed the accumulations of TGF-β, collagen, and α-SMA [17]. Paeoniflorin (PF), the key active constituent of *Paeonia lactiflora* Pallas, has previously been reported to prevent the progression of renal fibrosis in UUO mice. The antifibrosis efficacy of PF was mainly reflected in improving histopathological disorders and reducing collagen deposition in kidney tissues [18]. Additionally, PF successfully downregulated TGF-β1 expression and inhibited smad2/3 activation in fibrotic kidneys induced by UUO [18]. These findings strongly support the hypothesis that TXL may attenuate EMT in DN.

The aim of the present study was to explore the mechanisms of TXL-mediated renoprotection and determine whether TXL can inhibit TGF-β1-induced EMT in DN. Results showed that TXL successfully inhibited TGF-β1 expression and TGF-β1-induced EMT, and TXL may be a new possible therapy in diabetic nephropathy.

2. Material and Methods

2.1. Preparation of Tong xinluo Ultrafine Powder Solution. Tong xinluo ultrafine powder was procured by Shi-jiazhuang Yiling Pharmaceutical company (Lot no. 071201, Shijiazhuang Yiling Pharmaceutical Co., Shijiazhuang, China). It contains a group of medicines such as *Panax ginseng* C. A. Mey. Extract and *Paeonia lactiflora* Pall. extract. The detailed formulation of TXL is shown in Supplementary Materials available online at http://dx.doi.org/10.1155/2014/123497. The herbal drugs were authenticated and standardized on marker compounds according to the Chinese Pharmacopoeia (2005). To reduce the dose variability of TXL among different batches, the species, origin, harvest time, medicinal parts, and concocted methods for each component were strictly standardized. Moreover, high performance liquid chromatography (HPLC), high performance capillary electrophoresis, and gas chromatography were applied to quantitate the components of TXL. HPLC was taken to check fingerprint chromatograms of the aqueous extracts of the 10 batches for similarity analysis [12]. The detailed result of HPLC can be found in Supplementary Material. The herbal drugs were ground to ultrafine powder with the diameter $\leq 10\,\mu$m by a micronizer. In vivo experiment, 750 mg/kg TXL ultrafine powder dissolved in aquadistillate was intragastrically administered each day. In cell culture, TXL ultrafine powder was dissolved in serum-free DMEM/F12 (Dulbecco's modified Eagle's medium/F12). The solution was sonicated for 1 hour followed by centrifugation at 1164 g for 10 min. The supernatant was centrifuged for a second time. Finally, it was filtrated by 0.22 μm filters and stored at -20°C. Meanwhile, the precipitate was heated and dried at 60°C in order to calculate the practical volume of dissolved Tong xinluo ultrafine powder. The final concentration is 2000 μg/mL [19]. In in vitro experiment, it was diluted with DMEM/F12.

2.2. Animals. To explore the effect of TXL on TGF-β1 expression and renal tubular EMT in DN, KK-Ay mice were spontaneous animal models for the evaluation of pathogenesis and treatment in patients with DN [20]. DN was diagnosed when their random blood glucose (RBG) was $\geq$16.7 mmol/L and urine albumin creatine ratio (ACR) was $\geq 300\,\mu$g/mg. C57BL/6J mice were fed with common forage and KK-Ay mice were fed with high fat forage for 4 weeks. C57BL/6J mice gavaged with aquadistillate were set as normal control group (Normal, $n = 10$), KK-Ay mice gavaged with aquadistillate were set as diabetic nephropathy group (DN group, $n = 10$), and KK-Ay mice treated with TXL (750 mg/kg/per day, gavage) were set as TXL group (DN + TXL group, $n = 10$). After being treated for 12 weeks, blood and 24-hour urine were collected. Renal tissue was immediately frozen for western blot and RT-PCR and 4% (w/v) paraformaldehyde was used to fix renal tissues for immunofluorescence staining. C57BL/6J and KK-Ay mice were purchased from the Chinese Academy of Medical Sciences (8 weeks of age, Beijing, China). The experiment complied with the Animal Management Rule of the Ministry of Public Health, China, and the experimental protocol was approved by the Animal Care Committee of Capital Medical University, Beijing, China.

Table 1: Oligonucleotide primers used in the study.

Gene name	Forward	Reverse
mmu-TGF-β1	5′-atacgcctgagtggctgtct-3′	5′-ctgatcccgttgatttcca-3′
hsa-E-CA	5′-tcttcggaggagagcggtggtcaaa-3′	5′-gccgagcgtccaggcccctgtgcag-3′
mmu-E-CA	5′-gagtggagaacgaggaacccttga-3′	5′-acgtgtccggctctcgagcggtata-3′
hsa-α-SMA	5′-atcaaggagaaactgtgttatgtag-3′	5′-gatgaaggatggctggaacagggtc-3′
mmu-α-SMA	5′-gagtcagcgggcatccacgaaa-3′	5′-tgctgggtgcgagggctgtgat-3′
hsa-smad3	5′-gaggcgtgcggctctactacatc-3′	5′-gccaggagggcagcgaact-3′
mmu-smad3	5′-gcacagccaccatgaattac-3′	5′-gcacagccaccatgaattac-3′
hsa-smad7	5′-aggtgttccccggtttctcca-3′	5′-ttcacaaagctgatctgcacggt-3′
mmu-smad7	5′-gctttcagattcccaacttctt-3′	5′-gatatccagggagggctcttg-3′
hsa-Col IV	5′-tggtcttactgggaactttgctgc-3′	5′-ggtgggatctgaatggtctggc-3′
mmu-Col IV	5′-tggtcttactgggaactttgctgc-3′	5′-accctgtggtccaacgactcctctc-3′
hsa-FN	5′-agaagtgggaccgtcagggaga-3′	5′-caggagcaaatggcaccgaga-3′
mmu-FN	5′-tctgggaaatggaaaaggggaatgg-3′	5′-cactgaagcaggtttcctcggttgt-3′

2.3. Cell Culture. Human renal tubuloepithelial cells (HKCs) were purchased from Cell Resource Center in China. It was incubated with Dulbecco's modified Eagle's medium/F12 (DMEM/F12) containing 10% (v/v) fetal calf cerum (FBS). Cells were maintained at 37°C in a 5% (v/v) CO_2 water-saturated atmosphere. Recombinant human transforming growth factor β1 (TGF-β1, eBioscience, Santiago, USA) was used to induce EMT in HKCs in vitro. Five concentrations (5 ng/mL–25 ng/mL) of TGF-β1 and five time points (24–72 hours) were applied to observe the effect of TGF-β1 on EMT in vitro. Concentration of 10 ng/mL of TGF-β1 was used to induce EMT in the following study. Cells exposed to 10 ng/mL TGF-β1 for 48 hours were set as TGF-β1 group. In order to test the TXL-mediated protection in EMT, we first preconditioned the HKCs with TXL for 30 min before they were exposed to TGF-β1 [8]. Cells exposed to 10 ng/mL TGF-β1 and 250 μg/mL TXL for 48 hours were set as TGF-β1 + TXL group. Cells cultured in normal DMEM/F12 without TGF-β1 were set as normal group.

2.4. RT-PCR Analysis. Total RNA was extracted with Trizol (TIANGEN, Beijing, China) in accordance with the manufacturer's recommendations. Each sample was reverse transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen). Gene expression was quantified by means of the comparative Ct method ($\Delta\Delta$Ct) and the relative quantification (RQ) was calculated as $2^{-\Delta\Delta Ct}$ [21–23]. Relative mRNA levels of E-CA, α-SMA, smad3, smad7, Col IV, and FN were examined and normalized to β-actin mRNA. All RT-PCRs were performed in triplicate, and the data was presented as mean ± SD. The primers of TGF-β1, E-CA, α-SMA, smad3, smad7, Col IV, and FN are in Table 1.

2.5. Western Blot Analysis. TGF-β1, E-CA, α-SMA, smad3/p-smad3, and smad7 were detected by western blot as previously described [24, 25]. Western blot was performed with mouse monoclonal to TGF-β1 (Abcam, 1 : 500), rabbit monoclonal to E-CA (CST, 1 : 500), rabbit polyclonal to α-SMA (Abcam, 1 : 500), rabbit monoclonal to smad3 (Abcam, 1 : 500), rabbit polyclonal to p-smad3 (CST, 1 : 500), and rabbit polyclonal to smad7 (Epitomic, 1 : 500). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1 : 5000). Western blot analyses were performed at least in triplicate. Densitometry was detected by Image J [26, 27].

2.6. Immunofluorescence Staining. Renal tissue sections were prepared after fixation in 4% (w/v) paraformaldehyde and embedded in paraffin. For semiquantitative analysis, 20 high-power microscope fields of renal tissue were randomly selected. The mean fluorescence activity was analyzed by image-pro plus 6.0 software [28, 29]. Cells at 80% confluence on coverslips were fixed with 4% (w/v) paraformaldehyde. For semiquantitative analysis, 40 high-power microscope fields of cells were randomly selected, and the mean fluorescence activity was analyzed with image-pro plus 6.0 software [28, 29]. Antibodies and dilutions were as follows: rabbit monoclonal to E-CA (CST, 1 : 50), rabbit polyclonal to α-SMA (Abcam, 1 : 50), rabbit polyclonal to FN (Abcam, 1 : 250), and rabbit polyclonal to Col IV (Abcam, 1 : 250). DAPI was used to stain the cell nuclei (blue). Confocal microscope (Leica TCS SP5 MP, Heidelberg GmbH, Germany) was used in this experiment.

2.7. Biochemical Assays and Light Microscopy. Blood was drawn from mice fasting overnight at 24 weeks of age, and mouse metabolic cages were used to collect urine samples of 24 hours. Tissue for light microscopy was fixed in 4% (w/v) paraformaldehyde and then embedded in paraffin. Four-micrometer thick sections of renal tissue were processed for hematoxylin-eosin (HE) and Masson's trichrome staining.

2.8. Statistical Analysis. The results were presented as means ± SD of at least three independent experiments. Statistical analysis was carried out using SPSS17.0. Data were analyzed with the one-way ANOVA. $P < 0.05$ was considered statistically significant.

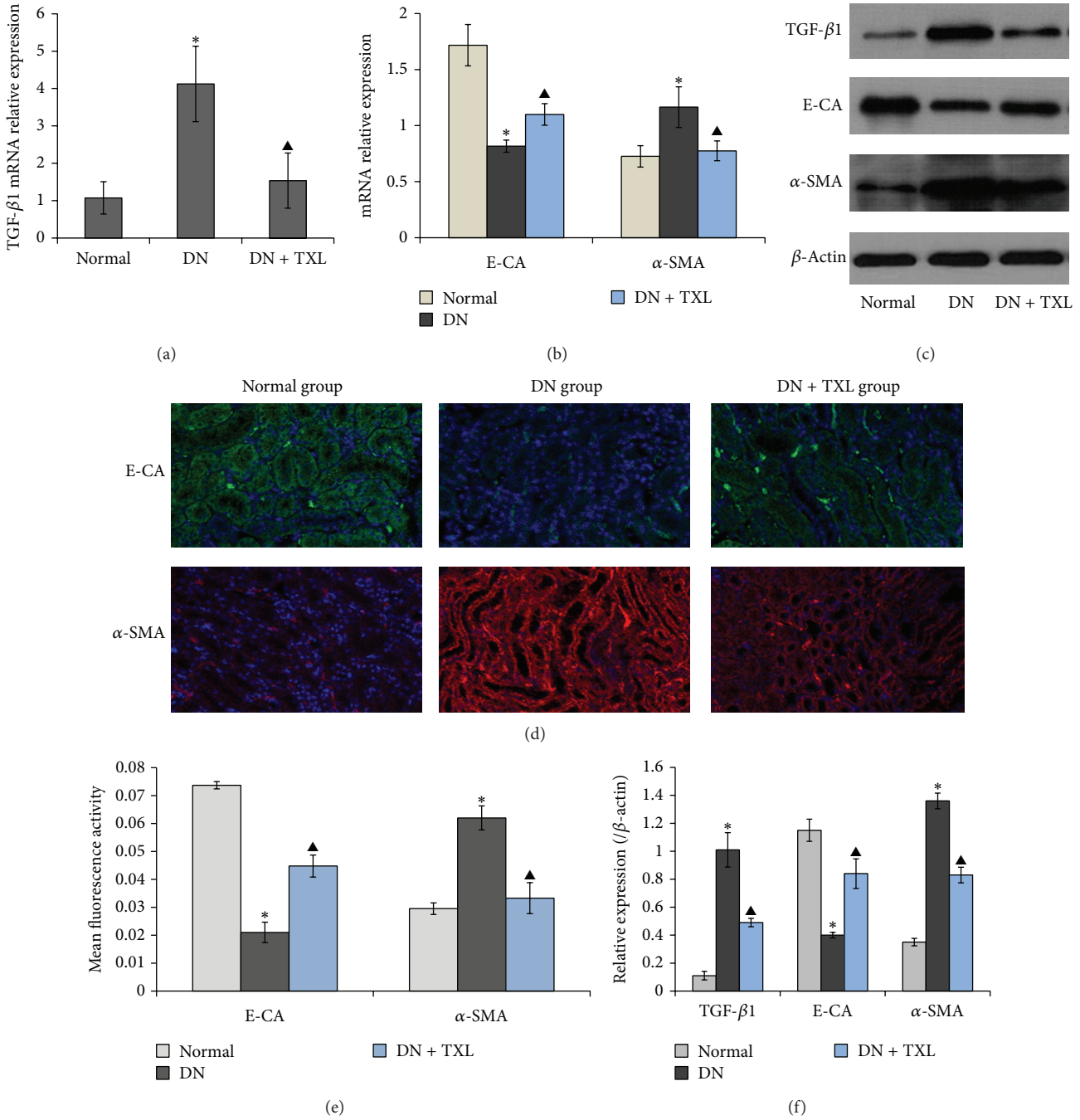

Figure 1: Effects of TXL on the expressions of TGF-β1, E-CA, and α-SMA in renal tissues of KK-Ay mice. (a) mRNA expression of TGF-β1 was determined by RT-PCR with β-actin as an internal control. (b) mRNA expressions of E-CA and α-SMA were determined by RT-PCR with β-actin as an internal control. (c) Representative bands of TGF-β1, E-CA, and α-SMA detected by western blot. (d) Representative immunofluorescence staining photographs of E-CA and α-SMA, visualized by confocal microscope. Images are shown at 20x. (e) Mean fluorescence activity of E-CA and α-SMA analyzed by image-pro plus 6.0 software. (f) Densitometry analysis of TGF-β1, E-CA, and α-SMA bands from (c), normalized to β-actin.

3. Results

3.1. Effects of TXL on TGF-β1 Expression and EMT Markers in Renal with DN. Overwhelming evidences implicate that TGF-β1 acts as the key mediator of tubular EMT [30]. To observe whether TXL affected TGF-β1 expression and markers of EMT in vivo, RT-PCR was first performed to examine the expression of TGF-β1 at 24 weeks of age. RT-PCR results showed that TGF-β1 was significantly enhanced in DN group compared with normal group (Figure 1(a), $^{*}P < 0.05$). After being treated with TXL for 12 weeks, expression of TGF-β1 was significantly decreased compared

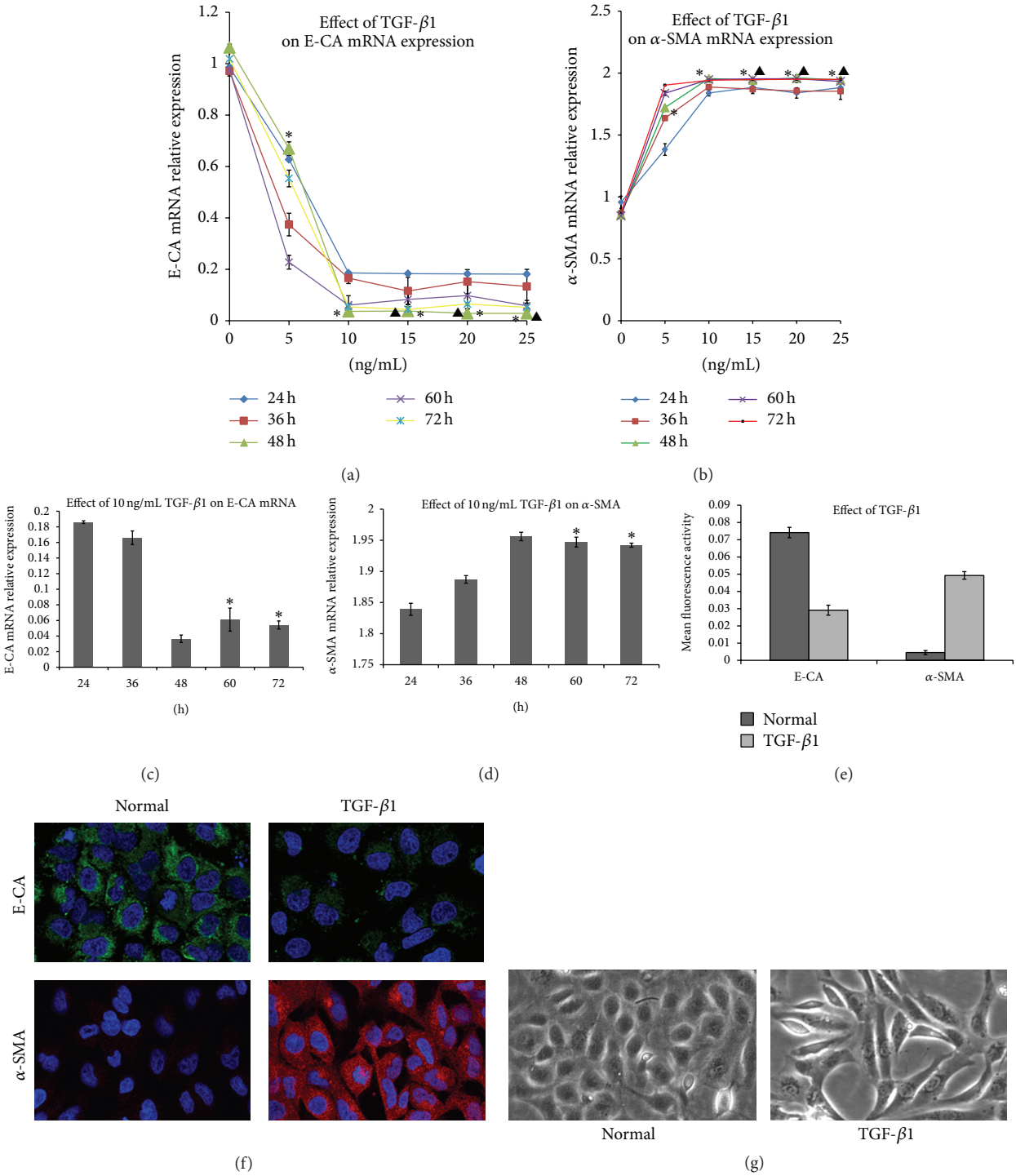

Figure 2: Time-course and dose-response of E-CA and α-SMA mRNA expressions in HKCs induced by TGF-β1. (a) HKCs were treated with five concentrations (5 ng/mL–25 ng/mL) of TGF-β1, and expression of E-CA mRNA was determined by RT-PCR with β-actin as an internal control. (b) Expression of α-SMA mRNA was determined by RT-PCR with β-actin as an internal control. (c) E-CA mRNA expression of cells exposed to 10 ng/mL TGF-β1 from 24 to 72 hours, normalized to β-actin. (d) α-SMA mRNA expression of cells exposed to 10 ng/mL TGF-β1 from 24 to 72 hours, normalized to β-actin. (e) Mean fluorescence activity of E-CA and α-SMA in normal HKCs and cells incubated with 10 ng/mL TGF-β1 for 48 hours. Photographs were analyzed by image-pro plus 6.0 software.(f) Representative immunofluorescence staining photographs of E-CA and α-SMA in normal HKCs and cells incubated with 10 ng/mL TGF-β1 for 48 hours. Images are shown at 40x and visualized by confocal microscope. (g) Representative photographs of phenotype change in HKCs induced by 10 ng/mL TGF-β1 compared with normal cells.

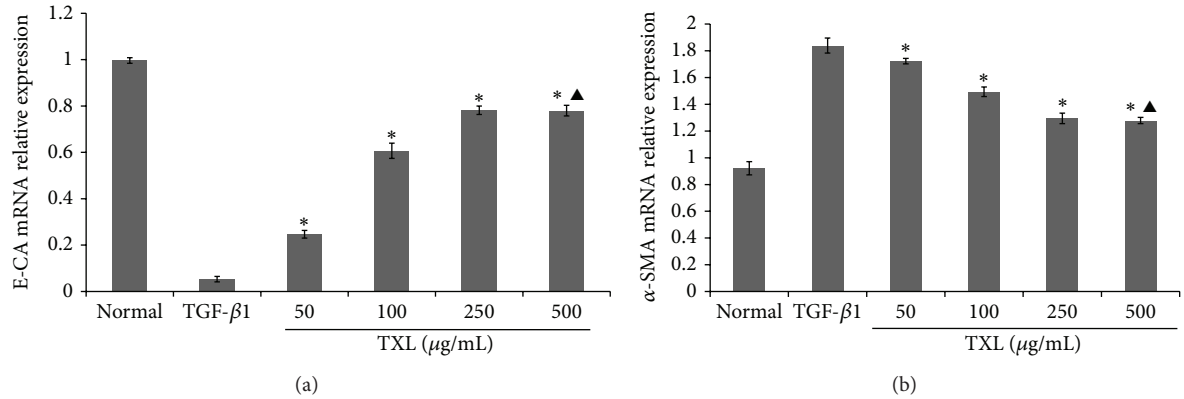

Figure 3: Effects of different concentrations of TXL on the expressions of E-CA and α-SMA in HKCs. (a) Cells were treated with different concentrations of TXL (50–500 μg/mL) for 48 hours, and E-CA mRNA expression was determined by RT-PCR with β-actin as an internal control. (b) Cells were treated with different concentrations of TXL (50–500 μg/mL) for 48 hours, and α-SMA mRNA expression was determined by RT-PCR with β-actin as an internal control.

with DN group (Figure 1(a), $^{\blacktriangle}P < 0.05$). Additionally, Western blot results were consistent with RT-PCR results (Figures 1(c) and 1(f)). TXL suppressed TGF-β1 expression both at mRNA and protein levels. Next, to further verify the effect of TXL on tubular EMT, E-CA and α-SMA were detected by RT-PCR, western blot, and immunofluorescence staining, respectively. RT-PCR and western blot results showed that α-SMA was enhanced and E-CA was decreased in DN group compared with normal group (Figures 1(b), 1(c), and 1(f), $^{*}P < 0.05$). More importantly, TXL significantly decreased α-SMA expression and increased E-CA expression compared with DN group both at mRNA and protein levels (Figures 1(b), 1(c), and 1(f), $^{\blacktriangle}P < 0.05$). Immunofluorescence staining showed the similar results that epithelial marker E-CA was significantly decreased, while α-SMA was increased in DN group compared with normal group (Figures 1(d) and 1(e), $^{*}P < 0.05$). More importantly, TXL treatment significantly restored E-CA and α-SMA expressions (Figures 1(d) and 1(e), $^{\blacktriangle}P < 0.05$). These results demonstrated that TXL can inhibit TGF-β1 expression and EMT in DN.

3.2. TGF-β1 Induces Epithelial-to-Mesenchymal Transition In Vitro. Five concentrations (5 ng/mL–25 ng/mL) of TGF-β1 and five time points (24–72 hours) were applied to observe the effect of TGF-β1 on EMT in vitro. Increase of α-SMA and loss of E-CA are the markers of EMT. RT-PCR and immunofluorescence staining were used to show the changes of EMT markers. RT-PCR showed a dose-dependent manner in the decrease of E-CA mRNA, lowest at 10 ng/mL (Figure 2(a), $^{*}P < 0.05$ versus normal, $^{\blacktriangle}P > 0.05$ versus 10 ng/mL), while α-SMA mRNA was significantly increased and peaked at 10 ng/mL (Figure 2(b), $^{*}P < 0.05$ versus normal, $^{\blacktriangle}P > 0.05$ versus 10 ng/mL). Cells exposed to 10 ng/mL TGF-β1 exhibited a time-dependent manner in the decrease of E-CA mRNA, lowest at 48 hours, while α-SMA mRNA was significantly increased and peaked at 48 hours (Figures 2(c) and 2(d), $^{*}P > 0.05$ versus 48 hours). Additionally, the results of immunofluorescence staining showed that fluorescence activity of α-SMA was obviously enhanced and E-cadherin was decreased in TGF-β1 group compared with normal group (Figures 2(e) and 2(f), $P < 0.05$). TGF-β1 also induced an elongated and fibroblast-like phenotype change in HKCs (Figure 2(g)). These changes suggested that the HKCs had begun to lose the epithelial phenotype and changed to express the myofibroblastic markers.

3.3. Effects of TXL on TGF-β1-Induced Epithelial-to-Mesenchymal Transition In Vitro. To determine whether TXL affects EMT in vitro, markers of EMT (α-SMA and E-CA) were examined by RT-PCR. HKCs were treated with different concentrations of TXL (50–500 μg/mL). 10 ng/mL TGF-β1 was selected to induce EMT. RT-PCR showed that TXL restored E-CA expression in a dose-dependent manner, peaked at 250 μg/mL (Figure 3(a), $^{*}P < 0.05$ versus TGF-β1 group, $^{\blacktriangle}P > 0.05$ versus 250 μg/mL), whereas α-SMA expression was inhibited and lowest at 250 μg/mL (Figure 3(b), $^{*}P < 0.05$ versus TGF-β1 group, $^{\blacktriangle}P > 0.05$ versus 250 μg/mL). TXL positively reversed TGF-β1-induced downregulation of E-CA and upregulation of α-SMA.

3.4. Effects of Tong xinluo on TGF-β1/Smads Signal Pathway In Vivo and Vitro. In EMT, various studies have explored the roles ofTGF-β1 in activating smads [31]. To explore whether TXL affects TGF-β1/smads signal pathway, renal tissue and HKCs were detected by RT-PCR and western blot, respectively. 10 ng/mL TGF-β1 was applied to induce EMT in HKCs. Cells exposed to 10 ng/mL TGF-β1 were set as TGF-β1 group, and cells exposed to 10 ng/mL TGF-β1 and 250 μg/mL TXL were set as TGF-β1 + TXL group. The normal cells cultured only with DMEM/F12 were set as normal group. In vivo, western blot showed that p-smad3/smad3 were remarkably increased and smad7 was decreased in DN group compared with normal group (Figures 4(a) and 4(b), $^{*}P < 0.05$), while p-smad3 and smad3 were significantly decreased and smad7 was increased in TXL group compared

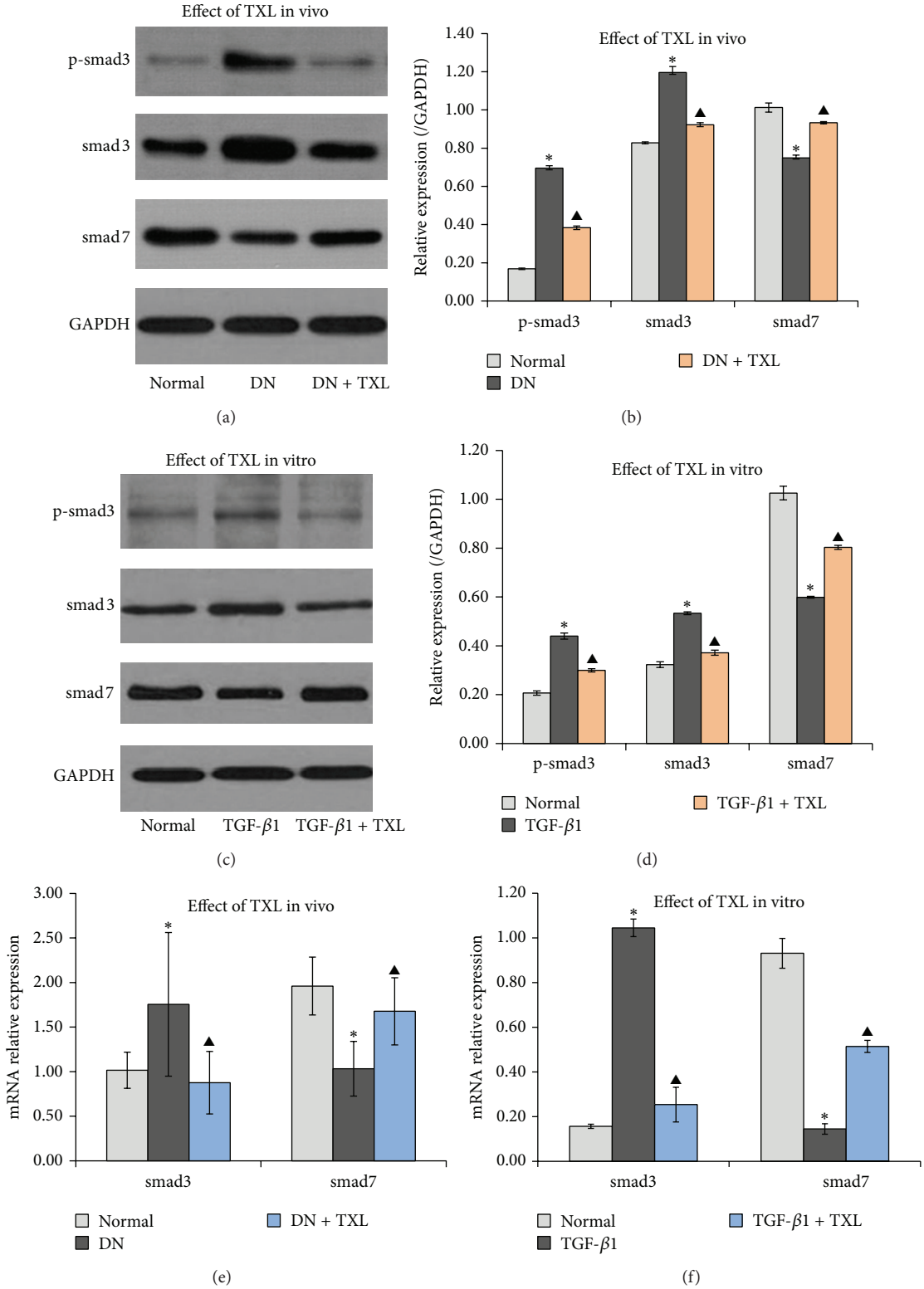

Figure 4: Effects of TXL on the expressions of p-smad3/smad3 and smad7 in both renal tissues and HKCs. (a) Representative bands of p-smad3/smad3 and smad7 detected by western blot of renal tissues. (b) Densitometry analysis of p-smad3/smad3 and smad7 bands from (a), normalized to GAPDH. (c) Representative bands of p-smad3/smad3 and smad7 detected by western blot of HKCs. (d) Densitometry analysis of p-smad3/smad3 and smad7 bands from (b), normalized to GAPDH. (e) Effects of TXL on smad3 and smad7 mRNA expressions in renal tissues, normalized to β-actin. (f) Effects of TXL on smad3 and smad7 mRNA expressions in HKCs, normalized to β-actin.

with DN group (Figures 4(a) and 4(b), $^{\blacktriangle}P < 0.05$). RT-PCR results were consistent with western blot results (Figure 4(e)). Additionally, in vitro, western blot and RT-PCR also showed the significant increase of p-smad3 and smad3 in TGF-β1 group compared with normal group. In contrast, smad7 was significantly decreased (Figures 4(c), 4(d), and 4(f), $^{*}P < 0.05$). Importantly, p-smad3/smad3 were remarkably decreased and smad7 was increased in the TGF-β1 + TXL group compared with TGF-β1 group (Figures 4(c), 4(d), and 4(f), $^{\blacktriangle}P < 0.05$). Thus, the present results showed that TXL positively deceased p-smad3/smad3 expressions and increased the expression of smad7.

3.5. Prevention of Enhanced Collagen IV and Fibronectin Expressions by Tong xinluo. EMT paves the way for extracellular matrix (ECM) deposition and ultimately renal fibrosis [32]. To explore the effect of TXL on ECM, RT-PCR was used to detect the changes of collagen IV (Col IV) and fibronectin (FN) mRNA. In vivo, results showed that Col IV and FN mRNA were markedly increased in DN group compared with normal group (Figure 5(a), $^{*}P < 0.05$), while Col IV and FN mRNA were significantly decreased in TXL group compared with DN group (Figure 5(a), $^{\blacktriangle}P < 0.05$). Meantime, results of immunofluorescence staining also exhibited that Col IV and FN proteins were observably increased in DN group compared with normal group (Figures 5(c) and 5(d), $^{*}P < 0.05$). Importantly, TXL successfully decreased the expressions of Col IV and FN protein (Figures 5(c) and 5(d), $^{\blacktriangle}P < 0.05$). Furthermore, in vitro, results showed that TXL significantly decreased the expressions of Col IV and FN mRNA compared with TGF-β1 group (Figure 5(b), $^{\blacktriangle}P < 0.05$). Immunofluorescence staining of HKCs showed the same results as RT-PCR (Figures 5(e) and 5(f), $^{\blacktriangle}P < 0.05$). Importantly, the results of Col IV and FN expressions in vitro were consistent with the results in vivo. The present results suggested that TXL may have the function of ameliorating ECM deposition.

3.6. Effects of TXL on 24 h-UAER, BUN, and Creatine Clearance Ratio and Morphological Changes. Elevated urine albumin excretion ratio (UAER) is a key risk factor for renal and cardiovascular disease in type 2 diabetes [33]. Creatinine clearance ratio (Ccr) is generally taken as an evaluation of renal filtration function. Renal morphology was observed by light microscopy at 24 weeks of age. Compared with the normal group, 24 h-UAER and blood urea nitrogen (BUN) in DN group were significantly increased, while Ccr was decreased (Figures 6(b), 6(c), and 6(d), $^{*}P < 0.05$). Interestingly, 24 h-UAER and BUN were decreased and Ccr was increased in TXL group compared with the DN group (Figures 6(b), 6(c), and 6(d), $^{\blacktriangle}P < 0.05$). HE staining showed the vacuoles degeneration of renal tubular epithelial cells in DN group and TXL partly ameliorated it. Masson staining exhibited the amount of collagen fibers and TXL positively mitigated these pathological variations compared with DN group (Figure 6(a)). The present results suggested that TXL exerted its positive effect in DN via decreasing UAER/BUN and increasing Ccr. Furthermore, it ameliorated renal structure.

4. Discussion

Diabetic nephropathy (DN), a leading cause of mortality in diabetic patients, affects approximately one-third of all diabetic patients and causes a heavy economic burden for them [34]. The crucial pathology underlying DN is interstitial fibrosis [25]. EMT of tubuloepithelial cells is a widely recognized mechanism that sustains interstitial fibrosis in DN [35]. EMT can be induced or regulated by various growth and differentiation factors, including TGF-β1 and connective tissue growth factor (CTGF). Among these, TGF-β1 has received much attention as a major inducer of EMT during fibrosis [31]. In the present study, RT-PCR results exhibited that TGF-β1 expression was significantly enhanced in DN group and pointed to the crucial role of TGF-β1 in DN. The present result was consistent with previous reports [36, 37]. Additionally, we chose to examine several well-established typical events of EMT in renal tissue and renal tubuloepithelial cells that have been widely used in the study of TGF-β1-induced EMT [25, 38]. Accompanied with the increase of TGF-β1 expression, the expression of E-CA was significantly decreased while α-SMA was elevated in DN group. In cell experiment, TGF-β1 evoked EMT in HKCs was obtained by assessment of morphological changes, increased expression of α-SMA, and down-regulation of the epithelial marker E-cadherin. More importantly, we present novel findings that TGF-β1 is able to induce EMT in a dose and time dependent manner. Cells exposed to 10 ng/mL TGF-β1 for 48 hours were used in vitro experiment to induce EMT.

Chinese medicine Tong xinluo (TXL) is extracted from a group of herbal medicines including *Panax ginseng* C. A. Mey. extract, *Paeonia lactiflora* Pall. extract, and *Borneolum syntheticum* [39]. Some active components extracted from *Panax ginseng* significantly suppressed TGF-β expression [17]. *Paeoniflorin* can exert antifibrogenic effects by down-regulating smad3 expression and phosphorylation through TGF-β1 signaling [40]. According to the potentially advantageous regulation of cell signaling events by components of TXL in kidney [16, 18], we tried to explore whether TXL could inhibit EMT in DN. Importantly, we now demonstrate that TXL strikingly inhibits TGF-β1 expression in DN and also inhibits TGF-β1-induced morphological and phenotypic changes in HKCs. RT-PCR results showed that TXL greatly decreased α-SMA mRNA expression and increased E-CA mRNA expression. The inhibition of E-CA downregulation and α-SMA upregulation seen in the current studies was maximal with 250 μg/mL concentration of TXL. This pharmacological mechanism of TXL inhibiting EMT is absolutely in accordance with previous studies; Hung et al. demonstrated that counteract TGF-β1 and its downstream signal transducers, like smad3 and smad7, may be an effective therapy in inhibiting EMT [24, 41]. The present study also showed that TXL is able to negatively modulate TGF-β1 signaling at several levels. Having demonstrated that TXL blocked TGF-β1 expression and TGF-β1-evoked changes in the cell

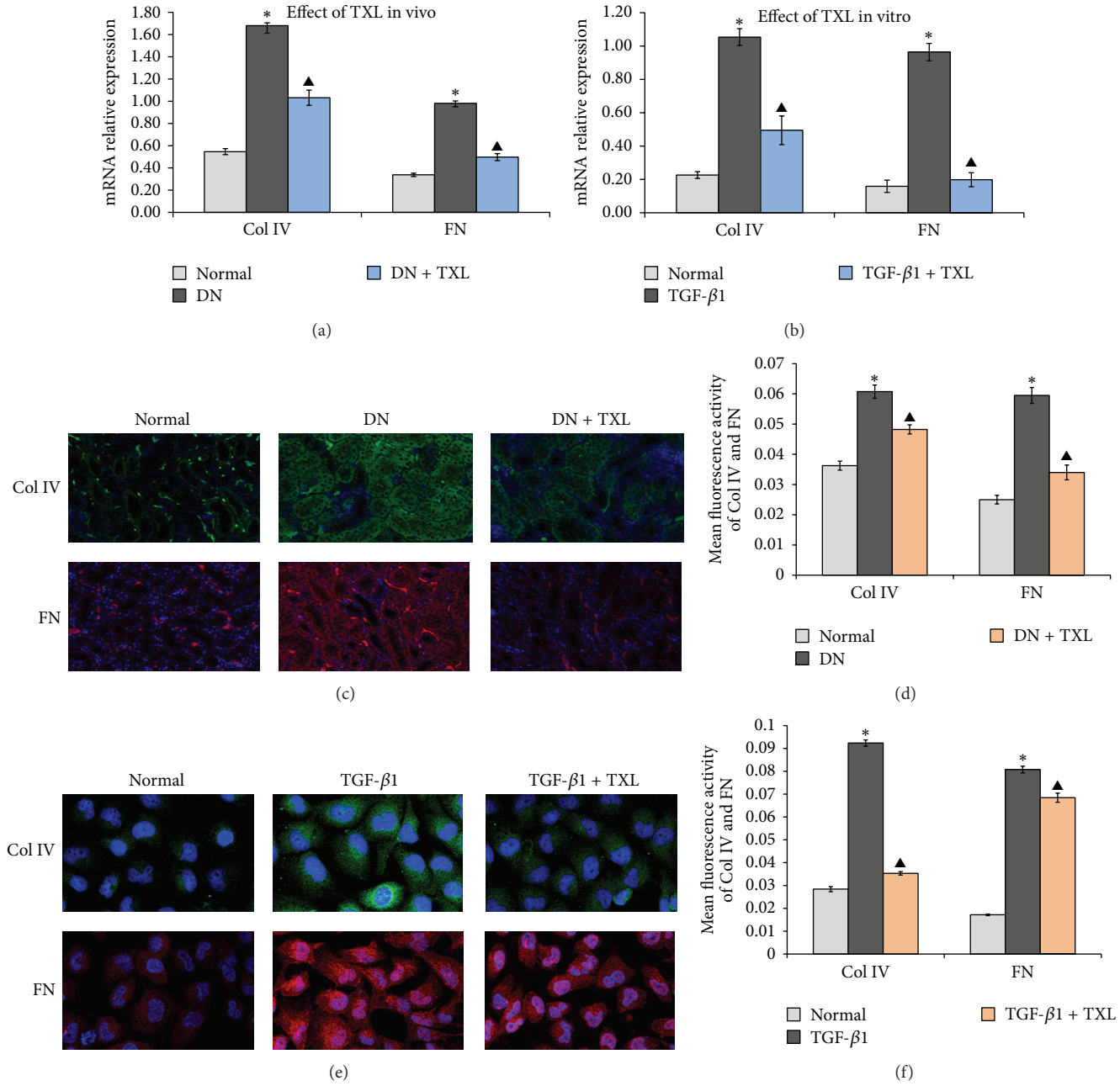

Figure 5: Effects of TXL on the expressions of Col IV and FN. (a) Col IV and FN mRNA expressions were detected by RT-PCR in vivo, normalized to β-actin. (b) Col IV and FN mRNA expressions were detected by RT-PCR in vitro, normalized to β-actin. (c) Representative photographs of Col IV and FN by immunofluorescence staining in renal tissues. Images are shown at 20x. (d) Mean fluorescence activity of Col IV and FN in renal tissues. Photographs were analyzed by image-pro plus 6.0 software. (e) Representative photographs of cells stained with primary antibody against Col IV and FN together with DAPI (blue). Images are shown at 40x. (f) Mean fluorescence activity of Col IV and FN detected by immunofluorescence staining. Photographs were analyzed by image-pro plus 6.0 software.

phenotype, we examined the ability of TXL to interfere with these TGF-β1 signaling elements. TGF-β1 caused upregulation of smad3 and p-smad3 expressions, while decreased smad7 expression. More importantly, the changes of smads were restored to a greater or lesser extent by TXL, reflected in the decrease of smad3/p-smad3 expressions and the increase of smad7 expression in TXL group. Previous works had demonstrated the involvement of smads in TGF-β1-mediated EMT and proven the positive effect of regulating these smads in inhibiting EMT. TXL appears to inhibit EMT via decreasing TGF-β1-induced smad3 and p-smad3 expression, which is consistent with the previous studies [24, 42]. smad7 was a negative regulator of TGF-β1-induced EMT [43]. In the present study, western blot and RT-PCR results showed that

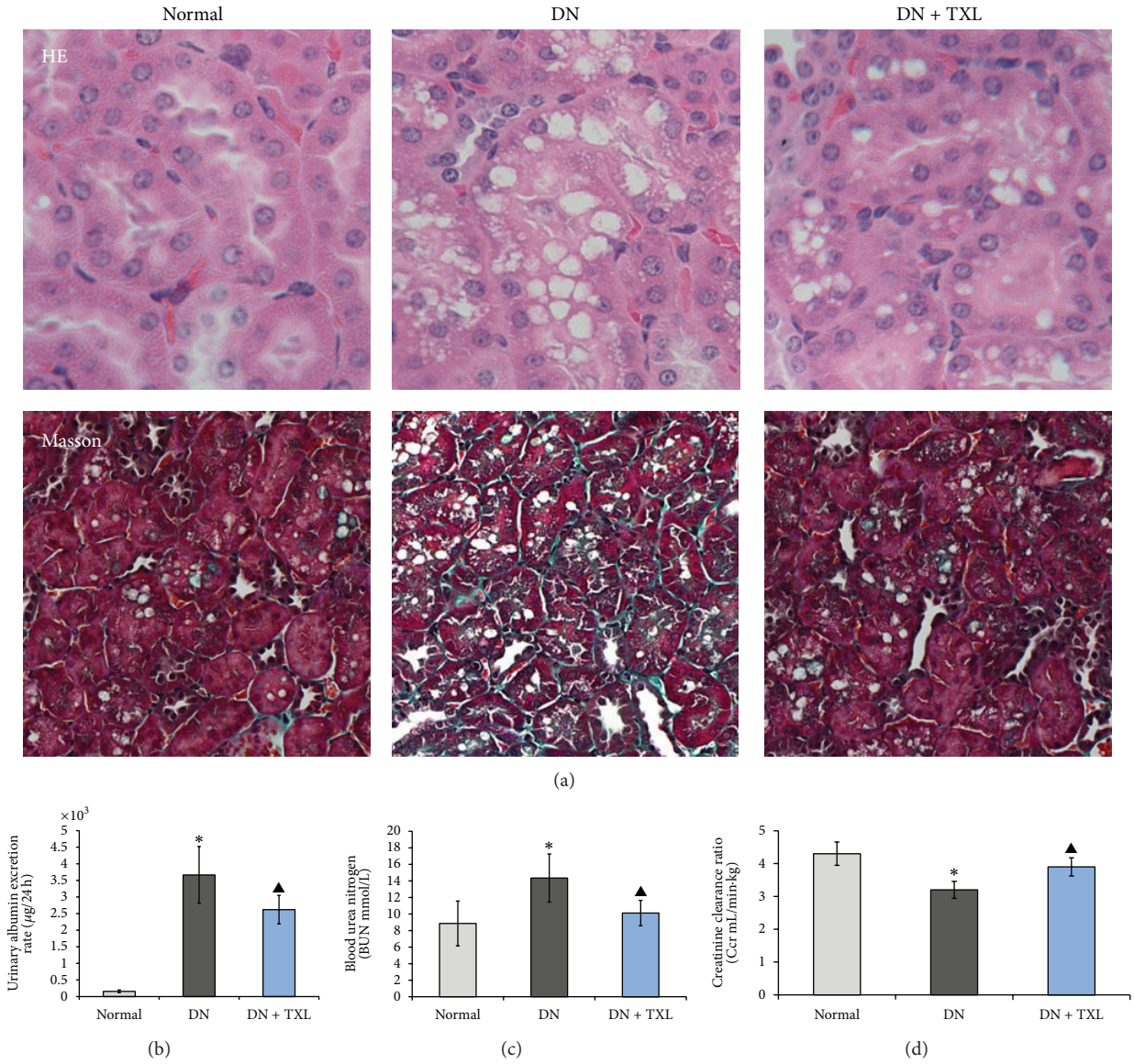

Figure 6: Effects of TXL on renal function and structure. (a) HE staining and Masson staining of renal tissues. HE staining showed the vacuoles degeneration of renal tubular epithelial cells in DN group and TXL partly ameliorated it. Masson staining exhibited the deposition of collagen fibers in DN group and TXL effectively alleviated it. (b) Effect of TXL on urine albumin excretion. (c) Effect of TXL on BUN. (d) Effect of TXL on creatinine clearance ratio.

TXL significantly increased smad7 expression both in vitro and in vivo. The most possible reason of TXL inhibiting EMT may be that TXL represses TGF-β1 expression and blocks downstream signaling cascades of TGF-β1 in HKCs. Taken together, the present results demonstrate that TXL may be a positive therapy in inhibiting TGF-β1-induced EMT. These results further support TXL to be of therapeutic potential in treating diabetic nephropathy via inhibiting EMT.

Excessive deposition of extracellular matrix (ECM) is a histological hallmark of DN, which is closely related to the progressive decline of renal function [44]. *Panax ginseng* is the primary component of TXL; researches showed that diabetes-induced upregulations of ECM proteins in the kidneys were significantly diminished by *Panax ginseng* administration; furthermore, albuminuria in the diabetic mice was prevented [45]. However, whether TXL ameliorated renal structure and function by inhibiting ECM accumulation in TGF-β1-induced EMT remains unclear. The present findings indeed supported the notion that TXL suppressed expressions of Col IV and FN both at mRNA and protein levels. In this sense, present results may account for part of the mechanisms that TXL improve renal structure and function. Indeed, we observed a significant decrease of 24 h-UAER and BUN, while Ccr was increased in TXL group, as compared to untreated diabetic mice. These findings suggest that administration of TXL may contribute to tubular repair and may accelerate the decay in renal function observed in the diabetic condition. The current results demonstrate that

TXL successfully ameliorates renal structure and function, and its novel therapeutic potential in diabetic nephropathy is highly attractive.

5. Conclusion

In summary, the present data provides a new perspective on the molecular effects of TXL on DN by showing that TXL treatment inhibits TGF-β1-induced EMT. But more basic researches and clinical studies are needed to future investigate the positive role of TXL in protecting DN. Moreover, the present data inspires further study to explore the effects and mechanisms of Chinese herbal compounds, which may have important therapeutic applicability in DN.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgment

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Research Article

Effect of "Jian-Pi-Zhi-Dong Decoction" on Gamma-Aminobutyric Acid in a Mouse Model of Tourette Syndrome

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The purpose of this study was to explore the positive effects of Jian-Pi-Zhi-Dong Decoction (JPZDD) on Tourette syndrome (TS) by investigating the expression of gamma-aminobutyric acid (GABA) and its type A receptor ($GABA_AR$) in the striatum of a TS mice model. The model was induced by 3,3′-iminodipropionitrile (IDPN) treatment; then mice were divided into 4 groups (n=22, each); control and IDPN groups were gavaged with saline and the remaining 2 groups were gavaged with tiapride and JPZDD. We recorded the stereotypic behaviors of TS mice and measured the content of GABA in striatum by HPLC and $GABA_AR$ expression by immunohistochemistry and real-time PCR. Our results showed that JPZDD inhibited the abnormal behaviors of TS model mice and decreased GABA levels and $GABA_AR$ protein and mRNA expression in the striatum of TS model mice. In brief, the mechanism by which JPZDD alleviates TS symptoms may be associated with $GABA_AR$ expression downregulation in striatum which may regulate GABA metabolism.

1. Introduction

Tourette syndrome (TS) is a developmental neurobehavioral disorder characterized by stereotypic, involuntary, repetitive movements and noises, referred to as chronic (more than one year) motor and vocal tics, respectively [1]. TS usually starts in childhood and tends to peak at onset of puberty and improve by the end of adolescence [2]. In addition, TS patients also show behavioral and emotional disturbances including symptoms of attention deficit hyperactivity disorder (ADHD), obsessive compulsive disorder (OCD), and pervasive developmental disorder [3].

Until now, the etiological and pathophysiological mechanism of TS remained unclear. It is generally agreed that the basal ganglia, including circuits that link the striatum to cortex, are dysfunctional [4]. The basal ganglia form a network of interconnected subcortical structures, including the striatum, globus pallidus, substantia nigra, and subthalamic nucleus. In the cortico-striato-thalamocortical (CSTC) circuit, the cerebral cortex provides the main input to the striatum; then the striatum relays information to the globus pallidus and substantia nigra through two pathways: one excitatory (direct pathway) and one inhibitory (indirect pathway). Malfunction of these circuits may contribute to the behaviors that manifest as tics. For a long time, abnormal dopaminergic activity in the synaptic cleft has been considered the most important factor in TS [1]. Most scholars postulate that hyperactivity of dopaminergic neurons and/or dopamine receptors underlie the pathophysiology of TS [5–7]. However, the results obtained in these previous studies were inconclusive. Both DA and GABAergic neurons are crucial for the maintenance of basal ganglia circuits. Therefore, other imbalances, such as in glutamatergic or GABAergic metabolism, seem probable in TS [8]. Recently, it was proposed that disrupted excitatory/inhibitory balance in key circuits might underlie many neurodevelopmental disorders including TS [9]. Dopaminergic and GABAergic systems interact in the basal ganglia system; GABAergic neurons in corticostriatal pathways have been reported to either directly or indirectly activate the dopaminergic system

[10]. Clinical studies also showed that metabolic disturbance of GABA was involved in the pathogenesis of TS [11, 12].

The amino acid neurotransmitter GABA is the principal inhibitory neurotransmitter in the central nervous system (CNS). GABA regulates brain excitability via its $GABA_A$ receptors [13]. GABA receptors are key targets for drug design to treat various tics, and $GABA_A$ receptor systems may play a major role in the pathophysiology of TS [14]. Levetiracetam, with a typical enhancing activity at $GABA_A$ receptors, could be of interest for treatment of tics [15]. $GABA_A$ receptors are composed of five subunits that together comprise more than 19 different classes, including α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3 [16]. The $GABA_A$ alpha 4 receptor was predicted to be spliced in the pathophysiology of TS and tics [12].

A previous study has proven that Jian-Pi-Zhi-Dong Decoction (JPZDD) can effectively inhibit abnormal behavior in a TS mouse model and increase the level of dopamine transporter in the striatum [17]. However, this study did not explain the phenomenon of higher expression of DAT in some TS patients and does not completely explain the pathophysiology. We therefore hypothesized that an abnormality in the function of CSTC circuits could also be caused by pathological changes in GABA and its receptors. The present study aimed to explore the possible mechanism of JPZDD on the GABAergic system of a TS mouse model, in particular the $GABA_A$ receptor.

2. Materials and Methods

2.1. Drugs and Reagents. 3, 3′-Iminodipropionitrile (IDPN) was purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA), and tiapride (Tia) from Jiangsu Nhwa Pharmaceutical Co., Ltd. (Xuzhou, Jiangsu, China). Gamma-aminobutyric acid (GABA, 5835) and o-phthaldialdehyde (OPA, P0657) were purchased from Sigma, beta-mercaptoethanol (βME, 0482) from Amresco Co., LLC. (Solon, OH, USA), anti-$GABA_A$ receptor alpha 4 antibody (primary antibody, 1 : 100) from Abcam Co., LLC. (Hong Kong, China), and poly-HRP anti-rabbit IgG secondary antibody (PV-9001) from ZSGB-BIO Co. (Beijing, China).

2.2. Preparation of JPZDD. Ten different Chinese medicinal herbs were included in the JPZDD. They were purchased from the Pharmaceutical Department at Dongfang Hospital affiliated to Beijing University of Chinese Medicine (BUCM). Director Qing-chun Hao identified components, and the voucher specimens were deposited. JPZDD contains ten ingredients: 10 g *Pseudostellaria heterophylla*, 10 g *Atractylodes macrocephala* Koidz, 10 g *Poria cocos* Wolf, 6 g *Pinellia ternata* Breit, 6 g *Citrus reticulata* Blanco, 6 g *Saposhnikovia divaricata* Schischk, 3 g *Gentiana scabra* Bge, 10 g *Angelica sinensis* Diels, 6 g *Ligusticum chuanxiong* Hort, and 10 g *Uncaria rhynchopylla* Jacks. All herbs were soaked for 1 h at room temperature and decocted with distilled water for 2 h. The filtrate was condensed and dried at 60°C using a vacuum-desiccator. The extracted granules were analyzed by infrared fingerprint compared with standards to guarantee a qualified rate of more than 90% and then packaged and stored at room temperature.

2.3. Animals. We used eighty-eight male ICR mice, weighing 18 ± 2 g, aged 4 weeks, purchased from Vital Rive Laboratories, Beijing, China (no. SCXK 2012-0001). All animal experimental protocols conformed to the Animal Management Rules of the Chinese Ministry of Health, and the study was approved by the Animal Ethics Committee of the Chinese Academy of Medical Sciences. Animals were kept in a standard animal feeding room with a room temperature of 21 ± 1°C and relative humidity of 30% to 40%, on a 12 h light-dark cycle (light for 12 h: 07:00 to 19:00 and darkness for 12 h: 19:00 to 07:00) and ad libitum access to purified water. The mice were fed for 1 w before generating the TS model. After one week, the mice were randomly divided into a saline group (control group, $n = 22$) and a TS model group ($n = 66$). The saline group was injected (i.p.) with 0.9% saline (15 mL kg^{-1}); the TS model group was injected (i.p.) with IDPN (350 mg kg^{-1}) once a day for 7 consecutive days. According to the behavioral measurements used for evaluating grades of stereotype, we made behavioral recording of the TS model group, as described by Wang et al. [17]. The TS mouse model group was further divided into 3 groups: IDPN model group ($n = 22$), IDPN+Tia group ($n = 22$), and IDPN+JPZDD group ($n = 22$). The saline and IDPN group were gavaged with saline (0.9%) at 20 mL Kg^{-1}, the IDPN +Tia group with Tia at 50 mg kg^{-1}, and the IDPN+JPZDD group with JPZDD at 20 g kg^{-1} once a day for 6 consecutive weeks. Behavioral recordings were conducted by 2 trained observers who were familiar with the measurements but blind to the group condition. Each animal was observed for 2 min [18], and the average score was calculated. On the last day of the trial, the mice were euthanized by cervical dislocation.

2.4. High Performance Liquid Chromatography (HPLC). The dorsolateral striatum from both the left and right sides ($n = 10$, per group) was dissected and frozen at −80°C. Brain tissue samples were weighed and then homogenized in 700 μL of ice-cold lysis buffer [19], containing o-phthalaldehyde 27 mg, anhydrous ethanol 1 mL, tetraborate buffer 9 mL, and β-mercaptoethanol 5 μL.

The homogenate was centrifuged at 14000 rpm for 15 min at 4°C and filtered through a 0.22 μm filter (Costar, Spin-X) and then centrifuged at 7000 rpm. Standard solution or the filtrate obtained from brain homogenate (20 μL) was injected into the HPLC. Chromatographic conditions were as follows. The precolumn was Shiseido (Guard Cartridge, Capcell C18 MG S-5, 4.0 × 10 mm). The chromatographic column was Waters XTerra MS (3.0 × 50 mm, 2.5 um, Part no. 186000598). The mobile phase was composed of 100 mM disodium hydrogen phosphate, 25% methanol, and 10% acetonitrile (pH 6.70). The flow rate was 0.6 mL/min. The column oven was held constant at 40°C. Working solutions of GABA (40, 20, 8, 4, 2, 1, and 0.5 μg/mL) were used. The calibration curve of each compound was determined by plotting the ratio of peak area to internal standard versus concentration of the spiked

standard solution. A linear regression equation ($y = ax + b$) was evaluated, where x is the concentration of the analytes and y is the peak area ratio. The correlation coefficient (R^2) was calculated.

2.5. Immunohistochemistry. Mice ($n = 6$, per group) were perfused and fixed with 4% PFA after anesthetizing with 10% chloral hydrate. The brains were removed and postfixed in 4% PFA for 14 h and then embedded in paraffin. The embedded brains were cut into 3 μm sections. After dewaxing, rehydration, and blocking, the sections were incubated with an alpha 4 primary antibody (ab4120, Abcam, Hong Kong) at a dilution of 1 : 100 overnight at 4°C [20]. After washing with PBS three times, the tissues were incubated with secondary antibody (PV-9001, ZSGB-BIO, China) for 30 minutes. Sections were visualized by incubating with by DAB (1 : 20) for 5 min and then coverslipped using neutral balsam. Six visual fields were chosen randomly from bilateral stained striatum under an upright microscope at 20 x magnification. The optical density (OD) of each field was calculated using the Image-Pro Plus 6.0 analysis system. The values of $GABA_A$ receptor alpha 4 protein in the striatum were measured in every three brain slices of each mouse.

2.6. Real-Time PCR. The dorsolateral striatum from both the left and right sides ($n = 6$, per group) was dissected and frozen at −80°C. Total RNA from the striatum was isolated with trizol reagent according to the manufacturer's protocol (Tiangen Biotech, Beijing, China). GABA alpha 4 primers were as follows: forward primer: 5′-GCTGACAGAGGGAAATAAATAAAG-3′ and reverse primer 5′-TGGATGATTCTGGTAGAGTGGG-3′. Beta actin primers were as follows: forward primer 5′-GCCTTCCTTCTTGGGTATGGAA-3′ and reverse primer 5′-CAGCTCAGTAACAGTCCGCC-3′. The basic protocol for real-time PCR was an initial denaturation at 95°C for 10 min, followed by 45 cycles of amplification. For cDNA amplification, the cycles consisted of denaturation at 95°C for 10 min, annealing at 95°C for 15 s, and elongation at 60°C for 60 s. The SYBR green signal was detected using IQ5 real-time PCR machine (Bio-Rad, Hercules, CA, USA). PCR products were analyzed by gel electrophoresis and melting curve analysis to confirm specific amplifications. mRNA expressions were normalized using β-actin. Transcript levels were quantified using the $\Delta\Delta Ct$ value method.

2.7. Statistical Analysis. Results were expressed as the mean ± S.E.M. The significance of differences was examined using ANOVA, followed by Student-Newman-Keuls (SNK) test. In all tests, the criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Behavior Study. IDPN could induce stereotyped behavior significantly without intervention compared with the saline control group. TS mouse model induced by IDPN showed abnormal stereotypes in different degrees. After the IDPN injection, we divided the TS mouse model into three groups according to the score to ensure that there was no statistical significance among these groups, and then we gave the different treatment. The IDPN+JPZDD and IDPN+Tia groups showed a significant decrease in severity of stereotyped behavior before and after treatment ($P < 0.05$), but the score of stereotypes for the IDPN group had no statistical significance ($P > 0.05$) (Figure 1).

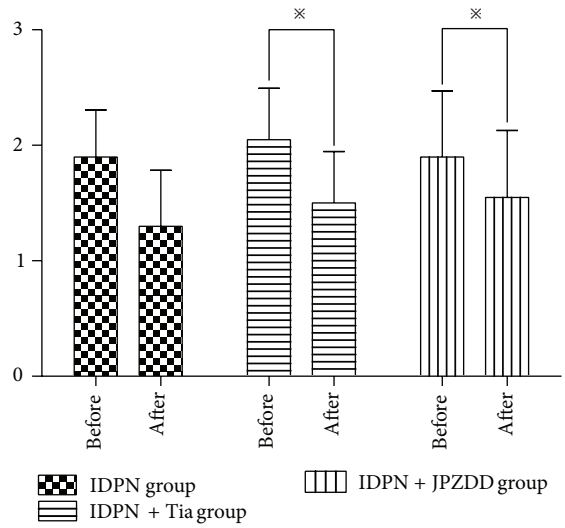

Figure 1: Evaluations of stereotypy of TS mice model in different groups before and after the 6 weeks of treatment. The stereotypy score decreased in all groups, but a significant effect of treatment was observed in the IDPN+Tia group and the IDPN+JPZDD group. Data were shown as mean ± SEM ($n = 22$ mice/group); ※ represents P values <0.05.

3.2. Content of GABA in Striatum by HPLC. Content of GABA in striatum was measured by HPLC. At the end of the treatment, the level of GABA in the IDPN group (261.24 ± 47.31) and IDPN+Tia group (258.57 ± 57.64) was significantly higher than the saline group (200.54 ± 23.94) ($P < 0.05$). The content of GABA in the IDPN+JPZDD group (220.90 ± 48.30) was lower than the IDPN group ($P < 0.01$) (Figure 2).

3.3. Level of $GABA_AR$ in Striatum by IHC. To further investigate the activity and quantity of $GABA_AR$ in the striatum, $GABA_AR$ protein expression was assessed by immunohistochemistry (Figure 3(a)). $GABA_AR$ protein in IDPN group was higher in the IDPN group compared with the saline group ($P < 0.01$). Moreover, IDPN+JPZDD decreased the abundance of GABA, and a notable improvement was observed in JPZDD treated mice compared to the saline group ($P < 0.05$). Tia had the same effect on the expression of $GABA_AR$ protein in striatum as JPZDD ($P > 0.05$) (Figure 3(b)).

3.4. Expression of $GABA_AR$ mRNA in the Striatum. Real-time quantitative PCR was used to measure the level of $GABA_AR$ mRNA in mouse striatum. A standard curve was drawn for $GABA_AR$ alpha 4 and β-actin genes. Melting curve

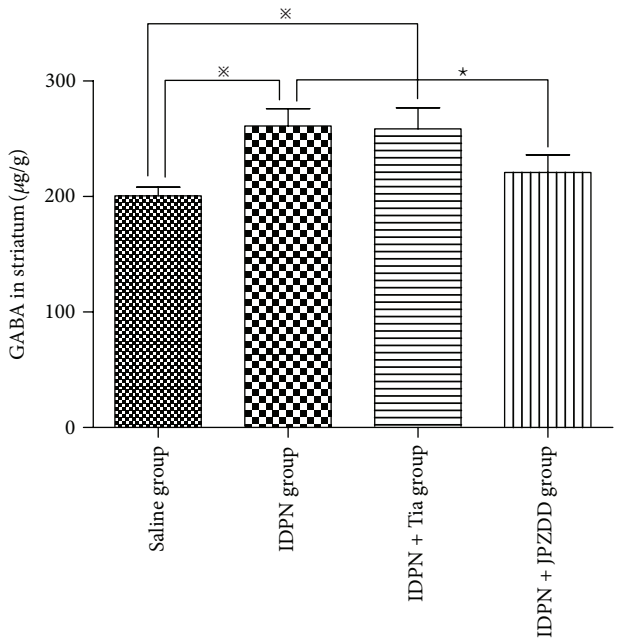

Figure 2: HPLC to evaluate the content of GABA in striatum. The bar graphs represent data from 40 different animals. Data were shown as mean ± SEM (n = 10 mice/group). ※ represents P values <0.05; ★ represents P values <0.01.

analysis confirmed that there were no primer dimers in the PCR products. Results show that $GABA_AR$ mRNA expression in the IDPN+JPZDD group, IDPN group, and IDPN+Tia group was higher than the control group, but there was no significant difference between these groups ($P > 0.05$) (Figure 4).

4. Discussion

In traditional Chinese medicine (TCM), TS is classified as chronic infantile convulsion or tugging and slackening. Its basis pathogenesis is excessive energy of the liver and deficiency of the spleen. Based on this theory, we hypothesized that deficiency of the spleen could cause spleen phlegm and excessive energy of the liver could cause liver wind. The liver wind agitated and spleen phlegm obstructed the channels. According to this hypothesis, the principle of treatment is strengthening the earth (spleen) and suppressing the wood (liver). Under this guide, we created a recipe named JPZDD that included two ancient formulas of Liu-Jun-Zi-Tang (LJZT) and Xie-Qing-Wan (XQW). The main function of XQW is suppressing the liver wind, while LJZT can strengthen the spleen. The herbs in JPZDD were strictly based on the compatibility theory of TCM. The chief drugs in JPZDD are *Pseudostellaria heterophylla* Pax, which nourishes Qi, and *Gentiana scabra* Bge, which purges liver fire to cease liver wind. *Poria cocos* Wolf and *Atractylodes macrocephala* Koidz may invigorate the spleen-qi and *Citrus reticulata* Blanco and *Pinellia ternata* Breit may eliminate the phlegm; these four herbs are adjuvant herbs. *Saposhnikovia divaricata* Schischk and *Uncaria rhynchopylla* Jacks may clear away the liver fire and cease liver wind. *Ligusticum chuanxiong* Hort and *Angelica sinensis* Diels may nourish the liver blood and harmonize the liver wind; these are assistant herbs.

Our previous studies have demonstrated that JPZDD was an effective decoction, which could reduce the times of spontaneous hyperkinesis [21, 22]. At the same time, we have focused on the JPZDD regulation of monoamine neurotransmitters and their metabolites in TS mice. A tenfold dosage was given and satisfactory results were achieved. By IHC and in situ hybridization, we found that JPZDD could increase the expression of DAT protein and mRNA in varying degrees. Its antitic effect might partly be attributed to the synergistic interactions consisting of antioxidation and cognitive improvement [17].

In this study, we chose to use the TS mouse model described by Wang et al. [17]. This model is induced by IDPN (3, 3-iminodipropionitrile). This mouse model displays a series of stereotyped behaviors, such as lateral and vertical head twitching, circling, sniffing, and glomming. These symptoms last at least 2 months without any intervention. Our behavioral studies have further verified the stability of this model. There were no statistical differences in the saline and IDPN groups while there were statistical differences in the INPN+JPZDD and IDPN+Tia groups before and after the treatment. Therefore, we infer that JPZDD could improve the symptoms of TS.

As is well known, many amino transmitters are involved in the CSTC circuits, including GABA and GLU. GABA is the major inhibitory neurotransmitter in mammalian brains. GABA is produced by GABAergic neurons and released at GABAergic synapses formed between GABAergic neurons and their targets [23]. Many GABAergic interneurons of the cerebral cortex migrate tangentially from the same embryonic regions in the ganglionic eminence and give rise to the GABAergic medium spiny projection neurons of the striatum [24]. GABAergic interneurons were shown to play a key role in regulating the development of the cortex, striatum, cerebellum, and hippocampus [25, 26]. Postmortem analysis of TS patients showed decreased numbers of PV positive GABAergic interneurons in striatum and increased numbers in the globus pallidus [27, 28]. Clinical study had also shown that several GABA and Ach-related genes were involved in the pathophysiology of TS and tics [12]. Our experimental results showed that the content of GABA was higher in IDPN, IDPN+Tia, and IDPN+JPZDD groups than in the control group. This result contradicted Lerner's study [14]. We inferred that GABA was produced by glutamic decarboxylation and the relative increase in the Glu activity exerted neurotoxic effect, so the content of GABA increased along with the increase of Glu to keep the new balance. A previous study also showed that high concentrations of Glu increased the extracellular pool of taurine and glycine and then increased extracellular GABA [29, 30].

The rapid inhibitory responses characteristic of GABAergic transmission are mediated by the activation of $GABA_AR$ receptors [31]. Dysfunction involving the $GABA_AR$ system may play a major role in the pathophysiology of TS [1]. Baclofen, a synthetic GABA analogue, exerted antispasmodic effects and had been found to benefit children with TS [32,

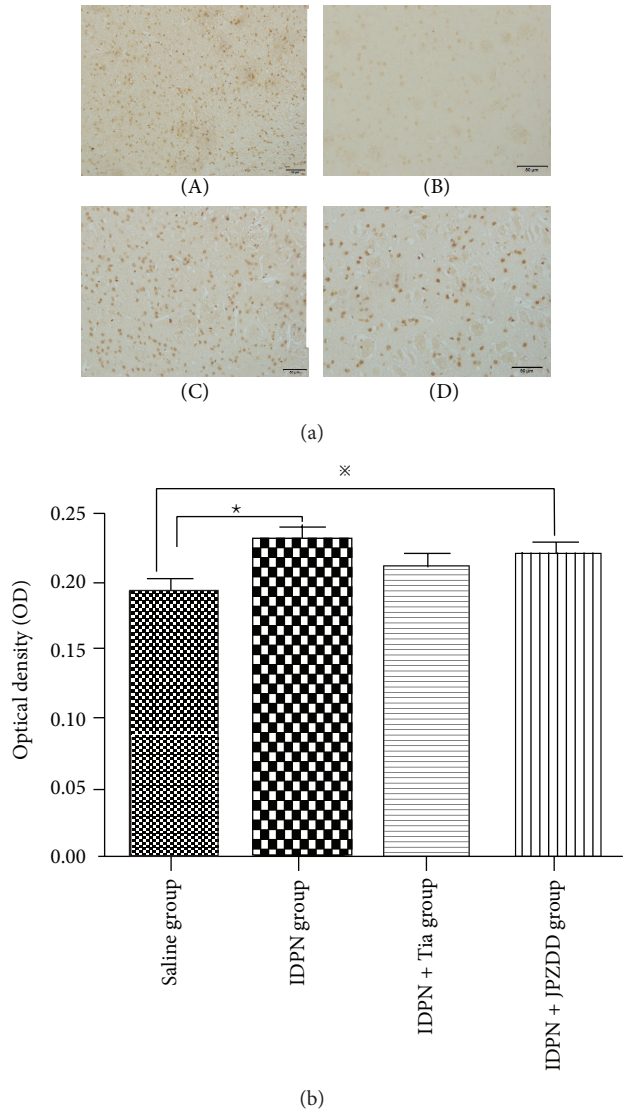

Figure 3: Immunohistochemistry of $GABA_AR$ in the striatum. (a) Comparison of the $GABA_AR$ in different groups, (A) IDPN group, (B) saline group, (C) IDPN+Tia group, and (D) IDPN+JPZDD group. Magnification 20 x. (b) The optical density was quantified. The bar graphs represent data from 24 different animals. Data were shown as mean ± SEM ($n = 6$ mice/group). ※ represents P values <0.05; ★ represents P values <0.01.

33]. Therefore, we hypothesized that abnormalities of GABA in TS may be associated with abnormal expression of GABA receptors. To the best of our knowledge, no other study has compared GABA receptors between TS models and controls. Therefore, our study may shed light on the GABAergic mechanism of TS. Our experimental results also revealed that more stereotypy behaviors correlated with higher expression of $GABA_A$ receptor, with statistical significance between the saline group and the other groups. The IHC indicated that JPZDD and Tia might decrease the bioactivity of $GABA_A$ receptors, so as to maintain balance. Combined with previous results, it is reasonable to postulate that the decrease of $GABA_A$ receptor protein expression in striatum which led directly to the altered GABA content was one potential antitic effect of JPZDD. PCR showed no statistical difference between these groups, so the reduction in $GABA_A$ receptor was mainly at the protein level rather than at the nucleic acid level. Although there was no difference between the JPZDD group and the Tia group, JPZDD-treated animals did not show adverse side effects, such as extrapyramidal symptoms, tardive dyskinesia, drowsiness, or hyperprolactinemia.

However, the present study has some limitations as follows. First, there are differences between humans and mouse; thus further experiments performed on TS patients can provide more clinical implication for TS treatment. Second, this study did not explain the phenomenon of lower expression of GABA in some patients. Third, other types of GABA receptor could be tested in the future.

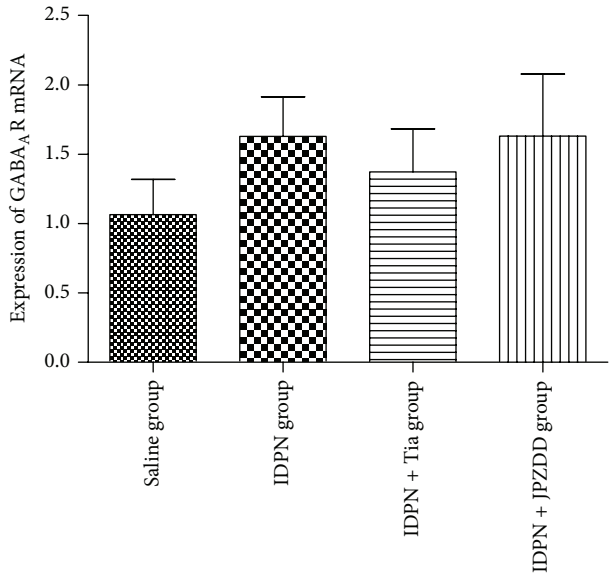

FIGURE 4: Expression of GABA$_A$R mRNA expression in the striatum. The bar graphs represent data from 24 different animals. Data were shown as mean ± SEM (n = 6 mice/group).

In conclusion, results from the study suggested that JPZDD might effectively inhibit stereotype actions and control TS symptoms by downregulating the GABA$_A$ receptor expression, which may directly decrease the excessive generation of GABA in the striatum. Thus therapeutic strategies targeting the GABAergic system could be effective in treating TS.

Authors' Contribution

Wen Zhang, Wenjing Yu, and Minkyoung Lee conducted the experiment. Daohan Wang provided the reagents and materials. Li Wei performed the statistical analysis; Sumei Wang performed the drafting and critical revision of the paper. The authors state that they have read and approved the paper submitted to the journal.

Conflict of Interests

The authors declare that they have no conflict of interests in the matter and no direct financial relation with the commercial identity mentioned in this work.

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Research Article

Comparison of the Effects of Acarbose and TZQ-F, a New Kind of Traditional Chinese Medicine to Treat Diabetes, Chinese Healthy Volunteers

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Ethnopharmacological Relevance. TZQ-F has been traditionally used in Traditional Chinese Medicine as a formula for the treatment of diabetes. *Aim of the Study*. This study aims to compare the pharmacologic effects and gastrointestinal adverse events between TZQ-F and acarbose. *Methods*. The double-blind randomized placebo-controlled fivefold crossover study was performed in 20 healthy male volunteers. Plasma glucose, plasma IRI, and plasma C-peptide were measured to assess the pharmacologic effects. Flatus and bowel activity were measured to assess the adverse event of gastrointestinal effect. *Results*. 3 and 4 tablets of TZQ decreased the C_{max} of plasma glucose compared with that of the previous day and with placebo. 3 tablets also decreased C_{max} of plasma C-peptide compared with placebo. 4 tablets increased C_{max} of plasma insulin after breakfast and the AUC of plasma C-peptide after breakfast and dinner. 2 tablets did not decrease plasma glucose and elevated the C_{max} and AUC of C-peptide after breakfast and dinner, respectively. Acarbose 50 mg decreased the C_{max} of plasma insulin and C-peptide after breakfast and the C_{max} of plasma glucose and C-peptide after dinner. The subjects who received TZQ did not report any abdominal adverse events. *Conclusions*. 3 tablets of TZQ have the same effects as the acarbose.

1. Introduction

The prevalence of type 2 diabetes is rising exponentially and it has become a global health priority [1]. The International Diabetes Federation has predicted that the number of individuals with diabetes is likely to increase from 382 million in 2013 to 592 million in 2035 [2]. All types of diabetes mellitus are characterized by an increased cardiovascular risk, which is mostly pronounced in type 2 diabetes. This has a special significance, as this most common type of the disease develops asymptomatically in the majority of the cases, and therefore the detection of type 2 diabetes is often delayed and the advanced complications are frequently presented at the time of the diagnosis.

Impaired glucose tolerance (IGT), as well as insulin resistance, is known to be associated with an increased risk of type 2 diabetes and hypertension, which are well-recognized risk factors for cardiovascular diseases [3–5]. Considering the heavy burden of these metabolic disorders on the public health, improvement of IGT and/or insulin resistance is a supremely important health issue.

Traditional Chinese Medicine (TCM) has been used in treating diabetes mellitus for almost twenty centuries in China. TangZhiQing Formula (TZQ-F) is a well-known

antidiabetic formula containing five herbs, which are *Paeonia lactiflora* Pall., root, *Morus alba* L., leaf, *Nelumbo nucifera* Gaertn., leaf, *Salvia miltiorrhiza bge*., roots, and *Crataegus pinnatifida bge*., leaf. TZQ-F comes from a prescription named *Salvia miltiorrhiza powder*, which was recorded in *Taiping Holy Prescriptions for Universal Relief* of Song dynasty of China. The results of antidiabetic studies showed TZQ-F can reduce blood glucose, total cholesterol, and triglyceride levels of KK-Ay mice after 4 weeks of oral administration [6].

α-Glucosidase inhibitors are commonly used for type 2 diabetes mellitus. α-Glucosidase inhibitors reduce the absorption of carbohydrates from the small intestine and thereby lower the levels of postprandial blood glucose. Plants and microorganisms are rich sources of α-glucosidase inhibitors. Screening of α-glucosidase inhibitors from plants and synthetic sources has been a hot research topic [7]. Our previous study [8] showed that TZQ-F possesses blood glucose lowering effects, possibly by inhibiting intestinal α-glucosidase. As a continuing study, this paper compares pharmacologic effects and gastrointestinal adverse events associated with TZQ-F and acarbose which is an α-glucosidase inhibitor being marketed for 30 years approximately.

2. Patients and Methods

The subjects were 20 male volunteers aged from 19 to 29 years (mean ± SD, 23.35 ± 2.62 years), who were in good health, as determined by history, physical examination, and routine laboratory investigations. Body mass index was 18.94 to 23.94 kg/m^2 (mean ± SD, 21.47 ± 1.59 kg/m^2). Informed written consents were given before the trial began, and the participants were free to withdraw at any time during the study. One subject withdrew after period 3, because he has to go back homeland to take care of his ill mother.

The drug TZQ-F and placebo were produced by Shan-Dong Buchang Shenzhou Pharmaceutical Co., Ltd., which was approved to produce tablets in November 2010 by CFDA (China Food and Drug Administration). In May 2012, the trial protocol was approved by the Ethics Committees of the Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine where the study was conducted. The registration number from the international clinical trial net is ChiCTR-TTRCC-12002866.

The subjects were hospitalized from the night before the first day of the study (on which no drugs were given) until the morning after the second day of the study (on which the drugs were administered). During hospitalization, only prescribed meals were allowed; meals were eaten at 8 am, 12 pm, and 6 pm. The same three meals were served on the first and second day of the study. Carbohydrate was supplied as bread at breakfast and rice at lunch and dinner. Plasma glucose, immunoreactive insulin (IRI), and C-peptide levels were monitored at breakfast and dinner. Energy available in breakfast, lunch, and dinner was 691 kcal (carbohydrate, 104 g; fat, 19 g; protein, 26 g), 922 kcal (carbohydrate, 138 g; fat, 26 g; protein, 34 g), and 687 kcal (carbohydrate, 103 g; fat, 19 g; protein, 26 g), respectively. Caffeinated and alcoholic drinks were prohibited during hospitalization.

Table 1: Dosage regimens of five groups.

Groups	Acarbose	TZQ			
Acarbose	●	□	□	□	□
TZQ 2 tablets	○	■	■	□	□
TZQ 3 tablets	○	■	■	■	□
TZQ 4 tablets	○	■	■	■	■
Placebo	○	□	□	□	□

"●" means one 50 mg tablet of acarbose; "○" means one 50 mg tablet of acarbose simulation agent; "■" means one 0.64 g tablet of TZQ; "□" means one 0.64 g tablet of TZQ simulation agent.

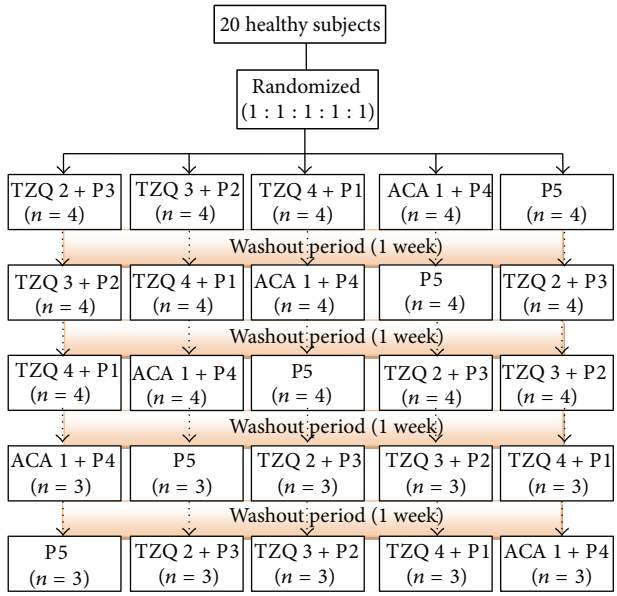

Figure 1: Flow of study subjects. ACA1 denotes 1 tablet of acarbose; TZQ 1–TZQ 5 denotes 1–5 tablets of TZQ; P1 denotes 1 tablet of acarbose simulation agent; P2 denotes 1 tablet of acarbose simulation agent and 1 tablet of TZQ simulation agent; P3 denotes 1 tablet of acarbose simulation agent and 2 tablets of TZQ simulation agent; P4 denotes 1 tablet of acarbose simulation agent and 3 tablets of TZQ simulation agent; P5 denotes 1 tablet of acarbose simulation agent and 4 tablets of TZQ simulation agent.

Subjects were prohibited from vigorous exercise during the study. No drug except the test drugs was administered from after the screening test (1 month before the study) until the end of the fifth treatment period of the study.

2.1. Study Design. The study was conducted according to a randomized, double-blind, placebo-controlled, fivefold, crossover design. No drugs were given on the first day, and the following drugs were administered on the second day: acarbose, 2 tablets, 3 tablets, and 4 tablets of TZQ, or placebo 3 times a day. The specification of TZQ is 0.64 g per tablet. See Table 1 of the dosage regimen of the five groups.

The subjects were divided into five groups; each group contains four subjects using a balanced Latin square of four subjects by five kinds of treatments. The drugs were administered with 200 mL water just before each meal during the five treatment periods. The drug-free washout period between each two treatment periods was 1 week (Figure 1).

To investigate pharmacologic effects, plasma glucose, IRI, and C-peptide levels were determined before and 0.25, 0.5, 1, 1.5, 2, and 3 hours after the breakfast and dinner. Area under the plasma concentration-time curve (AUC) for plasma glucose, IRI, and C-peptide was calculated using the trapezoidal rule.

To investigate the gastrointestinal effects, subjective symptoms, flatus, and bowel activity were monitored. The severity, time of onset, and time of disappearance of all symptoms of the subjects were recorded on the designated form. The frequency and severity of flatus as mild, moderate, or serious were also recorded. The flatus score was calculated by multiplying the frequency by 3 points for serious flatus, 2 for moderate flatus, and 1 for mild flatus. For assessment of bowel activity, the frequency of defection was recorded and the stools were photographed. The stools were then classified from the photographs as watery, loose, soft, firm, or hard bolus. Stool scores were calculated by multiplying the frequency by a score ranging from 5 points for watery stool to 1 point for hard bolus.

2.2. Analytic Method. IRI and C-peptide level were determined by chemiluminescence (ADVIA Centaur, Siemens). Plasma glucose level was determined by the glucose oxidase method.

2.3. Statistical Analysis. Analysis of variance (ANOVA) was used to test the effects of treatment on maximum concentration (C_{max}) and AUC for the change of plasma glucose, IRI, and C-peptide. If the drug effect was found to be significant by ANOVA, paired t-test was used to test the effects of each group before and after the treatment; multiple comparison of LSD was used to test the difference between the treatment and the placebo. For analysis of flatus and bowel activity, the one-sample Wilcoxon test was used. All tests were two-tailed, and the level of significance was set at 0.05.

3. Results

3.1. Plasma Glucose. The drug treatments significantly decreased C_{max} of the plasma glucose after dinner ($P = 0.0003$). Compared with placebo, reduction in C_{max} of plasma glucose was significant in acarbose 50 mg, 3 tablets and 4 tablets of TZQ after dinner, respectively (Figure 3, Table 2). Compared with before treatment, acarbose 50 mg, 3 tablets and 4 tablets of TZQ also showed statistically significant role in decreasing C_{max} of plasma glucose after dinner (Figure 3, Table 2). All of the drug treatments did not change plasma glucose after the breakfast significantly.

3.2. Plasma Insulin. Plasma insulin changed significantly after the treatment. The acarbose 50 mg decreased C_{max} of plasma insulin after breakfast and dinner, respectively. 3 tablets of TZQ decreased C_{max} of plasma insulin after dinner only. Compared with placebo, acarbose significantly decreased the C_{max} of plasma of IRI after breakfast (Figure 2, Table 3). 4 tablets of TZQ increased C_{max} of plasma insulin

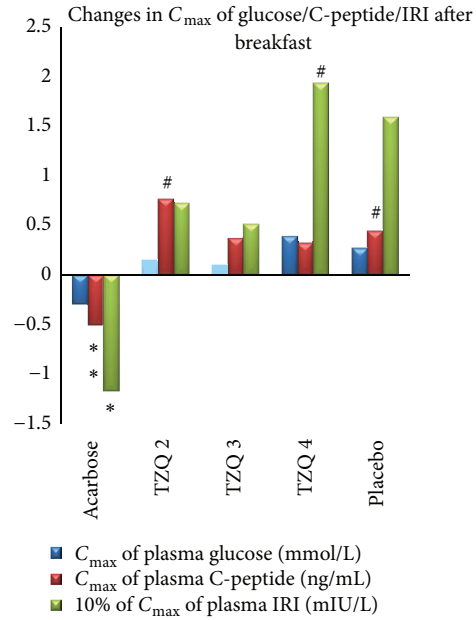

Figure 2: Mean change in C_{max} of glucose/C-peptide/IRI after breakfast from first day (no drug administration) to second day (drug administration). Reduction in plasma C-peptide and IRI was significant in acarbose group ($^{*}P < 0.05$ versus placebo; $^{\#}P < 0.05$ versus before treatment). Elevation of C_{max} of plasma IRI was significant in TZQ 4 group ($^{\#}P < 0.05$ versus before treatment). Elevation of C_{max} of plasma C-peptide was significant in TZQ 2 and placebo groups ($^{\#}P < 0.05$ versus before treatment).

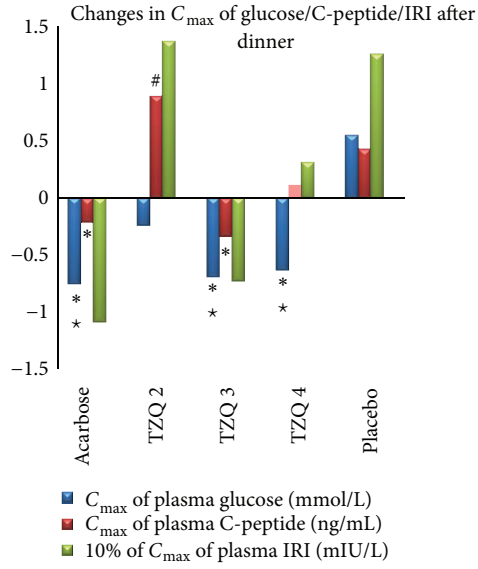

Figure 3: Mean change in C_{max} of glucose/C-peptide/IRI after dinner from first day (no drug administration) to second day (drug administration). Reduction in C_{max} of plasma glucose was significant in acarbose and TZQ 3 and TZQ 4 groups ($^{*}P < 0.05$ versus placebo; $^{\#}P < 0.05$ versus before treatment). Reduction in C_{max} of plasma C-peptide was significant in acarbose and TZQ 3 groups ($^{*}P < 0.05$ versus placebo; $^{\#}P < 0.05$ versus before treatment). Elevation of C_{max} of plasma C-peptide was significant in TZQ 2 group ($^{\#}P < 0.05$ versus before treatment).

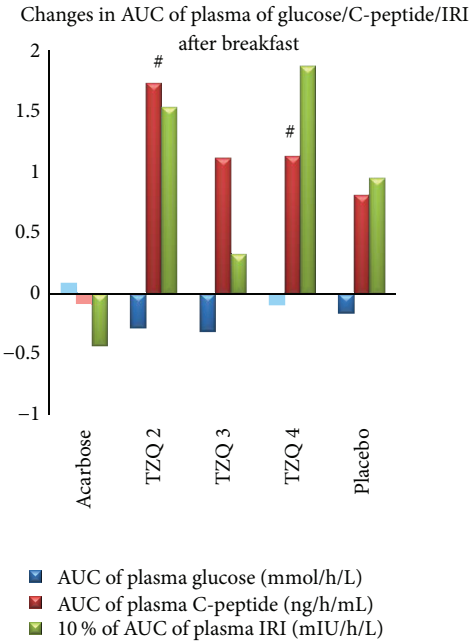

Figure 4: Mean change in AUC of glucose/C-peptide/IRI after breakfast from first day (no drug administration) to second day (drug administration). Elevation in AUC of plasma C-peptide was significant in TZQ 2 and TZQ 4 group ($^{\#}P < 0.05$ versus before treatment).

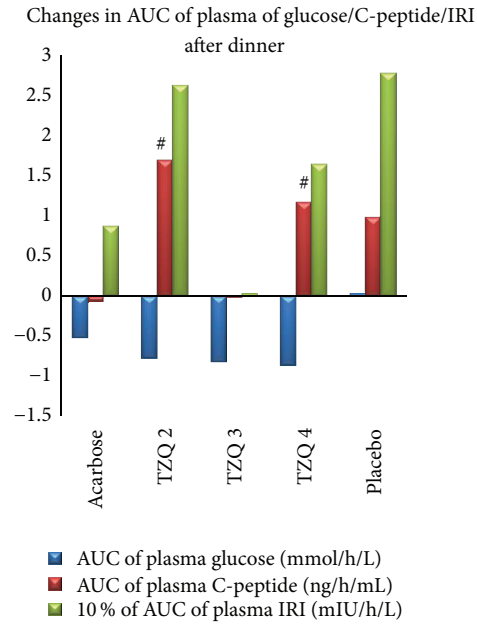

Figure 5: Mean change in AUC of glucose/C-peptide/IRI after dinner from first day (no drug administration) to second day (drug administration). Elevation of AUC of plasma C-peptide was significant in TZQ 2 and TZQ 4 groups ($^{\#}P < 0.05$ versus before treatment).

after breakfast ($P = 0.015$ versus before treatment) (Figure 2, Table 3).

3.3. C-Peptide. Plasma C-peptide changed significantly after the treatment. Compared with placebo, acarbose 50 mg significantly decreased C_{max} of plasma C-peptide after breakfast and dinner, respectively (Figures 2 and 3, Table 4). 3 tablets of TZQ significantly decreased C_{max} of plasma C-peptide only after dinner (Figure 3, Table 4).

Compared with before treatment, elevation of C_{max} of plasma C-peptide after breakfast was significant in 2 tablets of TZQ and placebo (Figure 2, Table 4). 2 tablets of TZQ significantly elevated C_{max} of plasma C-peptide after dinner (Figure 3, Table 4). Besides, 2 tablets of TZQ and 4 tablets of TZQ significantly elevated the AUC of plasma C-peptide after breakfast and dinner, respectively (Figures 4 and 5, Table 4).

3.4. Gastrointestinal Effects. Flatus scores did not increase significantly during the treatment compared with that of the previous day in subjects receiving TZQ and placebo but increased significantly in acarbose 50 mg group ($P = 0.036$). There were no significant differences in flatus scores between groups (Figure 6).

Stool scores did not increase significantly during the treatment compared with that of the previous day in all groups (Figure 7).

4. Discussion and Conclusions

Previous *in vitro* mechanism study of TZQ showed that three fractions of TZQ had strong inhibition effects on

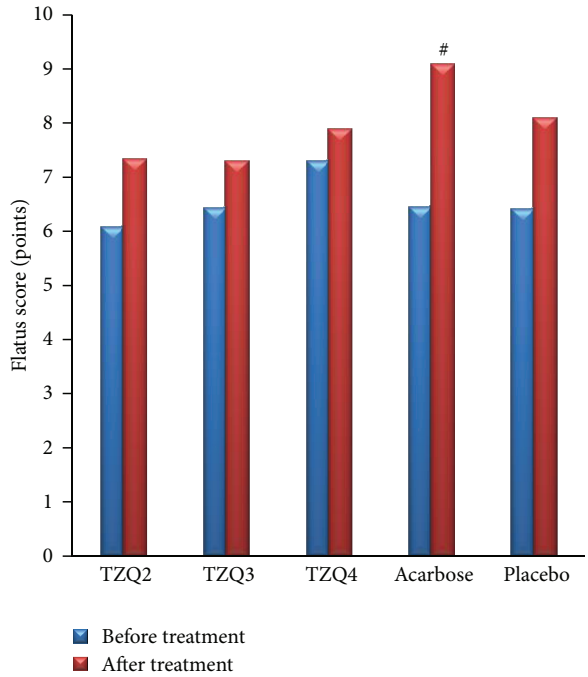

Figure 6: Mean flatus score before (blue column) and during (red column) administration of 2 tablets, 3 tablets, and 4 tablets of TZQ, acarbose, and placebo on 19 volunteers. Mean flatus score was significantly elevated in acarbose dose ($^{\#}P < 0.05$ versus first day (before treatment)).

Table 2: Maximum concentration (C_{max}) and area under the plasma concentration-time curve (AUC) of plasma glucose after breakfast and dinner on 19 healthy volunteers (mean ± SEM).

	Dose (mg/d, p.o.)		C_{max} of plasma glucose (mmol/L) after breakfast		
		n	First day	Second day	Change
Acarbose	50	19	6.93 ± 0.84	6.64 ± 0.86	−0.29 ± 0.74
TZQ- 2 tables	1280	19	6.75 ± 0.74	6.90 ± 1.06	0.15 ± 0.95
TZQ- 3 tables	1920	19	6.83 ± 1.03	6.92 ± 0.73	0.10 ± 0.84
TZQ- 4 tables	2560	19	6.78 ± 0.99	7.17 ± 0.92	0.39 ± 0.82
Placebo	—	19	6.97 ± 1.14	7.23 ± 0.95	0.27 ± 0.84
	Dose (mg/d, p.o.)		C_{max} of plasma glucose (mmol/L) after dinner		
		n	First day	Second day	Change[#]
Acarbose	50	19	7.28 ± 1.03	6.54 ± 0.89[*]	−0.75 ± 0.88
TZQ- 2 tables	1280	19	7.38 ± 0.84	7.14 ± 1.38	−0.24 ± 1.40
TZQ- 3 tables	1920	19	7.69 ± 0.91	7.00 ± 1.08[*]	−0.69 ± 0.90
TZQ- 4 tables	2560	19	7.35 ± 0.97	6.72 ± 0.77[*]	−0.63 ± 1.09
Placebo	—	19	7.06 ± 0.86	7.60 ± 1.53	0.55 ± 1.32
	Dose (mg/d, p.o.)		AUC of Plasma glucose (mmol·h/L) after breakfast		
		n	First day	Second day	Change
Acarbose	50	19	16.29 ± 2.01	16.38 ± 1.79	0.09 ± 1.45
TZQ- 2 tables	1280	19	16.60 ± 1.43	16.31 ± 1.54	−0.28 ± 1.19
TZQ- 3 tables	1920	19	16.65 ± 1.91	16.34 ± 1.08	−0.31 ± 1.50
TZQ- 4 tables	2560	19	16.79 ± 2.01	16.69 ± 1.77	−0.09 ± 1.44
Placebo	—	19	16.55 ± 1.69	16.39 ± 1.83	−0.16 ± 1.04
	Dose (mg/d, p.o.)		AUC of plasma glucose (mmol·h/L) after dinner		
		n	First day	Second day	Change
Acarbose	50	19	17.59 ± 1.99	17.06 ± 2.03	−0.52 ± 1.78
TZQ- 2 tables	1280	19	18.01 ± 0.41	17.23 ± 3.32	−0.78 ± 3.43
TZQ- 3 tables	1920	19	18.08 ± 1.87	17.25 ± 2.91	−0.82 ± 2.24
TZQ- 4 tables	2560	19	17.98 ± 1.63	17.10 ± 2.16	−0.87 ± 2.27
Placebo	—	19	17.29 ± 1.60	17.32 ± 2.84	0.03 ± 2.79

[*] $P < 0.05$ versus first day (before treatment). [#] $P < 0.05$ five treatments compared using ANOVA.

rat intestinal disaccharase, which are mulberry leaf total alkaloids fraction, mulberry leaf total flavonoid fraction, and hawthorn leaf total flavonoids fraction. Particularly, the mulberry leaf total alkaloids fraction (IC50 = 0.26 μg/mL for sucrase and 0.05 μg/mL for maltase) is stronger than the positive control of acarbose [8]. So in the clinical practice, TZQ may affect the plasma glucose at the similar style of acarbose.

3 tablets and 4 tablets of TZQ have significantly decreased the C_{max} of the plasma glucose compared with that of previous day and with placebo. Like acarbose, 3 tablets of TZQ also decreased C_{max} of plasma C-peptide compared with placebo. 4 tablets of TZQ significantly increased C_{max} of plasma insulin after breakfast and the AUC of plasma C-peptide after breakfast and dinner. Though 2 tablets of TZQ did not decrease plasma glucose significantly, it elevated the C_{max} and AUC of C-peptide after breakfast and dinner, respectively. Acarbose 50 mg decreased the C_{max} of plasma insulin and C-peptide after breakfast and the C_{max} of plasma glucose and C-peptide after dinner significantly. It shows that the 3 tablets of TZQ have the same effects as the acarbose which is to inhibit the postprandial increase in blood glucose levels by inhibiting and delaying digestion and absorption of carbohydrate.

3 tablets of TZQ and the alpha-glucosidase inhibitor, acarbose, inhibited the postprandial increase in plasma glucose levels and decreased insulin secretion to maintain normoglycemia in nondiabetic subjects. Inhibition of the postprandial increase in plasma glucose was more marked at dinner than at breakfast. This was partially due to cumulative effect of alpha-glucosidase inhibitors [9]. Although data are not available in humans, the turnover time of disaccharidases in the rat has been reported to be 11.5 hours. Thus it would appear reasonable that because TZQ or acarbose was given at every meal, the cumulative effects would be observed at dinner.

TZQ 2-tablet dose increased the C_{max} and AUC of plasma C-peptide after breakfast and dinner, respectively, and TZQ 4-tablet dose significantly increased the AUC of plasma C-peptide after breakfast and dinner, respectively, and thus the TZQ 2- and TZQ 4-tablet dose possibly increased the insulin secretion. Traditionally, Chinese herbs are used as a formulated decoction, a specific combination of different herbs, prepared using a unique methodology to achieve a specific efficacy. The herbs in the formula are not simply added together in a cumulative fashion. Instead, they are precisely combined according to a particular principle. The

Table 3: Maximum concentration (C_{max}) and area under the plasma concentration-time curve (AUC) of plasma IRI after breakfast and dinner on 19 healthy volunteers (mean ± SEM).

	Dose (mg/d, p.o.)	n	C_{max} of plasma IRI (mIU/L) after breakfast		
			First day	Second day	Change[#]
Acarbose	50	19	75.77 ± 50.30	64.10 ± 35.92	−11.67 ± 32.69
TZQ- 2 tables	1280	19	80.02 ± 51.86	86.83 ± 49.25	7.28 ± 29.33
TZQ- 3 tables	1920	19	85.65 ± 49.82	90.83 ± 44.86	5.18 ± 24.35
TZQ- 4 tables	2560	19	77.42 ± 40.10	96.85 ± 49.99[*]	19.43 ± 31.44
Placebo	—	19	85.36 ± 46.86	101.32 ± 54.16	15.96 ± 39.25
	Dose (mg/d, p.o.)	n	C_{max} of plasma IRI (mIU/L) after dinner		
			First day	Second day	Change[#]
Acarbose	50	19	54.85 ± 37.42	44.00 ± 30.64	−10.85 ± 35.69
TZQ- 2 tables	1280	19	42.78 ± 24.93	56.50 ± 29.19	13.72 ± 23.77
TZQ- 3 tables	1920	19	55.79 ± 29.29	48.25 ± 27.37	−7.27 ± 30.70
TZQ- 4 tables	2560	19	48.86 ± 30.92	51.99 ± 28.55	3.13 ± 20.49
Placebo	—	19	52.43 ± 26.17	65.09 ± 3.62	12.66 ± 36.66
	Dose (mg/d, p.o.)	n	AUC of plasma IRI (mIU/h/L) after breakfast		
			First day	Second day	Change
Acarbose	50	19	107.73 ± 70.16	103.45 ± 50.89	−4.28 ± 39.61
TZQ- 2 tables	1280	19	113.90 ± 61.00	129.24 ± 60.36	15.34 ± 37.52
TZQ- 3 tables	1920	19	127.50 ± 70.60	130.74 ± 61.47	3.24 ± 43.54
TZQ- 4 tables	2560	19	122.72 ± 71.76	141.48 ± 68.06	18.76 ± 39.57
Placebo	—	19	128.43 ± 79.93	137.97 ± 63.00	9.54 ± 47.88
	Dose (mg/d, p.o.)	n	AUC of plasma IRI (mIU/h/L) after dinner		
			First day	Second day	Change
Acarbose	50	19	66.01 ± 35.68	74.75 ± 43.69	8.73 ± 39.39
TZQ- 2 tables	1280	19	66.45 ± 31.29	92.68 ± 36.51	26.23 ± 28.88
TZQ- 3 tables	1920	19	84.95 ± 49.02	85.23 ± 43.16	0.28 ± 47.98
TZQ- 4 tables	2560	19	77.84 ± 45.67	94.32 ± 47.68	16.48 ± 35.71
Placebo	—	19	68.39 ± 29.40	96.21 ± 7.93	27.82 ± 26.31

[*] $P < 0.05$ versus first day (before treatment). [#] $P < 0.05$ five treatments compared using ANOVA.

characteristics of Chinese herbal medicine include multiple-component and multitarget actions [10]. TZQ is one of these typical Chinese herbal formulas that different dose may produce different effect.

The incidence of abdominal adverse events has previously been reported with acarbose. Our study also demonstrated the same result. The subjects receiving TZQ did not report abdominal adverse events. That may be because of the characteristics of Chinese herbal medicine, that is, multiple-component and multitarget actions. It will improve the patient's compliance.

Although the C_{max} and AUC of plasma glucose after dinner decreased significantly with acarbose and TZQ, the reduction rate was small. As the subjects were not diabetic, postprandial plasma glucose levels were maintained within a narrow range, resulting in a small reduction in plasma glucose levels when a normal amount of food was ingested. TZQ has been used for many centuries in China to treat diabetes, but the clinical evidence has not been established. Based on this study, a multicenter clinical trial will be carried out by our team to evaluate the effect of TZQ on the diabetes mellitus in the near future.

Appendices

A. Quality Control of TZQ-F

The traditional Chinese herbal medicine preparation TZQ-F is a combination of five herbal ingredients—*Paeonia lactiflora* Pall., root, *Morus alba* L., leaf, *Nelumbo nucifera* Gaertn., leaf, *Salvia miltiorrhiza Bge.*, root, *Crataegus pinnatifida* Bge., leaf—manufactured under the Code of Good Manufacturing Practice by Shandong Buchang Shenzhou Pharmaceutical Co., Ltd. [8]. The TZQ tablets employed in this research were batch 120606. All the test and quality control (QC) of this product act in accordance with the "Chinese Pharmacopoeia" (2010 version). Accordingly, the marker compounds of *Nelumbo nucifera* Gaertn., *leaf*, and *Paeonia lactiflora* Pall., *root*, arenuciferine and paeoniflorin, respectively. A TZQ tablet contains not less than 0.33 mg

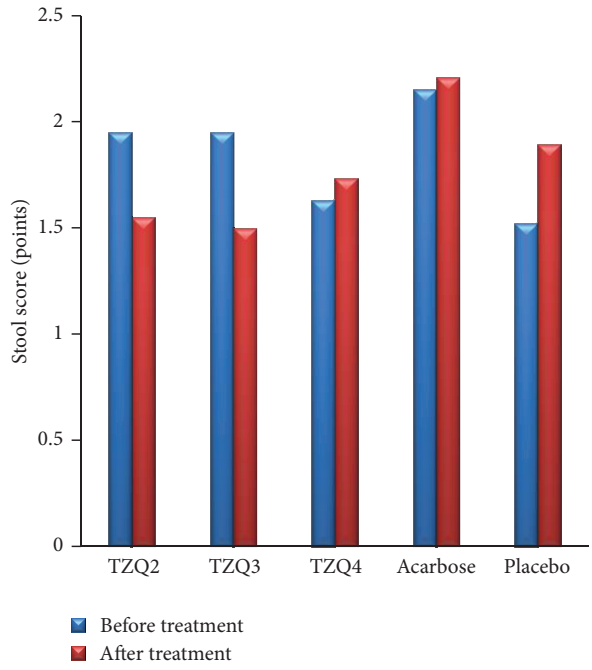

FIGURE 7: Mean stool score before (blue column) and during (red column) administration of 2 tablets, 3 tablets, and 4 tablets of TZQ, acarbose, and placebo on 19 volunteers.

of nuciferine ($C_{19}H_{21}NO_2$) and not less than 6.2 mg of paeoniflorin ($C_{23}H_{28}O_{11}$). Phenotypic trait—products are film coated tablets with a faint characteristic odour and a slightly bitter taste. The inner surface is brown after removing the coating layer. Identification—thin layer chromatographic identification test is employed to identify the five herbal ingredients. Checkup—disintegration time limited is not more than 1 hour; mass discrepancy is within the limits of 5%; microbial limit should also meet the specification.

B. The Quantification of Nuciferine in the Tablets of TZQ

The leaf of *Nelumbo nucifera* Gaertn. is a Traditional Chinese Medicine for losing weight and has been commonly used for clearing heat, removing heatstroke, cooling blood, and stanching blood. The major phytochemicals present in lotus leaf are three aporphine alkaloids, *N*-nornuciferine, *O*-nornuciferine, and nuciferine. According to the guiding principles of the "Chinese Pharmacopoeia" (2010 version), nuciferine is regarded as a QC compound to conduct the determination of folium nelumbinis.

B.1. High Performance Liquid Chromatography Analysis. HPLC analyses were performed using an Agilent HPLC system (Agilent 1100 Series, Agilent Technologies, CA, USA) composed of a column heater, a sample manager, a binary solvent manager, and a variable wavelength detector. The liquid chromatograph is equipped with a 4.6 mm × 250 mm column that contains 5 μm packing C18 (ZORBAX 300 Å Extend-C18, Agilent, CA, USA). The employed detection

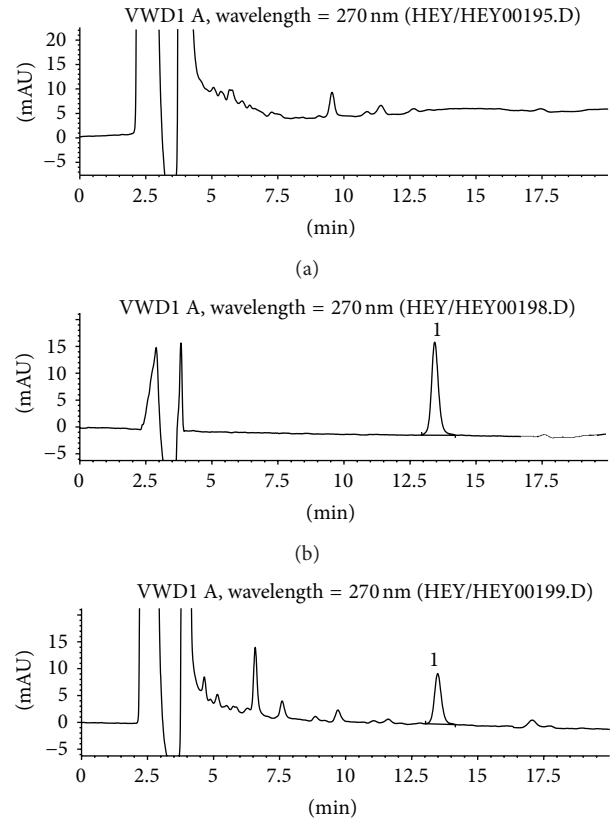

FIGURE 8: Typical HPLC chromatograms of marker compound nuciferine in TZQ: (a) blank control; (b) standard compound control; (c) TZQ sample. 1, nuciferine from *Nelumbo nucifera* Gaertn., leaf.

wavelength is 270 nm for nuciferine. A filtered and degassed mixture of acetonitrile, water, triethylamine, and acetic acid (33 : 64.8 : 1.5 : 0.7) is prepared. The flow rate is about 1.0 mL per minute. The column temperature is maintained at 25°C. Chromatograph the standard preparation and record the peak responses as directed for procedure: the column efficiency is not fewer than 2000 theoretical plates; the tailing factor is not more than 2.0; and the relative standard deviation for replicate injections is not more than 2.0% [11].

B.2. Sample Preparation. Weigh and finely powder not fewer than 10 tablets. Transfer an accurately weighed portion 1.0 g of the powder to a 100 mL volumetric flask, add 80 mL of methanol, and sonicate for about 10 minutes with intermittent shaking. Shake the flask on a mechanical shaker for about 30 minutes. Dilute with mobile phase to volume and mix. Pass a portion of this solution through a polytetrafluoroethylene membrane filter having a 0.45 μm porosity, discarding the first few mL.

B.3. Analytical Results. Based on the analytical results, the content of marker compounds was 0.84 mg/g for nuciferine in the TZQ tablets. The analytical chromatograms are shown in Figure 8.

Table 4: Maximum concentration (C_{max}) and area under the plasma concentration-time curve (AUC) of plasma C-peptide after breakfast and dinner on 19 healthy volunteers (mean ± SEM).

	Dose (mg/d, p.o.)		C_{max} of plasma C-peptide (ng/mL) after breakfast		
		n	First day	Second day	Change[#]
Acarbose	50	19	5.13 ± 2.20	4.63 ± 1.29[*]	−0.50 ± 1.19
TZQ- 2 tables	1280	19	4.68 ± 1.71	5.46 ± 1.98[*]	0.77 ± 1.09
TZQ- 3 tables	1920	19	5.20 ± 2.33	5.57 ± 2.11	0.37 ± 1.69
TZQ- 4 tables	2560	19	5.08 ± 2.17	5.41 ± 2.04	0.33 ± 1.11
Placebo	—	19	5.09 ± 2.11	5.54 ± 2.29[*]	0.45 ± 1.46
	Dose (mg/d, p.o.)		C_{max} of plasma C-peptide (ng/mL) after dinner		
		n	First day	Second day	Change[#]
Acarbose	50	19	4.56 ± 1.49	4.35 ± 1.80	−0.21 ± 1.20
TZQ- 2 tables	1280	19	4.31 ± 1.17	5.20 ± 1.56[*]	0.89 ± 0.76
TZQ- 3 tables	1920	19	4.98 ± 1.58	4.64 ± 1.50	−0.33 ± 1.00
TZQ- 4 tables	2560	19	4.50 ± 1.80	4.61 ± 1.45	0.11 ± 1.17
Placebo	—	19	4.70 ± 1.49	5.13 ± 1.57	0.43 ± 1.15
	Dose (mg/d, p.o.)		AUC of plasma C-peptide (ng/h/mL) after breakfast		
		n	First day	Second day	Change[#]
Acarbose	50	19	9.83 ± 3.40	9.76 ± 2.92	−0.08 ± 1.23
TZQ- 2 tables	1280	19	9.67 ± 3.04	11.40 ± 3.74[*]	1.73 ± 1.72
TZQ- 3 tables	1920	19	10.26 ± 4.26	11.39 ± 3.90	1.12 ± 2.45
TZQ- 4 tables	2560	19	10.32 ± 4.37	11.45 ± 4.33[*]	1.13 ± 2.07
Placebo	—	19	10.40 ± 4.16	11.22 ± 4.22	0.81 ± 1.78
	Dose (mg/d, p.o.)		AUC of plasma C-peptide (ng/h/mL) after dinner		
		n	First day	Second day	Change[#]
Acarbose	50	19	9.29 ± 2.32	9.23 ± 2.90	−0.07 ± 1.73
TZQ- 2 tables	1280	19	9.92 ± 2.41	11.62 ± 3.08[*]	1.70 ± 1.42
TZQ- 3 tables	1920	19	10.66 ± 3.65	10.64 ± 3.41	−0.02 ± 2.06
TZQ- 4 tables	2560	19	9.74 ± 2.96	10.91 ± 3.53[*]	1.17 ± 1.94
Placebo	—	19	10.30 ± 3.14	11.28 ± 2.82	0.98 ± 2.08

[*] $P < 0.05$ versus first day (before treatment). [#] $P < 0.05$ five treatments compared using ANOVA.

C. The Quantification of Paeoniflorin in the Tablets of TZQ

The dried peeled root of *Paeonia lactiflora* Pall. is one of the Chinese traditional tonic crude drugs. Paeoniflorin, a water soluble substance isolated from the root of *P. lactiflora*, is one of the bioactive components and has been reported to exhibit anticoagulant, neuromuscular blocking, cognition-enhancing, immunoregulating, and antihyperglycemic effects. Therefore, paeoniflorin is chosen as a second QC marker compound of TZQ tablet.

C.1. HPLC Analysis. HPLC analyses were performed using an Agilent HPLC system (Agilent 1100 Series, Agilent Technologies, CA, USA) composed of a column heater, a sample manager, a binary solvent manager, and a variable wavelength detector. The liquid chromatograph is equipped with a 4.6 mm × 250 mm column that contains 5 μm packing C18 (ZORBAX 300 Å Extend-C18, Agilent, CA, USA). The employed detection wavelength is 230 nm for paeoniflorin. A filtered and degassed mixture of acetonitrile and water (14 : 86) is prepared. The flow rate is about 1.0 mL per minute. The column temperature is maintained at 25°C. Chromatograph the standard preparation and record the peak responses as directed for procedure: the column efficiency is not fewer than 3000 theoretical plates; the tailing factor is not more than 2.0; and the relative standard deviation for replicate injections is not more than 2.0% [12].

C.2. Sample Preparation. Weigh and finely powder not fewer than 10 tablets. Transfer an accurately weighed portion 1.0 g of the powder to a 100 mL volumetric flask, add 80 mL of water, and sonicate for about 5 minutes with intermittent shaking. Shake the flask on a mechanical shaker for about 30 minutes. Dilute with mobile phase to volume and mix. Pass a portion of this solution through a polytetrafluoroethylene membrane filter having a 0.45 μm porosity, discarding the first few mL.

C.3. Analytical Results. Based on the analytical results, the content of marker compounds was 9.69 mg/g for paeoniflorin in the TZQ tablets. The analytical chromatograms are shown in Figure 9.

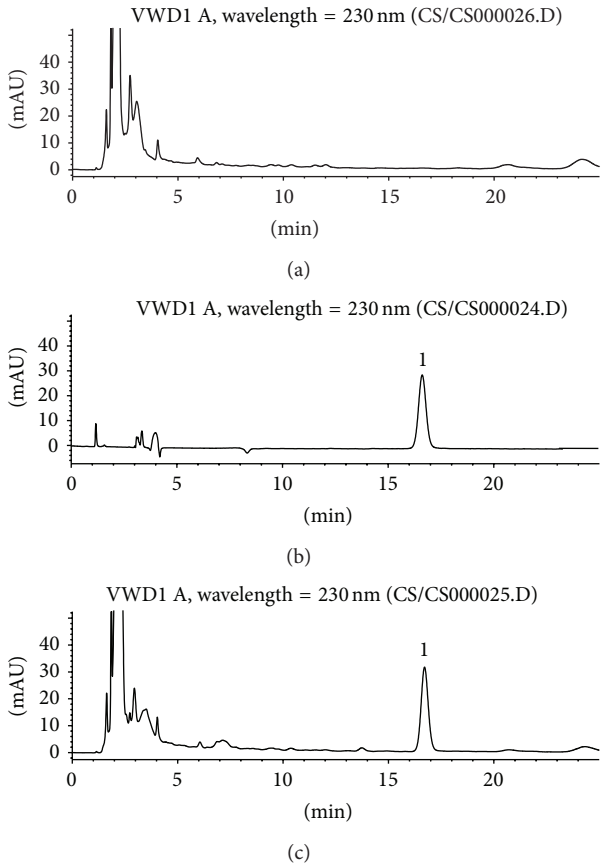

Figure 9: Typical HPLC chromatograms of marker compound paeoniflorin in TZQ: (a) blank control; (b) standard compound control; (c) TZQ sample. 1, paeoniflorin from *Paeonia lactiflora* Pall., root.

Conflict of Interests

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

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Research Article

Treatment of Posttraumatic Stress Disorder Using the Traditional Japanese Herbal Medicine Saikokeishikankyoto: A Randomized, Observer-Blinded, Controlled Trial in Survivors of the Great East Japan Earthquake and Tsunami

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The Great East Japan earthquake and tsunami caused immense damage over a wide area of eastern Japan. Hence, many survivors are at high risk for posttraumatic stress disorder (PTSD). This randomized, observer-blinded, controlled trial examined the efficacy and safety of the traditional Japanese herbal formula saikokeishikankyoto (SKK) in the treatment of PTSD among survivors of this disaster. Forty-three participants with an Impact of Event Scale-Revised (IES-R) score ≥ 25 were randomized into SKK ($n = 21$) and control ($n = 22$) groups. The primary endpoint was the change in IES-R scores from baseline till after 2 weeks of treatment. Intergroup statistical comparisons were performed. The magnitude of changes in total IES-R scores differed significantly between the two groups ($P < 0.001$). Post hoc analysis showed that the total IES-R score improved significantly in the SKK group from 49.6 ± 11.9 to 25.5 ± 17.0 ($P < 0.001$). Subscale scores improved significantly in the SKK group (avoidance, $P = 0.003$; hyperarousal, $P < 0.001$; intrusion, $P < 0.001$). Two-week treatment with SKK significantly improved IES-R scores among PTSD patients. This traditional medicine may be a valid choice for the treatment of psychological and physical symptoms in PTSD patients.

1. Introduction

Two years have passed since March 11, 2011, the day the Great East Japan earthquake and tsunami claimed 20,000 lives in a flash. Although people have been able to move from shelters to temporary housing, heaps of rubble and radioactive pollution prevent the rehabilitation of many areas. While some commercial fishing has resumed, inshore fish are polluted with radiation and cannot be sold. The towns and cities inundated by the tsunami are, even now, a wasteland. The disaster caused posttraumatic stress disorder (PTSD) in many people [1]. Many patients have visited our clinic for PTSD treatment, but psychotherapists are in short supply. We attempted to treat them using mood stabilizers, antidepressants, and hypnotics, but these medications are not always effective; some of these patients then suffer from intractable drug dependency. A Chinese herbal formula was reported to be effective for patients with PTSD after the Sichuan earthquake in 2008 [2]. Saikokeishikankyoto (SKK, Chaihu-Guizhi-Ganjiang-Tang in Chinese) is a traditional Japanese-Chinese herbal formula that has a marked effect on some PTSD patients. We therefore conducted a randomized observer-blinded trial of the efficacy and safety of SKK for the treatment of PTSD.

2. Methods

2.1. Participants. Subjects were recruited from the outpatient clinic at the National Sendai-Nishitaga Hospital in Sendai, Japan. The required sample size was calculated as 20 subjects for each arm, with an alpha error of 0.05 and power of 0.8. All participants completed an intake assessment that included medical history, physical examination, and standard blood examination as well as an Impact of Event Scale-Revised score (IES-R) (see the following pattern) to assess the severity of PTSD at baseline.

Scoring the Impact of Event Scale-Revised Questionnaire

Impact of Event Scale-Revised Questionnaire

Instructions. Below is a list of difficulties people sometimes have after stressful life events. Please read each item, and then indicate how distressing each difficulty has been for you during the past seven days with respect to _____, which occurred on _____. How much were you distressed or bothered by these difficulties?

Item Response Anchors are as follows: 0 = not at all; 1 = a little bit; 2 = moderately; 3 = quite a bit; 4 = extremely.

The Intrusion subscale is the mean item response of items 1, 2, 3, 6, 9, 14, 16, and 20. Thus, scores can range from 0 to 4. The avoidance subscale is the mean item response of items 5, 7, 8, 11, 12, 13, 17, and 22. Thus, scores can range from 0 to 4. The hyperarousal subscale is the mean item response of items 4, 10, 15, 18, 19, and 21. Thus, scores can range from 0 to 4. Consider the following.

(1) Any reminder brought back feelings about it.

(2) I had trouble staying asleep.

(3) Other things kept making me think about it.

(4) I felt irritable and angry.

(5) I avoided letting myself get upset when I thought about it or was reminded of it.

(6) I thought about it when I did not mean to.

(7) I felt as if it had not happened or was not real.

(8) I stayed away from reminders of it.

(9) Pictures about it popped into my mind.

(10) I was jumpy and easily startled.

(11) I tried not to think about it.

(12) I was aware that I still had a lot of feelings about it, but I did not deal with them.

(13) My feelings about it were kind of numb.

(14) I found myself acting or feeling like I was back at that time.

(15) I had trouble falling asleep.

(16) I had waves of strong feelings about it.

(17) I tried to remove it from my memory.

(18) I had trouble concentrating.

(19) Reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart.

(20) I had dreams about it.

(21) I felt watchful and on-guard.

(22) I tried not to talk about it.

Total IES-R score: _____.

The inclusion criteria were as follows: (1) survivors of the Great East Japan earthquake and tsunami who were older than 20 years and diagnosed with PTSD according to the Diagnostic and Statistical Manual (DSM)-IV TR; (2) IES-R score ≥ 25 (cutoff point).

Exclusion criteria were as follows: (1) major medical illness such as neoplastic disease, acute inflammation, or any other disease that would most likely prevent the completion of this study; (2) psychosis due to other disorders such as schizophrenia, depression, and/or dementia; (3) delirium due to drugs, alcohol, metabolic intoxication, or inflammation; and (4) use of neuroleptics, antianxiety drugs, antiepileptic drugs, antidepressants, or herbal remedies during the past 2 months.

Participants underwent a complete diagnostic assessment, including medical history, physical examination, laboratory tests, and the Structured Clinical Interview for DSM-IV TR and IES-R. Eligible subjects were randomized into either the SKK or control group. Random numbers were generated using computer software. Treatment codes were held by the corresponding investigator, who was isolated from the patients and outcome data.

The study protocol was performed with the intention to treat. SKK extract was prescribed to the patients in the SKK

group. The SKK extract (TJ-11) was processed by Tsumura (Tokyo, Japan), and it contained the following mixture of dried herbs: Bupleuri Radix (6 g), Trichosanthis Kirilowii (3 g), Cinnamomi Cassiae (3 g), Radix Scutellariae Baicalensis (3 g), Concha Ostreae (3 g), Glycyrrhizae Radix (2 g), and dried Zingiberis Rhizoma (2 g). These herbs are registered in the Pharmacopoeia of Japan version 15. Each participant in the SKK group received 2.5 g of SKK powder (1.17 g extract) 3 times a day for 2 weeks. The processes involved in the production and supply of SKKcomply with Good Manufacturing Practices for Kampo products and are also approved by the Ministry of Health, Labour, and Welfare of Japan.

Participants understood that those randomized into the control group could receive any treatment after completion of the whole trial if they wanted. Patients were free to withdraw at any time. Clinical assessments were performed at the baseline and at the endpoint.

The primary clinical outcome measure was the severity of PTSD symptoms as measured by the total IES-R. The secondary outcome measures were 3 IES-R subscale scores as defined below. The intrusion subscale is the mean response to items 1, 2, 3, 6, 9, 14, 16, and 20. The avoidance subscale is the mean response to items 5, 7, 8, 11, 12, 13, 17, and 22. The hyperarousal subscale is the mean response to items 4, 10, 15, 18, 19, and 21.

2.2. Statistical Analysis. Analyses were performed as a modified intention to treat. If a patient dropped out, all outcome measures were assessed within a week. Statistical analysis was performed using the SPSS software (version 16, SPSS Japan Inc., Tokyo, Japan). Measurements of the mean and SD were calculated at the baseline and at the endpoint for all continuous primary and secondary measures. Baseline comparisons of group differences were conducted using independent samples t-tests for continuous variables and chi-square test for categorical variables. Comparisons between the SKK and the control group were performed by the two-way analysis of variance (ANOVA). The changes in each group from baseline to endpoint were compared using the paired t-test when the intergroup difference was significant ($P < 0.05$) according to the post hoc test.

This study was carried out in compliance with the ethical principles embodied in the Helsinki Declaration. Written informed consent was obtained from each participant prior to participation in this study. The study protocol was approved by the Institutional Review Board of Sendai-Nishitaga National Hospital in Sendai, Japan, and registered with the UMIN clinical trial registry (UMIN000010890, http://www.umin.ac.jp/ctr/index.htm).

3. Results

Of the 48 enrolled subjects, 5 were excluded according to the exclusion criteria. The remaining 43 participants were randomized into SKK ($n = 21$) and control ($n = 22$) groups. The background factors for each group are shown in Table 1, with no significant difference except for the IES-R hyperarousal subscale score. One participant in the SKK group withdrew from the study on the third day because of coughing; all participants in the control group completed the study.

Table 1: Background factors for SKK and control groups.

	Group		P value
	SKK	Control	
n	21	22	
Sex (m/f)	9/12	13/9	0.45
Age (year)	52.3 ± 13.0	48.0 ± 20.9	0.42
IES-R (baseline)	49.6 ± 11.6	43.7 ± 13.7	0.14

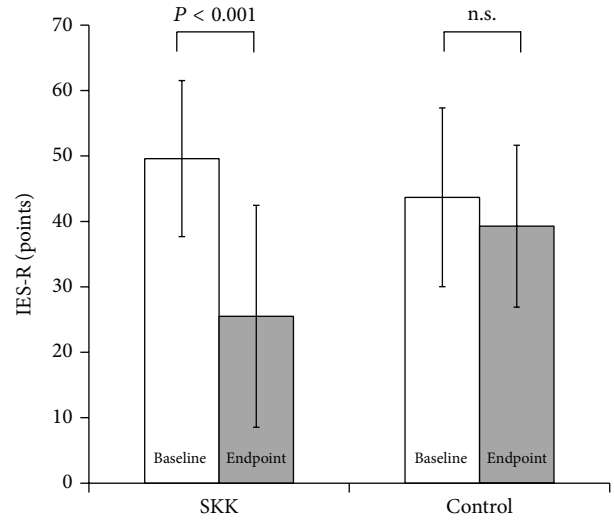

Figure 1: Changes in the total IES-R scores. The two-way analysis of variance (ANOVA) showed a significant difference between the groups ($P < 0.001$) and post hoc testing showed that total IES-R scores were significantly improved only in the SKK group.

3.1. Changes in Total IES-R Scores. Two-way ANOVA analysis showed that the changes in total IES-R scores differed significantly between groups ($P < 0.001$). The post hoc testing showed that the total IES-R score was significantly improved from 49.6 ± 11.9 to 25.5 ± 17.0 in the SKK group ($P < 0.001$) but decreased from 43.7±13.7 to 39.3±12.4 in the control group. The latter trend was not significant (Figure 1).

3.2. Changes in IES-R Subscale Scores. The changes in IES-R subscale scores are shown in Figures 2(a), 2(b), and 2(c). Two-way ANOVA showed significant differences between groups ($P = 0.025$ for avoidance, $P = 0.005$ for hyperarousal, and $P = 0.001$ for intrusion), although there were baseline differences in scores on the hyperarousal subscale. The post hoc testing showed that all subscales improved significantly from baseline to the endpoint in the SKK group ((a) $P = 0.003$, (b) $P < 0.001$, and (c) $P < 0.001$), whereas only avoidance showed a significant change in the control group ((a) $P = 0.032$, (b) n.s., and (c) n.s.).

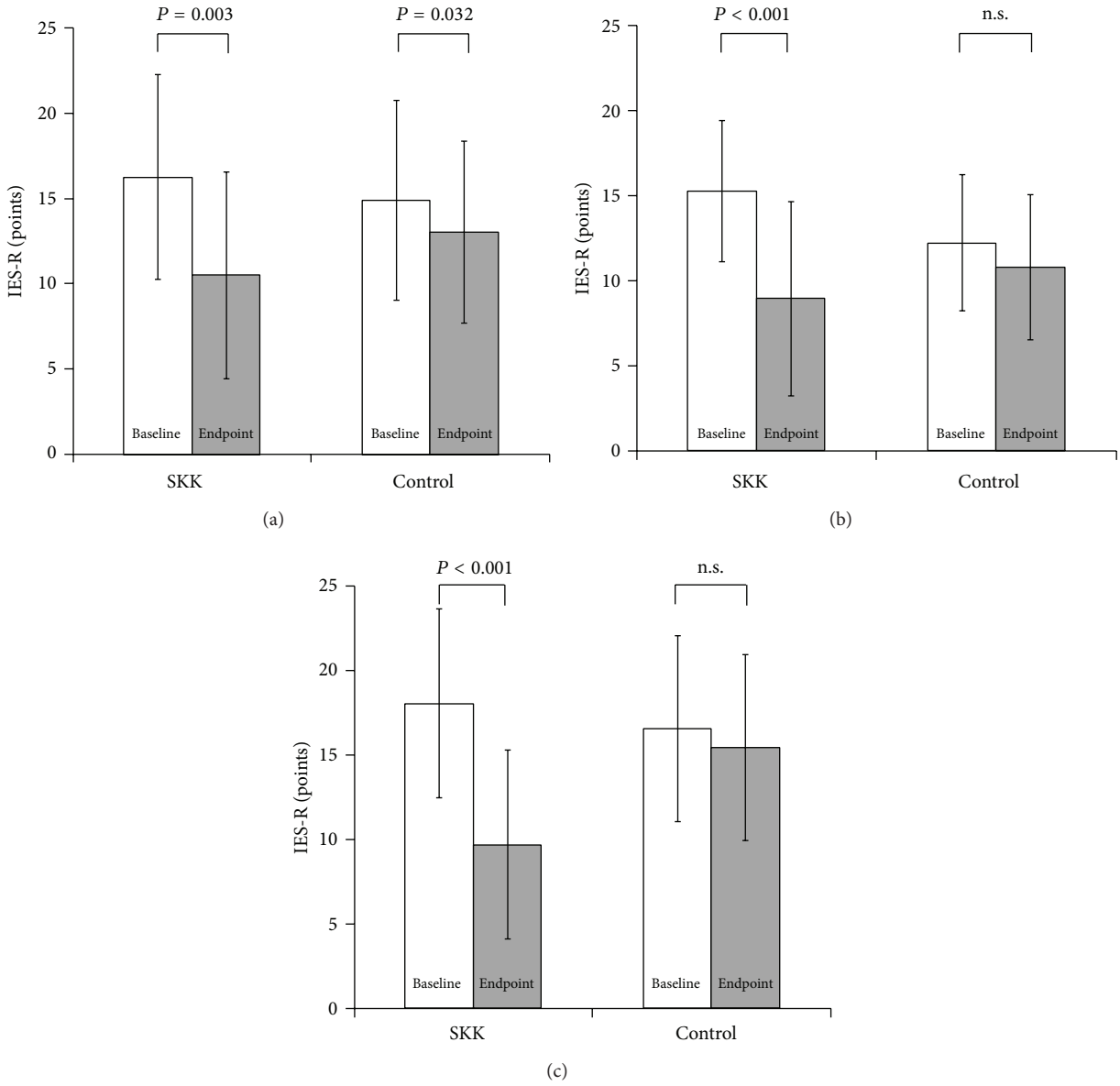

Figure 2: (a) Change in the IES-R avoidance subscale score. (b) Change in the IES-R hyperarousal subscale score. (c) Change in the IES-R intrusion subscale score. Two-way ANOVA showed that each subscale differed significantly between the groups ((a) $P = 0.025$, (b) $P = 0.005$, (c) $P = 0.001$). Post hoc testing showed that all subscale scores changed significantly from baseline to the endpoint in the SKK group ((a) $P = 0.003$, (b) $P < 0.001$, and (c) $P < 0.001$), whereas in the control group, only the avoidance subscale score showed a significant change ((a) $P = 0.032$, (b) n.s., and (c) n.s.).

3.3. Significant Improvements in Each Item in the IES-R. The IES-R items that differed significantly between groups were as follows: "any reminder brought back feelings about it" (Q1, $P < 0.001$); "other things kept making me think about it" (Q3, $P = 0.005$); "I thought about it when I did not mean to" (Q6, $P < 0.001$); "I found myself acting or feeling like I was back in that time" (Q14, $P = 0.003$); "reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart" (Q19, $P = 0.001$); "I had dreams about it" (Q20, $P = 0.002$); and "I felt watchful and on-guard" (Q21, $P = 0.001$).

3.4. Adverse Events and Follow-Up. No participant showed any abnormal change in the blood examination results. One participant in the SKK group withdrew from the study on the third day because of light coughing. The cough was minor and continued for 3 days. When he came back to the clinic 2 weeks later, he showed no symptoms or signs. The relationship between the treatment and the cough is unknown. After the trial, some participants in the control group were treated using traditional medicines according to the rules of the traditional Chinese-Japanese medical decision. They were treated with different medicines, including SKK. No systematic data are available.

4. Discussion

The present study showed that SKK significantly improved PTSD caused by the disaster. A recent study showed that PTSD was strongly suspected in about 10% of all high school students in the city of Sendai at 9 months after the Great East Japan earthquake [3]. Both psychological and pharmacological treatments are effective for PTSD, but there is a severe shortage of psychologists and psychotherapists in the disaster-stricken area. General physicians and primary care doctors must routinely care for PTSD patients. Antidepressants, benzodiazepine, and antipsychotics are used to treat PTSD, but these medications have adverse effects. Drug dependency is common with the use of benzodiazepine. Selective serotonin reuptake inhibitors are also used to treat PTSD, but continuous treatment is often necessary to prevent relapse. Better pharmacological treatments that are both safe and effective are thus needed. Since the disaster, we have tried traditional herbal medicines, such as yokukansan (Yigan San), known to be effective for the treatment of mental disorders [4]. We observed that patients treated with SKK showed very clear improvements in PTSD symptoms. These clinical observations lead us to this trial. This study shows that SKK treatment resulted in the marked, rapid, and tolerable amelioration of PTSD symptoms in all participants, with no severe adverse events. In particular, SKK improved patient responses to the following items: "any reminder brought back feelings about it," "other things kept making me think about it," "I thought about it when I did not mean to," "I found myself acting or feeling like I was back in that time," "reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart," and "I felt watchful and on-guard." These complaints may thus be indications for trying this medication. SKK also improved patient responses to "I had dreams about it." Sleep disturbance is commonly resistant to treatment [5] and can lead to drug abuse [6].

Several pharmacological mechanisms underlying the effects of SKK have been investigated [7–9]. Acute moderate to high stress activates serotonergic neurons in the hippocampus to release 5-hydroxytryptamine (5HT). 5HT then activates postsynaptic 5HT1A receptors that inhibit the process of hippocampal long-term potentiation [7]. The repeated administration of SKK significantly increases the 5HT level in the hippocampus and the corpus striatum and of NE and 5HT in the hippocampus [8]. SKK also regulates plasma interleukin-6 and soluble interleukin-6 receptor concentrations and improves depressed mood in climacteric women with insomnia [9]. These findings may partially explain the mechanisms of SKK action in the treatment of mental disorders.

4.1. Limitations. The present study has some limitations. First, we could not include a placebo for comparison. Japanese law requires that if a placebo is used, it must be produced using the same procedures as the actual drug. Placebo preparation is thus impractical in Japan for a small pilot study like ours. Placebo effects may have influenced our data. The number of participants in this study was rather small but nonetheless reached the statistically targeted sample size. The observation time was short (14 days), because this was the maximum waiting time allowed for participants in the control group. No serious adverse events were observed in the present study, which is noteworthy when considering that Radix Scutellariae Baicalensis is suspected of causing interstitial pneumonia [10]; this relationship is unclear because incidents are rare. Hypokalemia caused by *Glycyrrhiza* root is well known, and it is observed in 6% of elderly subjects [11]. Clinical signs should be carefully monitored because SKK contains these herbs. More elaborate studies are required in the future to elucidate the effect and mechanism of SKK on patients.

Despite these limitations, the present study reports that the familiar herbal medicine SKK is significantly efficacious for the treatment of PTSD, with no serious adverse events. This information may be valuable for general physicians and primary care doctors who care for PTSD patients in Japan because SKK is a well-known traditional herbal medicine. The history of its clinical use in eastern countries dates back to 2 AD. We previously reported that traditional medicine was beneficial in the disaster-stricken area, where the modern medical system and its infrastructures, such as electricity, hospitals, clinics, and logistics, were entirely destroyed [12–14]. In such a situation, traditional medicine could be used to treat daily symptoms that manifest as physical findings among evacuees. Traditional medicine should be considered as a powerful tool for the practice of disaster medicine.

5. Conclusions

SKK significantly improved IES-R scores after 2 weeks of treatment. This traditional medicine may be a treatment choice for psychological and physical symptoms in PTSD patients.

Abbreviations

PTSD: Posttraumatic stress disorder
IES-R: Impact of Event Scale-Revised score
DSM: Diagnostic and Statistical Manual
SKK: Saikokeishikankyoto
ANOVA: Analysis of variance.

Conflict of Interests

Shin Takayama has no personal competing financial or nonfinancial interests in this study; however, the Tohoku University Graduate School of Medicine did receive a grant from Tsumura, a Japanese manufacturer of Kampo medicine.

Authors' Contribution

Takehiro Numata took part in planning the study, performed the data analysis, and provided advice for writing the paper. Shen GunFan helped to plan the study and performed the data analysis. Shin Takayama took part in planning the study, provided advice for statistical analysis, and wrote the

paper. Satomi Takahashi and Yasutake Monma performed the data analysis. Soichiro Kaneko, Hitoshi Kuroda, Junichi Tanaka, Seiki Kanemura, and Masayuki Nara helped to plan the study and provided advice related to writing the paper. Yutaka Kagaya, Tadashi Ishii, Masahiro Kohzuki, and Nobuo Yaegashi were responsible for the study design and execution and assisted in writing the paper. Koh Iwasaki proposed the study. All authors read and approved the final paper. Takehiro Numata and Shen GunFan equally contributed to this work.

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Research Article

Synergetic Antimicrobial Effects of Mixtures of Ethiopian Honeys and Ginger Powder Extracts on Standard and Resistant Clinical Bacteria Isolates

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Purpose. To evaluate antimicrobial effects of mixtures of Ethiopian honeys and ginger rhizome powder extracts on *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (MRSA), *Escherichia coli* (R), and *Klebsiella pneumoniae* (R). *Methods*. Agar diffusion and broth assays were performed to determine susceptibility of these standard and resistant clinical bacteria isolates using honey-ginger powder extract mixtures. *Results*. Honey-ginger powder extract mixtures produced the highest mean inhibition (25.62 mm ± 2.55) compared to the use of honeys (21.63 mm ± 3.30) or ginger extracts (19.23 mm ± 3.42) individually. The ranges of inhibitions produced by honey-ginger extract mixtures on susceptible test organisms (26–30 mm) and resistant strains (range: 19–27 mm) were higher compared to 7–22 mm and 0–14 mm by standard antibiotic discs. Minimum inhibitory concentrations (MIC) of mixture of honeys-ginger extracts were 6.25% (0.625 v/mL) on the susceptible bacteria compared to 75% for resistant clinical isolates. Minimum bactericidal concentration (MBC) of honey-ginger extracts was 12.5% (0.125 g/mL) for all the test organisms. *Conclusion*. The result of this study showed that honey-ginger powder extract mixtures have the potential to serve as cheap source of antibacterial agents especially for the drug resistant bacteria strains.

1. Introduction

Plants have been used as a source of therapeutic agents in traditional medicinal system since ancient time due to bioactive compounds they contain [1, 2]. The world health organization (WHO) has described traditional medicine as cheap way to achieve total health care coverage of the world's population and has encouraged the rational use of plant based traditional medicines by member states [3, 4]. In Ethiopia, one of the developing countries, about 80% of the total population relies on traditional remedies as a primary source of health care [3, 5]. Recently, indiscriminate use of antimicrobial drugs to treat the infectious diseases resulted in the development of resistant pathogenic bacteria strains like drug resistant *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus aureus* [6]. Multidrug resistant pathogenic bacteria strains in hospitals and community are the main cause of mortality and morbidity [6–8]. The increasing resistance bacteria against the existing antibiotics resulted in many studies to focus on antimicrobial agents derived from plants [9–12]. Traditional medicine has become a form of complementary medicine and holds a great promise as source of effective therapy for multiple drug resistant strains of bacteria [13, 14].

Ginger has several ethnomedicinal and nutritional values as spice and flavoring agents in Ethiopia and elsewhere [15, 16]. In last few decades, gingeris extensively studied for its medicinal properties by advanced scientific techniques and a variety of bioactive compounds such as tannins, flavonoid, glycosides, essential oils, furostanol, spirostanol, saponins, phytosterols, amides, alkaloids have been isolated from the different parts of the plant which were analyzed pharmacologically [9, 10, 17–19]. The plant was reported

for antimicrobial [10, 18–22], nephroprotective [22], anti-inflammatory, and immunomodulatory [23] activities. Traditionally, ginger is reported totreatnausea, vomiting, asthma, cough, palpitation, inflammation, and dyspepsia, loss of appetite, constipation, indigestion, and pain in different parts of the world [24]. Similarly, ginger is used, traditionally, for treating common cold, stomachache, cough, fever, and influenza in Ethiopia [25, 26]. Mixtures of ginger rhizome powder and honeys are also used to treat different types of respiratory and gastrointestinal infections in traditional medicine of Ethiopia. The Ethiopian honeys have also been found effective in producing antibacterial effects on susceptible and resistant strains of bacteria from clinical isolates in Ethiopia [27]. The net effect of herbal drug interactions can be additive, synergetic, or antagonistic [28]. The additive and synergetic effects of phytochemicals in fruit and vegetables are responsible for their potent antioxidant and anticancer activities [29]. The combined antibacterial activity of honey-garlic (*Allium sativum*) or fresh ginger leaves or rhizome extract mixtures was reported superior over the use of these antimicrobial agents individually [12, 30]. Drug interactions of the antimicrobial agents contained in mixtures of Ethiopian honeys and dried ginger rhizome powder extracts were not evaluated. Study on the type of interactions of antimicrobial agents in mixtures of honey and ginger rhizome extract would give an insight about the advantage of using mixtures instead of using ginger or honeys individually. The aim of this study was to evaluate antibacterial effects of mixture of Ethiopian honeys (*Apis mellifera* and stingless bees honeys) and ginger powder ethanol/methanol extracts on standard and resistant clinical isolates of *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*.

2. Materials and Methods

2.1. Study Area and Period. Honeys and ginger rhizomes were purchased in September and October, 2012, G.C from Gondar and Tigray regions in Ethiopia and their antimicrobial effects, including honey-ginger powder extract mixtures, were analyzed in biotechnology laboratory in Gondar University from September 20, 2012, to January 1, 2013.

2.2. Chemicals and Reagents. Methanol (B. Number A5791), Ethanol (Alpha chemical B Number M120415, India), Chloroform (BDH chemicals Ltd, Poole, lot Number 27710, England), Acetone, and distilled water were among the different chemicals and solvents used during this study.

2.3. Test Organisms. Standard and clinical isolates microorganisms such as *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (R), Methicillin resistance *Staphylococcus aureus* (MRSA), and *Klebsiella pneumonia* (R) were obtained from Gondar University teaching hospital laboratory.

2.4. Preparation of Honey-Ginger Extracts Solutions. Ginger rhizomes were washed with tape and distilled water and sliced into uniform pieces using sterile knife before drying in microoven at 37°C for 24 hours. The dried ginger pieces were crushed using electric grinder to obtain ginger powder. Different ginger extracts were obtained by adding 20 g of ginger powder into 100 mL methanol and ethanol as previous study [31]. Fifty percent ginger solution (50% v/v) was obtained by dissolving 1 mL ginger extract in 1 mL. Ginger powder water extract was considered as negative control as no inhibition zones were found in previous study [31]. The Ethiopian honeys were filtered using sterile gauze to get 100% pure honey. Honey-ginger mixtures will be prepared by mixing 1 mL of the ginger extract and 1 mL honey extract which will be diluted in 2 mL distilled water to obtain honey-ginger extract solution (50% v/v).

2.5. Preparation of the Mueller Hinton Agar (MHA). Mueller Hinton agar (lot Number X4225F, oxoid, England) medium was prepared by dissolving 38 g of Mueller Hinton agar in 1000 mL distilled water and boil until complete dissolutions. The solution was sterilized in an autoclave (121°C, 1 bar) for 15 min. The suspension was poured (20 mL) into sterile petridishes in the hood to solidify at room temperature.

2.6. Preparation of the Nutrient Broth (7146). After dissolving 8 g nutrient broth powder in one liter of purified water, the mixture was mixed thoroughly to form a clear medium which would be incubated at 35°C for 18–24 hours after the bacterial specimens were inoculated. Turbidity indicates good growth. Nutrient broth culture medium could live longer under refrigeration.

2.7. Preparation of Inoculations and Assays of Antibacterial Activities. The inoculation of the bacteria was done by streaking the surface of the plates with swab in a zigzag manner to spread the bacteria until the entire surface was covered. With a previously sterilized cork borer (4 mm) size, wells of equal distance were bored to drop 100 μL of different ginger extracts and mixtures of honey-ginger powder extracts for agar diffusion assays. Hundred microliters (100 μL) of the honey, ginger powder extract, and honey ginger extract mixtures at 50% (v/v) concentration were inoculated into wells of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (R), *Staphylococcus aureus* (MRSA), and *Klebsiella pneumonia* (R). The culture plates were incubated at 37°C for 24 h. Inhibition zones were indicated by clear area around the wells which were measured in millimeters by caliper in order to evaluate the degree of susceptibility of the test organisms.

2.8. Preparation of 0.5 McFarland Standards and Standardization of Bacteria Concentration. In this study, 0.5 mL of 0.048 M $BaCl_2$ (1.175% W/V $BaCl_2{\cdot}2H_2O$) was added to 99.5 mL of 0.18 M H_2SO_4 (1% V/V) with constant stirring to make 0.5 McFarland Standards. The standard was distributed into a screw capped test tube for color comparison of the test inoculums. Hundred microliter (100 μL) bacteria sample from nutrient broth culture media (lot Himedia laboratory, pvt, ltd., India) was added into 5 mL saline and the concentration was adjusted to 1-2 $\times$ 10^8 colony forming unit

per milliliter (Cfu/mL) by comparing with McFarland 0.5 standardized.

2.9. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Hundred microliter (100 μL) of bacteria samples from nutrient broth culture medium was added into 5 mL saline and the concentration was adjusted to 1-2 $\times 10^8$ Cfu/mL by comparing with McFarland 0.5 standard with constant stirring before culturing in new broth medium to determine the lowest concentration of antimicrobial agent capable of preventing growth (Minimum inhibitory concentrations (MIC)). The inoculations of *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (R), *Staphylococcus aureus* (MRSA), and *Klebsiella pneumonia* (R) were done in different nutrient broth medium containing honey, ginger extract solutions, and mixtures honey-ginger extract solution. The tubes were incubated for 20–24 hours at 37°C to observe turbidity (growth) which indicated the MIC of the honey, ginger extract, and honey-ginger extract mixtures on the test organisms. The minimum bactericidal concentrations (MBCs) were determined by subculturing the contents of nutrient broth used for MIC tests on Mueller Hinton agar media using sterile wire loop and making a strike on the media to see bacteria growth after incubating at 37°C for 24 hours. Absence of growth indicated the minimum bactericidal concentrations (MBCs) of the antimicrobial agents.

2.10. Drug Susceptibility. Drug susceptibility of standard *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and resistant clinical isolates *Escherichia coli* (R), *Staphylococcus aureus* (MRSA), and *Klebsiella pneumonia* (R) cultures were determined using methicillin, amoxicillin, and penicillin discs by disc diffusion method. The result was interpreted as resistant, intermediate, or susceptible by comparing the results with what has already been reported by Clinical and Laboratory Standards Institute (CLSI) [32].

2.11. Statistical Analysis. The antibacterial effects (inhibitions) of honey, ginger extracts, and honey-ginger extracts mixtures were compared using descriptive statistics. All statistical analysis has been performed by using statistical package of social science (SPSS) version 20. Comparisons of honeys, ginger extracts, and honey-ginger extracts mixtures for their overall mean inhibitions were analyzed using one-way analysis of variance (ANOVA). P values less than 0.05 were considered as significantly different. Further Tukey's Honestly Significant Difference (HSD) test or post hoc test was performed to see the effect of the antimicrobial agents on the individual bacteria strain.

3. Results

The overall comparison of the antimicrobial agents has shown that honey-ginger extract mixtures produced the highest mean inhibition (25.62 mm ± 2.55) for the total test organisms compared to the use of honeys (21.63 mm ± 3.30) or ginger extracts (19.23 mm ± 3.42) individually. The least

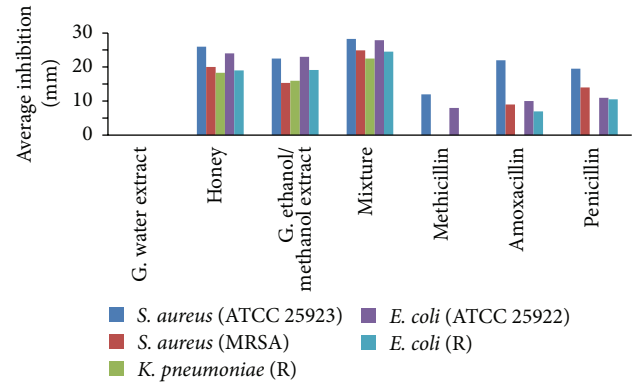

Figure 1: Average inhibition in mm for ginger powder water extracts, honeys, ginger powder ethanol/methanol extracts, mixture of honeys-ginger extracts, and standard antibiotic discs (Methicillin, Amoxicillin, and Penicillin).

mean inhibition result was obtained from the Methicillin antibiotic discs (4 mm ± 5.26) (Table 1). Mean inhibition of the honey-ginger extract mixture on susceptible bacteria isolates (27.74 mm ± 1.56) was higher compared to its effect on resistant isolates (23.97 mm ± 1.72). Similarly, the average inhibition of the honeys, ginger extracts, and honey-ginger extract mixtures was higher on susceptible bacteria strains compared to resistant clinical isolates (Figure 1). Ginger water extract did not produce any inhibition indicating the bioactive agents in the ginger powder are not water soluble. The overall mean inhibition of honeys of stingless bees and *Apis mellifera* was the same as statistical analysis using ANOVA and Tukey's Honestly Significant Difference (HSD) test (multiple comparison) which was greater than 0.05 ($P > 0.05$). Similarly, ginger extracts using methanol and ethanol solvents showed no differences for their inhibitions on the test bacteria strains. But statistically significant difference was observed when overall mean inhibitions of honeys, ginger extracts, and honey-ginger extract mixtures were compared ($P = 0.00$) (Table 1).

The highest inhibition (30 mm) was produced by honey-ginger extract mixtures on the susceptible bacteria strains. The range of inhibitions produced by honey-ginger extract mixtures on the susceptible bacteria isolates (26–30 mm) and resistant clinical isolates (19–27 mm) was also greater than 7–22 mm on susceptible or 0–14 mm on resistant isolates produced by the antibiotic discs (Methicillin, Amoxicillin, and Penicillin) (Table 1). When mean inhibition results on susceptible and resistant bacteria isolates were compared using ANOVA, there were statistically significant differences ($P = 0.000$) for honey, ginger extract, and honey-ginger extract mixture (Table 1).

Further Tukey's Honestly Significant Difference (HSD) test or post hoc test, however, showed absence of significant difference between mean inhibitions of susceptible on *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) when honey ($P = 0.964$), ginger extract ($P = 0.964$), and honey-ginger mixture ($P = 0.973$) were used (Table 2). Similarly, no differences in mean inhibitions were found when the results

Table 1: Results of one-way ANOVA and descriptive statistics of the inhibitions of honeys, ginger extracts, and honey-ginger extracts mixtures on the test bacteria isolates.

Agents	Test organisms	*N*	Mean	Standard deviation	Minimum	Maximum
Honey*	*Staphylococcus aureus* (ATCC 25923)	6	22.50	1.64	21.00	24.00
	Staphylococcus aureus (MRSA)	6	15.50	1.517	13.00	17.00
	Klebsiella pneumoniae(R)	6	16.00	0.63	15.00	17.00
	Escherichia coli (ATCC 25922)	6	23.00	1.27	21.00	24.00
	Escherichia coli (R)	6	19.17	1.33	18.00	21.00
	Total	**30**	**19.23**	**3.42**	**13.00**	**24.00**
Extract*	*Staphylococcus aureus* (ATCC 25923)	6	22.50	1.64	21.00	24.00
	Staphylococcus aureus (MRSA)	6	15.50	1.52	13.00	17.00
	Klebsiella pneumoniae (R)	6	16.00	0.63	15.00	17.00
	Escherichia coli (ATCC 25922)	6	23.00	1.26	21.00	24.00
	Escherichia coli (R)	6	19.17	1.33	18.00	21.00
	Total	**30**	**19.23**	**3.42**	**13.00**	**24.00**
Mixture*	*Staphylococcus aureus* (ATCC 25923)	12	28.25	1.14	27.00	30.00
	Staphylococcus aureus (MRSA)	12	24.92	1.56	22.00	27.00
	Klebsiella pneumoniae (R)	12	22.50	1.57	19.00	25.00
	Escherichia coli (ATCC 25922)	12	27.92	1.38	26.00	30.00
	Escherichia coli (R)	12	24.50	0.90	23.00	26.00
	Total	**60**	**25.62**	**2.55**	**19.00**	**30.00**
Methicillin*	*Staphylococcus aureus* (ATCC 25923)	3	12.00	2.55	11.00	13.00
	Staphylococcus aureus (MRSA)	3	0.00	1.00	0.00	0.00
	Klebsiella pneumoniae (R)	3	0.00	0.00	0.00	0.00
	Escherichia coli (ATCC 25922)	3	8.00	0.00	7.00	9.00
	Escherichia coli (R)	3	0.00	1.00	0.00	0.00
	Total	**12**	**4.00**	**0.00**	**0.00**	**13.00**
Amoxicillin*	*Staphylococcus aureus* (ATCC 25923)	3	21.00	5.26	20.00	22.00
	Staphylococcus aureus (MRSA)	3	10.00	1.00	9.00	11.00
	Klebsiella pneumoniae (R)	3	—	1.00	—	—
	Escherichia coli (ATCC 25922)	3	10.00	—	10.00	10.00
	Escherichia coli (R)	3	8.00	0.00	7.00	9.00
	Total	**12**	**12.25**	**1.00**	**7.00**	**22.00**
Penicillin*	*Staphylococcus aureus* (ATCC 25923)	3	20.00	5.40	19.50	20.50
	Staphylococcus aureus (MRSA)	3	13.00	0.50	12.00	14.00
	Klebsiella pneumoniae (R)	3	—	1.00	—	—
	Escherichia coli (ATCC 25922)	3	10.00	—	9.00	11.00
	Escherichia coli (R)	3	10.00	1.00	9.50	10.50
	Total	**12**	**13.25**	**0.50**	**9.00**	**20.50**
				4.32		

*The mean difference is significant at the 0.05 level.

between the resistant *S. aureus* (MRSA) and *E. coli* (R) were compared for honey-ginger mixture alone ($P = 0.940$).

The minimum bactericidal concentration (MBC) for honeys, ginger extracts, and honey-ginger extract mixtures was 12.5% (0.125 g/mL). The bactericidal effect of this concentration was 100% for all test organisms. The minimum inhibitory concentrations (MIC) of honeys, ginger extracts, and honey-ginger extract mixtures were shown in Table 3. Honey-ginger extract mixtures have 6.25% (0.0625 g/mL) MIC values for all susceptible strains compared to 75% for resistant clinical isolates (Table 3).

4. Discussion

The ginger powder water extract (negative control) did not show bacterial growth inhibition on the test organisms (Figure 1) as it has already been reported [31]. But higher inhibition results were found for ginger ethanol or methanol extracts (19.23 mm ± 3.42) on the test organisms. The highest inhibition (24 mm) produced by this ginger powder extract on *S. aureus* (ATCC 25923) (Table 1) was less than 30 mm for *S. aureus* using fresh ginger rhizome ethanol extract in similar study [11]. This difference could be explained by the loss

Table 2: Results of Tukey's Honestly Significant Difference (HSD) test of honey-ginger extract mixtures on the test bacteria isolates.

Post hoc test	(*I*) spp.	(*J*) spp.	Mean difference (*I* − *J*)	Std error	Sig
Mixture	*Staphylococcus aureus* (ATCC 25923)	*Staphylococcus aureus* (MRSA)	3.33333*	0.54518	0.000
		Klebsiella pneumoniae (R)	5.75000*	0.54518	0.000
		Escherichia coli (ATCC 25922)	0.33333	0.54518	0.973
		Escherichia coli (R)	3.75000*	0.54518	0.000
	Staphylococcus aureus (MRSA)	*Staphylococcus aureus* (ATCC 25923)	−3.33333*	0.54518	0.000
		Klebsiella pneumoniae (R)	2.41667*	0.54518	0.000
		Escherichia coli (ATCC 25922)	−3.00000*	0.54518	0.000
		Escherichia coli (R)	0.41667	0.54518	0.940
	Klebsiella pneumoniae (R)	*Staphylococcus aureus* (ATCC 25923)	−5.75000*	0.54518	0.000
		Staphylococcus aureus (MRSA)	−2.41667*	0.54518	0.000
		Escherichia coli (ATCC 25922)	−5.41667*	0.54518	0.000
		Escherichia coli (R)	−2.00000*	0.54518	0.005
	Escherichia coli (ATCC 25922)	*Staphylococcus aureus* (ATCC 25923)	−0.33333	0.54518	0.973
		Staphylococcus aureus (MRSA)	3.00000*	0.54518	0.000
		Klebsiella pneumoniae (R)	5.41667*	0.54518	0.000
		Escherichia coli (R)	3.41667*	0.54518	0.000
	Escherichia coli (R)	*Staphylococcus aureus* (ATCC 25923)	−3.75000*	0.54518	0.000
		Staphylococcus aureus (MRSA)	−0.41667	0.54518	0.940
		Klebsiella pneumoniae (R)	2.00000*	0.54518	0.005
		Escherichia coli (ATCC 25922)	−3.41667*	0.54518	0.000

*The mean difference is significant at the 0.05 level.

Table 3: Minimum inhibitory concentration (MIC) of honey, ginger extracts, and honey-ginger extract mixtures on susceptible and resistant isolates of the test bacteria species.

Susceptible bacteria isolate	Honey	G. extract	Mixture	Resistant clinical isolates	Honey	G. extract	Mixture
S. aureus (ATCC 25923)	6.25%	6.25%	6.25%	*S. aureus* (MRSA)	12.5%	12.5%	6.25% & 12.5%
E. coli (ATCC 25922)	6.25%	6.25% & 12.5%	6.25%	*E. coli* (R)	6.25% & 12.5%	12.5%	6.25% & 12.5%
				K. pneumoniae (R)	6.25% & 12.5%	12.5%	6.25% & 12.5%
at 6.25% (0.0625 gm/mL)	100%	75%	100%		60%	0%	75%
% at 12.50% (0.125 gm/mL)	0 %	25%	0 %		40%	100%	25%

of water soluble antioxidant volatile oils from the ginger powder up on dehydration [22, 33]. Despite the loss of some antibacterial agents by evaporation during making ginger powder [33], antibacterial agents extracted by the organic solvents were enough to produce inhibition on both susceptible *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 (24 mm) that was greater than inhibitions produced by the three positive control antibiotic discs (range: 7–22 mm) (Table 1). According to Clinical and Laboratory Standards Institute (CLSI) [32] standardization of antibiotic discs, inhibition is considered susceptible if it is ≥18 mm for Amoxicillin (25 μg), ≥14 mm for Methicillin (5 μg), and ≥29 mm for Penicillin (10 μg). The results obtained from amoxicillin discs showed *S. aureus* (ATCC 25923) to be susceptible as already reported by standardization manual [32]. Very low inhibition results from standard antibiotic discs identified the resistant clinical isolates. The mean (23.97 mm ± 1.72) and range (19–27 mm) of inhibitions produced by honey-ginger extract mixtures on

resistant clinical isolates were greater than mean (6.38 mm ± 5.24) and range (0–14 mm) of antibiotic discs (Methicillin, Amoxicillin, and Penicillin). The inhibitions of ginger powder ethanol/methanol extracts were enhanced (25.62 mm ± 2.55) by mixing with honeys due to their synergistic antibacterial effects of honey-ginger extract mixtures as already reported [10]. The ginger powder ethanol/methanol extracts were positive for known antimicrobial agents such as saponin, alkaloids, phlobatannin, flavonoids, and cardiac glycosides [10]. The antimicrobial effects of different honeys might be related to Phytochemicals such as Phenolic acids (benzoic and cinnamic acids) and flavonoids (flavanones, flavanols) which were reported for significant contribution of the antioxidant capacity of honey that varies greatly depending on the floral sources [34]. The presence of propolis makes stingless honeybees honey slightly different in antimicrobial effect from *Apis mellifera* honey [35]. Propolis (resinous protective barrier) contains flavonoids, aromatic acids, esters, aldehydes, ketones, fatty acids, terpenes, steroids, amino acids, polysaccharides, hydrocarbons, alcohols, hydroxybenzene, and several other compounds in trace amounts [36]. When *Apis mellifera* honey (eucalyptus) and stingless honeybees honey were compared, the former had higher phenolic and flavonoid contents than the stingless bee honey which in turn had the higher Antioxidant activity [37]. The antimicrobial effects of different Ethiopian honeys were already evaluated with stingless bees honey producing slightly greater mean inhibition compared to *Apis mellifera* honey [27]. But Tukey's Honestly Significant Difference (HSD) test (post hoc test) showed absence of significant difference between mean inhibitions of stingless honeybees honey and *Apis mellifera* honey ($P = 0.964$) on susceptible on *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). Many, but not all, of the bacterial strains commonly encountered by humans are killed by flavonoids, even though the mechanism is not yet known [38]. The synergistic antimicrobial effects of honey-ginger extract mixtures might be related chiefly to the increase in volume of these flavonoides in the mixture since both ingredients contained these antimicrobial agents.

Antimicrobial activities (antibacterial, antiviral, antifungal, and antiparasitic) of honeys were reported due to high osmolarity, acidity, hydrogen peroxide, and phytochemicals [39–45]. *In vivo* use of honey for human as therapeutic agent depends on the evaluation of the nonperoxide phytochemical components of honey as hydrogen peroxide can be destroyed by catalase in the body tissues and serum [46]. Similarly, the high osmolarity and acidity of honeys are destroyed in the digestion system or blood circulation of human. The nonperoxide phytochemical components of Manuka Apinae honey (after removing hydrogen per oxide by treating with enzyme catalase) from New Zealand have been found to have substantial levels of antibacterial activity [47]. Such manuka honey was tested against seven species of bacteria and was found to have MIC (minimum inhibitory concentration) that range from 1.8% to 10.8% (v/v) [48]. Probably, oral administration of honey-ginger extract mixture, after clinical evaluation and pharmacological standardization, might be therapeutic for some drug resistant disease causing bacteria strains. The minimum bactericidal concentration (MBC) for this study was 12.5% (0.125 g/mL) for both susceptible and resistant bacteria strains. The minimum inhibitory concentrations (MIC) of honey-ginger extract mixture were 6.25% (0.0625 g/mL) for all (100%) susceptible and 75% resistant bacteria strains (Table 3). The fact that both honey and ginger are used in human nutrition and the effectiveness of their mixture as antimicrobial agent at very low concentration make honey-ginger mixture a novel source of effective drug for resistant bacteria strains. Further clinical evaluation and pharmacological standardization of honey-ginger extract mixtures are recommended before using the mixtures against drug resistant bacteria strains for therapeutic purposes.

5. Conclusion

In conclusion, honeys-ginger powder extract mixtures were found to have more antimicrobial effect than the use of honeys or ginger extracts solutions individually. The use of honeys and ginger extracts mixtures for drug resistant bacteria such as *staphylococcus aureus* (MRSA), *Escherichia coli* (R), and *Klebsiella pneumonia* (R) is recommended.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Yalemwork Ewnetu, Wossenseged Lemma, and Nega Birhane participated in selecting the study area and preparing the proposal. Yalemwork Ewnetu carried out the experiment as part of M.S. thesis and Nega Birhane supervised the overall activities and reviewed the documents. Wossenseged Lemma was involved in statistical analysis and preparation of this paper.

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Research Article

Effects of *Ferulago angulata* Extract on Serum Lipids and Lipid Peroxidation

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Background. Nowadays, herbs they are considered to be the main source of effective drugs for lowering serum lipids and lipid peroxidation. The present experimental animal study aimed to assess the impact of *Ferulago angulata* on serum lipid profiles, and on levels of lipid peroxidation. *Methods*. Fifty male Wistar rats, weighing 250–300 g, were randomly divided into five equal groups (ten rats in each). The rat groups received different diets as follows: Group I: fat-rich diet; Group II: fat-rich diet plus hydroalcoholic extracts of *Ferulago angulata* at a dose of 400 mg/kg; Group III: fat-rich diet plus hydroalcoholic extracts of *Ferulago angulata* at a dose of 600 mg/kg; Group IV: fat-rich diet plus atorvastatin; Group V: common stock diet. The levels of serum glucose and lipids and the atherogenic index were measured. In addition, malondialdehyde (MDA), thiol oxidation, carbonyl concentrations, C-reactive proteins, and antioxidant capacity were evaluated in each group of rats. *Results*. Interestingly, by adding a hydroalcoholic extract of *Ferulago angulata* to the high-fat diet, the levels of total cholesterol and low-density lipoproteins (LDL) in the high-fat diet rats were both significantly reduced. This result was considerably greater compared to when atorvastatin was added as an antilipid drug. The beneficial effects of the *Ferulago angulata* extract on lowering the level of triglycerides was observed only when a high dosage of this plant extraction was added to a high fat diet. Furthermore, the level of malondialdehyde, was significantly affected by the use of the plant extract in a high-fat diet, compared with a normal regimen or high-fat diet alone. *Conclusion*. Administration of a hydroalcoholic extract of *Ferulago angulata* can reduce serum levels of total cholesterol, triglycerides, and LDL. It can also inhibit lipid peroxidation.

1. Introduction

Cardiovascular diseases are one of the main causes of death or life-threatening morbidities throughout the world. Hyperlipidemia is a major risk factor for these disorders and it is closely associated with the appearance and progression of coronary atherosclerosis [1]. Various antilipid medications are now used to lower serum lipids in patients with suspected cardiac ischemic disease; however, due to a range of associated serious adverse events such as liver disease, severe muscular disorders, fetal complications, drug interactions, and also the impossibility of using these drugs for long periods of time, the administration of these drugs has now been limited. Therefore, the use of herbal drugs, which have frequently shown promising effects in the treatment of various complications, such as diabetes mellitus [2], cancer [3, 4], Alzheimer's [5, 6], and gastrointestinal complications [7, 8], is preferable.

Herbs are now considered to be a main source for preparing the most effective drugs for lowering serum lipids. More than 200 types of herbal drugs have been identified to have antilipid effects; however, these effects have not been confirmed in a notable number of them.

Ferulago angulate (locally called Chavil) is widespread in the high altitudes of several Asian countries such as Turkey,

Table 1: Comparison of biochemical markers between the experimental study groups.

	Glucose (mg/dL)	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	VLDL (μmol/L)	Atherogenic index (mg/dL)
Normal diet	130.66 ± 48.52	55.22 ± 14.06	89.77 ± 27.01	22.48 ± 4.68	27.53 ± 9.27	17.95 ± 1.22	0.153 ± 0.062
High-cholesterol diet	187.55 ± 12.19	119.00 ± 7.19	178.55 ± 15.32	52.38 ± 5.08	42.27 ± 1.19	35.71 ± 1.56	0.266 ± 0.041
Plant diet (400 mg)	177.67 ± 38.15	81.40 ± 13.28	145.61 ± 12.97	34.71 ± 3.21	41.46 ± 6.02	29.12 ± 7.32	0.175 ± 0.012
Plant diet (600 mg)	166.87 ± 30.21	73.21 ± 10.93	102.33 ± 7.42	22.49 ± 4.05	42.65 ± 5.59	20.46 ± 0.21	0.020 ± 0.003
Atorvastatin	214.40 ± 44.80	76.20 ± 6.45	132.21 ± 14.14	32.58 ± 7.23	42.60 ± 6.26	27.38 ± 1.12	0.023 ± 0.054

HC: high cholesterol.
AIP < 0,11: low risk.
AIP (0.11–0.21): intermediate risk.
AIP >0.22: increased risk.

Iraq, and Iran [9]. Data in the existing literature state that oil originating from this plant has different antimicrobial and antioxidant properties, which can be beneficial in those who are susceptible to ischemic cardiac events [10, 11]. In addition, during the last decade, several lines of evidence have suggested that low-density lipoprotein (LDL) oxidation is a key mechanism for rendering lipoproteins atherogenic [11]. According to the demonstrated role of *Ferulago angulata* as an antioxidant plant [11], and due to the association of oxidized lipids with the progression of atherosclerosis, the role of *Ferulago angulata* in lowering serum lipids and thus preventing the formation of atherosclerosis is now hypothesized. Hence, the present experimental animal study aimed to assess the effects *of Ferulago angulata* on serum lipid profiles and on the levels of lipid peroxidation.

2. Methods

2.1. Animal Selection. In this experimental study, 50 Wistar male rats weighing 250–300 g were divided into five equal groups as follows: Group I received a fat-rich diet with a Presintra-M regimen (containing serum itralipid) (a type of rat diet); Group II received a fat-rich diet with Presintra-Mregimen, plus hydroalcoholic extracts of *Ferulago angulata* at a dose of 40 mg/kg; Group III received a fat-rich diet with a Presintra-M regimen, plus hydroalcoholic extracts of *Ferulago angulata* at a dose of 600 mg/kg; Group IV: received a fat-rich diet with a Presintra-M regimen plus atorvastatin (10 mg/kg); Group V: received only a routine rat diet. The housing conditions, including room temperature and light and dark cycles, were identical for all groups of rats throughout the study. All rats in the study survived for at least 40 days and were fed during this period. In order to prepare the high-fat diet, the Presintra-M regimen was prepared from egg yolk cholesterol (1 g cholesterol from 100 g egg yolk) and serum itralipid reaching 2% cholesterol combined with triglycerides and choline in a palm oil-based emulsion. Finally, an appropriate dose of this regimen to induce hypercholesterolemia in animals (25 mg/kg/day) was administered [12] orally by gavage.

2.2. Preparation of Plant Extract. A herbarium specimen of *Ferulago angulata* was prepared and deposited in the Medical Plants Research Center Herbarium of Shahrekord University of Medical Sciences, Iran.

Initially, the plant was ground into a powder and passed through a suitable sieve. Then, through the following percolation method, a hydroalcoholic extract of the plant was prepared. This process continued for 72 hours; when the extraction was completed, it was followed by evaporation of the alcohol using a rotary evaporator [13]. Finally, two different concentrations of a hydroalcoholic extract of *Ferulago angulate* (400 mg/cc and 600 mg/cc) were prepared.

2.3. Study Measurements. All of the blood samples were centrifuged at 3500 g for 20 min, after the blood had been collected directly from the rat's heart. Blood glucose and serum lipids were measured using commercially available kits from Pars Azmoon (Tehran, Iran). Serum samples were analyzed with a BT 3000 Plus biochemical analyzer (Biotecnica, Italy). To investigate the possible antioxidant effects of the plant, the amount of malondialdehyde (MDA) (as a marker for assessing lipid peroxidation) was measured using a reverse phase high-pressure liquid chromatography (HPLC) method, after derivatization with 2,4-dinitrophenylhydrazine. Thiol oxidation and carbonyl concentrations were measured by spectrophotometry. C-reactive protein (CRP) as an acute phase protein was determined by using commercial animal study kits (Pars-Azmoon, Iran). Antioxidant capacity was evaluated by the DPPH method [14]. The atherogenic index of plasma (AIP = Log(TG/HDL_C)) is defined as the zone of atherogenic risk.

2.4. Statistical Analysis. In order to compare the measured biomarkers among the animal groups, a one-way ANOVA test or a Kruskal-Wallis test was used. In statistical analysis of the data, P values of 0.05 or less were considered statistically significant. All the statistical analyses were performed using SPSS (version 19) for Windows (SPSS Inc., Chicago, IL, USA).

3. Results

As demonstrated in Table 1, by using a high-fat diet, either with a plant extract regimen or without it, blood sugar levels were increased significantly and thus adding plant extract to a high cholesterol regimen could not effectively reduce

Table 2: Comparison of biochemical activity between the experimental study groups.

	Normal diet	High-cholesterol diet	Plant diet (600 mg)	Plant diet (400 mg)	Atorvastatin	*P* value
Ferritin	2.7 ± 0.04	9.1 ± 1.43	2.9 ± 0.07	3.6 ± 0.15	2.7 ± 0.08	<0.05
Thiol plasma	201.3 ± 0.56	173.6 ± 0.38	217.8 ± 9.43	209.5 ± 8.01	207.05 ± 0.7	<0.05
Malondialdehyde	5.74321 ± 0.32	10.03581 ± 0.70	4.30072 ± 0.66	5.64488 ± 0.85	5.11525 ± 0.73	<0.05
Carbonyl plasma	0.72 ± 0.05	0.89 ± 0.08	0.71 ± 0.02	0.75 ± 0.04	0.72 ± 0.08	<0.05
CRP	3.14 ± 0.22	3.98 ± 0.3	3.00 ± 0.2	3.40 ± 0.078	3.00 ± 0.23	<0.05
Antioxidant capacity	282.2 ± 4.23	432.1 ± 1.72	462.2 ± 3.86	413.3 ± 5.54	399.7 ± 0.48	<0.05

CRP: C-reactive protein.

blood glucose levels. However, total cholesterol and also the level of LDL were both significantly reduced by adding plant extract to a high-fat diet, which was even more effective than when atorvastatin was added as an antilipid drug. This beneficial effect of plant extract was not revealed on HDL concentrations and was observed in triglycerides only with high dosages of this plant extraction. Furthermore, the level of MDA as an oxidant agent could be strongly affected by the use of the plant extract in a high-fat diet compared with a normal regimen or high-fat diet alone.

Table 2 summarizes the results and compares the biochemical factors in all of the study groups. The two groups which received a normal diet and a high-fat diet plus atorvastatin had approximately the same levels of ferritin, thiol, MDA, and carbonyl, whereas the group supplied with a high-fat diet had elevated levels of all of the above factors. In contrast, the groups which received a high-fat diet plus plant diet (400 or 600 mg) had lower levels of MDA, CRP, and ferritin, and higher levels of antioxidant capacity and thiol activity, compared to the other groups. Moreover, the rats fed with a high-fat diet plus the plant diet (600 mg) were shown to have significantly higher levels of antioxidant capacity, in comparison with the rats which received the high-fat diet plus lower doses of the plant diet (400 mg).

4. Discussion

Our study demonstrated that using *Ferulago angulata* is associated with lower MDA and CRP levels and higher antioxidant capacity and thiol activity in the study groups. Using higher doses of *Ferulago angulata* had a stronger effect on the hyperlipidemic rats than on the other groups. Epidemiological studies strongly suggest that the long-term consumption of diets rich in plant polyphenols offer protection against the development of cancers, cardiovascular diseases, diabetes, osteoporosis, and neurodegenerative diseases [15]. Along with a few previously published studies on the importance of the therapeutic properties of *Ferulago angulata* in the medicinal field, first we attempted to confirm the antioxidant effect of this plant extract and then we examined its antilipid properties, leading to a demonstration of its protective effects against cardiovascular risks.

According to previous results, this plant has 25 various components with different antibacterial, anti-inflammatory, and antioxidant effects [16]. Our study demonstrated a significant effect of *Ferulago angulata* on plasma MDA levels, which is a major oxidant marker in animal models that were fed with a high-fat regimen. This antioxidant effect was evident in those groups fed with higher doses of the plant extract**.** In a study by Amirghofran et al. [17] an extract of this plant, at a concentration of 50 μg/mL, demonstrated a significant decrease in nitric oxide production after a 24-hour treatment. This inhibitory effect was also observed after 48 hours. Vast experimental studies have proven the antioxidant properties of *Ferulago angulata*. In this regard, peroxide and thiobarbituric indexes of the samples were determined and compared with blank samples (without any antioxidants and with tertiary butyl hydroquinone (TBHQ)). The results indicated that the minimum concentration of extract for conserving the vegetable oil is about 0.02% under excremental conditions [18]. As a result of convincing evidences for the contribution of oxidative damage to the pathology of atherosclerosis and vascular defects, the antioxidant protective effects of this plant make an important contribution to its cardiovascular protective effects.

In addition to the antioxidant properties of *Ferulago angulata*, based on the key role of lipid peroxidation in the progression of atherosclerosis, for the first time, we hypothesized that the antioxidant effect of this plant might inhibit lipid peroxidation and thus the level of oxidized lipids might be reduced by administrating an extract of this plant. To demonstrate this hypothesis, we measured the serum levels of lipid profiles, as well as biochemical biomarkers of oxidative capacity, in animals fed with a high-fat regimen in combination with different doses of *Ferulago angulata* extract. The results indicated that the level of total cholesterol and also LDL were both significantly reduced by adding plant extract to a high-fat regimen, and this lipid lowering effect could be comparable with the effects of atorvastatin, which is a strong antilipid drug. We believe that the effects of *Ferulago angulata* might not originate from its direct effect on lipid metabolism; however, it could be mediated by its influence on lowering oxidized lipid products. However, this is the first observation on the antilipid effects of *Ferulago angulata* and thus it needs to be extensively evaluated in further studies.

In conclusion, an extract of *Ferulago angulata* can effectively increase the activity of thiol groups, reduce plasma levels of MDA as an oxidant agent, and increase antioxidant capacity and it can also decrease serum lipids. Therefore, using an extract of this plant might have a strong cardioprotective effect. However, in order to minimize its side effects, the optimal dosages and the time of administration of this plant extract need to be assessed in further trials.

Conflict of Interests

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

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Research Article

Changes of Peripheral Blood Lymphocyte Subtypes in Patients with End Stage Cancer Administered Localized Radiotherapy and Bojungikki-Tang

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Localized radiotherapy (RT) can cause immune dysfunction. Bojungikki-tang is known to restore immune function. We investigated the absolute counts and percentages of peripheral blood (PB) lymphocyte subtypes in end stage cancer patients before and after RT and after oral administration of Bojungikki-tang water extract (BJITE) and to evaluate the changes mediated by RT and BJITE. Absolute counts and percentages of lymphocyte and lymphocyte subsets were determined in whole blood using the TetraONE System (Beckman Coulter, USA). Flow cytometry results were compared before and after RT and after administration of BJITE. Absolute numbers of CD3+, CD4+, and CD8+ T cells and CD19+ B cells decreased significantly after RT ($P < 0.05$). Absolute numbers of CD3-CD56+ cells did not change in both groups. No significant differences were observed in the absolute counts of lymphocyte subtypes before and after administration of BJITE or vitamin group. When BJITE group was compared with vitamin group, absolute numbers of CD19+ B cells increased. RT-induced decrease in T cells and B cells in PB suggests that immune deterioration occurs after RT. Administration of BJITE might be effective in the restoration of number of B cells.

1. Introduction

Radiotherapy (RT) can be used to control symptoms of patients with end stage cancer. RT with palliative intent is administered in approximately one-half of cancer patients [1]. The purposes of palliative RT are to decrease symptoms, including pain, bleeding, and obstruction, and to improve the patient's quality of life [1, 2]. However, there is an adverse effect of palliative RT. It has been demonstrated that localized radiation, even if administered to limited target volume, causes immune dysfunction [3–5].

Bojungikki-tang (Hochuekkito in Japanese and Buzhong-yi-qi-tang in Chinese) is a traditional herbal formula used in Korea, Japan, and China and it is composed of 10 species of medicinal plants. It has been traditionally used to improve severe weakness in Asian countries. Recent studies have demonstrated that Bojungikki-tang water extract (BJITE) has effect on restoring immune function [6] and inducing an increased protection against microbial agents [7]. It is useful not only for an enhancement of natural killer (NK) activity [8] but also for restoration of antitumor T cell response from stress-induced suppression [9]. Bojungikki-tang is known to have protective effect of intestine and hematopoietic organs against radiation damage [10]. However, the immunological response of Bojungikki-tang in patients administrated RT has not yet been determined.

The aims of the present study were to determine the absolute counts and percentages of peripheral blood (PB) lymphocyte subtypes in end stage cancer patients before and after RT and after oral administration of Bojungikki-tang using single-platform technology and to evaluate the changes mediated by RT and Bojungikki-tang.

2. Methods and Materials

2.1. Patients. Thirteen patients were enrolled in this single center, randomised controlled study. Patients meeting the following criteria were included: age 40 years or older; ECOG performance score 0–2; end stage cancer; selected for palliative RT. Exclusion criteria were as follows: patients who have been treated with operation, chemotherapy, or curative radiotherapy in the 2 months prior to randomisation; patients with severe hepatic or renal dysfunction (AST > 80 IU/L, ALT > 80 IU/L, BUN > 50 mg/dL, and creatinine > 3.4 mg/dL); subjects with a history or hypersensitivity to functional foods; women who are pregnant or nursing; patients who are of child bearing age and are not willing to use contraception; patients who have had major surgery in the 3 months prior to randomization; patients with neuropsychiatric disease; patients who have had cardiovascular or cerebrovascular disease during the previous 6 months; patients who have taken drug in the 3 months prior to randomization. All patients had to provide written informed consent before registration and the trial protocol was approved by the Institutional Review Board of Daegu Catholic University Medical Center, Korea.

Eligible patients were randomly assigned (1 : 1) to palliative RT with Bojungikki-tang (BJITE group) or palliative RT with vitamin (vitamin group). Table 1 shows the patients' clinical characteristics. All patients except one suffered from metastatic tumor.

2.2. Procedures. All patients were treated with external RT using a linear accelerator with 6 and 10MV (Varian 21EX linear accelerator equipped with standard multileaf collimators) for 2 to 5 weeks. Fractions of 1.5–3.0 Gy were delivered 5 days/wk for a total dose of 30–50 Gy. After completing RT, BJITE group was orally administered 9.0 g of Bojungikki-tang everyday (4.5 g × 2) throughout the 4-week period. Bojungikki-tang was manufactured as a spray-dried powder of hot water extract obtained from 10 medical plants composed of Ginseng radix, Atractylodis rhizoma, Astragali radix, Angelicae radix, Aurantii nobilis pericarpium, Zizyphi fructus, Bupleuri radix, Glycyrrhizae radix, Zingiberis rhizoma, and *Cimicifugae* rhizoma. Placebo (vitamin) group was taken as non-Bojungikki-tang control. Vitamin tablet was Co-Q ten vitaalbu tab (Ilyang Pharmaceutical Co., Korea) containing 280.9 mg ascorbic acid, 2.5 mg cupric oxide, 5 mg 0.1% cyanocobalamin, 0.47 mg dried ergocalciferol powder, 20 mg dried retinol acetate powder, 60.8 mg ferrous fumarate, 41.4 mg magnesium oxide, 1.58 mg manganese dioxide, 100 mg nicotinamide, 15 mg pyridoxine hydrochloride, 30 mg riboflavin, 30 mg thiamine nitrate, 60 mg tocopherol acetate 50%, 10 mg ubidecarenone, and 1.87 mg zinc oxide. Vitamin was given orally daily throughout the 4-week period. The administration of any drugs known to affect the host immunity was avoided during this period.

Table 1: Patient clinical characteristics.

	Vitamin group ($N = 6$)	BJITE group ($N = 7$)
Age (range)	64 ± 6 (54–70)	66 ± 6 (59–76)
Sex (f/m)	3/3	3/4
Primary site	Esophagus (1)	Lung (3)
	Pyriform sinus (1)	Rectum (2)
	Breast (1)	Common bile duct (1)
	Liver (1)	Gastrointestine (1)
	Ovary (1)	
	Uterus (1)	
Metastatic tumor	Bone (1)	Bone (5)
	Lymph node (3)	Brain (1)
	Chest (1)	Liver (1)

2.3. Blood Collection. Whole blood samples were collected one day before starting RT (Time 0), one day after completing RT (Time 1), and four weeks after oral administration of BJITE or vitamin (Time 2) in sterile EDTA vacutainers.

2.4. Flow Cytometric Immunophenotyping Using Fluorochrome-Conjugated Antibodies. The percentages and absolute lymphocyte counts were determined in whole blood using a standard single-platform technique, the TetraONE System (Beckman Coulter, Miami, USA), based on four-color flow cytometry in the presence of counting beads. The following combinations were used during immunofluorescence analysis: tube1, anti-CD45-FITC/anti-CD4-PE/anti-CD8-ECD/anti-CD3-PC5, and tube 2, anti-CD45-FITC/anti-CD56-PE/anti-CD19-ECD/anti-CD3-PC5. For each specimen, 100 μL of EDTA-anticoagulated blood was added to 10 μL of tetraCHROME reagent containing the four-antibody-fluorochrome combinations and incubated for 20 min at room temperature in a dark room. Specimens were then lysed using the ImmunoPrep Reagent System at the Coulter Multi-Q-Prep Workstation. Immediately prior to analysis, 100 μL of Flow-Count Fluorospheres (Beckman Coulter) was added to each tube, and the beads were counted along with cells. The sample acquisition and flow cytometric immunophenotypic analysis were performed on the FCM, Cytomics (Beckman Coulter), with a fully automated software-reagent combination. The identification of lymphocytes by expression of bright CD45 and low side scatter signals was followed by the identification of T cell subtypes based on the expression of CD3, CD4, and CD8. B cell subsets and natural killer (NK) cell subsets were based on the expression of CD19 and CD3-CD56+, respectively. The absolute count of cells per microliter was obtained by calculating the number of cells counted × concentration of beads/number of beads counted.

Table 2: Lymphocyte subset counts (cells/μL) in the peripheral blood of end stage cancer patients during study period.

Cell type	Surface markers	Time 0		Time 1		Time 2	
		BJITE group	Vitamin group	BJITE group	Vitamin group	BJITE group	Vitamin group
Total lymphocytes		944 ± 287	707 ± 328	722 ± 355	399 ± 165	1263 ± 567	572 ± 261
T lymphocytes	$CD3^+$	612 ± 183	638 ± 401	406 ± 240*	284 ± 84*	772 ± 331	434 ± 193
Helper	$CD4^+$	348 ± 189	291 ± 106	227 ± 180*	144 ± 47*	381 ± 233	167 ± 64
Cytotoxic	$CD8^+$	268 ± 116	307 ± 300	157 ± 98*	128 ± 58*	342 ± 169	250 ± 145
	$CD4^+/CD8^+$ ratio	1.58 ± 1.15	1.33 ± 0.71	1.71 ± 1.04	1.51 ± 1.08	1.36 ± 1.05	0.81 ± 0.34
B lymphocytes	$CD19^+$	92 ± 63	95 ± 66	34 ± 27*	16 ± 19*	89 ± 68	33 ± 28*
NK cells	$CD56^+$	254 ± 158	138 ± 40	291 ± 322	80 ± 95	288 ± 257	136 ± 128

* P value < 0.05 was considered statistically significant (Time 0 versus Time 1 and Time 0 versus Time 2 by Wilcoxon's signed rank sum test).

Table 3: The percentages of lymphocyte subsets in the peripheral blood of end stage cancer patients during study period.

Cell type	Surface markers	Time 0		Time 1		Time 2	
		BJITE group	Vitamin group	BJITE group	Vitamin group	BJITE group	Vitamin group
Total lymphocytes		12.2 ± 3.8	11.6 ± 2.8	10.4 ± 5.1	10.5 ± 3.3	17.3 ± 8.7	14.9 ± 7.5
T lymphocytes	$CD3^+$	61.2 ± 11.3	70.2 ± 6.4	62.2 ± 19.7	76.3 ± 10.6	64.8 ± 13.1	71.4 ± 9.5
Helper	$CD4^+$	33.0 ± 14.1	35.1 ± 7.6	35.9 ± 18.1	39.4 ± 11.9	31.3 ± 7.7	28.8 ± 7.0
Cytotoxic	$CD8^+$	28.4 ± 13.7	31.1 ± 11.6	23.3 ± 7.0	34.3 ± 17.0	30.6 ± 13.8	40.3 ± 15.3
B lymphocytes	$CD19^+$	8.8 ± 6.0	10.2 ± 6.1	5.0 ± 2.9	4.0 ± 4.4*	7.2 ± 3.8	6.1 ± 5.0*
NK cells	$CD56^+$	25.7 ± 13.9	17.5 ± 7.8	30.1 ± 20.0	16.7 ± 11.1	24.8 ± 15.7	20.7 ± 11.2

* P value < 0.05 was considered statistically significant (Time 0 versus Time 1 and Time 0 versus Time 2 by Wilcoxon's signed rank sum test).

2.5. Statistical Analysis. The data are presented as mean and SD. Mann-Whitney U test and the chi-square test were used when appropriate to compare distribution of individual variable between groups. Wilcoxon's signed rank sum test was used to compare change of absolute lymphocyte count and lymphocyte subset. The two-sided P values were considered significant at $P < 0.05$. SPSS software version 19.0 (SPSS Inc., Chicago, IL) was used for statistical analysis.

3. Results

3.1. Percentages and Counts of Total Lymphocytes and Lymphocyte Subsets after Radiotherapy. Using the single-platform technology, we initially compared both the percentages and absolute counts of lymphocyte subsets in all patients. Absolute counts and percentages of lymphocytes and lymphocyte subsets after RT in the BJITE group ($n = 7$) versus vitamin group ($n = 6$) are shown in Tables 2 and 3, respectively. There were no significant changes in total lymphocyte counts or percentages of lymphocytes during period of time in both groups. No significant differences were observed in the percentages of lymphocyte subtypes. However, absolute numbers of CD3+, CD4+, and CD8+ T cells and CD19+ B cells decreased significantly after RT ($P < 0.05$) (Figure 1). Absolute numbers of CD3-CD56+ cells did not change in both groups.

3.2. Effects of BJITE on Total Lymphocytes and Lymphocyte Subsets. Absolute counts and percentages of lymphocytes and lymphocyte subsets after administration of BJITE or vitamin are shown in Tables 2 and 3, respectively. After administration of BJITE, there were no differences in total lymphocyte number and all lymphocyte subsets compared with baseline data. No differences were found, before and after administration of BJITE or vitamin group, in the CD3+, CD4+, and CD8+ T cells as well in of CD56+ cells. However, there were significant differences in CD19+ B cell counts and percentages of CD19 in vitamin group. In vitamin group, the percentages and absolute counts of CD19 cells did not increase compared with baseline status and remained the decreased status.

4. Discussion

Bojungikki-tang is known to restore immune functions and to improve anti-tumor activity [11–13]. It is usually indicated for patients with general weakness and anemia. Palliative RT relieves clinical symptoms in advanced cancer patients but it can also cause immunological changes. In the present study, we have investigated the percentages and absolute counts of PB lymphocytes and lymphocyte subtypes in patients with end stage cancer administered localized radiotherapy and Bojungikki-tang.

In our study, total lymphocyte in counts and percentages of lymphocytes did not change after palliative RT in both groups. This is contrast to the results of the earlier study of radiation-induced lymphocytopenia [3–5, 14]. This is probably due to differences of radiation dose and interval.

Absolute counts of T cells and B cells declined after localized RT. Absolute counts of NK cells were not affected by RT. Local radiation at therapeutic doses always triggered some activation of the innate and adaptive immune system

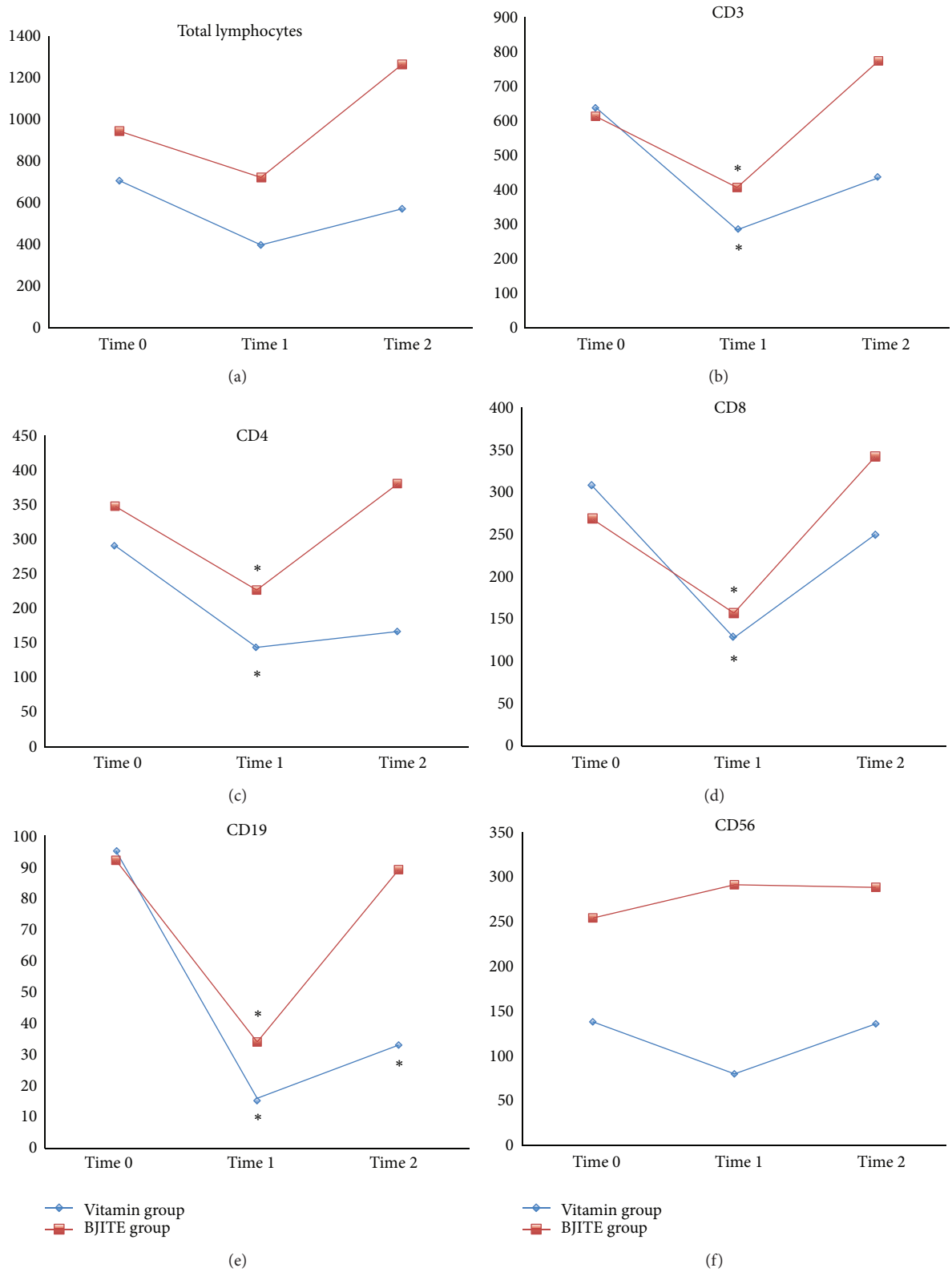

Figure 1: Lymphocyte subset counts during RT in vitamin group versus Bojungikki-tang extract (BJITE) group.

[15]. Results of present study correspond well with those of the earlier study, which reported that naive T cells and B cells are highly radiosensitive [16, 17]. No significant differences were observed in the percentages of lymphocyte subtypes in all patients. These findings differ from the results of the earlier study, which reported that, unlike absolute numbers, the percentages of all analyzed lymphocyte subsets of cervical cancer patients significantly elevated after RT [18]. Safwat et al. [14] reported that localized RT is associated with a significant increase in the percentage of CD4+ T cells and a significant reduction of the absolute number of lymphocyte subsets in patients with non-Hodgkin's lymphoma. This result is similar to our result except for the increase of CD4+ T cell percentages.

Absolute counts of lymphocyte subpopulations in PB have been traditionally measured by dual-platform technologies, which were standard, widely used methodologies. These methods couple percentages of positive cell subsets determined by flow cytometry with the absolute lymphocyte count obtained by automated hematology analyzers. However, they may be responsible for substantial differences in absolute lymphocyte counts determined by different analyzers [19]. Recently, single-platform technologies were developed and are performed entirely on flow cytometry. These methods have significantly improved the assay precision and accuracy and agreement of results between laboratories [19, 20]. Using the single-platform flow cytometry, it was possible to independently analyze the percentages and absolute counts of lymphocyte subsets. In this study, absolute counts of lymphocyte subpopulations decreased significantly after RT, although the percentages of lymphocyte subsets did not change. The values of percentages did not consider total WBC count, which might be changed in cancer patients, particularly after administration of RT. Therefore, it may be useful to determine absolute counts, not percentages, of lymphocyte subsets for exactly reflecting immune status of patients.

Absolute counts or percentages of lymphocytes from BJITE or vitamin group showed no significant differences before and after administration of BJITE or vitamin. Another study also reported that the total number of circulating lymphocytes; CD3+, CD4+, and CD8+ T cells; and CD20+ B cells remained unchanged [21].

Interestingly, CD19+ B cells increased significantly after administration of BJITE. These findings are in contrast to the results of the earlier study, which reported that BJITE is remarkably effective in the restoration of number of T cells and NK cells [8]. Bojungikki-tang is known to have beneficial effects on anti-tumor activity [9] or NK cell activity [21]. In this study, measurement of NK cell activity was not performed. The administration of Bojungikki-tang polysaccharide fraction was associated with elevated expression levels of CD19/CD40 specific for pre-B cells [22].

5. Conclusions

In conclusion, RT-induced decrease in helper T cells, cytotoxic T cells, and B cells in PB suggests that immune deterioration occurs after RT. Administration of BJITE might be effective in the restoration of number of B cells.

Conflict of Interests

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

Authors' Contribution

A-Jin Lee and Ho Jun Lee contributed equally to this work as first author.

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Review Article

Efficacy of Kampo Medicine in Treating Atopic Dermatitis: An Overview

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Atopic dermatitis (AD) is a common inflammatory skin disease with recurring episodes of itching and a chronic relapsing course. Current treatment options for AD include topical agents, such as topical corticosteroids and oral antiallergic drugs. Providing effective long-term treatment is sometimes difficult due to the chronic, relapsing nature of AD; therefore, there is a need to identify better therapeutic options with minimal side effects that are well tolerated over the variable course of the disease. Traditional herbal medicine, also known as Kampo medicine in Japan, has a long history and plays a role in the prevention and treatment of various diseases, including AD. Some Kampo medicines are useful for treating inflammatory skin diseases, and there has been increased interest in using Kampo medicine to develop new therapeutic agents for AD. Standard Kampo formulas for AD are effective in removing the symptoms of "*Netsu Sho*," "*Ketsu-Kyo*," "*Ki-Kyo*," and "*O-Ketsu*." This paper discusses the efficacy of Kampo medicines in treating AD. Knowledge of the mechanisms of action of Kampo medicines will result in greater choices of pharmacotherapeutic agents for AD.

1. Introduction

Atopic dermatitis (AD), also known as atopic eczema, is a common chronic inflammatory skin disease characterized by infiltration of inflammatory cells, extensive pruritus, and a clinical course of symptomatic flares and remissions [1]. The pathogenesis of the disease is now better understood, and important factors involved in the process include genetic factors, skin barrier dysfunction, and immune dysregulation [2]. The lesions are often erythematous with edematous, weeping papules, and vesicles in patients with acute AD, while lichenified thickened plaques were observed in patients in the chronic stage of the disease. Current treatment options for AD comprise topical agents, such as topical corticosteroids and oral antiallergic drugs, to overcome inflammation and moisturizing agents to improve physiologic skin dysfunction. Some AD patients are refractory to these conventional treatments, and current AD therapeutic guidelines recommend the use of further intensive treatment options, such as ultraviolet phototherapy or oral cyclosporine [3]. However, providing effective long-term treatment is sometimes difficult due to the chronic, relapsing nature of AD; therefore, there is a need to identify better therapeutic options with minimal side effects that are well tolerated over the variable course of the disease.

Traditional herbal medicine, also known as Kampo medicine in Japan, has a long history and plays a role in the prevention and treatment of various inflammatory skin diseases, including AD. Indeed, appropriate therapy with Kampo herbal drugs has been proven to be effective in patients with AD who are resistant to basic treatment. Moreover, many patients currently visit physicians seeking Kampo medicine treatment.

2. Kampo Medicine for AD in Japan

Recently, the use of alternative and complementary medicines has become popular in Western countries, where Kampo medicines are now available. The use of Kampo medicines is evolving as a comprehensive approach to treatment and is combined with established Western medical drugs. Kampo medicine was originally introduced from China more than 1,500 years ago. Due to its unique development in Japan, it is quite different from traditional Chinese medicine,

Table 1: Kampo formulas for atopic dermatitis.

Kampo formula	*Sho* (pattern of symptoms)	Skin symptoms
Byakkokaninjinto Oren-gedoku-to	*Netsu Sho* (dry mouth, hot flash, and perspiration)	Pruritus, erythema
Unsei-in	*Netsu Sho* + *Ketsu-Kyo* (dry mouth, hot flash, perspiration + pallor, and dizziness)	Pruritus, erythema, xerosis
Tokii-inshi	*Ketsu-Kyo* (pallor, dizziness)	Xerosis
Keishibukuryogan Kamisyoyosan	*O-Ketsu* (blood stasis/stagnant)	Lichenification
Hochu-ekki-to Juzen-taiho-to	*Ki-Kyo* (fatigue, drowsiness, or appetite loss)	(Fatigue, drowsiness)

and Kampo prescriptions have been used to treat various diseases for centuries in Japan. To date, the Japanese Ministry of Health, Labour, and Welfare has approved 148 Kampo prescription products for use in clinical practice.

Western medicine is anchored in the treatment of the pathogenesis and pathological condition of a disease, whereas Kampo medicine emphasizes totality, in particular improving constitutional aspects. The concept of "*Sho*" originates from traditional Chinese medicine; however, it has been simplified as a result of the Kampo theory [4]. "*Sho*" refers to the pattern of symptoms present at any moment, and Kampo herbal drugs are usually prescribed according to the patient's "*Sho,*" such as "*Yin*" (negativity) and "*Yang*" (positivity) or "*Kyo*" (deficiency) and "*Jitsu*" (fullness), in order to target components, such as "*Ki*" (energy, spirit, and function), "*Ketsu*" (blood and organs), and "*Sui*" (fluid), all of which are considered to be basic components constituting the human body [5]. When treating a patient, Japanese practitioners recognize the Kampo diagnosis of "*Sho*" and choose the most suitable formula.

Some Kampo medicines are useful for treating inflammatory skin diseases, and there has been increased interest in using Kampo herbal drugs to develop new therapeutic agents for AD. In 2010, the report, "Evidence Reports of Kampo Treatment 2010—345 Randomized Controlled Trials—," was published by the Committee for EBM, the Japan Society for Oriental Medicine (published on the website of the Japan Society for Oriental Medicine), promoting several Kampo medicine preparations as effective against AD. Standard Kampo formulas for AD are effective in removing the symptoms of "*Netsu Sho,*" "*Ketsu-Kyo,*" "*O-Ketsu*," and "*Ki-Kyo*" (Table 1). Byakkokaninjinto and Oren-gedoku-to have been used to remove "*Netsu Sho*" symptoms, Tokii-inshi has been used to eliminate "*Ketsu-Kyo*" symptoms, and Keishibukuryogan and Kamisyoyosan have been used to clear away "*O-Ketsu*" symptoms. Hochu-ekki-to and Juzen-taiho-to have been used to remove "*Ki-Kyo*" symptoms, which include a delicate nature, easy fatigability, and/or hypersensitivity in AD patients [6, 7]. To date, several clinical and general studies have been reported in which these preparations were used to treat AD, and new evidence in support of Kampo medicine therapy has been obtained.

3. Kampo Formulas

3.1. Byakkokaninjinto. Byakkokaninjinto contains the extract of five medicinal plants (*Gypsum fibrosum*, *Anemarrhenae rhizoma*, *Glycyrrhizae radix*, *Ginseng radix,* and *Oryzae fructus*) and is commonly used to improve symptoms of "*Netsu Sho,*" which include dry mouth, hot flashes, perspiration, and pruritus. A recently published report showed that Byakkokaninjinto treatment increases the expression of aquaporin 5, which is known to regulate salivary secretion from the submandibular gland [8]. It has also been suggested that Byakkokaninjinto may improve dry mouth, such as that observed in patients with Sjögren syndrome, by increasing the expression of aquaporin 5 and enhancing salivary secretion [8].

Byakkokaninjinto is effective in improving facial pruritus and erythema in many cases of AD. Indeed, in patients with severe facial erythema, it has been reported that hot flashes are significantly improved for one to two weeks after the administration of this drug. Seki et al. previously reported that thermographic images of the face obtained before and after the administration of Byakkokaninjinto to treat symptoms of "*Netsu Sho*" helped to objectively select effective drugs in each case [9]. In order to make the use of Byakkokaninjinto more appropriate and effective, a daily log of conditions was created to investigate the subjective symptoms and effects of the drug [9]. Subsequently, Natsuaki investigated facial skin temperatures using thermography before and after the administration of Byakkokaninjinto. The authors measured the resting skin temperature of the face in 20 AD patients with "*Netsu Sho*" symptoms, including dry mouth, hot flashes, and pruritus, using thermography [10]. The skin temperatures in the AD patients were significantly higher than those observed in the healthy controls ($P < 0.01$). The authors then examined the cooling effect of Byakkokaninjinto in the patients with AD and found that the oral administration of Byakkokaninjinto lowered the facial skin temperature after 90 minutes in the AD patients. Furthermore, the skin symptoms of hot flashes and pruritus were reduced in the AD patients with "*Netsu Sho.*" The results suggested that thermography is useful for evaluating the cooling effect of Byakkokaninjinto.

3.2. Keishibukuryogan. Keishibukuryogan is a traditional herbal medicine that is widely administered to patients with symptoms of "*O-Ketsu,*" which involves blood stasis, in order to improve blood circulation. Matsumoto et al. explored the use of a proteomic approach for diagnosing blood stasis in rheumatoid arthritis patients treated with Keishibukuryogan [11]. In addition, Keishibukuryogan is used to treat symptoms of peripheral ischemia, such as cold extremities [12]. Keishibukuryogan is now one of the most frequently used traditional medicines in Japan and has been used clinically to treat various diseases, including skin diseases.

Keishibukuryogan preparations demonstrate anti-inflammatory and free radical scavenging effects. Keishibukuryogan is composed of five medicinal plants (*Cinnamomi cortex*, *Paeoniae Radix*, *Moutan cortex*, *Persicae semen,* and *Hoelen*) [13]. *Paeoniae Radix* and *Moutan Cortex* contain many known active components that are common in both plants, including paeoniflorin, paeonol, oxypaeoniflorin, benzoylpaeoniflorin, and palbinone [14]. Paeoniflorin is a characteristic principal bioactive component of *Paeoniae Radix*, which contains approximately 5.57% (w/w) paeoniflorin, and *Moutan Cortex*, which contains approximately 3.96% (w/w) paeoniflorin [15]. Paeoniflorin has many pharmacological effects, including anti-inflammatory and antiallergic effects [16]. Keishibukuryogan and paeoniflorin suppress the production of macrophage migration inhibitory factor, interleukin (IL)-6, IL-8, and tumor necrosis factor-α in LPS-stimulated human dermal microvessel endothelial cells and prominent cells in the dermal skin [17]. Accordingly, Keishibukuryogan may have beneficial effects that result in the inhibition of inflammatory cytokines in human dermal microvessel endothelial cells.

Keishibukuryogan improves the conjunctional microcirculation in patients with cerebrospinal vascular diseases [18], thus suggesting that it may have beneficial effects on hematological parameters, such as blood viscosity and red blood cell deformability [19, 20]. In addition, Keishibukuryogan exerts useful effects on the endothelial function in patients with metabolic syndrome-related factors [21]. AD lesions are characterized by differences in the state of activation of endothelial cells and the release of inflammatory mediators by and toward the vasculature [22]. Longstanding inflammatory skin conditions result in itching-induced scratching, which causes cutaneous damage, including the manifestation of endothelial cells as lichenification. Recently, the long-term administration of Keishibukuryogan was found to achieve marked improvements in patients with a high level of lichenification [23]. Consequently, it is believed that such long-term administration is effective in patients who exhibit a tendency toward the remission of symptoms due to Keishibukuryogan administration for approximately one month [23]. Therefore, Keishibukuryogan has been found to be effective against AD, particularly in patients with lichenified lesions, and may become a useful therapy for intractable AD in patients previously treated with conventional modalities.

3.3. Hochu-Ekki-to. Hochu-ekki-to contains 10 medicinal plants (*Astragali Radix*, *Atractylodis Lanceae Rhizoma* , *Ginseng Radix*, *Angelicae Radix*, *Bupleuri Radix*, *Zizyphi Fructus*, *Aurantii Nobilis Pericarpium* , *Glycyrrhizae Radix*, *Cimicifugae Rhizoma,* and *Zingiberis Rhizoma*). Hochu-ekki-to has been used traditionally to treat weak patients with chronic diseases possessing the symptoms of "*Ki-Kyo*," which include loss of appetite, a mild fever, night sweats, palpitations, fear, restlessness, a weak and feeble voice, slurred speech, and a disturbance of vision [24]. This medicine is known to have an effect in easily improving infectious conditions and recovering the body's protective capacity by enhancing the immune function and is intended to be applied in patients exhibiting symptoms in the respiratory apparatus [25], given its clinical efficacy in treating chronic colds and preventing respiratory infection with MRSA in patients with impaired consciousness. The oral administration of Hochu-ekki-to in mice succeeds in enhancing antigen-specific antibody responses in the systemic immune system via the upper respiratory mucosal immune system [24]. Furthermore, Hochu-ekki-to has various immunopharmacological effects, particularly antiallergic properties, including suppressing the serum IgE level and eosinophil infiltration and improving dermatitis by controlling the Th1/Th2 balance, possibly by inducing interferon-γ production from intraepithelial lymphocytes [26–28].

Previous case reports have suggested that Hochu-ekki-to is effective in a certain subgroup of patients with AD [29]. Following the administration of Hochu-ekki-to in patients with recalcitrant AD, the levels of eosinophils were statistically decreased after three months of treatment with this formula. The serum IgE levels showed a tendency to decrease following the administration of this substance [29].

The use of Chinese herbal medicine therapy in clinical studies has various restrictions due to the difficulty of creating placebos. A recent double-blind, placebo-controlled study showed considerably effective benefits in managing the clinical signs of AD with Hochu-ekki-to [30]. In that study, 91 AD patients with "*Ki-Kyo*" symptoms were enrolled. Hochu-ekki-to or a placebo was orally administered twice daily for 24 weeks. The results showed that the total equivalent amount of topical agents, including topical steroids and/or tacrolimus, was significantly lower in the Hochu-ekki-to group than in the placebo group after the 24-week treatment period ($P < 0.05$), although the overall skin severity scores were not statistically different. That study demonstrated that Hochu-ekki-to is a useful adjunct to conventional treatments in AD patients with a "*Ki-Kyo*" constitution. The use of Hochuekki-to significantly reduces the dose of topical steroids and/or tacrolimus in AD patients without aggravating AD. Recently, Takemura et al. conducted a clinical study in which Hochu-ekki-to was used in combination with topical corticosteroids in AD patients [31]. Consequently, a significant decrease in the use of topical steroids was observed.

3.4. Juzen-Taiho-to. Juzen-taiho-to consists of 10 different herbs (*Astragali Radix*, *Cinnamomi Cortex*, *Rehmanniae Radix*, *Paeoniae Radix*, *Cnidii Rhizoma*, *Atractylodis Lanceae Rhizoma*, *Angelicae Radix*, *Ginseng Radix*, *Hoelen,* and *Glycyrrhizae Radix*) and has traditionally been used to treat symptoms of "*Ki-Kyo*," which include fatigue, loss of appetite and anorexia, in patients with various diseases in Japan. At present, Juzen-taiho-to is often clinically used to treat some

cancers and prevent adverse effects resulting from chemotherapy and radiation therapy, blood diseases, skin diseases, and so on [32]. Juzen-taiho-to suppresses primary melanocytic tumors by potentiating T-cell-mediated antitumor cytotoxic immunity *in vivo* [33]. AD patients successfully treated with Juzen-taiho-to have been reported in Japan [34, 35]. It has been suggested that the effects on Th1 responses achieved via innate immune signaling are one possible mechanism underlying the activity of Juzen-taiho-to in controlling morbid states of the skin in patients with AD [34].

4. Conclusion

Many Kampo medicines have anti-inflammatory and antiallergic effects. Several studies of the pharmacological effects of Kampo medicines and their constituent herbs have been conducted in recent years, and much evidence has been accumulated. The mechanisms of action of the herbs included in Kampo medicines are becoming clearer, in addition to the fact that these mechanisms are not found in Western medicines. Furthermore, the concept of "*Sho*" can be scientifically analyzed and is thus expected to become another accepted concept in Western countries. On the other hand, although considerable attention has been paid to traditional herbal medicines as a treatment option for AD, only a few reports have examined the efficacy of these medicines in a randomized, double-blind manner [30, 36, 37]. It was recently reported that herbal medicine significantly improves symptom severity and is well tolerated in patients with AD. However, the poor quality of the studies does not allow for valid conclusions to be drawn supporting the tolerability or routine use of these drugs [38].

Kampo medicines are now widely used worldwide. The general public tends to believe that these agents are safe because of their natural origin; thus, they are used frequently. However, administration of Kampo medicines has been reported to be associated with diverse side effects, such as interstitial pneumonia [39] and skin eruption [40]. We also need to pay attention to these side effects.

Additional studies addressing these methodological issues are warranted to determine the therapeutic benefits of Kampo medicine for AD.

Abbreviations

AD: Atopic dermatitis
IL: Interleukin.

Conflict of Interests

The author states that there is no conflict of interests.

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Research Article

Randomized Clinical Trial: The Clinical Effects of Herb-Partitioned Moxibustion in Patients with Diarrhoea-Predominant Irritable Bowel Syndrome

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Objective. To explore the efficacy of Herb-partitioned moxibustion in treating IBS-D patients. *Method*. 210 IBS-D patients were randomly assigned on a 3 : 3 : 2 basis to group HM, group FM, or group PB for 4-week treatment. The change of GSRS total score at weeks 4 and 8, the changes of GSRS specific scores, and adverse events were evaluated. *Results*. Patients in group HM and group FM had lower GSRS total score at week 4 (1.98 ± 0.303, 2.93 ± 0.302 versus 3.73 ± 0.449) and at week 8 (2.75 ± 0.306, 3.56 ± 0.329 versus 4.39 ± 2.48) as compared with patients' score in group PB. However, there was no significant difference of GSRS total score between group HM and group FM. The effect of HM was significantly greater than that of orally taking PB in ameliorating the symptoms of rugitus (0.38 versus 0.59, $P < 0.05$), abdominal pain (0.28 versus 0.57, $P < 0.01$), abdominal distension (0.4 versus 0.7, $P < 0.01$), and increased passage of stools (0.06 versus 0.25, $P < 0.01$) at the end of treatment period. In the follow-up period, patients' therapeutic effect in group HM remained greater than that in group FM (in abdominal pain, abdominal distension, and increased passage of stools) and that in group PB (in loose stools). *Conclusions*. HM appears to be a promising, efficacious, and well-tolerated treatment for patients with IBS-D.

1. Introduction

Irritable bowel syndrome (IBS), which is defined as "abdominal pain or discomfort that occurs in association with altered bowel habits over a period of at least three months" [1], is a common gastrointestinal (GI) disorder. Symptoms of IBS include abdominal pain, changes in bowel habits (diarrhea, constipation, or both), bloating, and incomplete defecation [2]. Although IBS does not end up with the development of serious disease and associated mortality, it does have a significant negative impact on patients' quality of life and social functioning [3] and can increase healthcare costs [4]. Unfortunately, IBS remains incurable and conventional medicine only provides some relief for individual symptoms [5]. Nowadays standard available therapies for IBS-D include antispasmodics, antidiarrhoeals, and 5-hydroxytryptamine$_3$ (5-HT3) receptor antagonist [6]. However, a series of systematic reviews conducted by the American College of Gastroenterology Task Force showed poor quality of evidence that certain antispasmodics and antidiarrheals can reduce the frequency of stools but cannot affect the overall symptoms of IBS. They also noted that $5HT_3$ and 5-hydroxytryptamine$_4$ ($5HT_4$) agonists carry a possible risk of ischemic colitis and cardiovascular events, respectively, which may limit their utility [1].

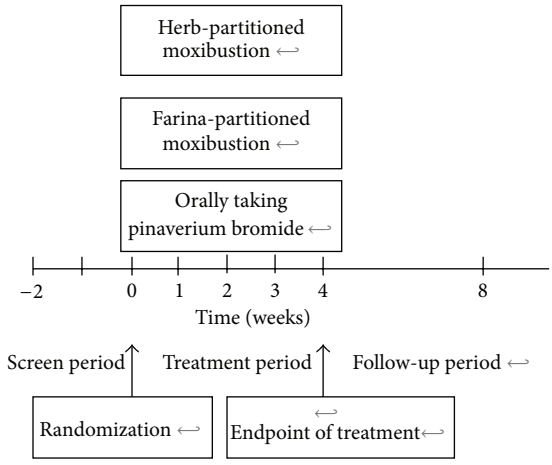

Figure 1: Schematic presentation of the study design.

It is common for IBS patients to seek complementary and alternative medicine (CAM) to treat their annoying bowel symptoms [7], and the percentage of IBS patients who have used CAM is 20–35% [8, 9]. A recent systematic review and meta-analysis of acupuncture for IBS found that patients reported greater benefits from acupuncture than from pharmacological therapies [10]. Another comprehensive review recorded that Chinese herbal prescription can decrease visceral hypersensitivity of IBS patients [11]. Both of them indicated the superiority of traditional Chinese medicine (TCM) in treating IBS.

Moxibustion therapy, which is used to treat diseases not less than acupuncture in China, is one of the three major therapies just as important as acupuncture and traditional Chinese herbal medicine, but less attention is paid to moxibustion therapy than acupuncture nowadays. Herb-partitioned moxibustion (HM) is a kind of moxibustion therapy, and it is used for treating many diseases but seldom used for treating IBS before. Whether there is curative effect of HM in ameliorating IBS symptoms remains unknown. Consequently, the aim of present trial is to explore the potential of HM to attenuate the symptoms of IBS-D.

2. Methods

2.1. Study Design. The present study was a multicenter (3 centers), randomized, controlled trial (Figure 1).

2.1.1. Patient Selection. Patients were recruited from the outpatients of Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Qilu Hospital Affiliated to Shandong University and Beijing Hospital of Traditional Chinese Medicine Affiliated to Capital Medicine University, between October 2008 and January 2011.

Eligible patients met the following inclusion criteria: adult patients (aged 18–60 years) suffer from IBS-D as defined by Rome III [12] criteria and IBS-D was caused by spleen-qi deficiency according to the diagnostic methods of the Chinese medicine. At the end of the screen period, IBS-D patients were randomized into one of the three groups if they exhibited diarrhea the occurred for at least 2 days/week.

Patients excluded from study participation had the following: stool with pus and blood or with mucus; females who were pregnant or nursing; previous gastrointestinal or abdominal surgery; presence of primary disease such as cardiovascular disease, kidney disease, or hematopoietic system disease; diagnosis of any psychiatric disease; recent excessive consumption of alcohol, usage of any medication aimed for the treatment of IBS within the 2 weeks preceding randomization, or involvement in any investigational medications during the 2 weeks prior to screening. Patients who were allergic to the drugs used in the trial were also excluded.

2.1.2. Randomization and Blinding. Randomization was performed by an independent statistician through generating allocation numbers based on a random number creation system. Eligible patients were randomly assigned on a 3 : 3 : 2 basis into herb-partitioned moxibustion group (group HM, herbs were used), farina-partitioned moxibustion group (group FM, farina was used as placebo for it contains no herb ingredients) or orally taking pinaverium bromide [13] group (group PB). The patients were randomly assigned in each center using a blocked randomization, and the block size is 16.

Double-blind could not be performed in this study. However, we can blind the patients between group HM and group FM. The evaluators, data collectors, and data statisticians were all blinded to treatment arm assignments.

The study protocol was approved by the Ethics Committee of Shandong University of Traditional Chinese Medicine Affiliated Hospital in 2010 (Registration no.: 20100110). All patients gave written informed consent. In order to insure the quality of this trial, treatment was performed by trained and certified clinicians who have the Chinese medicine practitioner license from the Ministry of Health of the People's Republic of China.

2.1.3. Interventions. The medicinal herbs used in Group HM (seen in Table 1) were purchased from Jianlian Medicine Company (Jinan, China) and were kindly authenticated by Dr. Baoguo Li the professor of pharmacognosy (College of Pharmacy, Shandong University of Traditional Chinese Medicine, Jinan, China). The voucher specimens were deposited in Shandong University of Traditional Chinese Medicine. The voucher specimen number of the herbs was 080916.

The herbs were mixed in proportions (seen Table 1) and were shattered into medicamental pulverata by pulverizer. In group HM and group FM, the patients' navel and its surrounding area were disinfected using 75% alcohol. A bowl made by dough with a hole (diameter 2 cm, depth 2 cm) in the middle was placed on patient' navel. Musk and medicamental pulverata (about 8–10 g) were filled in the hole. The medicamental pulverata was replaced by farina (only contained flour, about 8–10 g) in group FM. Then a burning moxa cone (diameter 2 cm, height 2 cm) was put on the medicamental pulverata and changed till it burned out. Ten

Table 1: Standard formula of ingredients of the medicamental pulverata.

Chinese name	Pharmaceutical name	Powered herb, %	Effect according to TCM
Bai Zhu	Atractylodis macrocephalae, rhizome	20	Invigorating spleen
Fu Ling	Poriae cocos, sclerotium (Hoelen)	15	Clearing damp and promoting diuresis
Ding Xiang	*Syzygium aromaticum*	15	Checking diarrhea
Shan Yao	*Dioscorea opposite*	10	Invigorating spleen-qi
Wu Bei Zi	Galla chinensis	40	Checking diarrhea
She Xiang	Moschus	A few	Motivating the penetration of herbs

moxa cones were used during each treatment time. At the end of the treatment, the medicamental pulverata was sealed with adhesive tape and was washed clean 2 days later. The treatment (both in group HM and group FM) was performed twice a week and lasted for 4 weeks (treatment period). Patients in group PB orally took pinaverium bromide 50 mg three times a day for 4 weeks.

2.2. Efficacy Endpoints

2.2.1. Primary Efficacy Endpoint. The primary efficacy endpoint is the total score of gastrointestinal symptom rating Scale [14] (GSRS total score). The GSRS was originally created and validated in Swedish [14, 15] for the assessment of GI symptoms. It contains seven items (abdominal pain, rugitus, abdominal distension, increased flatus, increased passage of stools, loose stools, and urgent need for defecation). In the present study, it was translated into Chinese and modified according to clinical practice. In this scale, each item was scored 0 (absence of the symptom), 1 (mild symptom), 2 (moderate symptom), or 3 (extreme degree of the symptom), rendering a total score between 0 and 21. The higher the score is, the more severe the symptom is.

2.2.2. Secondary Efficacy Endpoint. Secondary efficacy endpoints are the GSRS symptom specific scores of abdominal pain, rugitus, abdominal distension, increased flatus, increased passage of stools, loose stools, and urgent need for defecation. The occurrence (number of times and frequency) and severity of all these symptoms with regard to GSRS were recorded in a diary by the patients themselves. Adverse events (AEs) were also recorded by clinicians during the treatment period, such as skin burn related to moxibustion therapy (the dropped ashes from the burning moxa cone) and infection caused by burn or allergy caused by the medicamental pulverata. All of them were recorded in detail throughout the study.

To insure the safety of this study, patients also received standard heart, renal, liver function laboratory tests and routine examination of blood, urine, and stools in the screen period and at the end of the treatment period (week 4).

Patients were followed up one month after the treatment (at week 8) by investigators using phone, mail, or e-mail.

2.3. Statistical Analysis. In the present study, the efficacy analyses were primarily performed on the full analysis set (FAS) corresponding to the intention-to-treat (ITT) population which included all randomized patients and secondarily on the per protocol (PP) population (including eligible patients who completed the whole study treatment).

The primary efficacy variable was the changes from baseline in GSRS total score. As for secondary endpoints, the changes of the GSRS symptom specific scores from baseline were analyzed. All these variables, both between-group and within-group comparisons, were made for exploratory advantage of curative effect.

We used Wilcoxon rank-sum test in between-group comparisons while the Wilcoxon's sign rank test was used in within-group comparisons. P values reported in this paper are two-sided and P values of <0.05 were considered statistically significant. All statistical analyses were carried out by using the Statistical Analysis System (SAS) version 8.1 (SAS Institute, Inc., Cary, NC, USA).

3. Results

3.1. Baseline Patient Characteristics. In our study, patients were mostly females (74.29%) and had a mean age of 25.7 years. GSRS total scores were similar among three different groups at baseline. And there were also no significant differences in GSRS symptom specific scores among three groups. The baseline age, gender, race, and the severity of IBS-D symptoms of the patients were listed in Table 2 and they were all similar among different groups.

In the present study, 285 patients were recruited, and 75 patients were excluded because they did not meet the inclusion criteria. In total, 210 patients (FAS) were randomized. The majority of randomized patients ($n = 200$, 95.24%, PP) completed the trials, whereas 10 (4.76%) patients discontinued the study. The most common reasons for study discontinuation were the lack of efficacy in three patients (group FM: one patient; group PB: two patients) and AEs in two patients (group HM: one patient; group PB: one patient). Patients' disposition and the size of the analysis population were summarized in Figure 2. Similar analytic results of PP population and FAS population were detected. Here we are going to introduce the analytic results of FAS population.

3.2. Primary Endpoint. The GI symptom severity, as measured by the GSRS total score, was compared within-group and between-group. A decline in GI symptoms (GSRS total score) was identified throughout the treatment period in all the three groups ($P < 0.01$) (Figure 3). Within-group comparison between week 8 and week 4 (Table 3) showed no

Table 2: Baseline demographic characteristics and GSRS total score (ITT) in three groups.

Parameter	Group HM (75)	Group FM (75)	Group PB (60)	Total (210)	P value
Gender					
Female (%)	55 (73.33)	57 (76.00)	44 (73.33)	156 (74.29)	0.91
Male (%)	20 (26.67)	18 (24.00)	16 (26.67)	54 (25.71)	
Age (years)					
Mean (min, max)	26.69 (18.00, 59.00)	25.39 (19.00, 59.00)	24.97 (19.00, 60.00)		0.21
S.E.	1.045	0.908	1.012		
Race					
Han nationality (%)	74 (98.67)	73 (97.33)	60 (100.00)	207 (98.57)	0.43
Other nationalities (%)	1 (1.33)	2 (2.67)	0 (0)	3 (1.43)	
Marriage					
Married (%)	13 (17.33)	9 (12.00)	10 (16.67)	32 (15.24)	
Single (%)	62 (82.67)	65 (86.67)	50 (83.33)	177 (84.28)	0.50
Other (%)	0 (0)	1 (1.33)	0 (0)	1 (0.48)	
Course of disease (month)					
Mean (min, max)	63.49 (3.00, 480.00)	66.49 (3.00, 486)	42.67 (3.00, 360.00)		0.06
S.E.	8.231	10.947	6.859		
Combination of other drugs					
Yes (%)	0 (0)	0 (0)	0 (0)	0 (0)	
No (%)	75 (100.00)	75 (100.00)	60 (100.00)	210 (100)	
GSRS total score					
(Mean ± S.E.)	8.75 ± 0.251	8.60 ± 0.276	8.85 ± 0.287		0.71

S.E: standard error; GSRS: gastrointestinal symptom rating scale; ITT: intention-to-treat; HM: herb-partitioned moxibustion; FM: farina-partitioned moxibustion; PB: orally taking pinaverium bromide.

Table 3: Changes of GSRS total score in the three groups.

	Baseline (week 0) (mean ± S.E.)	Week 4 (mean ± S.E.)	Change at week 4	P value	Week 8 (mean ± S.E.)	Change at week 8	P value
Group HM	8.75 ± 0.251	1.98 ± 0.303	$-6.77^{\triangle}$	<0.01	2.75 ± 0.306	$-6.00^{\#}$	<0.01
Group FM	8.60 ± 0.276	2.93 ± 0.302	−5.67	<0.01	$3.56 \pm 0.329^{*}$	−5.04	<0.01
Group PB	8.85 ± 0.287	3.73 ± 0.449	−5.12	<0.01	$4.39 \pm 0.482^{**}$	−4.46	<0.01

S.E.: standard error; compared with week 4, $^{*}P < 0.01$, $^{**}P < 0.05$; compared with Group PB, $^{\triangle}P < 0.01$, $^{\#}P < 0.01$; GSRS: gastrointestinal symptom rating scale; HM: herb-partitioned moxibustion; FM: farina-partitioned moxibustion; PB: orally taking pinaverium bromide.

significant change of GI symptom in Group HM. However, in group FM (3.56 ± 0.329 versus 2.93 ± 0.302, $P < 0.01$) and group PB (4.39 ± 0.482 versus 3.73 ± 0.449, $P < 0.05$) the GI symptom was heavier at week 8 than at week 4. Between-group comparison (Table 3) showed that the relief of GI symptom was better in group HM than that in group PB at week 4 (−6.77 versus −5.12, $P < 0.01$) and at week 8 (−6.00 versus −4.46, $P < 0.01$), whereas no significant difference was found between group HM and group FM.

3.3. Secondary Endpoint. Figure 4 shows the changes in GSRS score for all seven IBS specific symptoms throughout the study time. There was significant relief of IBS specific symptoms from baseline to 4 and 8 weeks in all of the groups ($P < 0.01$). In group HM, the severity of all IBS specific symptoms was similar between week 4 and week 8. However, in group FM, IBS specific symptoms of abdominal pain, abdominal distension, and increased passage of stools were more serious ($P < 0.05$) from weeks 4 to 8. In group PB, IBS specific symptom of loose stools was more serious ($P < 0.01$) at week 8.

The comparison of effectiveness among three groups showed that at week 4, there was more relief of symptoms of rugitus ($P < 0.05$), abdominal pain, abdominal distension and increased passage of stools ($P < 0.01$) were seen in group HM compared to group PB. No difference was identified between group HM and group FM. At week 8, patients' symptoms of abdominal pain and abdominal distension were lighter ($P < 0.05$) in group HM than in group FM. Patients' symptoms of abdominal distension, loose stools ($P < 0.05$), abdominal pain, rugitus, increased passage of stools, and urgent need for defecation ($P < 0.01$) were lighter in group HM than in group PB.

3.4. Safety and Tolerability. No serious adverse events were reported during the whole study period. Only 1 patient (1.3%) in Group HM and 1 patient (1.7%) in group PB reported the allergy. Both of them were mild-to-moderate and were

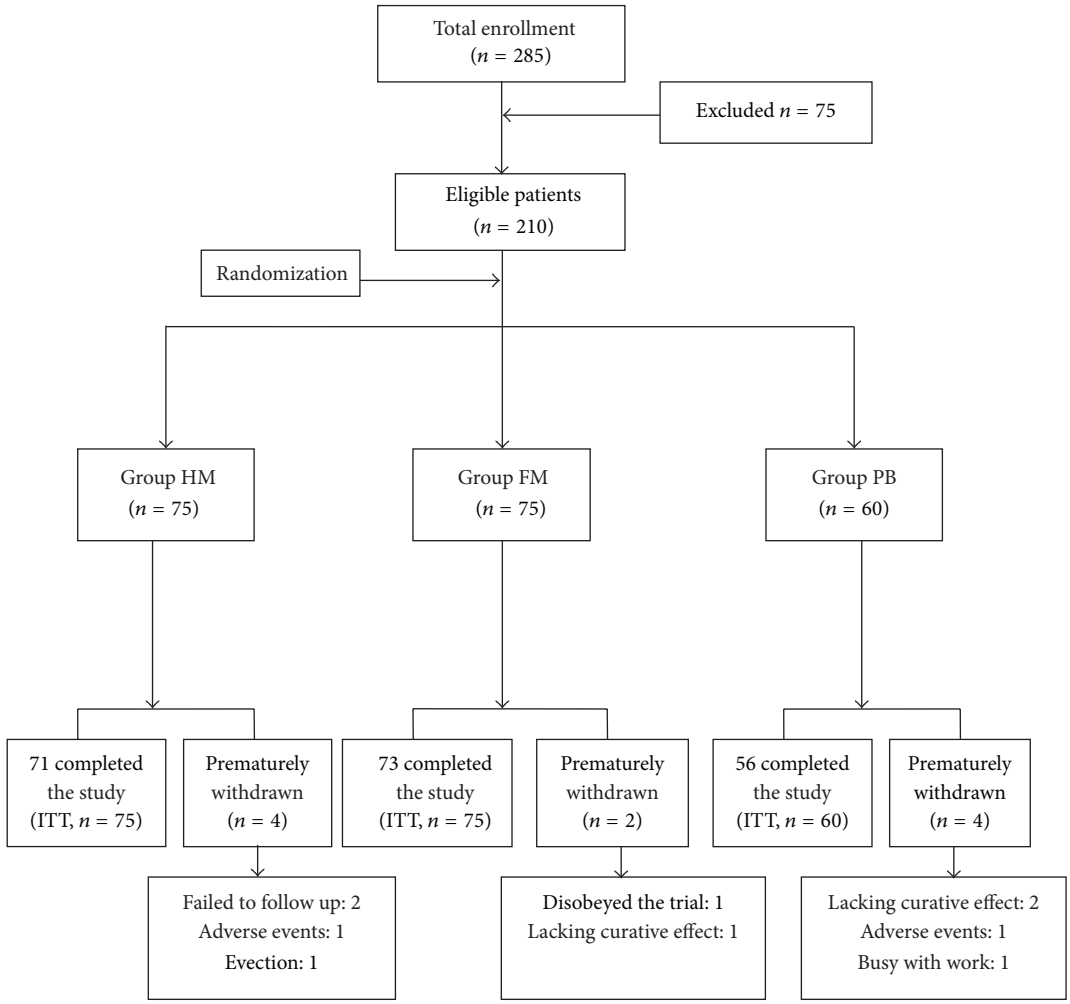

Figure 2: Flow chart of the trial. HM: herb-partitioned moxibustion; FM: farina-partitioned moxibustion; PB: orally taking pinaverium bromide; ITT: intention-to-treat.

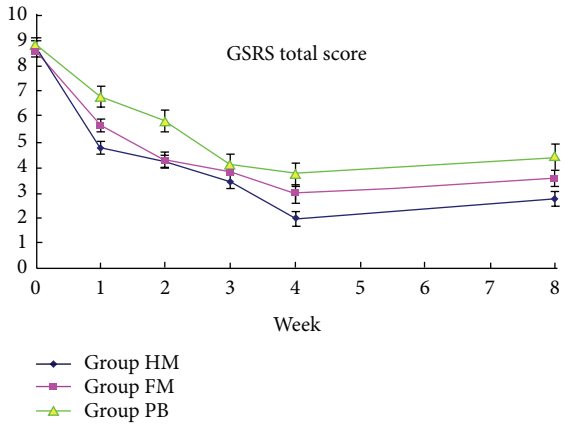

Figure 3: Primary endpoint: changes of the GSRS total score from baseline to week 4 (end of the treatment) and week 8 (follow-up period) for the three groups in the FAS population. HM: herb-partitioned moxibustion; FM: farina-partitioned moxibustion; PB, orally taking pinaverium bromide.

considered to be related to the study treatment. When we stopped the treatment on these two patients, the symptom of allergy disappeared. No clinically significant differences among the three patient groups were detected in the analysis of laboratory values, vital signs, and physical examination either.

4. Discussion

So far, the present study is the largest multicenter (3 centers) RCT with HM in IBS-D. Our results demonstrated that herb-partitioned moxibustion should be significantly better than orally taking pinaverium bromide in improving the GI symptoms of IBS-D. However, there was no significant difference between herb-partitioned moxibustion and farina-partitioned moxibustion. Additionally, the use of the HM was well tolerated and free from serious adverse effects. Only two patients were found to have light allergy during the study time and recovered without treatment.

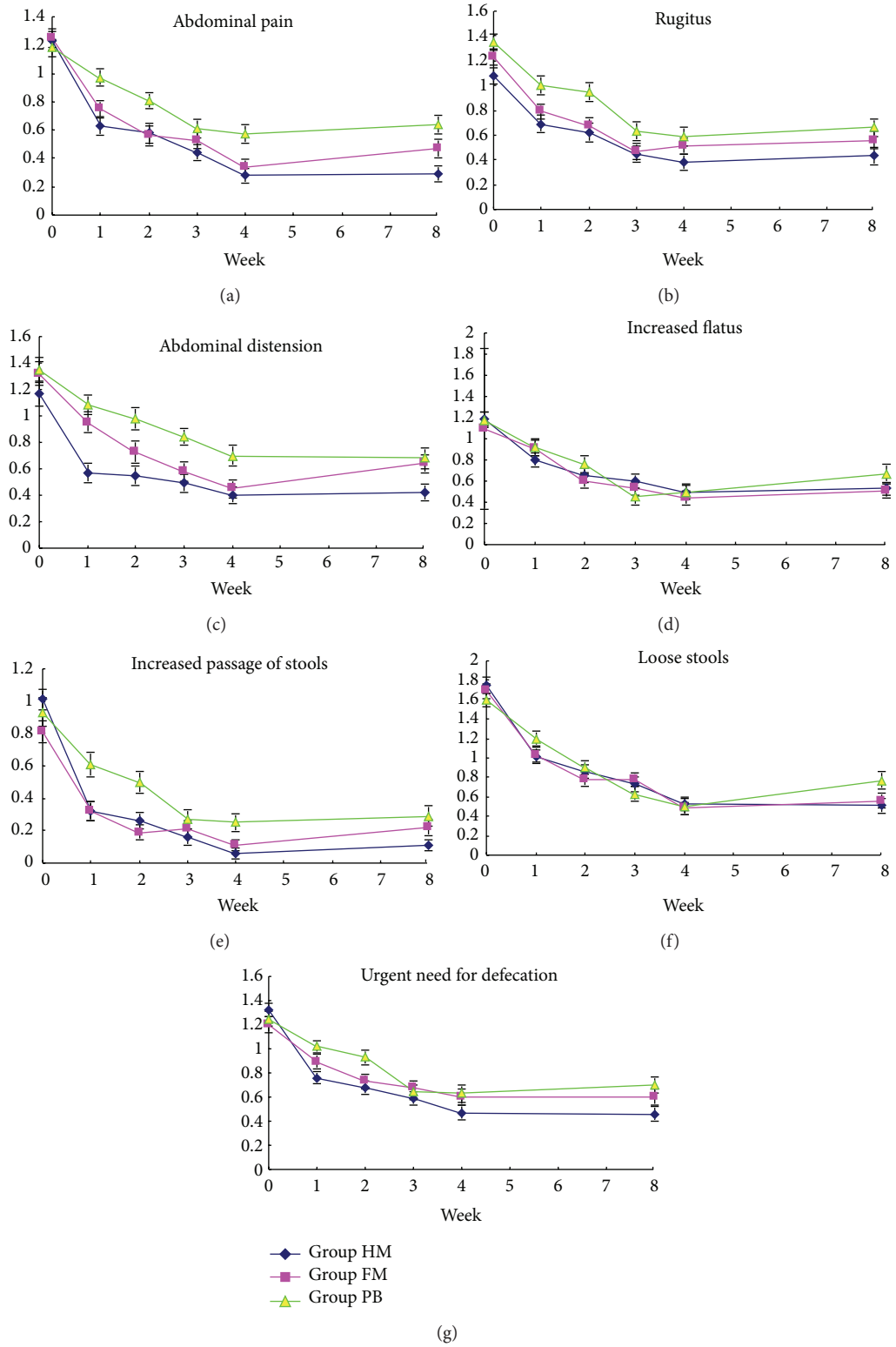

Figure 4: Secondary endpoint changes in GSRS specific score. (a) Abdominal pain; (b) rugitus; (c) abdominal distension; (d) increased flatus; (e) increased passage of stools; (f) loose stools; (g) urgent need for defecation during study time. HM: herb-partitioned moxibustion; FM: farina-partitioned moxibustion; PB: orally taking pinaverium bromide.

Our findings are consistent with the previous clinical trial [16] including 81 patients. In that trial, HM was more effective than pharmacological therapy (responder rate 92.7% versus 62.5%) in ameliorating IBS symptoms. The animal experiment [17] reported that HM could decrease visceral hypersensitivity of rats. Importantly, our study found that the improvement of IBS symptoms in patients receiving HM and FM is superior to that of patients receiving pinaverium bromide in contrast to the studies 16 and 17 mentioned above, which did not have a FM control (or moxibustion alone) arm. This indicated that moxibustoin on navel (Shenque CV8) is better than orally taking PB in ameliorating GI symptom of IBS-D patients. Additionally, we found that there was no significant difference ($P = 0.45$) of patients' GSRS scores between group HM and group FM at weeks 4, which indicated that the warmth of burned moxa cylinder played a more important role than herbs in improving GI symptom of IBS-D patients. What is more, compared to week 4, GSRS specific score in Group HM remained stable in the follow-up period (week 8). However, the rebounding of several IBS-D symptoms was seen in Group FM and Group PB. This indicated that the herbs played an important role in the maintenance of curative effect.

HM is one kind of moxibustion therapies, which combines all the functions of moxibustion, acupoints and herbs. The moxa cylinder is burned to warm up herb-partition to stimulate acupoints instead of acupuncture needle, accelerating the penetration of the herbs into human body. Navel (Shenque CV8) is closed to large intestine and small intestine in anatomical position. What is more, it is also associated with spleen, large intestine, and small intestine according to meridian theory. And it is easy for the effective constituent of herbs penetrating into body due to the thin skin around it. In addition, the herbs used in our study were aimed to ameliorate symptoms of IBS-D in different aspects. Some of them are characterized with the nature of aromatic and warm character, such as Dingxiang and Shexiang, which can motivate the penetration of herbs. The remaining herbs were used to either reinforce spleen-qi or check diarrhea.

The present study plays an important role in the development of safer effective alternatives for the management of IBS-D there is significant unmet clinical need. However, it had some limitations. First of all, the potential limitation of the study is that, as a pilot study, no power analysis was provided, which lowered the statistical power of the study to a certain extent. Additionally, in order to maintain the compliance of the patients, 8 sessions of HM over 4 weeks were applied in the present study. But it may have been insufficient to achieve maximum effect from HM, because IBS-D is often a lifelong condition typified by chronic and episodic symptoms [6]. What is more, it would be helpful if the present study had a longer follow-up period to explore whether HM has a therapeutic approach that works for a long term. Finally, it is known that IBS-D had a severe impact on the quality of life [18–21] (QOL), which is correlated with the appearance of symptoms, the protracted time, and severity of the disease. Nevertheless, the impact of HM on IBS-D patients' quality of life was not analyzed in the present study, whereas many other studies [22–24] did. As a consequence, it is hoped that further studies can be carried out in this field.

5. Conclusion

The present trial provided preliminary evidence that HM may be a promising, efficacious, and well-tolerated treatment for IBS-D patients. This finding encourages further investigation of the efficacy of this method in larger, well-designed clinical trials. Here are some directions for future researches: optimal treatment duration and follow-up period should be determined to investigate the possible mechanism of action of HM, based on time, in treating with IBS-D. The impact of HM on IBS-D patients' overall satisfaction or QOL is needed, as well as the correlation between the dose of medicamental pulverata used in HM and the overall effect of HM therapy.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Yu-xia Ma and Xiao Liu and their affiliations contribute equally to this paper.

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Research Article

Melinjo (*Gnetum gnemon* L.) Seed Extract Decreases Serum Uric Acid Levels in Nonobese Japanese Males: A Randomized Controlled Study

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Melinjo (*Gnetum gnemon* L.) seed extract (MSE) containing *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) and other derivatives exerts various beneficial effects. However, its mechanism of action in humans remains unknown. In this study, we aimed to investigate beneficial effects of MSE in healthy adult males. In this double-blind, randomized controlled study, 30 males aged 35–70 years with ≤10% flow-mediated dilatation received placebo or 750 mg MSE powder for 8 weeks, and twenty-nine males (45.1 ± 8.8 years old) completed the trial. There was a significant difference in the melinjo and placebo groups. Compared with the placebo control, MSE significantly reduced serum uric acid at 4 weeks and 8 weeks ($n = 14$ and 15, resp.). HDL cholesterol was significantly increased in the melinjo group. To clarify the mechanism of MSE for reducing uric acid, we investigated xanthine oxidase inhibitory activity, angiotensin II type 1 (AT1) receptor binding inhibition rate, and agonistic activities for PPARα and PPARγ. MSE, *trans*-resveratrol, and a resveratrol dimer, gnetin C (GC), significantly inhibit AT1 receptor binding and exhibit mild agonistic activities for PPARα and PPARγ. In conclusion, MSE may decrease serum uric acid regardless of insulin resistance and may improve lipid metabolism by increasing HDL cholesterol.

1. Introduction

Melinjo (*Gnetum gnemon* L.) belongs to the family Gnetaceae, native to Indonesia. The tree is small to medium in size, 15–20 m tall, with evergreen leaves. The fruit-like strobilus consists of little skin and a large nut-like seed that is 2–4 cm long inside, with both the fruits and leaves being very popular in Indonesian cuisines.

Kato et al. found that melinjo seed extract (MSE) contains various stilbenoids including *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), gnetin C (GC; resveratrol dimer), gnetin L (GC derivative), gnemonoside A (GC-diglucoside), gnemonoside C (GC-monoglucoside), and gnemonoside D (GC-monoglucoside) [1]. These derivatives are collectively referred to as "Melinjo resveratrol." Recently, *trans*-resveratrol has attracted considerable attention because it extended the lifespan of mice that were fed a high-calorie diet [2]. Moreover, human studies indicated that *trans*-resveratrol is beneficial in the management of diabetes [3] and cardiovascular diseases [4]. However, several *in vitro* studies on the resveratrol derivatives in MSE revealed its nutraceutical effects such as the inhibition of lipase and amylase, antibacterial properties [1], inhibition of angiogenesis [5], and immunostimulatory effects [6]. In mice, MSE was reported to suppress body weight gain and improve insulin resistance [7]. However, the clinical efficacy of MSE remains unknown in humans. Therefore, to evaluate the effects of MSE on humans, we designed this clinical study using healthy volunteers and evaluated the various biomarkers in association with metabolic syndrome.

2. Materials and Methods

2.1. Clinical Study Design. The present study was a randomized, double-blinded, and placebo-controlled trial with parallel groups. We conducted the study according to the guidelines in the Declaration of Helsinki. All procedures involving human participants were approved by the Shirasawa Clinical Research Center Ethical Review Board. Each participant provided written and informed consent prior to participation.

2.2. Participants. Adult males aged 35–70 years with ≤10% flow-mediated dilation (FMD), determined at the time of screening, were recruited for this study from August to September, 2011, through newspaper advertisements at the Shirasawa Clinical Research Center, Tatebayashi city, Gunma prefecture, Japan. Exclusion criteria included the following conditions: consumption of functional foods related to lipid and glucose metabolism; allergy to melinjo; drinking red wine that contains *trans*-resveratrol regularly; receiving medication for hypertension, diabetes, or hyperlipidemia; and preexisting severe liver, renal, or heart disease. In addition, we excluded the volunteers who participated in another clinical study within two months and were not judged to meet the conditions by the doctor responsible for the study.

The enrolled participants who met the inclusion criteria were randomly assigned into the melinjo and placebo groups by using computer-generated random numbers. Each participant in the melinjo group consumed five capsules containing 750 mg MSE powder every morning (once daily) for eight weeks, while each participant in the placebo group consumed five placebo capsules following the same protocol. The participants could not distinguish the difference between the two types of capsules with respect to their shape, size, weight, and color. The participants were advised not to consume other health foods during the study. They attended the Shirasawa Clinical Research Center for clinical assessment at the following three study time points: baseline (0), 4, and 8 weeks. Finally, 29 adult males (age: 45.1 ± 8.8 years, BMI: 24.4 ± 1.9 kg/m^2) participated in and completed the trial.

2.3. Test Substances. All MSE and placebo capsules were supplied by the Yamada Bee Company, Inc. The seeds (endosperms) of melinjo were collected in Indonesia (Desa Bangkok, Kecamatan Gurah Kabupaten Kediri, Kediri, Jawa Timur) in July 2009. The dried endosperms of melinjo (250 g) were powdered and soaked in 55% EtOH (750 mL) at room temperature for 3 days to obtain MSE (23 g). Dextrin (0.39 g) and water (5 g) were added to 6.25 g MSE and lyophilized to prepare the MSE powder used for biological experiments. In order to confirm the safety of the MSE powder, the Yamada Bee Company, Inc. conducted a repeated dose study in humans. Every morning for 28 days, 44 healthy volunteers aged 32–49 years were administered a maximum of 5,000 mg MSE powder. Throughout the study, no clinically noteworthy abnormalities were observed (unpublished data).

In our study, one melinjo capsule contained 150 mg MSE powder, 100 mg dextrin, 29 mg cellulose, and 9 mg sugar ester. The MSE powder contained >20% the resveratrol derivatives. Regarding *trans*-resveratrol, the content ratio was 0.1%. On the other hand, one placebo capsule contained 250 mg dextrin, 29 mg cellulose, and 9 mg sugar ester. The appearance of the capsules used for both groups was identical.

2.4. Clinical Assessments. The participants were instructed to arrive without having consumed anything for at least 8 h on the examination day. Before the initiation of the trial (week 0) and again at four and eight weeks, all participants went through FMD, pulse wave velocity (PWV), ankle-brachial index (ABI), body weight, fat percentage, BMI, and a general examination including blood pressure, pulse rate, and blood chemistry analysis (levels of total protein, albumin, albumin globulin ratio, total bilirubin, aspartate aminotransferase, alanine transaminase, γ-GTP, total cholesterol, HDL cholesterol, low-density lipoprotein (LDL) cholesterol, arteriosclerotic index, remnant-like particles (RLP) cholesterol, triglycerides, uric acid, urea nitrogen, creatinine, sodium, potassium, chlorine, HOMA-IR, fasting immunoreactive insulin, blood sugar, hemoglobin A1c, total homocysteine, and N-terminal prohormone of brain natriuretic peptide (NT-proBNP); the reactive oxygen metabolites-derived compounds (d-ROMs) test; the Biological Antioxidant Potential (BAP) test; white blood cell and red blood cell counts; hemoglobin levels; hematocrit value; mean cell volume; levels of mean cell hemoglobin, mean cell hemoglobin concentration, and platelets), and urinalysis (specific gravity, pH, protein, glucose, ketones, blood, bilirubin, and urobilinogen) (Table 1).

For the evaluation of endothelial function in metabolic syndrome, FMD was measured in the right brachial artery using UNEXEF38G (UNEX Corporation, Nagoya, Japan); specialized in measuring FMD, this device is a combination of ultrasonography and a sphygmomanometer. The probe of this device is composed of two probes to capture the minor axis of the vessel and one probe to capture the long axis of the vessels during the two probes. With the three probes, the position of a brachial artery and the long axis can be easily located, and the vessel diameter can be accurately measured. In addition, the equipment can automatically measure FMD after 5 min of avascularization. The participants were reclined on the bed in a supine position during the FMD test. They were fitted with a cuff, which was positioned on the right upper arm, abutting the cubital fossa. We maintained the laboratory room temperature at 25 ± 1°C, taking into account its effects on the examination [8].

The arteriosclerosis index was assessed using PWV and ABI by BP-203RPE III (Omron Healthcare Co., Ltd, Tokyo, Japan). This device has four cuffs that can simultaneously measure blood pressure levels in both arms and both legs and automatically calculate ABI. Moreover, the device can record pulse waves via sensors in the cuffs, calculate the transmission distance from the right arm to each ankle according to body height, and automatically compute and output the bilateral brachial-ankle PWV (baPWV) values using the transmission time and distance.

Table 1: The effects of melinjo seed extract administration for 8 weeks in adult men.

	Week 0		Week 4		Week 8		
	Melinjo ($n = 14$)	Placebo ($n = 15$)	Melinjo ($n = 14$)	Placebo ($n = 15$)	Melinjo ($n = 14$)	Placebo ($n = 15$)	P
	Means ± SD	Means ± SD	Means ± SD	Means ± SD	Means ± SD	Means ± SD	
Body weight (kg)	70.8 ± 9.5	70.4 ± 5.9	71.5 ± 9.9	71.0 ± 6.4	71.4 ± 10.0	70.8 ± 6.2	0.96
Fat percentage (%)	22.1 ± 3.8	22.5 ± 3.4	22.9 ± 4.6	23.5 ± 3.2	22.9 ± 4.7	23.4 ± 3.4	0.946
BMI (kg/m^2)	24.5 ± 2.6	24.3 ± 1.1	24.8 ± 2.6	24.5 ± 1.3	24.7 ± 2.6	24.4 ± 1.2	0.965
Systolic blood pressure (mmHg)	115.7 ± 10.7	118 ± 14.7	115.3 ± 9.2	118.9 ± 13.0	117.6 ± 10.5	119.5 ± 13.3	0.708
Diastolic blood pressure (mmHg)	74.9 ± 9.3	77 ± 11.8	75.7 ± 7.0	76.4 ± 10.4	75.2 ± 6.8	76.5 ± 9.3	0.756
Pulse (bpm)	60.6 ± 5.9	59.1 ± 8.7	61.3 ± 6.8	60.7 ± 8.6	62.9 ± 9.4	63.6 ± 11.8	0.711
FMD (%)	5.5 ± 2.6	5.8 ± 2.1	6.7 ± 1.6	6.1 ± 2.2	5.7 ± 2.4	6.8 ± 1.8	0.233
baPWV (right) (cm/s)	1210.4 ± 145.9	1297 ± 149.3	1236.5 ± 112.7	1282.5 ± 162.4	1283.5 ± 156.3	1314.9 ± 156.4	0.27
baPWV (left) (cm/s)	1226.1 ± 138.0	1324.1 ± 173.7	1241.9 ± 127.8	1305.1 ± 156.9	1299.7 ± 172.0	1337.5 ± 175.5	0.287
Ankle-brachial index (right)	1.14 ± 0.06	1.17 ± 0.06	1.13 ± 0.07	1.16 ± 0.04	1.13 ± 0.07	1.18 ± 0.04	0.549
Ankle-brachial index (left)	1.14 ± 0.05	1.16 ± 0.05	1.13 ± 0.07	1.14 ± 0.05	1.12 ± 0.08	1.16 ± 0.04	0.41
Total protein (g/dL)	7.1 ± 0.4	7.0 ± 0.3	7.12 ± 0.3	7.1 ± 0.3	7.2 ± 0.3	7.1 ± 0.4	0.156
Albumin (g/dL)	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.2	4.4 ± 0.3	4.5 ± 0.2	4.4 ± 0.1	0.771
Total bilirubin (mg/dL)	1.0 ± 0.4	1.0 ± 0.7	0.9 ± 0.3	1.0 ± 0.8	0.9 ± 0.4	0.9 ± 0.7	0.956
Aspartate aminotransferase (U/L)	28.1 ± 10.6	21.7 ± 7.4	22.8 ± 5.6	22.1 ± 5.2	22.7 ± 5.9	22.0 ± 5.6	0.074
Alanine aminotransferase (U/L)	34.9 ± 24.8	25.4 ± 13.2	27.5 ± 15.2	27.8 ± 14.3	27.0 ± 14.1	26.7 ± 13.9	0.096
Gamma-glutamyl transpeptidase (U/L)	44.1 ± 33.8	36.3 ± 22.8	42.9 ± 35.0	42.9 ± 34.5	42.5 ± 40.7	42.1 ± 35.2	0.289
Total cholesterol (mg/dL)	193.9 ± 44.8	214.3 ± 26.7	196.4 ± 45.9	219.1 ± 28.1	199.0 ± 37.8	213.9 ± 29.7	0.654
HDL cholesterol (mg/dL)	52.4 ± 11.4	51.2 ± 12.9	54.1 ± 11.6	50.7 ± 11.4	57.4 ± 12.6	51.4 ± 13.7	0.111
LDL cholesterol (mg/dL)	122.9 ± 38.9	135.3 ± 27.8	123.5 ± 42.2	138.0 ± 24.3	124.5 ± 34.7	135.7 ± 30.0	0.871
Triglycerides (mg/dL)	106.3 ± 65.0	144.5 ± 104.7	114.7 ± 64.7	99.0 ± 47.7	118.5 ± 74.5	173.1 ± 136.6	0.381
Arteriosclerotic index	2.9 ± 1.4	3.4 ± 1.0	2.8 ± 1.2	3.5 ± 1.0	2.6 ± 1.1	3.4 ± 1.1	0.346
Uric acid (mg/dL)	6.7 ± 1.5	6.6 ± 1.1	6.3 ± 1.4	6.7 ± 0.9	6.1 ± 1.4	6.6 ± 1.1	0.009*
Blood urea nitrogen (mg/dL)	13.6 ± 3.7	13.8 ± 4.4	13.3 ± 4.0	13.3 ± 2.3	13.1 ± 3.7	12.9 ± 2.7	0.992
Creatinine (mg/dL)	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.283
Sodium (mEq/L)	139.9 ± 2.1	138.9 ± 1.8	138.9 ± 2.2	138.9 ± 2.6	139.1 ± 2.3	139.1 ± 2.4	0.629
Potassium (mEq/L)	4.3 ± 0.5	4.2 ± 0.3	4.4 ± 0.4	4.5 ± 0.3	4.4 ± 0.3	4.5 ± 0.4	0.284
Chlorine (mEq/L)	102.5 ± 2.5	102.2 ± 1.9	101.1 ± 2.3	101.9 ± 2.6	101.3 ± 2.2	101.9 ± 2.0	0.74
Blood sugar (mg/dL)	92.8 ± 13.7	92.6 ± 4.4	93.6 ± 13.5	93.5 ± 3.9	94.3 ± 18.7	95.9 ± 8.2	0.837
Hemoglobin A1c (%)	5.1 ± 0.5	5.0 ± 0.3	5.0 ± 0.7	5.0 ± 0.2	5.0 ± 0.7	5.1 ± 0.3	0.58
Albumin globulin ratio	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.3	1.6 ± 0.2	1.6 ± 0.2	0.666
White blood cell ($\times 10^4/\mu$L)	4846.7 ± 1186.2	6266.7 ± 2728.1	4760.0 ± 1025.3	6066.7 ± 2138.0	4860.0 ± 1240.3	6040.0 ± 2233.4	0.894

Table 1: Continued.

	Week 0		Week 4		Week 8		
	Melinjo ($n = 14$)	Placebo ($n = 15$)	Melinjo ($n = 14$)	Placebo ($n = 15$)	Melinjo ($n = 14$)	Placebo ($n = 15$)	P
	Means ± SD	Means ± SD	Means ± SD	Means ± SD	Means ± SD	Means ± SD	
Red blood cell ($\times 10^4/\mu$L)	496.6 ± 31.6	480.1 ± 24.7	503.5 ± 34.6	491.1 ± 22.4	508.4 ± 28.3	483.3 ± 21.9	0.185
Hemoglobin (g/dL)	15.2 ± 0.6	14.9 ± 0.8	15.5 ± 0.7	15.4 ± 0.7	15.7 ± 0.6	15.2 ± 0.7	0.161
Hematocrit (%)	44.2 ± 2.1	43.2 ± 1.9	44.9 ± 2.0	44.6 ± 2.0	45.5 ± 1.7	44.0 ± 1.9	0.15
Mean cell volume (fL)	89.1 ± 2.7	90.3 ± 3.2	89.5 ± 3.1	90.9 ± 3.2	89.7 ± 3.5	91.3 ± 2.9	0.862
Mean cell hemoglobin (pg)	30.6 ± 1.4	31.0 ± 1.4	30.8 ± 1.5	31.5 ± 1.1	30.8 ± 1.5	31.5 ± 1.1	0.2
Mean cell hemoglobin concentration (g/dL)	34.3 ± 1.0	34.4 ± 0.8	34.4 ± 1.0	34.6 ± 0.7	34.4 ± 1.0	34.6 ± 0.7	0.756
Platelets ($\times 10^4/\mu$L)	23.2 ± 4.8	21.8 ± 5.4	24.0 ± 4.1	22.4 ± 6.3	24.3 ± 4.2	23.5 ± 7.7	0.685
Fasting IRI (μU/mL)	5.1 ± 2.9	4.9 ± 2.2	5.4 ± 3.4	7.0 ± 5.4	5.4 ± 3.5	7.1 ± 6.5	0.407
HOMA-IR	1.2 ± 0.8	1.1 ± 0.5	1.3 ± 0.8	1.6 ± 1.3	1.3 ± 0.9	1.7 ± 1.7	0.404
NT-proBNP (pg/mL)	76.5 ± 195.4	32.1 ± 20.0	26.0 ± 16.7	26.2 ± 22.2	20.3 ± 14.4	25.1 ± 23.1	0.505
RLP cholesterol (mg/dL)	6.9 ± 4.8	8.9 ± 8.4	7.6 ± 5.3	13.0 ± 11.9	6.9 ± 4.9	11.0 ± 11.6	0.242
Total homocysteine (mg/dL)	13.5 ± 5.2	14.6 ± 8.4	11.7 ± 2.5	14.7 ± 8.9	11.2 ± 2.8	12.5 ± 4.6	0.399
d-ROMs test (U.CARR)	330.8 ± 58.4	331.6 ± 55.2	338.0 ± 53.6	345.6 ± 52.2	356.5 ± 46.2	365.0 ± 60.5	0.781
BAP test (μmol/L)	2585.3 ± 174.2	2471.5 ± 189.3	2341.1 ± 91.9	2277.0 ± 115.7	2441.9 ± 178.5	2329.4 ± 179.9	0.633
Urine PH	5.7 ± 0.9	5.7 ± 1.2	5.5 ± 0.5	6.2 ± 1.1	6.1 ± 0.9	5.6 ± 0.8	0.008*
Urine specific gravity	1.021 ± 0.006	1.019 ± 0.008	1.023 ± 0.004	1.019 ± 0.008	1.022 ± 0.004	1.018 ± 0.007	0.689

BAP: biological antioxidant potential; d-ROMS: reactive oxygen metabolites-derived compounds; LDL: low-density lipoprotein; NT-proBNP: amino-terminal probrain natriuretic peptide; RLP: remnant-like particles. Values are given as means ± SD. P for interaction. *$P < 0.05$.

2.5. Statistical Analysis. Statistical analyses were performed using SPSS version 20 (IBM Corporation, NY, USA). Statistical comparisons between groups were calculated using two-way factorial ANOVA. The factors were the assignment and the survey period, and the dependent variables were the evaluation criteria. Multiple comparisons were performed using Tukey's HSD test. Values of $P < 0.05$ were considered significant. Results are presented as means ± SD.

2.6. In Vitro Experiments

2.6.1. Assay of Xanthine Oxidase Activity. The assay mixture consisting of 50 mL test solution and 50 mL enzyme solution (0.05 units/mL in 70 mM phosphate buffer, pH 7.5) was prepared immediately before use. After preincubation at 25°C for 15 min, the reaction was initiated by the addition of 100 mL substrate solution (800 mM xanthine in the same buffer). The assay mixture was incubated at 25°C for 30 min. The reaction was stopped by adding 20 mL of 1 M HCl and 20 mL volumes of diluted 20 mM potassium dihydrogenphosphate onto a Sunniest RP-AQUQ column (4.6 mm I.D. × 150 mm). The mobile phase was acetonitrile/20 mM potassium dihydrogenphosphate (1 : 99 v/v) at a flow rate of 0.8 mL/min. Further, uric acid was detected by its UV absorbance at 290 nm. The retention time of uric acid on this system was 4.9 min. A blank was prepared in the same way, but the enzyme solution was added to the assay mixture after adding 1 M HCl. One unit of XO is defined as the amount of enzyme required to produce 1 mmol of uric acid/min at 25°C. The XO inhibitory activity was expressed as the percentage inhibition of XO in the above assay system, calculated as $(1 - B/A) \times 100$, where A and B are the activities of the enzyme without and with test material, respectively. The IC_{50} values were calculated from the mean values of data from the four determinations. The extracts were dissolved initially in EtOH, followed by dilution with the buffer; the final EtOH concentration was <5%. Allopurinol, a known XO inhibitor, was used as a positive control.

2.6.2. Evaluation of the Binding Inhibition Rate of Angiotensin II Type 1 Receptor. Evaluation of the affinity of compounds for the human angiotensin II type 1 (AT1) receptor in the transfected HEK-293 cells was determined using a radioligand binding assay. The cell membrane homogenates (8 μg protein) were incubated for 120 min at 37°C with 0.05 nM [^{125}I][Sar^1-Ile8] angiotensin II, both in the absence or presence of the test compound, in a buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, and 0.1% BSA. Nonspecific binding was determined in the presence of 10 μM angiotensin II. Following incubation, the samples

were rapidly filtered under vacuum through glass fiber filters (GF/B, Packard) presoaked with 0.3% PEI and rinsed several times with ice-cold 50 mM Tris-HCl using a 96-sample cell harvester (Unifilter, Packard). Thereafter, the filters were dried and measured for radioactivity in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscint 0, Packard). The results were expressed as a percent inhibition of the control radioligand specific binding. The standard reference compound was saralasin, which was tested in each experiment at several concentrations to obtain a competition curve from which its IC_{50} was calculated.

2.6.3. Assay of Peroxisome Proliferator-Activated Receptor (PPAR) α and γ Agonist Activity. The COS-1 cells were collected by processing of trypsin, centrifuged at 1000 rpm for 3 min at 4°C. After removing the supernatant, the cells were seeded in 60 mm culture dishes at a density of 5×10^5 cell/well in a 2 mL medium and cultured for 24 h at 37°C with the presence of 5% CO_2. The Effectene Transfection Reagent (QIAGEN, Tokyo, Japan) was used to transform the cells. 150 μL Buffer EC, 0.25 μg pPPARα-Gal4 (or pPPARγ-Gal4), 1 μg pGal4-Luc, 1 μg pSEAP-control vector, and 18 μL Enhancer were added into a 1.5 mL tube, and the contents in the tube were stirred with the vortex for 10 s. Subsequently, after leaving for 3 min at 25°C, 25 μL Effectene was added to the tube. The contents were stirred with the vortex for 10 s and were left for 7 min at 25°C. The medium of the 60 mm culture dish was removed, and 4 mL fresh medium was introduced there during this time. Subsequently, 7 min later, 1 mL culture medium was added to the 1.5 mL tube and was suspended with a pipette. All the contents were dripped to the 60 mm culture dish, and the contents were incubated for 16 h at 37°C with the presence of 5% CO_2.

The transformed cells were collected by processing trypsin. The cells were centrifuged at 1000 rpm for 3 min at 4°C, and the supernatant was removed. The cells were suspended in 10 mL culture medium and were seeded in a 96-well plate with 125 μL medium for each well. The cells were then cultured for 1-2 h at 37°C with the presence of 5% CO_2. The test samples (1.25 μL) were added to each well and were cultured with gentle stirring for 24 h at 37°C in the presence of 5% CO_2.

The medium (25 μL) was removed from each well of the 96-well plate and was transferred to each well of a 96-well white plate. Thereafter, a solution for measurement of the luciferase activity was fused at 37°C, and the 100 μL solution was added to the 100 μL medium of the rest in each well. Each luminescence activity was measured after reacting for 35 min in a dark place. 25 μL 1 × dilution buffer was added to each 25 μL medium, which was collected from the 96-well plate. It was stirred gently and left for 30 min at 65°C. Thereafter, they were cooled down to 4°C and then back to 25°C. 90 μL assay buffer was added to each well, was gently stirred, and was left for 5 min at 25°C. 10 μL/MUP solution was added to each medium, and it was gently stirred. After reacting for 60 min at 25°C in a dark place, the fluorescence intensity (Ex = 360 nm, Em = 460 nm) based on 4-methylumbelliferone was measured.

3. Results

3.1. MSE Decreases the Serum Uric Acid Levels in Healthy Volunteers. In order to study the beneficial effects of MSE, we designed a clinical trial of MSE with healthy volunteers, wherein we evaluated the various biomarkers, including blood chemistry, CBCs, body weight, blood pressure, urinalysis, pulse wave velocity (PWV), flow-mediated dilatation (FMD), and HOMA-IR (a biomarker of insulin sensitivity) (Table 1).

Healthy volunteers with administration of 750 mg MSE powder revealed a significant decrease in the uric acid levels at four weeks (6.3 ± 1.4 versus 6.7 ± 0.9 mg/dL, $P < 0.05$; Figure 1(a)) and at eight weeks (6.1 ± 1.4 versus 6.6 ± 1.1 mg/dL, $P < 0.05$; Figure 1(a)) when compared to the placebo control. As presented in Figure 1(b), we confirmed a beneficial effect of MSE at four weeks, which was as well maintained at eight weeks, suggesting the stable clinical benefit of MSE in the long-term control of serum uric acid levels.

Although a previous clinical study on *trans*-resveratrol demonstrated the improvements in insulin resistance and lipid profiles with human subjects [9], we failed to demonstrate these clinical benefits for MSE. Interestingly, in this study, we found a novel clinical benefit of MSE on uric acid. Because uric acid not only plays an important role as an antioxidant molecule but also as a biomarker for cardiovascular diseases and gout, we explored the possibility that MSE confers health benefits in the prevention of cardiovascular diseases and gout.

3.2. MSE May Inhibit AT1 Receptor Binding. In order to clarify the mechanism of MSE in decreasing the serum uric acid levels, we investigated the potential inhibition of uric acid synthesis and uric acid reabsorption in the renal tubular epithelia. With regard to the synthesis of uric acid, we first investigated the inhibitory activity of MSE on xanthine oxidase. Allopurinol, a well-known chemical compound used for the treatment of gout, effectively inhibited the xanthine oxidase activity with IC_{50} at a concentration of 0.23 μg/mL (Figure 2(a)). However, MSE, GC, GC monoglucuronic acid conjugate, and *trans*-resveratrol failed to demonstrate any inhibitory activities on xanthine oxidase (Figures 2(b)–2(e), IC_{50} at the concentrations of 133 μg/mL, 157 μg/mL, and 350 μg/mL, resp.), suggesting that MSE decreases the serum uric acid levels by a mechanism other than the xanthine oxidase suppression. Next, we explored the possibility whether MSE inhibits the reabsorption of uric acid in the renal tubules. The inhibition of angiotensin decreases the serum uric acid levels by suppressing the reabsorption of uric acid from the renal tubular epithelia [10]. In this paper, we performed *in vitro* investigation to evaluate the inhibitory activity of MSE on angiotensin as well as GC, which revealed that MSE and GC have a significant inhibitory activity on AT1 receptor binding, whereas *trans*-resveratrol revealed no inhibitory activity. These data suggest that MSE inhibits the angiotensin signal, which then downregulates the transporter of uric acid; however, we cannot rule out the possibility that other pathways are involved in the regulation of uric acid

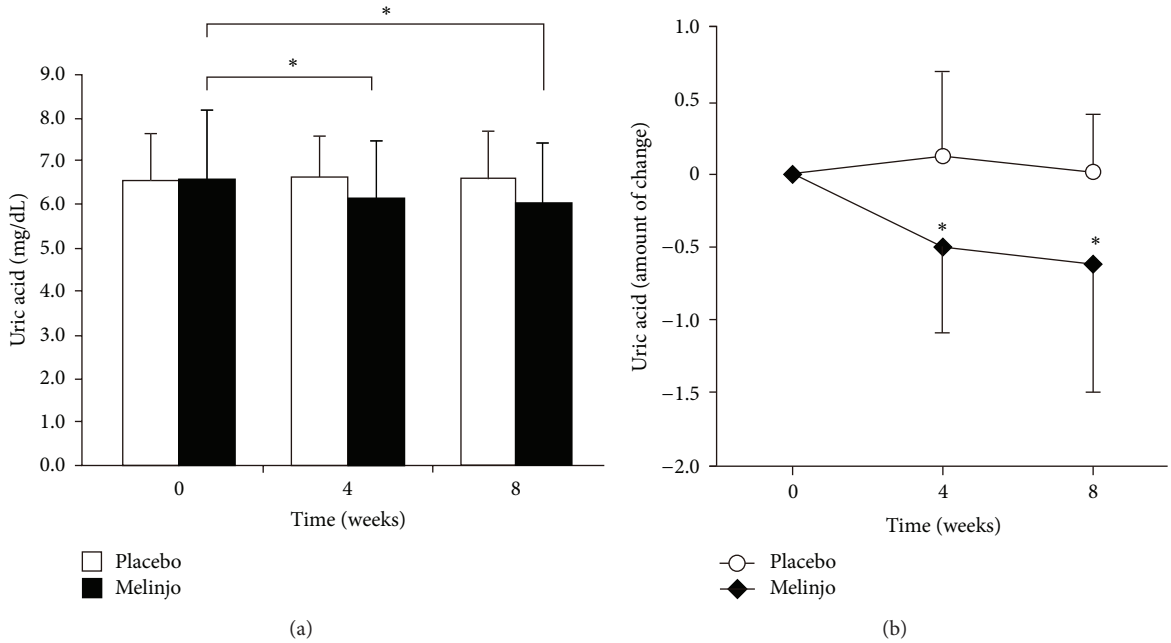

Figure 1: The effects of MSE on the serum uric acid levels before and four or eight weeks after the administration of 750 mg MSE or placebo. (a) The serum uric acid levels significantly decreased in the melinjo group ($n = 14$) than in the placebo group ($n = 15$). (b) The changes in the uric acid levels in the melinjo group were presented by an amount of changes in placebo group. The effect of MSE at four weeks was as well maintained at eight weeks. Statistical significance was calculated using Tukey's HSD test. Values are presented as means ± SD. $^*P < 0.05$.

because we have not evaluated all the relevant regulatory pathways.

3.3. MSE May Increase the Serum HDL Cholesterol Levels in Healthy Volunteers. Although we failed to detect any beneficial effects of MSE on LDL cholesterol, we found a significant increase in the HDL cholesterol levels in healthy volunteers who consumed 750 mg MSE powder for eight weeks as indicated in Figure 3(a) (52.4 ± 11.4 to 57.4 ± 12.6 mg/dL, $P < 0.05$). It is well known that HDL cholesterol transports the deposited cholesterol from atherosclerotic lesions of blood vessels to the liver [11], and it also counteracts the deleterious effect of LDL cholesterol in the pathogenesis of atherosclerosis. In this context, MSE may confer a benefit in the prevention of atherosclerosis without the alternation of LDL metabolism.

In order to clarify the molecular mechanisms that increase the HDL cholesterol levels on MSE administration, we evaluated the agonistic activities for PPARα and PPARγ because these receptors can increase the HDL cholesterol levels [12–15]. As displayed in Figures 3(b)–3(d), MSE or grapes extract revealed mild agonistic activities for PPARα and PPARγ (Figures 3(b) and 3(c)), which is similarly identified with *trans*-resveratrol (Figure 3(d)). The agonistic activities detected here revealed weaker signals compared with the positive controls, WY1643 for PPARα and troglitazone for PPARγ (Figures 3(b), 3(c), and 3(d)). For a better understanding of the mechanisms, further studies are warranted on the molecular mechanism involved in the metabolism of HDL cholesterol.

4. Discussion

The results of this study suggest that the MSE decreases the serum uric acid levels by inhibiting the reabsorption of uric acid in the renal tubular epithelia as well as by increasing the HDL cholesterol levels by PPAR agonistic activity. In this paper, we demonstrated, for the first time, the novel actions of MSE, which is distinct from *trans*-resveratrol. The actions demonstrated here have not been previously reported with *trans*-resveratrol [16–19].

In metabolic syndrome, insulin resistance causes hyperinsulinemia, which then leads to upregulation of serum uric acid by enhancing the reabsorption of serum uric acid in the renal tubules [20]. In addition, hyperinsulinemia downregulates GAPDH, one of the key glycolysis enzymes, which can then activate the pentose phosphorylation pathway with a concomitant increase of purine synthesis de novo [21]. It is unlikely that MSE downregulates uric acid by improving insulin resistance because we failed to identify any signs of improvement on HOMA-IR in the present study. This result suggests that MSE downregulates the serum uric acid levels independently of insulin resistance.

GC, gnemonoside C, and gnemonoside D in MSE are reported to inhibit the α-amylase activity [1]. This inhibitory activity could suppress the rapid postprandial insulin secretion, which inhibits the reabsorption of uric acid in the renal tubules. Notably, dysfunction of the ATP-binding cassette transporter subfamily G member 2 (ABCG2) suppresses the excretion of uric acid into intestine [22]. Another possibility is that MSE enhances ABCG2 to secrete more uric acid into the intestine, thereby decreasing the serum uric acid levels.

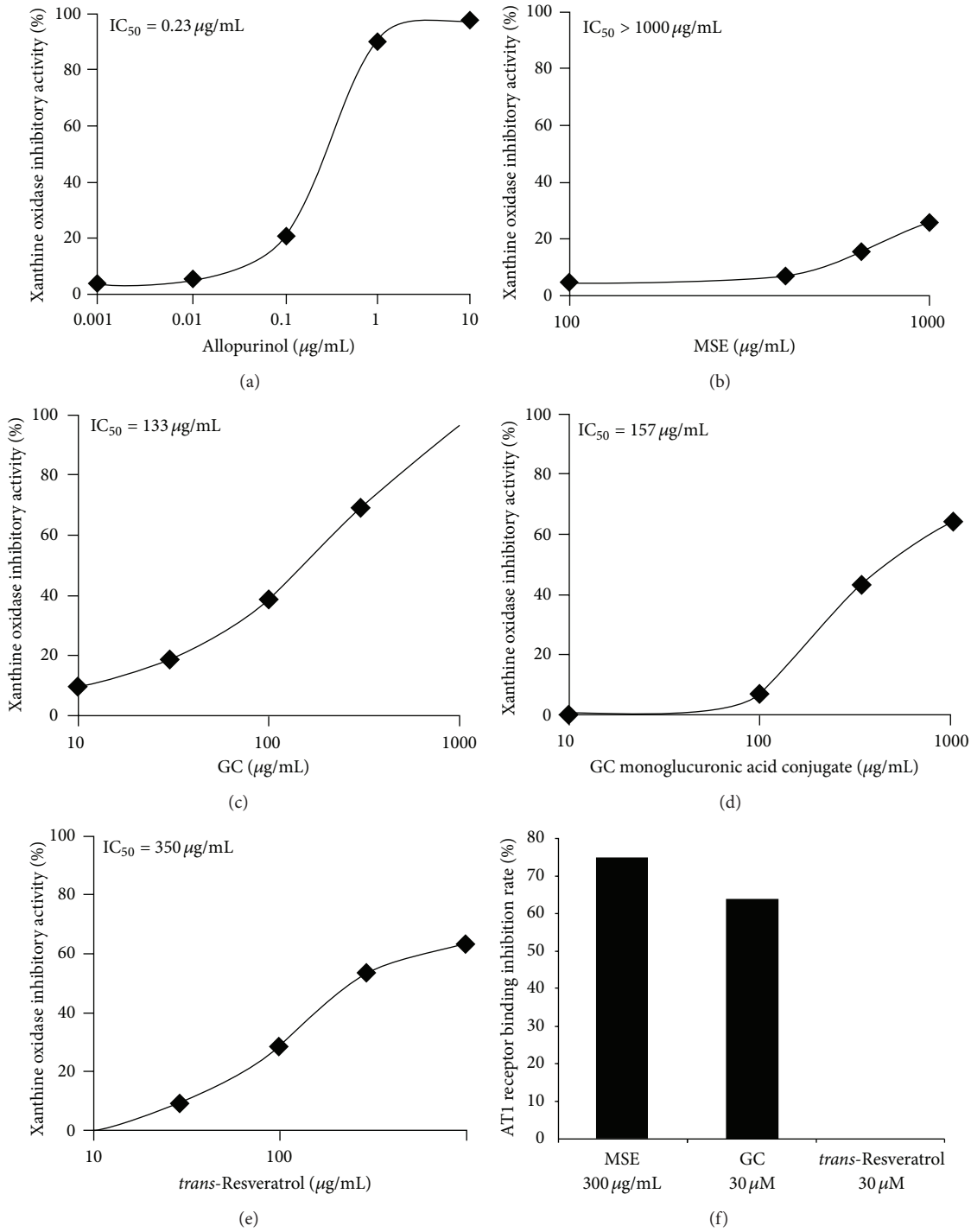

Figure 2: The effects of the 50% inhibitory concentration (IC_{50}) of MSE, GC, GC monoglucuronic acid conjugate, and *trans*-resveratrol on the xanthine oxidase inhibitory activity. Allopurinol was used as a positive control. (a) Allopurinol. (b) MSE. (c) GC. (d) GC monoglucuronic acid conjugate. (e) *trans*-Resveratrol. (f) AT1 receptor binding inhibition rate of MSE, GC, and *trans*-resveratrol.

Further clinical studies would be required to confirm the efficacy as well as the optimized MSE dose for an efficient control of uric acid and HDL cholesterol.

Regarding the influence of *trans*-resveratrol on uric acid, a few studies reported that in animals [23, 24]. In artificially induced hyperuricemia in mice, *trans*-resveratrol and its analogues decreased the serum uric acid levels and increased the uric acid excretion by regulating the renal organic ion transporters [23]. In addition, in diabetic rats, *trans*-resveratrol decreased the serum uric acid levels [24]. These findings suggest that *trans*-resveratrol decreases the serum uric acid levels in the presence of insulin resistance.

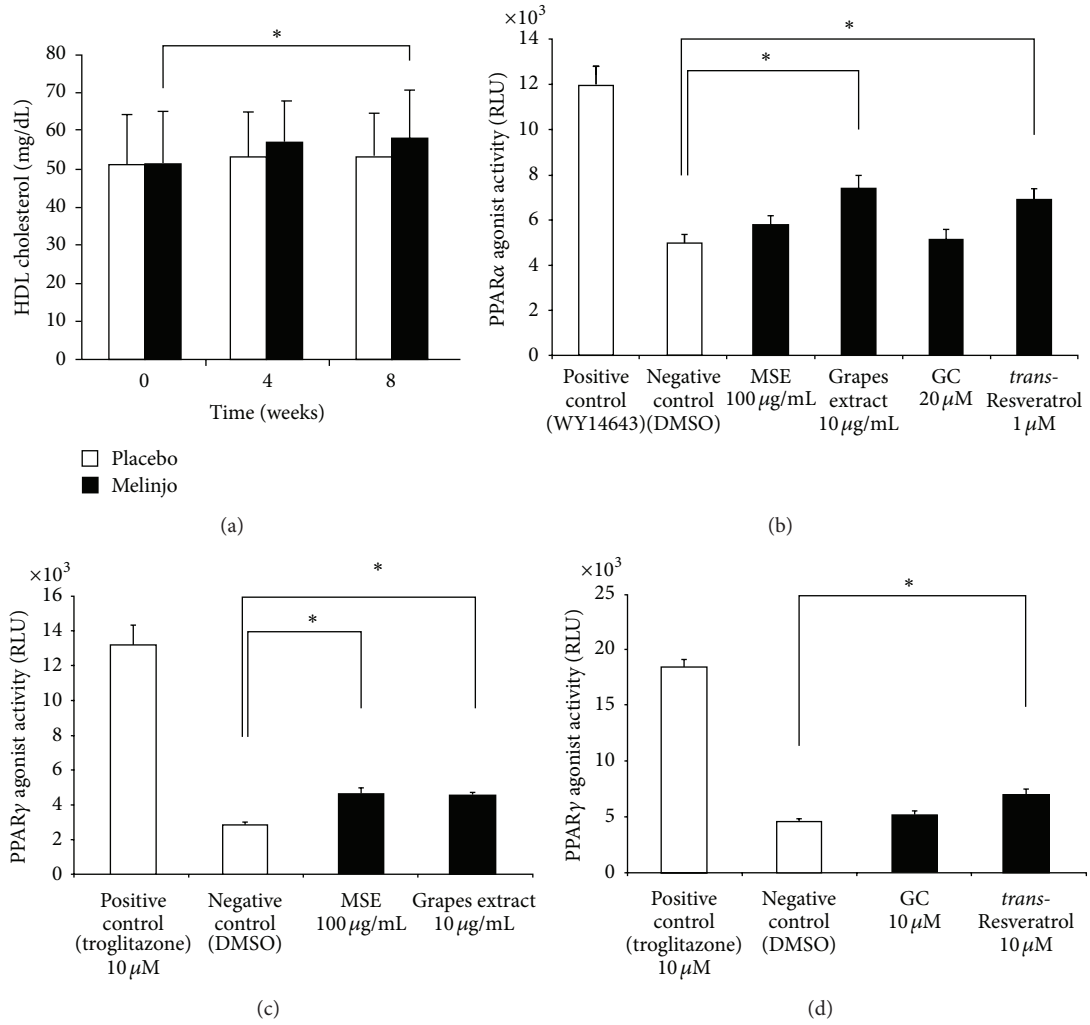

Figure 3: (a) The effects of MSE on the HDL cholesterol levels before and four or eight weeks after the administration of 750 mg MSE powder or placebo. Statistical analysis is presented in Figure 1. $^{*}P < 0.05$. (b) The PPARα agonist activity of MSE, grapes extract, GC, and *trans*-resveratrol. (c) The PPARγ agonist activity of MSE and grapes extract. (d) The PPARγ agonist activity of GC and *trans*-resveratrol. Values are presented as means ± SD. $^{*}P < 0.05$.

Considering these results of studies, *trans*-resveratrol would not have influenced serum uric acid level in our study.

About the effects of *trans*-resveratrol on HDL cholesterol, some clinical trials have shown that *trans*-resveratrol increased HDL cholesterol. However, Sahebkar recently concluded that *trans*-resveratrol does not have a significant effect of resveratrol supplementation on plasma lipid concentrations in the meta-analysis of randomized controlled trials (RCT) [18]. In addition, the range of *trans*-resveratrol doses was between 10 mg/day and 1,500 mg/day in the RCTs [18], while the dose in our study was about 0.75 mg/day; it was very less than the selected studies. Therefore, *trans*-resveratrol would not have contributed to the increase of HDL cholesterol level in MSE group. However, it might be possible that *trans*-resveratrol was one of the ingredients in MSE that affected the parameters in our study since the effects of *trans*-resveratrol on serum uric acid and HDL-cholesterol still remain unclear.

5. Conclusions

Since the relation between hyperuricemia and metabolic syndrome has been pointed out these days [25], our study shed light on the possibility that MSE decreases the serum uric acid levels. Furthermore, MSE may improve the lipid metabolism by increasing the HDL cholesterol levels. Nevertheless, further research would be required to understand the molecular mechanism for regulating uric acid and HDL cholesterol, which is more specific for MSE and distinct from *trans*-resveratrol.

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