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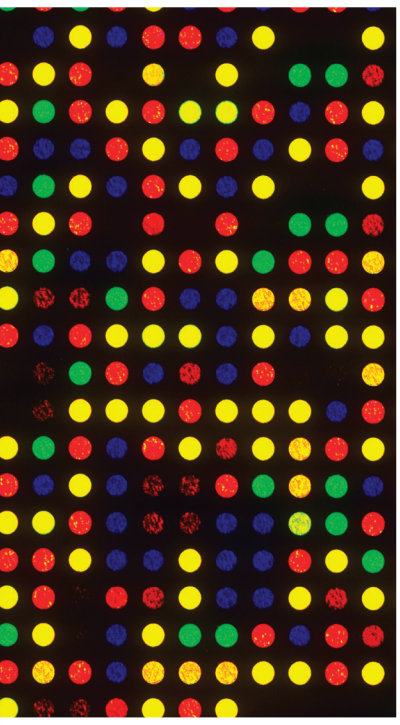
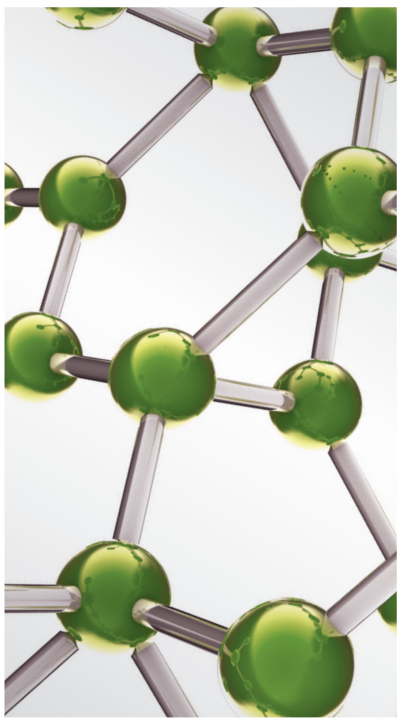
Complementary and Alternative Medicine for Diseases and Disorders in Digestive Tract: Basic to Clinics

Guest Editors: Chang Gue Son, Zhao Xiang Bian, Jing Hua Wang, and H. Balaji Raghavendran



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Editorial Board

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Editorial

Complementary and Alternative Medicine for Diseases and Disorders in Digestive Tract: Basic to Clinics

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The gastrointestinal (GI) tract is a long pathway of about 9 m, passing through the longitudinal center of the body. The disorders or diseases of GI tract are very commonly observed in clinical practices. As conventional medical therapies either do not produce satisfactory results or may have side effects, many patients seek complementary and alternative medicine (CAM) [1]. Furthermore, patients prefer additional CAM therapies to improve health-related quality of life through holistic concepts [2]. In the USA, it has been reported that 51% of the patients with GI tract disorders have tried some form of CAM [3], whereas, in the UK, 26% of the patients with GI tract symptoms and 48% of the patients with irritable bowel syndrome (IBS) have been noted to use CAM [4].

With the increasing numbers of patients and practitioners using CAM modalities, the number of studies on the application of CAM for the treatment of GI disorders has increased. CAM modalities include a wide variety of approaches, including acupuncture, moxibustion, herbal medicine, nutrition, microbial therapy using probiotics, meditation, chiropractic, cupping, massage, yoga, and Qigong. Among them, herbal medicine (single herb or mixture of multiple herbs) and acupuncture have been most extensively studied for GI disorders [5]. For example, clinical studies in China have indicated that several herbal drugs show superior effect to western drugs in the management of ulcerative colitis, a refractory and chronic inflammatory bowel disease (IBD) [6, 7]. In particular, functional GI disorders, such as functional dyspepsia and IBS, have been relatively main targets of study using acupuncture or moxibustion [8, 9]. Serial trials have proposed that modulation of GI motility by acupuncture could be one of the mechanisms responsible for its effects on functional disorders in the GI track [10, 11]. However, we still need to prove the efficacy, safety, and cost-effectiveness of CAM treatments for GI illnesses.

This special issue is an attempt to contribute to the knowledge on CAM treatments for GI diseases and disorders. We particularly called for articles that have explored the clinical or animal-based evidence demonstrating the effectiveness of CAM. A collection of seven original research articles and two reviews are presented, which address the clinical evaluation and animal-based pharmacological effects of herbal drugs on GI disorders, as well as the central neural mechanisms of acupuncture in the regulation of gastric motility. The two review articles have systematically analyzed the clinical benefits of two traditional Chinese herbal formulas on diabetic gastroparesis and functional dyspepsia. Interestingly, two research articles have simultaneously reported the clinical results of an identical herbal drug (*Ban Xia Xie Xin Tang* in China and *Banha-Sasim-Tang* in Korea) used for the treatment of functional dyspepsia, through randomized controlled trial (RCT). Furthermore, the subjects of five animal studies were colorectal cancer, peptic ulcer, colitis, and liver injury, and their treatments comprised various multiple herbal medicines including single herb.

This special issue provides valuable information to practitioners and researchers working in the field of GI tract. However, there are still some major challenges due to the lack of convincing clinical evidence and less standardized therapeutics of CAM for digestive tract problems. Accordingly, further evidence-based clinical trials should be developed and implemented.

Acknowledgments

We, the editorial team, sincerely thank all the authors for submitting their valuable manuscripts and for their patience, and we are also grateful to the reviewers for their timely responses. All the credits for developing this issue go to all its contributors and the editorial team.

Chang Gue Son
Zhao Xiang Bian
Jing Hua Wang
H. Balaji Raghavendran

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Research Article

Use of Propolis Hydroalcoholic Extract to Treat Colitis Experimentally Induced in Rats by 2,4,6-Trinitrobenzenesulfonic Acid

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This study focused on the therapeutic effect of a propolis SLNC 106^{PI} extract on experimental colitis. Wistar adult rats received 0.8 mL rectal dose of one of the following solutions: saline (group S), 20 mg TNBS in 50% ethanol (group TNBS), 20 mg TNBS in 50% ethanol and propolis extract in saline (group TNBS-P), propolis extract in saline (group SP), and 20 mg TNBS in 50% ethanol and 50 mg/kg mesalazine (group TNBS-M). The animals were euthanized 7 or 14 days after the colitis induction. Samples of the distal colon were harvested for the analysis of myeloperoxidase (MPO) enzyme activity and for morphometric analysis in paraffin-embedded histological sections with hematoxylin-eosin or histochemical staining. The animals treated with TNBS exhibited the typical clinical signs of colitis. Increased MPO activity confirmed the presence of inflammation. TNBS induced the development of megacolon, ulceration, transmural inflammatory infiltrate, and thickened bowel walls. Treatment with propolis moderately reduced the inflammatory response, decreased the number of cysts and abscesses, inhibited epithelial proliferation, and increased the number of goblet cells. The anti-inflammatory activity of the propolis SLNC 106 extract was confirmed by the reductions in both the inflammatory infiltrate and the number of cysts and abscesses in the colon mucosa.

1. Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are chronic idiopathic inflammatory disorders that represent the two major types of inflammatory bowel disease (IBD). These diseases affect the gastrointestinal tract, and their course is characterized by alternating periods of remission and flare-up. The flare-up is manifested by abdominal pain, severe diarrhea, rectal bleeding, fever, weight loss, and potential systemic complications [1].

Although its cause is multifactorial, IBD depends on the presence of one or more genetically determined disorders that alter the barrier function of the bowel epithelium and lead to a greater exposure of the mucosal immune system to the normal components of the intestinal flora [2, 3].

The main therapeutic approach includes generic or selective anti-inflammatory agents and immunosuppressants. Treatment induces remission of the acute symptoms but is unable to cure the disease. In addition, 60 to 70% of the

patients require surgical intervention due to complications [1].

Mesalazine (5-aminosalicylic acid) is one of the main agents used for flare-ups and to maintain remission in mild and moderate forms of UC and CD [1]. However, the biological activity of several other substances has been tested in recent decades [4–10]. Isolated propolis components, such as caffeic acid phenethyl ester (CAPE) and 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C), and complete propolis extracts are promising alternatives because their biological activities (which include anti-inflammatory, antioxidant, immunomodulating, antimicrobial, and wound healing effects) are directly associated with treating inflammatory processes, such as IBD [11–16].

More than 300 substances have been identified as chemical components of propolis. The proportions of these compounds in propolis depend on the local flora [17, 18]. Phenolic compounds (flavonoids, aromatic acids, and benzopyrenes), di- and tri-terpenes, essential oils, aromatic acids and esters, aldehydes, ketones, and phenylpropanoids (caffeic and chlorogenic acids) are among the main components of propolis. Although the flavonoids [19] and phenolic acids are the components that are most directly related to tissue regeneration and to antimicrobial, antioxidative, and anti-inflammatory activities, the biological potential of propolis is probably a result of synergy between its components [17, 18] because the isolated compounds do not induce the same effects as the total extract [20, 21].

Due to its wide range of biological activities, particularly those that may be useful for treating IBD, the present study sought to assess the therapeutic effect of a hydroalcoholic propolis extract on experimentally induced colitis in rats.

2. Materials and Methods

2.1. Animals. Fifty albino Wistar male adult rats (*Rattus norvegicus)* 90 days old and weighting 369.8 ± 26.25 g were obtained from the Central Biotery of the Universidade Estadual de Maringá (UEM), PR, Brazil. The animals were kept in polypropylene boxes (four animals per box) at a controlled temperature of 23 to 25°C and with a 12-hour light/dark cycle. The rats were fed standard rodent rations and water *ad libitum*. This study was approved by the Animal Experimentation Ethics Committee of UEM (Protocol 006/2008).

2.2. Propolis Extract Production. Propolis was produced in the apiary from Iguatemi Experimental Farm, UEM, Maringa, PR, Brazil, collected and stored at −22°C. The hydroalcoholic extract, prepared from the same batch, in order to avoid possible seasonal variations. The extract called SLNC106$^{(PI)}$ (patent in progress), was performed in one step, and all variables were controlled and standardized in the laboratory of Phytotherapy Apitherapy and Development Maringá, PR, Brazil [22].

2.3. Experimental Procedure. Animals were fasted overnight with free access to water. Colitis was induced by a single enema instillation containing 0.8 mL of 20 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Sigma Chemical Co., St. Louis, USA) in 50% ethanol [23].

The rats were treated with a 0.8 mL enema obtained by adding propolis extract to saline in the 8% (w/w) proportion or 0.8 mL of mesalazine solution (50 mg/Kg) (5-aminosalicylic acid-5-ASA) (EMS S/A, São Paulo, Brazil) as a reference drug [24]. Daily treatment started 48 hours after the colitis induction and lasted 5 or 12 days (ending at 7 or 14 days after the colitis induction), at which time the animals were euthanized. This delayed therapy approach was chosen to allow inflammation to develop [4]. The animals were randomly distributed among five groups of 10 animals each with $n = 5$ for each experimental time-point. Group S (control) received a single dose of 0.9% saline, group TNBS received TNBS solution, group SP received 0.9% saline and was treated with propolis, group TNBS-P received TBNS solution and was treated with propolis, and TNBS-M group received TNBS solution and were treated with mesalazine.

The following parameters were evaluated daily: body weight, vitality, stool appearance, and consistency. Numerical scores for stool consistency and rectal bleeding were calculated on a 0 to 2 scale: (0) stools with normal consistency; (1) liquid stools adhering to the anus without rectal bleeding; and (2) liquid stools and blood adhering to the anus (adapted from Lamprecht et al. 2001) [25]. Two hours before euthanasia, the animals received 0.5 mg/kg of intravenous (penian vein) vincristine sulfate (Tecnocris, Eurofarma, São Paulo, Brazil) as a mitotic blocker [26]. The animals were then euthanized with an overdose (40 mg/kg) of sodium thiopental. After laparotomy, the large intestine was removed, weighed, and measured. Distal colon samples were harvested for macroscopic assessment, measurement of myeloperoxidase activity, and histological processing for histomorphometric analysis.

2.4. Stereomicroscopy Assessment. Samples of the distal colon were opened by a longitudinal incision on the mesocolic margin, washed to remove feces, and observed under a stereomicroscope with trans-illumination (Olympus SZ 61, Tokyo, Japan). Alterations were scored on a 0 to 5 scale: (0) absence of inflamed areas; (1) localized hyperemia without ulcerations; (2) linear ulcers without significant inflammation and mild hyperemia; (3) ulcerations without necrosis (crusts) and 2 to 4 cm of inflammation; (4) ulcerations with crusts, megacolon, serosa adhering to organs, and 2 to 4 cm of inflammation; and (5) ulcerations with crusts, megacolon, serosa adherence involving several intestinal folds, stenosis and inflamed areas larger than 4 cm [23, 27].

2.5. Measurement of Myeloperoxidase (MPO) Activity. The progression of the inflammatory response was determined by measuring these myeloperoxidase (MPO) activity [28]. Samples from the distal colon samples were macerated and homogenized in a 50 mM pH 6.0 potassium phosphate buffer containing 0.5% (w/v) hexadecyltrimethylammonium bromide (HETAB) (Sigma Chemical Co., St. Louis, USA) with 50 mg of tissue in 1 mL of buffer solution. The samples

were then subjected to an ultrasonic bath for 30 s, heated for two hours in a water bath at 60°C and centrifuged at 5000 g and 25°C for 10 minutes. Triplicate 10 μL samples were removed from the supernatant and 200 μL of the staining reagent was added; the staining reagent contained 4.2 mg of o-dianisidine dihydrochloride (Sigma Chemical Co. St. Louis, USA), 22.5 mL of double-distilled water, 2.5 mL of pH 6.0 50 mM potassium phosphate buffer, and 12.5 μL of 1% hydrogen peroxide. The reaction was terminated after five minutes by adding 1.46 M sodium acetate, and the MPO activity was determined by the 450 nm absorbance, as measured by an ELISA reader (Lionheart Diagnostics, Status Labsystems, Multiskan RC, Uniscience, Brazil).

2.6. Histological Study. The samples from distal colon were washed with saline solution, fixed with Bouin's solution and embedded in paraffin. Semiserial 7 μm sections perpendicular to the long axis of the colon were obtained. The hematoxylin-eosin (H&E)-stained sections were used for the following purposes: (1) morphometric analysis of the bowel layers; (b) inflammation assessment; (c) tissue damage assessment [29]; and (d) epithelial cell proliferation assessment using the metaphase index (MetI). The sections were stained using the Periodic Acid-Schiff (PAS) histochemical method to allow the goblet cells to be counted. The thickness of the bowel mucosa, submucosa, muscular and serosa layers and complete intestinal wall was assessed under 4x magnification at 10 random sites from the histological sections (50 measures/animal/layer). The number of goblet cells was determined by counting 100 microscopic fields/animal over a total area of 9.06 mm^2 at 40x magnification.The morphometric analysis and goblet cell counting were performed with images obtained from a QColor 3 camera (Olympus American INC, Canada) coupled to an Olympus BX 41 optic trinocular microscope (Tokyo, Japan). The bowel layers were measured using Image-Pro plus 4.5 image analysis software (Media Cybernetics, Silver Spring, MD). The microscopic inflammation and tissue damage were scored on a 0 to 3 severity scale using the following criteria: (a) ulceration, (b) abscesses in crypts, (c) cysts, (d) damaged wall architecture, (e) inflammatory infiltrates, and (f) vascular dilatation; a detailed description is given in Fabia et al., 1993 [29]. The metaphasic index (MetI), the ratio of the nuclei in metaphase to the total number of counted nuclei, was determined for the longitudinal crypts that exhibited evident lumens; 2.500 cells were counted for each animal [30] using an Olympus BX41 optic binocular microscope (Tokyo, Japan) with 40x magnification. The MetI was multiplied by Tannock's factor to correct tissue geometry, thereby avoiding overestimates of the number of metaphase nuclei [31].

2.7. Statistical Analysis. The Kruskal-Wallis test followed by Dunn's posttest were used for the nonparametric numerical variables. The means of the parametric variables were compared using one- and two-way variance analysis (ANOVA) models and Tukey or Bonferroni posttest. The statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software, Inc. San Diego, CA, USA), and the results are expressed as mean ± standard deviation. Significance was established at 5%.

3. Results

3.1. Clinical Assessment. The animals in the TNBS, TNBS-M, and TNBS-P groups exhibited reduced vitality and piloerection and 100% developed severe diarrhea one day after the colitis was induced. Administering saline or saline + propolis did not result in clinical alterations, but the animals remained active, and the consistency and appearance of the stools were normal. Table 1 describes the stool appearance and consistency scores.

Throughout the study, the animals in the TNBS, TNBS-P and TNBS-M groups had a significantly greater weight loss ($P < 0.05$) than that of the animals in the control groups (Figure 1). Body weight recovery was at its lowest in the group treated with propolis solution.

3.2. Stereomicroscopy Analysis. The results of the stereomicroscopic analysis are described in Table 2. The animals in group S exhibited no alterations. Approximately 60% of the animals in group SP exhibited hyperemic areas after five days of treatment, and 20% had hyperemia after 12 days. One week after the colitis was induced, groups TNBS and TNBS-M exhibited tissue lesions and had scores significantly different from those of group S. Similar but milder lesions were present in group TNBS-P. Although there were lesions with differing degrees of severity (ranging from hyperemic areas in the mucosa to healed ulcers exhibiting whitish devitalized tissue) in the groups with induced colitis (independent of treatment), the differences in the lesions were not statistically significant 12 days after the onset of treatment.

3.3. Width and Weight/Length Ratios. The distal colon width and the large intestine weight/length ratios indicated edema and are described in Table 3.

3.4. Measurement of Myeloperoxidase (MPO). The MPO activity is described in Table 4. The MPO activity was significantly greater ($P < 0.05$) in groups TNBS, TNBS-P, and TNBS-M than in groups S and SP at 7 and 14 days. The MPO activity was higher in the animals treated with propolis than in the animals with untreated colitis at 14 days (Table 4).

3.5. Histomorphometric Assessment

3.5.1. Microscopic Analysis. The animals in groups S and SP exhibited normal histological characteristics in the distal colon and had lymphatic nodules in the submucosa (Figures 2(a), 2(b), 2(e) and 2(f)). The distal colons of the animals with colitis exhibited similar characteristics after the TNBS was administered, regardless of the treatment. Tissue damage and loss of superficial cells were observed on day 7, resulting in multiple ulcerations and dense inflammatory infiltrates with a predominance of neutrophils and eosinophils. A large number of crypts were deformed by cysts or microabscesses and exhibited neutrophilic exudates. In addition to edema,

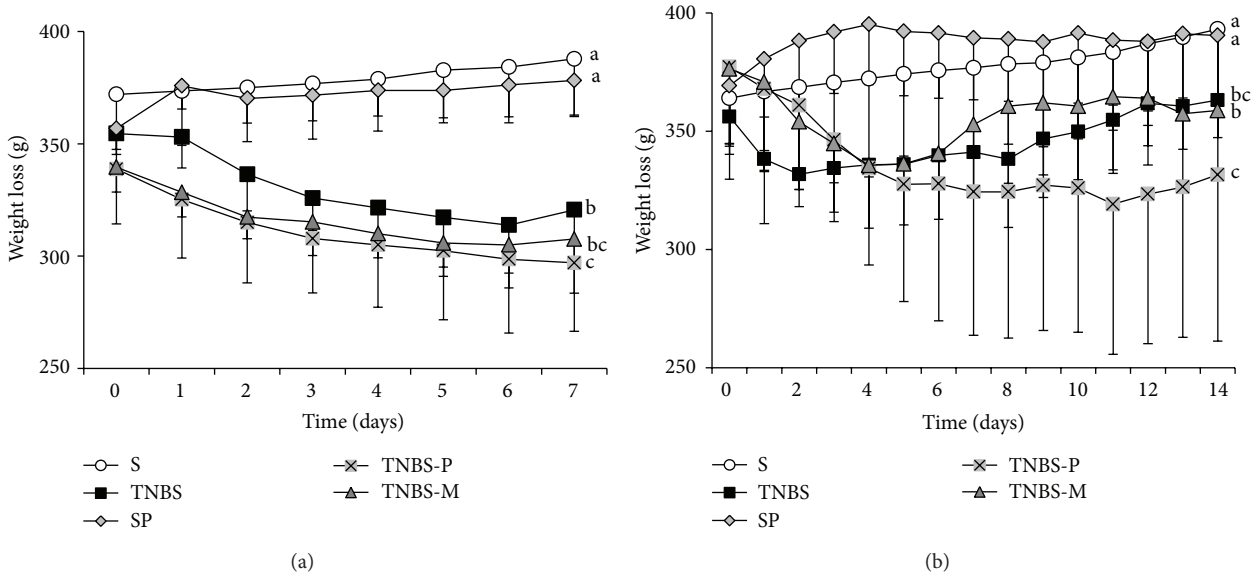

Figure 1: The body weight of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). (a) The animals euthanized 7 days after the colitis was induced. (b) The animals euthanized 14 days after the colitis was induced. Different letters indicate significant differences in a one-way ANOVA model with Tukey's post-test ($P < 0.05$) ($n = 5$).

Table 1: The characteristics of stools from rats that received rectal TNBS (to induce colitis) or saline solution (S) after 5 or 12 days of treatment with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). The animals were euthanized 7 or 14 days after the colitis was induced. The results are expressed as the mean ± standard deviation ($n = 5$).

Group/time (days)	Characteristics of stools (0–2) (0) Well-formed pellets (1) Liquid stools stuck to the anus, no bleeding (2) Liquid stools and blood stuck to the anus	
	Day 7	Day 14
S	0^{a}	0^{a}
TNBS	2.0 ± 0^{b}	1.6 ± 0.55^{b}
SP	0^{a}	0^{a}
TNBS-P	1.6 ± 0.55^{b}	1.0 ± 0^{ab}
TNBS-M	1.0 ± 0^{ab}	1.0 ± 0^{ab}

[a,b]Means followed by different letters in the same column are significantly different according to the Kruskal-Wallis test and Dunn's post-test ($P < 0.05$).

Table 2: Stereomicroscopic assessment of the distal colons of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). The animals were euthanized 7 or 14 days after the colitis was induced. The results are expressed as the mean ± standard deviation ($n = 5$).

Group/time (days)	Score (0–5) (0) Absence of inflamed areas (1) Localized hyperemia without ulcerations (2) Mild hyperemia, linear ulcers without significant inflammation (3) Ulcerations without necrosis (crusts), 2 to 4 cm of inflammation (4) Ulcerations with crusts, 2 to 4 cm of inflammation (5) Ulcerations with crusts (inflammation >4 cm)	
	Day 7	Day 14
S	0^{a}	0^{a}
TNBS	4.6 ± 0.55^{b}	3.2 ± 1.79^{b}
SP	0.6 ± 0.55^{a}	0.2 ± 0.45^{a}
TNBS-P	3.6 ± 1.14^{ab}	3.0 ± 2.0^{ab}
TNBS-M	4.2 ± 1.30^{b}	3.8 ± 1.79^{b}

[a,b]The means followed by different letters in the same column are significantly different according to the Kruskal-Wallis test and Dunn's post-test ($P < 0.05$).

these exudates also caused the loss of crypt epithelium and goblet cells (Figures 2(c) and 2(i)). The areas of regenerated mucosa exhibited bifurcated crypts with broadened bases, wide lumens, irregular shapes, cysts and abscesses. The submucosa contained dense transmural inflammatory infiltrates that consisted predominantly of neutrophils (with some eosinophils, plasmocytes, macrophages, and phagocytic epithelial cells), whereas only polymorphonuclear cells were observed in the lymph nodes of the submucosa and myenteric plexuses (Figures 2(d), 2(g), and 2(h)). An expanded serosa with adipose tissue exhibiting a lymphocyte and macrophage infiltrates and increased vascularization was also observed (Figures 2(h) and 2(j)). The distal colons of the rats in the TNBS, TNBS-P, and TNBS-M groups exhibited

Table 3: The width and weight/length ratio (mg/cm) of the distal colons of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). The animals were euthanized 7 or 14 days after the colitis was induced. The results are expressed as the mean ± standard deviation ($n = 5$).

Group/time (days)	Width (cm)		Weight/length ratio (mg/cm)	
	Day 7	Day 14	Day 7	Day 14
S	1.02 ± 0.18^{a}	1.00 ± 0.07^{a}	252.4 ± 61.1^{a}	268.0 ± 42.6^{a}
TNBS	1.56 ± 0.31^{b}	1.36 ± 0.15^{a}	558.2 ± 46.4^{b}	415.1 ± 105.9^{a}
SP	1.02 ± 0.18^{a}	1.04 ± 0.19^{a}	236.7 ± 41.6^{a}	259.8 ± 18.0^{a}
TNBS-P	1.26 ± 0.18^{a}	1.25 ± 0.18^{a}	500.7 ± 125.3^{bc}	687.3 ± 169.0^{c}
TNBS-M	1.16 ± 0.15^{a}	1.25 ± 0.11^{a}	423.2 ± 90.6^{c}	605.9 ± 67.1^{c}

[a,b,c]The means followed by different letters in the same column are significantly different according to a one-way ANOVA model and Tukey's post-test ($P < 0.05$).

characteristics on day 14 that were similar to those observed on day 7, although milder.

Figure 3 describes the frequencies at which the animals exhibited dense, moderate, mild, or no inflammatory infiltrate, and Figure 4 describes the frequency of the assessed histological variables: colon wall architecture, presence of cysts, abscesses, and ulcers.

3.5.2. Morphometry of the Bowel Layers. The thickness of the bowel layers and complete distal colon wall is described in Table 5.

3.5.3. Number of Goblet Cells. The number of goblet cells in the intestinal glands of the animals with induced colitis decreased. This decrease was more pronounced 7 days after induction. The animals in the TNBS-M group had the fewest goblet cells on day 7 (Figure 5(a)). By contrast, the number of goblet cells increased ($P < 0.05$) in the healthy animals that were administered the propolis solution (group SP). Similar results were observed on day 14, except that the TNBS group had goblet cells populations that were similar to those of the untreated control animals (group S) (Figure 5(b)).

3.5.4. Assessment of Epithelial Cells. Table 6 describes the epithelial proliferation results. Colitis treatments using the mesalazine (at 7 and 14 days) or propolis solutions (14 days) inhibited intestinal gland cell proliferation.

4. Discussion

We previously demonstrated the wound healing effect, effectiveness of anti-inflammatory, and antimicrobial of propolis ethanolic extracts [16, 32, 33], and therefore the interest in evaluating their activity in a model of experimental colitis.

Colitis was induced in rats by rectally administering a single dose of 20 mg TNBS in 50% (v/v) ethanol in the distal colon and was assessed for 14 days. The therapeutic effect of a hydroalcoholic propolis (SLNC 106) solution was assessed and compared to that of the standard medication, namely, mesalazine.

In the present study, all of the animals that received TNBS exhibited the typical clinical signs of colitis after 24 hours: piloerection, hypoactivity, weight loss, and diarrhea. These signs improved after 48 hours. Severe diarrhea with rectal bleeding is characteristic of inflammatory bowel disease (IBD). Severe diarrhea is the result of functional and structural alterations in the gastrointestinal tract and is associated with inflammation, nausea, and abdominal pain, which occur mainly in the active disease phase [34, 35].

Table 4: The myeloperoxidase activity (MPO) (nm) in the distal colons of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). The animals were euthanized 7 or 14 days after the colitis was induced. The results are expressed as the mean ± standard deviation ($n = 5$).

	MPO activity (OD 450 nm)	
Group/time (days)	Day 7	Day 14
S	0.185 ± 0.041^{a}	0.166 ± 0.071^{a}
TNBS	0.618 ± 0.161^{b}	0.659 ± 0.124^{b}
SP	0.068 ± 0.074^{a}	0.180 ± 0.097^{a}
TNBS-P	0.720 ± 0.051^{b}	0.823 ± 0.029^{c}
TNBS-M	0.749 ± 0.172^{b}	0.782 ± 0.046^{bc}

[a,b,c]The means followed by different letters in the same column are significantly different according a to two-way ANOVA model and the Bonferroni post-test ($P < 0.05$).

The inflammatory status of the distal colon was biochemically characterized by myeloperoxidase (MPO) enzyme activity and morphologically characterized by a series of indicators, including hyperemia, ulcerations, width and weight/length ratios of the distal colon, and histomorphometric assessment of the intestinal wall.

From the macroscopic perspective, megacolon was observed in all of the animals with colitis. However, there were no differences in bowel width among the treated and control animals, suggesting that propolis and mesalazine may have attenuated the development of megacolon during the first week. During the second week, the animals in the TNBS group exhibited a decrease in distal colon width that was similar to that of the treated animals, even though they did not receive treatment; therefore, this parameter improves over time, regardless of pharmacological treatment. A degree of adherence to adjacent organs and mesenteric

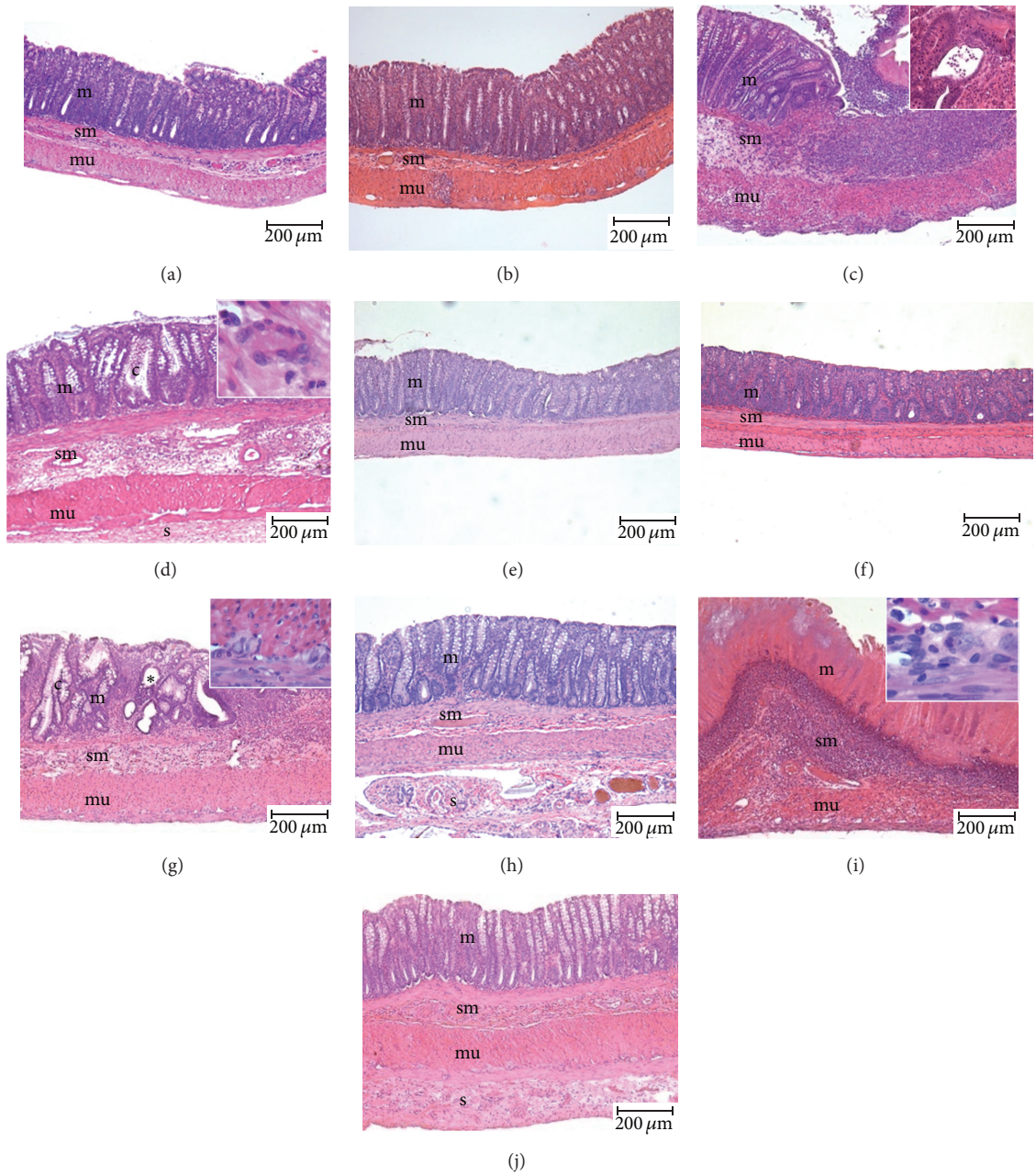

Figure 2: Photomicrography of the distal colons of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine solution (TNBS-M). The animals were euthanized 7 or 14 days after the colitis was induced. The animals in groups S ((a) and (b)) and SP ((e) and (f)) exhibited normal histological characteristics (m: mucosa, sm: submucosa, and mu: muscular). Group TNBS ((c) and (d)). (c) shows extensive ulceration and intense inflammatory infiltrate surrounded by normal mucosa; a crypt abscess containing polymorphonuclear cells is shown in detail. In (d), note the distorted crypts (c), presence of foreign body giant cells in the submucosa (detail), and expansion of the serosa (s). The group TNBS-P ((g) and (h)) mucosa shows regeneration with bifurcated crypts (c) and cysts (∗) in (g) and serosa expansion (S) in (h). Group TNBS-M ((i) and (j)). In (i), note the ulcerated area in the mucosa and intense transmural inflammatory infiltrate. In the detail, note the presence of eosinophils inside a myenteric ganglion. In (j), note the restored mucosa, absence of inflammatory infiltrate, and expanded serosa (s). Hematoxylin-eosin. Scale = 200 μm.

fat accumulation was observed at 7 and 14 days in a strong contrast to the appearance of the colon in the healthy animals.

In this experimental colitis model, megacolon develops as a consequence of dysmotility arising from changes in the structure and function of the enteric nervous system (ENS) [34]. TNBS-induced colitis is associated with a 20% loss of myenteric neurons, which occurs when neutrophils infiltrate the ganglia. Interestingly, this neuronal loss seems to persist for a period of time that is coincident with the resolution of inflammation. The reduction in myenteric neurons has

Table 5: A morphometric analysis (by group and time) of the bowel layers and complete wall in the distal colons of rats that received rectal TNBS (20 mg/50% ethanol) (TNBS, TNBS-P, TNBS-M) or saline solution (S and SP) and were rectally treated with mesalazine (TNBS-M) or propolis SLNC106 (SP and TNBS-P). The results are expressed as the mean ± standard deviation ($n = 5$).

Group/time	Mucosa (μm)	Submucosa (μm)	Muscle layer (μm)	Serosa (μm)	Total wall (μm)
Day 7					
S	308.7 ± 32.2^{a}	70.1 ± 21.9^{a}	193.9 ± 14.9^{a}	16.0 ± 1.4^{a}	563.9 ± 69.6^{a}
TNBS	388.9 ± 75.2^{ab}	382.2 ± 158.1^{b}	257.6 ± 81.4^{a}	35.9 ± 24.3^{a}	1062.0 ± 265.8^{b}
SP	325.4 ± 20.9^{a}	59.9 ± 9.3^{a}	187.8 ± 45.4^{a}	25.2 ± 7.6^{a}	580.3 ± 50.2^{a}
TNBS-P	447.7 ± 81.5^{b}	241.1 ± 148.6^{ab}	318.4 ± 76.2^{ab}	82.4 ± 74.7^{a}	1033.0 ± 119.2^{b}
TNBS-M	456.6 ± 60.3^{b}	271.4 ± 204.4^{ab}	470.8 ± 350.0^{b}	192.8 ± 153.3^{b}	1320.0 ± 650.1^{b}
Day 14					
S	347.8 ± 30.6^{a}	82.11 ± 27.7^{a}	250.4 ± 51.9^{a}	16.4 ± 1.5^{a}	677.8 ± 30.9^{a}
TNBS	362.0 ± 44.5^{a}	235.2 ± 101.5^{a}	200.5 ± 57.4^{a}	73.9 ± 21.6^{ab}	888.1 ± 174.3^{a}
SP	321.1 ± 5.6^{a}	126.9 ± 118.8^{a}	177.0 ± 55.7^{a}	21.0 ± 6.9^{a}	675.0 ± 302.9^{a}
TNBS-P	460.3 ± 50.7^{b}	257.9 ± 203.5^{a}	282.3 ± 84.8^{a}	132.7 ± 57.9^{b}	1121.0 ± 252.1^{b}
TNBS-M	464.8 ± 100.4^{b}	270.6 ± 204.3^{a}	342.0 ± 84.0^{a}	250.4 ± 111.4^{c}	1312.0 ± 391.7^{b}

^{a,b,c}The means followed by different lowercase letters in the same column were significantly different at 7 and 14 days according to a two-way ANOVA model and the Bonferroni post-test ($P < 0.05$).

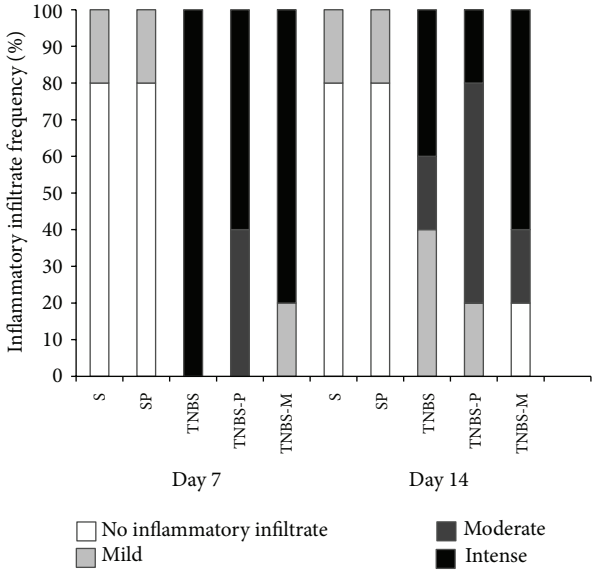

Figure 3: Frequency with which animals exhibited (a) intense, moderate, mild, or no inflammatory infiltrate after they received rectal TNBS (to induce colitis) or saline solution (S) and were treated for 5 or 12 days with propolis hydroalcoholic solution.

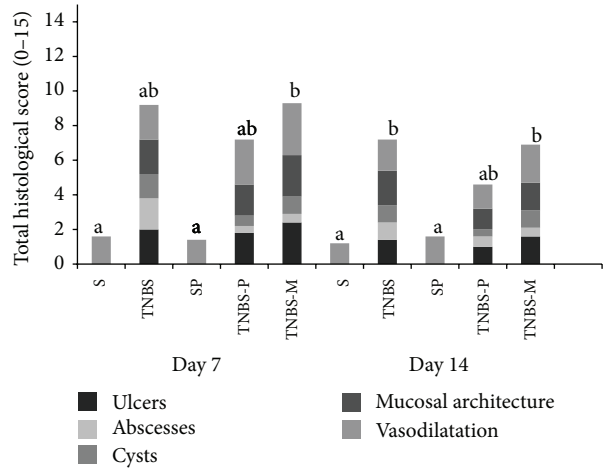

Figure 4: The total score of animals exhibiting histological changes (alterations in the architecture of the distal colon wall, cysts, abscesses, and ulcers) after they received rectal TNBS (to induce colitis) or saline solution (S) and were treated for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P) or mesalazine solution (TNBS-M). Means ± SD followed by different letters on the day of the animals' deaths indicate a significant difference according to the Kruskal-Wallis test and Dunn's post-test ($P < 0.05$); ($n = 5$).

not been associated with any particular subpopulation, which suggests that indiscriminate loss occurs at the onset of TNBS-induced colitis [36].

Inflammation is known to affect bowel function. In the present study, MPO activity remained high throughout the assessed period. Neither the propolis extract nor the mesalazine were able to reduce this enzyme activity, which was higher ($P < 0.05$) in the animals treated with propolis than in those treated with mesalazine. This difference may suggest aggravation of the inflammatory process, particularly in the animals treated with propolis; however, the anti-inflammatory effect of propolis was apparent after five days of administration when 40% of the animals already exhibited moderate inflammatory infiltrate. At five days, only 20% of the animals treated with mesalazine exhibited decreased inflammation, and any decreases were classified as mild.

Twelve days after the propolis administration, inflammation was reduced in 80% of the animals, 60% had moderate infiltrates and 20% had mild infiltrates. At this time, 60% of the animals treated with mesalazine still had dense infiltrates, 20% had mild infiltrates, and 20% no longer exhibited inflammation.

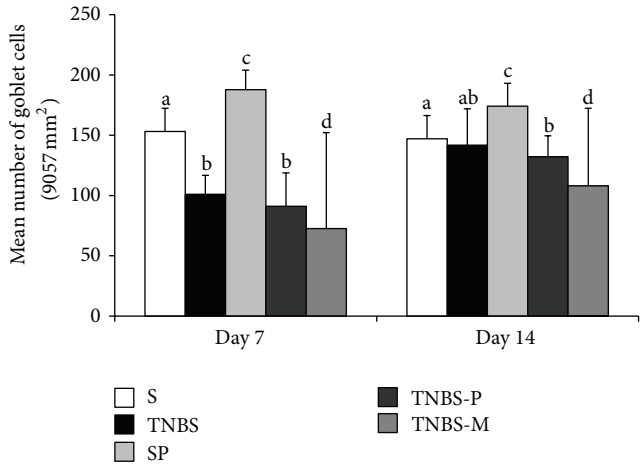

Figure 5: The number of goblet cells in the distal colons of rats (area of 9.06 mm^2 at 40x magnification) that received rectal TNBS (to induce colitis) or saline solution (S) and were treated for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P) or mesalazine solution (TNBS-M). The animals were euthanized on day 7 (a) or 14 (b) after the colitis was induced. Means ± SD followed by different letters on the day of the animals' deaths indicate a significant difference according to the Kruskal-Wallis test and Dunn's post-test ($P < 0.05$), ($n = 5$).

Table 6: The metaphase index (MetI) in the epithelium of the distal colons of rats that received rectal TNBS (to induce colitis) or saline solution (S) after treatment for 5 or 12 days with propolis hydroalcoholic solution (TNBS-P and SP) or mesalazine.

Group/time	Metaphase Index (MetI) (%)	
	Day 7	Day 14
S	6.945 ± 0.8575	8.872 ± 0.7792
TNBS	9.099 ± 2.722	10.51 ± 0.8000
SP	6.373 ± 1.042	5.68 ± 0.827
TNBS-P	7.468 ± 2.029	6.038 ± 1.102[a]
TNBS-M	4.528 ± 3.298[a]	5.663 ± 1.053[a]

[a]$P < 0.05$ compared to TNBS in the same column through a one-way ANOVA model and Tukey's post-test (mean ± standard deviation, $n = 4$).

These results suggest that the therapeutic effect of the propolis treatment was modulated, that is, its action was slower, but its scope was wider because it encompassed a higher number of animals than did the mesalazine treatment. The anti-inflammatory effect of mesalazine was more rapid and more pronounced only in the animals that responded well to it (less than half of the treated population). The modulated anti-inflammatory action of propolis and the restricted action of mesalazine may explain the high MPO activity levels that were observed. The inflammation persisted after 12 days of treatment with both drugs.

All of the animals with colitis that did not receive pharmacological treatments exhibited dense inflammatory infiltrate in the first week. On day 14, the inflammation had spontaneously decreased in most (60%) of the animals (20% had moderate and 40% had mild infiltrate), whereas 40% still exhibited dense infiltrate.

The combined and separate therapeutic effects of propolis and mesalazine in an acetic acid-induced colitis model were investigated. Most of the animals treated with propolis exhibited normal histology, and 50% and 33% of the rats treated with mesalazine alone or in combination with propolis, respectively, exhibited inflammatory infiltration. The authors concluded that the drugs are effective both alone and in combination but that the combined effect was not cumulative for experimental colitis [37].

The anti-inflammatory effect of propolis has been attributed to several active components: caffeic acid, quercetin, naringenin, CAPE [38], coumaric acid, ferulic acid, campherol, and galangin [39]. The possible mechanisms include prostaglandin suppression, leukotriene synthesis by macrophages, and inhibition of the myeloperoxidase, NADPH-oxidase, ornithine decarboxylase, and protein tyrosine kinase enzymes [40]. Propolis also stimulates macrophages and phagocytic activities [41].

Phenolic compounds, particularly flavonoids, are thought to remove the excess free radicals produced by inflammation. Although oxidative damage is already known to be associated with tissue destruction, modulating free radical production may represent a new direction in IBD treatment [41].

CAPE is an important antioxidant that also has anti-inflammatory properties because it inhibits arachidonic acid release from the cell membrane and thus suppresses cyclooxygenase (COX-1 and COX-2) activity [42]. It is also a powerful and specific inhibitor of nuclear factor-kappa B (NF-kB) [43], which is super-expressed in the lamina propria of Crohn's disease patients [3] and is a pathogenic factor in TNBS-induced colitis models [44].

The NF-kB is activated by several factors associated with IBD, such as inflammatory cytokines (interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α)), bacterial products and oxidative stress. The NF-kB controls IL-1β expression and inducible nitric oxide synthase (iNOS) in macrophages. The corresponding mRNA expression is suppressed at the transcriptional level after an ethanolic extract of propolis is added to a J774A macrophage cell culture line [45], suggesting that propolis or its components inhibit the inflammatory response via different mechanisms. Allied to this, it was found that TNBS-induced colitis in mice previously fed (14 days before induction of colitis) with standard food containing propolis extract showed an improvement of colitis symptoms in a dose-dependent manner by inhibiting the Th1 cells differentiations [46].

The histological analysis in the present study revealed a predominance of neutrophils in the early acute phase that was followed by eosinophilic domination of the transmural infiltrate. Polymorphonuclear cells were found near or even inside the myenteric ganglia and may be associated with enteric nervous system effects and neuronal death [47, 48].

Transmural inflammation may result in fistulas, abscesses, and fissures due to serosal infiltration of adjacent loops. Such an infiltration results in fibrin deposits and makes the mesenteric surfaces of the inflamed loops adherent between themselves and to other abdominal organs, thereby favoring the formation of fistulas and fibrous adherences [49].

Experimental data suggest that even moderate inflammation may cause persistent alterations in the nervous function and smooth muscle of the gastrointestinal tract. These alterations can result in colon dysmotility, hypersensitivity, and dysfunction, even when the inflammation is restricted to the proximal small intestine. Alterations in bowel function are observed after resolution of acute intestinal inflammation, suggesting that the changes it induces remain after recovery, and play an important role in producing IBD symptoms [50].

Macroscopically assessing colitis lesions underestimates their intensity; therefore, it should complement rather than replace microscopic analysis. Therefore, the present study microscopically assessed the frequency of the following histological variables: cysts, abscesses, ulcers, and alterations in the structure of the intestinal wall.

Crypt abscesses when totally developed, characteristically show neutrophils inside, in the crypt wall and adjacent lamina propria. In addition to edema, this exudate may also cause a loss of the crypt epithelium and goblet cells [51]. By contrast, cysts typically do not contain inflammatory cells inside. The propolis treatment was beneficial because it decreased the number of abscesses and cysts.

The hyperemia and ulcerations analyses determine the commitment of tissues. All of the induced colitis groups exhibited a range of alterations, from hyperemia to ulcerations, with various degrees of severity. The propolis and mesalazine treatments caused a small decrease in the lesion score only after 12 days of treatment, suggesting that neither of the drugs were able to reduce hyperemia and/or ulceration.

An increase in the weight/length ratio is a frequent finding in TNBS-induced colitis [9–11] and serves as an indicator of edema. In the present study, this ratio remained significantly higher in animals with colitis at days 7 and 14, indicating a persistent inflammatory state. This finding is consistent with other studies in which treatment also failed to reduce the colon weight increase and the weight/length ratio [8, 10]. The injuries caused by TNBS can sometimes reach a magnitude that is difficult to overcome by pharmacological treatment, which may explain why these treatments do not affect certain parameters [8].

All of the animals with colitis exhibited a thickening of the distal colon walls, mostly at the submucosa and serosa. In addition to the inflammatory infiltrate, the submucosa exhibited dilated vessels and edema, which explains its expansion. The thickening of the serosa was characterized by expansion, extensive vascularization, and infiltration by immune cells. A remarkable pathological angiogenesis, in which vessels originating in the submucosa penetrate the muscular layer towards the serosa, is associated with the chronic stages of inflammatory bowel disease [52].

In addition, the muscular layer increased significantly during the first week; this process is usually, partially, attributed to the accumulated inflammatory cells [23], inflammatory hypertrophy and hyperplasia of the muscular cells, and altered protein content. These factors explain the intestinal motility disorders observed in TNBS-induced inflammation models [53]. These alterations resulted in a notable increase in total wall thickness mainly on day 7. An increased total intestinal wall thickness is typical of this colitis model [23] and is probably associated with the inflammatory factors mentioned above.

Inflammation may interfere with the migration and proliferation of epithelial cells and thus modulates bowel epithelium repair [54]. Treatment with propolis and mesalazine reduced proliferative activity in the crypts relative to the TNBS group. The inhibitory effect of the propolis treatment was more noticeable after 12 days, whereas the effect inhibitory of the mesalazine was already perceptible on day 5. The untreated animals with colitis exhibited the highest proliferation rates.

The metaphasic index did not differ between the TNBS and control S groups; only the TNBS-P and TNBS-M groups differed significantly from that of the TNBS group, suggesting that the metaphasic index was increased (although not significantly) in the animals with untreated colitis. This hypothesis is strengthened by the presence of bifurcated crypts in the TNBS group, which suggests that a regenerative response may have been triggered by TNBS-induced epithelial erosion stimulating reproduction by the budding or branching of new crypts from surviving crypts. This phenomenon is a signature of the hyperplasic response and includes a reduction in the duration of the cell cycle and an expansion of the proliferative compartment [30]. The animals in the control group that were administered the propolis solution exhibited low proliferation indices, which suggests an inhibitory effect of propolis that is independent of inflammation condition.

Cell proliferation in experimental colitis models is controversial. Both, increased [55, 56] and decreased [57] proliferative indices have been reported.

The function of other epithelial cells may also be affected. Thus, fewer goblet cells were found in the animals with colitis during the first week, independent of treatment. With the exception of the group treated with mesalazine, the epithelial cell population recovered. In the controls, the propolis exhibited a stimulatory effect on goblet cells. Several studies have reported a remarkable reduction in goblets cells, more severe colitis, and reduced mucin content after TNBS-induced inflammation [4, 48, 58]. By contrast, Torres et al. (1999) [55] found an increase in the number and size of goblet cells and suggested that this phenomenon is characteristic of TNBS-induced cryptitis; the increased cellularity may reflect the increased proliferation as a tissue repair mechanism in response to TNBS.

In general, the literature suggests that experimental colitis treatments are more effective when applied preventively before colitis is induced [4, 9]. In the present study, treatment was initiated 48 hours after induction (i.e., when the inflammatory process was already established) and it was, thus, a late therapeutic approach [4]. In this context, we concluded that the therapeutic anti-inflammatory potential of the propolis hydroalcoholic extract could be established by the decreased intensity of the inflammatory infiltrate and by the reduction in the number of cysts and abscesses in the colonic mucosa.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Chinese Herbal Medicine Banxiaxiexin Decoction Treating Diabetic Gastroparesis: A Systematic Review of Randomized Controlled Trials

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Objective. To assess the current clinical evidence of Banxiaxiexin decoction for diabetic gastroparesis (DGP). *Methods*. Electronic databases were searched until December 2012. No language limitations were applied. We included RCTs using Banxiaxiexin decoction/modified Banxiaxiexin decoction for DGP. No restriction for the control group except acupuncture. Applying clinical effective rate as the main outcome index. Data extraction, analyses and quality assessment were conducted according to the Cochrane review standards. *Results*. 16 RCTs involving 1302 patients were finally identified, and the methodological quality was evaluated as generally low. The data showed that the effect of Banxiaxiexin decoction (BXXD) for DGP was superior to the control group ($n = 1302$, RR 1.23, 95% CI 1.17 to 1.29, $Z = 8.04$, $P < 0.00001$). Only one trial recorded adverse events, no obvious adverse event occurred. *Conclusions*. Banxiaxiexin decoction could regain the gastric emptying rate and improve diabetic gastrointestinal symptoms. However, the methodological quality of included studies is low, and long term efficacy and safety are still uncertain, which indicates that the findings above should be read with caution. Thereby, well-designed, large-scale, and high-quality randomized controlled clinical trials with scientific rigor are warranted for stronger evidence in future research.

1. Introduction

Diabetic gastroparesis (DGP) is a syndrome characterized by delayed gastric emptying in the absence of mechanical obstruction of the stomach in patients with diabetes, which is a well-established complication of diabetes, first reported in 1958 [1]. The cardinal symptoms include postprandial fullness, nausea, vomiting, and bloating [2, 3]. Symptoms attributable to gastroparesis are reported by 5% to 12% of patients with diabetes [4, 5]. This is consistent with the well-established observation that DGP which typically develops after diabetes mellitus (DM) has been established for ≥10 years [1, 6]. Once established, DGP tends to persist despite the improvement of glycemic control [7], which reduces quality of life on all the main aspects including physical, emotional, mental, social, and body functions [8]. DGP is also associated with higher mortality and morbidity [9]; DM patients with classic symptoms of gastroparesis (including early satiety, postprandial fullness, bloating, abdominal swelling, nausea, vomiting, and retching) and documented delay in gastric emptying are more likely to have cardiovascular disease, hypertension, and retinopathy [10], suggesting that DGP may be related to the complications which are known as complications of poor diabetic control.

The pathogenesis of DGP has not been clarified. Current pieces of research have found that smooth muscle degeneration in DGP is caused by multiple factors, including autonomic nervous dysfunction, hyperglycemia, gastrointestinal

hormone secretion disorder, abnormalities of interstitial cells of Cajal (ICC), and vascular lesions [11–14]. Based on blood glucose control, the available treatment options of modern medical treatment include nutritional support, improvement of gastric emptying using prokinetics, symptom control, and use of a gastric electric stimulator [15, 16]. The increasing number of drugs under development with different mechanisms of action improves clinical symptoms, whereas they are far from clinical satisfaction [17]. Metoclopramide, being one of the typical DGP treatments, has a well-recognized complication which is tardive dyskinesia [18]. Another commonly used drug is Erythromycin, which is a useful agent for short term treatment in hospital; however, its long term benefit is limited due to the development of tachyphylaxis [18]. Besides, the use of botulinum toxin injection and gastric electric stimulator is still controversial [19–21]. The high recurrence rate leads to the further deterioration of the disease [8]. Nowadays, with the incidence of diabetes increasing, more and more people will be perplexed by DGP [22]. Therefore, to seek effective measures of treatment has become a major health problem, which is beneficial to the people's livelihood.

Clinical practice has shown a bright future of traditional Chinese medicine (TCM) in treating diabetes and its complications [23]. Banxiaxiexin decoction (BXXD), a traditional Chinese herbal medicine containing seven commonly used herbs (*Pinellia ternata*, Radix Scutellariae, Rhizoma Zingiberis, Panax ginseng, Radix Glycyrrhizae, *Coptis chinensis*, and Fructus Jujubae), is widely used to treat gastrointestinal discomfort in clinical practice for a long time in China [24–26]. The mechanism of the prescription may be acrid to diffuse and bitter to descend, reinforcing and reducing according to the theory of TCM. A recent research showed that BXXD could improve gastrointestinal motility [27, 28]. Biochemically, BXXD also adds plasma motilin, gastrin, and nitric oxide; suppresses VIP; adjusts gastric myenteric plexus, c-kit positive, and ICC volume; copes against dysrhythmia; and promotes gastric emptying markedly [29–32].

According to TCM, diabetic patients are always having dysfunction in stomach and have disorder in ascending and descending. Asthenia and sthenia, cold and heat are mixed up when the course of DM becomes long. According to the theory of TCM, we could use BXXD as an alternative method for treating DGP [33, 34]. There have been numbers of research works indicating that BXXD is effective to DGP, whereas the data supporting the validity is not enough. This systematic review aims to assess the current clinical evidence of BXXD for DGP by conducting the literature reviews in databases for randomized controlled trials (RCTs).

2. Methods

2.1. Database and Search Strategy. A computer-based online search was conducted in the Medline, Cochrane Library, Chinese Biomedical Literature Database (CBM), Chinese National Knowledge Infrastructure (CNKI), Chinese Scientific Journal Database (VIP) and Wanfang Databases. Search terms used were ("diabetic gastroparesis" OR "gastrointestinal changes" OR "gastrointestinal disease") AND ("herb" OR "Banxiaxiexin Decoction" OR "BanXia Xie Xin" OR "Banxiaxiexin Tang") AND ("randomized controlled trial" OR "controlled clinical trial" OR "random" OR "randomly" OR "randomized" OR "control"). We searched all articles published before December, 2012.

2.2. Inclusion Criteria. All the RCTs that used BXXD in treatment group were included. RCTs used BXXD combined with conventional treatment (Domperidone, Mosapride, etc.) compared with conventional treatment were included as well. The study evaluated DGP patients in spite of gender, age, or nationality, but those who had other gastrointestinal diseases were excluded. The main outcome index was clinical effective rate, which was based on the gastric emptying test and gastrointestinal (GI) symptoms variation. The secondary outcome index was FPG. Adverse events would also be measured. Duplicated publications reporting the same groups of participants were excluded.

2.3. Data Extraction and Quality Assessment. Data extraction were independently proceeded by two authors (J. X. Tian and M. Li). The extracted data on study included the title of study, authors, year of publication, sample size, gender and age of the participants, name and component of Chinese herbs, details of the control interventions, treatment process, outcomes, adverse effects, and the details of methodological information. Discrepancies were resolved by consensus through discussion between the two authors and, if needed, by asking for the further evaluation of the third party (X. L. Tong). The methodological quality of trials was assessed independently by two authors (J. Q. Liao and J. L. Li) using criteria from the Cochrane Handbook for Systematic Review of Interventions [35]. The items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnels (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias), and other biases. We judged each item from three levels ("Yes" for a low risk of bias, "No" for a high risk of bias, and "Unclear" otherwise). Then we assessed the trials and categorized them into three levels: low risk of bias (all the items were in low risk of bias), and high risk of bias (at least one item was in high risk of bias), unclear risk of bias (at least one item was in unclear).

2.4. Statistical Analysis. RevMan 5.1 software was used for data analyses, which was offered by Cochrane collaboration. Dichotomous data were expressed as relative risk (RR) and continuous outcomes as weighted mean difference (WMD), both with 95% confidence intervals (CI). Heterogeneity was assessed using the I^2 test with the significance level set at I^2 over 50% or $P < 0.1$. If there was no heterogeneity ($I^2 < 50\%$), we selected the fixed effect model; otherwise we used random effects model in explaining the possible causes of heterogeneity ($I^2 > 50\%$). Publication bias would be explored by funnel plot analysis if sufficient studies were found [25].

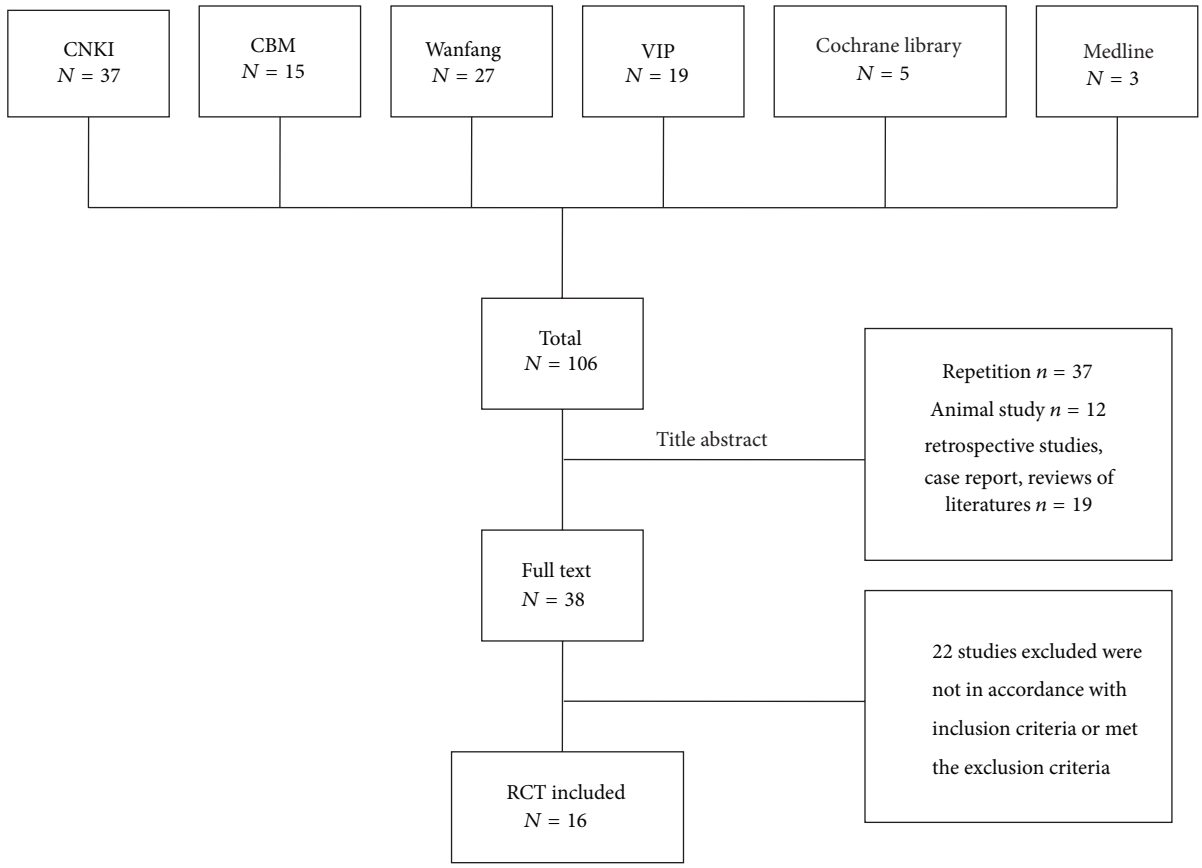

Figure 1: Flow chart of trials selection process.

3. Results

3.1. Description of Included Trials. A total of 106 studies were initially identified, all of them came from electronic database. The search results were summarized in Figure 1. After screening the titles and abstracts, 38 potentially relevant studies were found. Most of them were excluded due to repetitions, retrospective studies, animal study, case report, and reviews of the literature; 37 studies were excluded because of duplicated publication, 12 studies were excluded due to the animal studies, and the rest 19 studies were noncontrolled clinical trials including retrospective studies, case report, reviews of the literature. After a detailed evaluation of full text, 22 studies were excluded, 3 trials claimed that they were RCTs, while they actually were cohort studies using healthy people without intervention as control group, 8 trials were excluded because they only reported the difference after treatment, 6 trials were not evaluated because they included gastric emptying test or gastrointestinal (GI) symptoms, and the intervention of the rest 5 trials was not in accordance with inclusion criteria. Finally, 16 studies, involving 1302 patients, were in accordance with our inclusion criteria and did not met the exclusion criteria. All studies were conducted in China and published in Chinese between 2003 and 2012. The bibliographic details of included studies were given in Table 1.

Among the 16 studies, all participants came from inpatient and/or outpatient department of gastroenterology or endocrinology, and the experimental interventions were oral administration and included 666 males and 636 females. The age of participants ranged from 30 to 80. The diagnosis criteria of research included the following. Eight trails [39, 41, 44, 46–50] mentioned WHO DM diagnosis criteria, acquired certain duration of gastrointestinal discomfort such as postprandial fullness, nausea, vomiting, bloating, and gastrointestinal emptying, and delayed and excluded other gastrointestinal diseases. Four trails [36, 37, 40, 43] mentioned DM diagnosed and certain duration of gastrointestinal discomfort such as postprandial fullness, nausea, vomiting, bloating, and gastrointestinal emptying and delayed and excluded other gastrointestinal diseases. One trail [51] mentioned matching internal diseases diagnose criteria [52], and the rest 3 trials only demonstrated patients with essential DGP.

In the treatment group, there were 13 trails that used herbals alone and 3 trails that used herbals plus conventional western drugs as treatment. Despite the combination of herbals and western medicine in the treatment group, 4 studies used concentrated BXXD and 12 studies used modified BXXD in treatment group. In the control group, all studies used prokinetic medicine alone, 2 of them used Cisapride, and 1 used Mosapride and the others used Domperidone. The period of intervention ranged from 2 weeks to 9 weeks. Three

Table 1: Characteristics of included RCTs.

Trials	Sample size	Gender	Age (yr)	Intervention		Period	Outcome measure	Balance report of baseline
				Experimental group	Control group			
Fu (2006) [36]	83 (44/39)	(19 M : 25 F)/ (17 M : 22 F)	45–65/ 43–66	Modified Banxiaxiexin decoction (300 mL/d)	Domperidone (10 mg, tid)	9 W	Clinical effective rate Gastric emptying test	Not mentioned
Gao (2011) [37]	67 (35/32)	(21 M : 14 F)/ (17 M : 15 F)	34–72 (54)/ 37–71 (56)	Modified Banxiaxiexin decoction, bid	Domperidone (10 mg, tid)	8 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Li (2004) [38]	85 (45/40)	(28 M : 17 F)/ (28 M : 12 F)	50–72	Modified Banxiaxiexin decoction (400 mL/d)	Domperidone (10 mg, tid)	4 W	Clinical effective rate GI symptoms	Not mentioned
Liu et al. (2008) [39]	76 (38/38)	(36 M : 40 F)	58 ± 12	Concentrated Banxiaxiexin decoction, bid	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test	Not mentioned
Liu (2012) [40]	120 (60/60)	(58 M : 62 F)	43.52 ± 25.48	Concentrated Banxiaxiexin decoction, bid plus Domperidone (10 mg, tid)	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Luo et al. (2008) [41]	92 (46/46)	(25 M : 21 F)/ (24 M : 22 F)	54.3 ± 8.3/ 54.8 ± 8.6	Modified Banxiaxiexin decoction, bid plus Mosapride (5 mg tid)	Mosapride (5 mg tid)	4 W	Clinical effective rate Gastric emptying test GI symptoms Adverse events	$P > 0.05$
Qiu et al. (2004) [42]	65 (35/30)	(19 M : 16 F)/ (17 M : 13 F)	43–75/ 42–73	Modified Banxiaxiexin decoction, bid plus Cisapride (5 mg, tid)	Cisapride (5 mg, tid)	4 W	Clinical effective rate Gastric emptying test	No significant differences
Sun (2009) [43]	96 (51/45)	(24 M : 27 F)/ (18 M : 27 F)	38–69/ 35–72	Concentrated Banxiaxiexin decoction, bid	Domperidone (20 mg, tid)	2 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$
Wang (2011) [44]	100 (50/50)	(24 M : 26 F)/ (25 M : 25 F)	39–68/ 38–70	Modified Banxiaxiexin decoction (300 mL/d)	Domperidone (10 mg, tid)	2 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$
Wang (2011) [45]	56 (30/26)	(18 M : 12 F)/ (15 M : 11 F)	54.5 ± 9.6/ 53.8 ± 10.2	Modified Banxiaxiexin decoction (300 mL/d)	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test GI symptoms	$P > 0.05$
Wang (2010) [46]	96 (48/48)	(22 M : 26 F)/ (24 M : 24 F)	55 ± 5/ 53 ± 6	Modified Banxiaxiexin decoction (300 mL/d)	Domperidone (10 mg, tid) plus Roxithromycin (150 mg bid)	4 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Yin (2012) [47]	110 (57/53)	(35 M : 22 F)/ (30 M : 23 F)	58 ± 10.7/ 59 ± 11.5	Concentrated Banxiaxiexin decoction, bid	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Zhou (2003) [48]	72 (48/24)	(20 M : 28 F)/ (11 M : 13 F)	38–64 (56.2)/ 36–63 (56.1)	Modified Banxiaxiexin decoction, bid	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Zhou (2005) [49]	86 (43/43)	(20 M : 23 F)/ (19 M : 24 F)	53.01 ± 17.1/ 51.13 ± 18.1	Modified Banxiaxiexin decoction, bid	Cisapride (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test, FBG	$P > 0.05$
Zhu and Ji (2009) [50]	50 (26/24)	(14 M : 12 F)/ (11 M : 13 F)	38–64 (56.2)/ 36–63 (56.1)	Modified Banxiaxiexin decoction, bid	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test	$P > 0.05$
Zou (2009) [51]	48 (24/24)	(14 M : 10 F)/ (13 M : 11 F)	54 ± 10.57/ 55.1 ± 10.37	Modified Banxiaxiexin decoction (200 mL/d)	Domperidone (10 mg, tid)	4 W	Clinical effective rate Gastric emptying test, FBG	$P > 0.05$

Table 2: Quality assessment of included RCTs.

Trials	Randomization	Allocation concealment	Blinding of participants personnel and outcome assessors	Incomplete outcome data	Selective reporting	Other sources of bias	Risk of bias
Fu (2006) [36]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Gao (2011) [37]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Li (2004) [38]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Liu et al. (2008) [39]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Liu (2012) [40]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Luo et al. (2008) [41]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Qiu et al. (2004) [42]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Sun (2009) [43]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Wang (2011) [44]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Wang (2011) [45]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Wang (2010) [46]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Yin (2012) [47]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Zhou (2003) [48]	Table of random	Unclear	Unclear	Yes	No	Unclear	Unclear
Zhou (2005) [49]	Unclear	Unclear	Unclear	Yes	No	Unclear	High
Zhu and Ji (2009) [50]	Table of random number	Unclear	Unclear	Yes	No	Unclear	Unclear
Zou (2009) [51]	Table of random number	Unclear	Unclear	Yes	No	Unclear	Unclear

classes were used to evaluate treatment efficacy including significant effective, effective, and ineffective; all trials used clinical effective rate (including significant effective and effective) based on the gastric emptying test and gastrointestinal (GI) symptoms variation to evaluate efficacy, which was considered as the main outcome index. Two studies [49, 51] recorded FPG variation; we regarded it as secondary outcome index in this systematic review. Adverse events would also be measured.

3.2. Methodological Quality of Included Trials. The quality assessments were summarized in Table 2. The sample size of included trials varied from 40 to 120 patients; none of the 16 studies reported details for sample size calculations and none was double-blind, placebo controlled study. Three studies described adequate methods of randomization using random number tables [48, 50, 51], the rest 13 studies reporting "randomly allocating" participants as the method of randomization were not described. No trials had clear descriptions of their method of allocation concealment and blinding procedures. All of 16 trials provided patient characteristics and described similarity between comparison groups in baseline, but no trails reported participant losses, which was hard to determine whether these studies had attrition bias. Only 1 trial reported adverse events and 4 trials [40, 48–50] reported followup. The methodological quality of included studies was assessed to be of generally low according to the predefined quality assessment criteria, which indicated that further investigations might influence the confident intervals of this meta-analysis and the result would likely be reversed.

3.3. Effect of the Interventions

3.3.1. Clinical Effective Rate. All included studies compared the clinical effective rate between treatment group and control group after intervention, which was based on the variation of gastric emptying test and gastrointestinal (GI) symptoms. Three classes were used to evaluate treatment effects as significant effective, effective, and ineffective. Different studies had similar evaluation standards, and we pooled varies kinds of measurements together to evaluate the general effective rate. Total effective rate was the combination of significant effective and effective rates, which was considered as the main outcome index. Included trials showed homogeneity in the consistency of the trial results ($\chi^2 = 9.64$, $P = 0.84$, $I^2 = 0\%$). Thus, fixed effects model should be used for statistical analysis. The treatment group scored significantly higher than the control group ($n = 1302$, RR 1.23, 95% CI 1.17 to 1.29, $Z = 8.04$, $P < 0.00001$).

To compare the efficacy of the BXXD with the control group, subgroup analysis had been introduced. Four trails used concentrated BXXD in the treatment group, while 12 trails used modified BXXD. All of the subgroups had shown that treatment group was more effective than control group ($n = 330$, RR 1.35, 95% CI 1.21 to 1.50, $Z = 5.50$, $P < 0.00001$) and ($n = 972$, RR 1.19, 95% CI 1.13 to 1.26, $Z = 6.03$, $P < 0.00001$) (Figure 2). Thirteen studies used herbals alone as treatment group, while 3 studies used herbals plus conventional western drug as treatment. All of the subgroups had shown that treatment group was more effective than control group ($n = 1025$, RR 1.23, 95% CI 1.16 to 1.30, $Z = 7.14$, $P < 0.00001$) and ($n = 277$, RR 1.23, 95% CI 1.10 to 1.38, $Z = 3.69$, $P < 0.00001$) (Figure 3).

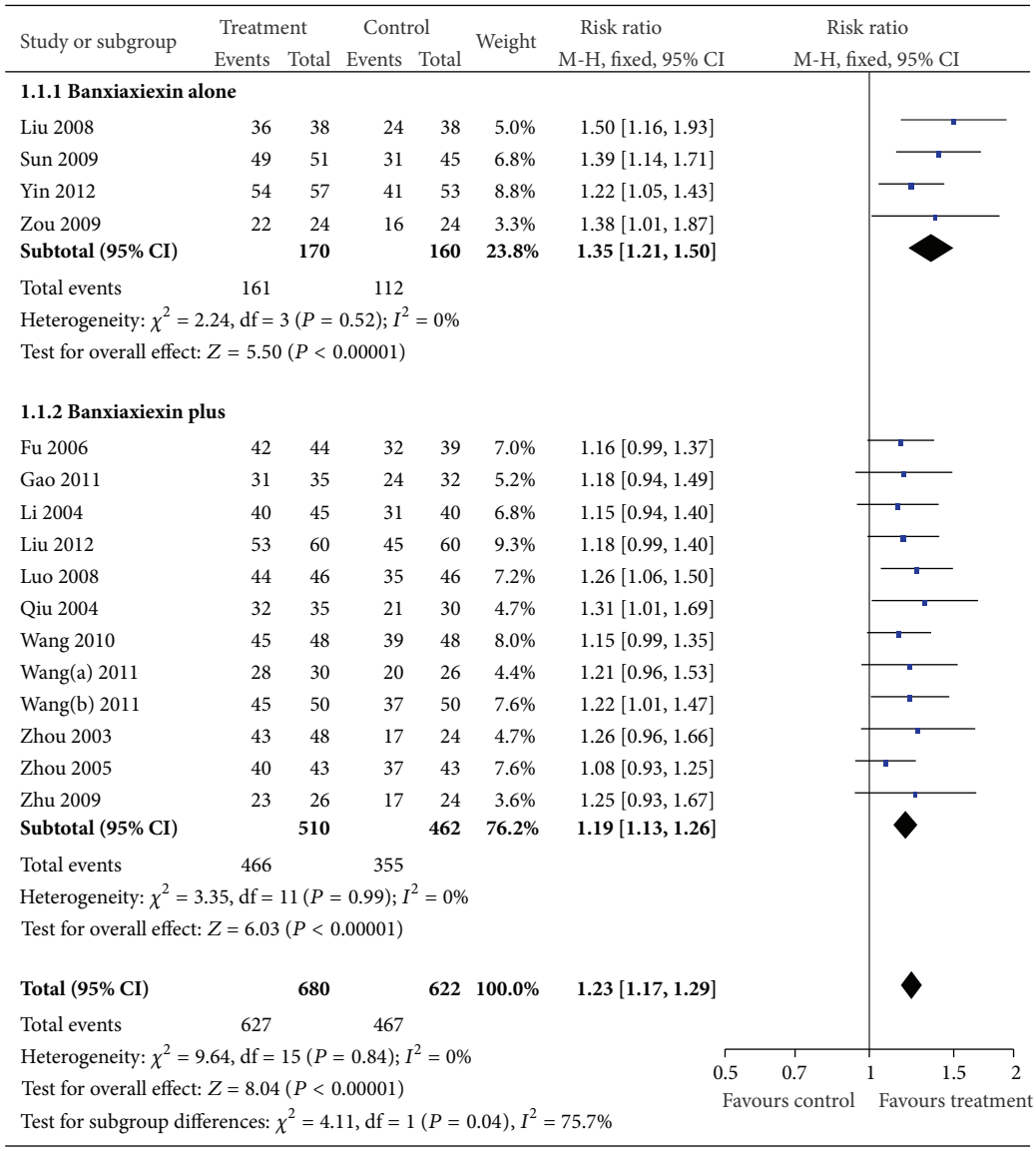

Figure 2: Effective rate comparison between concentrated BXXD and modified BXXD in treatment group.

3.3.2. Blood Glucose. Two trials provided data for FBG variation [49, 51], and they did not show homogeneity (χ^2 = 16.24, P < 0.0001, I^2 = 94%). Thus, random effects model should be used for statistical analysis. The meta-analysis of 2 trials showed that there were no significant differences on decreasing FPG between the treatment group and the control group (n = 67, MD −1.40, 95% CI from −3.85 to 1.05, Z = 1.12, P = 0.26) (Figure 4).

3.4. Publication Bias. Funnel plots based on the data of effective rate were elaborated in Figure 5. The figure was asymmetrical, which indicated that potential publication bias might influence the results of this paper. Although we conducted comprehensive searches and tried to avoid bias, since all trials were published in Chinese, we could not exclude potential publication bias.

3.5. Adverse Events. Only 1 trail [41] listed safety reports, but no adverse event had been observed in both groups.

3.6. Followup. 2 trails [48, 50] included followup. Zhou [48] reported 3 recurrences out of 26 patients (11.5%) in treatment group, while 2 out of 6 patients (33.3%) were reported in control group 6 months after intervention stopped. Zhu and Ji [50] reported 3 recurrences out of 14 patients (21.4%) in treatment group, while 2 out of 6 patients (33.3%) were reported in control group 6 months after intervention stopped.

4. Discussion

The life quality of those who had diabetic gastroparesis symptoms was severely interfered [9]. Most patients improved glycemic control and symptoms by conventional treatment of western medicine [16, 17]. However, these managements

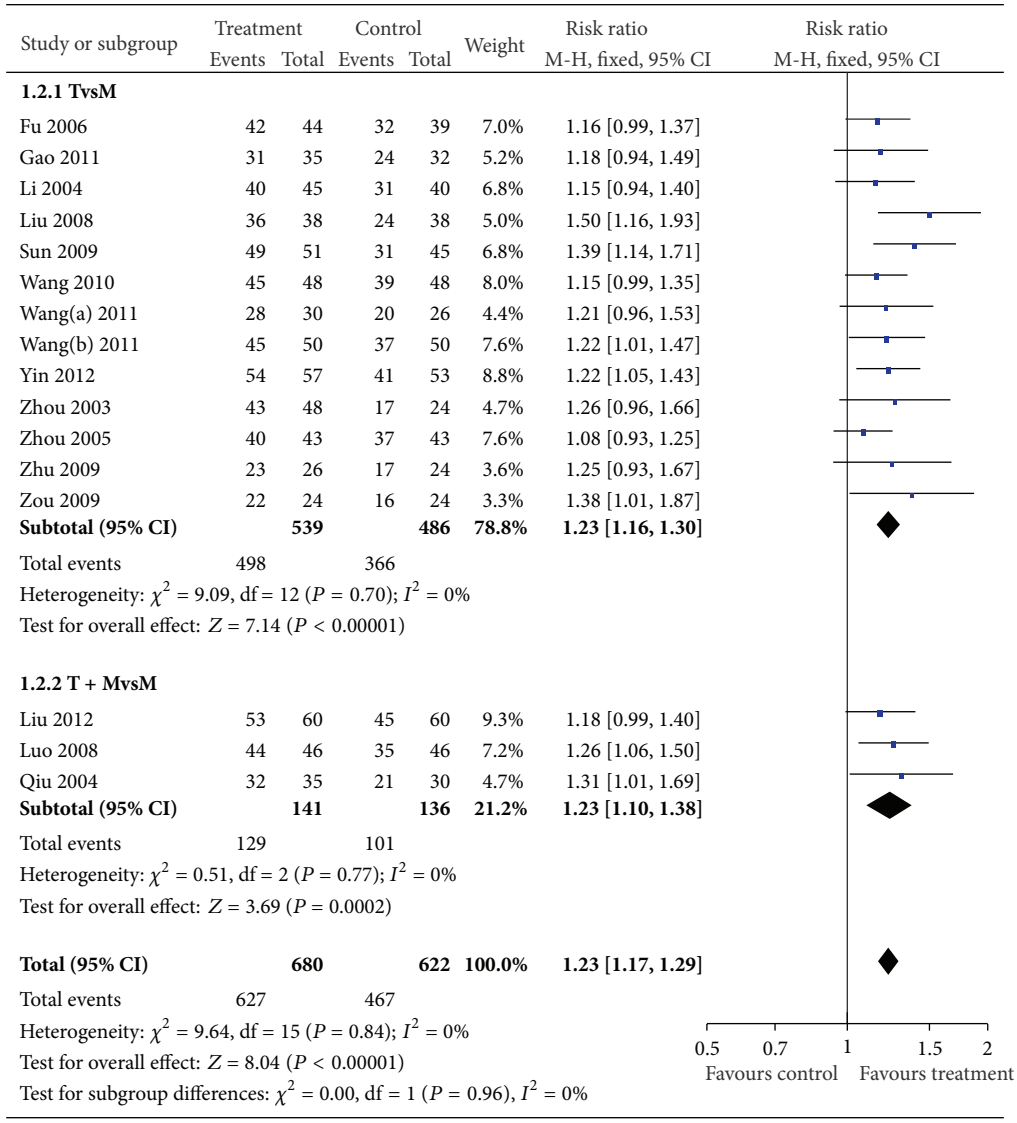

Figure 3: Effective rate comparison between herbals alone and herbals plus conventional western drugs as treatment.

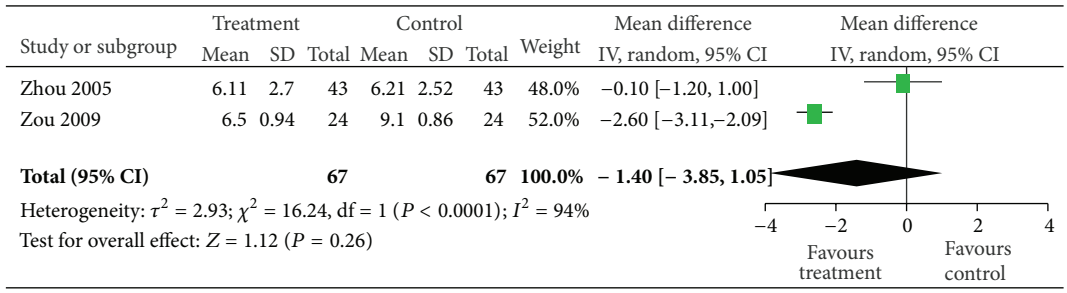

Figure 4: FBG comparison between treatment group and control group.

are far from clinical satisfaction [18]. Therefore, it is very important to seek for more safe and effective prevention and treatment. There are researches and clinical trials about TCM treating DGP, including herbs and acupuncture [44, 53, 54]. As BXXD is widely used to treat gastrointestinal discomfort in clinical practice for a long time in China [24–26], we conduct a systematic review to assess the current clinical evidence of BXXD for DGP.

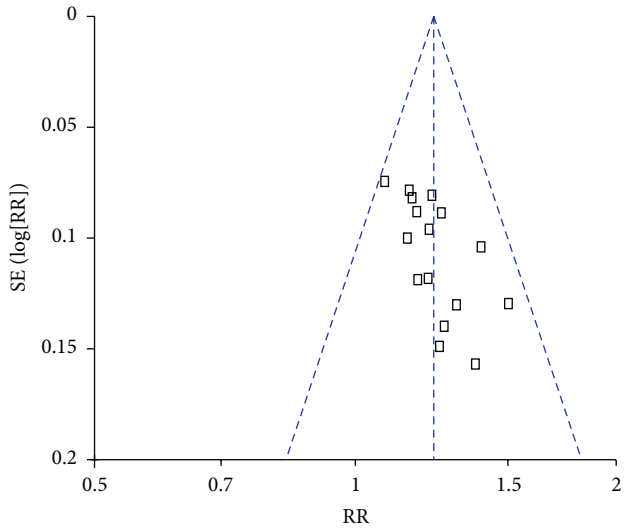

Figure 5: Funnel plot of publication bias.

This research is the first systematic review about Chinese herbal medicine treating DGP. In this systematic review, 16 studies involving 1302 participants were included. The review applied clinical effective rate based on the gastric emptying test and gastrointestinal (GI) symptoms variation as the main outcome indexes. The data showed that the effectiveness of BXXD for DGP was superior to the control group. This result is encouraging which indicates new optional treatment for DGP, but the methodological quality of the trials was evaluated generally as low, and the conclusion needs to be confirmed by further study.

The limitations of this review include the following aspects. Though the included researches had detailed including criteria, the participants had years of DM and certain period of gastrointestinal discomfort such as postprandial fullness, nausea, vomiting, bloating, and gastrointestinal emptying delayed; what is worth attention is that there are different appearances in different degrees of DGP. Those who are severely suffering from gastrointestinal symptoms might have serious nausea and vomiting, difficulty swallowing and even could not proceed the gastric emptying test or regular drug taking. Gastric emptying test is a gold standard of DGP diagnosis nowadays, which makes researches of DGP would have limitation in participants. We suggest that it is necessary to accumulate effective treatment for those who are too severe to proceed the gastric emptying test and use vomiting times and duration as auxiliary indicators. It could provide more strong evidence for more widely clinical application.

In this research, the efficacy indicator was effective rate based on the gastric emptying test and syndrome variation. About the gastric emptying test, 5 studies used runtime for determination of efficacy [40, 46, 48–50], while 7 studies used variation level of gastric emptying; the exact runtime was absent [36, 39, 42–44, 47, 51]. One study [45] used gastric emptying residues, and 2 studies [36, 40] used improvement percentage for determination of efficacy. The efficacy determination of DGP is different from blood pressure, lipids, and blood sugar which have explicit numerical index, which makes efficacy determination of gastrointestinal lesions complicated. On the other hand, gastrointestinal discomfort is the most important clinical characteristic. Though the gastric emptying test is very important in efficacy determination as an objective indicator, the variation of gastrointestinal syndromes is also irreplaceable. It corresponds to the fact that judgment of treatment should not be made by only some objective indicators; syndrome improvement is also important to patients. The efficacy determination in the researches included contained evaluation of syndrome variation. But most included trails simply described the syndrome variation. One study [45] used Semiquantitative questionnaire, and the rest 15 studies were lacking unified syndrome questionnaire to evaluate the syndrome variation. Gastroparesis Cardinal Symptom Index (GCSI) is widely used to evaluate the gastrointestinal lesions [55], but none of the included researches used this questionnaire. Thus, it is urgent to standardize the evaluation of gastrointestinal lesions. It could also improve the consistency in future researches.

In those included researches, 2 studies [49, 51] recorded FPG variation, and the meta-analysis showed that there were no significant differences on decreasing FPG between the treatment group and the control group. The rest 14 studies did not mention the variation of blood sugar. We were unable to evaluate potential influences of the blood sugar variation in our analyses. BXXD is used to improve the gastrointestinal dysfunction and restore the normal gastrointestinal peristalsis [28, 29], and research works have proven the mechanisms [30–33]. But few reported the hypoglycemic effect. Though researches have proven the hypoglycemic effect of Coptis Chinensis and Radix Scutellariae, which are contained in BXXD [56–59], whether the whole formula could affect blood sugar and whether the effectiveness in improving gastrointestinal function is related to blood sugar improvement are not clear.

The decocting of BXXD in all included studies was unified twice per day, but the variation among trials was apparent in terms of dosage, treatment course, and sample size. Three studies used combination herbals with prokinetic medicine and, others used herbals alone as treatment. While 4 studies used concentrated Banxiaxiexin decoction the rest used modified. The period of intervention ranged from 2 weeks to 9 weeks. None of them reported sample size calculations, and the efficacy could not be clarified on some outcome measurements due to the small number of studies, thus the reliability of the outcome might be questionable.

All trials included were lacking description of randomization method; only 3 pieces of research mentioned random form [48, 50, 51], and the other 13 trials just mentioned "randomly allocating" with no detailed information. It is difficult to identify whether those researches proceed randomization adequately. No researches mentioned allocation concealment. Therefore, it may introduce some false "RCTs" in the review and may mislead the results. We have tried to contact the authors for further information about the trials, but regrettably no information provided until now.

No research mentioned blind method which could lead to performance bias and detection bias that patients and

researchers were aware of the therapeutic interventions for the subjective outcome measures. The operability of blind method was very low because of using Chinese herbal and western medicine as treatment and control. Only 1 research [41] mentioned safety and described no adverse event after intervention. Even no reports of adverse event and safety should be concerned and recorded in detail. No trails reported participant losses or used intention to treat method, which was hard to determine whether these studies had attrition bias. Only 2 researches [48, 50] mentioned followup. Diabetes gastrointestinal disease is easy to recur, thus it is necessary to proceed a long term followup in research. We tried to avoid language bias and location bias, but all the included researches were published in China; the result was limited in worldwide application. The quality of the methodology was low; future researches should enhance the randomization, safety report, detailed followup, and blind method to improve the quality. In order to explore the efficacy and safety of BXXD treating diabetes gastrointestinal disease, it is urge to proceed more well-designed, complete efficacy indicator, larger scaled, and multiple center randomized clinical trials.

5. Conclusion

From this systematic review, we find that BXXD could regain the gastric emptying rate and improve diabetic gastrointestinal symptoms; thus it could be considered as an alternative way to treat DGP. But the efficacy determination system of TCM treating DGP is not established, also the long term efficacy and safety of BXXD treating DGP are still uncertain, the methodological quality is assessed to be of general low and some posible biases exist. The previous results should be read with caution. Thereby, the efficacy determination system of TCM treating DGP should be established soon, and well-designed, large-scale, high-quality randomized controlled clinical trials with scientific rigor are warranted for stronger evidence in the future, while the followup and adverse events should also be clarified in detail. Accumulating clinical evidence of severe gastroparesis is very necessary.

Conflict of Interests

No conflict of financial interests existed.

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Research Article

Aqueous Extract of *Solanum nigrum* Leaves Induces Autophagy and Enhances Cytotoxicity of Cisplatin, Doxorubicin, Docetaxel, and 5-Fluorouracil in Human Colorectal Carcinoma Cells

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Colorectal cancer is a common cancer worldwide, and chemotherapy is a mainstream approach for advanced and recurrent cases. Development of effective complementary drugs could help improve tumor suppression efficiency and control adverse effects from chemotherapy. The aqueous extract of *Solanum nigrum* leaves (AE-SN) is an essential component in many traditional Chinese medicine formulas for treating cancer, but there is a lack of evidence verifying its tumor suppression efficacy in colorectal cancer. The purpose of this study is to evaluate the tumor suppression efficacy of AE-SN using DLD-1 and HT-29 human colorectal carcinoma cells and examine the combined drug effect when combined with the chemotherapeutic drugs cisplatin, doxorubicin, docetaxel, and 5-fluorouracil. The results indicated that AE-SN induced autophagy via microtubule-associated protein 1 light chain 3 A/B II accumulation but not caspase-3-dependent apoptosis in both cell lines. The IC_{50}s after 48 hours of treatment were 0.541 and 0.948 mg/ml AE-SN in DLD-1 and HT-29, respectively. AE-SN also demonstrated a combined drug effect with all tested drugs by enhancing cytotoxicity in tumor cells. Our results suggest that AE-SN has potential in the development of complementary chemotherapy for colorectal cancer.

1. Introduction

Colorectal cancer is one of the most common types of cancer worldwide with particularly high incidences in developed countries [1]. In Taiwan, colorectal cancer is already the most common type of cancer and the third most common cause of cancer deaths [2]. Currently, surgery is still the only curative treatment for colorectal cancer. Although 75–80% of newly diagnosed cases are localized or regional tumors, around 50% of patients suffer recurrence after surgery [3, 4]. adjuvant therapy such as postoperative chemotherapy is used to eliminate remaining lesions and help control the risk of recurrence. Chemotherapy is also one of the main treatment approaches in advanced and recurrent cases. However, chemotherapy is often associated with adverse side effects in patients, particularly in the elderly population. Various drug

resistance problems in colorectal cancer cases also reduce the response rates. These clinical features limit the performance of chemotherapy in patients. Hence, in order to reduce systematic side effects and improve the tumor suppression capability of chemotherapy, the use of complementary and alternative medicine has become increasingly popular during the last few decades, particularly in Western countries [5–7]. Any effective drug which promotes the tumor suppression efficacy of chemotherapeutic regimens or eases the associated adverse effects may serve as an appropriate candidate to establish an integrated chemotherapy and improve clinical outcomes in cancer patients.

One approach in developing integrated chemotherapy is to choose a drug which enhances tumor cell suppression efficiency by increasing cytotoxicity using a different cell death mechanism from other drugs used in the regimen. In general, the tumor suppression mechanisms of current chemotherapeutic drugs are mainly based on disruption of cell cycle processes, resulting in cell apoptosis. For example, well-studied chemotherapeutic drugs such as the alkylating agents, cisplatin, and carboplatin inhibit DNA synthesis in tumor cell growth by forming DNA adducts [8, 9], whereas plant alkaloids, such as the taxanes, block tumor cell mitosis by stabilizing tubulin [10, 11]. Other common drugs such as doxorubicin function as topoisomerase II inhibitors and interfere with DNA/RNA synthesis in tumor cells [12, 13]. The currently recommended chemotherapeutic drug for colorectal cancer is 5-fluorouracil (5-Fu) [3, 14–16], which activates apoptosis by incorporation of DNA/RNA on cancer cells [17]. Treatments using these common chemotherapeutic drugs normally lead to cell cycle arrest and activate the apoptotic process in tumor cells. Combining standard chemotherapeutics with antitumor drugs to induce tumor cell death via other molecular pathways would not only improve tumor suppression efficiency but also reduce the doses of chemotherapeutic drugs, which could help control adverse effects and may slow the development of drug resistance.

Traditional Chinese medicine (TCM) is based on the use of natural products and well-established theoretical approaches. TCM provides many potential candidates as effective drugs for integrated cancer chemotherapy, such as TJ-41 (Bu-Zhong-Yi-Qi-Tang) and PHY906 (Huang-Qin-Tang) [18–20]. In TCM practice, a therapeutic formula is normally prepared as an aqueous extract mixed with various medical herbs. One major herb in this formula is responsible for relieving the target symptom, whereas other medicinal herbs are added to enhance the therapeutic effects or reduce the side effects of the major herb. *Solanum nigrum* (SN) is frequently used as an elemental ingredient for clinical TCM cancer therapy [8]. Recently, many *in vitro* studies have demonstrated the antitumor effects of SN extracts on various cancer types, including leukemia and prostate, liver, breast, lung, stomach, colon, bladder, and endometrial cancers [8, 21–24]. In these studies, the SN-related antitumor effect was thought to occur via activation of apoptosis and autophagy in human cancer cells, particularly when using the aqueous extract of SN (AE-SN) [22, 25]. However, most studies on the treatment effect on colon cancer have mainly assessed the tumor suppression capability of pure compounds such as solamargines and degalactotigonin [21], rather than evaluating the antitumor efficiency and mechanism of AE-SN. The tumor suppression efficacy of AE-SN in colon cancer cells therefore remains unclear. We previously observed that AE-SN has a combined drug effect with the standard chemotherapeutic drug docetaxel in human endometrial cancer cells [24]. This observation suggests that AE-SN may also enhance cytotoxicity of chemotherapeutic drugs in human colorectal cancer cells.

The aim of the present study is to evaluate the tumor suppression efficacy of AE-SN alone and combined drug effects of AE-SN with the common chemotherapeutic drugs cisplatin, doxorubicin, and 5-Fu docetaxel on human colorectal cells. This information could be helpful in improving the tumor suppression efficiency of chemotherapy for colorectal cancer.

2. Materials and Methods

2.1. Plant Materials and Preparation of AE-SN. In TCM practice, the drug form of SN is generally prepared as the aqueous extract of SN leaves (AE-SN). The preparation of AE-SN for the present study was therefore based on the TCM processing method. Briefly, 50 g of the dried leaf part of *Solanum nigrum* was immersed in 750 mL distilled water. This raw solution was gradually heated to 100°C within 50 min and maintained at 100°C for one hour. This AE-SN solution was further concentrated to 1 g/mL.

2.2. Cell Culture. The human colorectal carcinoma cell lines, HT-29 and DLD-1, were a gift from Dr Pei-Yi Tsai (Department of Animal Pharmacology, Development Center for Biotechnology, Taipei, Taiwan) and purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan), respectively. Both HT-29 and DLD-1 cells contain a p53 mutation. Cells were cultured in Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium (Gibco, Grand Island, NY, USA) with 100 U/mL of penicillin and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, USA) at 37°C in a 5% CO_2 humidified incubator.

2.3. Cytotoxicity Assay and Microscopic Observation. HT-29 cells or DLD-1 cells were seeded into 96-well microplates at a density of 5×10^3 cells per well overnight and then treated with 0, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 mg/mL AE-SN for 24 or 48 hr. In order to clarify the autophagic cell death on AE-SN treated cells, three autophagy inhibitors, 3-methyladenine (3-MA), bafilomycin A, or pepstatin A/E64d (Sigma-Aldrich, St Louis, MO, USA), were treated with AE-SN on HT-29 and DLD-1 cells. The cytotoxicity of AE-SN on tumor cells was then determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A trypan blue exclusion test was also performed to confirm the cell viability determined by MTT assay. HT-29 cells or DLD-1 cells were seeded into 24-well plates at a density of 3×10^4 cells per well overnight and then treated with 0, 0.2,

0.5, or 1 mg/mL AE-SN. After 48 hr incubation, cells were harvested by trypsinization and centrifuged at 100 ×g for 5 min to collect the cell pellet which was then resuspended in prewarmed phosphate-buffered saline with trypan blue (Sigma-Aldrich, St Louis, MO, USA) at a 1 : 1 ratio for 3 min at room temperature. The number of live cells without trypan blue staining was counted by two independent observers using a hemocytometer under a microscope. In the study of AE-SN combined with cisplatin, doxorubicin, and docetaxel, cells were treated with a series of cisplatin, doxorubicin, or docetaxel with 0, 0.5, or 1 mg/mL AE-SN for 48 hr. Cisplatin, doxorubicin and 5-fluorouracil (5-Fu) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and docetaxel (Tyxan) from TTY Biopharm (Taipei, Taiwan). Cell morphology was observed with a Nikon Eclipse TS100 optical microscope (Nikon Instruments, Melville, NY, USA) and photographed at 100x magnification. Cytotoxicity was also determined by MTT assay.

2.4. Western Blotting Analysis of Cell Death Markers. HT-29 and DLD-1 cells (5×10^5 cells per dish) were seeded in 6 cm dishes overnight and incubated with 0 or 1 mg/mL AE-SN alone, or in combination with cisplatin, doxorubicin, or docetaxel for 48 hr. Cells were harvested by RIPA buffer (150 mM NaCl, 50 mM pH 7.5 Tris-HCL, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/mL leupeptin and 100 μg/mL aprotinin). The total protein concentration of the cell extracts was determined by a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Each cell extract was then equalized to 30 μg and separated using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred onto a polyvinylidene fluoride membrane (Pall Corp, Port Washington, NY, USA) and probed with the primary antibodies, caspase-3 (1 : 1,000), mammalian microtubule-associated protein 1 light chain 3 A/B (LC3A/B, 1 : 1,000), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1 : 10,000), followed by donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (1 : 10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). This anti-LC3 A/B identifies the LC3 A/B I and II forms with molecular masses of 16 and 14 kDa, respectively. The accumulation of LC3 A/B II forms is considered a protein marker for activation of autophagy [26], whereas the anticaspase-3 recognizes procasapse-3 3 with a mass of 35 kDa and cleaved caspase-3 with masses of 19 and 17 kDa. Anti-caspase-3 and LC3 A/B were purchased from Cell Signaling Technology (Danvers, MA, USA), and anti-GAPDH was purchased from Abfroniter (Seoul, Republic of Korea). Immunoreactivity was then detected with a Western-Bright electrochemiluminescence Western blotting detection kit (Advabsta, Menlo Park, CA, USA). Semiquantitative analysis of the intensity of the immunoreactive bands was performed by ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5. Statistical Analysis. Data from cell viability and semiquantitative Western blotting analysis were presented as mean ± stand and derivation (SD). Statistical significance was analyzed by Student's t-test when comparing two different groups and one-way ANOVA when examining the dose-dependent effect. Statistical analysis was performed by SPSS (SPSS Inc, Chicago, IL, USA).

CalcuSyn software (Biosoft, Cambridge, UK) was used for the statistical analysis of the half maximal inhibitory concentration (IC_{50}) of AE-SN determined by MTT assay and for the combined effects of AE-SN with chemotherapeutic drugs. Statistical analysis of the combined drug effects with the CalcuSyn software is based on the median-effect method and evaluated by the combination index (CI) value; a synergistic effect of two drugs was determined when the calculated CI value was less than 1 [27].

3. Results

3.1. AE-SN Cytoxicity and Induction of Autophagy in HT-29 and DLD-1 Human Colorectal Carcinoma Cells. As shown in Figure 1, AE-SN induced cytotoxicity in both DLD-1 and HT-29 cells in a dose-dependent manner. According to the cytotoxicity results obtained from MTT assay, the IC_{50}s after 48 hr AE-SN treatment were 0.541 and 0.948 mg/mL AE-SN in DLD-1 and HT-29 cells, respectively (Table 1). In contrast, the trypan blue exclusive test indicated that the cell viability of DLD-1 under 0.5 mg/mL AE-SN treatment was 50.1%, whereas that of HT-29 under 1.0 mg/mL AE-SN treatment was 37.4%. These conflicting results indicated that the MTT assay may underestimate the real cytotoxicity of AE-SN because of disruption of mitochondrial metabolism. Under microscopic inspection, lipid-like droplets were observed in AE-SN-treated DLD-1 and HT-29 cells (Figures 2(a)–2(d)). This morphological feature suggested that AE-SN-treated cells were in the autophagic process which may be related to AE-SN-induced cytotoxicity. On the other hand, no apoptosis-related morphological changes such as cell shrinkage or chromatin condensation were observed in AE-SN-treated colorectal carcinoma cells. To further examine the cell death mechanisms involved in AE-SN-induced cytotoxicity, the activation of caspase-3, the protein marker for apoptosis, and the accumulation of LC3 A/B II, the protein marker for autophagy, were determined by western blotting analysis. Figures 2(e) and 2(f) demonstrate that accumulation of LC3 A/B II was significantly increased in AE-SN-treated colorectal carcinoma cells, whereas activation of caspase-3 was not observed. The accumulation of LC3 A/B II is a protein marker which identifies autophagy in cells, and Western blotting analysis indicated that AE-SN treatment increased LC3 A/B II accumulation 12.83- and 7.08- fold in DLD-1 and HT-29 cells, respectively (Figure 2(e)). These results suggested that AE-SN was effective in suppressing tumor cell growth in DLD-1 and HT-29 human colorectal carcinoma cells and induced accumulation of LC3 A/B II and the autophagic process, but not caspase-3-dependent apoptosis.

To clarify the role of AE-SN-activated autophagy on HT-29 and DLD-1 cells, AE-SN-treated HT-29 and DLD-1 cells were also cotreated with autophagic inhibitors: 3-MA, bafilomycin A, or pepstatin A/E64d. 3-MA is a class III

Table 1: IC_{50} of AE-SN alone and in combination with cisplatin, doxorubicin, docetaxel, or 5-Fu in DLD-1 and HT-29 cells.

		DLD-1						HT-29					
			AE-SN						AE-SN				
			0.2 mg/ml		0.5 mg/ml				0.5 mg/ml		1.0 mg/ml		
Regimens	Dose	IC_{50}[a]	IC_{50}[b]	CI	IC_{50}[b]	CI	Dose	IC_{50}[a]	IC_{50}[b]	CI	IC_{50}[b]	CI	
AE-SN (mg/mL)		0.541						0.948					
Cisplatin (μM)		35.565	14.682		0.354			19.032	2.533		0.545		
	0.5			0.207		4.582	2			0.604		0.611	
	1			0.205		4.108	5			0.689		0.414	
	5			0.248		3.511	10			0.669		0.288	
	10			0.129		1.207	20			0.561		0.34	
	50			0.063		0.426	50			0.986		0.643	
	100			0.053		0.281	100			1.844		1.267	
Doxorubicin (μM)		3.556	0.621		0.091			11.863	7.945		0.131		
	0.05			7.221		17.851	0.2			1.012		2.838	
	0.1			2.576		4.91	0.5			3.033		4.382	
	0.5			0.592		1.394	1			1.473		1.904	
	1			0.293		0.165	2			0.679		0.428	
	5			0.674		0.429	5			1.21		0.429	
	10			1.081		0.822	10			1.097		0.981	
Docetaxel (nM)		6.166	2.041		1.887			4.127	1.186		0.021		
	0.05			3.791		7.585	0.05			16.083		18.362	
	0.1			3.216		5.008	0.1			2.336		7.342	
	0.5			0.888		2.005	0.5			2.577		6.031	
	1			0.441		0.545	1			2.564		4.714	
	5			0.479		0.235	5			0.331		0.211	
	10			0.825		0.397	10			0.729		0.245	
5-Fu		NA[c]	NA[c]		6.131			NA[c]	18.51		2.747		
	1			1.229		1.409				1.464		2.747	
	5			1.446		1.265				1.202		0.537	
	10			0.083		0.429				0.479		0.316	
	50			1.139		0.948				0.84		1.018	
	100			1.987		1.869				1.85		2.376	

[a]Cells treated by AE-SN alone; [b]cells treated by a combination of AE-SN and the respective chemotherapeutic drug.
AE-SN: aqueous extract of *Solanum nigrum*; IC_{50}: half maximal inhibitory concentration.
CI < 1 = synergistic effect.
[c]NA: data not available.

phosphoinositide-3 kinase inhibitor to block LC3 A/B I to II conversion [28], bafilomycin A is a lysosomal inhibitor to prevent the fusion of lysosomes and autophagosomes [29], and pepstatin A/E64d is a cathepsins inhibitor to interfere with autolysosomal digestion [30]. As shown in Figures 3(a), slightly recovery of cell viability was observed on AE-SN and autophagic inhibitors cotreated HT-29 cells, whereas no difference was observed in DLD-1 cells. Cotreatment of AE-SN and 3-MA only reduced AE-SN-induced accumulation of LC-3 A/B II from 3.33- to 2.5-fold of control in HT-29 cells (Figure 3(b)). These results suggested that autophagy was partly involved in AE-SN-induced cell death in HT-29, but not DLD-1 cells.

3.2. Combined Drug Effects of AE-SN and Cisplatin, Doxorubicin, Docetaxel, and 5-Fu. Since AE-SN effectively induced caspase-3- independent cell death in DLD-1 and HT-29 cells, the present study further examined the potential of AE-SN in combination with chemotherapeutic drugs which are associated with apoptotic cell death. Tumor cells were treated by serial doses of four chemotherapeutic drugs, cisplatin, doxorubicin, docetaxel or 5-fu alone or in combination with AE-SN for 48 hr. According to previous results, HT-29 cells are less sensitive to AE-SN and therefore the selected AE-SN doses for combination treatment were 0.5 and 1.0 mg/mL, whereas doses of 0.2 and 0.5 mg/mL were chosen for DLD-1 cells. Cytotoxicity was determined by MTT assay and the combined drug effect was further analyzed. AE-SN presented an enhanced cytotoxicity in combination with all four chemotherapeutic drugs in both cell lines (Figure 4). Analysis showed the synergy of AE-SN combined with each chemotherapeutic drug (CI less than 1) (Table 1). The IC_{50} of each regimen also significantly decreased in

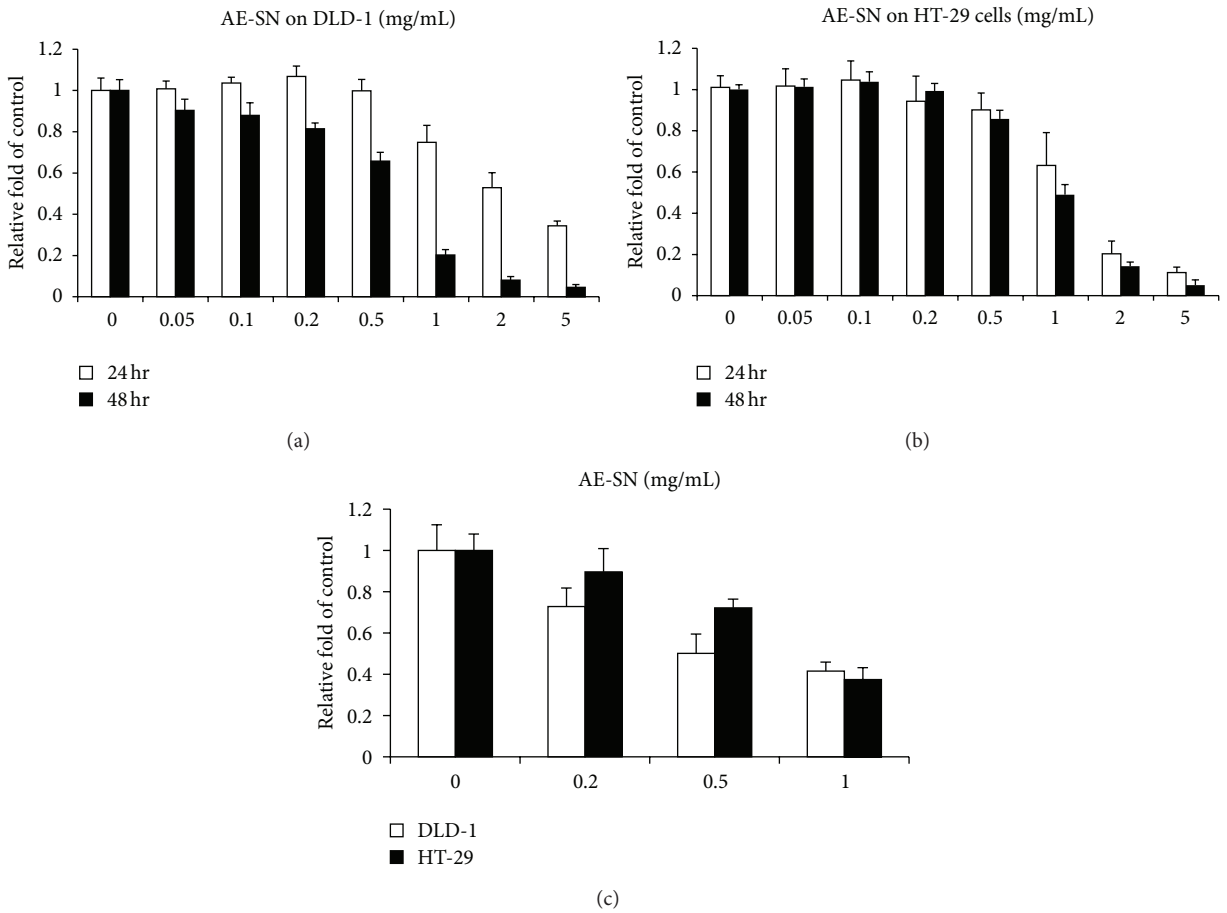


Figure 1: Cytotoxicity of AE-SN in DLD-1 and HT-29 human colorectal carcinoma cells (a); DLD-1 cells were treated with 0.05 to 5 mg/mL AE-SN for 24 or 48 hr; (b), HT-29 cells were treated with 0.05 to 5 mg/mL AE-SN for 24 hr; the cytotoxicity of (a) and (b) was analyzed by MTT assay; (c)DLD-1 or HT-29 cells were treated with 0.2 to 1 mg/mL AE-SN; and the cytotoxicity was analyzed by a trypan blue exclusion test. Experiments were performed in triplicate and data are shown as mean ± SD. Both cell lines showed dose-dependent effects with AE-SN treatment at 24 and 48 hr in the MTT assay and trypan blue exclusion test (one-way ANOVA, $P < 0.001$). AE-SN: aqueous extract of *Solanum nigrum*: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

AE-SN-combined treatment in comparison with each chemotherapeutic drug alone (Table 1). Together, these *in vitro* results suggest that AE-SN can potentially enhance the tumor suppression effect in human colorectal carcinoma cells when combined with cisplatin, doxorubicin, docetaxel and, 5-Fu.

3.3. Activation of LC3 A/B and Caspase-3 in AE-SN and Chemotherapeutic Drug-Treated Cancer Cells. The four tested chemotherapeutic drugs are well known to induce apoptosis via activation of caspase-3 in many tumor cells. This raised a possibility that AE-SN-enhanced cytotoxicity is due to a combined effect of AE-SN-induced caspase-3- independent cell death and chemotherapeutic drug-induced apoptotic cell death. In order to examine this hypothesis, DLD-1 cells or HT-29 cells were treated with 0 or 1 mg/mL AE-SN in combination with cisplatin (50 or 20 μM), doxorubicin (5 μM for both), docetaxel (1 nM for both), and 5-Fu (50 μM) for 48 hr and total protein extracts were harvested. The apoptotic protein marker, cleaved caspase-3, and autophagic protein marker, LC3 A/B II, were then determined in the total protein extracts of the cells. As shown in Figure 5(a), all tested drugs activated caspase-3 and produced cleaved caspase-3 in DLD-1, but not HT-29 cells, whereas AE-SN increased LC3 A/B II in both cell lines. Semiquantitative data further confirmed that AE-SN treatment induced LC3 A/B II accumulation in chemotherapeutic drug-treated cells (Figure 5(b)). In cisplatin-treated cells, cotreatment with 1 mg/mL AE-SN increased LC3 A/B II 3.35- and 4.78-fold, whereas in docetaxel treatment, AE-SN also increased the autophagic marker by 3.36- and 3.15-fold in DLD-1 and HT-29 cells, respectively. In combination with 5-Fu, AE-SN induced 3.03- and 2.65-fold of LC3 A/B II accumulation, similar to docetaxel. In doxorubicin-treated DLD-1 and HT-29 cells, the fold induction of LC3 A/B II was 2.09- and 2.05-fold, respectively. It is slightly lower than cisplatin, docetaxel, and 5-Fu. When both cell lines were exposed to a combination of AE-SN and the test drugs, activation of caspase-3 and LC3 A/B II were observed together (Figure 5(a)).

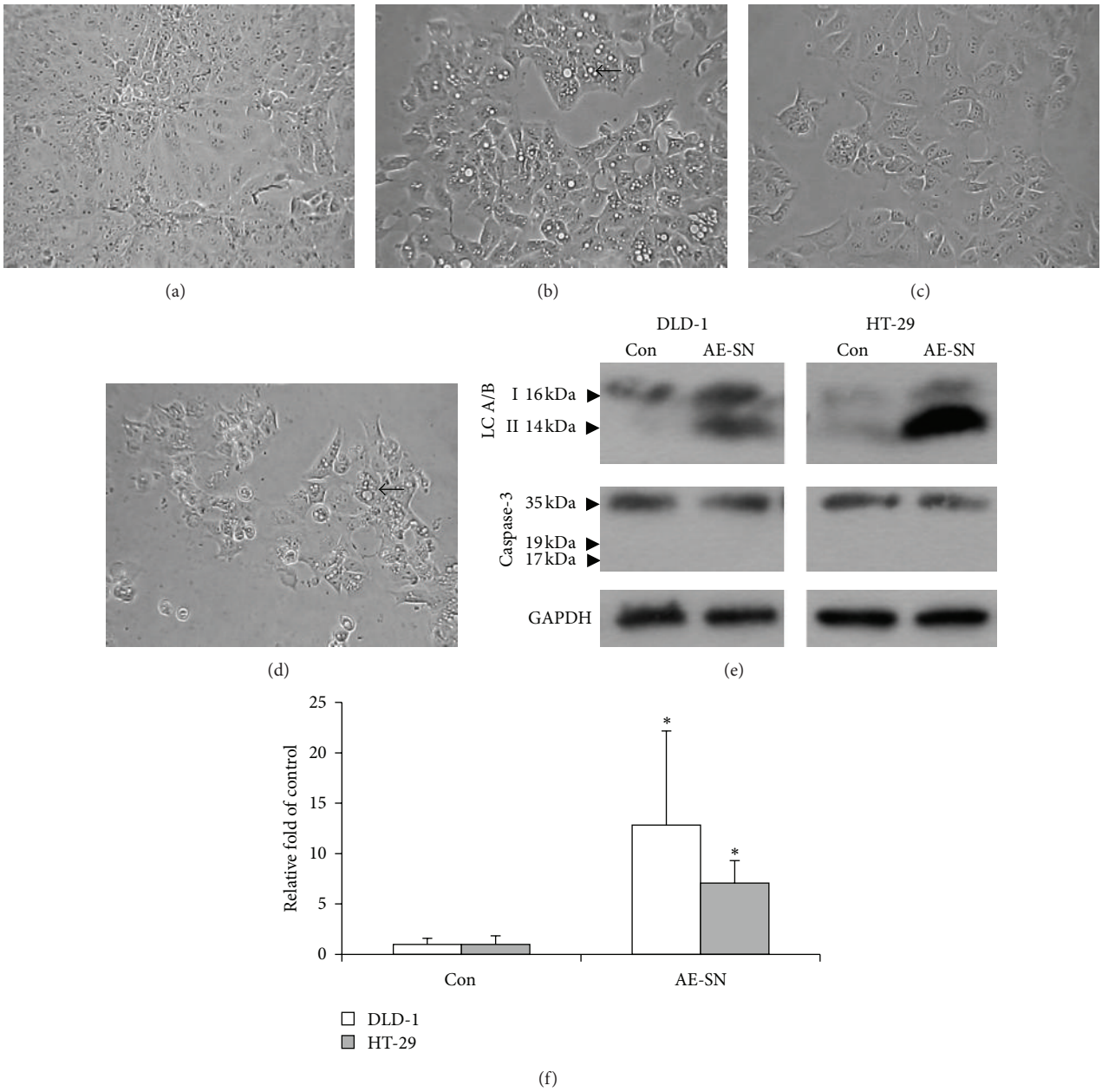


Figure 2: AE-SN induced morphological changes and LC-3 activation in DLD-1 and HT-29 cells. (a) and (b), DLD-1 cells were treated with 0 or 1 mg/mL AE-SN for 48 hr, respectively; (c) and (d), HT-29 cells were treated with 0 or 1 mg/mL AE-SN for 48 hr. Arrows indicate lipid droplet-like morphological changes in AE-SN-treated cells. Magnification = 100x. (e) Total protein extracts were harvested from DLD-1 cells or HT-29 cells which were treated with 0 or 1 mg/mL AE-SN (control versus AE-SN) for 48 hr. Activation of caspase-3 and LC3 A/B in AE-SN-treated cells was determined by western blotting analysis. (f) Semiquantification of LC-3 A/B II in AE-SN-treated cells. Data presented are mean ± SD ($n = 5$). indicates statistical significance compared with 0 mg/mL AE-SN treatment using Student's t-test ($P < 0.05$). AE-SN: aqueous extract of *Solanum nigrum*; Con: control; GADPH: glyceraldehyde 3-phosphate dehydrogenase; LC-3 A/B II: mammalian microtubule-associated protein 1 light chain 3 A/B II.

This result suggested that the AE-SN enhanced cytotoxicity with chemotherapeutic drugs and also induced LC3 A/B II accumulation in colorectal carcinoma cells.

4. Discussion

The tumor suppression efficacy and related mechanisms of AE-SN have been examined in some cancer types. For instance, AE-SN induced both cleavage of caspase-3 and accumulation of LC3 A/B II in HepG2 human hepatocellular carcinoma cells [25], and similar results were observed in AU565 human breast carcinoma cells [22]. Interestingly, AE-SN treatment only induced LC3 A/B II accumulation but not cleavage of caspase-3in HEC-1A, HEC-1B, and KLE human endometrial cells [24], with similar results demonstrated in DLD-1 and HT-29, human colorectal carcinoma cells.

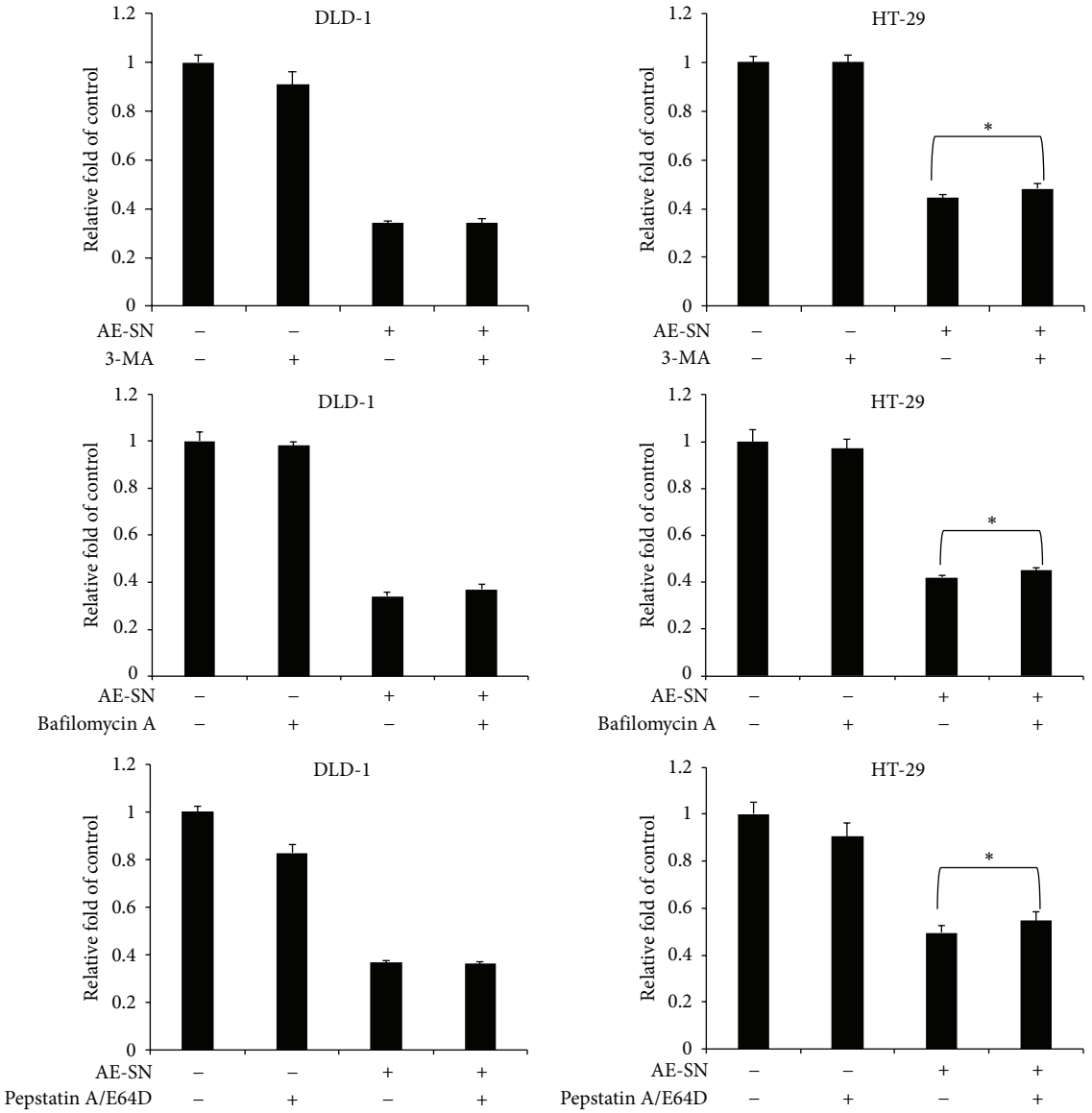


Figure 3: Inhibition of AE-SN induced autophagy by using 3-MA, bafilomycin (a), and pepstatin A/E64d on DLD-1 and HT-29 cells. (a) DLD-1 and HT-29 cells were treated with 0.5 or 1.0 mg/mL AE-SN, respectively, in combination with 1 μM 3-MA, 2 nM bafilomycin A, or 2 μg/mL pepstatin A/E64d for 48 hr. Cytotoxicity was analyzed by MTT assay. Experiments were performed in triplicate and the data shown are mean ± SD. * indicates statistical significance compared with 0 mg/mL AE-SN treatment using Student's t-test ($P < 0.05$). (b) DLD-1 and HT-29 cells were treated with control, AE-SN (1 mg/mL), 3-MA, or AE-SN plus 3-MA (μM) for 48 hr and accumtrluation of LC3 A/B II was determined by Western blotting. Numbers indicated fold induction of LC3 A/B II compared with control. AE-SN: aqueous extract of *Solanum nigrum*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

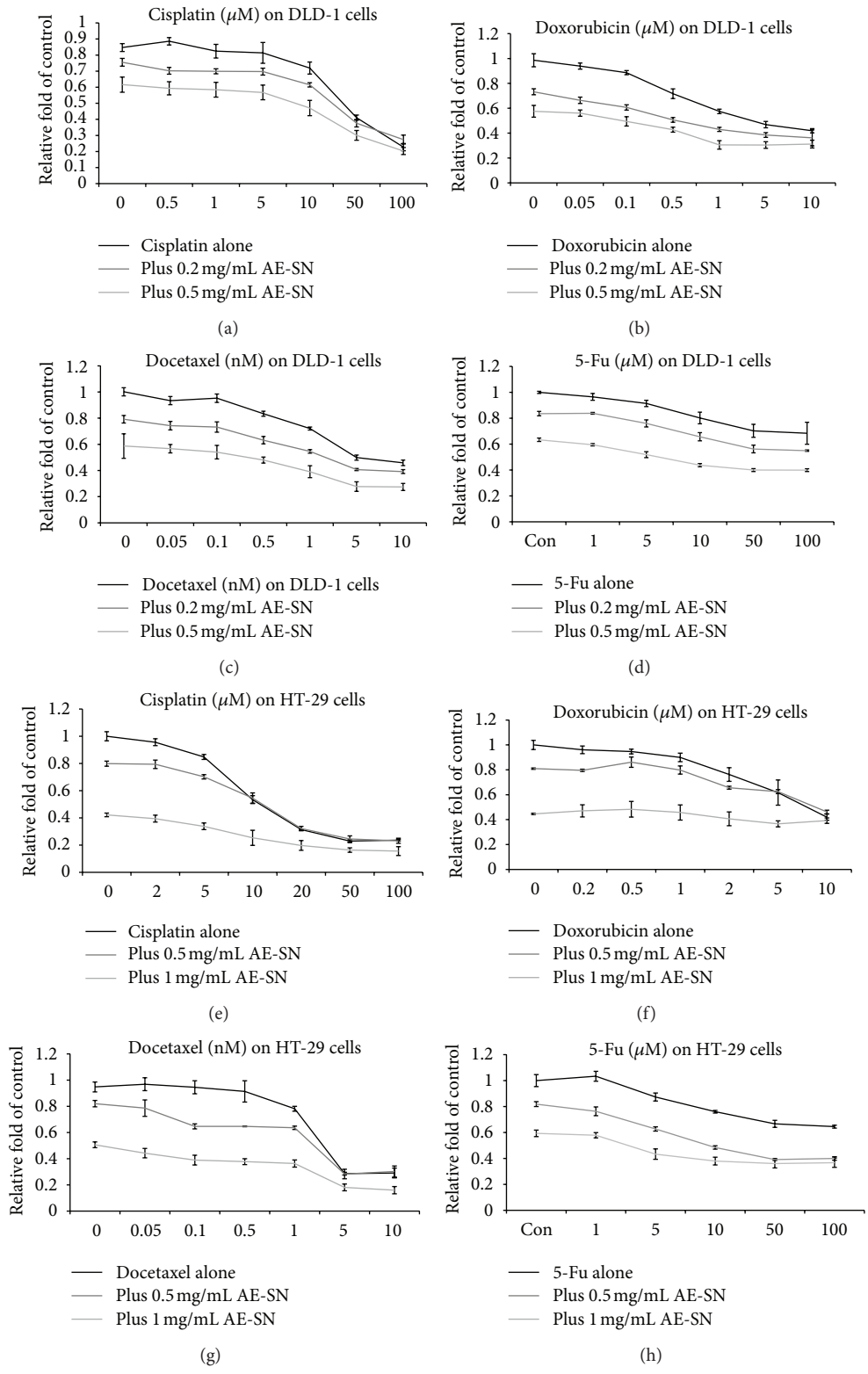
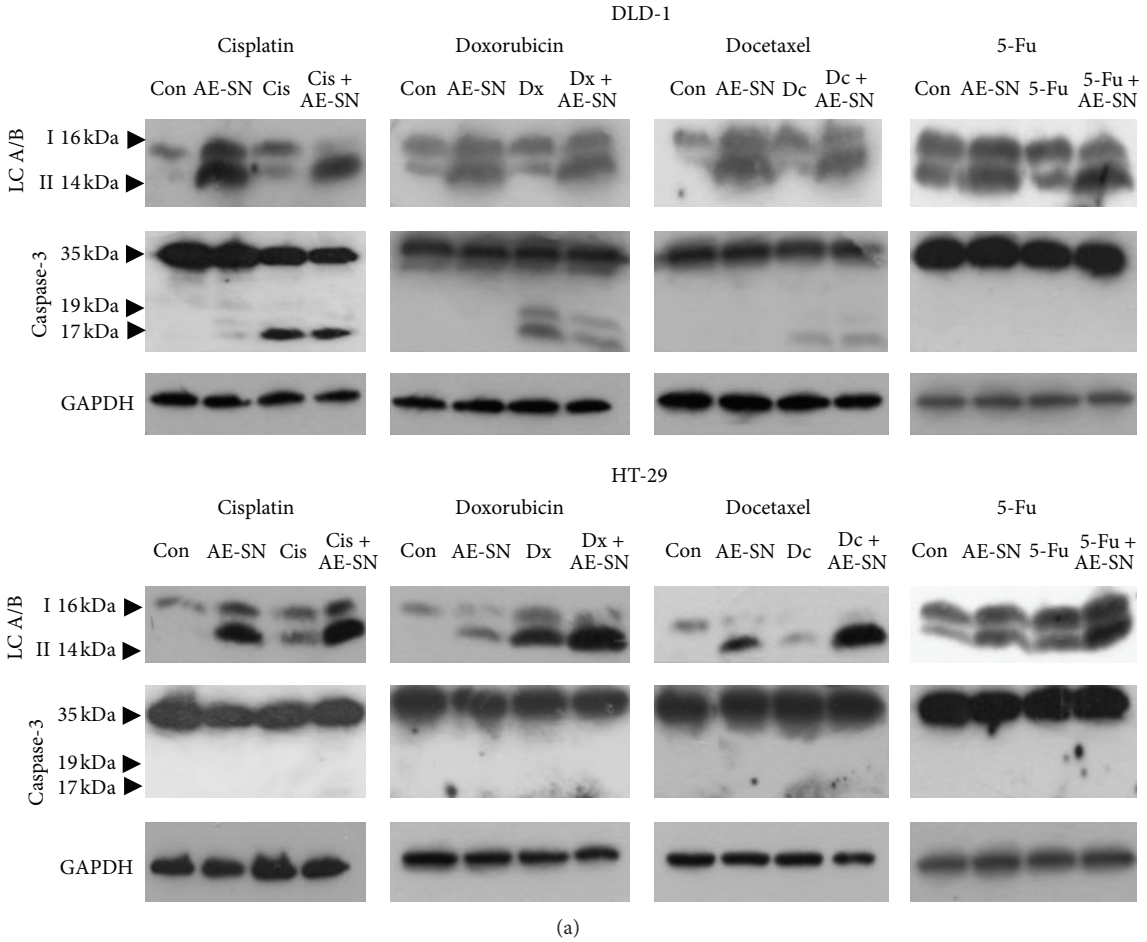
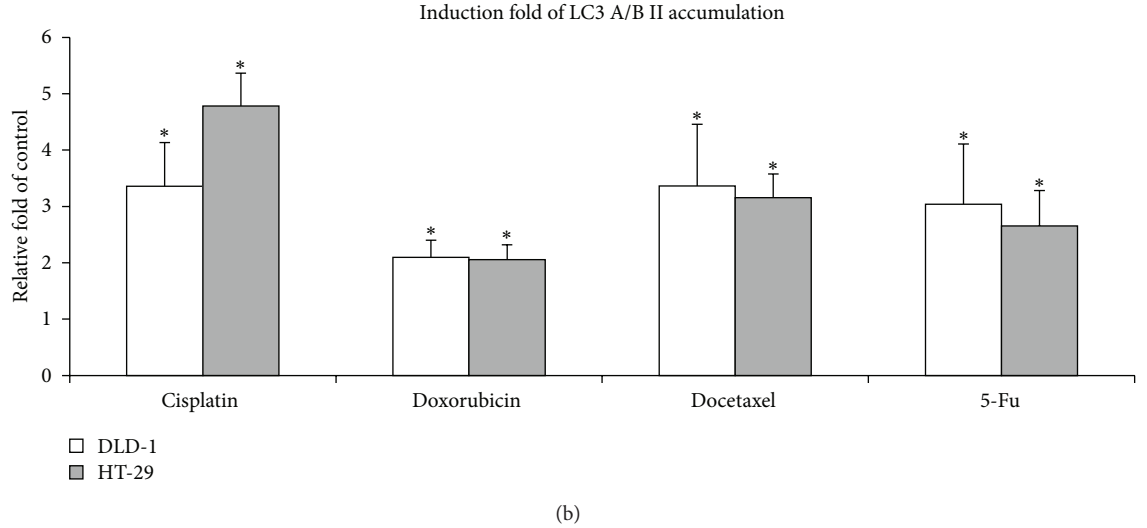


Figure 4: Cytotoxicity of a combination of AE-SN and the chemotherapeutic drugs, cisplatin, doxorubicin, docetaxel, or 5-Fu. (a), (b), (c), and (d), DLD-1 cells were treated with 0 to 100 μM cisplatin, 0 to 10μM doxorubicin, 0 to 10 nM docetaxel, 0 to 100 μM 5-Fu in combination with 0, 0.2 or 5 mg/mL AE-SN for 48 hr; (e), (f), (g), and (h) HT-29 cells were treated with 0 to 100 μM cisplatin, 0 to 10μM doxorubicin, and 0 to 10 nM docetaxel, or 0 to 100 μM 5-Fu in combination with 0, 0.5 or 1 mg/mL AE-SN for 48 hr. Cytotoxicity was analyzed by MTT assay. Experiments were performed in triplicate and the data shown are mean ± SD. AE-SN: aqueous extract of *Solanum nigrum*; MTT: 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.



(a)



(b)

Figure 5: Activation of cell death markers, LC3 A/B and caspase-3 in DLD-1 and HT-29 cells treated with AE-SN and cisplatin, doxorubicin, or docetaxel. Both DLD-1 and HT-29 cells were treated with 0, 1 mg/mL AE-SN, chemotherapeutic drugs alone, and a combination of 1 mg/mL AE-SN with chemotherapeutic drugs. The doses of cisplatin used were 50 μM in DLD-1 and 20 μM in HT-29. The dose of doxorubicin used was 5μM in DLD-1 and HT-29. The dose of docetaxel used was 1 nM in DLD-1 and HT-29, whereas the dose of 5-Fu was 20μM. After 48 hr incubation, total protein extracts were harvested from cells. (a) Activation of LC3 A/B and caspase-3 in DLD-1 and HT-29 cells was determined by western blotting analysis; (b) semiquantification of LC3 A/B II is presented as the relative fold induction of the control as mean ± SD ($n = 3$). *indicates statistical significance compared with 0 mg/mL AE-SN treatment using Student's t-test ($P < 0.05$). AE-SN: aqueous extract of *Solanum nigrum*; GADPH: glyceraldehyde 3-phosphate dehydrogenase; LC-3 A/B II: mammalian microtubule-associated protein 1 light chain 3 A/B II.

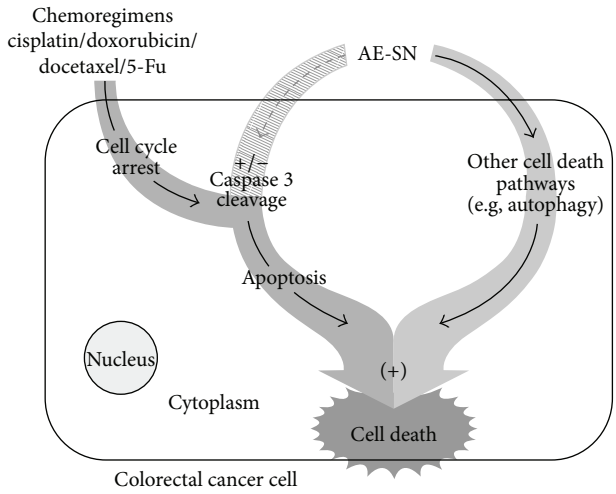


Figure 6: Proposed mechanism for the programmed cell death activated by AE-SN and chemotherapeutic drugs in human colorectal carcinoma cells. AE-SN-activated cell death can increase with cisplatin/doxorubicin/docetaxel/5-Fu-activated caspase-3-dependent or independent cell death (+/− caspase-3 cleavage) and result in further enhanced tumor cell death. (+) indicates synergistic effect of AE-SN with chemotherapeutic drugs. Solid line: activated pathway; the dotted line: inactive pathway. AE-SN: aqueous extract of *Solanum nigrum*.

AE-SN-induced autophagy was suggested to cause cancer cell death, particularly in human hepatocellular carcinoma cells, HepG2 [25], and breast adenocarcinoma cells, AU565 [22]. However, autophagic cell death was only identified in HepG2 cells by using 3-MA, a phosphoinositide 3-kinase inhibitor [25]. In cancer cells, autophagy plays a dual role in performing cytoprotection or programmed cell death (reviewed by [31, 32]. For example, autophagy protected colorectal cancer cells HCT-116 and DLD-1 from 5-Fu-induced apoptosis [33]. In present study, autophagic inhibitors such as 3-MA, bafilomycin, and pepstatin A/E64d only slightly recovered cell viability and reduced accumulation of LC3 A/B II on HT-29 cells. These results indicated that AE-SN activated autophagy may partly contribute to AE-SN-induced cell death on HT-29 cells, but not DLD-1 cells. On the other hand, treatment of autophagic inhibitors cannot further promote cell death suggesting that AE-SN-induced autophagy may not be involved in cytoprotective effect on HT-29 and DLD-1 cells. Since both caspase-3- dependent apoptosis and autophagy were not fully responsible for AE-SN-induced cell death on HT-29 and DLD-1 cells, the exact cell death mechanism activated by AE-SN still remained unclear.

In the present study, AE-SN demonstrated an effective cytotoxicity in human colorectal carcinoma cells. Both DLD-1 and HT-29 cell lines have p53 mutations and are partially resistant to the p53-mediated apoptotic pathway [34, 35]. AE-SN may therefore fail to activate apoptosis via p53 but may still be capable of activating cell death in both cell lines. Lee and colleagues suggested that SN-isolated glycoprotein induced caspase-3-dependent apoptosis in HT-29 cells [36], whereas the present results indicate that AE-SN treatment cannot activate cleavage of caspase-3 in HT-29 cells. These conflicting results provide an important reminder that the biological effects of medicinal herbs may vary according to different preparation and purification methods. Moreover, several studies suggested that the AE-SN-induced apoptosis is due to the presence of steroid alkaloids and phytoglycoproteins in AE-SN [21, 37–39], whereas the chemical compound responsible for AE-SN-induced tumor cell death remained unknown. In order to clarify the exact tumor suppression mechanism, further investigation for identification of effective compounds in AE-SN is therefore required. Nevertheless, the present data suggest that AE-SN may be a potential drug for dealing with colorectal cancer cells, particularly when they are resistant to apoptosis-based chemotherapeutic drugs.

Since AE-SNinduced cytotoxicity in a caspase-3- independent manner, cotreatment of AE-SN with apoptotic activators may further enhance cytotoxicity in tumor cells. AE-SN co-treatment significantly enhanced cytotoxicity in all tested chemotherapy drugs. Although HT-29 is a more AE-SN-resistant cell line, a low dose of AE-SN at 0.5 mg/mL still demonstrated a synergistic effect as good as a higher dose of 1 mg/mL with all tested drugs. AE-SN-induced LC3 A/B II accumulation was also present in cisplatin/doxorubicin/docetaxel-treated cells, and cleaved caspase-3 was induced by these chemotherapeutic drugs in DLD-1, but not HT-29 cells. Meanwhile, 5-Fu treatment induced cell death independent of activation of caspase-3- in both cell lines. Both caspase-3 dependent/independent cell death induced by chemotherapeutic drugs can be further enhanced by AE-SN treatment. Compared with AE-SN treatment alone, co-treatment with chemotherapeutic drugs decreased the fold induction of LC3 A/B II in both cell lines. For example, cotreatment of 1 mg/mL AE-SN and 5 μM doxorubicin decreased the induction fold from 12.83 and 7.08-fold to 2.09 and 2.05-fold in DLD-1 and HT-29 cells, respectively. Moreover, higher fold induction of LC3 A/B II accumulation was observed in HT-29 compared with that absaved in DLD-1 cells (3.35- versus 4.78-fold) with co-treatment of cisplatin and AE-SN. This suggests that treatment with chemotherapeutic drugs may disrupt AE-SN-induced autophagy in colorectal carcinoma cells regardless of the synergistic cytotoxic effects observed under AE-SN co-treatment, and this disruption may differ by cancer cell types and selected chemotherapeutic drugs. Interestingly, autophagy activated by AE-SN on DLD-1 and HT-29 cells had no protective effect against all tested chemotherapeutic drugs. This finding supported the previous study using autophagic inhibitors, and suggested AE-SN induced autophagy did not play a protective role in HT-29 and DLD-1 cells. Further investigation on the relationship of AE-SN-induced autophagy and cell death may help clarify the molecular mechanism involved in the drug cross-interaction. Collectively, these results indicate that AE-SN is a potential candidate for integration in chemotherapeutic regimens.

Although AE-SN demonstrated an observed tumor suppression efficacy alone and was also capable of enhancing tumor cell death induced by chemotherapeutic drugs the *in*

vitro evidence provided here requires further verification *in vivo*. In order to assess the tumor suppression efficiency *in vivo*, the optimal administration approach and dosage of AE-SN alone or in combination with individual chemotherapeutic drugs must be further examined with appropriate animal cancer models. Any unexpected adverse effects of the application of AE-SN must be carefully investigated *in vivo* prior to consideration for further clinical trials. AE-SN dramatically reduced the IC_{50} doses of all tested chemotherapeutic drugs. Reduced dosage of chemotherapeutic drugs also suggests potentially more tolerance of adverse effects by colorectal cancer patients.

5. Conclusion

In this study, AE-SN demonstrated a tumor suppression efficacy against DLD-1 and HT-29 human colorectal carcinoma cells and further enhanced tumor cell death with tested chemotherapeutic drugs in human colorectal carcinoma cells. AE-SN induced tumor cell death in a caspase-3- independent manner on HT-29 and DLD-1 cells with activation of autophagy. Although the full mechanism of AE-SN-induced cell death was still unclear, AE-SN was capable of enhancing cell death activated by cisplatin/doxorubicin/docetaxel/5-Fu (Figure 6). In conclusion, AE-SN is a potential candidate to development of the complementary for colorectal cancer.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Chen-Jei Tai and Chien-Kai Wang contribute equally to this work.

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Research Article

The Neural Mechanism by Which the Dorsal Vagal Complex Mediates the Regulation of the Gastric Motility by Weishu (RN12) and Zhongwan (BL21) Stimulation

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A large number of studies have been conducted to explore the mechanism of Back-Shu and Front-Mu points. While several lines of evidence addressed the acupuncture information of Shu acupoints and Mu acupoints gathering in the spinal cord, whether the convergence is extended to the high centre still remains unclear. The study selected gastric Mu points (RN12) and gastric Shu points (BL21) regulating gastric motility and its central neural mechanisms as the breakthrough point, using the technique of immunochemistry, nuclei lesion, electrophysiology, and nerve transection. Here, we report that gastric motility regulation of gastric Shu and Mu acupoints and their synergistic effect and the signals induced by electroacupuncture (EA) stimulation of acupoints RN12 and RN12 gather in the dorsal vagal complex (DVC), increasing the levels of gastrointestinal hormones in the DVC to regulate gastric motility through the vagus. In sum, our data demonstrate an important role of DVC and vagus in the regulation of gastric motility by EA at gastric Shu and Mu points.

1. Introduction

RN12 is the gastric Front-Mu point, while BL21 is the gastric Back-Shu point. The distribution of Back-Shu and Front-Mu points in the body has two characteristics: that is Yin and Yang in opposition and the other is, they are close to the Zang-fu viscera. The Back-Shu points are distributed in the back and waist, belonging to Yang, while the Front-Mu points are distributed in the chest and abdomen, belonging to Yin. The distribution of the two depends on the anatomic positioning of the viscera. In particular, the Front-Mu points are closer to the viscera, forming a relationship of Front-Mu points-viscera-Back-Shu points. According to the principles of traditional Chinese medicine, acupuncture at gastric Shu and Mu points can regulate gastric functions. Further, clinical practice demonstrates that EA at acupoints RN12 and BL21 can treat gastrointestinal (GI) diseases [1]. However, the mechanism(s) by which stimulation of RN12 and BL21 regulates gastric motility through central nuclei, as well as the neural mechanism employed in this process, still remain(s) unclear. Addressing this question can provide valuable clues for the development of effective therapeutics against gastrointestinal motility disorders.

The DVC is composed of the dorsal motor nucleus of the vagus (DMV) and the nucleus of the solitary tract (NTS), which, respectively, contain neurons providing vagal efferent innervation to the major portion of the gastrointestinal tract and neurons receiving vagal afferent input from the viscera [2, 3]. Therefore, the DVC is considered to be a parasympathetic preganglionic center regulating gastrointestinal functions. Previous studies indicated that the DVC plays very important roles in the modulation of gastric motility by acupuncture [4, 5]. Neurophysiological studies have demonstrated that

modulation of gastric motility is dependent on an intact vagus [6–8].

In this study, we performed EA stimulation of the gastric Front-Mu points (RN12) and gastric Back-Shu points (BL21) to investigate the regulation of gastric motility as well as the neural mechanisms mediating this effect. Our study centered on the DVC-vagus nerve-gastric channel, using immunohistochemistry to monitor the expression levels of *c-fos*, MTL, and GAS in the DVC. Employing the techniques of central nuclei lesion and peripheral nerve transection, we endeavored to verify the central target and neural channel mediating the regulation of gastric motility by EA at RN12 and BL21.

2. Materials and Methods

2.1. Materials. The following software, equipment, and reagents were employed in this study: PowerLab 8/30 data acquisition system; LabChart software; lesion-making device; double digital stereotaxic apparatus; anatomic microscope; self-made balloon; SDZ-IV type electronic acupuncture instrument of Hua Tuo brand; acupuncture needles; immunohistochemical kit; polyclonal rabbit primary antibody; and anti-rabbit secondary antibodies.

2.2. Animals and Groups. Male Sprague-Dawley rats weighing between 250~300 g were housed under controlled conditions (22~24°C, light on from 6:00 a.m. to 6:00 p.m.) with free access to food and water. They were randomly divided into the following groups: MOD group (induced gastric distention), RN12 group (EA at RN12), BL21 group (EA at BL21), RN12 + BL21 group (EA at RN12 plus BL21), vagotomy group (cutting of the bilateral subdiaphragmatic vagus), vagotomy + EA group (cutting vagus + EA at RN12 plus BL21), DVC lesion group (damaged DVC), and DVC lesion + EA group (damaged DVC + EA at RN12 plus BL21). Before each experiment, the animals were deprived of food for 18 h. All experimental protocols have been approved by the Committee of Animal Use for Research and Education of China and conformed to the National Institute of Health Guide for the Care and Use of Laboratory animals.

2.3. Electroacupuncture. EA stimulation was delivered via a pair of acupuncture needles at left BL21 and RN12 (BL21 is located 5 mm on the side of the twelfth thoracic vertebra, with needle electrodes inserted to a depth of 4 mm into the skin; RN12 is located on the median line of the upper abdomen, 20 mm above the umbilicus, with needle electrodes inserted to a depth of 2 mm into the skin). These needles were connected to an SDZ-IV type electronic acupuncture instrument. The frequency switched back and forth between 20 Hz and 100 Hz; the current intensity was set at 2–2.5 mA.

2.4. Measurement of Intragastric Pressure. Intragastric pressure was measured with the use of a rubber balloon that was tied around a polyethylene tube (PE 160) and inserted into the body of the stomach through a small incision in the duodenum. The balloon was secured at the pylorus with a suture to avoid movement, and the tube was connected to a pressure transducer (MLTO380), which was connected to a bridge amplifier of the PowerLab 8/30 system. The balloon was filled with water at 37°C (1.5–2.0 mL, the volume determined to be necessary to induce an intragastric pressure). Pressures were recorded and analyzed by LabChart, the data acquisition system, for online analysis. The exact location of the balloon was verified after each experiment.

2.5. DVC Lesion. The animal was deeply anesthetized with chloral hydrate and placed in a stereotaxic frame; the dorsal surface of the brain stem was then exposed. We placed the electrode tip in the DVC using the coordinates of Paxinos and Watson, and the electrode was advanced into the DVC (coordinates: *Ap* 11.3–14.3 mm, *L* 0.7–1.7 mm, and *H* 7.5–8.7 mm); the bilateral DVC was destroyed with a DC current (2 mA, 10 s) by a lesion-making device.

2.6. Bilateral Subdiaphragmatic Vagotomy. To demonstrate that DVC acts via stimulation of the vagal pathways, acute bilateral subdiaphragmatic vagotomy was performed: a midline incision was made in the abdominal wall, and the stomach was carefully manipulated to expose the esophagus. The subdiaphragmatic vagal trunks were exposed halfway between the diaphragm and the gastric cardia. Both anterior and posterior trunks of the vagal nerves were transected.

2.7. Brain Tissue Preparation. The brains were postfixed for 24 h at 4°C in the same fixative. Paraffin sections (5 μm) were cut at the interaural levels of −4.24 to −5.08 mm (DVC) according to the atlas of Paxinos and Watson.

2.8. Immunohistochemical Analysis of c-fos, MTL, and GAS. Briefly, the brain sections were rinsed in PBS and incubated with 0.3% H_2O_2 for 30 min to remove endogenous peroxidase activity. All of the sections were incubated for 24 h at 4°C with the corresponding polyclonal rabbit antibody diluted in PBS containing 0.1% sodium azide and 0.3% Triton X-100 (PBS-T, pH 7.4). Following this step, sections were then rinsed in PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit secondary antibody. Finally, brain sections were processed using the standard biotin-avidin-horseradish peroxidase method. *c-fos*, MTL, and GAS immunoreactivity was detected as a dark brown nuclear staining.

2.9. Statistical Analysis. All values were expressed as mean ± SE. Statistical analysis was performed with one-way ANOVA and LSD tests. A difference with a P value < 0.05 was considered statistically significant.

3. Results

3.1. The Effect of EA at RN12 and BL21 on IGP. To determine whether EA at RN12 and BL21 could affect gastric motility in rats, we designed an experiment in which the EA stimulation protocol described above was applied at RN12 and BL21, both alone and in combination. As shown in Figure 1(a), IGP was dramatically increased in the three EA groups, especially in the RN12 + BL21 group ($P < 0.01$, $n = 8$); further, compared

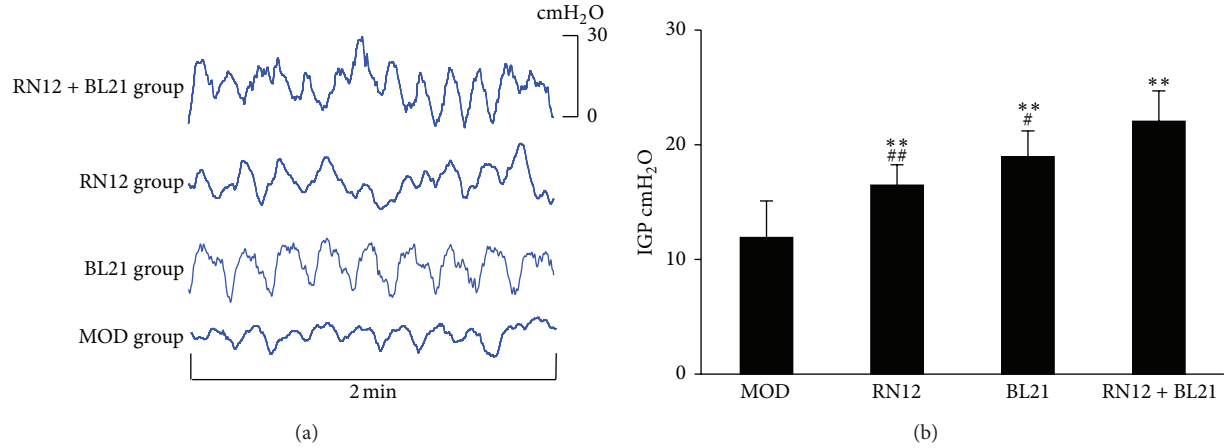

Figure 1: The effects of EA at RN12 and BL21 on IGP. (a) Representative waves of IGP of rats induced by stimulating RN12 and BL21. (b) Summarized data for the effect of stimulation at RN12 and BL21 on intragastric pressure. $n = 8$ for each group, using one-way ANOVA followed by LSD test. $^{**}P < 0.01$ compared with MOD group; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ compared with RN12 + BL21 group.

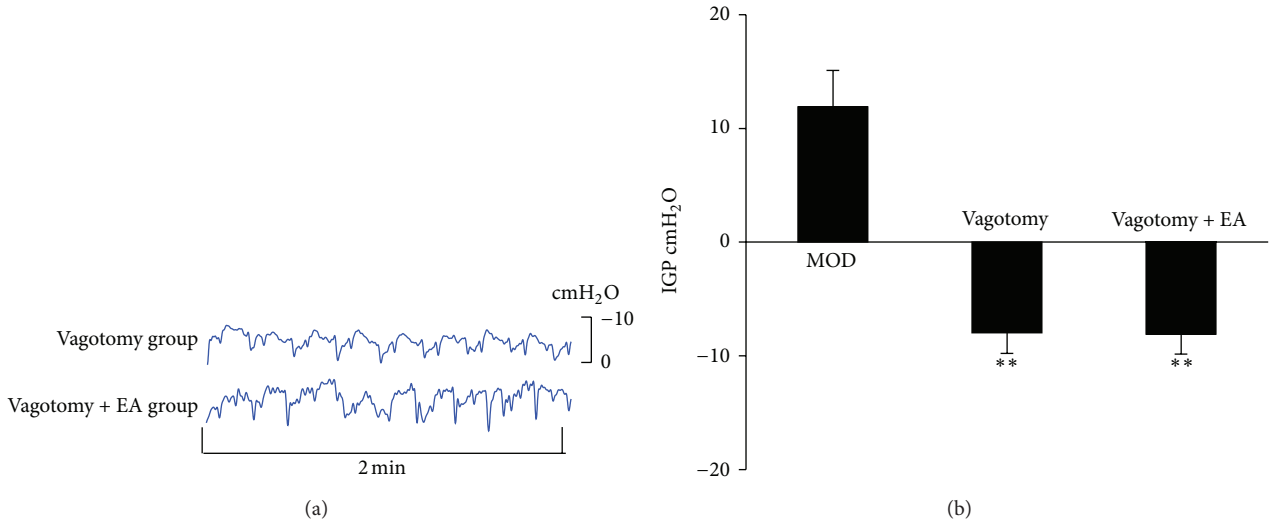

Figure 2: The effects of vagus in regulating IGP by stimulating RN12 + BL21. (a) Representative waves of IGP of rats induced by cutting bilateral subdiaphragmatic vagus with or without stimulating RN12 + BL21. (b) Summarized data for the effect of stimulation at RN12 + BL21 with bilateral subdiaphragmatic vagotomy on intragastric pressure. $n = 8$ for each group, using one-way ANOVA followed by LSD test. $^{**}P < 0.01$ compared with MOD group.

with the RN12 and BL21 groups, the increase in IGP was significantly greater in the RN12 + BL21 group ($P < 0.05$, $n = 8$). Thus, these data suggest that EA at RN12 and BL21 can regulate gastric motility, with stimulation of both of these acupoints eliciting a synergistic effect.

3.2. The Role of the Vagus in the Regulation of Gastric Motility by EA at RN12 + BL21. To determine the role of vagus in mediating the enhancement of gastric motility by EA at RN12 + BL21, we cut the bilateral subdiaphragmatic vagus under an anatomic microscope. We found that the IGP was decreased compared with MOD group ($P < 0.01$, $n = 8$), and the waves were changed. The decreased IGP induced by vagotomy was not restored by EA at RN12 + BL21 (Figures 2(a) and 2(b)). This suggested that the vagus potentially plays a role in mediating the effect of EA at RN12 + BL21 on gastric motility.

3.3. The Role of the DVC in the Regulation of Gastric Motility by EA at RN12 + BL21. To elucidate whether the DVC was responsible for modulating the effects of EA at RN12 + BL21 on gastric motility, we stereotaxically damaged the DVC with a lesion-making device. We found that this intervention caused IGP to be decreased as compared with the MOD group ($P < 0.01$, $n = 8$), with the waves becoming disordered. The decreased IGP induced by DVC lesion was not restored by EA at RN12 + BL21 (Figures 3(a) and 3(b)). These results suggest that the DVC plays critical roles in regulating gastric motility and that EA at RN12 + BL21 increases gastric motility through the DVC.

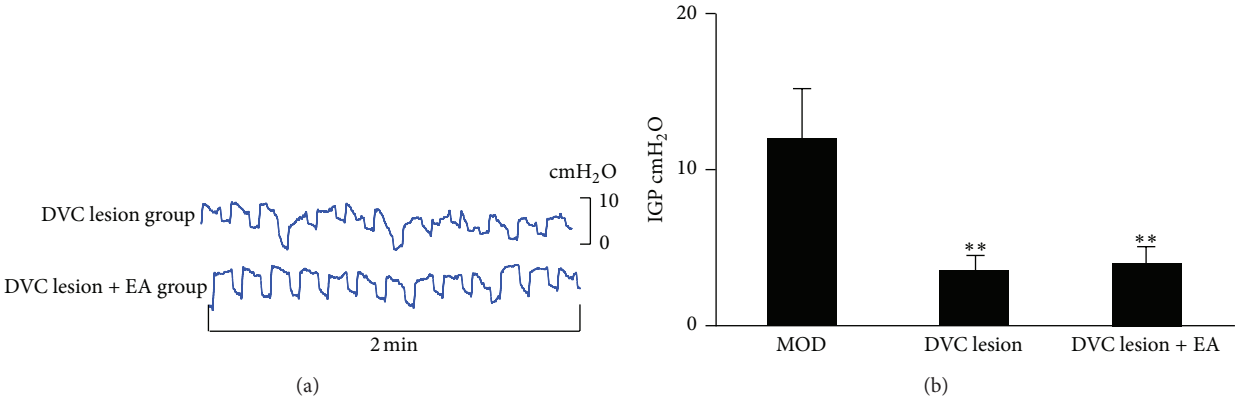

FIGURE 3: The effects of DVC in regulating IGP by stimulating RN12 + BL21. (a) Representative waves of IGP of rats induced by damaging DVC with or without stimulating RN12 + BL21. (b) Summarized data for the effect of stimulation at RN12 + BL21 with DVC lesion on intragastric pressure. $n = 8$ for each group, using one-way ANOVA followed by LSD test. $^{**}P < 0.01$ compared with MOD group.

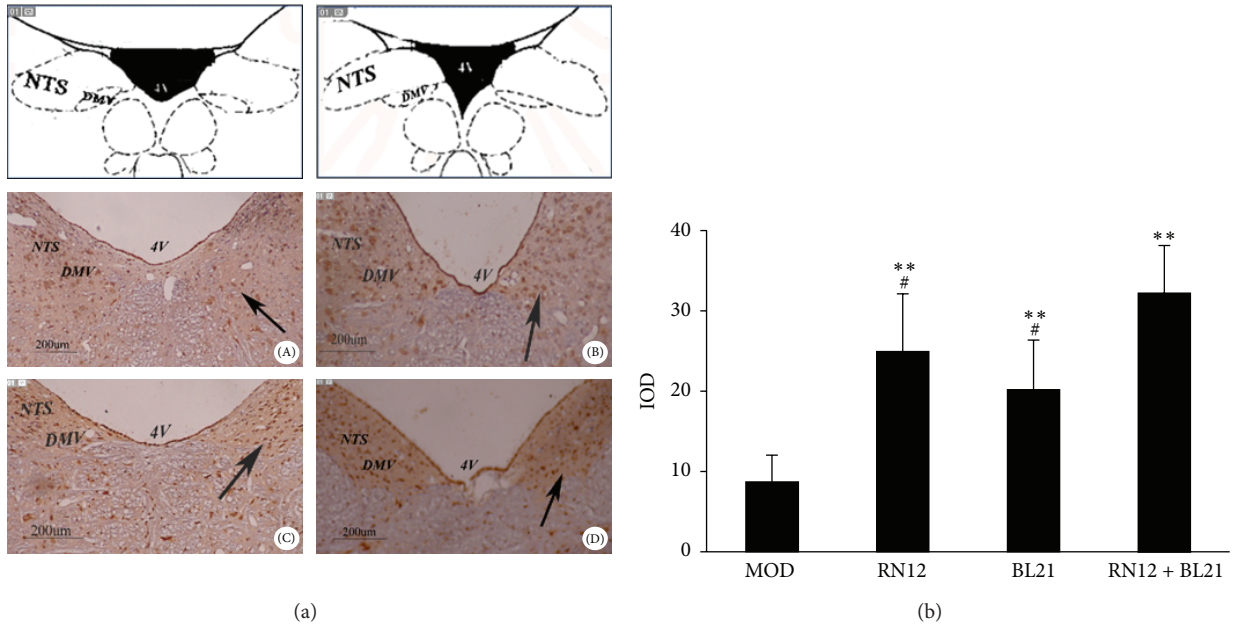

FIGURE 4: The expression of *c-fos* in DVC. (a) Photomicrographs of the medullary sections showing *c-fos* immunoreactivity in the DVC (NTS and DMV) in model rats (A) and in rats with EA at RN12 (B), BL21 (C), and RN12 + BL21 (D). The anatomic locations of the photomicrographs are indicated in the top, adapted from the atlas of Paxinos and Watson [30]. *c-fos*-positive neurons are presented as dark brown staining in the cell nuclei. Scale bars: 200 μm. 4 V: fourth ventricle. (b) Integral optical density (IOD) of *c-fos*-positive neurons in the DVC. Each column represents the mean ± SE of IOD. $^{**}P < 0.01$ compared with MOD group; $^{\#}P < 0.05$ compared with RN12 + BL21 group.

3.4. EA at RN12 and BL21 Activates DVC Neurons. In the model rats, the number of *c-fos*-positive neurons in the DVC was low, while EA markedly induced *c-fos* expression in the DVC as compared with the model animals ($P < 0.01$); further, *c-fos* expression was statistically increased in the RN12 + BL21 group as compared with the RN12 and BL21 groups ($P < 0.05$) (Figures 4(a) and 4(b)). These findings indicate that the acupuncture signals induced by stimulation of RN12 and BL21 gather in the DVC.

3.5. EA at RN12 and BL21 Enhances the Production of Gastrointestinal Hormones in the DVC. Compared with the MOD group, the expression levels of motilin and gastrin in rat DVC were significantly increased in the three EA groups ($P < 0.05$) (Table 1). Our data suggest that gastrointestinal hormones in the DVC participate in the modulation of gastric motility by EA stimulation of RN12 and BL21.

4. Discussion

Combined stimulation of the Shu and Mu points is commonly used in clinical practice. The Back-Shu point and Front-Mu point are matched to treat diseases of the viscera and of the bowel. Treatment of hepatopathy requires the selection of BL18 (Shu) with LR14 (Mu), while the treatment of gastropathy necessitates the selection of BL21 (Shu) with RN12 (Mu).

Table 1: Effects of EA on the expression of MTL and GAS in DVC.

Groups	n	MTL	GAS
MOD	8	8.20 ± 2.46	13.38 ± 2.87
RN12	8	15.47 ± 3.98*	23.33 ± 4.12*
BL21	8	17.52 ± 3.54*	21.04 ± 3.58*
RN12 + BL21	8	14.68 ± 4.02*	19.92 ± 4.07*

Each value represents the mean ± SE of IOD and the numbers indicated in the table. * $P < 0.05$ compared with MOD group.

While the traditional Chinese medicine literature defines BL21 and RN12 as the points for functional gastrointestinal disorders, insufficient attention has been paid to its effect on these diseases. In this study, we investigated whether EA at RN12 and BL21 could improve gastric motility. We discovered that the combined EA stimulation of gastric Shu and Mu points has synergistic effects. It is worth noting that, although most cases showed an upregulation of gastric motility by EA at BL21 and RN12 [9], in some cases EA at these points failed to accelerate gastric motility, or even inhibited gastric motility, which is similar to previous reports [10].

With regards to modern anatomy, the nerve segment relationship between internal organs and their Back-Shu and Front-Mu points is very consistent. Modern research shows that there is a specific pathway connecting the stomach with its Back-Shu and Front-Mu points, with visceral and somatic afferent impulses gathering at the spinal cord [11]. A topic of intense investigation is whether or not the convergence is extended to the high centre. On the basis of the acupuncture literature and the modern research of Shu and Mu points combination, the following "targeted convergence" hypothesis has been put forth: the acupuncture signals of Shu and Mu points gather not only in the spinal cord, but also have "targeted convergence" in the brain stem and hypothalamus, achieving integration of high hub through the neural microcircuitry. The essence of Shu and Mu points combination is the convergence of the effector organ and the target.

In recent years increasing attention has been paid to studying the interconnections between acupuncture acupoints and brain targets. It has been previously reported that the nervous system, neurotransmitters, and endogenous substances respond to EA [12]. Among these components, the functional activity of the hypothalamus and brainstem is the core foundation of acupuncture-modulated effects [13, 14]. Our finding that EA at RN12 and BL21 could induce the expression of *c-fos* in the DVC and that the upregulation in the RN12 + BL21 group was even more marked demonstrates that the acupuncture signals of the gastric Shu and Mu points gather in the medullary DVC.

The DVC lies in the dorsomedial part of the medulla oblongata and consists of the NTS and DMV. Sensory inputs from the stomach, both mechanical and chemical, are transmitted mainly to the dorsomedial, medial, and commissural subnucleus of the NTS via the vagus [15]. Both the NTS and DMV play critical roles in regulating gastric activity [16]. The gastric efferent neurons are concentrated in the central portion of the DMV and project to the stomach via gastric branches of the vagus [17]. Laugghton [18] and Semba et al. [19] found that stimulating the DMV and other areas could improve or restrict gastric activity. Some studies showed that the vagal pathway and related DVC potentially play a role in mediating the effect of EA on gastric activities [20–23]. In our study, we found that DVC lesion or bilateral subdiaphragmatic vagotomy caused gastric motility to be decreased, and EA at RN12 + BL21 was not able to restore this decreased gastric motility. It was demonstrated that the DVC was the central target of the gastric Shu and Mu points combination regulating gastric motility, and the effects of the combined stimulation depended upon an intact vagus nerve.

Furthermore, a variety of neurotransmitters and neuromodulators (especially neuropeptides) distributed in the central nervous system are involved in regulating gastric function. Gastrointestinal peptides can regulate gastrointestinal motility through the nervous system [24]. In the nervous system, gastrointestinal peptides, such as motilin, can regulate gastrointestinal spontaneous rhythmic contraction [25]. Motilin is a polypeptide composed of 22 amino acids. It has been found in the central and peripheral nervous system of the gastrointestinal wall. The main physiological functions of motilin involve the regulation gastrointestinal motility. We found that EA could promote gastric motility, and this phenomenon might involve the action of motilin and cholecystokinin (CCK) in the periphery [26]. The present study demonstrated that acupuncture could change the level of motilin in bulbus medullae. It has been suggested that acupuncture may regulate level of motilin in bulbus medullae to influence gastric motility [27]. Gastrin is a vital gastrointestinal hormone distributed in the central nervous system and gastrointestinal tract. It acts as a neuromodulator in the central nervous system and as a hormone in gastrointestinal tract, participating in the regulation of gastric motility [28]. It belongs to the family of brain-gut peptides and it promotes gastrointestinal motility. Studies have shown that EA can increase gastrin of the central nervous system and excite the vagal and peptidergic nerves of the peripheral nervous system, with gastrin being one of the important mediators of the effects of acupuncture [29]. Our finding that the expression levels of MTL and GAS in DVC were increased significantly in the three EA groups suggests that gastrointestinal hormones in the DVC participate in EA-regulated gastric motility.

5. Conclusion

Based on the principles of traditional Chinese medicine and with the use of modern technology, this study demonstrated that EA stimulation of the gastric Shu and Mu points can regulate gastric motility, with combined stimulation of these acupoints eliciting a synergistic effect. The effect was closely connected with the DVC, as acupuncture signals generated by EA at gastric Shu and Mu points gather in the DVC, elevating gastrointestinal hormones in the DVC, which in turn play a role in regulating gastric motility through the vagus. Thus, we suggest that the effects of combined EA stimulation of gastric Shu and Mu points may be achieved through the combined efforts of the DVC-vagus nerve-gastric channel.

Conflict of Interests

The authors declare that there are no conflict of interests.

Acknowledgment

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Research Article

Evaluation on the Pharmacological Effect of Traditional Chinese Medicine SiJunZiTang on Stress-Induced Peptic Ulcers

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Purpose. To explore the effects of SiJunZiTang (SJZT) on central neurotransmitters and the inhibition of HCl hypersecretion, along with the role of the vagus nerve. From this, the effects of SJZT and its constituent ingredients on inhibiting stress-induced peptic ulcers will be determined. *Methods*. Methods used to determine SJZT's effectiveness included (1) measuring the antipeptic ulcer effects of varying combinations of the constituents of SJZT; (2) evaluations of monoamine (MA) level in the brain; and (3) measuring the effects of longer-term SJZT treatment. *Results*. Comparing the control and experimental groups where the rats' vagus nerves were not cut after taking SJZT orally (500 mg/kg and 1000 mg/kg), the volume of enterogastric juice, free HCl and total acidity all reduce dose-dependently. The group administered SJZT at 1000 mg/kg showed significant reductions ($P < 0.05$). For the experimental groups where the vagus nerves were cut, a comparison with the control group suggests that the group receiving SJZT (500 mg/kg) orally for 21 days demonstrated a cure rate of 34.53%. *Conclusion*. The results display a correlation between the therapeutic effects of SJZT on stress-induced peptic ulcers and central neurotransmitter levels. Further to this, SJZT can inhibit the hypersecretion of HCl in the stomach, thus inhibiting stress-induced peptic ulcers.

1. Introduction

The occurrence of self-reported ulcer over a nine-year period was more likely in subjects who reported any of several concrete life stressors or psychological distress at baseline. The ulcerogenic effects of stress have been shown to be robust enough to survive adjustment for behavioral and physical confounding factors [1]. True stress ulcers are primarily superficial gastric fundus lesions that occur in the clinical setting of severe shock, trauma, burns, and sepsis, especially peritonitis.

SiJunZiTang (SJZT) is a basic prescription, consisting of four components: *Ginseng Radix, Poria Cocos, Atractylodis Rhizoma*, and *Glycyrrhizae Radix*. SJZT is a common Chinese herbal prescription, tonifying the spleen and stomach, is traditionally used for the treatment of gastrointestinal diseases in oriental countries.

The antiulcer activities of SJZT also have been mentioned in recent research [2]. However, the antiulcer mechanism of SJZT is not clear. Several studies indicated the CNS in modulating gastrointestinal function and response to injury [3, 4]. Dopamine (DA) plays a critical role in the protection

of gastric mucosa and is mediated through corresponding receptors [5]. Dopamine D_1 (central)/DA_1 (peripheral) receptors are believed to influence gastrointestinal functions and pathologies. Centrally administered D_1 agonists exert effects of greater magnitude than when administered peripherally [3, 4]. The hypothesis of a "brain-gut axis" was proposed to account for the growing body of evidence showing that a variety of peptides, neurotransmitters and neuromodulators profoundly affect not only gastric secretory responses but also gastric and duodenal ulcerogenesis [3, 4].

The goal of this study was to better understand the correlation between the inhibitory effects of SJZT on hypersecretion of free HCl, and how the pharmacological effect of SJZT could inhibit stress-induced peptic ulcers, and whether this was associated with the central neurotransmitters, such as dopamine (DA), norepinephrine (NE), and serotonin (5-hydroxytryptamine, 5-HT), and the different combinations of its ingredients on stress-induced peptic ulcers.

2. Materials and Methods

2.1. Materials

2.1.1. Experimental Animals. The study used healthy male Wistar Kyoto (WKY) rats with a body weight of 180 ± 20 g, obtained from the National Laboratory Animal Breeding and Research Center for experiments. The rats were fed with maintenance diet (Altromin 1320) and housed in a room of specific pathogen-free (SPF) facility of the Experimental Animal Center of Chung Shan Medical University, in which the room temperature maintained at 25 ± 1°C, relative humidity 55 ± 5%, air change rate (ventilation rate) 12 times/hour, and the artificial light application time was from 07:00 to 19:00. The rats were kept in the room for at least 7 days before the experiment to allow them to adapt to the environment.

Our study protocol and experimental design were approved by the Institutional Animal Care and Use Committee (IACUC), Chung Shan Medical University Experimental Animal Center.

2.1.2. Medication Used. SiJunZiTang (SJZT) herb formulas were prepared according to the official compendia "Tai Ping Hui Min Her Ji Jyu Fang" of the Song Dynasty in China. These included *Ginseng Radix*, *Poria cocos*, *Atractylodis Rhizoma,* and *Glycyrrhizae Radix*. The mixing ratio of the ingredients was 1 : 1 : 1 : 1.

All herbal ingredients were of a high quality, without damage by moths or worms, preserved and frozen in alcohol-free fluid extracts. The preparation procedure is illustrated in Figure 1. SJZT extract was procured from a good manufacturing practices (GMP) supplier. Purity and contaminant tests for the presence of toxic metals, pesticide residues, mycotoxins, and microorganisms were performed in our study. Qualitative chemical fingerprint analysis and quantitation of marker compounds of these traditional Chinese medicines (TCM) were carried out with high-performance liquid chromatography (HPLC). Extraction and manufacture of these

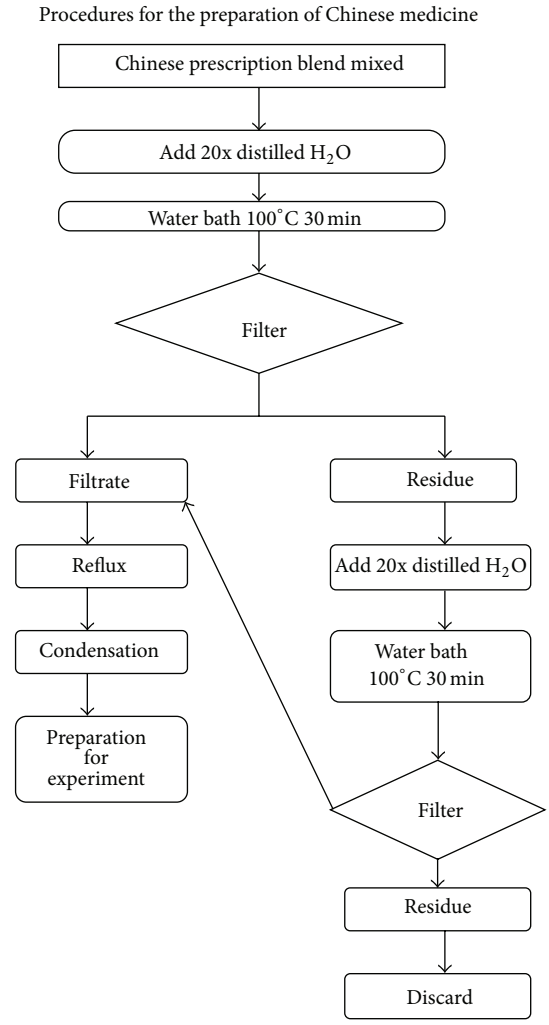

Figure 1: Procedures for the preparation of SJZT.

TCM were carried out under GMP and in accordance with The Japanese Pharmacopoeia 15th edition (JP15). Chemical standardization was performed with liquid chromatography-mass spectrometry (LC-MS) analysis. Stability of the formula was monitored with HPLC in real time. A HPLC flow chart is illustrated in Figure 2.

The SJZT was maintained in a refrigerator under 4°C and was prepared as a medicament (10 mL/kg) with dist. H_2O. The medicament would be used for oral administration (p.o.) or for administration through duodenum (i.d.).

DA, NA, 5-HT, and the metabolite VMA (vanillylmandelic acid), HVA (homovanillic acid), and 5-HIAA (5-hydroxyindole acetic acid) were adopted for analysis on the content of monoamine in brain. All the reagents were purchased from Sigma.

The HPLC-grade distilled water used in this experiment was purchased from a pharmaceutical factory.

2.1.3. Apparatuses Used. A barrel which was made of a 4 cm the diameter wire net was used for stressed peptic ulcers induced by water immersion. With regard to the analysis of

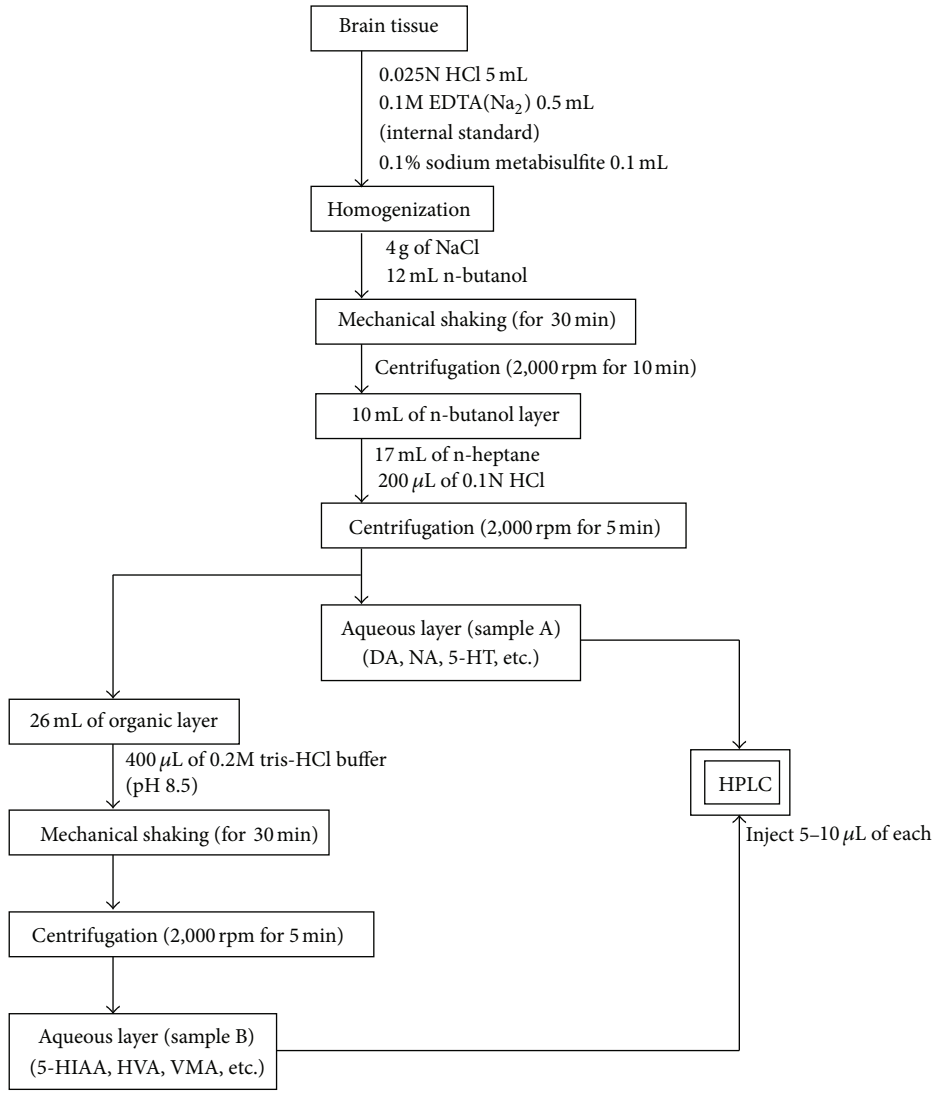

Figure 2: Flow chart of the preparation process for monoamine.

monoamine content changed in brain, a Model 440 HPLC Absorbance Detector (uv 280 nm, Model 717Autosample) with Data Model 746 produced by Waters was used.

A Stereomicroscope (Leica WILD M3Z) was used for detecting the ulcerative areas.

2.2. Methods

2.2.1. Blockade of Gastric Vagus Nerves and Relevant Effects. In this experiment, we fixed a rat on the operation panel and anesthetized with ether (WAKO of Japan), and then a cut was made from the point about 1.5 cm below the xiphoid and separated along the chest midline to the lower abdomen. The stomach of the rat was pulled out with apex circle-shaped tweezers. Next step, we separated the vagus nerves that were close to the cardia and nearby both sides of the esophagus, dissociated the nerves with a thin hook-shaped glass bar, and used the ophthalmological surgical fine scissors to cut off the vagus nerves. Finally, the stomach was put back into the rat's body and then sutured. These rats would be used for experiments until recovery. A total of 10 rats were used during the experiment.

2.2.2. Determination on Secretion of Gastric Juice and Relevant Effects. 8 healthy rats and 8 gastric vagus nerves cut-off rats were used. In the experiment, the rats were fed with dist. H_2O (5.0 mL/kg) and SJZT (500 mg/kg and 1000 mg/kg) by oral administration, respectively. 30 minutes after feeding, the rats were anesthetized with ether. Then we cut the belly open for exposing the pylorus, and a 100% cotton silk was used to ligate the junction of the pylorus and the duodenum. Finally the stomach was put back into the abdomen and we sutured the wound. 8 hours later, the animals were sacrificed and we cut the bellies open again for removing their stomachs out. Before taking the stomach out, both the cardia and the pyloric sphincter were nipped with hemostatic forceps. Finally, a cylinder was used to measure the volume of gastric juice.

2.2.3. Determination on Free HCl and Total Acidity in Gastric Juice and Relevant Effects. After processing the procedures stated in Section 2.2.2, we collected the gastric fluid, and then the fluid was centrifuged at a speed of 2000 rpm for 10 minutes. A 10 mL supernatant was taken out, placed into a beaker, and added with 2 drops of indicator A and B, respectively (A: 0.5% dimethylaminoazobenzene alcohol solution, B: 1% phenolphthalein alcohol solution). Then 0.1 N NaOH was used for titration. The amount of titrant used was recorded as A mL when the solution presented orange color. The amount of titrant consumed was recorded as B mL when the orange color solution turned into roseate color. According to A and B, the free HCl and the total acidity in the gastric juice were calculated. The unit of acidity is mEq/mL.

2.2.4. Antipeptic Ulcer Effects of Different Combinations of Ingredients of SJZT. The different combinations of ingredients of SJZT, *Radix Ginseng* + *Glycyrrhizae Radix, Atractylodis Rhizoma* + *Glycyrrhizae Radix, Radix Ginseng* + *Poria Cocos, Atractylodis Rhizoma* + *Poria Cocos, Radix Ginseng* + *Atractylodis Rhizoma, Poria Cocos* + *Glycyrrhizae Radix, Poria Cocos* + *Atractylodis Rhizoma* + *Glycyrrhizae Radix, Radix Ginseng* + *Atractylodis Rhizoma* + *Radix Glycyrrhizae, Poria Cocos* + *Radix Ginseng* + *Glycyrrhizae Radix, Poria Cocos* + *Atractylodis Rhizoma* + *Radix Ginseng, and Radix Ginseng* + *Poria Cocos* + *Atractylodis Rhizoma* + *Glycyrrhizae Radix* + dist. H_2O, were made into medicines 500 mg/kg (B.W.), respectively. All possible combinations of the constituent ingredients of SJZT were investigated. The medicines were prepared into doses 5.0 mL/kg (B.W.) and fed to the experimental rats by oral administration. 30 minutes later, the rats were placed in a water immersion restraint device designed by Takagi and Okabe [6] and immersed up to their xiphoid for 8-hour water bath at the temperature of 20 ± 1°C. Finally the animals were sacrificed and their stomachs were taken out. After sacrificing the animals, their stomachs were removed out, washed with 37°C normal saline, inflated with 37°C 10 mL 2% formalin, and fixed in the formalin solution for 5 minutes. The stomachs were cut, opening from the pylorus to the cardia along the greater curvature and washed in normal saline. After blotting up with filter papers, we observed the stomachs with a stereo microscope (Leica WILD M32) to determine the ulcerative areas, and then we measured and compared the total gastric ulcer length.

2.2.5. Effects of SJZT on Long-Term Continuous Treatment for Peptic Ulcers. We placed 40 healthy male Wistar rats in a water immersion restraint device designed by Takagi for 7 days to induce stress gastric ulcers. Then 20 rats were sacrificed randomly to check the gastric ulcerative areas. After checking and verifying all the 20 rats' ulcerative areas, we could identify the others as morbid rats with stress-induced gastric ulcer. Then 20 rats were randomly divided into Groups A and B with 10 rats in each group. The rats in Group A took SJZT (500 mg/kg/day) orally at 08:00 A.M. for 14 days. The rats in Group B took SJZT (500 mg/kg/day) orally at 08:00 A.M. for 21 days. Then the rats were sacrificed and their stomachs were taken out when the terms expired. Afterwards the stomachs were cleaned up with normal saline and were inflated with 10 mL formalin (2%) at 37°C. All the procedures were mentioned in Section 2.2.4.

2.2.6. Effects of SJZT on Monoamine in Brains of the Rats. A total of 6 rats from each group were used in the experiment. The rats heads were beheaded in 1 hour after feeding with SJZT (500 mg/kg) and 5.0 mL/kg dist. H_2O by oral administration. Then we removed their brains and quickly separated the cortex and brain stem (including the lower part of brain stem) in a cold room at 4°C. According to the approach of Shibuya et al. [7], HPLC of Waters Associates was used to determine the content of DA, NE, and 5-HT as well as relevant typical metabolite involving homovanillicacid (HVA), 3-methoxy-4-hydroxy-phenyl-ethyleneglycol (MHPG), and 5-hydroxy-indole acetic acid (5-HIAA) in each part. Finally the data collected were compared with the control group. Considering that the content of MA in brain might be affected by time, the animals were sacrificed at 11:00 A.M. in the experiment.

2.2.7. Statistical Method of Data. Data obtained in the experiments were expressed in the format of mean ± S.E., and the statistical method of one-way ANOVA was adopted for comparisons between experimental groups and control groups. $P < 0.05$, $P < 0.01$, and $P < 0.001$ refer to the significant difference statistically.

3. Results

3.1. Determination on Secretion of Gastric Juice and Relevant Effects. As Table 1 shows, in the group vagus nerve not yet cut off, the comparison between the control group fed with dist. H_2O (5.0 mL/kg) orally and the experimental groups fed with SJZT (500 mL/kg and 1000 mg/kg individually) orally suggests that gastric juice reduces dose dependently. The group fed with SJZT (1000 mg/kg) presents an effective significant difference ($P < 0.05$). For the nerve transection groups, both the control group and the experimental group, the amount of gastric juice is less than the groups where the nerves were not cut reducing down to approximately 1/3.

3.2. Determination on Free HCl and Total Acidity in Gastric Juice and Relevant Effects. As Table 1 shows, The vagus nerve was not cut group; the comparison between the control group fed with Dist. H_2O (5.0 mL/kg) orally and the experimental groups fed with SJZT (500 mL/kg and 1000 mg/kg individually) orally suggests that free HCl and total acidity in gastric juice reduce dose dependently. The experimental group fed with SJZT (1000 mg/kg) presents an effective significant difference ($P < 0.05$). For the nerve transection groups, both the control group and the experimental group, the gastric juice within free HCl and total acidity, are less than the groups where the vagus nerves were not cut, only the group administrated with SJZT (1000 mg/kg) presents a significant reduction ($P < 0.05$) in free HCl.

Table 1: Effects of SJZT on volume of gastric juice, free HCl, and total acidity of gastric acid for rats of vagus nerves were cut and not cut.

Types	Groups	UN-vagotomy	Vagotomy
Volume	Dist. H_2O 5.0 mL/kg	9.92 ± 0.62 mL	3.82 ± 0.30 mL
	SJZT 500 mg/kg	6.21 ± 0.93 mL	1.82 ± 0.52 mL
	SJZT 1000 mg/kg	5.43 ± 0.47* mL	2.89 ± 0.64 mL
Free HCl	Dist. H_2O 5.0 mL/kg	49.67 ± 3.11 mEq/mL	1.73 ± 0.51 mEq/mL
	SJZT 500 mg/kg	25.06 ± 2.80 mEq/mL	1.65 ± 0.63 mEq/mL
	SJZT 1000 mg/kg	21.02 ± 2.02* mEq/mL	0.91 ± 0.26* mEq/mL
Total acidity	Dist. H_2O 5.0 mL/kg	81.17 ± 4.08 mEq/mL	7.18 ± 0.87 mEq/mL
	SJZT 500 mg/kg	74.03 ± 2.55 mEq/mL	6.59 ± 0.96 mEq/mL
	SJZT 1000 mg/kg	39.31 ± 3.06* mEq/mL	7.82 ± 0.82 mEq/mL

*$P < 0.05$; $N = 6$; mean ± S.E.; peroral, control group: Dist. H_2O 5.0 mL/kg (B.W.)

Table 2: Effects of SJZT and relevant ingredients on stress-induced peptic ulcers.

Number	Groups	Results
a	5.0 mL/kg Dist. H_2O	40.57 ± 0.78 mm
b	SJZT	14.10 ± 0.51 mm***
c	*Poria Cocos* + *Glycyrrhizae Radix*	48.58 ± 1.42 mm
d	*Atractylodis Rhizoma* + *Glycyrrhizae Radix*	20.29 ± 0.69 mm*
e	*Radix Ginseng* + *Glycyrrhizae Radix*	15.32 ± 0.64 mm**
f	*Radix Ginseng* + *Poria Cocos*	21.50 ± 0.55 mm*
g	*Radix Ginseng* + *Atractylodis Rhizoma*	41.59 ± 1.06 mm
h	*Atractylodis Rhizoma* + *Poria Cocos*	34.99 ± 0.96 mm
i	*Glycyrrhizae Radix* + *Poria cocos* + *Atractylodis Rhizoma*	23.85 ± 0.31 mm
j	*Radix Ginseng* + *Poria Cocos* + *Glycyrrhizae Radix*	46.78 ± 0.53 mm
k	*Radix Ginseng* + *Poria Cocos* + *Atractylodis Rhizom*a	15.94 ± 0.29 mm*
l	*Radix Ginseng* + *Atractylodis Rhizom*a + *Glycyrrhizae Radix*	114.66 ± 1.33 mm

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; $N = 6$; mean (mm) ± S.E.

3.3. Antipeptic Ulcer Effects of Different Combinations of Ingredients of SJZT. As Table 2 and Figure 3 show, total peptic ulcer length of the control group that took dist. H_2O (5.0 mL/kg) orally is 40.57 ± 0.78 mm; total peptic ulcer length of the experimental group that took SJZT (500 mg/kg) orally is 14.10 ± 0.51 mm ($P < 0.001$). For the group of *Radix Ginseng* + *Glycyrrhizae Radix* (500 mg/kg), total peptic ulcer length is 15.32 ± 0.64 mm ($P < 0.01$). For the group of *Atractylodis Rhizoma* + *Glycyrrhizae Radix* (500 mg/kg), total peptic ulcer length is 20.29 ± 0.69 mm ($P < 0.05$). For *Radix Ginseng* + *Poria Cocos* (500 mg/kg) group, total peptic ulcer length is 21.50 ± 0.55 mm ($P < 0.05$), and for *Poria Cocos* + *Atractylodis Rhizoma* + *Radix Ginseng* (500 mg/kg) group, total peptic ulcer length is 15.94 ± 0.29 mm ($P < 0.05$).

Table 3: Therapeutic effects of continuous administration of SJZT on peptic ulcers.

Models	Ulcerative length	Ratio	Amount
Ulcer induced by water immersion for 7 days	120.17 ± 2.23 mm	100%	$N = 20$
SJZT (500 mg/kg) P.O. for 14 days	97.50 ± 1.88 mm	81.25%	$N = 10$
SJZT (500 mg/kg) P.O. for 21 days	78.67 ± 1.47 mm*	65.47%	$N = 10$

*$P < 0.05$; Mean (mm) ± S.E.; P.O. means per os or oral administration; Control group: Ulcer induced by water immersion for 7 days.

3.4. Effects of SJZT on Long-Term Continuous Treatment for Peptic Ulcers. As Table 3 shows, for the experimental group that was administrated SJZT (500 mg/kg) for 14 days successively, total peptic ulcer length is 97.5 ± 1.88 mm. For the experimental group that was administrated SJZT (500 mg/kg) for 21 days successively, total peptic ulcer length is 78.67 ± 1.47 mm ($P < 0.05$). Comparing with the control group that was administrated Dist. H_2O (5.0 mL/kg), prolong administration SJZT (500 mg/kg) can be obtained 34.53% benefits for the treatment of stress-induced peptic ulcers.

3.5. Effects of SJZT on Monoamine in Brain of Rat. As Tables 4(a) and 4(b) show, whether to give SJZT 500 mg/kg or SJZT 1000 mg/kg, NA, DA, and 5-HT contents in the experimental group of rat brain cortex or brain stem are lower than those in the control group and present a dose-dependent reduction ($P < 0.01$). Monoamine metabolite including VMA, HVA, and 5-HIAA content of the sites as well as the difference in the amount administered has different levels of change.

4. Discussion

Psychological stress is not only empirically associated with ulcers but is a very plausible risk factor for ulcer disease. Gastric acid output is correlated with psychological distress in patients with and without ulcers and increased enormously during intense military training [1]. Peptic ulcer disease is a deep gastrointestinal erosion disorder that involves

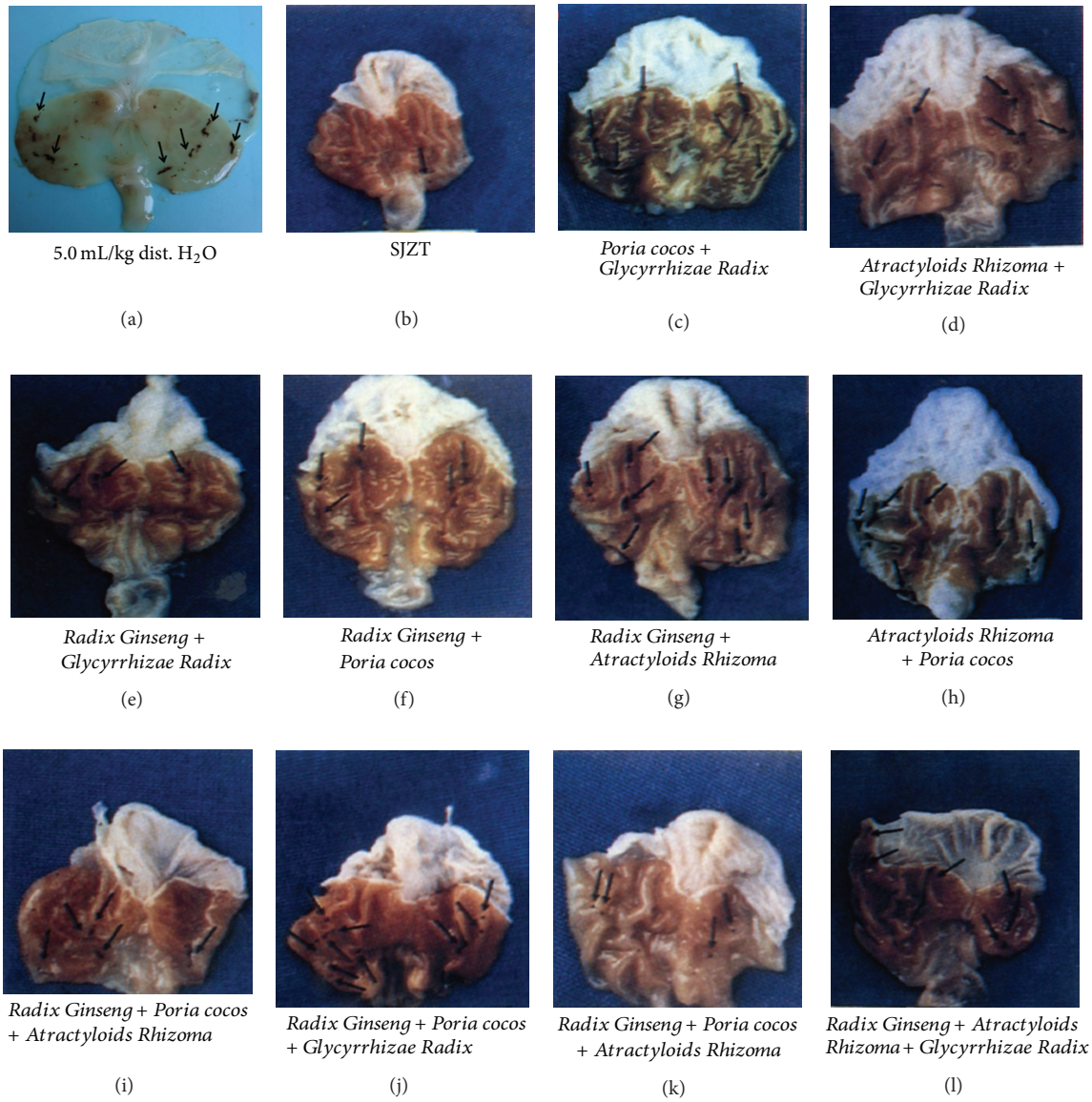

Figure 3: Effects of SJZT and relevant ingredients on stress-induced peptic ulcers.

the entire mucosal thickness and can even penetrate the muscular mucosa.

Numerous natural products have been evaluated as therapeutics for the treatment of a variety of diseases. These sources of products usually are derived from plants and animals that contain active constituents such as alkaloids, flavonoids, terpenoids, and tannins. The alkaloids are natural nitrogen-containing secondary metabolite mostly derived from amino acids and found in about 20% of plants [8]. Intragastric or intradermal administration of an ethanol extract of *Radix Ginseng* to rats decreased histamine, pentagastrin, carbachol, and vagal stimulation-induced gastric secretion and inhibited gastric ulcers induced by stress or by pyloric ligation [9–11]. *Poria Cocos* is used as a diuretic, sedative, and tonic; Triterpene acids and polysaccharides are the principal ingredients of *Poria Cocos* that are responsible for diverse bioactivities, including antitumor, anti-inflammatory, antioxidant, and antiemetic effects [12]. The dried rhizome of *Atractylodes macrocephala* Koidz is used as a digestive and a tonic, in which volatile oils, polysaccharides, sesquiterpenes, and flavonoids were identified with anti-inflammatory, hypoglycemic, and gastrointestinal inhibitory effects [12]. One study showed that the acetylene compound from *Atractylodes rhizome* significantly suppressed xanthine oxidase (X.O.) activity in the stomach tissue. The suppressive effects of this compound on lesion formation induced by indometacin and ischemia-reperfusion injury models would thus appear partly due to the inhibition of X.O. activity in the stomach tissue [13]. The antiulcer activity of *Glycyrrhizae Radix* has been demonstrated both experimentally and clinically. Intraperitoneal, intraduodenal oral administration of aqueous or alcoholic extracts of *Glycyrrhizae Radix* reduced

Table 4: (a) Content changes of monoamine. (b) Content changes of monoamine metabolite.

(a)

Brain	Reagents	N A	D A	5-HT
Cortex	Dist. H_2O 5.0 mL/kg	40.9 ± 2.3	102.4 ± 3.7	50.3 ± 2.2
	SJZT 500 mg/kg	31.3 ± 1.4	86.2 ± 3.3	34.1 ± 4.9
	SJZT1000 mg/kg	17.3 ± 1.1*	62.8 ± 5.6*	25.9 ± 2.8*
Brain stem	Dist. H_2O 5.0 mL/kg	47.9 ± 0.9	131.7 ± 3.4	57.2 ± 3.1
	SJZT 500 mg/kg	41.6 ± 0.3	79.4 ± 1.9*	39.2 ± 0.8*
	SJZT 1000 mg/kg	35.6 ± 0.5	77.8 ± 2.1*	35.1 ± 1.8*

*$P < 0.05$; mean ± S.E.; $N = 6$; peroral, ng/g (wet weight); control group: Dist. H_2O 5.0 mL/kg (B.W.).

(b)

Brain	Reagents	HVA	5-HIAA	VMA
Cortex	Dist. H_2O 5.0 mL/kg	7.2 ± 0.4	48.2 ± 2.5	10.2 ± 0.9
	SJZT 500 mg/kg	8.1 ± 0.2	48.2 ± 1.7	10.9 ± 1.8
	SJZT1000 mg/kg	6.8 ± 0.3	21.3 ± 0.8*	10.1 ± 1.3
Brain stem	Dist. H_2O 5.0 mL/kg	7.2 ± 0.4	48.2 ± 2.5	10.2 ± 0.9
	SJZT 500 mg/kg	7.8 ± 0.7	80.4 ± 1.6*	8.9 ± 0.2
	SJZT 1000 mg/kg	6.6 ± 0.4	73.8 ± 1.0	7.6 ± 0.3

*$P < 0.05$; mean ± S.E.; $N = 6$; peroral, ng/g (wet weight).

gastric secretions in rats, and it inhibited the formation of gastric ulcers induced by pyloric ligation, aspirin, and ibuprofen [14]. Glycyrrhizin and its aglycone (glycyrrhetic acid, enoxolone), two of the active constituents of *Glycyrrhizae Radix*, both have antiphlogistic activity and increase the rate of mucus secretion by the gastric mucosa. Deglycyrrhizinated liquorice (97% of glycyrrhizin is removed) effectively treated stress-induced ulcers in animal models [14–16]. The mechanism of antiulcer activity involves acceleration of mucin excretion through increasing the synthesis of glycoprotein at the gastric mucosa, prolonging the life of the epithelial cells and antipepsin activity [14].

Stress has been shown to alter normal dopaminergic neurotransmission, and exposure to stress profoundly increases the dopaminergic activity and induces relevant adaptive response of DA receptors in specific brain regions. Stress also activates the hypothalamus-pituitary-adrenal (HPA) axis and releases glucocorticoids. The stress-induced adaptation of brain DA function involves receptors, and it has also been demonstrated that DA receptor densities are affected by altered extracellular DA levels [17]. The great majority of studies in vivo have reported that DA or dopaminergic compounds inhibit the secretion of gastric acid or pepsin, stimulate the secretion of mucus or bicarbonate, and regulate mucosal blood flow [5]. Smooth-muscle cells express 5-HT_1 and 5-HT_2 effector serotonin receptors. Intramural ganglionar neurons and enterochromaffin cells have surface 5-HT_3 and 5-HT_4 receptors. Through these receptors, 5-HT regulates the contractile activity of smooth muscles. Serotonin induces contractions of the smooth-muscle cells of the fundal compartment of the stomach during reaction with 5-HT_{2B} receptors [18]. During ulcer relapse, we noted significantly raised levels of NA, DA and free 5-HT. After healing ulcer, significant reductions in NA, DA, free 5-HT, and significant increases in platelet serotonin values were observed. NA remained higher and platelet serotonin lower, both significantly more than the normal. The results demonstrate that some baseline autonomic system imbalance exists in ulcer patients, amplified and accentuated during relapse [19].

The secretion of gastric acid (HCl) is intimately related to peptic ulcer disease. Gastrin G-cells and somatostatin D-cells are important regulators of gastric acid secretion, and alterations in their relative numbers may play a key role in gastroduodenal disease [20]. In humans, gastrin is a peptide hormone that stimulates secretion of gastric acid (HCl) by the parietal cells of the stomach and aids in gastric motility. In this study, the vagus nerves of experimental animals were not cut; their secretion of gastric juice, free HCl, and total acidity in gastric juice present a dose-dependent reduction no matter they were fed with Dist. H_2O or SJZT (500 mg/kg). With regard to the group that was fed with SJZT (1000 mg/kg), a significant inhibition effect ($P < 0.05$) on secretion of gastric juice, free HCl, and total acidity in gastric juice is presented; but other groups have not indicated any valid significant difference. As the experimental animals whose vagus nerves were cut, although their secretion of gastric juice, free HCl, and total acidity in gastric juice increased or decreased to different extents, there is no any meaningful significant difference. The only exception is that the group fed with SJZT (1000 mg/kg) presents a remarkable inhibition effect on free HCl ($P < 0.05$) and 1/3 secretion volume of gastric juice, compared with the groups which vagus nerves were not cut. Therefore, we confirm the same findings as demonstrated in the groups of which vagus nerves were not cut; SJZT has the effect of inhibiting free HCl in gastric juice. SJZT may inhibit G-cell to secret gastrin, and free HCl in gastric juice can be reduced.

We also found that the effect of SJZT on content change of neurotransmitters in nervus centralis SJZT could reduce the content of 5-HT and DA in brain cortex and brain stem. This is consistent with SJZT having a more pronounced sedatory effect than Diazepam as concluded in our previous research report [21]. This result is consistent with after healing ulcer; significant reductions of DA, 5-HT were observed [19]. The results demonstrated that SJZT has antiulcer effect.

Chinese traditional prescription was made according to monarch, minister, assistant, and guide mode. This mode connects the modern thoughts of treating a disease by differentiating the disease and the symptoms, also determining a formula according to both the disease and syndrome. The proportions used in this study are based upon the source book of SJZT [Tai Ping Hui Min Her Ji Jyu Fang]. No changes have been made to this traditional predetermined formulation with *Radix Ginseng, Poria Cocos*, *Atractylodis Rhizoma,* and *Glycyrrhizae Radix* combined in equal measures.

In traditional Chinese culture, *qì* (also *chi*) is an active principle forming part of any living thing. *Qi* is frequently translated as "life energy," "life force," or "energy flow." Qi is the central underlying principle in traditional Chinese medicine. *Radix Ginseng* offers a sweet flavor and warming effects on the spleen and lung meridians, invigorating primordial *qi*, and is the principal herb in this formula. *Atractyloids Rhizoma works* as an assistant herb with a sweet and bitter flavor and offers warming properties and strengthens the spleen in order to dry dampness and invigorates the stomach to harmonize the middle. *Poria Cocos* with a sweet flavor, excretes damp and strengthens the spleen. It assists *Atractyloids Rhizoma* in invigorating the spleen function to remove dampness and is an adjuvant herb. *Glycyrrhizae Radix* with a sweet flavor and a warming property, replenishes *qi* and is used as a guiding herb to harmonize all herbs in the formula [22].

One of the purposes of the study is to determine the effects of the differing combinations of the constituent parts of SJZT. Every possible combination was tested through from the testing of individual ingredients working alone to all possible combinations of the three ingredients (in equal measure). In our study, we could observe that antiulcer effect was significant in *Radix Ginseng* + *Glycyrrhizae Radix* ($P < 0.01$), *Atractylodis Rhizoma* + *Glycyrrhizae Radix* ($P < 0.05$), *Radix Ginseng* + *Poria Cocos* ($P < 0.05$), *Poria Cocos* + *Atractylodis Rhizoma* + *Ginseng Radix* ($P < 0.05$), and in SJZT group ($P < 0.001$). This represents that the composing formula mode is an important meaning for promoting the research of treating diseases by differentiating integrated syndrome and symptom, enriching composing formula theory, and creating new formula in the clinic. As gastric mucosa contains mucous glycoprotein which is a kind of polymeric glycoprotein, it has the protection function to avoid autopepsia of pepsin which may cause peptic ulcer. Ginsenoside Rb1 was a component from *Radix Ginseng* which was investigated for its antiulcer effect [23]. Glycyrrhizin was a component from *Glycyrrhizae Radix* which was investigated for its antiulcer effect [24]. *Radix Ginseng* and *Glycyrrhizae Radix* of SJZT contain the ingredients such as ginsenoside and glycyrrhizin those are the main predecessors for synthesizing glycoprotein. The theory also is verified in the experiment that application of the combination of *Radix Ginseng* and *Glycyrrhizae Radix* achieved the curative effect on stress-induced peptic ulcers similarly to SJZT.

We conclude that SJZT not only reduces the content of free HCl in gastric juice to inhibit the attack factors and stimulate gastric mucosa secreting gastric mucus to protect the defense factors of gastric mucosa, but also reduces the content of 5-HT and DA in brain cortex and brain stem. SJZT has an excellent preventive and therapeutic effect on stress-induced peptic ulcers. SJZT could be considered as an alternative for the treatment of stress-induced peptic ulcers.

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Research Article

Huang Qi Jian Zhong Pellet Attenuates TNBS-Induced Colitis in Rats via Mechanisms Involving Improvement of Energy Metabolism

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Huang Qi Jian Zhong Pellet (HQJZ) is a famous Chinese medicine formula for treatment of various gastrointestinal tract diseases. This study investigated the role of HQJZ in 2,4,6-trinitrobenzene sulfonic acid- (TNBS-) induced colitis and its underlying mechanism. Colonic mucosal injury was induced by TNBS in the Sprague-Dawley rats. In the HQJZ treatment group, HQJZ was administered (2 g/kg) for 14 days starting from day 1 after TNBS infusion. Colonic mucosal injury occurred obviously 1 day after TNBS challenge and did not recover distinctively until day 15, including an increase in macro- and microscopic scores, a colonic weight index, a decrease in colonic length, a number of functional capillaries, and blood flow. Inverted intravital microscopy and ELISA showed colonic microcirculatory disturbances and inflammatory responses after TNBS stimulation, respectively. TNBS decreased occludin, RhoA, and ROCK-I, while increasing Rac-1, PAK-1, and phosphorylated myosin light chain. In addition, ATP content and ATP5D expression in colonic mucosa decreased after TNBS challenge. Impressively, treatment with HQJZ significantly attenuated all of the alterations evoked by TNBS, promoting the recovery of colonic injury. The present study demonstrated HQJZ as a multitargeting management for colonic mucosal injury, which set in motion mechanisms involving improvement of energy metabolism.

1. Introduction

Inflammatory bowel disease (IBD) is chronic and relapsing inflammatory conditions, characterized by mucosal ulceration [1–3]. Previous studies indicated that the destroyed integrality of colonic epithelium and disturbances of colonic microcirculation occur in the colonic mucosal injury [4–6]. IBD is thought to be caused by impaired innate immunity. Treatment with anti-inflammatory drugs, immunosuppression, and biological therapy targeting specific components of the immune response is thus currently used, in addition to surgery, in clinic for the patients with IBD. However, the efficiency of these strategies remains unsatisfying, appealing to development of novel management.

Energy status is a fundamental regulator of cellular function, and its deficit has been considered to be a

pathogenic factor in various conditions including IBD in human. Malnutrition and energy expenditure in IBD lead to energy deficit (ATP depletion) in colonic mucosa, resulting in the restitution of subnormal epithelial cell with hyperpermeability, edema, and the infiltration of inflammatory cell [7–10]. In experimental colitis, the concentration of adenine nucleotides is decreased in the colon, while administration of the adenine nucleotides ADP and ATP promotes epithelial cell restitution in damaged tissues [11, 12]. In addition, AMP-activated protein kinase (AMPK) was reported being down-regulated in the inflammatory colonic mucosa [13, 14]. AMPK is an energy-sensing enzyme. A recently published study revealed that AMPK activity supports endothelial barrier function by activating Rac/Cdc42/PAK pathway [15], which are known to play a critical role in endothelial barrier function via regulating cell adhesion and cytoskeleton dynamics. Activation of RhoA has been reported not only to evoke phosphorylation and degradation of occludin [16], but also to promote phosphorylation of myosin light chain (p-MLC), which interacts with actin generating cell contraction and leading to an impairment of barrier function [17–21]. Collectively, current evidence suggests that manipulating energy metabolism either by increasing ATP availability or by activating AMPK may be a potential management for IBD.

Huang Qi Jian Zhong Pellet (HQJZ) is composed of *Astragalus*, *Ramulus cinnamomi*, *White Peony root*, *Zingiber officinale* Roscoe, *Fructus jujubae*, *Radix glycyrrhizae*, and *Saccharum granorum* (Table 1). As a famous Chinese medicine formula, it has been used to treat various gastrointestinal tract diseases, such as gastritis and stomach ulcer. However, the mechanism responsible for its beneficial role is poorly understood. On the other hand, increased study has been published to explore the pharmacology of the composed herbs of HQJZ, showing the potential of this formula in anti-inflammation [22–24], antioxidative stress [25–27], and endothelial and mucosal protection [28, 29]. These results support the application of HQJZ in IBD [30]. Furthermore, study showed that *Astragalus* (one of main components of HQJZ) extract increases the levels of ATP and ADP and the activity of Na(+)-K(+)-ATPase, improves energy metabolism, and inhibits apoptosis, alleviating neuron injury after cerebral ischemia [31]. We speculated that HQJZ may be beneficial for IBD by acting at multiple targets involving regulation of energy metabolism. The present study was to address the role of HQJZ in 2,4,6-trinitrobenzene sulfonic acid- (TNBS-) induced colitis in rats and its underlying mechanism.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats weighing 180 to 220 g were purchased from the Animal Center of Peking University Health Science Center (The animal certificate number was SCXK 2006-0008). All animals were caged at 22 ± 2°C with a humidity of 50% ± 5% in a 12 h light/dark cycle and were provided standard diet and water *ad libitum*. The animals were fasted for 12 h before experiment but were allowed free access to water, and handled according to the guidelines of the Peking University Animal Research Committee. The surgical procedures and experimental protocols were approved by Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (LA2011-66).

2.2. Drugs. HQJZ (Batch no. 110310) was produced by TianQi pharmaceutical company (ChiFeng, China). The processing of the product followed strict quality control, and the ingredients were subjected to standardization. TNBS was purchased from Sigma (St. Louis, MO, USA).

2.3. Colonic Mucosal Injury. Colonic mucosal injury was induced by TNBS in rats as reported previously [33]. Rat was anesthetized with pentobarbital (60 mg/kg, i.p.) and was administrated with TNBS through enema at a dose of 100 mg/kg. For this purpose, a 3% TNBS solution (w/v) was prepared by mixing 5% TNBS water solution with 30% ethanol at 4 : 3, and the freshly prepared solution was rectally instilled into the colon 8 cm proximal to the anus, in a volume depending on the rat, by a polyvinyl rubber catheter 2 mm in diameter. The rat was maintained in a head-down position for 15 min.

2.4. Experimental Protocol. Total seventy rats were randomly divided into 5 groups, 16 rats each: Sham 15 d group (the rats received physiological saline by enema and 24 h thereafter physiological saline by gavage for 14 days), Sham 15 d + HQJZ group (the rats received physiological saline by enema and 24 h thereafter HQJZ at 2 g/kg by gavage for 14 days), TNBS 1 d group (the rats received TNBS by enema and were sacrificed after 24 h), TNBS 15 d group (the rats received TNBS by enema and 24 h thereafter physiological saline by gavage for 14 days), and TNBS 15 d + HQJZ group (the rats received TNBS by enema and 24 h thereafter HQJZ at 2 g/kg everyday by gavage for 14 days). On day 15, all animals were sacrificed after anesthesia with urethane. The number of animals for assessment of various parameters in each group is detailed in Table 2.

2.5. Measurement of Colonic Blood Flow. Colonic blood flow ($n = 8$ for each group) was measured by a Laser Doppler perfusion image system (PeriScan PIM3 System; PERIMED, Stockholm, Sweden). On day 15, an incision was made through abdominal wall to expose peritoneal cavity under anesthesia with intraperitoneally administrated urethane (2.0 g/kg). Epicolic tissues were covered with black soft leather, and a segment of 1 to 5 cm colon above the anus was exposed. The scanning procedures were performed according to the studies of Paris et al. [34] and Huang et al. [35].

2.6. Macroscopical Evaluation. Colon was removed immediately from animal after being sacrificed, measured for its length, and opened longitudinally along colonic mesentery to clear its contents. Colon weight index (colonic weight/body weight × 100%) was calculated ($n = 8$). The scoring of colonic macroscopic damage ($n = 8$) was undertaken as described by Butzner et al. [36]. The criteria for assessment of

Table 1: Characterization of the herbs included in HQJZ Pellet.

Herbs	Percentage content (%)	Identified compounds	Effects	References
Radix astragali mongolici (Huang Qi)	12.50	Astragaloside	Inhibiting NF-κB signaling and triggering T cell activation, antioxidative	[22, 23, 26]
Cortex cinnamomi cassiae (Rou Gui)	8.33	Cinnamaldehyde	Antisepsis	[24]
Radix paeoniae alba (Bai Shao)	12.50	Paeoniflorin	Antioxidant	[25]
Fructus jujubae (Da Zao)	8.33	Oleanolic acid	Antioxidative, antiglycative, and antiapoptotic effects	[27]
Rhizoma zingiberis recens (Sheng Jiang)	8.33	Volatile oil	Gastroprotective effects	[28]
Glycyrrhiza uralensis fisch (Gan Cao)	8.33	Glycyrrhizic acid	Against endothelial dysfunction	[29]
Saccharum granorum (Yi Tang)	41.66	Maltose and dextrin	Increasing free-energy (ATP) conservation	[32]

Table 2: The number of animals in different experimental groups for various parameters.

	Sham 15 d	Sham 15 d + HQJZ	TNBS 1 d	TNBS 15 d	TNBS 15 d + HQJZ	Total
Colonic microcirculation	6	6	6	6	6	30
Colonic blood flow, macroscopical and microscopical evaluation, expression of CD11b and, cytokines	8	8	8	8	8	40
Immunohistochemistry and immnofluorescence staining	(3)	(3)	(3)	(3)	(3)	
Western blot analysis	(5)	(5)	(5)	(5)	(5)	
Total	14	14	14	14	14	70

The animals were separated as two batches in the present study. Only one batch of animals (30 rats) was used to observe colonic microcirculation. And the other parameters were analyzed using the second batch of animals (40 rats). The same animals were used for detection of colonic blood flow, macroscopical and microscopical evaluation, and expression of CD11b and cytokines. Brackets: the tissues for immunohistochemistry, immnofluorescence staining, and Western blotting analysis were removed from the second batch of animals (40 rats). Sham 15 d: the rats received physiological saline by enema and 24 h thereafter physiological saline by: gavage for 14 days; Sham 15 d + HQJZ: the rats received physiological saline by enema and 24 h thereafter HQJZ at 2 g/kg by gavage for 14 days; TNBS 1 d, the rats received TNBS by enema, and were sacrificed after 24 h; TNBS 15 d: the rats received TNBS by enema and 24 h thereafter: physiological saline by gavage for 14 days; TNBS 15 d + HQJZ: the rats received TNBS by enema and 24 h thereafter: HQJZ at 2 g/kg everyday by gavage for 14 days.

macroscopic colonic damage were as follows: 0 score: normal appearance; 1 score: focal hyperaemia, no ulcers; 2 scores: ulceration without hyperaemia or bowel wall thickening; 3 scores: ulceration with inflammation at one site; 4 scores: ≥two sites of ulceration and inflammation; 5 scores: major sites of damage extending >1 cm along the length of the colon; and 6–10 scores: damage extended to >2 cm along the length of the colon, increasing the score by one for each additional cm of damage.

2.7. Microscopical Evaluation. The specimens were processed for paraffin sectioning and hematoxylin-eosin (HE) staining ($n = 8$). The microscopical evaluation was undertaken as described [37], taking into consideration both inflammatory cell infiltration and tissue damage. Scores for infiltration are as follows: 0: no infiltration; 1: increased number of inflammatory cells in the lamina propria; 2: inflammatory cells extending into the submucosa; and 3: transmural inflammatory cell infiltration. The scores for tissue damage are as follows: 0: no mucosal damage; 1: discrete epithelial lesions; 2: erosions or focal ulcerations; and 3: severe mucosal damage with extensive ulceration extending into the bowel wall.

2.8. Microcirculation in Colonic Chorion and Mucous Layer. Colonic microcirculation ($n = 6$ for each group) was observed under an inverted intravital microscope as described [38, 39]. The rats were anesthetized with urethane (2.0 g/kg body weight, intramuscularly). FITC (50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), rhodamine 6 G (0.1 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), or physiological saline was infused via right internal jugular vein. Following a median laparotomy, the colon segment was exteriorized and placed on a special stage. The colon was antimesentericly opened to assess functional capillary density, leukocyte rolling and adhering, and albumin leakage in chorion and mucous layer. The animals were placed on thermostat-controlled heating pads to keep the body temperature at 37°C and protect from drying by warm physiological saline. Fluorescent images were acquired by an inverted fluorescence microscope (DM-LFS, Leica, Mannheim, Germany) 3 min after infusion of FITC-albumin and rhodamine 6 G. Leukocyte adhering and rolling and albumin leakage were evaluated as described previously [35, 39, 40].

2.9. Expression of CD11b on Neutrophils and Concentration of TNF-α in Colonic Tissue. Blood ($n = 6$) was collected and anticoagulated with heparin. Fifty microliters of blood was incubated with 0.5 μg FITC-conjugated anti-CD11b antibody (BD Biosciences, San Jose, CA, USA) for 20 min.

The mean fluorescence intensity of CD11b was accessed with a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA, USA). Neutrophils were sorted by characteristic forward/side-scatter expression. Five thousand neutrophils were evaluated for each sample [41].

Colonic tissue homogenate ($n = 6$) was prepared for measuring level of cytokines and Western blot analysis [42]. Level of TNF-α in colonic tissue homogenate was assessed by flow cytometry (FACS Calibur, BD Biosciences, San Jose, CA, USA) with a BD cytometric bead array kit (BD Biosciences Pharmingen, San Jose, CA, USA). The data were analyzed by the BD cytometric bead array analysis software [43].

2.10. Immunohistochemistry Staining of MPO, F-Actin, and Occludin in Colon. The tissue sections ($n = 3$) were treated with 0.3% H_2O_2 in methanol for 15 min and blocked by 3% normal goat serum. MPO immunohistochemistry was conducted as routine using a rabbit anti-MPO antibody (1 : 200, Santa Cruz, CA, USA). The images were captured by a digital camera connected to a microscope (BX512DP70, Olympus, Tokyo, Japan) [39]. For observation of F-actin and occludin, immunofluorescence staining and confocal microscopy ($n = 3$ for each group) were performed as described [35]. The sections were treated with 0.01 M sodium citrate for antigen retrieval, blocked with 3% normal goat serum at room temperature for 15 min, and then incubated with rabbit anti-occludin antibody (1 : 80, Abcam, Cambridge, UK) overnight at 4°C. After washing, colonic tissues were incubated with a secondary antibody, Dylight 488-labeled goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) for 2 h at 37°C in the dark. Hoechst 33342 (BD Biosciences Pharmingen, San Jose, CA, USA) was applied to stain nucleus. F-actin in colonic tissues was stained with phalloidine (1 : 40, Abcam, Cambridge, UK). All sections were photographed under a laser scanning confocal microscope (TCS SP5, Leica, Mannheim, Germany).

2.11. Enzyme-Linked Immunosorbent Assay. Enzyme-linked immunosorbent assay (ELISA) ($n = 8$) was performed according to the manufacturers' instruction (GBD, San Diego, CA, USA). Colonic tissues were lysed in RIPA buffer (50 mM Tris-HCl at pH 7.4, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) with protease and phosphate inhibitor cocktail (Merk, Ashland, MA, USA) using a sonicator. Crude lysates were centrifuged at 19357 g for 20 min. The supernatant was used to measure the level of ATP, ADP, and AMP (GBD, San Diego, CA, USA), as well as MPO, IL-10, and IL-6 (GBD, San Diego, CA, USA). Absorbance was read at 450 nm.

2.12. Western Blot Analysis. Western blot analysis ($n = 5$ for each group) was preformed as described previously [35]. Briefly, proteins were separated using a 10% Tri-HCL precast gel, and polyacrylamide electrophoresis (Bio-Rad Laboratories, Hercules, CA, USA) was conducted at 80 V for 90 to 120 min, and then the proteins were transferred to polyvinylidene fluoride membranes with 220 mA at 4°C for 2 h. The membranes were blocked with 5% nonfat milk or 5% BSA diluted in TBST for 1 h at room temperature and were incubated overnight with primary antibodies. The primary antibodies used were as follows: rabbit anti-GAPDH (1 : 2000), RhoA (1 : 2000), ROCK-I (1 : 2000), AMPK-α (1 : 1000), phospho-AMPK-α (1 : 800), PAK-1 (1 : 1000), phospho-MLC2 (1 : 800), occludin (1 : 500), and mouse anti-Rac-1 (1 : 1000), which were all from Abcam, Cambridge, UK, as well as goat anti-ATP5D (1 : 200) (Santa Cruz, CA, USA). The membrane was incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 60 min. Blots were developed using ChemiLucent Detection System Kit (Millipore Chemicon International Inc., Temecula, CA, USA), and protein bands were visualized on X-ray film. Semiquantitation of the protein was performed using Image-Pro Plus 5.0 software (Media Cybernetic, Bethesda, MD, USA).

2.13. Statistical Analysis. All parameters were expressed as mean ± SE. Statistical analysis was performed using one-way ANOVA followed by the Tukey test for multiple comparisons. Differences with $P < 0.05$ were considered to be significant.

3. Results

3.1. HQJZ Protects against the Colonic Mucosal Macroscopic and Histological Injuries by TNBS. TNBS challenge for 1 d provoked apparent colonic mucosal injuries, including serious hyperemia, edema, and ulcers, some of which were covered with cruor or grimy sphacelus on the surface of colonic mucosa (Figure 1(a), a3). These injuries persisted till day 15 (Figure 1(a), a4) but attenuated by treatment with HQJZ (Figure 1(a), a5). Of notice, the colonic length in TNBS 1 d and TNBS 15 d groups was shorter than those in the 2 Sham groups, as well as shorter than that in TNBS 15 d + HQJZ group, indicating the protective role of HQJZ (Figures 1(a) and 1(c)). The colonic weight index and macroscopical injury scores were higher (Figures 1(b) and 1(d)) in TNBS 1 d and TNBS 15 d groups, compared to Sham groups, all of which were ameliorated significantly by HQJZ treatment. The representative microscopic images in different groups are shown in Figure 2(a). The histology in TNBS 1 d (a3) and TNBS 15 d (a4) groups exhibited pronounced alterations compared to Sham groups, including epithelial necrosis, epithalaxy, impaired mucosa involving submucosa with hyperemia and edema, and ulceration accompanied with numerous inflammatory cell infiltrations. Noticeably, all these alterations were alleviated in TNBS 15 d + HQJZ group (a5). Evaluation by histological scores confirmed this result, revealing a significant improvement of histology in TNBS 15 d + HQJZ group compared with TNBS-alone group (Figure 2(d)).

3.2. HQJZ Inhibits MPO Expression in Colonic Mucosa. MPO expression in colonic mucosa was assessed by ELISA while MPO-immunoreactive cells were evaluated by immunohistochemistry. MPO-positive cells with buffy particles were observed more frequently in the colonic stratum supravascular and submucosa in TNBS 1 d and TNBS 15 d groups

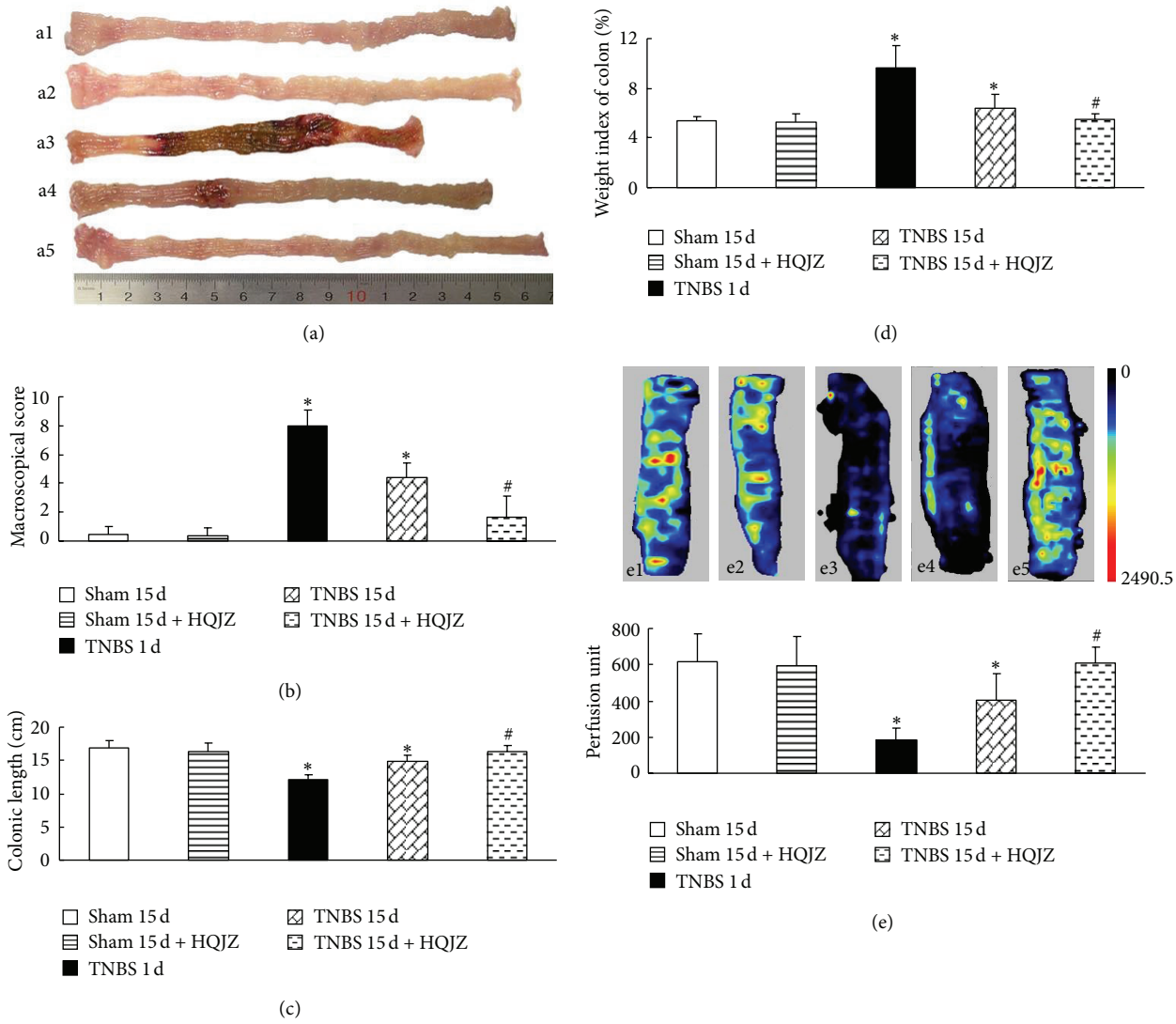

FIGURE 1: Macroscopic observation and colonic blood flow. (a) Representative images of colon, a1: Sham 15 d; a2: Sham 15 d + HQJZ; a3: TNBS 1 d; a4: TNBS 15 d; a5: and TNBS 15 d + HQJZ. (b) Macroscopic scores. (c) Colonic length. (d) Weight index of colon. (e) Representative images and quantitative analysis of colonic blood flow, e1: Sham 15 d; e2: Sham 15 d + HQJZ; e3: TNBS 1 d; e4: TNBS 15 d; and e5: TNBS 15 d + HQJZ. The magnitude of colonic blood flow is represented by different colors, with blue to red denoting low to high. Data were mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

compared to Sham group, whereas the number of MPO-immunoreactive cells decreased in TNBS 15 d + HQJZ group obviously (Figures 2(b) and 2(c)). Similarly, concentration of MPO tested by ELISA in the colonic tissue supernatant in TNBS 1 d and TNBS 15 d groups increased notably, which was significantly inhibited by HQJZ treatment (Figure 2(e)). These results indicated most of infiltrated cells as neutrophils.

3.3. HQJZ Improves Microcirculation of Colon. Assessment by a laser Doppler perfusion imager (Figure 1(e)) showed a significant reduction in colonic blood flow in TNBS 1 d and TNBS 15 d groups compared to Sham group, which was reversed by HQJZ treatment. In parallel with this result, TNBS significantly reduced functional capillary density ($P < 0.05$ versus Sham), which was also ameliorated by HQJZ (Figures 3(a), a1–a5, and 3(b)).

Leukocyte rolling and adhesion were evaluated in capillaries of mucous layer and venules of chorion layer (Figures 3(a), b1–b5, c1–c5, and 3(c), and 3(d)). Clearly, few rolling and adherent leukocytes were observed in Sham and HQJZ-alone groups. In contrast, the number of rolling and adhered leukocytes increased remarkably in TNBS 1 d and 15 d groups. Treatment with HQJZ significantly attenuated TNBS-provoked leukocyte rolling and adhesion. Transvascular efflux of FITC-labeled albumin from capillaries of mucous layer and venules of chorion layer was detected in all groups (Figures 3(a), d1–d5, e1–e5, and 3(e)). The results demonstrated that albumin leakage in TNBS 1 d and TNBS 15 d groups remarkably increased, which was also attenuated significantly by treatment with HQJZ.

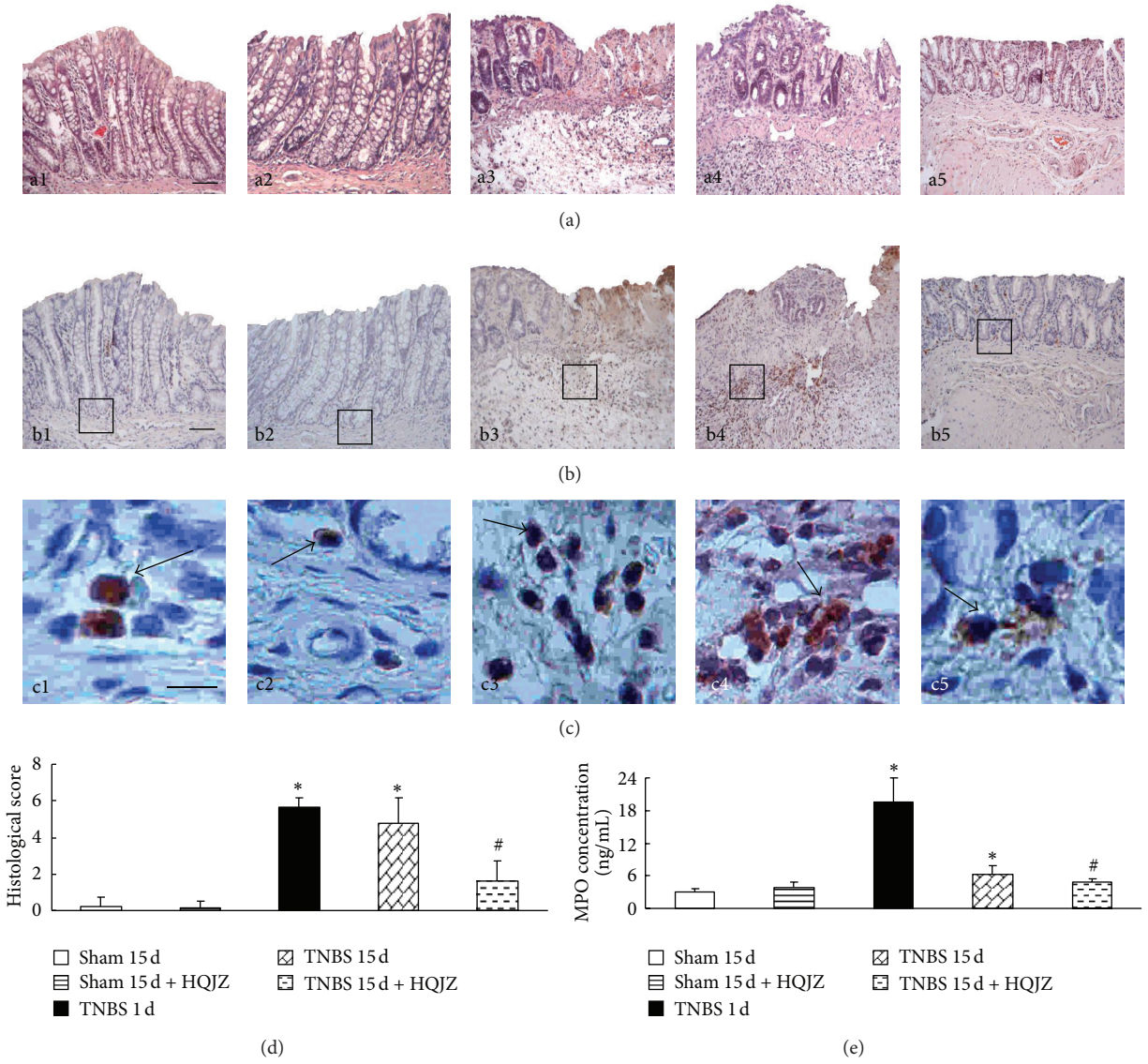

Figure 2: Representative histological images and scores and immunostaining and ELISA for MPO. (a) Representative histological images stained by HE, a1: Sham 15 d; a2: Sham 15 d + HQJZ; a3: TNBS 1 d; a4: TNBS 15 d; and a5: TNBS 15 d + HQJZ. Bar =100 μm. (b) and (c) Immunostaining for MPO, b1 and c1: Sham 15 d; b2 and c2: Sham 15 d + HQJZ; b3 and c3: TNBS 1 d; b4 and c4: TNBS 15 d; and b5 and c5: TNBS 15 d + HQJZ. The arrowheads indicate MPO-positive cells. Bar (b1) = 100 μm; Bar (c1) = 25 μm. (d) Histological scores. (e) The concentration of MPO determined by ELISA. Data were mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

3.4. HQJZ Inhibits CD11b Expression on Neutrophils, Decreases the Level of TNF-α and IL-6, and Increases the Level of IL-10 in Colonic Mucosa. Assessment by flow cytometry revealed an enhanced expression of adhesion molecule CD11b on neutrophils in TNBS-induced rats. This enhancement was blunted significantly by HQJZ treatment (Figures 4(a) and 4(b)).

The concentrations of the cytokines TNF-α, IL-6, and IL-10 in colonic tissues determined by cytometric bead array are presented in Figures 4(c) and 4(d). Compared with Sham groups, concentrations of TNF-α and IL-6 were elevated significantly in TNBS 1 d group and were reduced, but still statistically higher than those of Sham groups, in TNBS 15 d group. On the other hand, IL-10 was reduced in TNBS 15 d group, but not in TNBS 1 d group. TNBS-induced alteration in the concentration of cytokines observed in TNBS 15 d group was alleviated significantly by HQJZ treatment.

3.5. HQJZ Attenuates the Degradation of Occludin in Colonic Mucosa. Occludin was examined by confocal microscopy and Western blot. Confocal microscopy revealed a nearly continuous distribution of occludin on the surface of colonic epithelium and the junctions of colonic epithelial cells, as well as on the endothelium of microvessels in Sham groups. The distributions of occludin became discontinuous in TNBS

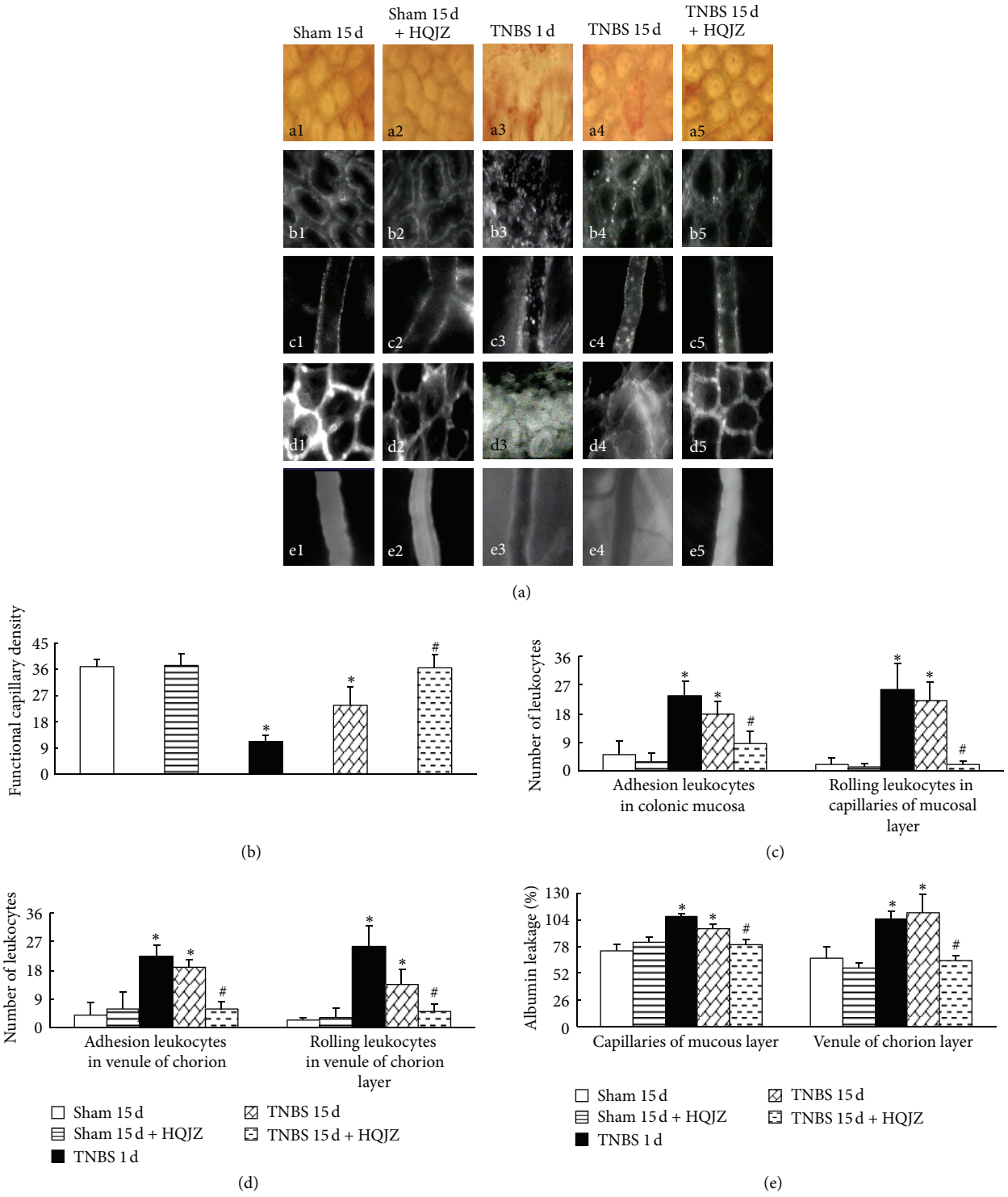

Figure 3: Colonic microcirculation. (a) Representative images of colonic microcirculation in different groups. a1–a5: Representative distribution of capillaries in colonic mucosa from different groups. b1–b5: Leukocyte adhesion to venules in mucous layer. c1–c5: Representative images of leukocyte adhesion to the wall of venules in chorion layer. d1–d5: Albumin leakage from venules in mucous layer. e1–e5: Representative images of albumin leakage from venules in chorion layer. Bar = 50 μm. (b) The density of functional capillaries in different groups. (c) The number of rolling and adherent leukocytes in venules in mucous layer. (d) The number of rolling and adherent leukocytes in venules of chorion layer. (e) Statistic analysis of albumin leakage from venules in chorion and mucous layer. Data were mean ± SEM ($n = 6$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

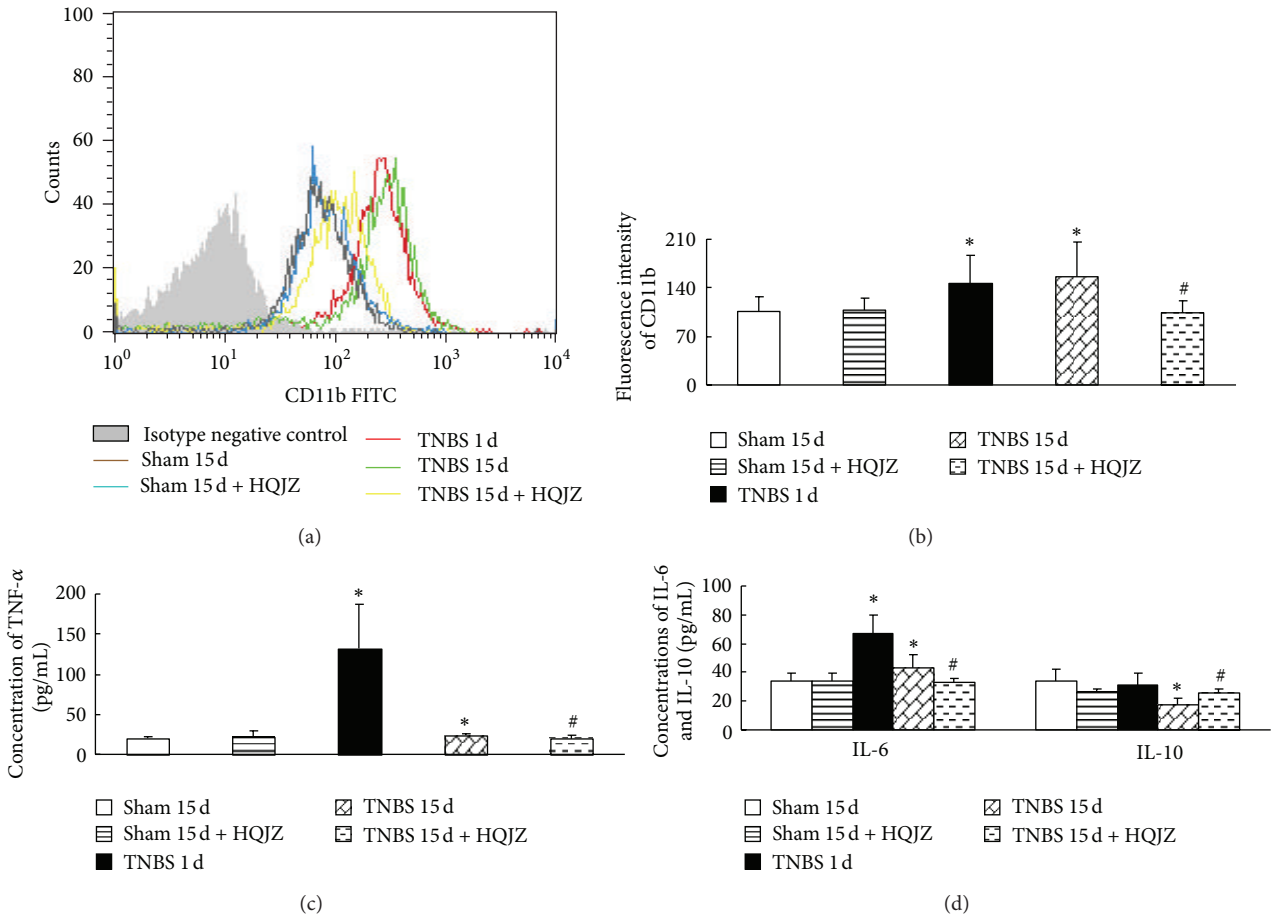

FIGURE 4: Expression of CD11b on neutrophils and the concentrations of cytokines in colonic mucosa. (a) Representative flow plots of CD11b on neutrophils in different groups. Histograms show the distribution of immunofluorescence labeling intensity of CD11b expression in each group. Ordinate indicates cell counts; Abscissa represents fluorescent intensity. (b) Fluorescence intensity of CD11b on neutrophils from different groups. (c) Concentration of TNF-α in colonic mucosa from different groups. (d) Concentrations of IL-6 and IL-10 in colonic mucosa from different groups. Data were mean ± SEM ($n = 8$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

1 d and TNBS 15 d groups. HQJZ treatment for 14 days apparently restored the alteration in occludin distribution caused by TNBS (Figures 5(a) and 5(b)). The results were confirmed by Western blot analysis (Figures 5(c) and 5(d)), showing a noticeable decrease in occludin expression in colonic mucosa from rats in TNBS 15 d group, while HQJZ treatment significantly relieved the decrease of occludin in the colonic mucosa 15 days after TNBS challenge.

3.6. HQJZ Regulates the Colonic Energy Status and Distribution of F-Actin. The content of ATP, ADP, and AMP was analyzed by ELISA (Figures 6(a) and 6(b)). The concentration of ATP in colonic tissue supernatant decreased notably after TNBS challenge for 1 day and 15 days. Correspondingly, the ratio of ADP/ATP and AMP/ATP was upregulated apparently. These TNBS-induced alterations in concentration of ATP and ratio of ADP/ATP and AMP/ATP were restored significantly by treatment with HQJZ (Figures 6(a) and 6(b)).

AMPK-α expression and phosphorylation and ATP5D expression were analyzed by Western blot (Figures 6(c), 6(d), 6(e), and 6(f)), revealing a considerable decrease in the expression of ATP5D protein in TNBS-challenged rats without HQJZ treatment. On the other hand, TNBS-evoked alteration in AMPK-α was more perplexing in that AMPK-α was decreased prominently in TNBS 1 d group, recovered somewhat but still statistically lower than Sham groups in TNBS 15 d group. While phospho-AMPK-α increased obviously in TNBS 1 d group, it decreased in TNBS 15 d group, as compared to Sham groups. Nonetheless, HQJZ treatment for 14 days significantly attenuated all of the alterations in ATP5D protein, AMPK-α, and phospho-AMPK-α caused by TNBS challenge.

We investigated the expression of F-actin by confocal microscopy, and MLC phosphorylation by Western blot. Confocal microscopy (Figure 7(a)) revealed that F-actin expression decreased in the colonic epithelium from TNBS-challenged rats compared to that from Sham group. HQJZ treatment apparently attenuated F-actin expression (Figure 7(a), a5). Moreover, MLC phosphorylation enhanced pronouncedly in response to 15 days of TNBS challenge, which was restored significantly by HQJZ treatment (Figures 7(b) and 7(c)).

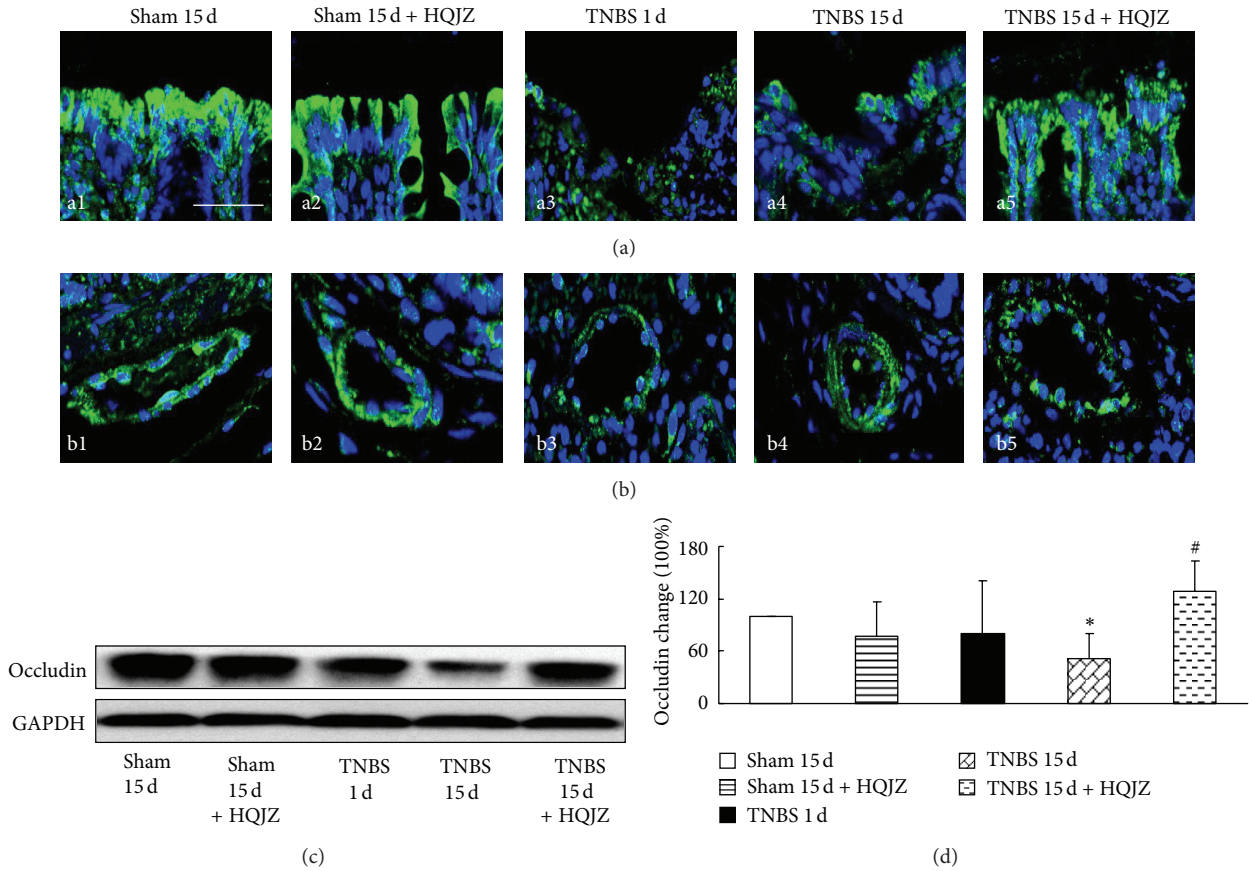

FIGURE 5: Representative immunofluorescence confocal images and Western blot analysis of occludin. Representative immunofluorescence confocal images of occludin in colonic mucosa (a) and venules from different groups (b). The green zone represents the distribution of occludin and the blue zone nuclei. Bar = 25 μm. (c) Representative Western blot of occludin protein. (d) Quantitative analysis of occludin protein in colonic mucosa from different groups. Data were mean ± SEM ($n = 5$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

3.7. HQJZ Regulates the Balance of RhoA/Rac in Colonic Mucosa. The expressions of RhoA, ROCK-I, Rac-1, and PAK-1 proteins were assessed by Western blot. Compared to Sham groups, TNBS challenge for 15 days evoked a significant increase in the expressions of RhoA (Figures 8(a) and 8(b)) and ROCK-I (Figures 8(a) and 8(c)), while it showed a decrease in Rac-1 (Figures 8(a) and 8(b)) and PAK-1 (Figures 8(a) and 8(e)). HQJZ restrained all of the TNBS-evoked alterations significantly.

4. Discussion

TNBS-induced murine colitis is an extensively used animal model for human IBD. In the present study, TNBS administration successfully evoked colonic inflammation in rats, as evidenced by both macro- and microscopic manifestations, as well as by the colonic microcirculatory disturbance and alterations in inflammatory cytokine production in colonic tissue. Importantly, all of the TNBS-evoked insults were attenuated by HQJZ treatment, highlighting its therapeutic effects on TNBS-induced colonic mucosal injury.

As a famous Chinese medicine formula, although HQJZ has been used in China almost for two thousand years, the study on the mechanisms responsible for its role in inflammatory diseases remains limited, and most of works were focusing on its major component *Astragalus*. Consistent with the results from studies on *Astragalus*, the present study revealed HQJZ to be able to downregulate the production of proinflammatory cytokines and upregulate anti-inflammatory cytokines. Furthermore, the current study demonstrated that HQJZ inhibited TNBS-induced CD11b expression on leukocytes and leukocyte adhesion to venular wall. The mechanism responsible for the anti-inflammatory potential of HQJZ is not clear at present. Nonetheless, the antioxidant ability of its compositions may contribute, at least in part, to the beneficial role of HQJZ in this regard [25–27].

In addition, HQJZ was found to attenuate the albumin leakage and leukocyte emigration from venules in the presence of TNBS, indicating its role in protection against vascular hyperpermeability. The significance of this finding resides in that, in colonic inflammation, hyperpermeability occurs not only in vascular endothelium but also in mucosal epithelium. Patients with colonic inflammatory disease typically present with relapsing diarrhea, which has been attributed to increased paracellular permeability in the colonic epithelium [44]. Thus, a management able to restore the increased

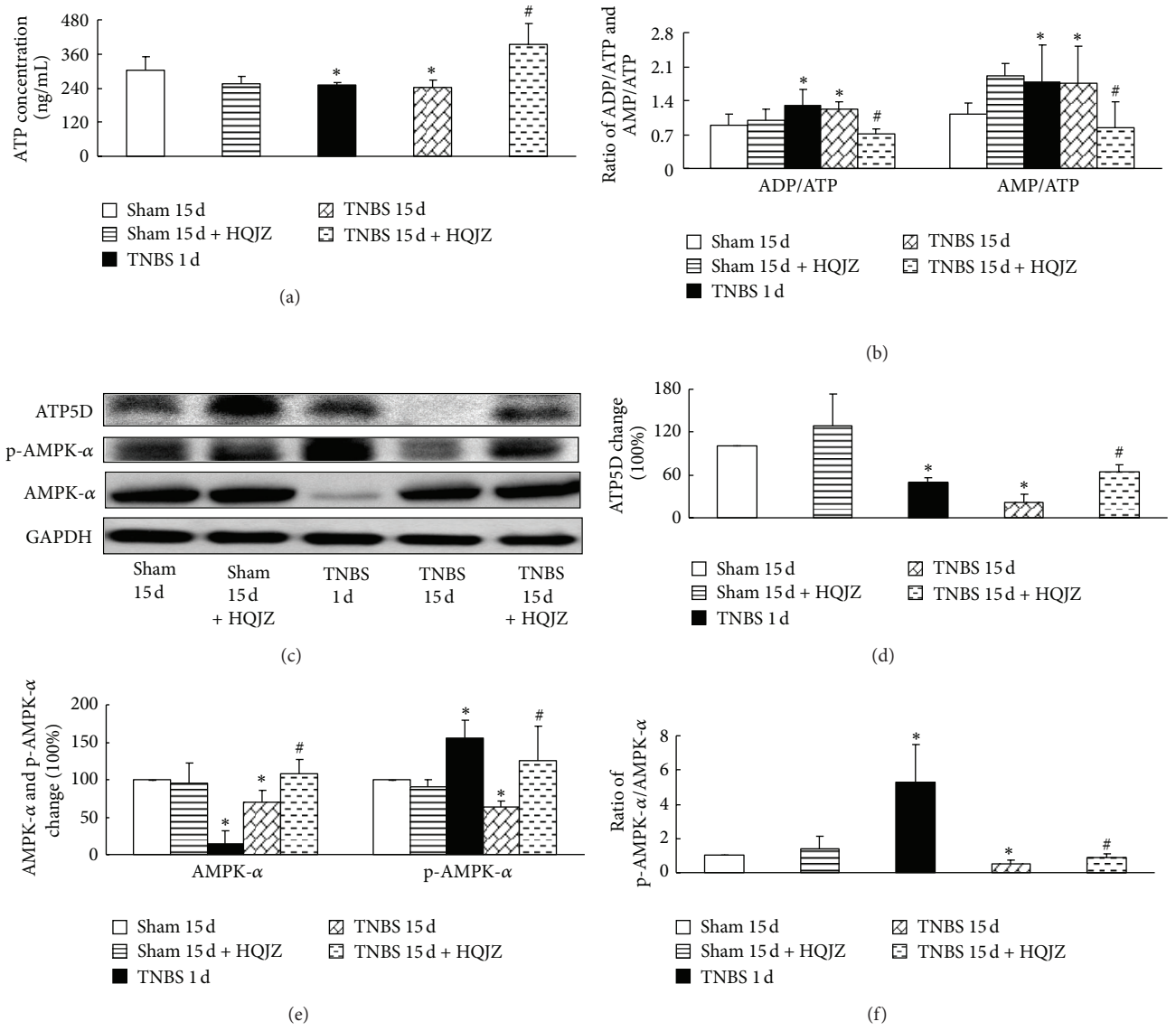

Figure 6: Energy status of colonic mucosa and Western blot analysis of ATP5D, AMPK-α, and p-AMPK-α. (a) The concentration of ATP in colonic mucosa ($n = 8$). (b) The ratios of ADP/ATP and AMP/ATP ($n = 8$). (c) Representative Western blot of ATP5D, AMPK-α, p-AMPK-α and GAPDH ($n = 5$). (d) Quantitative analysis of ATP5D protein ($n = 5$). (e) Quantitative analysis of AMPK-α and p-AMPK-α proteins. (f) The ratio of p-AMPK-α/AMPK-α ($n = 5$). Data were mean ± SEM. $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

colonic epithelium permeability is of great significance for relieving the symptom, particularly the malnutrition that patients suffer from. The permeability of the endothelial and epithelial barrier is controlled by intercellular junctions. The present study assessed the effect of HQJZ on the occludin, a protein that stabilizes tight junction through interaction with ZO-1 and actin cytoskeleton [45]. As expected, the result showed that HQJZ affected barrier permeability via mechanism(s) involving modulation of both expression and distribution of occludin.

We next explored the signaling pathway for the role of HQJZ in regulating barrier function. In regulation of barrier of both endothelium and epithelium, the Rho family, RhoA, Rac, and Cdc42 have been recognized as a major player [16–21]. The results from the present study showed that TNBS challenge evoked an increase in RhoA and ROCK-1, as well as in p-MLC, while it showed a decrease in Rac and PAK-1. Interestingly, all of these alterations were attenuated by treatment with HQJZ. These results highlight an implication of Rho family in the role of HQJZ in maintaining endothelial and epithelial barrier function.

Complete remission of colonic inflammatory diseases requires both the relief of inflammation and the repair of damaged epithelium. The repair of damaged colonic mucosa initiates with cell restitution, characterized by cell spreading and migration into the wound to restore epithelial continuity. We observed that HQJZ promoted the repair of damaged epithelium as evidenced by macro- and microscopic findings, implying a potential of HQJZ to accelerate cell restitution. It is likely that HQJZ exerts this effect via modulation of

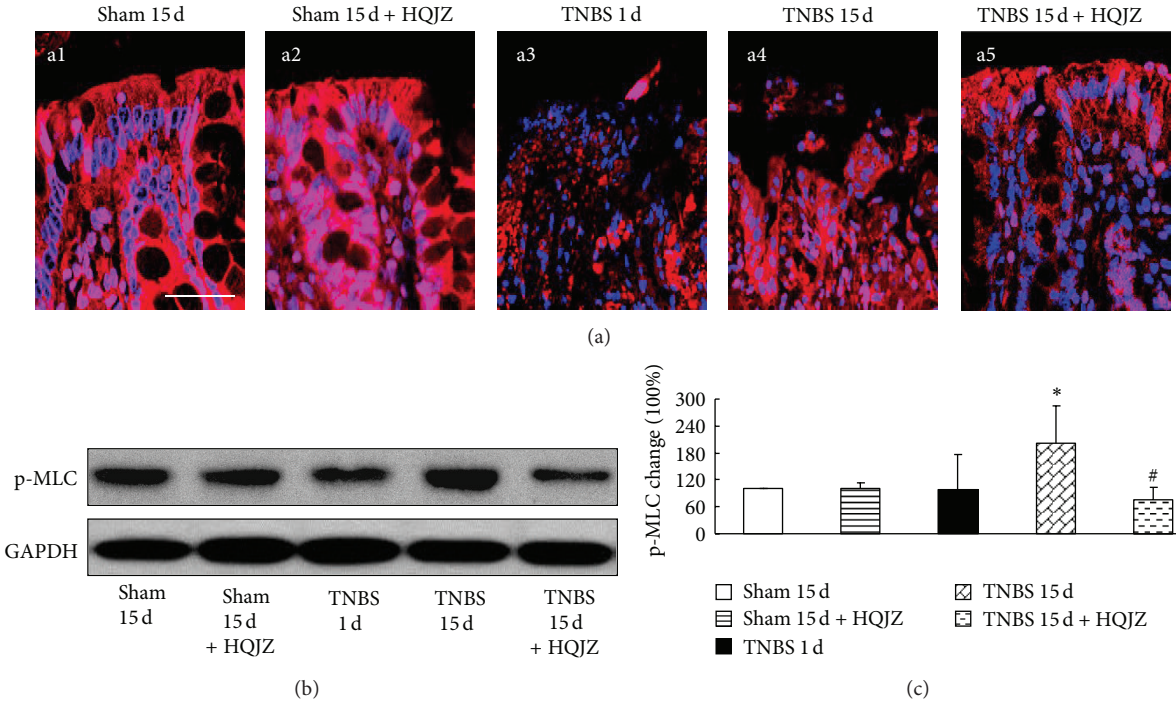

Figure 7: Distribution of F-actin in colonic mucosa and Western blot analysis of p-MLC. (a) Distribution of F-actin in colonic mucosa. The red zone represents the distribution of F-actin, and the blue zone represents nuclei. Bar = 25 μm. (b) Representative Western blots of p-MLC and GAPDH. (c) Quantitative analysis of p-MLC proteins. Data were mean ± SEM ($n = 5$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

Rho family, since cell restitution is a process of cellular locomotion, which has been shown to be driven by Rho family [20].

It appears that the signaling pathways mediating the role of HQJZ in attenuating endothelial and epithelial barrier function and in promoting cell restitution both converge on Rho family. The question then arises: How does HQJZ regulate Rho family? In view of the critical importance of energy metabolism in regulation of Rho family, we assumed that HQJZ may regulate Rho family by interference in energy metabolism.

We tested this assumption first by assessment of ATP, ADP, and AMP content in different condition. The result revealed a significant increase in ATP content in TNBS-injured rats when subjected to HQJZ treatment, suggesting the capacity of HQJZ to increase the energy supply, consistent with the result from others [31]. Energy metabolism is a process involving multiple reactions coordinated by numerous proteins, among which ATP synthase plays a central role, while AMPK acts as an energy sensor to monitor the energy status of the cell [46]. In the present study we assessed ATP5D, a critical subunit of ATP synthase [31], and revealed a significant decrease in ATP5D in colonic mucosa tissue after TNBS challenge, which accounts for the insufficient ATP supply observed then. The examination of AMPK showed an increase in activated AMPK in the early phase in injuring process by TNBS (1 day), reflecting an attempt of the cell to compensate the lack of energy, and it showed a decrease in the late phase (15 days), implying a failure of this attempt which led to delayed tissue injury repair. Interestingly, treatment with HQJZ resulted in an increased expression of ATP5D in colonic tissue damaged by TNBS, while it had little, though statistically significant, influence on the activated AMPK content compared to TNBS-alone group. This result implied that HQJZ modulated energy metabolism mostly by enhancing the expression of ATP5D, and, to a less extent, by activating AMPK. The mechanism for HQJZ to affect the expression of ATP5D and activation of AMPK needs to be elucidated by further study.

5. Conclusions

In summary, using TNBS-induced rat colonic mucosal injury as a model, the present study verified the favorable role of HQJZ in IBD, which manifested as attenuation of microcirculatory disturbance, relief of inflammation and colonic epithelium barrier function, and improvement of energy supply. HQJZ exerted its effects most likely by acting as an antioxidant and an energy metabolism modulator, suggesting it as a multitargeting strategy. Nonetheless, the detailed mechanism remains to be identified by further study.

Conflict of Interests

The authors have declared that there is no conflict of interests.

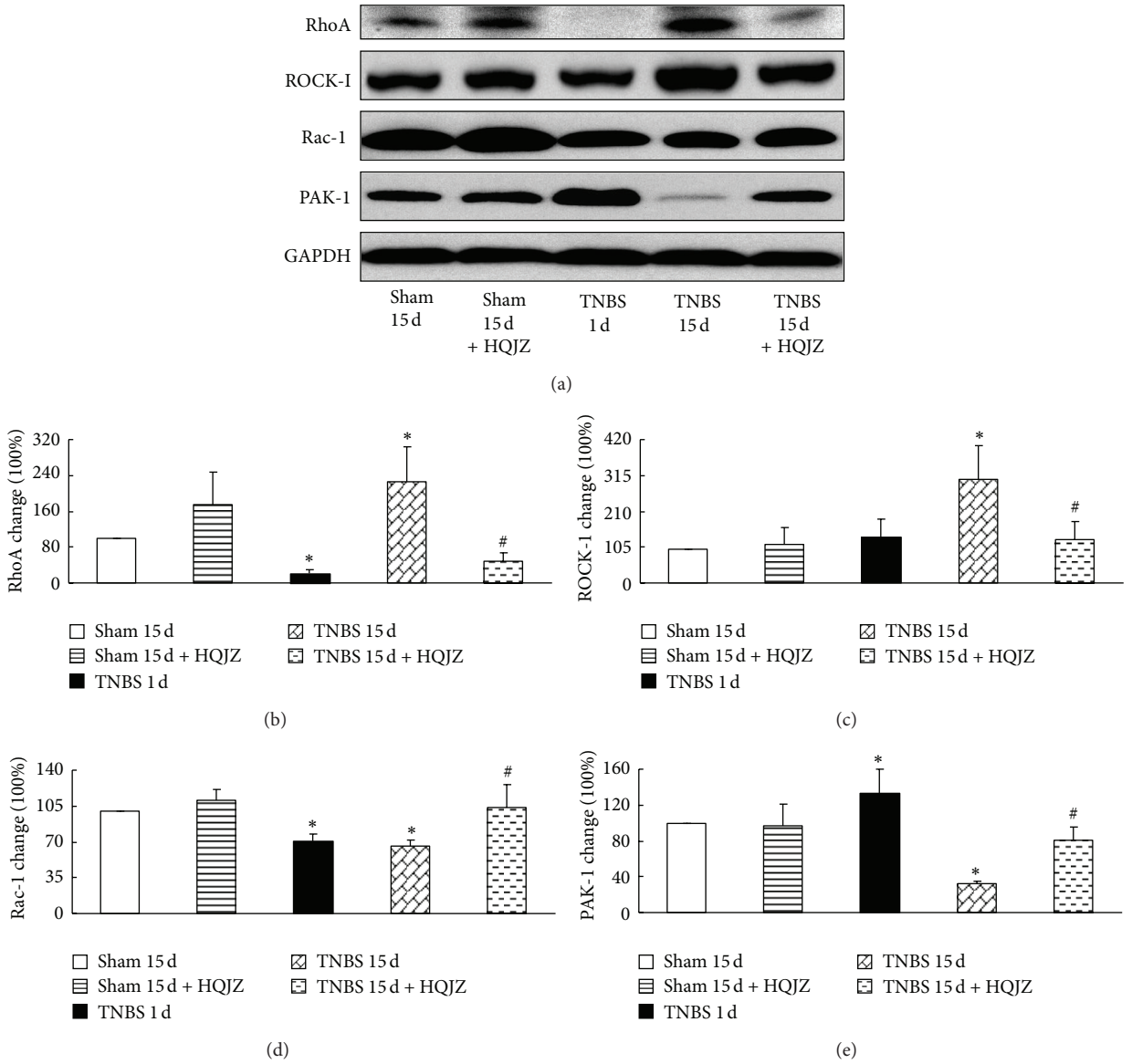

Figure 8: Western blot analysis of RhoA, ROCK-I, Rac-1, and PAK-1. (a) Representative Western blots of RhoA, ROCK-I, Rac-1, PAK-1, and GAPDH. (b) Quantitative analysis of RhoA protein. (c) Quantitative analysis of ROCK-I protein. (d) Quantitative analysis of Rac-1 protein. (e) Quantitative analysis of PAK-1 protein. Data were mean ± SEM ($n = 5$). $^{*}P < 0.05$ versus Sham group; $^{\#}P < 0.05$ versus TNBS 15 d group.

Acknowledgment

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Research Article

The Effects of *Banha-sasim-tang* on Dyspeptic Symptoms and Gastric Motility in Cases of Functional Dyspepsia: A Randomized, Double-Blind, Placebo-Controlled, and Two-Center Trial

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Introduction. Functional dyspepsia (FD) is highly prevalent, and no standard treatments exist for this condition. Herbal prescriptions are widely used to treat FD. In traditional Korean medicine, *Banha-sasim-tang* (BST) is a famous herbal prescription for dyspepsia. This study aimed to evaluate the efficacy of BST and to examine the relationship between gastric slow waves and dyspeptic symptoms. *Materials and Methods*. In total, 100 FD patients were recruited; BST or placebo was administered for 6 weeks. The gastrointestinal symptom scale, FD-related quality of life scale, and frequency or power variables regarding gastric slow waves were measured at 0, 6, and 14 weeks. *Results*. There were no significant differences in the overall dyspeptic symptoms or quality of life between the BST and placebo groups. However, early satiety was significantly improved in the BST group ($P = 0.009$, at 6 weeks by intention-to-treat analysis). Abnormal gastric dysrhythmias and power ratios were also significantly improved by BST. *Conclusion*. BST had no significant effects on FD. However, early satiety appeared to improve after BST administration. Electrogastrography may be a useful technique for assessing changes in gastric motility dysfunction after interventions for FD. Further investigation focused on specific symptoms or subtypes of FD is required.

1. Introduction

Functional dyspepsia (FD) is chronic or recurrent dyspepsia without evidence of organic disease [1]. In western countries, the prevalence of FD is 11.5–14.7% in the general population [2]. In South Korea, an epidemiologic survey showed that 25% of the population was afflicted by FD [3].

At present, the causes of FD are believed to be multifactorial, and pathogenesis-based treatments, including administration of acid secretion inhibitors and prokinetics and eradication of *Helicobacter pylori*, have been used without success [4–6]. Therefore, many FD patients seek alternative and more effective treatments such as herbal prescriptions, nutritional supplements, acupuncture, or moxibustion [1, 7–9].

Banha-sasim-tang (BST; *Hange-shashin-to* in Kampo medicine; *Ban xia xie xin tang* in traditional Chinese medicine) is one of the most famous herbal prescriptions in the old herbal prescription literature, where it is referred to as "*Shan-han-za-bing-lin* (傷寒雜病論)" [10]. In traditional Korean medicine, BST, which consists of 7 herbs, has been used to treat "gastric stuffiness" [10], a condition similar to dyspepsia. Recently, some studies have reported that BST has anti-inflammatory effects on the gastric mucosa and affects gastric function by influencing gut hormones or plasma peptides [11–13]. A recent clinical study investigating modified BST, conducted in China, demonstrated beneficial effects on only a subgroup of FD patients [14]. BST can be easily obtained without a prescription in Korea. Despite many previous experimental and clinical research studies, including a randomized controlled trial (RCT) in China [14], more reliable evidence about BST as a therapeutic alternative for FD are needed. However, few relevant RCTs have examined the effects of BST on FD or its mechanisms. The aim of

the present study was to investigate the effect of BST on dyspeptic symptoms and the quality of life in FD patients. To assess the changes in gastric motility dysfunction caused by BST, as a mechanical study, cutaneous multichannel electrogastrography (EGG), which detects gastric myoelectric activity (GMA), was used. The relationship between EGG frequency variables and FD symptoms was analyzed before and after oral administration of BST.

2. Materials and Methods

2.1. Study Design. This study was conducted as a randomized, placebo-controlled, double-blind, and 2-center trial at the Oriental Hospital, Kyung Hee University Medical Center, and at the Kyung Hee University Hospital at Gangdong in Seoul, Korea. The protocol of the trial was approved by the ethics review boards of both hospitals. The permission numbers were KOMC IRB 2009-05 for the Oriental Hospital and KHNMC-OH-IRB 2009-001 for the Kyung Hee University Hospital. Written informed consent was obtained from each participant before enrollment.

2.2. Study Subjects. Patients aged 19–75 and meeting the Rome III criteria for FD [15] were recruited from the clinics at both hospitals and from responders to local advertisements. In this trial, FD patients were classified into 1 of 3 subtypes: PDS, EPS, or mixed type: (1) meal-induced postprandial distress syndrome (PDS), characterized by postprandial fullness and early satiety; (2) epigastric pain syndrome (EPS), characterized by epigastric pain and burning; (3) PDS mixed with EPS [15]. The severity of dyspepsia was assessed using a validated gastrointestinal symptom (GIS) scale, which consists of 10 dyspeptic symptom subscales and is used to measure the severity of individual dyspeptic symptoms [16]. The existence of "moderate" severity on at least 3 subscales of the GIS resulted in the patient meeting the study's inclusion criteria [1]. However, patients with medical histories of peptic ulcers, reflux diseases, previous abdominal surgeries, mental diseases, such as major depression, predominant irritable bowel syndrome, severe organ diseases, or continuous administration of analgesic agents were excluded as were lactating or pregnant women. In addition, patients who were using antibiotics, proton-pump inhibitors, bismuth salts, prokinetic agents, and herbal prescriptions were excluded, as previously described [17].

2.3. Randomization and Blinding. Randomization was performed by an independent clinical research coordinator (CRC). The randomization document with the subject's basic information was transmitted by facsimile to the independent statistician (IKY) without a confirmed randomization number. The statistician determined the randomization number based upon the allocation sequence generated in advance by a random number program. Subsequently, the statistician returned the randomization document with the confirmed number to the CRC. The random allocation ratio for the 2 sites was 1 : 1. The randomization process was assured by the authorized contract research organization (CRO; Marinet, Seoul, Korea), as previously described [17].

During the study, the investigators did not contact the CRC, the clinical pharmacist, or the statistician. In particular, the CRC was separated from all researchers to ensure that the researchers did not have any influence on enrollment or randomization. The statistician received the randomization document with the number and returned it to CRC in order. Thus, the statistician's contact with other investigators was avoided. This blinding procedure was also verified by the authorized CRO.

Thus far, no standard therapy has been established for FD; therefore, the placebo used in this study did not have any active components. As experimental agents, Bansasin granules (Hanpoong Pharm & Food, Jeonju, Korea) in the current study are not artificial chemical therapeutics; a relevant placebo with an identical appearance, color, and flavor was required for effective blinding. After several attempts, a placebo that could not be distinguished from the real Bansasin granule by 6 healthy persons was successfully produced. Placebo was made of starch, lactose, brown caramel food coloring (BF2481, SaeRom BNF Co., Korea), and flavor (BF24781, SaeRom BNF Co., Korea) similar to color, flavor, and scent with Bansasin granule. Samples of the placebo were also kept by Hanpoong Pharm & Food. Additionally, at the end of the study, all subjects were asked whether the experimental agents that they had been given were real or placebo in order to evaluate the success of blinding.

2.4. Interventions. The intervention of the current study, BST (1/3 pack of herbal medicine (貼): pinelliae tuber (the rhizome of *Pinellia ternata* (Thunb.) Breit., family Araceae), 1.67 g; scutellariae radix (the root of *Scutellaria baicalensis* Georgi, family Labiatae), 1.00 g; ginseng radix (the root of *Panax ginseng* C.A. Meyer, family Araliaceae), 1.00 g; glycyrrhizae radix (the root of *Glycyrrhiza uralensis* Fisch., family Leguminosae), 1.00 g; zizyphi fructus (the fruit of *Zizyphus jujuba* Mill. var. inermis Rehder, family Rhamnaceae), 1.00 g; zingiberis rhizoma (the rhizome of *Zingiberis officinale* Roscoe, family Zingiberaceae), 0.83 g; coptidis rhizoma (the rhizome of *Coptis chinensis* Franch., family Ranunculaceae), 0.33 g) was extracted (0.91 g) with boiled water and mixed with starch (1.57 g) and lactose (0.52 g) then given the Bansasin granule (3 g). The manufacture was processed according to Korean Good Manufacturing Practice and permitted and regulated by the Korean Food & Drug Administration. The standard chemical components in the Bansasin granule (3 g) are berberin (11.6 mg), glycyrrhizin acid (25.0 mg), and baicalin (100.0 mg). Sample specimens were kept at the laboratory of Hanpoong Pharm & Food. Generally, 3 g of Bansasin granules 3 times per day is the recommended adult dosage for dyspeptic symptoms, including nausea, vomiting, diarrhea, abdominal pain, or anorexia [17].

This clinical trial consisted of a 6-week oral administration of BST or placebo (3 g, 3 times/day) and a 2-month follow-up period. Before randomization, all participants underwent a 7-day washout phase for elimination of any traces of previous medications. During the administration phase, subjects were prohibited from taking any

kind of dyspepsia-relieving agents. However, conventional treatments for dyspepsia were permitted if the dyspeptic symptoms were severe and could not be tolerated. During the follow-up period, any other type of treatment provided to the subjects was reported and documented.

2.5. Outcomes. Dyspepsia severity and quality of life were measured at baseline; at 2, 4, and 6 weeks after randomization; and at 1 and 2 months after completion of the BST or placebo administration.

The GIS scale was the primary outcome variable in this study [16], and the change in the total score of the GIS scale or significant changes in the GIS subscale scores at 6 weeks were considered as efficacy parameters. The GIS scale is composed of the following 10 dyspeptic symptoms: epigastric pain/upper abdominal pain, abdominal cramps, fullness, early satiety, loss of appetite, malaise, nausea, vomiting, retrosternal discomfort, and acidic regurgitation/heartburn. The severity of each subscale symptom was assessed using a 5-point Likert scale: none, 0; slight, 1; moderate, 2; severe, 3; very severe, 4. The total sum of the 10 GIS subscale scores presents patient's overall severity of dyspeptic symptoms. If the total sum of the GIS scale is higher, then the dyspeptic symptoms are more severe. All subjects assessed their own symptom severity by themselves. The GIS scale is very simple and easy for subjects to understand and to fill in.

As a secondary outcome, a visual analog scale (VAS) was used to determine the patient's overall judgment about dyspepsia (ranging from 0, no discomfort, to 100, the most intense discomfort).

For assessing the quality of life, the functional dyspepsia-related quality of life (FD-QoL) questionnaire, as a secondary outcome, was used for all subjects. The questionnaire consisted of 4 categories: diet (5 items), daily activity (4 items), emotion (6 items), and social functioning (6 items) [18]. The severity assessment for each item was the same as that for the GIS subscale. The FD-QoL questionnaire used in this trial was previously validated for use with Korean FD patients [18]. The total sum of the FD-QoL item scores presents patient's overall quality of life related dyspepsia. If the total sum of the FD-Qol item scores is lower, then the quality of life related to dyspepsia is better. All subjects assessed their own state by themselves.

2.6. GMA Measurement. In the present study, surface multichannel EGG (Polygraf ID, Medtronic A/S, Copenhagen, Denmark) was used to measure GMA in each subject at baseline and at 6 weeks of BST or placebo administration. As previously described [19], EGG measurements were conducted in the following sequence. First, the epigastric skin at the electrode attachment site was shaved and abraded with a sandy skin preparation jelly to reduce impedance. Then, 3 active surface electrodes were positioned at the following sites: the corpus of the stomach (channel 1), the proximal antrum (channel 2), and the distal antrum and pylorus regions (channel 3). A ground electrode and a reference electrode were also appropriately placed. EGG measurements were obtained in a quiet room after the subjects had fasted over night for ≥8 hours. To avoid motion artifacts, subjects were asked not to talk and to remain as still as possible during the EGG assessments. Each subject underwent a fasting (preprandial) EGG measurement for 20 minutes in the supine position and then ate the standard solid test meal (500 Kcal, 2 scrambled medium eggs, and 2 pieces of toasted bread with 500 mL of water). A postprandial EGG measurement was then performed for 40 minutes. The dominant EGG frequency, the percentage or percentage distribution of normal or abnormal gastric slow waves (tachygastria, bradygastria, and dysrhythmia), and the postprandial-to-preprandial power ratio which is defined as the ratio of the postprandial to fasting power of the dominant frequency (DF) were assessed.

2.7. Sample Size Calculation. To determine the efficacy of BST on FD, the superiority of BST over placebo had to be verified. Although there were no relevant previous studies using BST for calculating sample size, a previous herbal prescription trial for FD treatment had assessed efficacy using the GIS scale and a 2-sided test, yielding a 5% significance level [20]. The formula for estimating the sample size was as follows:

$$n_t = n_c = \frac{\left\{\left(Z_{\alpha/2} + Z_{\beta}\right)^2 \sigma^2 (\lambda + 1)/\lambda\right\}}{\left(\mu_c - \mu_t\right)^2}. \quad (1)$$

From the previous study, 3.5 points of GIS scale ($\mu_c - \mu_t = \Delta$) improved after herbal treatments compared with placebo, and a mean standard deviation (SD = σ) was 5.37 [21]. In our study, the ratio (λ) of BST to placebo group was 1 : 1. With an 80% power ($1-\beta$) and 5% significance level (α), assuming $\Delta = 3.5$ and $\sigma = 5.37$, a sample size of $n_t = n_c = 37$ subjects per group was needed (n_t, number of patients in the BST group; n_c, number of patients in the placebo group). Considering an expected dropout rate of 25%, 100 patients were required, as previously described [17].

2.8. Statistical Analysis. All analyses in this study were based on the intention-to-treat principle. Quantitative- and frequency-related variables were presented as means ± SD and number (%), respectively. The baseline characteristics of both groups were compared by the chi-square test or an independent t-test. As primary outcomes, the changes in the GIS total score or subscale scores from the beginning (day 0) to the end (6 weeks) of the study period were compared using an independent t-test. In addition, the VAS results for overall dyspeptic symptoms and the total FD-QoL scores were also compared in the same manner as the GIS scores. Frequency parameters in the EGG measurement, such as the dominant frequency, percentages of normal or abnormal gastric slow waves, and power ratio variables, were compared before and after treatment using the chi-square test or Fisher's exact test. Correlations between changes in the GIS scale results and the EGG parameters were analyzed by Pearson's correlation coefficients. Statistical analyses were conducted in a blinded manner by an independent statistician using SPSS 16.0 (SPSS, Chicago, IL, USA).

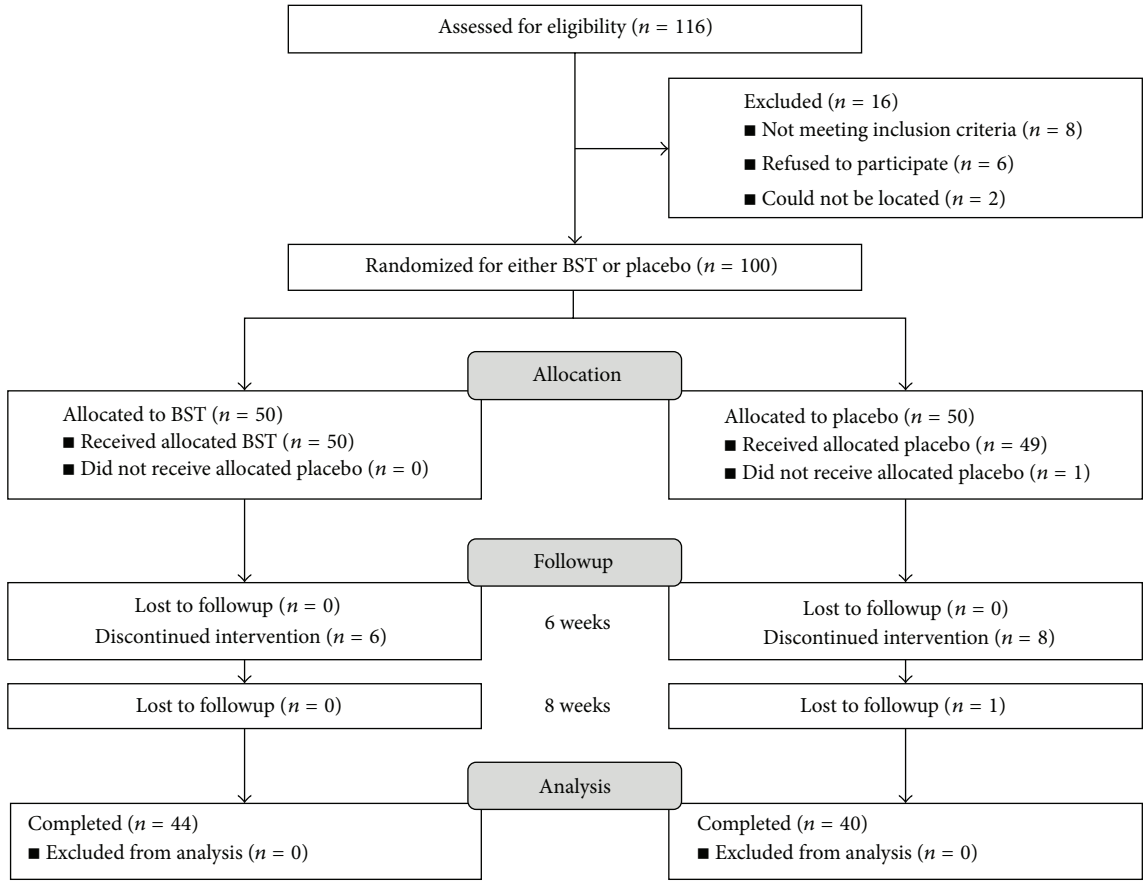

Figure 1: Flow chart of the trial.

3. Results

3.1. Study Participants. Of the 116 eligible subjects, 100 patients were included and randomly allocated to the BST or placebo groups in a 1 : 1 ratio between May 2009 and January 2011. Sixteen subjects withdrew during the study because of lack of efficacy or failure to followup. A flow chart of the trial is presented in Figure 1.

3.2. Baseline Characteristics. The general characteristics of the subjects are presented in Table 1. No significant differences were observed in any of the parameters between the 2 groups, except for body weight. However, the characteristics of dyspepsia in the BST group tended to be more continuous than those in the placebo group.

3.3. Safety and Adverse Events. Before randomization and after completing administration of BST or placebo, we assessed complete blood cell counts; levels of aspartate aminotransferase/alanine aminotransferase, gamma-glutamyl transpeptidase, blood urea nitrogen, and creatinine; erythrocyte sedimentation rates; electrocardiograms to ensure the subjects' safety. Throughout the trial, all adverse events were also monitored by the subjects' reports or the case report form documentation.

There were no safety issues in this trial. Eight adverse events, including epigastric pain or discomfort ($n = 3$), insomnia ($n = 2$), dry mouth ($n = 2$), and itching ($n = 1$), were reported by 4 subjects in the BST group. Thirteen adverse events, including abdominal pain or discomfort ($n = 2$), insomnia ($n = 2$), constipation ($n = 2$), itching ($n = 3$), dizziness ($n = 1$), and muscle pain ($n = 3$), were reported by 9 subjects in the placebo group. However, there were no serious adverse events during the study period.

3.4. Outcome Variables

3.4.1. GIS Scores and Individual Dyspeptic Symptoms. After 6 weeks of treatment, the total GIS scores for both groups had improved markedly (13.06 ± 4.82 at 0 week to 8.77 ± 6.87 at 6 weeks in BST group versus 13.94 ± 5.12 at 0 week to 6.83 ± 5.42 at 6 weeks in the placebo group); no significant difference was observed between the 2 groups (Figure 2). However, at 6 weeks, the early satiety subscale score was significantly improved in the BST group compared with the placebo group ($P = 0.009$, by intention-to-treat analysis; Table 2). However, there were no significant differences among other subscale scores at 6 weeks between the 2 groups (Table 2). During the follow-up period, the improved GIS scores were retained. However, there were no significant differences in the GIS scores or the individual dyspeptic symptoms between the groups.

Table 1: Characteristics of the subjects.

Parameters	BST ($n = 50$)	Placebo ($n = 50$)	P value
Gender (male/female)	25/25	22/28	0.55
Age (years)	49.54 ± 14.72	48.00 ± 12.62	0.53
Weight (kg)	61.88 ± 11.56	57.35 ± 9.23	**0.03**[*]
Height (cm)	163.67 ± 7.32	163.92 ± 7.73	0.86
Heart rate/min	73.76 ± 11.63	75.02 ± 11.42	0.70
BP systolic (mmHg)	123.44 ± 5.08	118.27 ± 11.67	0.06
BP diastolic (mmHg)	78.94 ± 13.36	74.12 ± 11.23	0.08
Smoking (yes/no)	7/43	6/44	0.77
Alcohol (yes/no)	25/25	24/26	0.84
Coffee (yes/no)	34/16	33/17	0.83
Rome criteria			
Postprandial distress syndrome (PDS) (%)	21 (42.00)	27 (54.00)	
Epigastric pain syndrome (EPS) (%)	12 (24.00)	8 (16.00)	0.43
Mixed (PDS + EPS) (%)	17 (34.00)	15 (30.00)	
Duration of symptom (years)	13.82 ± 3.25	13.37 ± 13.68	0.88
Characteristics of dyspepsia (%)			
Continuous	21 (40.91)	17 (32.50)	
Periodic	16 (34.09)	10 (20.00)	0.10
Irregular	13 (25.00)	23 (47.50)	
Helicobacter pylori infection			
In history (%)	8 (16.00)	11 (22.00)	0.74
Total GIS score	13.44 ± 4.87	13.82 ± 5.05	0.69
VAS for overall symptom	55.36 ± 18.63	53.08 ± 17.29	0.38
Total FD-QoL score	27.74 ± 18.63	32.18 ± 17.31	0.54
Total BDI score	22.70 ± 12.82	25.04 ± 13.49	0.30

BST: *Banha-sasim-tang*; BP: blood pressure; GIS: gastrointestinal symptom scale; VAS: visual analogue scale; FD-QoL: functional dyspepsia-related quality of life; BDI: Beck's depression inventory.
[*]Statistically significant.

3.4.2. VAS for Overall Symptoms. After 6 weeks of treatment, the VAS for overall symptoms in both groups had improved (55.36 ± 18.63 at 0 week to 41.32 ± 18.21 at 6 weeks in BST group versus 53.08 ± 17.29 at 0 week to 34.54 ± 20.62 at 6 weeks in the placebo group); no significant difference was observed between the 2 groups ($P = 0.09$, by intention-to-treat analysis, Figure 2).

3.4.3. FD-QoL Scores. After 6 weeks of treatment, the FD-QoL scores in both groups had improved (27.74 ± 18.63 at 0 week to 18.91 ± 17.58 at 6 weeks in BST group versus 32.18 ± 17.31 at 0 week to 18.51 ± 14.68 at 6 weeks in the placebo group); no significant difference was observed between the 2 groups ($P = 0.89$, by intention-to-treat analysis, Figure 2).

3.4.4. Changes in GMA. The quality of the EGG recordings was high, and no high levels of motion artifacts were observed. The mean value of the DF was 3.03 ± 0.24 cpm among all of the subjects (3.04 ± 0.25 in the BST group versus 3.02 ± 0.23 in the placebo group), which is considered to be within the normal range. The percentages of normal gastric slow waves or gastric dysrhythmias for each channel in both groups are described in detail in Table 4.

In this study ($n = 100$), some dyspeptic symptoms were significantly correlated with EGG parameters (preprandial DF and bloating, $r = 0.262$, $P = 0.017$; preprandial percentage of tachygastria in channel 3 and abdominal cramps, $r = 0.206$, $P = 0.040$; preprandial percentage of arrhythmia in channel 3 and vomiting, $r = 0.258$, $P = 0.010$; postprandial percentage of tachygastria in channel 1 and sickness, $r = 0.288$, $P = 0.004$; postprandial percentage of arrhythmia in channel 3 and vomiting, $r = 0.231$, $P = 0.021$; postprandial percentage distribution of tachygastria in channel 3 and early satiety, $r = 0.241$, $P = 0.016$, Pearson's correlation coefficient).

There was a pattern of decreasing tachygastria in the BST group compared with the placebo group at 6 weeks (preprandial tachygastria in channel 3, $P = 0.068$; postprandial tachygastria in channel 2, $P = 0.065$; postprandial tachygastria in channel 3, $P = 0.041$). No significant differences among the pre- and postprandial EGG recordings were observed between the 2 groups at 6 weeks.

Abnormal EGG findings were defined as a reading of dysrhythmia in ≥30% (normogastria <70%) of the total readings in the fed state or a power ratio of less than 1 [22].

Considering the definition of abnormal EGG findings, at 6 weeks, the power ratios in channels 1 and 2 for the BST

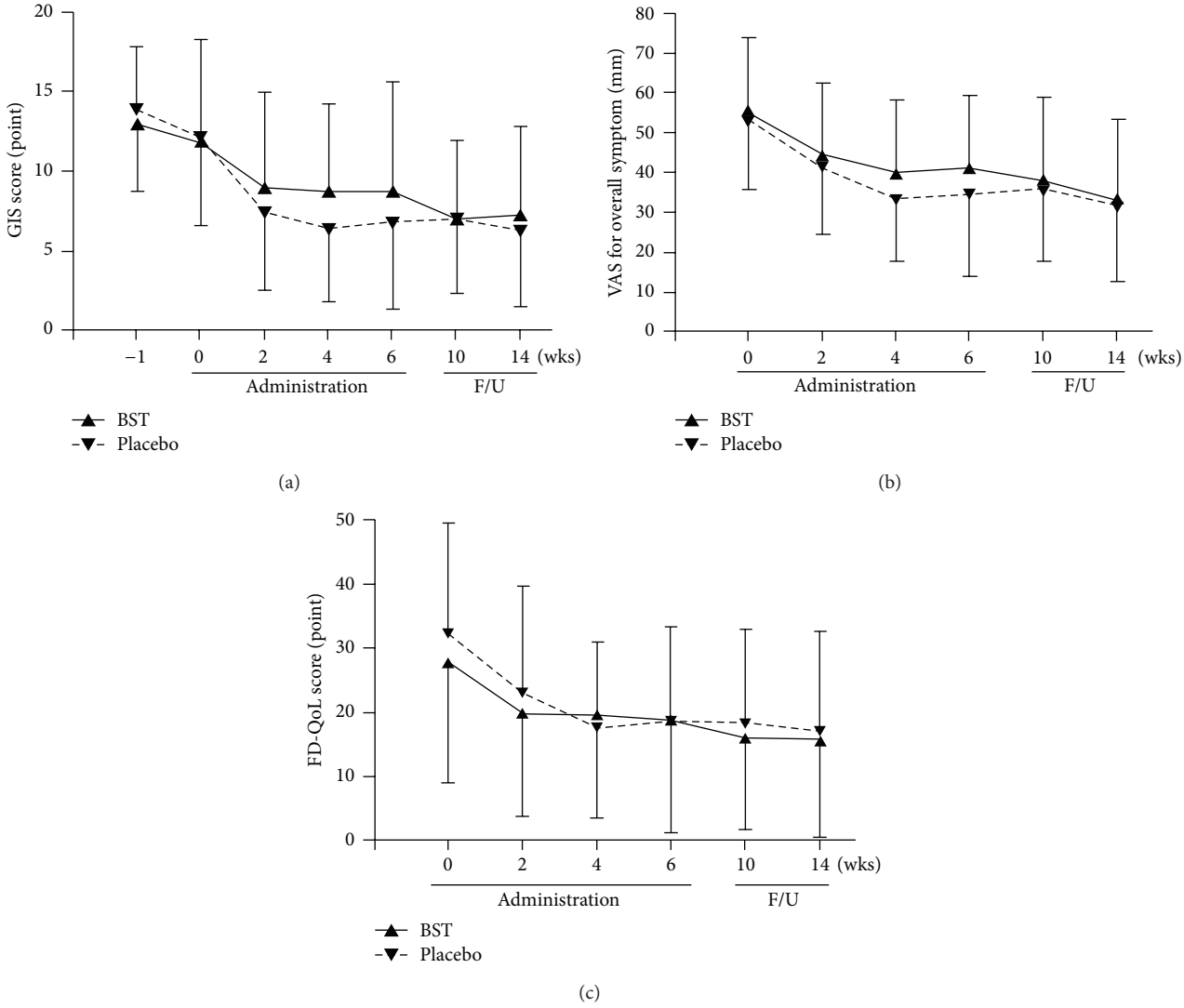

Figure 2: Changes in main outcomes of the trial. (a) Changes in the total scores of gastrointestinal symptom (GIS) scale between BST and placebo groups during the whole trial. (b) Changes in visual analogue scale (VAS) for overall symptoms between BST and placebo groups during the whole trial. (c) Changes in the total scores of functional dyspepsia-related quality of life (FD-QoL) between BST and placebo groups during the whole trial. BST: *Banha-sasim-tang*. F/U: follow-up period.

group tended to be improved, whereas those for the placebo group were aggravated (Table 4).

4. Discussion

This study demonstrated that BST administration for 6 weeks improved dyspeptic symptoms and the quality of life for FD patients. However, verification of the significant effects of BST on the overall FD symptoms and quality of life compared with placebo was difficult, because the placebo response rate was relatively high in this trial. Nonetheless, in the BST group, early satiety was significantly ameliorated at 6 weeks, and there were significant positive changes in the GMA parameters.

In traditional Korean medicine, BST is a well-known treatment for "epigastric stuffiness (心下痞)," as described in the ancient herbal prescription literature [10]. Epigastric stuffiness appears to be similar to the early satiety and abdominal discomfort symptoms reported among the symptoms of dyspepsia. Delayed gastric emptying or antral dysmotility is considered to be the main cause of this dyspepsia symptom in FD patients [19, 22].

Previous studies have indicated that BST increases levels of some gut hormones, such as motilin, gastrin, and somatostatin, which are closely correlated with gastric motility [11]. In addition, BST may regulate the hypothalamic-pituitary-adrenal (HPA) axis by increasing levels of calcitonin gene-related peptide and substance P or decreasing levels of adrenocorticotropic hormone and cortisol [12, 13]. Pinelliae tuber is a major component among the herbal components of BST and has been reported to accelerate gastrointestinal motility and gastric emptying in human studies [11, 12].

Table 2: Changes in individual dyspeptic symptoms of gastrointestinal symptom (GIS) scale.

Symptoms	Baseline		6 weeks		14 weeks	
	BST ($n = 50$)	Placebo ($n = 50$)	BST ($n = 44$)	Placebo ($n = 41$)	BST ($n = 44$)	Placebo ($n = 40$)
Nausea	1.04 ± 1.10	1.32 ± 1.02	0.70 ± 0.93	0.59 ± 0.84	0.59 ± 0.84	0.60 ± 0.71
Sickness	0.60 ± 0.90	0.62 ± 0.88	0.50 ± 0.82	0.49 ± 0.81	0.48 ± 0.73	0.30 ± 0.65
Vomiting	0.56 ± 0.86	2.38 ± 1.27	0.36 ± 0.72	0.22 ± 0.52	0.25 ± 0.53	0.15 ± 0.43
Bloating	2.42 ± 0.93	2.38 ± 1.03	1.43 ± 0.97	1.15 ± 0.96	1.57 ± 1.11	1.20 ± 0.88
Abdominal cramps	1.02 ± 0.94	1.32 ± 1.30	0.66 ± 0.94	0.54 ± 0.87	0.48 ± 0.59	0.45 ± 0.78
Early satiety	2.30 ± 1.28	2.26 ± 0.92	**1.09 ± 0.86***	1.59 ± 0.84	1.16 ± 1.14	1.28 ± 0.96
Heart burn	1.50 ± 1.16	1.50 ± 1.28	0.89 ± 0.89	0.71 ± 0.87	0.77 ± 0.94	0.78 ± 0.97
Loss of appetite	1.40 ± 1.21	1.34 ± 1.21	0.91 ± 1.01	0.71 ± 0.90	0.77 ± 0.94	0.83 ± 0.96
Retrosternal discomfort	1.00 ± 1.14	0.84 ± 1.17	0.66 ± 1.03	0.39 ± 0.67	0.59 ± 0.84	0.38 ± 0.63
Epigastric or upper abdominal pain	1.60 ± 1.01	1.68 ± 1.33	0.98 ± 1.07	0.71 ± 0.87	0.66 ± 0.96	0.53 ± 0.75

BST: *Banha-sasim-tang*.
*Statistically significant.

Table 3: Comparison between actual administration of experimental agents and subjects' expectation about their own groups at the end of the study.

		Subjects' expectation			P value
		BST n, (%)	Placebo n, (%)	Total n, (%)	
Actual administration	BST n, (%)	24 (28.6)	20 (23.8)	44 (52.4)	0.15
	Placebo n, (%)	28 (33.3)	12 (14.3)	40 (47.6)	
	Total n, (%)	52 (61.9)	32 (38.1)	84 (100)	

BST: *Banha-sasim-tang*.

Therefore, BST or its major component, pinelliae tuber, may improve the impaired gastric motility (delayed gastric emptying or antral dysmotility) in FD patients by changing the levels of gut hormones or regulating the HPA axis, thereby alleviating the early satiety symptom in this trial.

Although the pathogenic factors of FD remain unclear, up to 50% of FD patients have gastric motility dysfunction [23]. Gastric motility dysfunction, including gastric hypomotility or uncoordinated antral-duodenal contractions, may lead to delayed gastric emptying or impaired accommodation reflexes in the proximal stomach [24]. Gastric hypomotility and uncoordinated antral-duodenal contractions in FD patients are closely associated with gastric myoelectrical dysrhythmias. These dysrhythmias arise from dysregulation of gastric slow waves, which normally occur at a frequency of 3 cpm [19]. Therefore, GMA of the FD patients in this study was measured to investigate the correlation between dyspeptic symptoms and GMA variability and to elucidate the impacts of BST on gastric slow wave before and after administration of BST or placebo.

EGG is a diagnostic technique that can record GMA obtained from cutaneous abdominal electrodes [22]. Although some controversy exists regarding the correlation between GMA obtained from cutaneous EGG and dyspeptic symptoms [25], other clinical researchers have demonstrated a positive correlation between the frequency information obtained from cutaneous EGG and myoelectrical signals acquired directly from gastric serosal leads [26, 27].

During the fasting or fed states, the normal stomach muscles emit regular gastric slow waves and spike potentials and then periodically contract like heart muscles [22]. Multichannel, cutaneous EGG is a useful and noninvasive diagnostic technique that records GMA, consisting of gastric slow waves and spikes, through the abdominal surface [22]. In healthy subjects, a normal GMA is defined as approximately 2–4 cpm of the dominant frequency or >70% of normal gastric slow waves in a total EGG recording or a power ratio of >1 (defined as the ratio of the postprandial to fasting power of the DF) [22]. However, in FD patients, abnormal EGG findings, including excessive gastric dysrhythmia (especially tachygastria) or lowered postprandial dominant power, have been reported in many studies. These abnormal findings are closely correlated with delayed gastric emptying, impaired gastric accommodation, or antral hypomotility [19, 22, 28]. In our study, the DFs of both groups were within the normal range, whereas abnormal findings were observed in the pre/postprandial gastric slow waves (gastric dysrhythmia) and power ratios. Gastric dysrhythmia in channel 3 was one of the most common findings in this study. These results were quite similar to those of many previous EGG studies examining FD or gastric motility disorders [19, 22]. Moreover, the percentage of the normal power ratio was relatively low in the BST group compared with the placebo group; this may be due to a significantly high body mass index in the BST group. This observation was similar to those in a previous study examining the relationship between EGG power ratio

Table 4: Comparison of main parameters in electrogastrography (EGG) between BST and placebo groups.

EGG parameters	Groups	Normal/abnormal	Electrode sites of EGG					
			Channel 1		Channel 2		Channel 3	
			0 week	6 weeks	0 week	6 weeks	0 week	6 weeks
Preprandial dominant frequency *n*, (%)	BST	Normogastria	37 (74.0)	30 (69.8)	29 (58.0)	25 (56.8)	29 (58.0)	25 (56.8)
		Dysrhythmia	13 (26.0)	13 (30.2)	21 (42.0)	19 (43.2)	21 (42.0)	19 (43.2)
	Placebo	Normogastria	40 (80.0)	31 (75.6)	41 (82.0)	28 (68.3)	34 (68.0)	31 (75.6)
		Dysrhythmia	10 (20.0)	10 (24.4)	9 (18.0)	13 (31.7)	16 (32.0)	10 (24.4)
Postprandial dominant frequency *n*, (%)	BST	Normogastria	35 (70.0)	35 (79.5)	35 (70.0)	32 (72.7)	33 (66.0)	36 (81.8)
		Dysrhythmia	15 (30.0)	9 (20.5)	15 (30.0)	12 (27.3)	17 (34.0)	8 (18.2)
	Placebo	Normogastria	38 (76.0)	31 (75.6)	34 (68.0)	30 (73.2)	40 (80.0)	34 (82.9)
		Dysrhythmia	12 (24.0)	10 (24.4)	16 (32.0)	11 (26.8)	10 (20.0)	7 (17.1)
Power ratio *n*, (%)	BST	≥1	25 (59.5)	24 (74.3)	22 (57.9)	22 (67.6)	24 (75.0)	24 (67.6)
		<1	17 (40.5)	11 (25.7)	16 (42.1)	12 (32.4)	12 (25.0)	13 (32.4)
	Placebo	≥1	31 (72.7)	22 (59.5)	30 (73.2)	17 (50.0)	33 (85.0)	24 (72.2)
		<1	13 (27.3)	15 (40.5)	11 (26.8)	19 (50.0)	7 (15.0)	12 (27.8)

Abnormal EGG findings are defined as a reading of gastric dysrhythmia (including tachygastria, bradygastria, and arrhythmia) in ≥30% (or normogastria < 70%) of the total recordings of EGG in the fed state or a power ratio of >1 [22].
BST: *Banha-sasim-tang*.

and BMI (Table 1) [29]. After a 6-week administration of BST, early satiety was significantly ameliorated, the percentage of gastric dysrhythmia (including tachygastria) decreased, and the abnormal power ratios were improved (Tables 2 and 4). Early satiety symptoms are related to impaired gastric accommodation or delayed gastric emptying following a meal [19, 22]. Under these pathogenic conditions in FD patients, tachygastria or a lowered power ratio (<1) in the EGG findings has been frequently observed in many studies [19, 22]. In addition, EGG channel 3 is considered to be approximately located over the surface of the distal antrum. The distal antrum contains an ectopic pacemaker that generates the gastric slow waves in the stomach [19]. Therefore, BST may be postulated to modulate the irregularities in the ectopic pacemaker in the gastric antrum and decrease gastric dysrhythmia (especially tachygastria) or increase the power ratio and then improve dyspeptic symptoms such as early satiety.

In contrast, there were no significant differences in EGG findings according to the FD subtypes (data not shown). PDS or mixed FD subtypes were dominant in both groups (76% in the BST group and 84% in the placebo group), and these FD subtypes may be correlated to gastric motility dysfunction. Alternately, each subtype group may not have represented a large enough sample size to allow determination of statistically meaningful results. Therefore, a larger sample size for comparison of EGG parameters in other FD subtypes, such as EPS, is required in future studies. Our results suggest that EGG can be used for the assessment of interventional efficacy by detection of abnormal gastric slow waves.

In our study, there was a high placebo response rate with regard to the improvement in FD symptoms and QoL. In general, a 30–40% placebo response rate has been reported in many FD clinical trials [21]. The possible factors contributing to a high placebo response include natural history, Pavlovian conditioning, regression to the mean, small sample sizes, high expectations, longer administration durations, and a high number of visits and augmented relationships with doctors [30, 31]. As shown in Table 3, a relatively high percentage of subjects in the placebo group thought that they had received BST in our study. Additionally, other factors, such as an augmented relationship with doctors, may lead to a higher placebo response rate. Therefore, recruitment of subjects not exposed to experimental interventions, or clinical trial designs that control the relationship between the doctor and the patients will be needed in future studies.

Our study may be contrasted to a previous report regarding BST (*Ban xia xie xin* decoction in traditional Chinese medicine) [14]. First, we did not show a significantly beneficial effect of BST on FD patients compared with placebo; significant improvements in overall symptoms of FD were reported in the earlier study. This may be due to differences in the components of the herbal preparation used in the previous study. Although the name of the herbal preparation used in both studies was the same, the components were different. Furthermore, the use of 3 additional herbs (cortex *Magnolia officinalis*, medicated leaven, and ark shell) in the previous study may have positively or synergistically caused the improvement in FD symptoms; these herbs have been reported to have beneficial effects on functional gastrointestinal disorders [8]. Second, there was an obvious difference in the study populations between the 2 studies. Our study was aimed towards all FD patients, whereas the previous study targeted a specific group of FD patients, based on traditional Chinese medicine classifications. In addition, the duration of symptoms in our study (13.82 ± 3.25 years in the BST group) was much longer than that in the previous study (46.67 ± 59.41 months in the modified BST group). This indicated that the FD patients in our study had more severe dyspepsia symptoms, possibly affecting the efficacy of BST in our study.

Third, there were markedly different placebo response rates in the 2 studies. Our study showed a high placebo response rate (about 50% improvement), whereas only a 30% improvement in the placebo group was observed in the previous study. As mentioned above, other factors may have caused the high placebo response rate observed in our study. The high placebo response rate caused the BST to be statistically ineffective for the treatment of FD, despite similar improvements in FD symptoms compared with the previous study. Fourth, a specific diagnostic assessment, namely, GMA measurements by EGG, was conducted for FD patients in our study. There are several causes of FD development; however, gastric motility dysfunction, including delayed gastric emptying, antral dysmotility, or impaired meal accommodation, is the major cause of FD in Asians [28]. Therefore, the findings of our study will be useful for treating FD from the viewpoint of gastric motility dysfunction.

5. Conclusions

Compared with placebo, BST did not show a significant effect on FD. However, early satiety in FD patients may have improved after BST administration. In addition, EGG may be a useful modality for assessing the effects of therapeutics on gastric motility dysfunction. Further investigation focused on specific dyspeptic symptoms and related gut hormones, larger scale studies of FD subtypes, or development of the Zheng subgroup scale are required.

Conflict of Interests

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

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Review Article

Modified Chaihu Shugan Powder for Functional Dyspepsia: Meta-Analysis for Randomized Controlled Trial

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Context. Modified Chaihu Shugan powder (MCSP) is a popular traditional Chinese herbal formula for functional dyspepsia, which is revised from Chaihu Shugan San and recorded in a medical classic works of China. However, its role and effect in treating functional dyspepsia have not been well established. *Objective*. To assess the effect and safety of modified Chaihu Shugan powder for functional dyspepsia. *Methods*. We searched the published and unpublished studies up to August 2012. Only RCTs of modified Chaihu Shugan powder with or without prokinetic drugs versus prokinetic drugs in the patients diagnosed with functional dyspepsia were included. *Results*. Twenty-two clinical trials involving 1998 participants were included. There were evidences that modified Chaihu Shugan powder (RR = 1.20, 95%, CI 1.14 to 1.27) and modified Chaihu Shugan powder plus prokinetic drugs (RR = 1.18, 95%, CI 1.11 to 1.25) were significantly better treatment options than prokinetic drugs alone in improving symptoms. No serious adverse events were described in the included trials. *Conclusions*. This meta-analysis showed that modified Chaihu Shugan powder alone or in combination with prokinetic drugs might be more effective than prokinetic drugs alone. However, with poor methodological quality, all the included trials were at high risk of bias. Further large-scale high-quality trials are required for assessment.

1. Introduction

1.1. Rationale. Functional dyspepsia (FD), namely, functional gastrointestinal disorders or nonulcer dyspepsia, refers to symptoms centered in the upper abdominal region in absence of organic disease, such as epigastric pain, early satiety, fullness, belching, nausea, and vomiting [1–3]. It is a highly prevalent disorder. With influence of the definition applied, the global prevalence of FD had varied between 11.5% and 45% [4–6]. Although it is not a life-threatening condition, a number of out-patient studies suggested that FD markedly impaired patients' work and quality of life and laid a significant economic burden to the healthcare system [7–9]. Multiple factors, like motility abnormality, visceral hypersensitivity, psychosocial factors, excess secretion of gastric acid, duodenal acidity, helicobacter pylori, environment, diet, postinfectious factors, and genetics, were likely involved, but the pathogenesis of FD remains obscure [2, 6, 10, 11]. For this reason, no single medicine is effective for all patients with FD. In the area of medical therapy, traditional Chinese medicine (TCM) plays an important part, besides prokinetics, antacids, H_2-receptor antagonists, proton pump inhibitors, helicobacter pylori eradication, and antidepressants [12]. It was reported that at least one-third the US population used some form of TCM on a routine basis [13].

Chaihu Shugan San (CSS) is a classical and effective prescription recorded in a medical classic, Jingyue Quanshu also known as Jingyue's Complete Works, written in Ming Dynasty (1368–1644 year) of China, which has been used to improve some symptoms similar to FD by soothing liver, regulating qi, and relieving pain according to TCM theory. CSS are composed of Chinese Thorowax, Rhizoma Cyperi, Szechwan Lovage Rhizome, Pericarpium Citri Reticulatae,

Table 1: Inclusion and exclusion for the selected studies.

Inclusion criteria	Exclusion criteria
The patients diagnosed with FD according to Rome II [25], Rome III [26] consensus, or functional dyspepsia traditional Chinese medicine diagnosis standard [16]	Compared with other TCMs or control group combined with acid-suppressive drugs, eradication of *H. pylori*, fundus-relaxing drugs,and 5-HT_3 receptor antagonists
Control group with prokinetic drugs	Successful treatment without measuring in terms of illness severity scores or the intensity of individual symptoms
Clearly outlined criteria for successful treatment	Course of treatment ≤ 2 weeks
Random allocation	

Fructus Aurantii, white peony root, and licorice. As we know every formula of TCM is an organic whole. A basic structure of formulas includes monarch, minister, assistant, and guide herbal medicines. According to TCM theory, so long as monarch herbal medicines and combination relationship of a classical prescription do not change, there is no change in the main clinical indications of the prescription [14]. In the procedure of TCM treatment, nearly all of the clinical prescriptions are modified by classic formulas [15]. In the prescription of CSS, Chinese Thorowax as monarch herbal medicine plays a principal role in therapeutic effect; Rhizoma Cyperi and Szechwan Lovage Rhizome are minister herbal medicine increasing the effect of Chinese Thorowax; Pericarpium Citri Reticulatae, Fructus Aurantii, and white peony root are used to harmonize the interaction between the ingredients; as a guide herbal medicine, licorice could guide the ingredients to the lesions. In the light of TCM theory, MSCP added Chinese Angelica or Radix Curcumae to the FD patients with qi stagnation and blood stasis, Cape Jasmine Fruit or Radix Scutellariae to the FD patients with transformation of depressed liver qi into fire, and Fructus Lycii or Radix Adenophorae to the FD patients with liver yin deficiency based on CSS [14]. Therefore, MCSP is now a popular traditional Chinese herbal formula for improving some symptoms similar to FD and recommended by functional dyspepsia traditional Chinese medicine diagnosis standard (2001 edition) [16].

The Study showed that MCSP could significantly increase propulsive rate of the small intestine (77.16 ± 3.42%) and decrease the residual amount of the pigment in the stomach in the rats [17]. Study from Qiu et al. suggested that ferulic acid and meranzin hydrate found in MCSP had the significant effect on promoting gastrointestinal motility in rats [18]. Saikosaponin (main activity of Chinese Thorowax) has anti-inflammatory activity and raised the painful threshold value [19]. Fructus Aurantii can significantly inhibit the spontaneous movement of isolated duodenum from rabbits and reduce the contraction force which presents concentration-response relationship [20]. Zhu et al.'s study proved that *Cyperus Rotundus* can delay gastric emptying, protecting gastric mucosa and reduce incidents of ulcer in the model of rats' gastric ulcer [21]. White Peony root can reduce internal high sensitivity and regulate the function of brain-gut axis [22, 23]. Animal studies have proved that Pericarpium Citri Reticulatae and licorice root promoted gastric emptying and small intestinal vermiculation and protected gastric mucosa [24].

1.2. Objectives. Evidence that clearly demonstrates effect and safety of MCSP has not yet been systematically studied. In this study, we evaluated the effects of MCSP in monotherapy or in combination with other prokinetic agents on FD through a rigorous systematic review and meta-analysis of randomized trial.

2. Methods

2.1. Eligibility Criteria. To make sure of the validity, applicability, and comprehensiveness, we specified the eligibility of inclusion and exclusion criteria for the review (Table 1).

2.2. Information Sources. We searched the following electronic database: Cochrane Library (issue to August 2012), MEDLINE (1995 to August 2012), EMBASE (1995 to August 2012), SCI database (Science Citation Index Expanded), CNKI Database (China Knowledge Resource Integrated Database, 1979 to August 2012), Wanfang Data (1998 to August 2012), VIP Information (1985 to August 2012), CBMDisc (Chinese Biology Medical disc, August 2012), and Chinese Clinical Trials Registry (issue to August 2012). We also screened the relevant trials and identified review listed in the references. We restricted the language of publications to English and Chinese.

2.3. Search Strategy. We used the Boolean logic search for the databases as follows: (modified chaihu shugan *OR chaihu shugan *OR chai hu shu gan *OR Bupleurum Soothing*) and (functional dyspepsia OR nonulcer dyspepsia OR functional gastrointestinal disorders OR dyspepsia).

2.4. Study Selection. Two reviewers (N. Yang and X. Qiu) independently screened the information contained in the title, abstract, key words, and description of each searched paper according to the inclusion and exclusion criteria. Any difference during assessment between the two reviewers was discussed or resolved by a third dependent reviewer (X. Jiang).

2.5. Data Collection Process. We developed a data extraction sheet for the included study. To avoid bias in the data abstraction, two reviewers (X. Qiu and Z. Hu) independently abstracted the data from the papers and compared the results. Disagreements were resolved by discussion between the two reviews; if no agreements could be reached, it was resolved by the third dependent reviewer (X. Jiang).

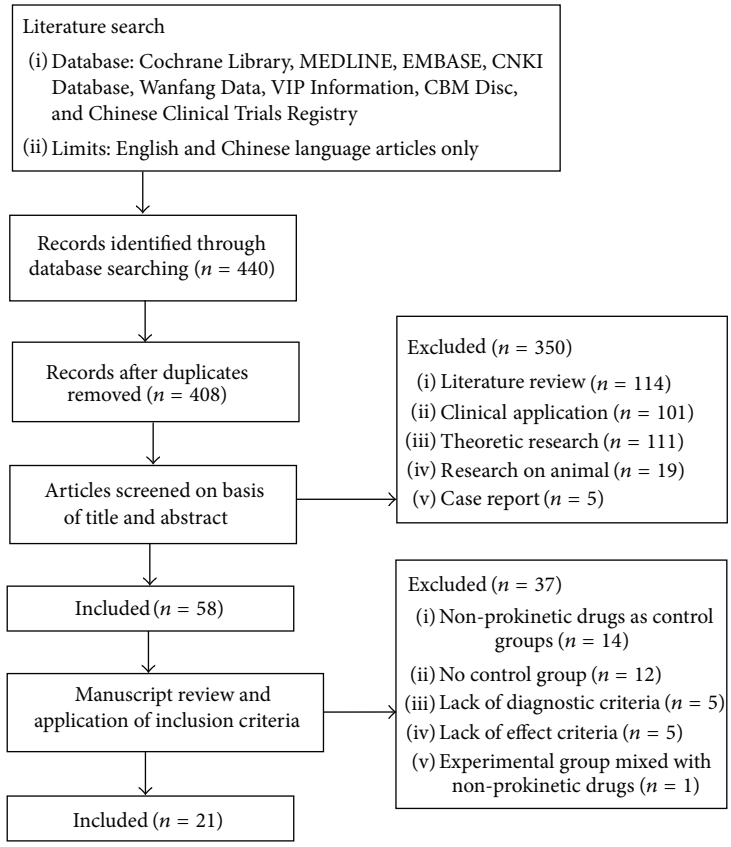

Figure 1: Flow diagram of selective for systematic review of MCSP for FD.

2.6. Data Items. Items extracted from each study include citations of studies, method of the trials, simple size, gender and average age of the participants, treatment duration, each group's interventions, symptom improvement index and adverse drug reaction.

2.7. Risk of Bias. Two reviewers (N. Yang and Z. Hu) independently accessed the risk of bias for each trial according to the Cochrane Handbook for Systematic Reviewers of Interventions version 5.1.0 [48]. Cochrane collaboration addressed the following seven specific domains to describe the risk of bias, including random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective outcome reporting, and other biases. Each trail was categorized as "Low risk" of bias, "High risk" of bias, or "Unclear risk" of bias. Disagreements were resolved by discussion and by adjudicated by a third reviewer (Jiang) when necessary.

2.8. Summary Measures. Our comparisons included MCSP versus prokinetic drugs and MCSP plus prokinetic drugs versus prokinetic drugs. We analyzed the main outcomes data of the trials according to Cochrane Handbook. We reported risk ratio (RR) with 95% confidence intervals (CI) for the dichotomous data, and mean differences (MD) with 95% CI for continuous data. We used Chi-square statistic to assess the heterogeneity. Fixed effect model can be appropriate when there is statistical homogeneity ($P > 0.1$, $I^2 < 50\%$) among the studies, and random effect model has to be pursued when statistical heterogeneity ($P < 0.1$, $I^2 > 50\%$) exists in the trials. Publication bias was assessed by the funnel plot.

3. Results

3.1. Study Selection. The study selection process, the reasons for excluding, and the search results at various stages were shown as a flow diagram (Figure 1) according to the planed search strategy. Successive rounds of review yielded 21 final studies, and one of those studies contained 2 RCTs [33]. The total of 22 RCTs were included, involving 1939 participants with FD. All studies were conducted in Chinese. In the trials, 13 RCTs compared MCSP versus prokinetic drugs, and 9 RCTs compared MCSP plus prokinetic drugs versus prokinetic drugs. There were no placebo controlled studies.

3.2. Study Characteristics. Of the 22 selected trials, 21 described the comparability analysis of source of participants, gender, age, and course of FD. The remaining 1 trail did not mention the information. The mean age of participants ranged from 33.9 to 56.0 years. Trial duration lasted for 3 weeks to 12 weeks. MCSP was prepared as decoction with traditional method of being boiled with water. All

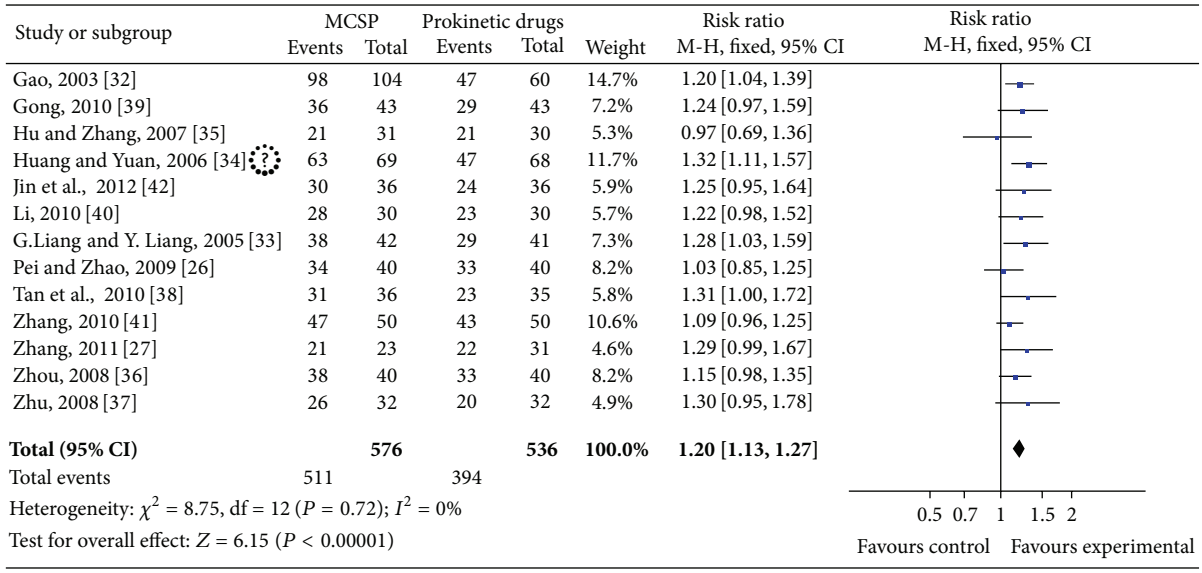

Figure 2: MCSP versus prokinetic drugs; outcomes: the total effectiveness.

the interventions were taken orally. Further details of the included RCTs were presented in Table 1. Incidence of adverse reactions of 22 trials was no related reports.

3.3. Risk of Bias within Studies. Overall the studies were at high risk of bias, which were shown in Table 2. All the trials claimed randomization, but only three RCTs [33, 38] reported that random number table was used. The remaining studies failed to provide information of how randomization was carried out. No allocation concealment and blinding were described. There were not notifications of dropouts and withdraws. No intention-to-treatment analyses were presented.

3.4. Results of Individual Studies

3.4.1. The Total Effective Rates of MCSP versus Prokinetic Drugs for FD. Thirteen trials compared the clinical total effectiveness of MCSP versus prokinetic drugs for FD (n = 1112). The test for heterogeneity was insignificant statistically ($P = 0.72$, $I^2 = 0\%$). Therefore, fixed effect model was used in the meta-analysis. The risk ratio for improvement of FD for MCSP treated versus prokinetic drugs treated was 1.20(95% CI 1.13 to 1.27), which achieved statistically significant (see Figure 2).

3.4.2. The Total Effective Rates of MCSP Plus Prokinetic Drugs versus Prokinetic Drugs for FD. Nine studies compared the clinical total effectiveness of MCSP versus prokinetic drugs for FD (n = 827). The test for heterogeneity was insignificant statistically ($P = 0.85$, $I^2 = 0\%$). Therefore, fixed effect model was used in the meta-analysis. MCSP plus prokinetic drugs had a greater probability of relieving the symptom of FD compared with prokinetic drugs alone (RR = 1.18, 95%, CI 1.11 to 1.25) (see Figure 3).

3.5. Risk of Bias across Studies. Figure 4 showed the reporting bias of trails on MCSP versus prokinetic drugs for FD. Each dot represented one study. The distribution of dots on the either side of center line was asymmetrical, which meant that there was a potential reporting bias.

To avoid distinguishing chance from real asymmetry because of fewer trials with too low power according to the Cochrane Handbook, we did not use test for funnel plot to detect the reporting biases of trails on MCSP plus prokinetic drugs versus prokinetic drugs for FD.

4. Discussion

4.1. Summary of Evidence. With the development of new effective treatments, herbal medicines have been increasingly used in many countries especially for benign and chronic conditions such as FD [49, 50]. Some studies showed that artichoke leaf extract [51], peppermint and caraway oil [52], MCSP [27–47], and Rikkunshito (Liu Jun Zi Tang) [53] were advocated for FD. However, there had been no systematic research to indicate that MCSP did worse or better than other medicines against FD.

FD is dyspepsia without evidence of an organic disease that is likely to explain the symptoms. There is no certain cure for it thus far. A vast number and variety of pharmacological treatment strategies was introduced to relieve the symptoms of FD. But some problems exist in allmost treatments. The efficacy of *H. pylori* eradication for FD remains controversial. Some meta-analyses concluded that H. pylori eradication had significant advantage over placebo [54, 55], but there were other studies which found insufficient or no benefit existing in treating FD [56, 57]. Histamine-type 2 receptor had superiority over placebo for patients with FD in clinical trials [13], however, which were merely limited to the symptom of epigastric pain and did not apply in global dyspepsia symptoms [58]. Some prokinetic agents showed more significant

Table 2: Characteristics of randomized controlled trials of MCSP for functional dyspepsia.

Study	Method	Duration	N (M : F)	Mean age	Interventions	Symptom improvement
Gao, 2003 [27]	RCT, not blinded Comparison: individuals	4 w	164 (76 : 88)	37.3	(1) MCSP (bid) (2) Domperidone (10 mg, tid)	(1) TER: 94.2% (98/104) (2) TER: 78.3% (47/60)
G. Liang and Y. Liang, 2005 [28]	RCT, not blinded Comparison: individuals	4 w	83 (38 : 45)	39.6	(1) MCSP (bid) (2) Cisapride (10 mg, tid)	(1) TER: 90.5% (38/42) (2) TER: 70.7% (29/41)
Huang and Yuan, 2006 [29]	RCT, not blinded Comparison: individuals	4 w	137 (64 : 73)	40.4	(1) MCSP (bid) (2) Domperidone (10 mg, tid)	(1) TER: 92.5% (63/69) (2) TER: 70.6% (47/68)
Hu and Zhang, 2007 [30]	RCT, not blinded Comparison: individuals	12 w	61 (16 : 45)	39.5	(1) MCSP (bid) (2) Cisapride (5–10 mg, tid)	(1) TER: 67.7% (21/31) (2) TER: 70.0% (21/30)
Zhou, 2008 [31]	RCT, not blinded Comparison: individuals	4 w	80 (42 : 38)	52.4	(1) MCSP (bid) (2) Cisapride (5 mg, tid), Vitamin B6 (20 mg, tid), Oryzanol tablets (20 mg, tid)	(1) TER: 95.0% (38/40) (2) TER : 82.5% (33/40)
Zhu, 2008 [32]	RCT, not blinded Comparison: individuals	4 w	64 (28 : 36)	36.4	(1) MCSP (bid) (2) Domperidone (10 mg, tid)	(1) TER: 81.3% (26/32) (2) TER: 62.5% (20/32)
Pei and Zhao, 2009 [33]	RCT, not blinded Comparison: individuals	4 w	80 (41 : 39)	51.9	(1) MCSP (bid), (2) Trimebutine maleate tablets (100 mg, q.d)	(1) TER: 85.0% (34/40) (2) TER: 82.5% (33/40)
Tan et al., 2010 [34]	RCT, not blinded Comparison: individuals	4 w	71 (34 : 37)	36.5	(1) MCSP (bid) (2) Flupentixol melitracen tablets (2 pills, q.d)	(1) TER: 86.1% (31/36) (2) TER: 65.7% (23/35)
Gong, 2010 [35]	RCT, not blinded Comparison: individuals	4 w	86 (30 : 56)	36.8	(1) MCSP (bid) (2) Domperidone (10 mg, tid)	(1) TER: 83.7% (36/43) (2) TER: 65.1% (29/43)
Li, 2010 [36]	RCT, not blinded Comparison: individuals	4 w	60 (24 : 36)	40.6	(1) MCSP (bid) (2) Cisapride (5 mg, tid)	(1) TER: 93.3% (28/30) (2) TER: 76.7% (23/30)
Zhang, 2010 [37]	RCT, not blinded Comparison: individuals	3 w	100	N/A	(1) MCSP (bid) (2) Cisapride (5 mg, tid)	(1) TER: 94.0% (47/50) (2) TER: 86.0% (43/50)
Zhang, 2011 [38]	RCT, not blinded Comparison: individuals	4 w	54 (23 : 31)	N/A	(1) MCSP (bid) (2) Domperidone (10 mg, tid), Compound Azintamide tablets (2 pills, tid)	(1) TER: 91.3% (21/23) (2) TER: 70.9% (22/31)
Jin et al., 2012 [39]	RCT, not blinded Comparison: individuals	4 w	72 (32 : 40)	39.3	(1) MCSP (bid) (2) Mosapride citrate tablets (5 mg, tid)	(1) TER: 83.3% (30/36) (2) TER: 66.7% (24/36)
Liu et al., 2005 [40]	RCT, not blinded Comparison: individuals	4 w	180 (72 : 108)	47.5	(1) MCSP (bid), Cisapride (5 mg, tid) (2) Cisapride (5 mg, tid)	(1) TER: 93.3% (84/90) (2) TER: 76.7% (69/90)
Shen et al., 2005 [41]	RCT, not blinded Comparison: individuals	4 w	57 (26 : 31)	33.9	(1) MCSP (bid), Cisapride (10 mg, tid) (2) Cisapride (10 mg, tid)	(1) TER: 83.3% (25/30) (2) TER: 62.9% (17/27)
Zhang and Liu, 2007 [42]	RCT, not blinded Comparison: individuals	4 w	94 (38 : 56)	56.0	(1) MCSP (bid), Mosapride (5 mg, tid) (2) Mosapride (5 mg, tid)	(1) TER: 93.8% (45/48) (2) TER: 73.9% (34/46)
Liu et al., 2008 [43]	RCT, not blinded Comparisonindividuals	4 w	48 (19 : 29)	35.0	(1) MCSP (bid), Flupentixol melitracen tablets (1 pill, q.d) (2) Flupentixol melitracen tablets (1 pill, q.d)	(1) TER: 95.8% (23/24) (2) TER: 87.5% (21/24)
Feng and Liu 2008 [44]	RCT, not blinded Comparison: individuals	4 w	75 (33 : 42)	36.1	(1) MCSP (bid), Domperidone (10 mg, tid) (2) Domperidone (10 mg, tid)	(1) TER: 89.5% (34/38) (2) TER: 81.1% (30/37)
Pei and Zhao, 2009 [33]	RCT, not blinded Comparison: individuals	4 w	80 (42 : 38)	52.3	(1) MCSP (bid), Trimebutine maleate tablets (100 mg, q.d) (2) Trimebutine maleate tablets (100 mg, q.d)	(1) TER: 95.0% (38/40) (2) TER: 82.5% (33/40)

Table 2: Continued.

Study	Method	Duration	*N* (M : F)	Mean age	Interventions	Symptom improvement
Qiu, 2010 [45]	RCT, not blinded Comparison: individuals	4 w	75 (28 : 47)	43.5	(1) MCSP (bid), Domperidone (10 mg, tid) (2) Domperidone (10 mg, tid)	(1) TER: 94.7% (36/38) (2) TER : 86.5% (32/37)
Tian, 2010 [46]	RCT, not blinded Comparison: individuals	4 w	98 (43 : 55)	40.9	(1) MCSP (bid), Domperidone (10 mg, tid) (2) Domperidone (10 mg, tid)	(1) TER: 87.8% (43/49) (2) TER : 71.4% (35/49)
Fan, 2010 [47]	RCT, not blinded Comparison: individuals	1 m	60 (21 : 39)	46.5	(1) MCSP (bid), Cisapride (5 mg, tid) (2) Cisapride (5 mg, tid)	(1) TER : 96.7% (58/60) (2) TER : 85.0% (51/60)

RCT: randomized clinical trial; F: female; m: male; w: week; M: month; 1: experimental group; 2: control group; TER: total effective rate.

Study or subgroup	MCSP + prokinetic drugs Events	Total	Prokinetic drugs Events	Total	Weight	Risk ratio M-H, fixed, 95% CI
Fan, 2010 [50]	58	60	51	60	15.7%	1.14 [1.01, 1.28]
Feng and Liu, 2008 [47]	34	38	30	37	9.4%	1.10 [0.91, 1.33]
Liu, 2005 [43]	84	90	69	90	21.3%	1.22 [1.07, 1.38]
Liu et al., 2008 [46]	23	24	21	24	6.5%	1.10 [0.92, 1.30]
Pei and Zhao, 2009 [26]	38	40	33	40	10.2%	1.15 [0.98, 1.35]
Qiu, 2010 [48]	36	38	32	37	10.0%	1.10 [0.94, 1.27]
Shen et al., 2005 [44]	25	30	17	27	5.5%	1.32 [0.95, 1.84]
Tian, 2010 [49]	43	49	35	49	10.8%	1.23 [1.00, 1.51]
Zhang and Liu, 2007 [45]	45	48	34	46	10.7%	1.27 [1.05, 1.53]
Total (95% CI)		**417**		**410**	**100.0%**	**1.18 [1.11, 1.25]**
Total events	386		322			
Heterogeneity: $\chi^2 = 4.06$, df = 8 ($P = 0.85$); $I^2 = 0\%$						
Test for overall effect: $Z = 5.68$ ($P < 0.00001$)						

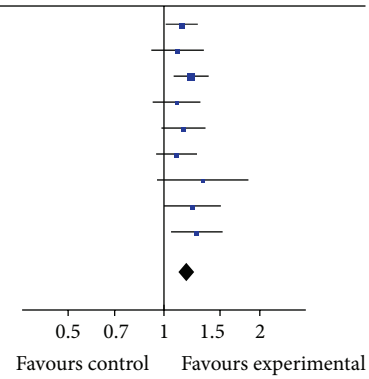

Figure 3: MCSP + prokinetic drugs versus prokinetic drugs; outcomes: the total effectiveness.

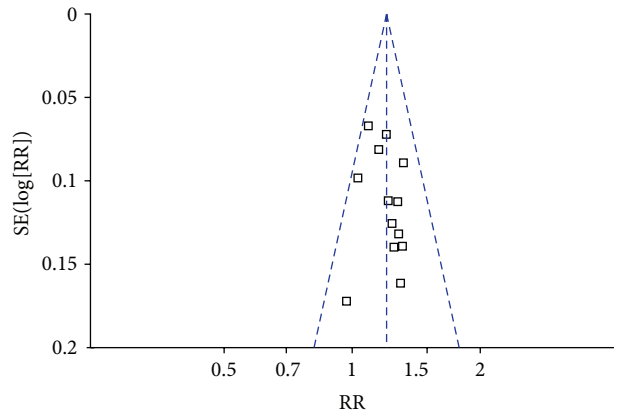

Figure 4: Funnel plot for MCSP versus prokinetic drugs for FD.

decrease in FD than placebo, which were widely prescribed in Canada, Mexico, and Australia like domperidone [13, 59]. But some of these such as metoclopramide and cisapride were of limited use because of the central nervous system and cardiac side-effects [60, 61]. Proton pump inhibitors (PPI) have been widely evaluated in the confined patients who have ulcer-like symptoms [62, 63]. Also antidepressants were reported to be used in treating FD, but there have been very limited data on it [64]. Thus treatment of patients with FD has been still a challenge and more effects should be made to develop new effective interventions.

MCSP is based on an ancient formula that has been clinically used in China since 1600s. Since 1990s, published clinical trials have been reporting that MCSP has good therapeutic effects on FD. Our meta-analysis truly showed that MCSP might be a benefit for the patients suffering from FD. MCSP plus prokinetic drugs appeared to be more effective than prokinetic drugs alone. Although every ingredient of preparation does help to get rid of symptoms in FD, MCSP reflects the uncertainty about the clear mechanisms. It is believed that patients who are proved to be intractable to drug therapies likely suffer psychological disturbances [65]. A study supported that Chaihu Shugan powder was effective and safe in treating depression [66]. And the present meta-analysis proved significant effectiveness of MSCP in FD, which expressed a consistency between Western and Chinese medicine.

4.2. Limitations. There are several limitations in our study. Firstly, all the included trials were at high risk of bias. All the studies were in Chinese. Of the 23 trials, only two described the method of randomization, which weakened the reliability and repeatability of the research. None of the trails provided the information about allocation concealment and blinding. No multicenter and large-scale RCTs were identified. Sample size and allocation of samples among the groups are optional. Most of the literatures had no follow-up records. Lack of intention-to-treat analysis can also lead to biased judgment of efficacy. Secondly, except one study with treatment course of 3 weeks and another with 12 weeks, the length of course in the other included trials was 4 weeks. According to the diagnostic criteria for FD, it is a chronic condition with symptoms that recur frequently over time [16, 25, 26, 49]. Shortened therapeutic period of FD might impact the treatment and make it difficult to find adverse drug reactions. Moreover, included studies of MCSP did not change the monarch, minister, assistant, and guide herbal medicines of CSS's prescription, which only added several herbs, but it still needs experimental evidence to establish the effect of added ingredients. Last but not the least, MCSP in all of the included trials were prepared by boiling or decocting, which is traditional way of preparing herbal medicines in China. It contributed to no placebo used in clinical trials of traditional Chinese medicine. The composition of the same prescription, in fact, was flexible, and thus caused performance bias. Yet a research suggested that granules and decoction of 20 traditional Chinese formulas had no significant statistical difference in their effectiveness [67]. In the view of drug development, conventional forms of TCMs are beneficial for improving compliance and quality of clinical trials.

Table 3: Assessment of risk of bias of included studies.

Study	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other biases
Gao, 2003 [27]	U	H	U	U	U	U	H
G. Liang and Y. Liang, 2005 [28]	U	H	U	U	U	U	H
Huang and Yuan, 2006 [29]	U	H	U	U	U	U	H
Hu and Zhang, 2007 [30]	U	H	U	U	U	U	H
Zhou, 2008 [31]	U	H	U	U	U	U	H
Zhu, 2008 [32]	U	H	U	U	U	U	H
Pei and Zhao, 2009 [33]	L	H	U	U	U	U	H
Tan et al., 2010 [34]	U	H	U	U	U	U	H
Gong, 2010 [35]	U	H	U	U	U	U	H
Li, 2010 [36]	U	H	U	U	U	U	H
Zhang, 2010 [37]	L	H	U	U	U	U	H
Zhang, 2011 [38]	U	H	U	U	U	U	H
Jin et al., 2012 [39]	U	H	U	U	U	U	H
Liu, 2005 [40]	U	H	U	U	U	U	H
Shen et al., 2005 [41]	U	H	U	U	U	U	H
Zhang and Liu, 2007 [42]	U	H	U	U	U	U	H
Liu et al., 2008 [43]	U	H	U	U	U	U	H
Feng and Liu 2008 [44]	U	H	U	U	U	U	H
Pei and Zhao, 2009 [33]	L	H	U	U	U	U	H
Qiu, 2010 [45]	U	H	U	U	U	U	H
Tian, 2010 [46]	U	H	U	U	U	U	H
Fan, 2010 [47]	U	H	U	U	U	U	H

L: low risk of bias; U: unclear; H: high risk of bias.

4.3. Conclusion. The result of this review provides preliminary data suggesting that either MCSP or MCSP plus prokinetic drugs achieved statistically significant improvement of symptoms of FD than prokinetic medications alone. However, the poor methodological quality made it difficult to determine the real role of MCSP in management of FD. After all, this review produced the rational evidence for the further use, research, and development of MCSP. Further large-scale high-quality clinical trials are required for assessment (see Table 3).

Conflict of Interests

There is no conflict of interests in relation to the study discussed in the paper.

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Research Article

Efficacy of Modified Ban Xia Xie Xin Decoction on Functional Dyspepsia of Cold and Heat in Complexity Syndrome: A Randomized Controlled Trial

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Background. Chinese herbal medicine (CHM) has been used in China and elsewhere to treat patients with functional dyspepsia (FD). However, controlled studies supporting the efficacy of such treatment are lacking. *Objective*. To assess the efficacy and safety of modified Ban xia xie xin decoction in patients with FD of cold and heat in complexity syndrome. *Methods*. We performed a randomized, double-blind, placebo-controlled trial involving patients from five centers. Patients with FD of cold and heat in complexity syndrome ($n = 101$) were randomly assigned to groups given either CHM modified Ban Xia Xie Xin decoction or placebo in a 2 : 1 ratio. Herbal or placebo granules were dissolved in 300 mL of boiled water cooled to 70°C. Patients in both groups were administered 150 mL (50°C) twice daily. The trial included a 4-week treatment period and a 4-week followup period. The primary outcomes were dyspepsia symptom scores, measured by the total dyspepsia symptom scale and the single dyspepsia symptom scale at weeks 0, 1, 2, 3, 4, and 8. *Results*. Compared with patients in the placebo group, patients in the CHM group showed significant improvements according to the total and single dyspepsia symptom scores obtained from patients ($P < 0.01$) and investigators ($P < 0.01$). *Conclusions*. CHM modified Ban Xia Xie Xin decoction appears to offer symptomatic improvement in patients with FD of cold and heat in complexity syndrome. *Trial Registration*. Chinese Clinical Trial Registry (ChiCTR): ChiCTR-TRC-10001074.

1. Introduction

Functional dyspepsia (FD) is a common functional gastrointestinal disorder characterized by chronic or recurrent upper abdominal fullness, epigastric pain, eructation, bloating, early satiety, nausea, vomiting, regurgitation, burning, loss of appetite, and other symptoms. FD accounts for a significant proportion of patients seen in gastroenterology offices. The global prevalence of FD is estimated to be 11.5% to 29.2% [1–4]. The direct and indirect economic burden caused by FD is huge and has a considerable negative impact on productivity [5, 6]. The pathophysiology of FD is poorly understood, although various mechanisms are thought to play a role in the development of symptoms [7–10]. No single available

treatment is reliably effective for this condition. Many studies have suggested the potential effectiveness of Chinese herbal medicine (CHM) in the treatment of FD [11]. Ban Xia Xie Xin decoction has been widely used for the treatment of patients with FD of cold and heat in complexity syndrome [12, 13]. However, most previous clinical trials have lacked rigor and used poor techniques for randomization and blinding. To date, relatively few multicenter, prospective, randomized, placebo-controlled, double-blind studies on using CHM to treat FD have been performed.

In Traditional Chinese Medicine (TCM), FD is considered to be nearly equivalent to the TCM term "stuffiness and fullness" [14], which is divided into different syndromes according to the clinical symptoms and signs. In our previous research, we studied the distribution of the different syndromes in 565 patients with FD and found that "cold and heat in complexity" is one of the most common syndromes of FD [15]. Ban Xia Xie Xin decoction is a traditional Chinese compound herbal recipe for mild regulation of cold and heat. We added related herbal medicines (Cortex Magnoliae officinalis, Medicated Leaven, Ark Shell) to that recipe to identify the formula of "modified Ban Xia Xie Xin decoction" that had a satisfactory clinical effect. Moreover, previous studies have shown that the active ingredients in the modified Ban Xia Xie Xin decoction can reinforce the protective function of the mucosa, regulate gastrointestinal function, and induce anti-inflammatory action against *Helicobacter pylori* [16–20].

In this trial, we tested the efficacy of the modified Ban Xia Xie Xin decoction in patients with FD and cold and heat in complexity syndrome using a randomized, double-blind, placebo-controlled study design.

2. Materials and Methods

2.1. Design. This study was a double-blind, placebo-controlled clinical trial. Patients were randomized into CHM or placebo groups in a 2 : 1 ratio. Because it would be unethical to assign an equal number of ill subjects to the ineffective placebo treatment, the 2 : 1 randomization plan was chosen to protect the rights of the subjects. The trial protocol was approved by regional ethics review boards, including the National Review Board for Clinical Drug Research in the Beijing Hospital of Chinese Medicine Hospital affiliated to Capital Medical University. There were no major changes in the study protocol after initiation of the study.

2.2. Participants. Patients were screened by investigators at five sites in China: the Beijing Hospital of Traditional Chinese Medicine affiliated to Capital Medical University, the Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, the Second Affiliated Hospital of Guangdong University of Traditional Chinese Medicine, the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, and the Beijing Xuanwu Hospital of Traditional Chinese Medicine. The study was conducted between April 2009 and March 2011. Patients were assessed according to the Rome III criteria and *The Guiding Principle for Clinical Research on New Drugs of Traditional Chinese Medicine* [14]. The inclusion and exclusion criteria are shown in Table 1. Written informed consent was obtained from all patients prior to inclusion in the trial. Patients were free to withdraw from the study at any time.

2.3. Randomization and Blinding. Randomization was performed with SAS9.10 (block size 6). Patients and investigators were all blinded. Eligible patients were assigned a randomization number according to a predetermined list at each center. These numbers were allocated to patients in sequential order and registered in the patient enrolment list, and the allocation was concealed. Emergency envelopes containing the randomization code were provided to the investigators and were examined at the end of the trial to ensure that the blinded conditions had been maintained.

2.4. Interventions. Patients in the CHM group were provided granules of Chinese herbal extracts prepared by Tcmages Pharmaceutical Co., Ltd. (Beijing, China). The standard herb formula (Table 2) was a modified Ban Xia Xie Xin decoction. Patients in the placebo group were given placebo granules that had been prepared by the same supplier and were designed to taste, smell, and look similar to the Chinese herbal formula granules. To ensure that the patients were not able to discriminate between placebo and active treatments, 20 healthy volunteers participated in a randomized taste and visual assessment of the placebo and active medication. Eight volunteers correctly identified the active compound as active, whereas 12 volunteers considered the placebo preparation to be the active compound. Thus, it is reasonable to assume that the medication was given in an appropriately blinded manner. Granules were dissolved in 300 mL of boiled water cooled to 70°C. Patients in both groups were required to take 150 mL (50°C) twice daily. For the duration of the trial, the patients were not allowed to take any concomitant medications associated with the treatment of FD. Treatment continued for 4 weeks and was followed by a 4-week followup period.

2.5. Outcomes. We assessed FD symptoms using two scales: (1) the total dyspepsia symptom (TDS) scale and (2) the single dyspepsia symptom (SDS) scale. Ratings were completed by both the investigators and patients at baseline and at weeks 1, 2, 3, 4, and 8.

2.5.1. Total Dyspepsia Symptom Scale. The TDS scale assessed eight items (postprandial fullness and bloating, early satiety, epigastric pain, epigastric burning, nausea, vomiting, eructation, and "other symptoms"), each with four scoring options (absent = 0, mild = 1, moderate = 2, or severe = 3). The percentage of TDS score improvement was calculated using the following formula: (TDS score of week 0−TDS score of week 4)/TDS score of week 0.

2.5.2. Single Dyspepsia Symptom Scale. The SDS scale measured three aspects of four principal symptoms of FD:

Table 1: Inclusion and exclusion criteria.

Inclusion criteria
(1) Patients who meet the Rome III diagnosis standard of functional dyspepsia.
(2) Patients who have cold and heat in complexity syndrome.
(3) Patients aged 18 to 65 without gender limitation.
(4) Singed the informed consent.
Exclusion criteria
(1) Patients who combined with GI ulcer, erosive gastritis, atrophic gastritis, severe dysplasia of gastric mucosa, or suspicious malignant lesion.
(2) Patients who have overlap syndrome combined with gastroesophageal reflux disease or irritable bowel syndrome.
(3) Patients whose syndrome is difficult to differentiate.
(4) Patients who have connective tissue diseases, diabetes or other endocrine disease, climacteric syndrome, or severe diseases in heart, liver, lung, kidney, or blood.
(5) Pregnant or lactating women. Disabled people.
(6) Patients with history of alcoholic or drug abuse.
(7) Patients who have allergic constitution or known to be allergic to the drug used in this trial.
(8) Patients who are involved in other trials.
(9) Patients with poor compliance or other reasons that the researcher considered not to be appropriate to participate in this trial.
(10) Patients with severe depression and have suicidal tendency.

Table 2: Chinese herb formula.

Chinese name	Pharmaceutical name	Powdered herb, %	Extraction yield, %
Ban Xia	Pinellia Tuber	9.1%	20%–30%
Huang Qin	Radix Scutellariae	9.1%	20%–30%
Huang Lian	Rhizoma Coptidis	4.5%	10%–20%
Gan Jiang	Dried Ginger	9.1%	10%–20%
Dang Shen	Pilose Asiabell Root	13.6%	40%–70%
Gan Cao	Liquorice Root	4.5%	20%–30%
Hou Po	Cortex Magnoliae Officinalis	9.1%	10%–20%
Shen Qu	Medicated Leaven	13.6%	20%–30%
Wa Lengzi	Ark Shell	27.3%	40%–70%

epigastric pain, epigastric burning, postprandial fullness and bloating, and early satiety. The three aspects were frequency, intensity, and level of discomfort and were rated by four scoring options (absent = 0, mild = 1, moderate = 2, or severe = 3). The total score obtained using this scale was called the SDS score. The percentage of SDS score improvement was calculated using the following formula: SDS score of week 0−SDS score of week 4)/SDS score of week 0.

2.6. Safety Monitoring. To assess the safety of the 4-week treatment, routine blood, urine, and stool sample tests as well as electrocardiogram and blood biochemical tests (ALT, AST, BUN, and Scr levels) were conducted before randomization and immediately after the completed treatment. During the trial, adverse events were observed in detail and documented using case report forms.

2.7. Sample Size. We performed sample size calculations in two ways. To guarantee the reliability of the trial, the calculation yielding the larger sample size was used. The sample size was calculated according to the following formula [21]:

$$n_1 = \left[u_\alpha \sqrt{\frac{\pi_c (1-\pi_c)(1+c)}{c}} + u_\beta \sqrt{\pi_1 (1-\pi_1) + \frac{\pi_2 (1-\pi_2)}{c}} \right]^2 \times \left((\pi_1 - \pi_2)^2 \right)^{-1}, \quad (1)$$

$$n_2 = c n_1$$

n_1 CHM, n_2 placebo,

$$\pi_c = \frac{\pi_1 + c\pi_2}{1+c}, \quad u_\alpha = 1.64, \quad u_\beta = 1.28, \quad c = 2,$$

$$\pi_1 = 0.5, \quad \pi_2 = 0.80.$$

Table 3: Patient characteristics.

Variables	CHM ($n = 67$)	Placebo ($n = 34$)	P values
Characteristic			
Mean age ± SD, year	39.87 ± 12.89	40.50 ± 12.44	$P > 0.05$
Sex ratio (male : female)	21 : 46	13 : 21	$P > 0.05$
Mean height ± SD, cm	164.15 ± 8.27	165.15 ± 6.27	$P > 0.05$
Mean weight ± SD, kg	58.67 ± 10.79	60.47 ± 13.25	$P > 0.05$
Mean course of disease ± SD, month	46.67 ± 59.41	37.68 ± 38.73	$P > 0.05$

The patients were assigned to either the CHM group or the placebo group (in a 2 : 1 ratio). The effective rates of treatment and placebo were assumed to be 80% and 50%, respectively [22, 23]. The calculation indicated that a sample size of 90 would be sufficient ($n = 60$ in the treatment group, $n = 30$ in control group). To allow for a 15% rate of dropouts and missing data, the sample size was 105 ($n = 70$ in the treatment group, $n = 35$ in control group). However, due to time limitations, we recruited 67 patients for the treatment group and 34 patients for the control group.

2.8. Statistical Analysis. We performed intention-to-treat analyses using all available data at each time point and the baseline-observation-carried-forward approach for missing data. The statistical analysis was performed by the Center of Clinical Epidemiology of the Third Hospital of Peking University. Parametric Student's t-tests or nonparametric Wilcoxon tests were used to quantitatively compare variables according to distribution characteristics. Quantitative variables are reported as mean ± SD. In this trial, there were two primary endpoints (TDS and SDS scores). Therefore, for multiple testing problems, the significance level underwent Bonferroni correction at $P < 0.025$.

3. Results

3.1. Study Population. Between April 2009 and March 2011, a total of 101 patients were recruited; 67 were randomized into the CHM group and 34 into the placebo group. Ten patients withdrew from the trial due to a lack of efficacy. No serious adverse events were reported. The physiological tests obtained after 4 weeks of treatment showed no abnormal values.

3.2. Participant Flow. The flow of participants in the study is summarized in Figure 1.

3.3. Baseline Data. The general characteristics of the patients are shown in Table 3. No significant differences were identified between the two groups in terms of parameters such as gender, age, course of disease, or symptom scores before treatment.

3.4. Primary Outcome Variables

3.4.1. Total Dyspepsia Symptoms Scale Score. After 4 weeks of treatment, the TDS score assessed by investigators was significantly better for the CHM group than for the placebo group ($Z = -4.547$, $P < 0.01$). At week 8, the score was also significantly better for CHM than for placebo ($Z = -3.878$, $P < 0.01$). The TDS scores provided by the patients themselves were similar to those given by the investigators (Table 4). The percentage of TDS score improvement after 4 weeks of treatment is summarized in Table 5.

The results were clinically meaningful. Ratings of the clinical global impression of improvement after the treatment showed the following significant results for the treatment versus placebo group, respectively: very much improved (47.8% versus 5.9%), much improved (28.4% versus 26.5%), slightly improved (10.4% versus 23.5%), and unchanged or deteriorated (13.4% versus 44.1%) ($P < 0.001$).

3.4.2. Single Dyspepsia Symptom Scale Score. SDS scores assessed by investigators. After 4 weeks of treatment, the scores of epigastric pain, postprandial fullness and bloating, early satiety, and burning sensation were significantly better for the CHM group than for placebo ($P < 0.01$). At week 8, the scores of epigastric pain, postprandial fullness and bloating, early satiety, and burning sensation were significantly better for CHM than for placebo ($P < 0.01$).

The SDS scores provided by patients were similar to those given by investigators. The percentage of SDS score improvement after 4 weeks of treatment is summarized in Table 5.

4. Discussion

FD is a heterogeneous disorder. It involves many pathogenic factors and different pathophysiological disturbances, including delayed gastric emptying, impaired accommodation, and hypersensitivity to gastric distention. Treatment of the underlying pathophysiological abnormality seems logical, but the main pharmacotherapeutic options include acid suppression, prokinetic drugs, and antidepressants [6, 24–26], all of which have limited effects. Herbal formulations are widely used to treat FD in China and many other areas in the world. However, the available evidence of the efficacy of these formulas is inadequate.

This multicenter, randomized, double-blind, placebo-controlled study indicated that modified Ban Xia Xie Xin decoction is effective in the management of symptoms associated with FD. The effects appeared to last for up to 4 weeks after completion of treatment and were particularly beneficial for epigastric pain, postprandial fullness and bloating, early

Table 4: TDS and SDS scores.

Variables	CHM ($n = 67$) Mean ± SD	Placebo ($n = 34$) Mean ± SD	P values
Baseline date (week 0)			
Gastroenterologist TDS scores	7.12 ± 2.71	7.68 ± 2.83	$P > 0.05$
Patient TDS scores	7.12 ± 2.69	7.59 ± 2.79	$P > 0.05$
Gastroenterologist SDS scores			
Epigastric pain	3.85 ± 2.18	3.47 ± 2.63	$P > 0.05$
Epigastric burning	2.36 ± 2.66	2.76 ± 2.63	$P > 0.05$
Postprandial fullness and bloating	4.96 ± 1.78	4.89 ± 2.05	$P > 0.05$
Early satiety	3.10 ± 2.32	3.32 ± 2.92	$P > 0.05$
Patient SDS scores			
Epigastric pain	3.90 ± 2.19	3.41 ± 2.64	$P > 0.05$
Epigastric burning	2.43 ± 2.68	2.76 ± 2.64	$P > 0.05$
Postprandial fullness and bloating	4.96 ± 1.78	4.88 ± 2.13	$P > 0.05$
Early satiety	3.09 ± 2.34	3.29 ± 2.94	$P > 0.05$
Week 4			
Gastroenterologist TDS scores	2.37 ± 2.15	5.09 ± 3.00	$P < 0.01$
Patient TDS scores	2.43 ± 1.98	5.13 ± 3.32	$P < 0.01$
Gastroenterologist SDS scores			
Epigastric pain	1.22 ± 1.72	2.59 ± 2.38	$P < 0.01$
Epigastric burning	0.78 ± 1.55	2.47 ± 2.30	$P < 0.01$
Postprandial fullness and bloating	1.79 ± 1.99	3.32 ± 1.84	$P < 0.01$
Early satiety	0.76 ± 1.62	1.82 ± 2.05	$P < 0.01$
Patient SDS scores			
Epigastric pain	1.23 ± 1.76	2.46 ± 2.34	$P < 0.01$
Epigastric burning	0.78 ± 1.55	2.42 ± 2.67	$P < 0.01$
Postprandial fullness and bloating	1.73 ± 1.89	3.45 ± 1.97	$P < 0.01$
Early satiety	0.77 ± 1.64	1.79 ± 2.04	$P < 0.01$
Week 8			
Gastroenterologist TDS scores	2.42 ± 2.75	4.41 ± 2.49	$P < 0.01$
Patient TDS scores	2.61 ± 2.15	4.31 ± 2.45	$P < 0.01$
Gastroenterologist SDS scores			
Epigastric pain	1.12 ± 1.57	2.35 ± 2.27	$P < 0.01$
Epigastric burning	0.73 ± 1.53	1.62 ± 2.00	$P < 0.05$
Postprandial fullness and bloating	1.75 ± 1.92	3.62 ± 1.79	$P < 0.01$
Early satiety	0.61 ± 1.48	1.47 ± 1.78	$P < 0.01$
Patient SDS scores			
Epigastric pain	1.17 ± 1.54	2.35 ± 2.17	$P < 0.01$
Epigastric burning	0.72 ± 1.54	1.64 ± 2.30	$P < 0.05$
Postprandial fullness and bloating	1.76 ± 1.90	3.62 ± 1.79	$P < 0.01$
Early satiety	0.60 ± 1.44	1.45 ± 1.79	$P < 0.01$

satiety, and burning sensation. Patients treated with modified Ban Xia Xie Xin decoction demonstrated significantly better outcomes (both clinically and statistically) for all outcome measures compared with patients receiving placebo. Moreover, no serious adverse events were reported during the study.

The evaluation of treatment effects in patients with FD is difficult, and there is currently no gold standard. In our study, we used two different parameters as the target variables. The TDS scale included almost all symptoms associated with FD, and the SDS scale included information on the four principal symptoms of FD, measured in terms of the

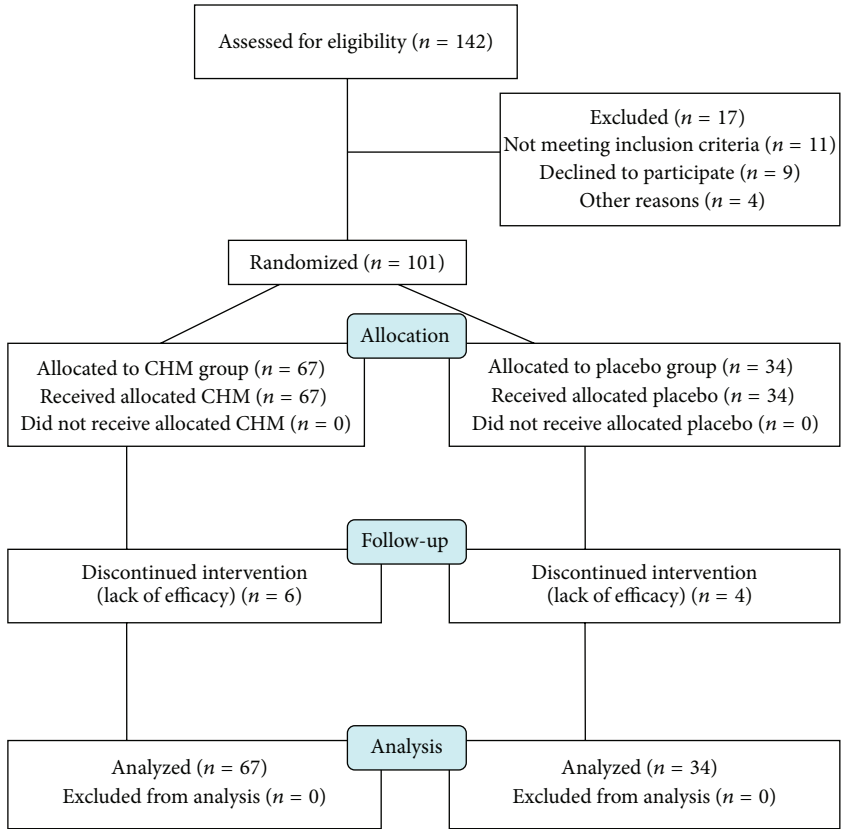

Figure 1: Flow of participants in the study.

Table 5: Percentage of TDS and SDS score improvements after 4 weeks of treatment.

Variables	CHM ($n = 67$)	Placebo ($n = 34$)
Gastroenterologist TDS scores	66.7%	33.7%
Gastroenterologist SDS scores		
Epigastric pain	68.3%	25.4%
Epigastric burning	66.9%	10.5%
Postprandial fullness and bloating	63.9%	32.1%
Early satiety	75.5%	45.2%
Patient TDS scores	65.9%	32.4%
Patient SDS scores		
Epigastric pain	68.5%	27.9%
Epigastric burning	67.9%	12.3%
Postprandial fullness and bloating	65.1%	29.3%
Early satiety	75.1%	45.6%

frequency, intensity, and level of discomfort. The target variables were recorded by both investigators and patients. Another difficulty in clinical trials involving patients with FD is the remarkable placebo response. It has been shown that one-third of patients with FD will respond to placebo in short-term trials [27], and the proportion may be even higher in long-term studies. In our study, we made a great effort to make the treatments in the two groups indistinguishable to the patients. A placebo of similar appearance, smell, and taste to the active concoction was used. To ensure that the patients were not able to discriminate between placebo and active treatment, 20 healthy volunteers participated in a randomized taste and visual assessment of the placebo and active medication. Eight volunteers correctly identified the active compound as active, whereas 12 volunteers considered the placebo preparation to be the active compound. Thus, it is reasonable to assume that the medication was given in an appropriately blinded manner. Despite the well-known high response rate to placebo in patients with FD, we found significantly greater improvements in dyspepsia symptoms in patients receiving the CHM compared with those receiving placebo.

In TCM, injury by food or drink, emotional injury, and congenital defects are the main pathogenic factors of FD. All pathogenic factors cause abnormal function of the upper abdominal spleen and stomach and the complexity of cold and heat. The herbal formula provided to patients in this study was a modified Ban Xia Xie Xin decoction. Ban Xia Xie Xin decoction is a traditional Chinese compound herbal recipe used to regulate cold and heat. We added related herbal

medicines (Cortex Magnoliae officinalis, Medicated Leaven, Ark Shell) to the recipe to identify the formula of "modified Ban Xia Xie Xin decoction" that had a satisfactory clinical effect. All of the herbs matched well, so the complexity of cold and heat was regulated and the spleen-stomach function was recovered. Therefore, all dyspepsia symptoms would be abated. This is in accordance with previous studies that showed physiological effects of the active ingredients in the modified Ban Xia Xie Xin decoction. In China, Ban Xia Xie Xin decoction is used for FD, and some of the active ingredients in the modified Ban Xia Xie Xin decoction have been shown to reinforce the protective function of the mucosa, regulate gastrointestinal function, and induce anti-inflammatory action against *H. pylori* [16–20]. However, herbal preparations are complex and contain a number of active ingredients that may work together. The multiple effects of different active ingredients may be of benefit for the variety of different symptoms that occur in functional gastrointestinal disorders. However, more studies are needed to explore the mechanisms of action and properties of the identified components. FD is a common, chronic, and recurrent functional gastrointestinal disorder. This study used a short treatment period and followup and a relatively small number of patients; so, there is ample room to enhance the evaluation of efficacy and safety by further studies.

5. Conclusions

We conclude that modified Ban Xia Xie Xin decoction may offer symptomatic improvements in patients with FD. In this randomized, double-blind, placebo-controlled trial, modified Ban Xia Xie Xin decoction was shown to be effective in the management of FD. Further studies are needed to determine the precise mechanisms of action.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contributions

S. Zhang, L. Zhao, Z. Wang, H. Wang, and B. Wu contributed to the conception and design of the study. S. Zhang and L. Zhao drafted the paper. All authors contributed to further writing of the paper. All authors read and approved the final paper.

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Research Article

The Effects of High-Dose Qinggan Huoxue Recipe on Acute Liver Failure Induced by D-Galactosamine in Rats

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Qinggan Huoxue Recipe is a traditional Chinese medicine, which has been usually used to improve liver function in hepatitis. In order to investigate the effects of high-dose Qinggan Huoxue Recipe on acute liver failure and explore the potential mechanism, we had built acute liver failure models in rats by intraperitoneal injection of D-galactosamine (D-GalN). High-dose Qinggan Huoxue Recipe was delivered by gavage. After treatment, the blood alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), cholinesterase (CHE), and prothrombin time (PT) were determined. The pathological score of liver tissue was recorded. Proliferating cell nuclear antigen (PCNA) immunohistochemistry staining and fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR) of high mobility group box 1 (HMGB1), toll-like receptor 4 (TLR4), nuclear factor-kappa B (NF-κB), and Caspase-3 were performed. The survival curve was also depicted. Our results demonstrated that high-dose Qinggan Huoxue Recipe could significantly improve liver function and increase survival rates in rats with acute liver failure. These effects were supposed to be mediated by suppressing inflammatory reaction and apoptosis.

1. Introduction

Acute liver failure (ALF) is a life-threatening medical emergency and occurs when the liver rapidly loses its function within a short period. ALF can develop secondary to a variety of causes and occurs when the extent of hepatocyte death exceeds the liver's regenerative capacity [1]. Currently, liver transplantation is the "Gold Standard" therapy for the disease. However, due to the limited availability of donor organs and rapid progression of the disease, the mortality of ALF remains high [2]. Therefore, it is imperative to develop novel therapeutic reagents for ALF.

Qinggan Huoxue Recipe is a traditional Chinese medicine prescription which has been used in China for a long time [3]. Previous literature had indicated that it could improve the liver function in alcohol liver disease models [4–6], but the effects of Qinggan Huoxue Recipe on acute liver failure were rarely explored.

In one of our previous prospective clinical cohort studies [7], we found that high-dose Qinggan Huoxue Recipe could significantly improve liver function and coagulation function, reduce complications, and reduce mortality in patients with hepatitis B-related acute-on-chronic liver failure. In order to confirm the effects of high-dose Qinggan Huoxue Recipe on acute liver failure and explore the potential mechanism, we had conducted this experiment.

2. Methods

2.1. Materials and Methods. All specific pathogen-free (SPF) male Wistar rats weighing 150 ± 20 g were purchased from Shanghai Experimental Animal Co., Ltd (Shanghai, China). D-galactosamine (D-GalN), a commonly used liver injury inducing drug [8, 9], was purchased from Hongbang Medical Technology CO., Ltd (Shanghai, China). Stronger Neo-Minophagen C (SNMC), a classic liver protection drug [10],

Table 1: The primer sequences used in fluorescence quantitative RT-PCR.

Parameters	5′ primer sequence	3′ primer sequence
HMGB1	5′TGTTCTGAGTACCGCCCAAA3′	5′TTTCGCTGCATCAGGTTTTC3′
TLR4	5′CCAGGAAGGCTTCCACAAGA3′	5′AATTCGACCTGCTGCCTCAG3′
NF-κB	5′GCACGAGGCTCCTTTTCTCAA3′	5′CGTTTTTCTTCAATCCGGTGG3′
Caspase-3	5′ACCGATGTCGATGCAGCTAA3′	5′AGGTCCGTTCGTTCCAAAAA3′
β-Actin	5′AAGGAGGCAAAGGACACCAA3′	5′AATGGCCCCCTTCACAGTTA 3′

was purchased from Minophagen Pharmaceutical Co., Ltd (Tokyo, Japan), and fixed into a concentration of 1.56 mg/mL with distilled water. Qinggan Huoxue Recipe which was boiled using *Artemisia capillaris*, *Patrinia, Scutellaria baicalensis, Polygonum cuspidatum, rhubarb*, and red *Peony* (2 : 4 : 4 : 4 : 1 : 4) was purchased from Affiliated Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China) with a concentration of 2.97 g/mL.

2.2. Design of Animal Experiment. 70 rats were randomized into four groups: negative control group (Control, 10 rats) fed with distilled water by gavage; model group (Model, 20 rats) fed with distilled water by gavage and injected with D-GalN 1.4 g/kg intraperitoneally three days after the gavage; Stronger Neo-Minophagen C group (SNMC, 20 rats) fed with SNMC 15.6 mg/kg/d by gavage and injected with D-GalN 1.4 g/kg intraperitoneally three days after the gavage; Qinggan Huoxue Recipe group (Experiment, 20 rats) fed with Qinggan Huoxue Recipe 29.7 g/kg/d (which equals 6.25× clinical dose) by gavage and injected with D-GalN 1.4 g/kg intraperitoneally three days after the gavage. The gavage last for 5 days. The dosage of Qinggan Huoxue Recipe used on rats was calculated by the formula $\text{Dose}_{\text{rat}} = \text{Dose}_{\text{human}} \times (\text{habeas index}_{\text{rat}}/\text{habeas index}_{\text{human}}) \times (\text{body weight}_{\text{human}}/\text{body weight}_{\text{rat}}) \times 2/3$ [11]. Based on this formula, the translational coefficient 6.25 was produced. In our previous clinical trial, the dose of Qinggan Huoxue Recipe used on human was 285 g/60 kg/d (4.75 g/kg/d). At last, the dose of 29.7 g/kg/d was achieved through the multiplication of human dose (4.75 g/kg/d) and translational coefficient (6.25). The dose of 29.7 g/kg/d is a relatively very high-dose used in rats compared with the other studies of Qinggan Huoxue Recipe reported [4, 12]. Besides, a dose response study was carried out in preexperiment to confirm the usage of this high-dose (data not shown). 36 hours after the D-GalN injection, 6 mL blood was collected through femoral artery of alive rats for detection of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), cholinesterase (CHE), and prothrombin time (PT). Then the rats were sacrificed and the left lobe of the liver was collected for further studies such as hematoxylin and eosin (HE) staining, proliferating cell nuclear antigen (PCNA) immunohistochemistry assay, and fluorescence quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2.3. Serum ALT, AST, TBIL, ALB, CHE, and PT Determination. The serum biochemical parameters ALT, AST, TBIL, ALB, CHE, and PT which closely reflect the liver function [4] were analyzed by the Department of Laboratory Medicine, Affiliated Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China).

2.4. Pathological Scores. The liver specimens were fixed, paraffin embedded, and cut into 3- to 5-μm sections. The sections were used for HE and PCNA staining. The method of HE staining has been introduced in the previous literature [13, 14]. All slides were read by three investigators who were blinded to the allocation arm of the animal. They were asked to grade the microscopic injuries seen in the liver using a semiquantitative scoring system [15], with zero indicating no discernable injury and 4 indicating the presence of severe injury. These scores were allocated based on their assessment of the following histological features: cellular oedema, interstitial oedema, neutrophil infiltration, capillary congestion, and structural distortion. For each slide, the average score of at least three observations was considered as the pathological score.

2.5. PCNA Immunohistochemistry. The PCNA immunohistochemistry kit was purchased from Boster Bioengineering CO., Ltd (Wuhan, China). Immunohistochemical staining of PCNA was performed according to the manufacturer's instructions. PCNA-positive cells were counted in 5 random visual fields under 40x magnifications for each section, and the number was expressed as the percentage of PCNA positive cells to the total number of cells counted [16]. Sections were examined microscopically for specific staining, and photographs were taken with a digital image-capture system (Olympus CX40, Tokyo, Japan).

2.6. Fluorescence Quantitative RT-PCR. The expression of high mobility group box 1 (HMGB1), toll-like receptor 4 (TLR4), nuclear factor kappa B (NF-κB), and Caspase-3 was detected by qRT-PCR. Total RNAs were extracted with Trizol reagent (Invitrogen) and reverse transcribed into cDNA by the ABI Step-One Plus Real-Time PCR System (Applied Biosystem Co., CA, USA). The primer sequences of the above parameters including β-actin were listed in Table 1.

2.7. Survival Curves. Another 55 rats were divided into the above four groups with animal numbers of 10, 15, 15, and 15, respectively, for observation of survival. The treatments were the same as mentioned before. The observation was begun with the time of D-GalN injection, while the endpoint was set at 96 hours after the injection.

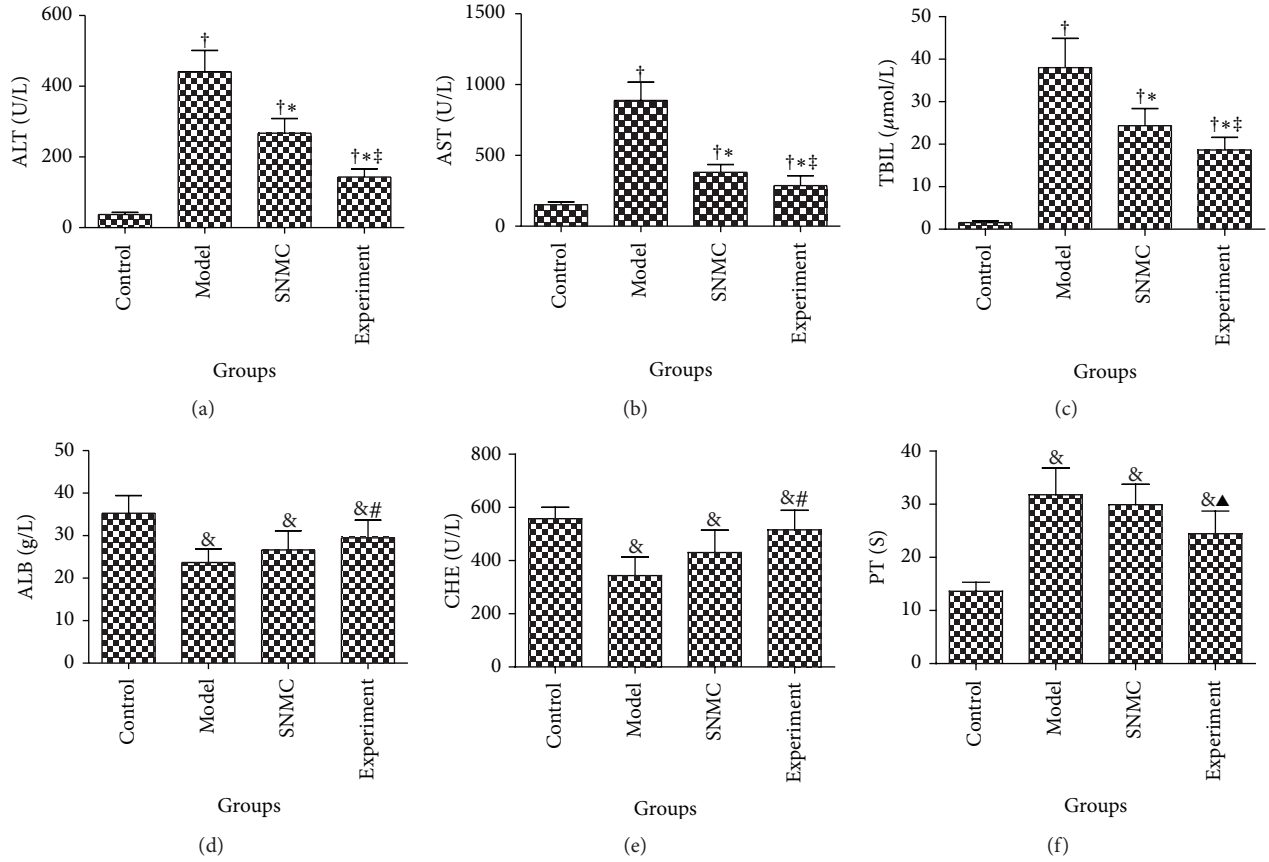

Figure 1: The serum ALT, AST, TBIL, ALB, and CHE levels and PT after treatment. (a) ALT; (b) AST; (c) TBIL; (d) ALB; (e) CHE; (f) PT. Control: negative control group; Model: the group injected with D-GalN; SNMC: the group injected with D-GalN and treated with Stronger Neo-Minophagen C; Experiment: the group injected with D-GalN and treated with Qinggan Huoxue Recipe. For Control, Model, SNMC, and Experiment groups, the ALT levels were 35.15 ± 6.01 U/L, 441.10 ± 60.36 U/L, 267.18 ± 41.45 U/L, and 143.22 ± 22.96 U/L, respectively; the AST levels were 151.61 ± 20.87 U/L, 887.80 ± 128.47 U/L, 380.49 ± 55.38 U/L, and 287.36 ± 68.97 U/L, respectively; the TBIL levels were 1.55 ± 0.43 μmol/L, 38.04 ± 6.84 μmol/L, 24.37 ± 4.03 μmol/L, and 18.65 ± 2.96 μmol/L, respectively; the ALB levels were 35.25 ± 4.19 g/L, 23.67 ± 3.21 g/L, 26.65 ± 4.50 g/L, and 29.46 ± 4.19 g/L, respectively; the CHE levels were 557.40 ± 43.23 U/L, 343.92 ± 68.93 U/L, 430.50 ± 83.53 U/L, and 515.82 ± 73.31 U/L, respectively; and the PT was 13.60 ± 1.73 s, 31.80 ± 5.02 s, 29.93 ± 3.83 s, and 24.46 ± 4.25 s, respectively. For (a), (b), and (c), $^{\dagger}P < 0.05$ comparing Control group, $^{*}P < 0.05$ comparing Model group, and $^{\ddagger}P < 0.05$ comparing SNMC group. For (d), (e), $^{\&}P < 0.05$ comparing Control group and $^{\#}P < 0.05$ comparing Model group. For (f), $^{\&}P < 0.05$ comparing Control group and $^{\blacktriangle}P < 0.05$ comparing Model group and SNMC group.

2.8. Statistical Analysis. The biochemical parameters, pathological scores, PCNA immunohistochemistry, and mRNA expressions were analyzed by one-way ANOVA, followed by the Student's t-test. Survival curves were plotted using the Kaplan-Meier method and analyzed applying the Log-rank test. All statistical analyses were performed using the SPSS 17.0 software package. All P values were two sided, and $P < 0.05$ was considered as the significant level of difference.

3. Results

3.1. The Serum ALT, AST, TBIL, ALB, and CHE Levels and PT. The serum ALT, AST, TBIL, ALB, and CHE levels and PT were shown in Figure 1. From the results, we could find that the ALT, AST, and TBIL were significantly increased, while ALB and CHE were significantly decreased after the injection of D-GalN. PT was remarkably elongated. All these parameters have indicated severe liver damage. However, Stronger Neo-Minophagen C and high-dose Qinggan Huoxue Recipe could improve the liver function by decreasing ALT, AST, TBIL, and PT and increasing ALB, CHE levels. And the effects were more significant in Qinggan Huoxue Recipe group.

3.2. Pathological Scores. As shown in Figure 2, there were massive necroses in the liver tissues of the model group. The necroses were reduced in the SNMC and experiment groups, especially in the experiment group.

3.3. PCNA Immunohistochemistry. The PCNA positive rates were 7.48 ± 0.90%, 17.55 ± 2.4%, 25.57 ± 2.94%, and 35.68 ± 4.75%, respectively, in control, model, SNMC, and experiment groups (Figure 3). The PCNA positive rate of experiment group was significantly larger than the other three groups, indicating high regeneration rates after the treatment of high-dose Qinggan Huoxue Recipe.

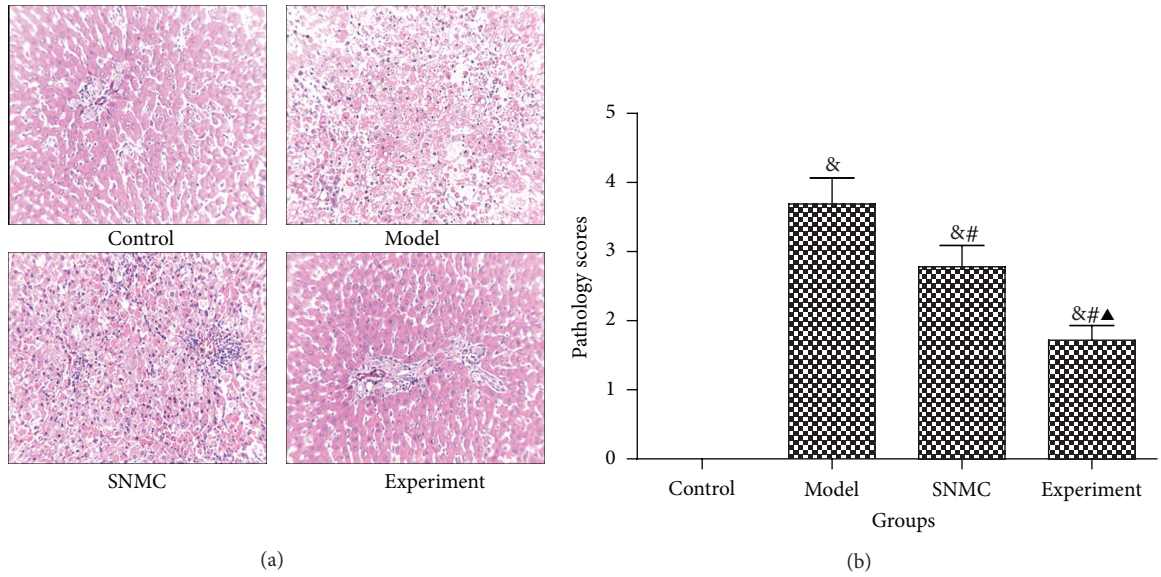

Figure 2: Pathological scores. (a) HE staining; (b) pathological scores. Control: negative control group; Model: the group injected with D-GalN; SNMC: the group injected with D-GalN and treated with Stronger Neo-Minophagen C; Experiment: the group injected with D-GalN and treated with Qinggan Huoxue Recipe. The pathological scores were 0, 3.69 ± 0.38, 2.78 ± 0.31, and 1.72 ± 0.21, respectively, in Control, Model, SNMC, and Experiment groups. $^{\&}P < 0.05$ comparing Control group, $^{\#}P < 0.05$ comparing Model group, and $^{\blacktriangle}P < 0.05$ comparing SNMC group.

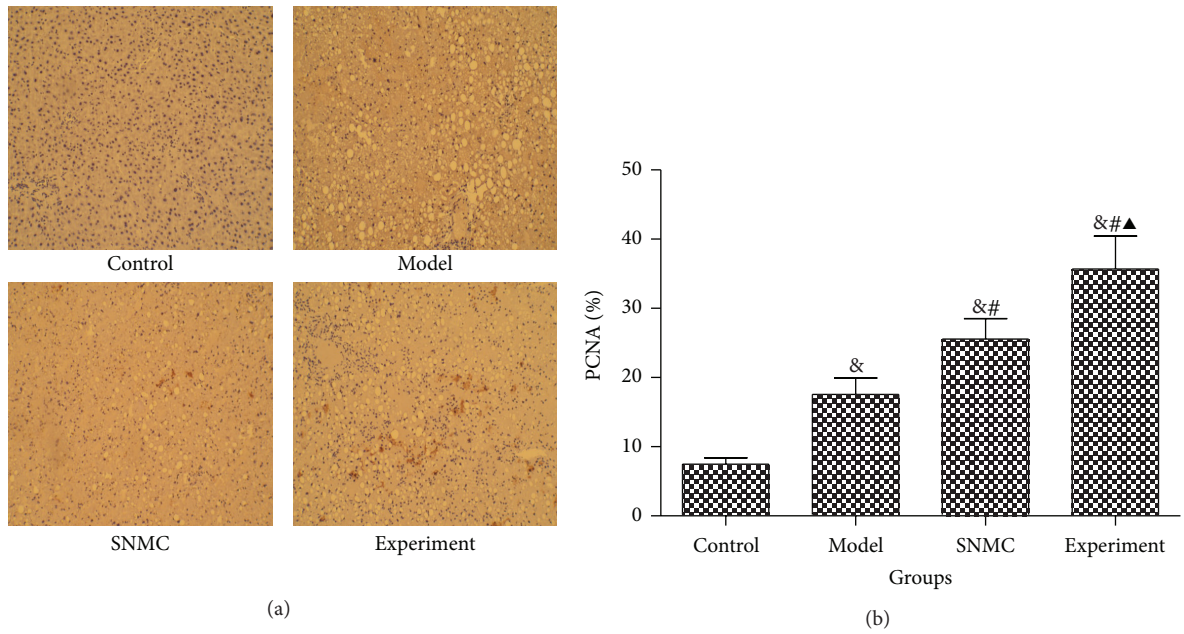

Figure 3: PCNA immunohistochemistry. (a) PCAN immunohistochemistry; (b) histogram of immunohistochemistry. Control: negative control group; Model: the group injected with D-GalN; SNMC: the group injected with D-GalN and treated with Stronger Neo-Minophagen C; Experiment: the group injected with D-GalN and treated with Qinggan Huoxue Recipe. The PCNA positive rates were 7.48 ± 0.90%, 17.55 ± 2.4%, 25.57 ± 2.94%, and 35.68 ± 4.75%, respectively, in Control, Model, SNMC, and Experiment groups. $^{\&}P < 0.05$ comparing Control group, $^{\#}P < 0.05$ comparing Model group, and $^{\blacktriangle}P < 0.05$ comparing SNMC group.

3.4. The mRNA Expressions. As shown in Figure 4, high-dose Qinggan Huoxue Recipe could remarkably decrease the mRNA expressions of HMGB1, TLR4, NF-κB, and Caspase-3, indicating that it could decrease inflammatory reaction and apoptosis of liver tissues.

3.5. Survival Curves. We excluded the control group from survival curve because there was no rat died at the endpoint. From Figure 5, we could found that high-dose Qinggan Huoxue Recipe could improve the survival of acute liver failure model significantly.

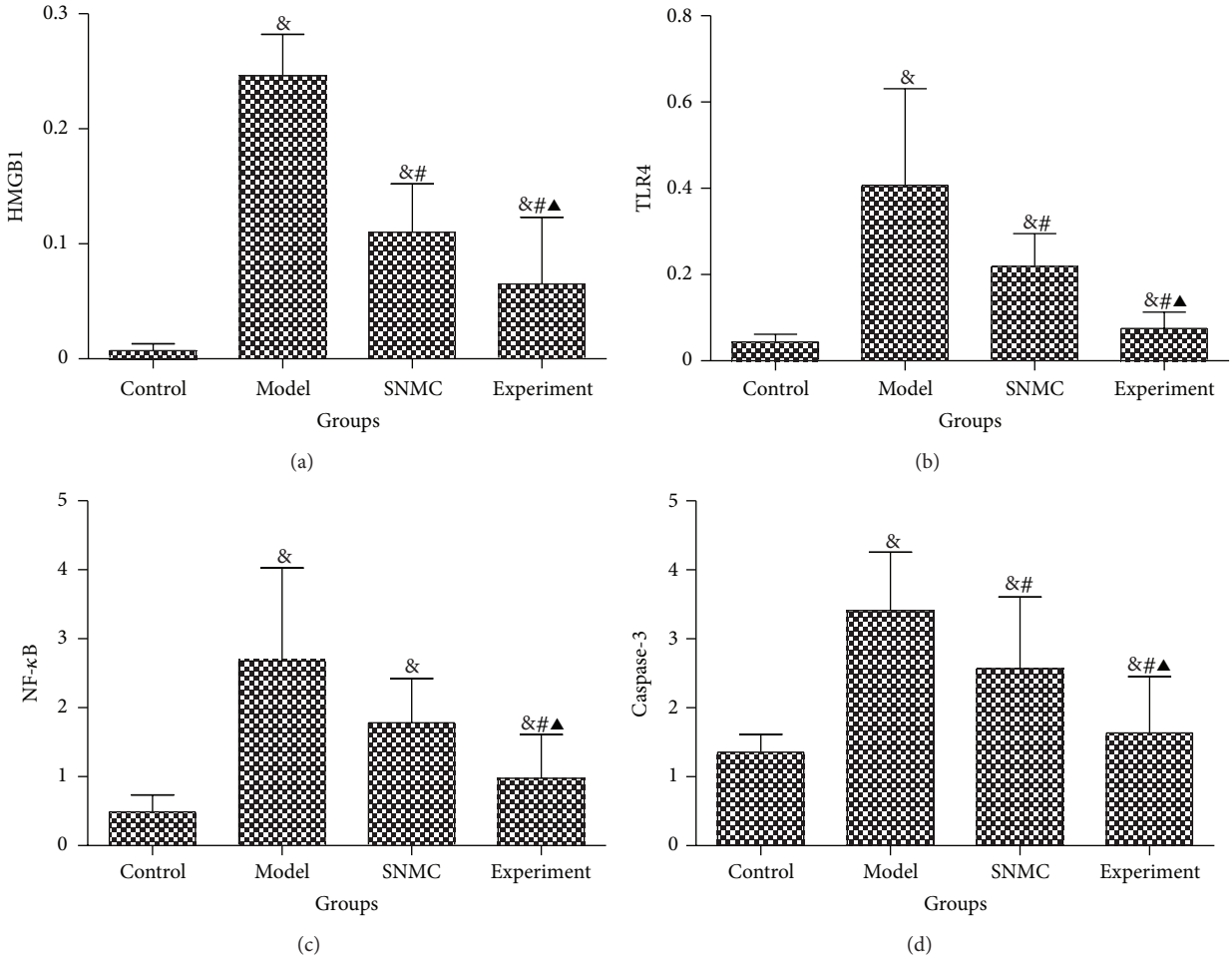

Figure 4: The mRNA expression. (a) HMGB1; (b) TLR4; (c) NF-κB; (d) Caspase-3. Control: negative control group; Model: the group injected with D-GalN; SNMC: the group injected with D-GalN and treated with Stronger Neo-Minophagen C; Experiment: the group injected with D-GalN and treated with Qinggan Huoxue Recipe. For Control, Model, SNMC, and Experiment groups, the HMGB1 mRNA expressions were 0.01 ± 0.01, 0.25 ± 0.04, 0.11 ± 0.04, and 0.07 ± 0.06, respectively; the TLR4 mRNA expressions were 0.04 ± 0.02, 0.41 ± 0.22, 0.22 ± 0.08, and 0.08 ± 0.04, respectively; the NF-κB mRNA expressions were 0.49 ± 0.25, 2.68 ± 1.35, 1.78 ± 0.64, and 0.98 ± 0.63, respectively; and the Caspase-3 mRNA expressions were 1.36 ± 0.26, 3.41 ± 0.85, 2.57 ± 1.04, and 1.64 ± 0.81, respectively. $^{\&}P < 0.05$ comparing Control group, $^{\#}P < 0.05$ comparing Model group, and $^{\blacktriangle}P < 0.05$ comparing SNMC group.

4. Discussion

In this study, the remarkable increased serum ALT, AST, and TBIL levels, elongated PT, decreased serum ALB, CHE levels, increased pathological scores, and rapid death had confirmed that acute liver failure models were successfully built by D-GalN injection. SNMC and high-dose Qinggan Huoxue Recipe could both ameliorate liver function and increase survival times; however, high-dose Qinggan Huoxue Recipe had significant stronger effects.

The Qinggan Huoxue Recipe used in this study has been practiced for many years in the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine and was found to be very effective in relieving hepatic complications. However, the protective effects of Qinggan Huoxue Recipe on acute liver failure patients were unsatisfactory when used in general dose. So we enhanced the dosage of Qinggan Huoxue Recipe in acute liver failure patients gradually, and we found that liver functions were remarkably improved without discovering any drug-related side effects. In order to confirm the protective effects of high-dose Qinggan Huoxue Recipe on acute liver failure, we had carried out our previous prospective clinical trial [7]. For the Chinese medicine included in this recipe, *Artemisia capillaris* could ameliorate the hydrophilic bile acids-induced hepatic injury which is probably related to a reduced oxidant stress and degree of hepatic fibrosis [17]. *Patrinia* could inhibit the biomarkers related to inflammation through the blocking of NF-κB activation and potentiate anti-inflammatory effects [18]. *Scutellaria baicalensis* could inhibit cyclooxygenase-2 overexpression and therefore alleviates cantharidin-induced rat hemorrhagic cystitis [19]. *Polygonum cuspidatum* was found to be an effective hepatoprotective agent and a promising candidate for the treatment of oxidative stress- and inflammation-related

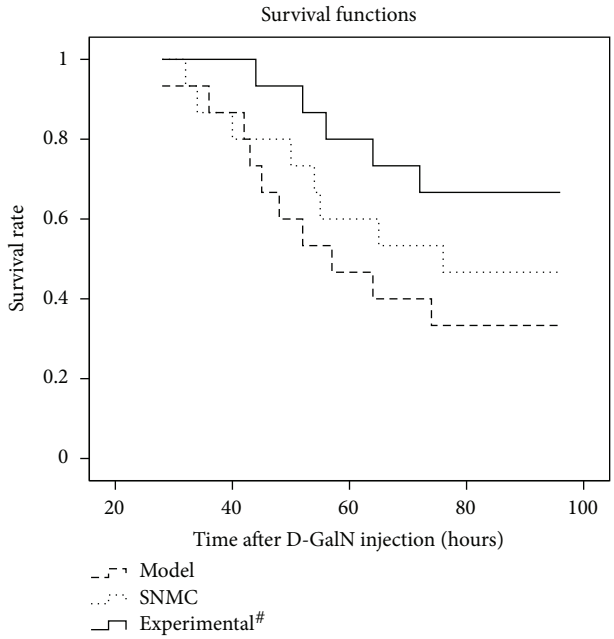

Figure 5: The survival curve. Model: the group injected with D-GalN; SNMC: the group injected with D-GalN and treated with Stronger Neo-Minophagen C; Experiment: the group injected with D-GalN and treated with Qinggan Huoxue Recipe. $^{\#}P < 0.05$ comparing Model group.

diseases [20, 21]. *Rhubarb* was found to have antioxidant, antiplatelet, and anticoagulant activities and could be used to treat experimental jaundice in rats [22]. Red *Peony* could inhibit inflammation and scavengs free radicals and was found to be effective in treating severe acute pancreatitis [23, 24]. Qinggan Huoxue Recipe has combined these Chinese herb medicines following the principle of clearing heat and resolving stasis. Finally, our previous clinical trial proved that high-dose Qinggan Huoxue Recipe could significantly improve hepatic function in acute liver failure [7], but the potential mechanism is still not for sure.

HMGB1, a highly conserved, ubiquitous protein that presents in the nuclei and cytoplasm of nearly all cell types, is a necessary and sufficient mediator of inflammation during sterile- and infection-associated responses [25]. Most cells constitutively express HMGB1 and release it on injury or death [26]. It has been suggested that HMGB-1 itself can signal through receptor for advanced glycation end products (RAGEs) and through the toll-like receptors TLR2, TLR4, and TLR9. Activation of these receptors results ultimately in the activation of nuclear factor-kappa B (NF-κB), inducing the upregulation of leukocyte adhesion molecules, production of proinflammatory cytokines, and angiogenic factors in both hematopoietic and endothelial cells, thereby promoting inflammation [27]. Although HMGB1 exerts its cellular and biologic inflammatory responses by binding to three members of TLRs family, namely, TLR2, TLR4, and TLR9, as well as RAGE, TLR4 is the primary receptor of endogenous HMGB1 in mediating cytokine release and tissue damage in various conditions, such as ischemia/reperfusion injury, hemorrhage, and trauma, and this mechanism of injury is attenuated or prevented by deficiency in TLR4 [28]. So we had detected the mRNA expressions of HMGB1, TLR4, and NF-κB in this study. We found that the mRNA expressions of HMGB1, TLR4, and NF-κB were remarkably increased in acute liver failure model group. However, they were significantly decreased after the treatment of SNMC or high-dose Qinggan Huoxue Recipe, especially in Qinggan Huoxue Recipe treatment group. Therefore, we think that high-dose Qinggan Huoxue Recipe could improve liver function in acute liver failure by suppressing inflammation, and this effect was most probably mediated by inhibiting HMGB1/TLR4/NF-κB pathway, though further studies were needed to exclude the interactions between HMGB1 with other receptors. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, Caspase-3 frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins [29]. After the activation of NF-κB, the activities of antigen presenting cells could be enhanced, and then cytotoxic T lymphocytes (CTL) were largely activated, which mediated the apoptosis of hepatocytes [30]. So high-dose Qinggan Huoxue Recipe could also improve liver function in acute liver failure by decreasing the activation of NF-κB and therefore decrease the apoptosis of hepatocytes.

In this study, we had also found that the PCNA positive rates were significantly higher in high-dose Qinggan Huoxue Recipe group, indicating high regeneration rates. However, whether the promotion of regeneration was produced by direct effects of high-dose Qinggan Huoxue Recipe or the subsequent effects of inflammation suppression was unknown. Besides, further studies were needed to explore the relationship of inflammation, apoptosis, and regeneration in the treatment of acute liver failure.

Although high-dose Qinggan Huoxue Recipe was used in this study, no drug-related side effects were discovered. In our previous prospective clinical study, the incidence rates of adverse events in the treatment group and the control group were 0.00% and 12.50%, respectively, and the difference was statistically significant. No drug-related adverse events were found in blood, urine and stool routine tests, renal function test, and electrocardiography [7]. During this experiment, we also observed the rats' appetite, behavior change, reaction to stimulation, and so on. We found that the appetites and behaviors of rats in acute liver failure model group were decreased evidently. Besides, they had developed a series of symptoms such as urinary incontinence, yellow urine, listlessness, lethargy, irritability, convulsion, and hemorrhage. However, these symptoms were remarkably decreased after the treatment of high-dose Qinggan Huoxue Recipe, and more important, no treatment-related side effects were discovered.

5. Conclusion

High-dose Qinggan Huoxue Recipe could significantly improve liver function and increase survival rates in rats with acute liver failure. These effects were supposed to be mediated by suppressing inflammatory reaction and apoptosis.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

H. Zhu and Y. Zhang equally contributed to the paper.

Acknowledgments

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