Immunoregulatory effects of the traditional Dai prescription Yajieshaba on food allergic mice

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Abstract. The Dai prescription Yajieshaba is widely used in Traditional Dai Medicine to treat food allergies and intolerance. However, information on the active chemical ingredients, effects and mechanisms of action of Yajieshaba is limited. The present study aimed to elucidate the effects and underlying mechanisms of Yajieshaba in the treatment of food allergies. Liquid chromatography with a diode array detector was used to measure the levels of palmatine and berberine, the active ingredients of Yajieshaba. A food allergy model was established in female BALB/c mice by three injections of ovalbumin (OVA) at 0, 48, and 96 h. OVA-sensitized mice recieved no treatments (control), Yajieshaba, loratadine, palmatine or berberine. The scratching frequency, serum immunoglobulin (Ig)G, IgE, interleukin (IL)-4, IL-10, IL-17, IL-21, interferon-γ and tumor necrosis factor-α levels were assessed at 50 and 98 h. The percentage of regulatory T cells (Tregs) was evaluated by flow cytometry at 98 h. The scratching frequency induced by OVA was significantly suppressed in mice treated with loratadine, palmatine, berberine or 3.50 and 4.70 g/kg Yajieshaba. The frequency of $CD4^+CD25^+$Treg in the spleen increased from 6.80% in mice in the control group to 12.50% in mice treated with 4.70 g/kg body weight Yajieshaba. Mice treated with palmatine or 4.70 g/kg body weight Yajieshaba had increased forkhead box p3 expression compared with those in the control group. Treatment with Yajieshaba decreased the scratching frequency and increased $CD4^+CD25^+Foxp3^+$ Treg frequency in the spleen. This indicated that symptoms of allergic reaction were alleviated following Yajieshaba treatment. Palmatine was identified as one of the major active components of Yajieshaba. The present study identified the possible mechanism through which Yajieshaba treatment may alleviate food allergy symptoms.

Introduction

Traditional Dai Medicine (TDM) is a 2,500-year-old ethnic medicine system practiced in Xishuangbanna (latitude 21˚08'-22˚36', longitude 99˚56'-101˚50'), Yunnan Province, China and by Dai nationals all over the world. TDM has been applied in the treatment of various diseases for thousands of years (1). In 1984, the government of China defined TDM as one of China's four important systems of ethnomedicine, the other three being Mongolian, Tibetan and Uygur medicine (2).

The ethnic group Dai has used unique plant species found in the Xishuangbanna region to develop their own medicine. The healing benefits of these plants have been discovered over time and novel therapeutic applications for Dai herbs are still being assessed. Although there is now a wealth of medical knowledge regarding TDM, the prevalence of TDM has decreased due to the introduction and increasing prevalence of western medicine throughout China (3). A lack of scientific rigor and absence of any identified mechanisms through which TDM herbs act to alleviate diseases also contributes to the diminishing prevalence of TDM.

The medical application of Yajieshaba was first recorded in the Pattra Leaf Scripture 'Dang-Ha-Ya-Meng-Dai (Experienced prescription of Dai medicine)' in 1100 AD and the various applications of Yajieshaba have been documented (4,5). The Yunnan Food and Drug Administration Agency has authorized the use of Yajieshaba [authorization no. (Z)05K02252] in >100 hospitals in Yunnan Province due to its beneficial clinical effects (6).

Yajieshaba is one of the primary TDMs prescribed and is effective at treating symptoms induced by food allergies and food intolerance (7-9). It has also been suggested that

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Abbreviations: CD4, cluster of differentiation 4; CD25, cluster of differentiation 25; FITC, fluorescein isothiocyanate; Foxp3, forkhead box p3; HPLC, high performance liquid chromatography; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; OVA, ovalbumin; PE, phycoerythrin; Th1, type 1 T helper cell; TDM, Traditional Dai Medicine; TNF-α, tumor necrosis factor α; Treg, regulatory T cell

Key words: Yajieshaba, $CD4^+CD25^+Foxp3^+$ regulatory T cells, ovalbumin

Yajieshaba may relieve edema, asthma and gastrointestinal symptoms induced by heterogeneous food proteins (4,5,8).

The herbal components of Yajieshaba (4) include *Fibraurea recisa,* which contains the active components palmatine and berberine (10). Palmatine and berberine are therefore considered to be the active components of Yajieshaba.

Previous studies have focused on the chemical and pharmacological properties of Yajieshaba (11-13). To the best of our knowledge, the present study was the first to detect palmatine and berberine in the Yajieshaba formulation. In addition, the present study assessed the regulatory effects of Yajieshaba with regard to cellular and humoral immunity, using acute ovalbumin (OVA)-sensitized female BALB/c mice. The results provided information to enhance the current understanding of the mechanisms of action of Yajieshaba in the treatment of food allergies and may enable the expansion of its application to other medical conditions.

Materials and methods

Materials. The individual plants required to produce Yajieshaba were identified by Professor Chao Zhang, Yunnan University of Traditional Chinese Medicine (Kunming, China). All the plants were purchased from Xishuangbanna Dai Autonomous Prefecture People's Hospital (Xishuangbanna, China). Specimens were deposited in the Herbarium of Pharmacognosy, Yunnan University of Traditional Chinese Medicine (Kunming, China). Yajieshaba powder was prepared using the following components: 30 g *Pueraria lobata Ohwi*, 15 g root of *Dregea sinensis* Hemsl, 15 g whole plant of *Arundina graminifolia* (D. Don) Hochr, 15 g stem of *Fibraurea recisa* Pierre, 30 g stem of *Mappianthus iodioides* Hand. Mazz, 10 g vine of *Bousigonia mekongensis*, 15 g root of *Clerodendrum chinese* (Osbeck) Mabb and 10 g root of *Glycyrrhiza uralensis* Fisch. The aforementioned herbal components were then ground and mixed. The powder of Yajieshaba was extracted three times at 100˚C with water (12 volumes, 30 min; 8 volumes, 20 min; and 8 volumes, 20 min, respectively). Extracts were combined, condensed, lyophilized and stored at 4˚C until use with a yield of 5.2%.

Palmatine and berberine (purity ≥98%) were purchased from Nanjing Jingzhu Bio-technology Co., Ltd. (Nanjing, China), lyophilized ovalbumin (OVA; purity ≥98%) was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). Loratadine was purchased from Xi'an Janssen Pharmaceutical Ltd. (Shaanxi, China). Alexa Fluor 647-anti-Forkhead box P3 (Foxp3) (catalog no., 560401), fluorescein isothiocyanate (FITC) anti-CD4 (catalog no., 553047), phycoerythrin (PE)-anti-CD25 (catalog no., 558642) and the mouse Foxp3 fixation and permeabilization buffers (catalog no., 560409) were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

High performance liquid chromatography analysis to determine palmatine and berberine content. All experiments were performed on an Agilent 1200 series high performance liquid chromatography (HPLC) system (Agilent Technologies Inc., Santa Clara, CA, USA), which included a binary pump, an autosampler, a column oven and a diode array detector with an on-line degasser. The separations were achieved using a TSK gel octadecyl silane-100 V (internal diameter, 4.6x250 mm; particle diameter, 5 μm; Tosoh Corp., Tokyo, Japan).

The samples were ultrasonically extracted (53 KHz, room temperature) in 100 ml acetonitrile-0.4% phosphate buffer (32:68) for 30 min. The extracts were further eluted with acetonitrile-0.4% phosphate buffer (32:68) for 15 min during the mobile phase. The detection wavelength was set at 354 nm, the oven temperature was set at 40˚C and the flow rate was set to 1.0 ml/min. A 10-μl injection volume was used in all analyses. Palmatine and berberine were dissolved in the mobile phase. Palmatine and berberine peaks were identified based on their retention time, as well as the ultraviolet spectral properties of the standards.

Animals. A total of 48 specific pathogen-free female BALB/c mice (age, 7-8 weeks; mean weight, 20±2 g), were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). The animals were housed in a stainless steel cage containing sterile paddy husk as bedding and were kept in a ventilated animal room. The mice had *ad libitum* access to water and commercial laboratory mice food and were kept at 22±1˚C and 60±10% humidity under a 12-h light/dark cycle. The animals were given 10 days to acclimatize to the environment. The present study was approved by the *Institutional Ethical Committee* on *Animal* Care and Experimentations of Yunnan University of Traditional Chinese Medicine (Kunming, China; no. R-062014015). Reasonable efforts were made to minimize animal suffering.

OVA sensitization and Yajieshaba treatment. Mice were divided into 8 groups, each consisting of 6 animals (Table I) and treated as follows: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. Food allergy was induced by subcutaneous injection of 3 mg OVA in 150 μl aluminum hydroxide (Xilong Chemical Co., Ltd., Shantou, China) gel and 150 μl 0.9% sodium chloride solution in the neck region. This was repeated at 48 and 96 h (14-16). Treatments were administered by oral gavage at 30 min following the second and third OVA injections. Half of the animals were sacrificed under diethyl ether (ShanDian Pharmaceutical Co., Ltd., Yunnan, China) inhalation anesthesia by cervical dislocation 2 h following the second OVA injection and the remaining ones were sacrificed 2 h following the third OVA injection to collect samples. A schematic diagram of the treatment schedule is presented in Fig. 1.

Measuring of scratching behavior. Scratching behavior was observed in a quiet experimental room. Each animal was placed in a plastic observation cage (29x18x16 cm) for ~10 min prior to scoring of scratching behavior. One observer, who was blinded to the experimental design, counted the total number of scratches on the injection site in each treatment group. A scratch was defined as lifting of the forelimb

towards the injected area followed by placing of the limb back to the floor, regardless of how many scratching strokes took place between those two movements. However, if the scratching bout extended beyond 1 min, it was counted as two events (17).

Measuring OVA-specific serum immunoglobulin (Ig)E, IgG and other cytokines. Blood samples (1-1.5 ml) were collected from each mouse via the retro-orbital vein under diethyl ether inhalation anesthesia and centrifuged at 393 x *g* for 10 min at room temperature. The serum was stored at -20˚C until further analysis. The amounts of IgE (ml037602), IgG (ml037601), interleukin (IL)-4 (ml002149), IL-10 (ml037873), IL-17 (ml037866), IL-21 (ml037878), interferon (IFN)-γ (ml002277), and tumor necrosis factor (TNF)-α (ml002095) were measured using respective ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

Flow cytometric analysis of $CD4^+CD25^+Foxp3^+$ regulatory T-cells (Tregs). At the end of the treatment, all mice were sacrificed under diethyl ether inhalation anesthesia by cervical dislocation. Spleens were collected in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The spleens were minced and dissociated for flow cytometry by pressing them through a 70-μm filter membrane to produce a single-cell suspension. The red blood cells were lysed (BD Pharm Lyse™ lysing solution; BD Biosciences) and the remaining white blood cells were diluted with staining buffer (FBS; BD Biosciences) to a concentration of $1x10^6$ cells/ml. The cells were incubated with FITC-conjugated anti-mouse CD4 (1:20) and PE-conjugated anti-mouse CD25 (1:40) at room temperature in the dark for 20 min. The cells were fixed with Foxp3 fixing solution (1:20) at 4˚C for 30 min and permeabilized with Foxp3 permeabilization solution (1:20) at 37˚C for another 30 min according to manufacturer's protocol, and were stained with Alexa Fluor 647 anti-mouse Foxp3 (1:20) prior to analysis using a flow cytometer (BD FACSCalibur, BD Biosciences) to quantify Treg frequncies.

Statistical analysis. Values are expressed as the mean ± standard deviation. One-way analysis of variance was performed to compare the treatment effects on dependent variables. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Palmatine and berberine content in Yajieshaba. The linear association between injection quantity (mg/10 μl, X-axis) and peak area (Y-axis) was Y=34.31X+21.82 for palmatine (R^2=0.999) and Y=33.51X+0.2250 for berberine (R^2=0.999). The limits of detection for palmatine and berberine were 0.1496 and 0.06432 μg/ml, respectively. The limits of quantification of palmatine and berberine were 0.3990 and 0.3216 μg/ml, respectively. The average recovery rates of palmatine and berberine were 99.37 and 97.15%, respectively.

The HPLC elution times for palmatine and berberine were 10.2 and 11.0 min, respectively (Fig. 2). The palmatine and berberine content in Yajieshaba formulation were 1.49 and 0.50 mg/g, respectively.

Table I. Experimental design of the present study.

Group	OVA challenge	Treatment	Dosage (g/kg/body weight)
A	No	Physiological saline	-
B	Yes	Physiological saline	-
C	Yes	Palmatine	0.1048
D	Yes	Berberine	0.0352
E	Yes	Loratadine	1.37
F	Yes	Yajieshaba	2.35
G	Yes	Yajieshaba	3.50
H	Yes	Yajieshaba	4.70

Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. OVA, ovalbumin.

Effect of Yajieshaba on scratching frequency. Subcutaneous injection of 3 mg OVA into the neck region elicited a clear scratching response in mice. The scratching frequencies of mice in different treatment groups at 50 (Fig. 3A) and 98 h (Fig. 3B) were evaluated. Scratching frequencies increased from 0.325 and 1.43 scratches/minute in the control group to 2.58 and 2.23 scratches/minute in the untreated OVA-sensitized mice at 50 and 98 h, respectively. Mice treated with loratadine, palmatine, berberine or Yajieshaba at 3.50 or 4.70 g/kg body weight exhibited a markedly lower scratching frequency than the OVA-sensitized mice at both time points. Mice receiving a low dose of Yajieshaba (2.35 g/kg body weight) did not have a markedly different scratching frequency compared with the mice in OVA-sensitized group at 50 and 98 h. Mice in the palmatine (0.1048 g/kg) and Yajieshaba (3.50 and 4.70 g/kg) treatment groups had a markedly lower scratching frequency compared with mice in the loratadine group at 50 h. Palmatine treatment reduced the scratching frequency by ~40%, indicating that it may be the major active anti-allergic component of Yajieshaba.

Effect of Yajieshaba on IgE, IgG and serum cytokines. The serum levels of IgE and IgG (Fig. 4) as well as IL4, IL-10, IL-21, IFN-γ and TNF-α (Table II) were measured at 50 and 98 h to identify the mechanism through which Yajieshaba suppressed the scratching frequency in OVA-sensitized mice.

IgE levels were similar in the normal group and the model group at 50 h (Fig. 4A); however, IgE levels in Groups D, F and G were higher than the normal group at 50 h (Fig. 4A). Notably, the IgE level in the model group was significantly lower than that in the normal group at 98 h ($P<0.05$; Fig. 4B). Palmatine, loratadine, and low- and middle-dose Yajieshaba were able to reverse this OVA-induced reduction at 98 h (Fig. 4B). IgE was increased in group D after 50 h; however, it reduced after 98 h. Similarly, at 50 h, IgE levels were higher in group D than group C, whereas the opposite was observed after 98 h.

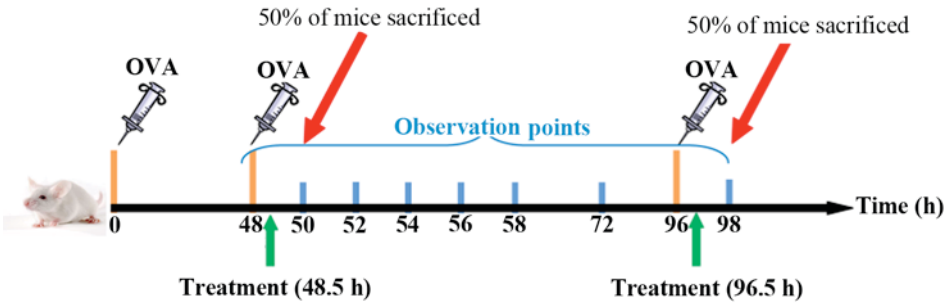

Figure 1. Schematic diagram of the treatment schedule used in the present study. OVA, ovalbumin.

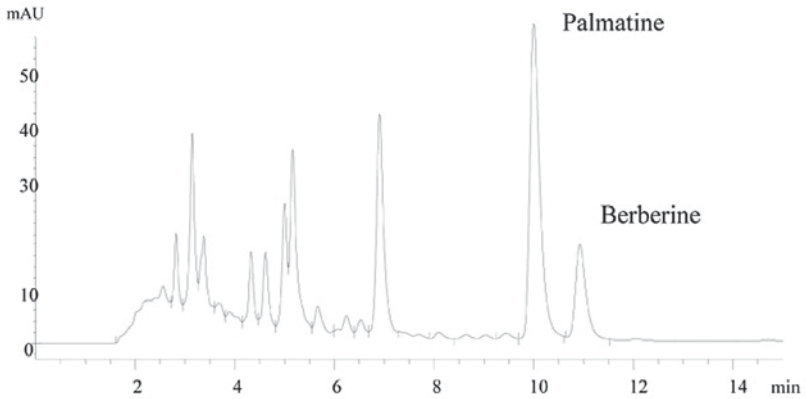

Figure 2. Representative HPLC spectrum of palmatine and berberine in the acetonitrile-0.4% phosphate buffer extract of the Yajieshaba formulation. HPLC with a diode array detector was used. HPLC, high performance liquid chromatography.

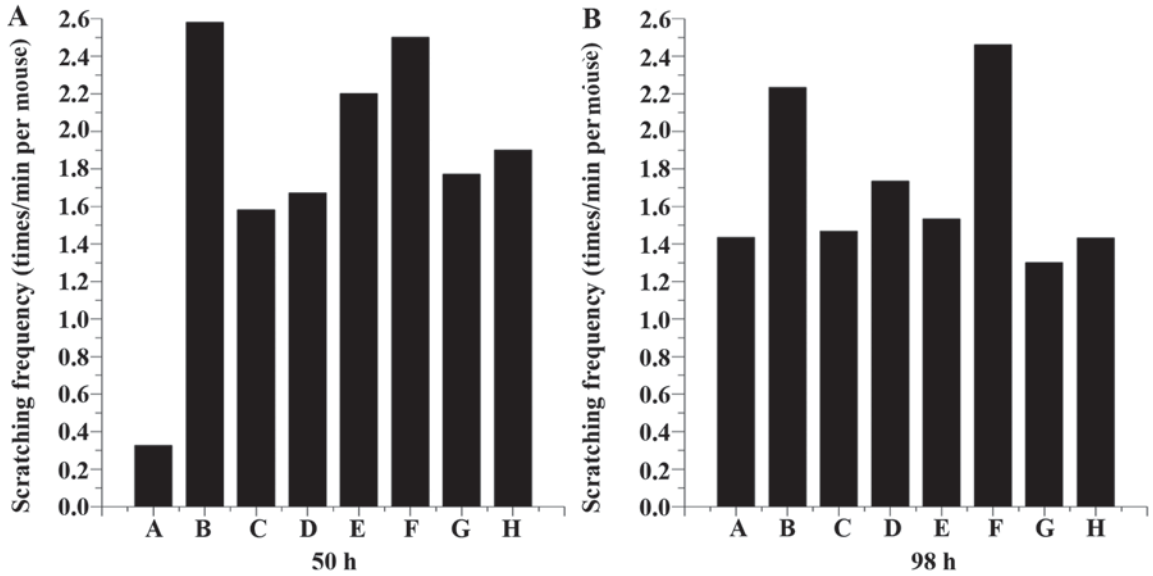

Figure 3. Average scratching frequency at (A) 50 and (B) 98 h. Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. OVA, ovalbumin.

OVA sensitization decreased serum IgG levels at 50 h (Fig. 4A), which was partially reversed in mice treated with Yajieshaba; however, no significant difference was detected. At 98 h, berberine significantly ($P<0.01$) increased serum IgG levels. The amount of serum cytokines, including IL4, IL-10, IL-17, IL-21, IFN-γ and TNF-α, did not significantly change in OVA-sensitized mice compared with those in the control group at 50 or 98 h (Table II). Yajieshaba treatment in the high dosage group significantly increased ($P<0.05$ vs. OVA-sensitized mice) serum IL-4, IL-10, IL-21, IFN-γ and

Table II. Effect of Yajieshaba on major inflammation cytokines in food allergic mice at 50 and 98 h.

Group	IL-4 (pg/ml)	IL-10 (pg/ml)	IL-17 (pg/ml)	IL-21 (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)
	50 h					
A	117±1.27	282±15.8	39.7±5.64	45.6±2.91	0.788±0.0835	645±57.9
B	124±5.21	295±17.1	41.7±2.64	47.5±2.41	0.845±0.128	691±25.4
C	123±4.16	301±17.0	37.6±3.50	50.7±3.52	0.643±0.240	730±7.45
D	123±6.39	317±14.5	40.8±3.01	50.7±3.53	1.89±0.167[c]	757±15.8[a]
E	131±4.34[b]	304±9.34	41.5±4.57	50.0±2.00	0.931±0.0934	740±36.7
F	135±6.82[a]	314±18.4	42.2±4.21	50.2±4.97	1.89±0.151[c]	736±12.9
G	140±7.99[b]	325±13.8[a]	43.5±0.812	54.4±1.20[b]	1.05±0.145	747±30.2
H	132±3.96[b]	349±13.8[a]	46.8±2.92	57.6±2.15[b]	1.12±0.0257[b]	754±10.2[b]
	98 h					
A	137±21.2	326±43.1	41.0±4.51	47.8±4.65	0.849±0.111	733±99.8
B	140 ±21.2	325±54.5	46.2±5.98	51.6±3.62	0.911±0.107	763±119
C	145±23.9	332±55.1	44.2±7.18	49.8±6.22	2.16±0.586[a]	835±125
D	146±27.8	354±67.1	46.0±5.14	53.3±9.24	1.71±0.230[b]	851±117
E	149±22.7	348±50.8	46.6±8.76	54.0±7.45	2.03±0.0963[c]	806±111
F	155±19.8	363±32.9	38.8±13.1	53.8±6.27	1.67±0.163[c]	807±104
G	168±40.1	378±82.2	50.7±6.90	56.8±11.2	2.23±2.11	837±134
H	174±38.7	403±53.3	45.0±9.91	66.3±11.0	1.19±0.0902[a]	839±118

Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. Values are expressed as the mean ± standard deviation. [a]P<0.05; [b]P<0.01; [c]P<0.001 vs. group B (OVA sensitized). IL, interleukin; IFN, interferon; TNF, tumor necrosis factor.

Table III. Effect of Yajieshaba on the $CD4^+CD25^+/CD4^+$ and $CD4^+CD25^+Foxp3^+/CD4^+$ cell ratio in the spleen.

Group	$CD4^+CD25^+/CD4^+$ (%)	$CD4^+CD25^+Foxp3^+/CD4^+$ (%)
A	12.10	1.86
B	6.80	1.03
C	7.20	0.89
D	8.20	0.72
E	10.30	1.55
F	8.50	0.53
G	7.50	1.18
H	12.50	5.50

At 50 and 98 h of OVA sensitization, spleens were collected and stained with fluorescent-labeled anti-CD4, anti-CD25, and anti-Foxp3 and analyzed for $CD25^+/CD4^+$ and $CD4^+CD25^+Foxp3^+/CD4^+$ cell ratio by flow cytometry. Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. CD, cluster of differentiation; Foxp3; OVA, ovalbumin.

TNF-α at 50 h. However, at 98 h, Yajieshaba treatment in high dosage group only significantly increased serum IFN-γ (P<0.05 vs. OVA-sensitized mice).

Effect of Yajieshaba on $CD4^+CD25^+Foxp3$ Tregs. As indicated in Fig. 5 and Table III, OVA sensitization markedly decreased the frequency of $CD4^+CD25^+$ Tregs in the spleen (6.80%) compared with that in the control group (12.10%; Table III). This decreased $CD4^+CD25^+$ Treg frequency in the OVA-sensitized mice may impair Treg-mediated immune inhibition, which is involved in suppressing the development of allergic-effector T cells from naive T cells and may therefore lead to an increase in the severity of allergic symptoms (18).

The high dose of the Yajieshaba prescription markedly increased the frequency of $CD4^+CD25^+$ Tregs in the spleen from 6.80%, in the OVA-sensitized group to 12.50% (Table III). Mice in the high-dose Yajieshaba group had an increased frequency of $CD4^+CD25^+$ Tregs in the spleen compared with mice in the loratadine group. However, mice in the palmatine and berberine group exhibited only a marginal increase in the frequency of $CD4^+CD25^+$ Tregs. This suggested that Yajieshaba contains a number of active components other than palmatine and berberine, which may contribute to its anti-allergic effects.

Mice in the OVA-sensitized group had a 44.6% decrease in Foxp3 expression in splenic $CD4^+CD25^+$ cells compared

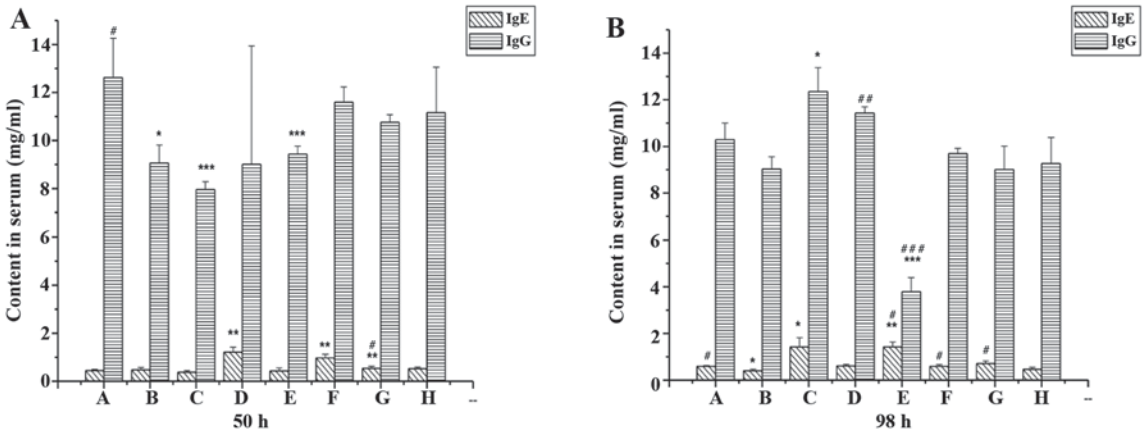

Figure 4. Effect of Yajieshaba treatments on the amount of total serum IgE and IgG in OVA-sensitized mice as determined by ELISA. (A) 50 h and (B) 98 h following OVA sensitization. Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. Values are expressed as the mean ± standard deviation. *P<0.05, **P<0.01 and ***P<0.001 vs. the group A; $^{\#}$P<0.05 $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. the group B. IgE, immunoglobulin E; OVA, ovalbumin.

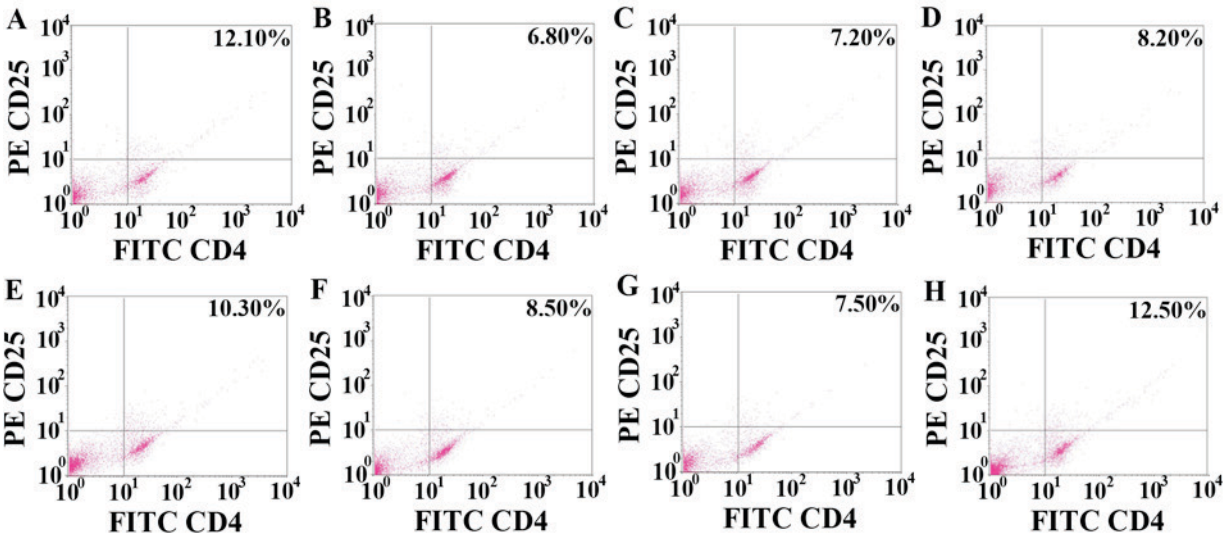

Figure 5. Representative flow cytometry figures of CD4 and CD25 staining of splenic cells from treatment groups. Groups: A, mice were not sensitized (control); B, mice were sensitized but not treated; C, mice were treated with palmatine (0.1048 g/kg); D, mice were treated with berberine (0.0352 g/kg); E, mice were treated with loratadine (1.37 g/kg); F, mice were treated with 2.35 g/kg body weight Yajieshaba; G, mice were treated with 3.50 g/kg body weight Yajieshaba; H, mice were treated with 4.70 g/kg body weight Yajieshaba. Cells were stained with fluorescent-labeled anti-CD4 and CD25 and analyzed in a flow cytometer. CD, cluster of differentiation; OVA, ovalbumin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

with that in the control group (Table III). Yajieshaba treatment reversed the OVA-induced Foxp3 suppression in a dose-dependent manner. Mice in the high-dose Yajieshaba group had a $CD4^{+}CD25^{+}Foxp3^{+}/CD4^{+}$ ratio of 5.50%, which was markedly higher than that in the OVA-sensitized group (1.03%; Table III).

Discussion

Food allergies are adverse immune responses to certain types of food, with food proteins being the most common allergens. Allergies occur when the body's immune system identifies certain proteins as harmful. OVA is a rich source of biologically active peptides, several of which may stimulate an immune response (19). Therefore, the OVA-sensitized allergy model is commonly used to study and identify the effects of various treatments for alleviating food allergies and their underlying mechanisms (14-16).

Allergic reactions to food are mediated via two major mechanisms: IgE-mediated (allergic) and non-IgE-mediated food reactions (20-22). The IgE food reactions are primarily type 2 T helper cell (Th2)-mediated and typically occur immediately following exposure. Symptoms include swelling, itching, reddening and smooth muscle contraction, which may result in asthma, atopic dermatitis, gastrointestinal symptoms, edema and a potentially life-threatening anaphylactic reaction (20).

Other allergic reactions to food may occur h or days following ingestion. These reactions are predominately IgG reactions (21), causing different symptoms that may include

bloating or sluggishness, gastrointestinal symptoms, sinus congestion, dark circles under the eyes and chronic nasal secretion (21).

In the present study, an acute food allergy model was established by sensitizing mice to OVA over three exposures. This food allergy model was characterized by a significant increase in scratching frequency and a decrease in Treg frequency in the spleen. The serum IgE in model group was reduced at 98 h. Palmatine, loratadine, and low- and middle-dose Yajieshaba were able to reverse this reduction at 98 h. Serum IgG levels decreased in the model group at 50 h. However, no significant difference of serum IgG was found in the treated groups at both 50 and 98 h. The model may therefore be considered to simulate the symptoms of human acute food allergies. The present investigation of the anti-allergic effects and mechanisms of Yajieshaba, one of the most widely applied prescriptions in TDM, was performed using this murine model of food allergy.

Yajieshaba and its major active constituent, palmatine, markedly inhibited the OVA-induced food allergic symptoms and markedly increased Treg frequency. Tregs are a subset of immune cells that specialize in immune suppression. The increased $CD4^+CD25^+$ Tregs in Yajieshaba- and palmatine-treated groups may therefore suppress the host's immune response to food allergens (18). These results indicated that the Yajieshaba had a direct stimulatory effect on $CD4^+CD25^+Foxp^{3+}$ Tregs. Increased Tregs in mice treated with Yajieshaba may be expected to inhibit the OVA-induced food allergy symptoms.

In addition, the $CD4^+CD25^+Foxp3^+/CD4^+$ ratio increased in a dose-dependent manner in mice treated with Yajieshaba. Palmatine treatment increased Foxp3 expression in $CD4^+CD25^+$ cells. Foxp3, a member of the forkhead/winged-helix family of transcription factors, is expressed specifically in naturally occurring Tregs (23) and is a key regulator in the development and function of Tregs. Therefore, alleviation of food allergy reactions by Yajieshaba and palmatine was potentially due to an increased Treg frequency.

Palmatine was identified as a component of Yajieshaba. It may have contributed to the pharmacological effects of Yajieshaba, as it has been demonstrated to have a number of pharmacological activities, including antifungal activity (24) as well as acetyl cholinesterase (15) and protonophore-inhibiting activities (25). The anti-allergic activity of palmatine has been reported in previous studies (26,27) and confirmed in the present study. It has been reported that palmatine inhibits degranulation of RBL-2H3 mast cells, a basophilic leukemia cell line, via the suppression of tyrosine kinase phosphorylation (26). In the present study, it was indicated that palmatine decreased the scratching frequency, reversed the OVA-induced increase in serum IgE and increased the Treg frequency. Palmatine may be absorbed into the blood stream within 45 min of consumption (28) and was therefore able to inhibit allergy symptoms within 1.5 h of allergen sensitization. Thus, the palmatine component of the Yajieshaba formulation may have contributed to its anti-allergic effect.

The present study illustrated the potential mechanism by which Yajieshaba alleviates food allergy reactions. Furthermore, the present study presented scientific evidence supporting the clinical use and further application of Yajieshaba. However, constituents other than palmatine and berberine may have contributed to the anti-allergic effects of Yajieshaba. Further studies on the anti-allergic effects of Yajieshaba are required to provide additional information on the mechanisms underlying its activity.

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