



Forsythia suspensa extract alleviates hypersensitivity induced by soybean β -conglycinin in weaned piglets

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ARTICLE INFO

Article history:

Received 22 July 2009

Received in revised form 4 January 2010

Accepted 11 January 2010

Available online 18 January 2010

Keywords:

Forsythia suspensa

Soybean allergy

Mast cells

Histamine

Cytokines

Swine

ABSTRACT

Aim of the study: Soybeans are known to stimulate food allergies; however, current therapies are lacking in alleviating hypersensitivity. The present study investigated whether *Forsythia suspensa* extract could attenuate purified soybean β -conglycinin-induced anaphylactic reactions in weaned piglets.

Materials and methods: Eighteen 14-day-old piglets were sensitized and boosted by oral administration of purified β -conglycinin. *Forsythia suspensa* extract was supplemented in the diet after the initial sensitization and continued for the remainder of the experiment. Piglets were challenged on day 28, and anaphylactic symptoms, passive cutaneous anaphylaxis, plasma histamine and intestinal microbial flora were analyzed. T-cell proliferative responses and cytokine production were also determined.

Results: *Forsythia suspensa* extract alleviated β -conglycinin-induced anaphylactic symptoms by markedly reducing anaphylactic antibodies, mast cell degranulation, and histamine release while improving intestinal microbial flora. Furthermore, *Forsythia suspensa* extract significantly suppressed β -conglycinin-induced T lymphocyte proliferation and IL-4 synthesis.

Conclusion: *Forsythia suspensa* extract protected β -conglycinin-sensitized piglets from anaphylactic reactions. *Forsythia suspensa* extract may provide a novel effective therapy for soybean allergy.

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1. Introduction

Soybean is a major protein source for humans and animals alike; however, soybean has also been reported to contain inflammatory allergens (Cordle, 2004). Clinical allergic reactions caused by the ingestion of soybean products range from mild gastrointestinal distress to life-threatening asthma and death (Foucard and Malmheden, 1999). β -Conglycinin, a primary storage protein that accounts for about 30% of total storage protein in soybeans (Utsumi, 1992), has been found to bind IgE in subjects with soy allergies (Rozenfeld et al., 2002; Mittag et al., 2004).

Currently, strict avoidance of soybean products is the only way to prevent allergic reactions; however, in practice this is difficult to achieve due to the widespread use of soybean products in many

foods. Anti-IgE therapy to alleviate food allergies has shown only modest and transient benefits (Leung et al., 2003). Conventional allergen-specific immunotherapy is also currently dissatisfactory because of adverse side effects and poor maintenance of tolerance afterward (Nelson et al., 1997). As the occurrence of food allergies increases, it is of great importance to find safe, effective and long-lasting therapies to alleviate allergic reactions to specific foods. Increasing attention is being given to the use of traditional Chinese medicines to find additional therapies to alleviate food allergies. Li et al. (2001) and Srivastava et al. (2005) reported that the Chinese herbal medicines named 'Food Allergy Herbal Formula-1' and 'Food Allergy Herbal Formula-2' blocked anaphylactic reactions in mice allergic to peanuts. These studies suggest that herbal medicines may be used as novel therapies to combat food allergies.

Forsythia suspensa Vahl (Oleaceae) is a climbing plant, which is widely distributed throughout China, Korea and Japan. The extract of the dried fruit from this plant has been widely used as an antipyretic, antidotal and anti-inflammatory agent for the treatment of infections, such as acute nephritis, erysipelas and ulcers (Guo et al., 2007a). Moreover, crude extracts exhibit resistance to hepatic injury, antibacterial, antiviral, antioxidant and anti-inflammatory activities (Ozaki et al., 2000; Guo et al., 2007a; Wang et al., 2008). A number of compounds including phenylethanoid glycosides, lignans, flavonoids, terpenes and volatile oils have been isolated from *Forsythia suspensa*. Among these, the first 3 com-

Abbreviations: cAMP, cyclic adenosine monophosphate; CBB, Coomassie Brilliant Blue; ELISA, enzyme-linked immunosorbent assay; FSE, *Forsythia suspensa* extract; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IL-4, interleukin-4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRC, National Research Council; PBS, phosphate buffered saline; PCA, passive cutaneous anaphylaxis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM, standard error of mean; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-thiogalactopyranoside.

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pounds were found to be responsible for the various biological effects of the plant extract (Piao et al., 2008).

Liu (2008) reported that phillyrin isolated from *Forsythia suspensa* could enhance immunological function and alleviate delayed hypersensitivity in mice. However, the role of *Forsythia suspensa* extract (FSE) in alleviating the adverse effects from food allergies has not yet been investigated. The physiological and immunological similarities between pigs and humans have made the pig an important large-animal model for biomedical research (Murtaugh, 1994; Helm et al., 2002). Previous studies found that soybean β -conglycinin induced immediate hypersensitivity in early weaned piglets (Hao et al., 2009), while FSE promoted growth and well-being without any adverse effects. Therefore, the objective of the current study was to further investigate whether oral administration of FSE could alleviate soybean β -conglycinin anaphylaxis in pigs.

2. Materials and methods

2.1. Preparation of β -conglycinin

Purified β -conglycinin suspension was donated from the Food Institute of China Agricultural University (Patent number, 200410029589.4, China). After lyophilization, protein content was determined by the Kjeldahl method and β -conglycinin purity was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Brilliant Blue R-250 staining (Laemmli, 1970). The protein concentration contained within the gel was determined using a Syngene GeneGenius gel documentation and analysis system (Syngene, Cambridge, United Kingdom) (Guo et al., 2007b). The degree of purity was measured to be >85%.

2.2. Extract preparation from *Forsythia suspensa*

Fructus forsythiae used in this study was purchased from Beijing Tong Ren Tang Pharmacy, China, and were authenticated by Xiang-Lan Piao, which was dried fruit of *Forsythia suspensa* (Thunb.) Vahl. A voucher specimen was deposited at State Key Laboratory of Animal Nutrition, China Agricultural University, China (Voucher No. FS 0801). Dried and powdered fruit of *Forsythia suspensa* (500 g) was extracted with 2500 mL of 80% methanol and sonicated for 1 h twice. The combined extracts were concentrated under reduced pressure below 45 °C on a rotary evaporator. The solution was freeze-dried to give a powdered (62.2 g). The crude extract was re-dissolved in water, extracted with CH_2Cl_2 three times and *n*-BuOH four times in succession. The CH_2Cl_2 fractions and the *n*-BuOH fractions were combined, respectively. The two fractions were dried on a rotary vaporization (Büchi, Rotavapor R-124, Flawil, Switzerland; Wang et al., 2008) to give, respectively, 26.4 g solid residue from the CH_2Cl_2 and 21.8 g from the *n*-BuOH. An aliquot (20 g) from CH_2Cl_2 was loaded on a silica gel column (Qingdao Chem., 200–400 mesh, 600 g) with CHCl_3 –MeOH as eluants with increasing polarity from 100:1 to 2:1 CHCl_3 –MeOH ratio. Repeated silica column chromatography of the fraction yielded 4 compounds. They were identified, based on their mass spectral, ^1H NMR, and ^{13}C NMR data, as phillygenin (16.7 mg), phillyrin (81.7 mg), forsythialan A (41.3 mg) and forsythoside A (16.5 mg) (Lu et al., 2010).

2.3. Animals, diets and experimental design

Eighteen newly weaned barrows (Large White $\times$ Landrace; initial body weight 4.42 ± 0.08 kg; all 14 days of age) were selected from a commercial pig farm (Beijing, China) and transported to China Agricultural University. All piglets were individually housed

Table 1

Composition and nutrient level of the basal diet.

Ingredient	Composition (%)
Corn	60.50
Skimmed milk powder	4.00
Whey powder	14.00
Casein	13.50
Spray dried porcine plasma	4.50
Limestone	0.90
Dicalcium phosphate	1.30
Salt	0.30
1% Premix ^a	1.00
Chemical analysis	
Crude protein	22.74
Lysine	1.61
Calcium	0.92
Phosphorus	0.75
Calculated DE, MJ/kg	14.59

^a 1% premix provided per kilogram of the complete diet: vitamin A, 10,000 IU; vitamin D₃, 1,500 IU; vitamin E, 30 IU; vitamin K₃, 2.5 mg; vitamin B₁, 1.5 mg; vitamin B₂, 10 mg; vitamin B₆, 10 mg; vitamin B₁₂, 0.05 mg; folic acid, 1 mg; biotin, 0.5 mg; niacin, 30 mg; pantothenic acid, 20 mg; Cu, 20 mg; Fe, 100 mg; Zn, 110 mg; Mn, 40 mg; Se, 0.3 mg; and I, 0.54 mg.

in 1.3 m $\times$ 1.2 m pens with slotted stainless steel floors. A mechanically ventilated nursery room was supplied with 16 h light/8 h dark, and maintained at 25–27 °C. Water and feed were available *ad libitum*. Piglets were fed a soybean- and peanut-free diet, using skimmed milk powder and casein as major protein sources. The basal diet was formulated to meet the nutrient requirements suggested by the NRC (1998) and contained no antibiotics (Table 1). Animal experiments were conducted at the Ministry of Agriculture Feed Safety and Bio-availability Evaluation Center (Beijing, China). All procedures were approved by the China Agricultural University Animal Care and Use Committee.

Piglets were randomly allocated into three treatment groups ($n=6$ for each group) according to litter and body weight; and housed individually. β -Conglycinin was dissolved in phosphate-buffered saline (PBS, pH 7.4), 50 mL/piglet. Group 2 was sham group, in which piglets were sensitized by means of oral gavage with 2 g purified β -conglycinin daily from day 0 to day 6. Following initial sensitization, piglets were boosted orally with 8 g β -conglycinin on day 21, and were then challenged with 12 g β -conglycinin (divided into 2 doses administered 30–40 min apart) on day 28. Piglets in group 3 had the same immunization schedule as group 2, and were treated with FSE (100 mg/kg) in the diet after the first sensitization and continued everyday thereafter. Naive piglets served as controls and were gavaged with equal volumes of PBS using the same schedule. 3 h after the second challenge dose, all piglets were slaughtered with an intracardial injection of sodium pentobarbital (50 mg/kg body weight) followed by jugular exsanguination. Sera from all groups of piglets were collected 1 day before β -conglycinin challenge and stored at -80°C .

2.4. Assessment of systemic anaphylactic markers

Anaphylactic markers were evaluated 30–40 min after the second challenge dose by three investigators using a scoring system previously described (Helm et al., 2002) with minor modifications: (0) no symptoms; (1) scratching and rubbing around the nose and head; (2) coughing, gagging, evidence of stomach contractions, reduced activity, tendency to lie down, easily stimulated to move, minor rashes; (3) vomiting, lethargy/malaise, tremors, diarrhea, convulsions, reduced activity that could not be stimulated by provocation; (4) wheezing, respiratory distress and symptoms requiring epinephrine; and (5) death.

2.5. Passive cutaneous anaphylaxis (PCA)

PCA testing was performed following modified procedures previously described by Helm et al. (2002). In this test, 30 additional 28-day-old normal piglets were used. Their abdomens were shaved 24 h before injection. Twelve piglets were used for sham group sera, in which 6 piglets were intradermally injected 500 μ L unheated serum (1:2 dilution), and the other 6 with 500 μ L heat-treated serum (56 °C for 3 h to inactivate IgE). Another 12 piglets were injected with serum from FSE treatment group using the same schedule as the sham group. The rest of the 6 piglets in the group of 30 were injected with an equal amount of unheated, diluted naive serum. 24 h after the injections, all the piglets were injected intravenously with purified β -conglycinin (5 mg/mL) and 100 μ L 0.5% Evan's blue dye. Wheal and flare measurements were made 30 min following injection of the dye. A reaction was scored as positive if the bluing of the skin at the sites of injection were greater than 5 mm in diameter.

2.6. Measurement of plasma histamine levels

To determine plasma histamine levels, plasma was collected 30 min after the second gavage challenge. Histamine concentration was determined using an enzyme immunoassay kit (USCN Life Science, Double Lake, MO, United States), following the manufacturer's protocol.

2.7. Histological analysis of jejunum mast cells

Longitudinal sections of jejunum tissue (3 cm obtained from the middle of the jejunum) were obtained and flushed with 5 mL of cold 0.9% NaCl solution. Tissue sections were fixed in Carnoy's solution for 24 h, followed by routine dehydration, paraffin embedding and cut to 6 μ m histological slices. Mast cell number was determined using toluidine blue (Gurr, Poole, United Kingdom) staining under a microscope (BX 50, Olympus Co., Japan). The number of mast cells in the mucosa was quantified by averaging the number of mast cells in eight 1 mm² areas of one histological slide with the aid of a Microcheck Grid (Beijing KeYi Optical Ltd., China). This was repeated on three independent histological slides for each tissue sample and represented as mast cell number per square millimeter.

2.8. Bacterial analyses of cecal digesta

In vitro survival of cecal microflora was determined using the methods of Liu et al. (2008a) with some modifications. Briefly, 1 g of fresh cecal digesta was taken from each animal and serially diluted 10-fold at a time with sterile physiological saline, resulting in dilutions ranging from 10⁻¹ to 10⁻⁸. *Escherichia coli* were cultivated on MacConkey agar (Beijing Haidian Microbiological Culture Factory, China). Total aerobes were cultured in yeast extract agar (Oxoid Ltd., Hampshire, United Kingdom), and total anaerobes were cultured in fastidious anaerobes agar (FAA; LAB M, Bury, United Kingdom). *Lactobacillus* was cultured in MRS agar (De Man, Rogosa, Sharpe agar; Oxoid Ltd., Hampshire, United Kingdom) with *Bifidobacteria* also cultured in MRS agar with 40 μ g/mL 5-bromo-4-chloro-3-indolyl- β -D-thiogalactopyranoside (X-gal) (Sigma Chemical Inc., St. Louis, MO) (Álvarez-Martín et al., 2007). Each digesta dilution was determined in triplicate. Total anaerobes and *Lactobacillus* were inoculated in Hungate roll tubes, while total aerobes, *Escherichia coli* and *Bifidobacteria* (anaerobically incubated) were fostered on plates. All tubes and plates were incubated at 37 °C for 24–48 h. The microbial enumerations of digesta were expressed as log 10 colony-forming units per gram. Bacteria were counted by a visual count of colonies using the best replicate

set from dilutions that resulted in 30–300 colonies per plate or tube.

2.9. T-cell culture and proliferation assays

On day 28 following the last oral administration of the antigen, all piglets were sacrificed, their spleens were aseptically removed, minced, and cell suspensions were prepared. After erythrocyte lysis, spleen cell suspensions were washed three times in Hanks' balanced salt solution (Gibco, Grand Island, NY, United States), resuspended in complete medium (RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin and 25 mM Hepes), and counted using trypan blue dye exclusion. Proliferation assays were performed by plating cell suspensions at a concentration of 1 $\times$ 10⁶/well in 96-well round-bottom plates (Costar, Corning Life Sciences, Acton, MA, United States) in triplicate with or without purified β -conglycinin (50 μ g/mL) (Gizzarelli et al., 2006). Cells stimulated with ConA (2 μ g/mL) served as a positive control. Cells were incubated in 5% CO₂ and 100% humidity for 66 h at 37 °C. 10 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) (Sigma Chemical Inc., St. Louis, MO, United States) was added to each well, and the plates were incubated for another 6 h. After incubation, 100 μ L of 10% sodium dodecyl sulfate in 0.04 mol/L HCl solution was added to lyse the cells and solubilize MTT crystals. Finally, the plates were read on an automated ELISA reader (Sunrise, Tecan, Austria) at 570 nm (Guo et al., 2007b). Results are expressed as a stimulation index, calculated as the absorbance of wells incubated with the antigen divided by the absorbance of wells containing unstimulated cultures.

2.10. Quantification of cytokines

Splenocytes were cultured in 24-well plates 2 $\times$ 10⁶/well/mL in the presence or absence of β -conglycinin (50 μ g/mL). Supernatants were collected after 72 h of culture. Expression of the cytokines IFN- γ and IL-4 were determined by ELISA, according to the manufacturer's instructions (Biosource, Camarillo, CA, United States).

2.11. Statistical analysis

All statistical analysis was performed using SAS v8.2 (Cary, NC, United States). Data are reported as the mean $\pm$ S.E.M. Data from the PCA reaction were analyzed by an unpaired *t*-test. For anaphylaxis scores, the difference between groups was analyzed by a Kruskal–Wallis one-way ANOVA. All other data comparisons were made using one-way ANOVAs followed by the Duncan multiple range test where appropriate. Differences between means were considered statistically significant when *P* < 0.05.

3. Results

3.1. Symptom prevention of FSE-induced anaphylactic reactions after oral β -conglycinin challenge

On day 28, anaphylactic symptom scores were made 30 min–3 h after oral β -conglycinin challenge. As displayed in Table 2, all sham-treated piglets developed systemic anaphylactic reactions. Two piglets showed evidence of minor rashes, reduced activity and a tendency to lie down (score 2, 33.3%); 3 piglets displayed symptoms of vomiting, lethargy/malaise and diarrhea (score 3, 50.0%). The 1 remaining piglet from the sham group exhibited severe symptoms of wheezing, respiratory distress and anaphylactic shock requiring epinephrine (score 4, 16.7%). In contrast, FSE-treated piglets exhibited a more mild anaphylactic reaction. Two piglets developed score 1 symptoms, and 1 piglet developed score 2 symptoms. Naive

Table 2Assessment of systemic anaphylactic reactions after oral β -conglycinin administration.

Treatment	Anaphylactic score, median (range)	Positive reaction, n/total	Positive reaction rate (%)
Naive	0 (0)	0/6	0
Sham	3 (2–4)*	6/6	100
FSE	1 (0–2)**	3/6	50

* $P < 0.05$ compared with naive piglets.** $P < 0.05$ compared with sham piglets.**Table 3**

PCA reactions after injection of heated or unheated immune serum.

Immunization	Heated serum (56 °C for 3 h)	Diameter, mm (mean $\pm$ S.E.M.)	Positive reaction	
			n/total	%
Naive	–	1.46 $\pm$ 0.20	0/6	0
Sham	–	9.56 $\pm$ 0.68*,**	6/6	100
	+	3.55 $\pm$ 0.32***	0/6	0
FSE	–	4.36 $\pm$ 0.47*,****	2/6	33.3
	+	1.95 $\pm$ 0.20	0/6	0

* $P < 0.05$ heated immune serum vs. unheated serum.** $P < 0.05$ vs. naive piglets.*** $P < 0.05$ vs. heated serum from FSE group piglets.**** $P < 0.05$ vs. sham piglets.

piglets did not exhibit any symptoms of anaphylaxis. These results demonstrate FSE possesses potent protective properties against β -conglycinin-induced anaphylaxis.

3.2. Effects on PCA reactions

Anaphylactic antibodies are associated with the severity of hypersensitivity. Thus, PCA testing was performed to study the effects of FSE on β -conglycinin-induced hypersensitivity. Injection of sera from intragastrically β -conglycinin-sensitized piglets induced PCA reactions, which were eliminated by heat (56 °C for 3 h) inactivation of immune sera. PCA reactions induced by unheated or heated sera from β -conglycinin-sensitized animals significantly decreased when treated with FSE. However, the reactions induced by heated sera were negative (<5 mm). Sera from naive animals did not react in the passive cutaneous test (Table 3). These results suggest that the clinical anaphylactic reactions were mediated by anaphylactic antibodies (mainly IgE), and FSE may alleviate anaphylactic reactions by suppressing anaphylactic antibody production.

3.3. Plasma histamine levels and mast cell number in the small intestine

Mast cell degranulation and histamine release are major markers of anaphylaxis, therefore, histamine levels were determined 30 min after administration of β -conglycinin. As shown in Table 4, plasma histamine levels were markedly elevated in sham-treated piglets compared to naive piglets (285.8 ng/mL vs. 17.4 ng/mL; $P < 0.001$). In contrast, histamine levels in FSE-treated piglets were lower than sham-treated piglets (85.3 ng/mL vs. 285.8 ng/mL; $P < 0.001$). Moreover, the number of mast cells found in the jejunum

Table 4

Effect of FSE on mast cell degranulation in the jejunum and plasma histamine concentration.

Item	Naive	Sham	FSE
Mucosal mast cell number/mm ²	20.72 $\pm$ 1.76	34.05 $\pm$ 0.82*	27.28 $\pm$ 1.12**
Plasma histamine concentration (ng/mL)	17.38 $\pm$ 4.56	285.76 $\pm$ 15.01*	85.31 $\pm$ 8.85**

* $P < 0.05$ compared with naive piglets.** $P < 0.05$ compared with sham piglets; $n = 6$, mean $\pm$ S.E.M.**Table 5**

Effect of FSE on the number of cecal microflora (log cfu/g of wet digesta).

Item	Naive	Sham	FSE
Total aerobes	5.47 $\pm$ 0.49	6.60 $\pm$ 0.22*	6.37 $\pm$ 0.26
<i>Escherichia coli</i>	4.33 $\pm$ 0.52	5.74 $\pm$ 0.29*	5.24 $\pm$ 0.44
Total anaerobes	7.94 $\pm$ 0.40	6.64 $\pm$ 0.62	7.32 $\pm$ 0.37
<i>Lactobacilli</i>	7.22 $\pm$ 0.32	5.90 $\pm$ 0.16*	6.76 $\pm$ 0.44
<i>Bifidobacteria</i>	6.73 $\pm$ 0.30	5.54 $\pm$ 0.28*	6.51 $\pm$ 0.43

* $P < 0.05$ compared with naive piglets ($n = 6$, mean $\pm$ S.E.M.).

was lower in the FSE-treated piglets compared to sham-treated piglets (27.3 vs. 34.1; $P < 0.001$). These results suggest that FSE treatment attenuated β -conglycinin-induced allergic reactions by blocking degranulation and histamine release from β -conglycinin-stimulated mast cells.

3.4. Effect of FSE on the number of beneficial bacteria in the intestine following β -conglycinin administration

To test whether orally administered FSE could improve the composition of the intestinal microflora in allergic piglets, microflora was analyzed in the cecal digesta. As displayed in Table 5, a greater number of aerobes were found in sham piglets, particularly *Escherichia coli*, when compared to piglets treated with FSE or the naive group. In contrast, the FSE treated group comprised higher counts of *Lactobacilli* and *Bifidobacteria* compared with the allergic piglets. No change in the total number of anaerobes was detected.

3.5. β -Conglycinin-induced T cell proliferation and Th2 cytokine production

To determine the mechanisms of FSE on the anaphylactic response induced by β -conglycinin, we investigated splenocyte cytokine profiles in vitro. Our results found that splenocytes from sham-treated, β -conglycinin-allergic piglets had a marked proliferative response to β -conglycinin stimulation. In contrast, the proliferative response to β -conglycinin in splenocytes from FSE-treated piglets was significantly less than that of splenocytes from sham-treated piglets ($P < 0.001$), and approximately equal to splenocytes from naive spleen cells (Fig. 1). Following β -conglycinin stimulation, splenocytes from FSE-treated piglets secreted significantly less IL-4, but significantly more IFN- γ than splenocytes from sham-treated piglets (both $P < 0.001$) (Fig. 2). These results suggest that FSE can suppress both β -conglycinin-induced T-cell proliferation and β -conglycinin-activated Th2 cytokine secretion.

4. Discussion and conclusions

Soybean allergies present a significant health problem for many, most commonly occurring early in life (Herian et al., 1993). With the increased use of soybean and soybean extracts in commercial food products and animal feed, the incidence of allergic reactions to foods has increased in both children and adults (Ballmer-Weber et al., 2007), as well as in young animals (Lallès et al., 1996). Previous studies employed young pigs as an animal model to investigate the anaphylactic response to soybean extracts, commonly resulting in

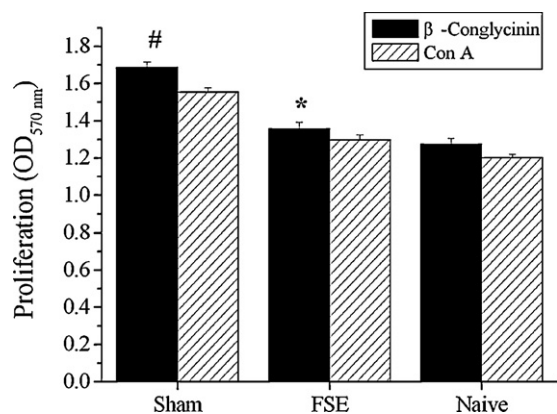


Fig. 1. Splenocyte proliferative response to β -conglycinin. Triplicate cultures of spleen cells from allergic, FSE treated and naive piglets were stimulated with 50 μ g/mL soybean β -conglycinin. Cells cultured with Con A (2 μ g/mL) serve as a control. Results are expressed as a stimulation index (mean $\pm$ S.E.M., $n = 6$). [#] $P < 0.05$ compared with naive piglets; ^{*} $P < 0.05$ compared with sham piglets.

disorders of immune function and reduced performance (Li et al., 1990; Dréau et al., 1994).

β -Conglycinin is one of the major storage proteins found in soybeans and has been previously identified as a potential diagnostic marker for severe allergic reactions to soy (Christensen et al., 2003; Liu et al., 2008b; Holzhäuser et al., 2009). In the present study, we generated a swine model of anaphylaxis in which the production of β -conglycinin-specific anaphylactic antibodies was induced by oral sensitization. Anaphylactic symptoms were then provoked by ingestion of high doses of β -conglycinin. The resulting symptoms involved multiple target organs, including the skin, gastrointestinal tract and respiratory system, mimicking the clinical characteristics of anaphylactic reactions exhibited by humans. Although the precise mechanisms regulating the pathogenesis of systemic anaphylaxis after ingestion of β -conglycinin are largely unknown, our findings that β -conglycinin markedly increased specific anaphylactic antibodies, increased intestinal mast cell degranulation and elevated plasma histamine concentration in this animal model suggest that histamine released from degranulated mast cells contributed to the symptoms of systemic anaphylaxis.

A traditional method for treating IgE-mediated allergic responses is the use of anti-histamine medication (Thornhill and Kelly, 2000); however, these can also produce many adverse side effects. Other novel approaches include anti-IgE, probiotics and food allergy vaccines. Compared with these therapies, the use of traditional Chinese herbal medicine demonstrates a number of advantages, such as being safe to ingest, economical, well tol-

erated and having long-term effects on the suppression of IgE antibodies (Li et al., 2001; Nowak-Węgrzyn, 2003; Srivastava et al., 2005). In this study, we found that FSE largely reversed established β -conglycinin-induced allergic symptoms as well as protecting sensitized piglets from anaphylactic reactions after β -conglycinin challenge. The protective effects were confirmed by several well-established parameters, including anaphylactic symptom scores, the production of anaphylactic antibodies, elevated plasma histamine levels and mast cell degranulation, which together demonstrate a potent therapeutic effect of FSE in ameliorating and preventing β -conglycinin-induced hypersensitivity.

It is plausible that FSE targets multiple elements of the β -conglycinin-induced allergic reaction cascade. In type 1 hypersensitive subjects, the Th2 cytokine, IL-4, promotes B-cell switching to IgE production and activates mast cells. The large quantity of IgE antibodies generated by individuals during an allergic reaction to specific foods immediately bind to the Fc ϵ R1 receptors located on the surface of the mast cells in the gastrointestinal tract and other tissues. This provokes mast cell degranulation and subsequent release of active mediators, including histamine and specific cytokines, leading to hypersensitivity (Lorentz et al., 2000; Chan et al., 2008). Furthermore, serum IgG1 antibody also can cause mast cell degranulation and target organ injury by binding to Fc ϵ R1 receptors located on the surface of the mast cells (Miyajima et al., 1997). As the ability of IgE to bind to Fc ϵ R1 is lost after heating, passive cutaneous reactions induced by heat-inactivated sera are only due to IgG1 antibodies (Faquim-Mauro et al., 1999). The results of PCA test showed that antigen-specific IgE and IgG1 were both produced in β -conglycinin-induced hypersensitivity, however, IgE played a key role in this model. So it is conceivable that FSE inhibits mast cell degranulation by suppressing the production of anaphylactic antibody, or directly suppresses β -conglycinin-induced B-cell activation. Previous studies have shown that β -conglycinin can cause severe intestinal damage in sensitized piglets (Hao et al., 2009), thus oral administration of FSE may actively reduce the amount of β -conglycinin allergens capable of interacting with mast cells by reducing intestinal permeability.

Numerous studies have demonstrated that a shift towards Th2 in the Th1/Th2 ratio plays a pivotal role in the pathogenesis of allergic disorders, including food allergies (Shida et al., 2000; Turcanu et al., 2003). In this study, FSE significantly suppressed the key Th2 cytokine IL-4, as well as increased IFN- γ production in the cultured splenocyte above sham levels. This suggests that FSE can regulate the activity of specific cytokines, providing an underlying immunotherapeutic mechanism to attenuate β -conglycinin-induced hypersensitivity. Furthermore, in the present study FSE was found to suppress β -conglycinin-specific T-cell proliferation, consistent with the report that FSE inhibits the activation

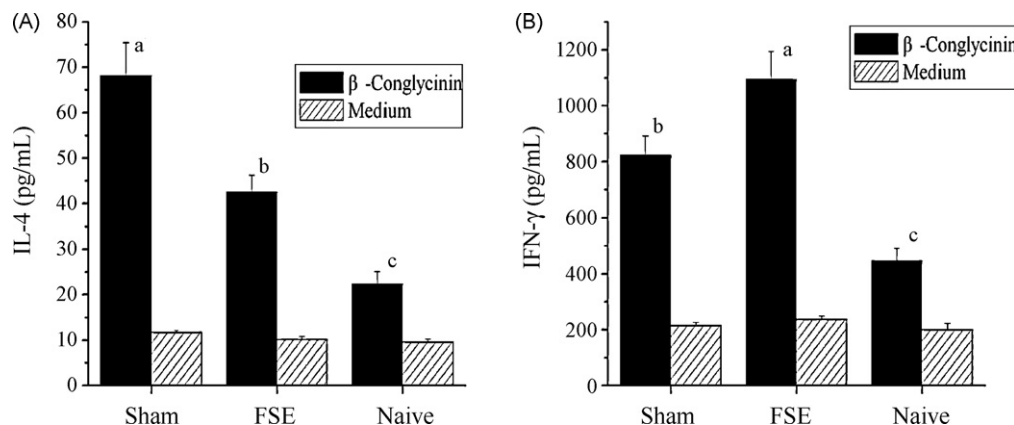


Fig. 2. Cytokine IL-4 (A) and IFN- γ (B) production in spleen cells from sham-treated, FSE-treated and naive piglets after in vitro stimulation with 50 μ g/mL β -conglycinin for 72 h. [#] $P < 0.05$ compared with naive piglets; ^{*} $P < 0.05$ compared with sham piglets; $n = 6$, mean $\pm$ S.E.M.

and proliferation of T lymphocytes (Yin et al., 2008). A recent study reported an active constituent of FSE to exert activity by suppressing cAMP phosphodiesterase activity, P38MAPK activation and inhibiting NF- κ B (Kim et al., 2006). We suggest that FSE may suppress T-cell proliferation via these reported signaling pathways. Further studies are required to confirm this hypothesis and to further explore the potential mechanisms of the therapeutic effects of FSE in anaphylaxis.

In addition, the results of this study found FSE to promote the proliferation of *Lactobacillus* and *Bifidobacteria*, and inhibit the growth of *Escherichia coli* in allergic piglets. In the gut, *Lactobacilli* and *Bifidobacteria* are common nonpathogenic microorganisms reported to promote the mucosal immune response (Erickson and Hubbard, 2000), to modulate cytokine expression and to stimulate production of cytokines Tr1 and Th3 (Smits et al., 2005; Frossard et al., 2007), and maturation surface markers (Christensen et al., 2002). *Forsythia suspensa* is rich in forsythiaside, suspensaside, lignans and glycosides, all known to promote beneficial antibacterial activity (Nishibe et al., 1982; Kang et al., 2008), advantageous to the growth of *Lactobacillus* and *Bifidobacteria*, consequently optimizing the balance between Th1- and Th2-like immunity.

In conclusion, we demonstrated that FSE, a herbal extract of *Forsythia suspensa*, inhibited purified soybean β -conglycinin-induced anaphylaxis in young pigs. This protective effect was associated with the suppression of anaphylactic antibodies and histamine release, an improved balance of intestinal microbial flora, as well as reversion of β -conglycinin-induced T cell proliferation and β -conglycinin-activated Th2 cytokine secretion. These results present FSE as an effective herbal therapy for alleviating soybean allergy.

Acknowledgments

This investigation was financially supported by the National Natural Science Foundation of China (No. 30430520, 30973960), SKLAN (2004DA125184-T-0810) and 2006 BAD12B05-10.

References

- Álvarez-Martín, P., Flórez, A.B., Mayo, B., 2007. Screening for plasmids among human bifidobacteria species: sequencing and analysis of pBC1 from *Bifidobacterium catenulatum* L48. *Plasmid* 57, 165–174.
- Ballmer-Weber, B.K., Holzhauser, T., Scibilia, J., Mittag, D., Zisa, G., Ortolani, C., Oesterballe, M., Poulsen, L.K., Vieths, S., Bindslev-Jensen, C., 2007. Clinical characteristics of soybean allergy in Europe: a double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology* 119, 1489–1496.
- Chan, B.C.L., Hon, K.L.E., Leung, P.C., Sam, S.W., Fung, K.P., Lee, M.Y.H., Lau, H.Y.A., 2008. Traditional Chinese medicine for atopic eczema: PentaHerbs formula suppresses inflammatory mediators release from mast cells. *Journal of Ethnopharmacology* 120, 85–91.
- Christensen, H.R., Frokiaer, H., Pestka, J.J., 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *Journal of Immunology* 168, 171–178.
- Christensen, H.R., Bruun, S.W., Frokiaer, H., 2003. Antigenic specificity of serum antibodies in mice fed soy protein. *International Archives of Allergy and Immunology* 132, 58–67.
- Cordle, C.T., 2004. Soy protein allergy: incidence and relative severity. *Journal of Nutrition* 134, 1213S–1219S.
- Dréau, D., Lallès, J.P., Philouze-Rome, V., Toullec, R., Salmon, H., 1994. Local and systemic immune responses to soybean protein ingestion in early-weaned pigs. *Journal of Animal Science* 72, 2090–2098.
- Erickson, K.L., Hubbard, N.E., 2000. Probiotic immunomodulation in health and disease. *Journal of Nutrition* 130, 403S–409S.
- Faquim-Mauro, E.L., Coffman, R.L., Abrahamsohn, I.A., Macedo, M.S., 1999. Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. *Journal of Immunology* 163, 3572–3576.
- Foucard, T., Malmheden, Y.I., 1999. A study on severe food reactions in Sweden—is soy protein an underestimated cause of food anaphylaxis? *Allergy* 54, 261–265.
- Frossard, C.P., Steidler, L., Eigenmann, P.A., 2007. Oral administration of an IL-10-secreting *Lactococcus lactis* strain prevents food-induced IgE sensitization. *Journal of Allergy and Clinical Immunology* 119, 952–959.
- Gizzarelli, F., Corinti, S., Barletta, P., Iacovacci, P., Brunetto, B., Butteroni, C., Afferni, C., Onori, R., Miraglia, M., Panzini, G., Di Felice, G., Tinghino, R., 2006. Evaluation of allergenicity of genetically modified soybean protein extract in a murine model of oral allergen-specific sensitization. *Clinical and Experimental Allergy* 36, 238–248.
- Guo, H., Liu, A.H., Li, L., Guo, D.A., 2007a. Simultaneous determination of 12 major constituents in *Forsythia suspensa* by high performance liquid chromatography–DAD method. *Journal of Pharmaceutical and Biomedical Analysis* 43, 1000–1006.
- Guo, P.F., Piao, X.S., Ou, D.Y., Li, D.F., Hao, Y., 2007b. Characterization of the antigenic specificity of soybean protein β -conglycinin and its effects on growth and immune function in rats. *Archives of Animal Nutrition* 61, 189–200.
- Hao, Y., Zhan, Z.F., Guo, P.F., Piao, X.S., Li, D.F., 2009. Soybean β -conglycinin-induced gut hypersensitivity reaction in a piglet model. *Archives of Animal Nutrition* 63, 1–15.
- Helm, R.M., Furuta, G.T., Stanley, J.S., Ye, J., Cockrell, G., Connaughton, C., Simpson, P., Bannon, G.A., Burks, A.W., 2002. A neonatal swine model for peanut allergy. *Journal of Allergy and Clinical Immunology* 109, 136–142.
- Herian, A.M., Taylor, S.L., Bush, R.K., 1993. Allergenic reactivity of various soybean products as determined by RAST inhibition. *Journal of Food Science* 58, 385–388.
- Holzhauser, T., Wackermann, O., Ballmer-Weber, B.K., Bindslev-Jensen, C., Scibilia, J., Perono-Garoffo, L., Utsumi, S., Poulsen, L.K., Vieths, S., 2009. Soybean (Glycine max) allergy in Europe: Gly m 5 (β -conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *Journal of Allergy and Clinical Immunology* 123, 452–458.
- Kang, H.S., Lee, J.Y., Kim, C.J., 2008. Anti-inflammatory activity of arctigenin from *Forsythia fructus*. *Journal of Ethnopharmacology* 116, 305–312.
- Kim, J.H., Kim, D.H., Baek, S.H., Lee, H.J., Kim, M.R., Kwon, H.J., Lee, C.H., 2006. Rengyolone inhibits inducible nitric oxide synthase expression and nitric oxide production by down-regulation of NF- κ B and p38 MAP kinase activity in LPS-stimulated RAW 264.7 cells. *Biochemical Pharmacology* 71, 1198–1205.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lallès, J.P., Dréau, D., Salmon, H., Toullec, R., 1996. Identification of soybean allergens and immune mechanisms of dietary sensitivities in preruminant calves. *Research in Veterinary Science* 60, 111–116.
- Leung, D.Y., Sampson, H.A., Yunginger, J.W., Burks Jr., A.W., Schneider, L.C., Wortel, C.H., Davis, F.M., Hyun, J.D., Shanahan Jr., W.R., 2003. Effect of anti-IgE therapy in patients with peanut allergy. *The New England Journal of Medicine* 348, 986–993.
- Li, D.F., Nelissen, J.L., Reddy, P.G., Blecha, F., Hancock, J.D., Allee, G.L., Goodband, R.D., Klemm, R.D., 1990. Transient hypersensitivity to soybean meal in the early-weaned pig. *Journal of Animal Science* 68, 1790–1799.
- Li, X.M., Zhang, T.F., Huang, C.K., Srivastava, K., Teper, A.A., Zhang, L., Schofield, B.H., Sampson, H.A., 2001. Food Allergy Herbal Formula-1 (FAHF-1) blocks peanut-induced anaphylaxis in a murine model. *Journal of Allergy and Clinical Immunology* 108, 639–646.
- Liu, J., 2008. Experimental study on the immunological function and anti-stress of phyllirin. *Journal of Shanxi Institute of Education* 24, 59–61.
- Liu, P., Piao, X.S., Kim, S.W., Wang, L., Shen, Y.B., Lee, H.S., Li, S.Y., 2008a. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of *Escherichia coli* and *Lactobacillus* in weaning pigs. *Journal of Animal Science* 86, 2609–2618.
- Liu, X., Feng, J., Xu, Z.R., Wang, Y.Z., Liu, J.X., 2008b. Oral allergy syndrome and anaphylactic reactions in BALB/c mice caused by soybean glycinin and β -conglycinin. *Clinical and Experimental Allergy* 38, 350–356.
- Lorentz, A., Schwengberg, S., Sellge, G., Manns, M.P., Bischoff, S.C., 2000. Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *Journal of Immunology* 164, 43–48.
- Lu, T., Piao, X.L., Zhang, Q., Wang, D., Piao, X.S., Kim, S.W., 2010. Protective effects of *Forsythia suspensa* extract against oxidative stress induced by diquat in rat. *Food and Chemical Toxicology* 48, 764–770.
- Mittag, D., Vieths, S., Vogel, L., Becker, W.M., Rihs, H.P., Helbling, A., Wuthrich, B., Ballmer-Weber, B.K., 2004. Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *Journal of Allergy and Clinical Immunology* 113, 148–154.
- Miyajima, I., Dombrowicz, D., Martin, T.R., Ravetch, J.V., Kinet, J.P., Galli, S.J., 1997. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc ϵ RIII. *The Journal of Clinical Investigation* 99, 901–914.
- Murtaugh, M.P., 1994. Porcine cytokines. *Veterinary Immunology and Immunopathology* 43, 37–44.
- National Research Council. 1998. Nutrient Requirements of Swine, 10th ed. National Academic Press, Washington, DC.
- Nelson, H.S., Lahr, J., Rule, R., Bock, A., Leung, D., 1997. Treatment of anaphylactic sensitivity to peanuts by immunotherapy with injections of aqueous peanut extract. *Journal of Allergy and Clinical Immunology* 99, 744–751.
- Nishibe, S., Okabe, K., Tsukamoto, H., Sakushima, A., Hisada, S., Baba, H., Akisada, T., 1982. Studies on the Chinese crude drug "Forsythiae Fructus." VI. The structure and antibacterial activity of suspensaside isolated from *Forsythia suspensa*. *Chemical and Pharmaceutical Bulletin* 30, 4548–4553.
- Nowak-Węgrzyn, A., 2003. Future approaches to food allergy. *Pediatrics* 111, 1672–1680.
- Ozaki, Y., Rui, J., Tang, Y.T., 2000. Antiinflammatory effect of *Forsythia suspensa* Vahl and its active principle. *Biological and Pharmaceutical Bulletin* 23, 365–367.
- Piao, X.L., Jiang, M.H., Cui, J., Piao, X.S., 2008. Lignans from the fruits of *Forsythia suspensa*. *Bioorganic and Medicinal Chemistry Letters* 18, 1980–1984.

- Rozenfeld, P., Docena, G.H., Anon, M.C., Fossati, C.A., 2002. Detection and identification of a soy protein component that cross-reacts with caseins from cow's milk. *Clinical and Experimental Immunology* 130, 49–58.
- Shida, K., Hachimura, S., Ametani, A., Ishimori, M., Ling, M., Hashiguchi, M., Ueda, Y., Sato, T., Kumagai, Y., Takamizawa, K., Habu, S., Kaminogawa, S., 2000. Serum IgE response to orally ingested antigen: a novel IgE response model with allergen-specific T-cell receptor transgenic mice. *Journal of Allergy and Clinical Immunology* 105, 788–795.
- Smits, H.H., Engering, A., van der Kleij, D., de Jong, E.C., Schipper, K., van Capel, T.M., Zaat, B.A., Yazdanbakhsh, M., Wierenga, E.A., van Kooyk, Y., Kapsenberg, M.L., 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *Journal of Allergy and Clinical Immunology* 115, 1260–1267.
- Srivastava, K.D., Kattan, J.D., Zou, Z.M., Li, J.H., Zhang, L., Wallenstein, S., Goldfarb, J., Sampson, H.A., Li, X.M., 2005. The Chinese herbal medicine formula FAHF-2 completely blocks anaphylactic reactions in a murine model of peanut allergy. *Journal of Allergy and Clinical Immunology* 115, 171–178.
- Thornhill, S.M., Kelly, A.M., 2000. Natural treatment of perennial allergic rhinitis. *Alternative Medicine Review* 5, 448–454.
- Turcanu, V., Maleki, S.J., Lack, G., 2003. Characterization of lymphocyte responses to peanuts in normal children, peanut-allergic children, and allergic children who acquired tolerance to peanuts. *Journal of Clinical Investigation* 111, 1065–1072.
- Utsumi, S., 1992. Plant food protein engineering. *Advances in Food and Nutrition Research* 36, 89–208.
- Wang, L., Piao, X.L., Kim, S.W., Piao, X.S., Shen, Y.B., Lee, H.S., 2008. Effects of *Forsythia suspensa* extract on growth performance, nutrient digestibility, and antioxidant activities in broiler chickens under high ambient temperature. *Poultry Science* 87, 1287–1294.
- Yin, L.L., Zeng, Y.Y., Huang, X.Y., Hou, H.N., 2008. Effects of *Forsythia suspensa* extract on cell proliferation and activation of the mouse T lymphocytes in vitro. *Chinese Journal of Cell and Molecular Immunology* 24, 10–12.