

Enhancement of Anti-Allergic Effects Mediated by the Kampo Medicine Shoseiryuto (Xiao-Qing-Long-Tang in Chinese) with Lysed *Enterococcus faecalis* FK-23 in Mice

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SUMMARY Kampo is a traditional Japanese medicine originating from ancient Chinese medicine which included the administration of herbal prescription, lifestyle advice and acupuncture. Orally administered Kampo prescriptions are believed to be influenced by diet and intestinal microbiota. However, reports on the Kampo administration effects are still limited. Shoseiryuto (TJ-19), which has anti-allergic and anti-inflammatory properties, is a Kampo prescription used clinically for the treatment of allergic bronchial asthma. We examined whether Shoseiryuto administration is affected by a probiotic product, lysed *Enterococcus faecalis* FK-23 (LFK). BALB/c mice were sensitized with cedar pollen allergen, and the peritoneal accumulation of eosinophils was induced. During a sensitization period of 21 days, varying amounts of Shoseiryuto (and saline as a control) were administered to the mice. The accumulation of eosinophils was significantly reduced by 30 mg/day doses of Shoseiryuto but not by 3 or 9 mg/day doses. Similarly, 3 mg/day Shoseiryuto, 30 mg/day LFK, 3 mg/day of Shoseiryuto co-administered with 30 mg/day of LFK, and saline control were compared. A significant reduction in the accumulation of eosinophils was observed at 3 mg/day Shoseiryuto co-administered with 30 mg/day of LFK. These results suggest that Shoseiryuto-mediated anti-allergic effects are enhanced by the probiotic (LFK). Although not significant statistically, serum allergen-specific and total IgE levels in the treatment group exposed to the mixed agent (*i.e.* Shoseiryuto and LFK) were generally lower than those receiving either one alone. The results indicate a synergistic effect of a Kampo medicine (Shoseiryuto, Xiao-Qing-Long-Tang in Chinese) and lysed *Enterococcus faecalis* FK-23 on allergic responses in mice.

The term 'Kampo' is a traditional Japanese medicine derived from an ancient Chinese herbal medicine.¹ It has been developed in Japan over the past 1,500 years, and constitutes multiple approaches that affect lifestyle such as diet,²⁻⁵ administration of herbal prescriptions and acupuncture. Many traditional Japanese herbal prescriptions have been used in combination with modern Western medicines. The effect of orally administered Kampo differs between patients if dietary advice is lacking. This may be caused by individual differences in intestinal conditions.⁶

Shoseiryuto (Xiao-Qing-Long-Tang in

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Chinese) is a Kampo preparation used for the treatment of allergic rhinitis, bronchitis and asthma.⁷ Several *in vitro* experiments have shown that Shoseiryuto inhibited histamine release and degranulation of mast cells,⁸ the proliferation of eosinophils,⁹ the growth and differentiation of basophils¹⁰ and the synthesis of tumor necrosis factor- α (TNF- α) by peripheral blood mononuclear cells.¹¹ *In vivo* experiments revealed that Shoseiryuto inhibited passive cutaneous anaphylaxis (PCA) in rats¹² and was efficacious towards allergic rhinitis¹³ and asthma¹⁴ in animal models.

Clinical trials have demonstrated the benefits of probiotic supplementation, made from lactic acid bacteria, in allergy prevention and therapy.¹⁵⁻²⁰ We previously demonstrated that sufficient amounts of a probiotic product of lysed *Enterococcus faecalis* FK-23 (LFK) has inhibitory effects on allergen-induced local accumulation of eosinophils¹⁸ and active cutaneous anaphylaxis¹⁹ in mouse models. In a clinical pilot study, we observed that the numbers of peripheral blood eosinophils were significantly reduced after oral LFK in patients with perennial allergic rhinitis.²⁰ However, no reports are available on the synergistic effects of Kampo and lactic acid bacteria in suppressing allergic response.

In the present study, we examine the combined effect of Shoseiryuto and LFK on allergen-induced peritoneal accumulation of eosinophils in

mice, and also determine the total and allergen-specific IgE profiles in this animal model.

MATERIALS AND METHODS

Shoseiryuto

Dried extract preparations of Shoseiryuto were obtained from Tsumura Co (Tokyo, Japan). There were 8 herb components in the medicine. These constituents and their actions are described in Table 1. Shoseiryuto (1.0 g) was extracted with methanol (20 ml) under ultrasonication for 30 minutes, and centrifuged at 1,620 x g for 5 minutes. The supernatant was filtered through a sterile 0.45 μ m membrane and subjected to HPLC analysis. The three-dimensional high-performance liquid chromatography (HPLC) profile of the methanolic Shoseiryuto solution is shown in Fig. 1.

Preparation of LFK

LFK was prepared as described previously.¹⁸ Briefly, *E. faecalis* strain FK-23 was cultured for 24 hours at 37°C in a broth medium consisting of 2% (w/v) glucose, 2% (w/v) yeast extract, 2% (w/v) meat extract and 4% (w/v) K₂HPO₄. Cells were harvested by centrifugation, washed three times with distilled water and treated with lysozyme (1 mg/ml) at 37°C for 2 hours. Following incubation at 105°C for 10 minutes, cells were lyophilized and used as the LFK preparation.

Table 1 Components of Shoseiryuto (Japanese; Xiao-Qing-Long-Tang in Chinese) extract granules

Japanese	Amount (g)	English	Actions
Hange	6.0	JP Pinellia tuber	Anti-emetic
Kankyo	3.0	JP Processed ginger	Warm, relieves a cough, anti-emetic
Kanzo	3.0	JP Glycyrrhiza	Antitoxic, sedative, protects digestive system
Keihi	3.0	JP Cinnamon bark	Promotes blood circulation
Gomishi	3.0	JP Schisandra fruit	Warm, relieves a cough
Saishin	3.0	JP Asiasarum root	Warm, relieves a cough, relieves pain
Shakuyaku	3.0	JP Peony root	Relieves spasm, relieves pain
Mao	3.0	JP Ephedra herb	Anti-inflammatory, sweats

JP, the Japanese Pharmacopoeia.

Preparation of cedar pollen allergen

Pollen allergen was purified from Japanese cedar (*Cryptomeria japonica*) as previously described.²¹

Experimental animals

Female BALB/c mice were obtained from Charles River Japan Inc. (Yokohama, Japan). Three-week-old mice used for experiments were fed on a pellet diet (CE-2, Clea Japan Inc., Tokyo, Japan) and had free access to tap water which had been passed through a PF filter (Organo Co., Tokyo, Japan). Animals were housed in cages with a 12-hour light/dark cycle. Temperature was maintained at $25.0 \pm 1.0^\circ\text{C}$ and humidity levels at $55.0 \pm 5.0\%$. Experiments were performed in accordance with the Japanese Association for Laboratory Animals Science in 1987 and the National Research Council Guide for the Care and Use of Laboratory Animals.

Allergen-induced cell accumulation

Cells were prepared according to the procedure described by Kaneko *et al.*²² BALB/c mice were sensitized with the cedar pollen allergen *via* subcutaneous injection of 100 μl of the allergen dilution (Cry j 1; 327 $\mu\text{g}/\text{ml}$) on days 0 and 1, followed by 200 μl subcutaneous injections on days 6, 8 and 14. Mice were challenged on day 20 by intraperitoneal injection of 200 μl of the allergen dilution. Peritoneal cells were harvested 24 hours later using 4 ml of 10 mM phosphate buffered saline pH 7.35 (PBS) supplemented with 1.0% fetal calf serum (FCS) and 5 U/ml heparin (Mochida Pharmaceutical Co., Tokyo, Japan). An appropriate PBS dilution of the infusion was added to Turk's solution and the total number of peritoneal cells counted using a hemocytometer under a microscope. For this purpose, 50 μl of peritoneal cell suspension (5×10^5 cells/ml) was smeared on a microscope slide after centrifugation. A differential cell count was carried out following cell fixation and staining with May-Grunwald Giemsa dye.

Administration of Shoseiryuto and LFK

1) Comparison of Shoseiryuto doses

Shoseiryuto at 3, 9 or 30 mg/animal/day (each 0.5 ml) were orally administered ($n = 7$ for all

groups) daily for 21 days of the sensitization period. Saline (0.5 ml) was administered to another group of mice for the same duration as the control ($n = 7$).

2) Comparison between low doses of Shoseiryuto, LFK and their combination

Shoseiryuto (3 mg/mouse/day), LFK (30 mg/mouse/day), or Shoseiryuto (3 mg/mouse) co-administered with LFK (30 mg/mouse/day) were given orally ($n = 7$ for all groups) daily during the 21-day sensitization period. Saline (0.5 ml) was administered to another group of mice for the same duration as the control ($n = 7$).

Measurement of total and allergen-specific IgE in sera

Serum levels of total and allergen-specific IgE were determined *via* a sandwich ELISA.²³ The serum samples were separated by centrifugation ($3,000 \times g$ for 5 minutes) from blood taken from the retro-orbital venous plexus at day 21. Allergen-specific IgE was measured in 1:2 and 1:16 dilutions of samples placed in wells of ELISA plates previously coated with cedar pollen allergen.²³ Antibody levels were expressed as absorbance at 450 nm after 30 minutes development.

Statistical analysis

Data was expressed as mean \pm standard deviation (SD). SPSS 10.0J was used for analysis. An initial one-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn method comparison test was used to determine differences between groups for all data obtained in this study. The p -value < 0.05 was considered statistically significant.

RESULTS

Dose-dependent effects of Shoseiryuto on peritoneal accumulation of eosinophils in cedar pollen allergen-sensitized mice

The total number of leukocytes in the control and 3 mg (low-dose), 9 mg (mid-dose), and 30 mg (high-dose) Shoseiryuto were 1.34×10^6 , 1.17×10^6 , 1.71×10^6 and 1.12×10^6 cells/ml, respectively. There were no significant differences among groups. The ratio of eosinophils in the control and low-dose,

mid-dose and high-dose Shoseiryuto-treated mice was 15.7%, 12.8%, 14.0% and 7.5%, respectively. There was a significant difference between the control and high-dose groups ($p = 0.003$). The total number of eosinophils in the control and low-dose, mid-dose and high-dose groups was 2.14×10^5 , 1.50×10^5 , 2.59×10^5 and 0.84×10^5 cells/ml, respectively (Fig. 2). There was a significant difference between the control and high-dose Shoseiryuto-treated mice ($p = 0.007$). There were no significant differences in the number of neutrophils, monocytes and lymphocytes between any of the mouse groups (Table 2).

Effect of Shoseiryuto and LFK on peritoneal accumulation of eosinophils in cedar pollen allergen-sensitized mice

The total numbers of leukocytes in the control, 3 mg Shoseiryuto (low-dose), 30 mg LFK (low-dose LFK), and 3 mg Shoseiryuto + 30 mg LFK (*i.e.* the mixed-agent) groups were 1.33×10^6 , 1.17×10^6 , 1.11×10^6 , and 1.06×10^6 cells/ml, respectively. There were no significant differences among all mouse groups. The percentages of eosinophils in the control and low-dose Shoseiryuto, low-dose LFK, and the mixed-agent groups was 15.7%, 12.8%, 16.0%, and 8.1%, respectively. There was a significant difference between the control and the mixed-agents group ($p < 0.0001$). As shown in Fig. 3, the total numbers of eosinophils in the control and low-dose Shoseiryuto, low-dose LFK, and the mixed-agents group were 2.60×10^5 , 1.50×10^5 , 2.00×10^5 , and 0.85×10^5 cells/ml, respectively. There was a significant difference between the control and the mixed-agents group ($p = 0.005$). No significant dif-

ference in the numbers of neutrophils, monocytes and lymphocytes was detected between any of the mouse groups.

Effect of Shoseiryuto and LFK on total and allergen-specific IgE in sera

Serum total IgE levels in the control and low-dose Shoseiryuto, low-dose LFK, and mixed-agents group were 6,926, 8,236, 7,207, and 4,179 ng/ml, respectively. There were no significant differences among the groups. The level in the mixed-

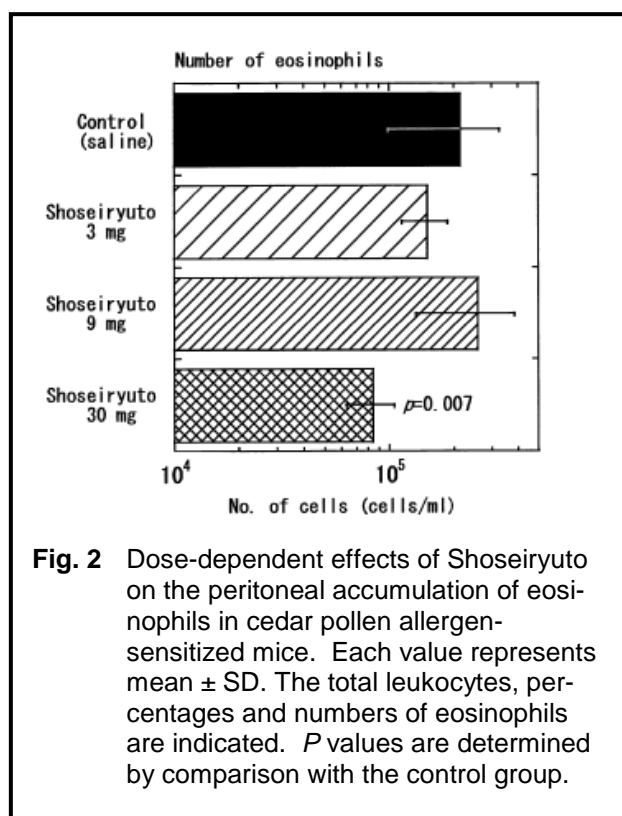


Fig. 2 Dose-dependent effects of Shoseiryuto on the peritoneal accumulation of eosinophils in cedar pollen allergen-sensitized mice. Each value represents mean \pm SD. The total leukocytes, percentages and numbers of eosinophils are indicated. P values are determined by comparison with the control group.

Table 2 Synergistic effects of Shoseiryuto and LFK on number of leukocytes, neutrophils, lymphocytes and monocytes

	Control	low-dose	mid-dose	high-dose
WBC	$1.34 \pm 0.48 \times 10^6$	$1.23 \pm 0.23 \times 10^6$	$1.71 \pm 0.49 \times 10^6$	$1.12 \pm 0.23 \times 10^6$
Neutrophils	$5.14 \pm 2.97 \times 10^4$	$3.83 \pm 3.52 \times 10^4$	$8.86 \pm 5.10 \times 10^4$	$4.44 \pm 2.06 \times 10^4$
Lymphocytes	$1.81 \pm 0.70 \times 10^5$	$2.13 \pm 0.38 \times 10^5$	$1.44 \pm 0.43 \times 10^5$	$1.95 \pm 0.59 \times 10^5$
Monocytes	$8.98 \pm 3.30 \times 10^5$	$8.01 \pm 1.30 \times 10^5$	$10.6 \pm 3.18 \times 10^5$	$7.99 \pm 1.73 \times 10^5$

Each value represents mean \pm SD.

agents group was generally lower than the control, although this was not statistically significant ($p = 0.071$). Serum allergen-specific IgE levels in the control and the low-dose Shoseiryuto, low-dose of LFK, and the mixed-agents group were 0.159, 0.192, 0.166 and 0.166 ng/ml, respectively. There were no significant differences among the groups (Table 3).

DISCUSSION

Shoseiryuto has long been prescribed for the treatment of allergic diseases^{24,25} and its clinical effectiveness was recently assessed in a double-blind randomized study for patients with allergic asthma and rhinitis.²⁶ Furthermore, the suppressive activity of Shoseiryuto on chemical mediators released from peritoneal mast cells has also been demonstrated *in vitro*.²⁷ Thus, Shoseiryuto has been suggested to have inhibitory effects on chemical mediators comparable to many anti-allergic drugs currently used in Western medicine. Nagai *et al.*²⁸ reported that the oral administration of Shoseiryuto reduced the production of IL-4 and IL-5 in airway inflammatory-model mice. Therefore, Shoseiryuto may inhibit the excess accumulation of eosinophils.

Shoseiryuto has been found to cause side effects in rare instances, such as interstitial pneumonia, myopathy or impaired liver function.²⁹ However, these effects were not apparent in our present study, as no differences between groups in body weight, and phenotypic abnormalities or other health problems were observed during the trials.

Our previous observations showed that orally effective doses of LFK can inhibit allergen-induced local eosinophilia. However, the lower do-

sage of LFK did not cause an inhibitory effect on eosinophilia.¹⁸ In the present study, we confirmed that 30 mg/animal of LFK (lower dosage of LFK) does not inhibit the accumulation of eosinophils. Nevertheless, significant inhibitory effects were observed when combining Shoseiryuto and LFK, which contrasts with the single administration of each of these agents. The mixed formula caused further reductions in the allergen-induced peritoneal accumulation of eosinophils.

Glycyrrhizae Radix and *Paeoniae Radix* are active components of Shoseiryuto. They are more effective in their immuno-modulatory activities *via* their metabolism by intestinal microbiota.^{30,31} Our previous work showed that LFK improved the dis-

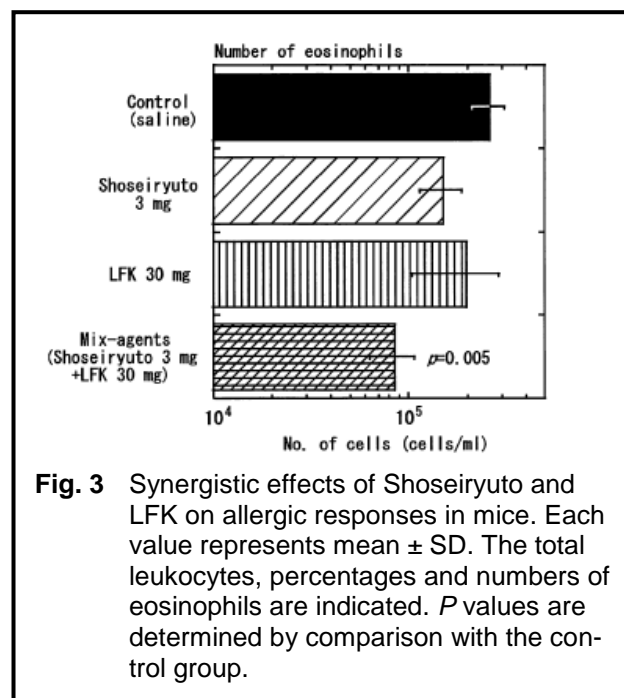


Fig. 3 Synergistic effects of Shoseiryuto and LFK on allergic responses in mice. Each value represents mean \pm SD. The total leukocytes, percentages and numbers of eosinophils are indicated. P values are determined by comparison with the control group.

Table 3 Synergistic effect of Shoseiryuto and LFK on serum levels of total and allergen-specific IgE in cedar pollen allergen-sensitized mice

	Control	Shoseiryuto-treated	LFK	Mixed-agents
Total IgE (ng/ml)	6,926 \pm 1,588	8,236 \pm 3,460	7,207 \pm 3,264	4,179 \pm 3,160
Specific IgE (absorbance at 450 nm)	0.159 \pm 0.023	0.192 \pm 0.059	0.166 \pm 0.050	0.166 \pm 0.052

Each value represents mean \pm SD.

turbance of intestinal microbiota induced by erythromycin in mice.³² This indicated the possibility that LFK enhances the effect of Shoseiryuto to inhibit allergic responses, by acting upon intestinal microbiota. This suggestion leads to another possibility in that probiotics may reduce the dose of Kampo which is necessary to attain the desired effects. The inter-individual variations in the effect of Kampo medicine may, in part, be explained by their metabolic relationships with intestinal microbiota in individual subjects. Kobashi *et al.*^{31,33,34} reported that some constituents of Kampo medicine are hydrolyzed to glycosides by intestinal microbiota, and that these plant glycosides act as prodrugs which are metabolized to their active form *via* deglycosylation carried out by the intestinal microbiota. Indeed, Wakabayashi *et al.*^{35,36} also showed that the effect of one particular kind of Kampo crude drug requires mediation by intestinal microbiota.

In conclusion, the combined administration of low dose of Shoseiryuto (a Kampo prescription) and a low dose of LFK (a probiotics) inhibits the allergic responses in BALB/c model mice. These results suggest that LFK is integral in enhancing the effect of Kampo medicine, reducing the inter-individual variation in their effects, and reducing their effective doses. Furthermore, it shows that the efficacy of orally taken Kampo prescriptions is influenced by diet and other supplements. Further clinical studies are warranted to examine this phenomenon while attention should be given to the precise involvement of the intestinal environment. These efforts would assist in a more rigorous evaluation of the optimal use of Kampo medicine.

CONFLICT OF INTEREST

T. Shimada, the primary author of this article, is employed by Nichinichi Pharmaceutical Co. Ltd. This research, however, was not conducted as part of Nichinichi Pharmaceutical's research initiative. All other authors declare no conflict of interest.

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