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Note

Anti-allergic Properties of a Fruit Extract of Prune (*Prunus domestica* L.) in Mite-sensitized BALB/c Mice

Koji Karasawa[1, 2], Ryoji Miyashita[3] and Hajime Otani[1, 3*]

[1] *Interdisciplinary Graduate School of Science and Technology, Shinshu University, Minamiminowa-mura 8304, Kamiina-gun, Nagano 399-4598, Japan*

[2] *Ina Food Industry Co., Ltd., Nishiharuchika 5074, Ina-shi, Nagano 399-4498, Japan*

[3] *Graduate School of Agriculture, Shinshu University, Minamiminowa-mura 8304, Kamiina-gun, Nagano 399-4598, Japan*

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The effects of oral ingestion of a hot water extract of prune (*Prunus domestica* L.) fruit on allergic responses were investigated in mite-sensitized BALB/c mice. The number of sneezing events in mice given the extract-added diet was significantly lower than in mice given the extract-free diet. Serum mite allergen-specific immunoglobulin E levels were significantly lower in mice given the extract-added diet than in mice given the extract-free diet. In contrast, the ratio of splenic IFN-γ^+CD4$^+$ cells/IL-4$^+$CD4$^+$ cells was higher in mice given the extract-added diet. Expression levels of Stat6, Btk and Stim1 mRNAs in spleen cells of mice given the extract-added diet were significantly lower than in mice given the extract-free diet. These results suggest that the prune extract may reduce type I allergic symptoms in mice via the adjustment of type 1 helper T cell/type 2 helper T cell balance and the suppression of mast cell degranulation.

Keywords: anti-allergic property, prune extract, immunoglobulin E, helper T cell subsets, degranulation

Introduction

Prune (*Prunus domestica* L.) was initially harvested in the Caucasus region in Western Asia, and its cultivation has now spread to South and East Asia and East Europe (Kayano *et al.*, 2003). The fruit has been used medicinally in South Asia for the treatment of leucorrhea, irregular menstruation and debility following miscarriage (Kayano *et al.*, 2003), and has also been reported to possess potential functionalities for improving lifestyle-related diseases (Tinker *et al.*, 1991). However, little is known regarding how prune fruit influences immune function.

Recently, the authors observed that the number of type 1 helper T (Th1) cells and natural killer cells in Peyer's patches was higher in C3H/HeN mice given a diet containing a hot water extract of prune fruit than in mice given an extract-free diet. In addition, the ratio of Th1 cells/type 2 helper T (Th2) cells in the spleens and Peyer's patches was higher in mice given the extract-added diet than in mice given the extract-free diet (Karasawa *et al.*, 2011).

Type I allergic diseases are generally characterized by an elevation in the serum immunoglobulin (Ig) E level. It is established that interleukin (IL)-4 produced from Th2 cells stimulates IgE production. In contrast, Th1 cells mainly secrete interferon (IFN)-γ, which inhibits IL-4 production by Th2 cells. It is, therefore, considered that type I allergic responses could be suppressed by a regulation of the balance between Th1 and Th2 activities (Platts-Mills, 2001).

These facts suggested that the prune extract might have a suppressive effect on type I allergic responses via the adjustment of Th1 cell/Th2 cell balance. Thus, this study evaluated the effect of a prune extract on the allergic responses induced by a mite (*Dermatophagoides farinae*) allergen in BALB/c mice.

Materials and Methods

Materials Phycoerythrin (PE)-labeled anti-mouse IL-4 monoclonal antibody (mAb, clone 11B11), PE-labeled anti-mouse IFN-γ mAb (clone XMG1.2), biotin-labeled anti-mouse CD4 mAb (clone RM4-5) and phycoerythrin/cyanine

*To whom correspondence should be addressed.
E-mail: otani84@shinshu-u.ac.jp

5 (PE/Cy5)-labeled streptavidin were obtained from BioLegend (San Diego, CA, USA). Brefeldin A (BFA), ionomycin, streptomycin and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was obtained from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT, USA). Penicillin was obtained from MP Biomedicals (Costa Mesa, CA, USA). A medium for cell culture, Roswell Park Memorial Institute (RPMI)-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan). TRIzol Reagent, dNTP mixture and M-MLV reverse transcriptase were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Two aliquots of SYBR premix Ex Taq mixture were purchased from Takara Bio Inc. (Shiga, Japan). Mite (*D. farinae*) allergen was obtained from LSL Co., Ltd. (Tokyo, Japan). ELISA kit for quantitation of total IgE was purchased from Bethyl Laboratories (Montgomery, TX, USA). 3,3′,5,5′-Tetramethyl benzidine (TMB) was obtained from KPL (Gaithersburg, MD, USA). Bovine serum albumin (BSA) and aluminum hydroxide gel were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers for Stat6, FcεR1γ, Btk and Stim1 were obtained from Hokkaido System Science Co., Ltd. (Sapporo, Japan). All chemicals used in this study were of the highest analytical grade commercially available.

Preparation of prune extract Dried prune was obtained from Shoei Foods Corporation (Tokyo, Japan). The fruit (200 g) was cut into pieces of approximately 5 × 5 mm and boiled in 500 mL of hot distilled water for 1 h under reflux. The supernatant was collected by centrifugation (5,000 × *g*, 30 min) and ultrafiltrated using a Stirred Ultrafiltration Unit (Model UHP-150K; Advantec Toyo Kaisha, Ltd., Tokyo, Japan) with a Molecular/Por Ultrafiltration Membrane MWCO 500 (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) to eliminate low molecular substances such as fructose, sucrose, minerals, etc. in the extract. The residue on the membrane was then dissolved in 100 mL of distilled water, freeze-dried and used as the prune extract (6 g dry weight).

Feeding procedure Four-week-old male BALB/c mice were obtained from Japan SLC (Shizuoka, Japan). The mice were given a commercial mouse powder feed (Oriental Yeast Co., Tokyo, Japan) for 1 week, and subsequently given the defined protein-free purified diet P5765 (Purina Mills, St. Louis, MO, USA) supplemented with 25% ovalbumin (extract-free diet) or a mixture of 24% ovalbumin and 1% prune extract (extract-added diet) between 5 and 11 weeks of age (n = 5). The detailed composition of each diet is shown in Table 1. The mice were intraperitoneally injected with 200 μL of saline solution containing 100 μg mite (*D. farinae*) allergen and 2 mg aluminum hydroxide gel at 5, 6 and 7 weeks of age. The mice were intranasally challenged by instillation with 20 μL of distilled water containing 20 μg of the mite allergen per week between 8 and 11 weeks of age. The number of sneezing events was then counted for 30 min after instillation. Diet and water were supplied *ad libitum* throughout the course of the experiment. The mice were housed at a temperature of 23 ± 2°C under a standard 12-h light-dark cycle. Blood and spleen samples were collected immediately following a lethal dose of ether at 11 weeks of age. Serum was obtained by centrifugation at 450 × *g* for 60 min at 4°C, and was stored at −30°C until use. All animal experimentations undertaken during this study were conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University, and according to Law no. 105 and Notification no. 6 of the Japanese government.

Spleen cell suspensions Spleen cell suspensions were prepared as described previously (Karasawa *et al.*, 2011). The spleen cell suspensions were used for cell function analysis.

Cell function analysis Cell surface markers and intracellular cytokines were labeled according to a previously described procedure (Karasawa *et al.*, 2011). The cell number was determined by means of a Guava personal cell function analyzer (Guava PCA, Guava Technologies, Hayward, CA, USA).

Antibody analysis Serum total and mite allergen-specif-

Table 1. Composition of diets.

	Extract-free diet	Prune extract-added diet
	%	
Prune extract	0.00	1.00
Ovalbumin	25.00	24.00
Protein-free diet		
Dextrin	32.74	32.74
Sucrose	27.11	27.11
RP Mineral Mix #10[a]	3.75	3.75
Corn oil	3.75	3.75
Lard	3.75	3.75
Powdered Cellulose	2.25	2.25
RP Vitamin Mix[b]	1.50	1.50
Choline chloride	0.15	0.15
Total	100.00	100.00

[a] RP Mineral Mix #10: calcium, 0.60%; phosphorus, 0.40%; potassium, 0.40%; magnesium, 0.07%; sodium, 0.21%; chlorine, 0.24%; fluorine, 5.0 ppm; iron, 63 ppm; zinc, 21 ppm; manganese, 65 ppm; copper, 15.0 ppm; cobalt, 3.2 ppm; iodine, 0.57ppm; chromium, 3.0 ppm; molybdenum, 0.82 ppm; selenium, 0.23 ppm.
[b] RP Vitamin Mix: vitamin A, 22.1 IU/g; vitamin D3, 2.2 IU/g; vitamin E, 50.1 IU/kg; vitamin K, 10.41 ppm; thiamine hydrochloride, 20.6 ppm; riboflavin, 20.0 ppm; niacin, 90 ppm; pantothenic acid, 55 ppm; folic acid, 4.0 ppm; pyridoxine, 16.5 ppm; biotin, 0.4 ppm; vitamin B12, 20 mg/kg; choline chloride, 1400 ppm.

ic IgE levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to a previously described procedure (Tobita *et al.*, 2009).

Preparation of total RNA and real-time reverse transcription (RT)-polymerase chain reaction (PCR) Total RNA from spleen cells was extracted and real-time RT-PCR was performed according to a previously described procedure (Karasawa *et al.*, 2011). The primer sequences for Stat6, FcεR1γ and Stim1 amplifications were according to the descriptions of Zaheer *et al.* (2007), Maier (2007) and Wang *et al.* (2006), respectively. The primer sequence for Btk amplification was reported by Lucas *et al.* (2007), and that for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was reported by Tobita *et al.* (2006). The relative amount of each mRNA was normalized using GAPDH expression as an internal control. An expression index was calculated from the normalized relative amount in the absence of the prune extract to the normalized relative amount in the presence of the prune extract.

Statistical analysis Data are expressed as the mean ± standard deviation (SD). Differences were assessed using a one-way analysis of variance (ANOVA). All analyses were performed using the two-sided tests. Differences were considered significant when *P*-values were less than 0.05.

Results

Allergic symptoms of mice Five-week-old BALB/c mice were given the prune extract-added diet or the extract-free diet for 6 weeks. No significant differences in body weight were observed between mice given these diets (data not shown). Figure 1 presents the number of sneezing events of the mice between 8 and 11 weeks of age. The number was significantly lower in mice at 10 and 11 weeks of age that were given the extract-added diet than in mice given the extract-free diet.

Total and mite allergen-specific IgE levels in serum Figure 2 shows the serum levels of total (A) and mite allergen-specific (B) IgE in 11-week-old mice given the prune extract-added diet or the extract-free diet. Both the total and mite allergen-specific IgE levels were significantly lower in mice given the extract-added diet than in mice given the extract-free diet.

Numbers of splenic immunocompetent cells Figure 3 presents the numbers of splenic IFN-γ^+CD4^+ (A) and IL-4^+CD4^+ (B) cells in 11-week-old mice given the prune extract-added diet or the extract-free diet. The number of IFN-γ^+CD4^+ cells was significantly higher in mice given the extract-added diet than in mice given the extract-free diet, although the number of IL-4^+CD4^+ cells did not significantly differ between the diets. Hence, as shown in Figure 3C, the ratio of IFN-γ^+CD4^+ cells/IL-4^+CD4^+ cells was significantly higher in the mice given the extract-added diet.

mRNA expression levels of proteins associated with degranulation in spleen cells Figure 4 shows the expression levels of Stat6 (A), FcεR1γ (B), Btk (C) and Stim1 (D) mRNAs in spleen cells of 11-week-old mice given the prune extract-added diet or the extract-free diet. The expression levels of Stat6, Btk and Stim1 were significantly lower in mice given the extract-added diet, while that for FcεR1γ showed a tendency to be reduced.

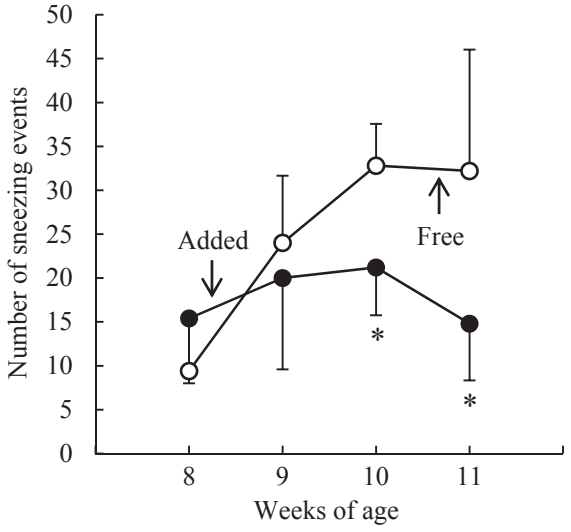

Fig. 1. Numbers of sneezing events in mice given the prune extract-added (closed circles) and extract-free (open circles) diets. Sneezing events were counted for 30 min after intranasal instillation with mite allergen. Data are presented as the mean ± SD ($n = 5$). *$P < 0.05$.

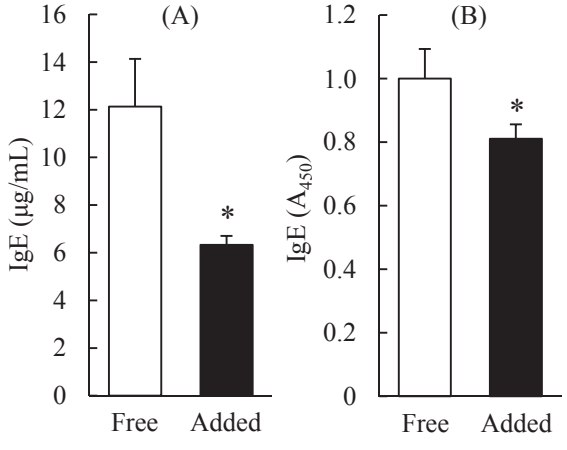

Fig. 2. Serum levels of total (A) and mite allergen-specific (B) IgE in mice given the prune extract-added (solid column) and extract-free (open column) diets. Data are presented as the mean ± SD ($n = 5$). *$P < 0.05$.

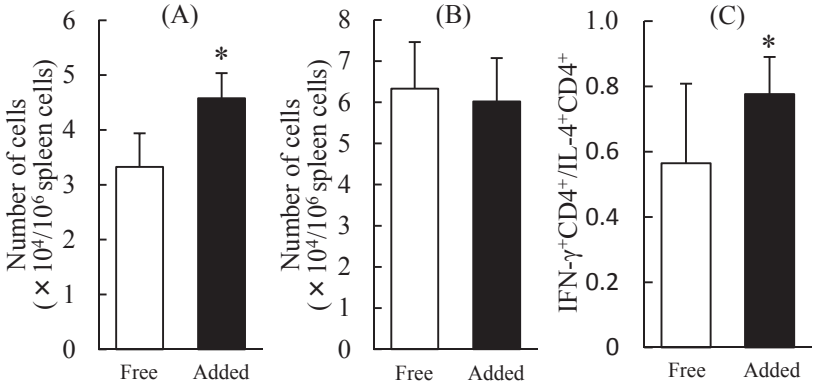

Fig. 3. Numbers of splenic IFN-γ^+CD4$^+$ (A) and IL-4$^+$CD4$^+$ (B) cells, and the ratio of IFN-γ^+CD4$^+$ cells/IL-4$^+$CD4$^+$ cells (C) in mice given the prune extract-added (solid column) and the extract-free (open column) diets. Data are presented as the mean ± SD (n = 5). *P < 0.05.

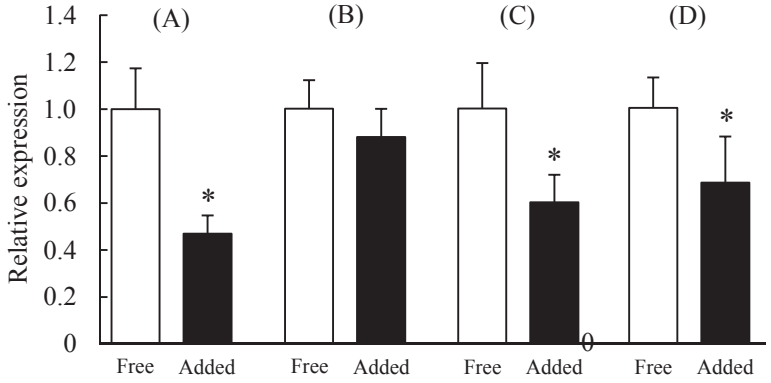

Fig. 4. Expression levels of Stat6 (A), FcεR1γ (B), Btk (C) and Stim1 (D) mRNA in spleen cells of mice given the prune extract-added (solid column) and extract-free (open column) diets. Data are presented as the mean ± SD (n = 5). *P < 0.05.

Discussion

As mentioned in the Introduction section, we recently observed that the ratio of Th1 cells/Th2 cells in the spleen and Peyer's patches was higher in C3H/HeN mice given the prune extract-added diet than in mice given the extract-free diet (Karasawa *et al.*, 2011). This result suggested that the prune extract might have a suppressive effect on type I allergic responses via the adjustment of Th1 cell/Th2 cell balance. Thus, this study evaluated the effect of the prune extract on the allergic responses in mice. Zhou *et al.* (2008) reported that type I allergic reactions were induced by intranasal administration of a mite allergen after intraperitoneal injection of the mite allergen in BALB/c mice. They also observed that the amount of total serum IgE in the mice without intraperitoneal immunization was approximately 0.2 μg/mL, while that in mice with the immunization was approximately 4 μg/mL or more. Hence, in this study, we treated BALB/c mice with the mite allergen according to the procedure described by Zhou *et al.* The total serum IgE amount in mice given the prune extract-free diet was 12.13 ± 2.00 μg/mL (Figure 2A). It was confirmed that type I allergic reactions could be appropriately induced in mice treated with the mite allergen in this study.

The number of sneezing events was significantly lower in mice given the prune extract-added diet at 10 and 11 weeks of age than in mice given the extract-free diet (Figure 1). In addition, the serum level of mite allergen-specific IgE in mice given the prune extract-added diet was significantly lower than that in mice given the extract-free diet (Figure 2B). These results indicate that the feeding of mice with the prune extract-added diet suppresses the development of type I allergy via the reduction of mite allergen-specific IgE production.

IgE is produced under a Th2 cell-dominant condition (Platts-Mills, 2001). In this study, the ratio of splenic Th1/Th2 cells was significantly higher in mice given the prune extract-added diet than in mice given the extract-free diet (Figure 3C). Stat6 is known to promote Th2 effector immune responses and IgE production in plasma cells (Forbes *et al.*, 2010). In this study, the expression level of Stat6 mRNA

was significantly lower in the spleen cells of mice given the prune extract-added diet than in those of mice given the extract-free diet (Figure 4A). These facts suggest that the lower production of mite allergen-specific IgE in the serum of mice given the prune extract-added diet might be due to an adjustment of splenic Th1 cell/Th2 cell balance and a reduction in the expression of Stat6 in plasma cells.

IgE potentially connects with high-affinity surface receptors (FcεR1) on mast cells. When an allergen binds to its IgE on mast cells, the IgE-FcεR1 complex leads to Btk activation. This kinase further activates multiple pathways, such as degranulation, transcription factor activation and cytokine production in mast cells (Kawakami and Galli, 2002). On the other hand, Stim1 is important for promoting Ca^{2+} influx, which is essential for degranulation in mast cells (Baba *et al.*, 2008). In this study, the expression levels of Btk and Stim1 mRNAs were significantly lower in the spleen cells of mice given the prune extract-added diet than in those of mice given the extract-free diet (Figure 4C and 4D). These results suggest that the anti-allergic effect of the prune extract might be partly due to a reduction of mast cell degranulation, resulting from the decrease in Btk and Stim1 expression.

Tinker *et al.* (1991) demonstrated that the ingestion of prune fruit reduced blood cholesterol levels in humans via the action of flavonoids. In this study, we demonstrated for the first time that the prune extract suppressed type I allergic responses in mice, although we did not identify any anti-allergic component(s) in the prune extract. However, we observed that the ultrafiltrated fractions of prune extract, with a molecular mass smaller than 1,000 Da and a molecular mass greater than 10,000 Da, increased the number of IFN-$\gamma^{+}CD4^{+}$ cells in mouse spleen cell cultures (data not shown). Zuercher *et al.* (2010) reported that an apple extract suppressed type I allergic reactions in mice via the action of procyanidins and quercetin glycosides. The molecular masses of procyanidin C1 and quercetin 3-glucoside are 867 and 465, respectively. Prune contains procyanidins, quercetin 3-rutinoside and quercetin 3-glucoside (Nunes *et al.*, 2008; Slimestad *et al.*, 2009). Therefore, the anti-allergic effect of prune extract observed in this study may be partly attributable to polyphenols, such as procyanidins, quercetin 3-rutinoside and quercetin 3-glucoside. On the other hand, we have no information about immunoregulatory component(s) with a molecular mass greater than 10,000 Da in prunes. We are now characterizing the anti-allergic components in the prune extract.

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