

Eppikajutsuto Protects against Food Allergy Induced by Ovalbumin in a Murine Model

Teruaki Matsui^{a, b} Hirotaka Yamashita^{a, c} Mikito Mori^a Hiroyuki Tanaka^{a, c}
Naoki Inagaki^{a, c}

^aLaboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Gifu,

^bDepartment of Pediatrics, Nagoya University Graduate School of Medicine, Nagoya, and ^cUnited Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Japan

Keywords

Eppikajutsuto · Food allergy · Herbal medicine · Kampo medicine · Ovalbumin

Abstract

Background: Currently, there are no efficient medications available for the prevention and treatment of food allergy (FA). Herbal medicines, including traditional Japanese Kampo medicines (TJKMs), are promising therapeutic drugs. **Methods:** We screened 18 TJKMs for treatment of FA symptoms in a mouse FA model induced by ovalbumin (OVA). BALB/c mice were sensitized intraperitoneally by an OVA/aluminum hydroxide gel mixture followed by 4 booster doses of oral OVA and FA symptom induction by 50 mg of OVA. TJKMs were orally administered for 28 days from the day of sensitization to the day before FA symptom induction. Evaluated FA symptoms included a decrease in body temperature and allergic diarrhea. Allergic sensitization was determined by plasma OVA-specific IgE levels. Cytokine mRNA levels in mesenteric lymph nodes, plasma mouse mast cell protease-1, and the number of mast cells in the small and large intestines were analyzed. Additionally, the therapeutic effect of the TJKM eppikajutsuto (EJT) on mast cell degranulation was determined in active anaphylaxis and passive cu-

taneous anaphylaxis models. **Results:** EJT effectively prevented FA symptoms. Although OVA-specific IgE levels and the intestinal mast cell numbers were not different between the EJT-treated and untreated FA mice, plasma mMcpt1 and IL-4 levels were lower in EJT-treated FA mice than untreated FA mice. EJT could alleviate symptoms in both active and passive anaphylaxis models. **Conclusion:** EJT prevented OVA-induced FA symptoms in a mouse model, suggesting that EJT might exert its therapeutic activity via IL-4 suppression and the inhibition of mucosal mast cell degranulation.

© 2017 S. Karger AG, Basel

Introduction

Food allergy (FA) affects 3–6% of children and is increasing in developed countries [1]. It was shown to negatively influence the quality of life of not only patients, but also their families [2]. The current standard to manage FA relies mainly on allergen avoidance and includes the prescription of antihistamines and an epinephrine auto-

The work was conducted at the Laboratory of Pharmacology, Department of Bioactive Molecules, Gifu Pharmaceutical University, Gifu, Japan.

injector to treat allergic symptoms in cases of accidental antigen exposure [1, 3]. However, there are no standard therapies or medications that prevent or cure FA [4]. Consequently, novel FA therapies, including herbal remedies, are currently being explored [5]. Food allergy herbal formula-2 (FAHF-2) and kakkonto are both herbal medicines reported to alleviate FA in a murine FA model. FAHF-2, a Chinese herbal medicine, was reported to protect mice from anaphylactic reaction to peanut in a murine FA model [6]. In addition, human peripheral blood mononuclear cells treated with FAHF-2 in the presence of a food allergen produced significantly lower levels of interleukin (IL)-5 and significantly higher levels of IL-10; the number of regulatory T cells were increased as well [7].

Kakkonto, a traditional Japanese Kampo medicine (TJKM), suppressed the incidence of allergic diarrhea caused by ovalbumin (OVA) and induced Foxp3⁺/CD4⁺/CD25⁻ regulatory T cells in the colon in a FA model [8, 9]. There are no TJKMs except kakkonto that were reported to alleviate FA, but we hypothesized that other TJKMs are promising therapeutic candidates for treating FA for the following reasons. First, TJKMs are shown to relieve allergic symptoms including atopic dermatitis [10–12] and bronchial asthma [13, 14]. Second, TJKMs comprise multiple crude medicinal compounds with complex mechanisms of action [15]; conversely, the definite cause and pathogenesis of allergic diseases, including FA, remain unclear, but complex environmental, genetics, and immunological factors are suggested to be involved in FA pathogenesis [3, 16]. Thus, TJKMs might exert multiple therapeutic effects to provide protection from FA, a disorder with a multifactorial pathogenesis.

In this study, we screened 18 TJKMs for their potential therapeutic efficacy in FA. We found that eppikajutsuto (EJT) suppressed FA symptoms in a mouse model. Our findings suggest that the mechanism of EJT's therapeutic effect included the suppression of degranulation from mucosal mast cells and reduction in IL-4 expression.

Material and Methods

Animals

Female BALB/c mice or male ICR were purchased from Japan SLC (Hamamatsu, Japan) and were housed in the experimental animal facility of Gifu Pharmaceutical University. All studies were approved by the Animal Experiment Committee of Gifu Pharmaceutical University. Four to 7 mice per group were used in all experiments.

FA Model

The FA model used in this study was previously reported [17]. Briefly, female BALB/c mice were intraperitoneally injected on days 0 and 7 with either 1 µg of OVA (Sigma-Aldrich, St. Louis, MO, USA) mixed with aluminum hydroxide gel (Alum; Thermo Fisher Scientific, Waltham, MA, USA) for sensitization or phosphate-buffered saline (PBS) with alum for control. Between days 14 and 21, 10 mg or 30 mg of OVA dissolved in 0.2 mL of PBS was orally administered 4 times. On day 28, 1 week after the last OVA administration, FA symptoms were induced by oral administration of 50 mg of OVA (Fig. 1a, 5a).

Drug Treatment

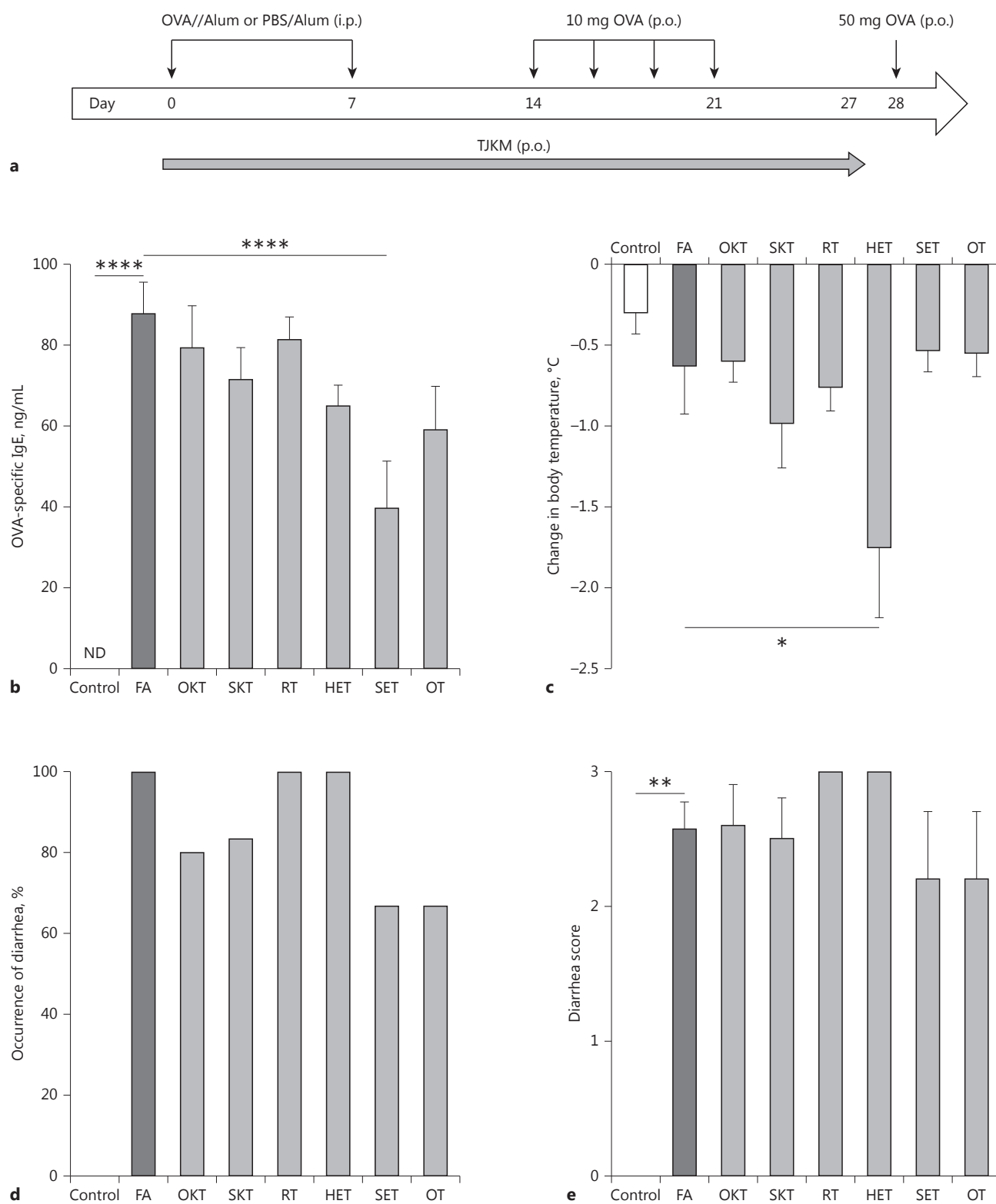
The TJKMs used in this study were powdered extracts provided by Tsumura & Co. (Tokyo, Japan). All TJKMs were manufactured by a standardized process which included spray drying of hot water extracts of constituted medical herbs. The quality of all TJKMs were guaranteed by the quantities of ingredients set by the Japanese Ministry of Health, Labour, and Welfare and have been widely prescribed in clinical conditions including allergic diseases. A high-performance liquid chromatography profile of EJT, one of the TJKMs provided by Tsumura & Co., is shown in online supplementary Figure 1 (see www.karger.com/doi/10.1159/000472715 for all online suppl. material). The following TJKMs were used: ogikenchuto, shokenchuto, keishikashakuyakuto, tokikenchuto, daikenchuto, rikkunshito, hochuekkito, juzentaihoto, ninjinyoeito, shikunshito, keihito, seishoekkito, orento, jiinkokato, keigai-rengyoto, EJT, orengekuto, and saikoseikanto. A total of 20 mg of each TJKM dissolved in 0.2 mL distilled water was administered orally per day for 28 days from day 0 to day 27 (Fig. 1a). Control and FA groups were administered only 0.2 mL of distilled water per day. We evaluated these 18 TJKMs in 4 independent experiments to identify the candidate therapeutic medications for FA.

Evaluation of Allergic Symptoms

FA symptoms included decrease in body temperature, determined by measurement of rectal temperature with a KN-91 thermometer (Natsume, Tokyo, Japan) 1 h after the administration of

Fig. 1. Experimental design and evaluation of the therapeutic effects of TJKMs in FA. **a** BALB/c mice were sensitized intraperitoneally (i.p.) with ovalbumin (OVA) mixed with aluminum hydroxide gel (OVA/Alum) and received booster doses of OVA per os (p.o.) 4 times. FA symptoms were elicited by 50 mg of OVA (p.o.) on day 28. TJKMs were orally administered for 28 days from day 0 to day 27. Control mice constituted the reference group for sensitization and received PBS/Alum (i.p.), followed by OVA (p.o.) and distilled water (p.o.) for treatment. FA mice were sensitized with OVA/Alum (i.p.) and administered OVA (p.o.), followed by distilled water (p.o.) as the reference group for treatment. For treatments, ogikenchuto (OKT), shokenchuto (SKT), rikkunshito (RT), hochuekkito (HET), seishoekkito (SET), and orento (OT) were administered. OVA-specific IgE on day 27 (**b**), change in body temperature (**c**), occurrence of diarrhea (**d**), and diarrhea score (**e**) in mice that were administered 50 mg of OVA (p.o.) were compared between the untreated FA and TJKM-treated FA mice. Values represent means ± standard error of mean (5–7 mice/treatment). ND, not detected. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$.

(For figure see next page.)



OVA on day 28, and allergic diarrhea, as previously reported [17]. Diarrhea was assessed by the following diarrhea scoring system: 0, solid; 1, funicular form; 2, slurry; and 3, watery. Diarrhea scores of 2 and 3 were considered to indicate allergic diarrhea.

Passive Cutaneous Anaphylaxis

Six-week-old male ICR mice were injected 1 µg of anti-2,4-dinitrophenylated (DNP) IgE (SPE-7; Sigma-Aldrich) in 10 µL of 0.9% saline or 10 µL of saline alone as control intradermally into both ears for sensitization. After 24 h, the mice were challenged with an intravenous injection of 100 µg of DNP-BSA (Molecular Probes, Eugene, OR, USA) in 250 µL of saline and 0.5% Evans blue dye. Then, 20 mg of EJT in 0.2 mL of distilled water or 0.2 mL of distilled water alone as treatment control was orally administered before anti-DNP IgE and DNP-BSA injections. The extravasation of the Evans blue into the ears was monitored for 30 min, at which time both ears were dissected and incubated overnight at 37°C with 0.7 mL of 1 N KOH. Evans blue dye was extracted in 9.3 mL of a mixture containing 0.6 N H₃PO₄ and acetone (5:13). The absorbance of Evans blue-containing H₃PO₄-acetone at 620 nm was then measured by a spectrophotometer (U-1900; Hitachi High-Technologies Corporation, Hitachinaka, Japan). The amount of extravasated Evans blue dye was calculated according to the standard curve.

Active Anaphylaxis

Six-week-old female BALB/c mice were intraperitoneally injected with 1 µg of OVA mixed with alum on days 0 and 7. Anaphylaxis was elicited by intravenous injection of 1 µg of OVA at day 14. A total of 20 mg of EJT in 0.2 mL of distilled water or 0.2 mL of distilled water alone as control was orally administered 1 h before anaphylaxis induction. Rectal temperature was measured every 15 min for 90 min after the administration of OVA.

Detection of OVA-Specific Immunoglobulin E in Plasma

OVA-specific immunoglobulin E (OVA-sIgE) was measured using enzyme-linked immunosorbent assay (ELISA) according to previously reported protocols [18, 19]. Briefly, a high protein-binding capacity ELISA plate (Nunc, Roskilde, Denmark) was coated with an anti-mouse IgE antibody (MCA419, clone LO-ME-3; Serotec, Oxford, UK) to capture OVA-sIgE. Enzymatic detection of biotinylated OVA binding to OVA-sIgE in plasma samples was achieved with measurement of horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark) at an absorbance at 492 nm (reference 690 nm) using an automatic ELISA plate reader (Titertek Multiscan MS, ver 8, Labsystems Oy, Helsinki, Finland).

Detection of Cytokine mRNA Expression

After the evaluation of allergic symptoms, animals were euthanized and mesenteric lymph nodes (MLNs) were collected. Total RNA was extracted using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA), and complementary DNA was prepared using PrimeScript RT Master Mix (Takara Bio, Ohtsu, Japan). Cytokine mRNA levels in MLNs were measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq II (Takara Bio) on a Takara Thermal Cycler Dice. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as internal control. The following primer sets were used: 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-TTGCTGTT-

GAAGTCGCAGGAG-3' for *Gapdh*; 5'-TCTCGAATGTACCAG-GAGCCATATC-3' and 5'-AGCACCTTGGAAAGCCCTACA-GA-3' for *IL-4*; 5'-CGGCACAGTCATTGAAAGCCTA-3' and 5'-GTTGCTGATGGCCTGATTGTC-3' for interferon-gamma (*Ifn-γ*); 5'-GCAGAGAAGCATGGCCAGAAA-3' and 5'-GGAG-AAATCGATGACAGCGCT-3' for *IL-10*; and 5'-GTGTGGAG-CAACATGTGGAAGTCTA-3' and 5'-TTGGTTCAGCCACT-GCCGTA-3' for transforming growth factor-beta (*Tgf-β*).

Detection of Intestinal Mast Cells

Intestinal mast cells were detected by naphthol AS-D chloroacetate esterase staining [20, 21]. Briefly, small and large intestines collected following euthanasia were flushed by 0.9% normal saline to remove luminal contents. The intestines were then prefixed with 10% neutral buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin wax II 60 (Sakura Finetek Japan, Tokyo, Japan) using path cassette M (Matsunami Glass Ind., Ltd., Osaka, Japan). Then, 5-µm tissue sections were prepared, and mucosal mast cells were identified by naphthol AS-D chloroacetate esterase staining kit (Sigma-Aldrich). The number of mast cells per 1 high-power field (1,000×) was determined using a Labophot 2 microscope (Nikon Corporation, Tokyo, Japan).

Measurement of Mouse Mast Cell Protease-1 and IL-4 in Plasma

Mouse mast cell protease-1 (mMcp1) and IL-4 levels in plasma samples that were collected after the evaluation of FA symptoms were measured using the mMCP-1 and mouse IL-4 ELISA Ready-SET-Go! kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

All statistical analyses were performed with EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [22]. Data were expressed as means with standard error of means. A Dunnett test was used to compare factors, other than those for diarrhea, such as decreasing body temperature, OVA-sIgE, mMcp1, IL-4, mRNA levels, and mast cell counts, of each mice group to those of FA group. A Steel test was used to analyze the difference in diarrhea scores between the FA and each mice group. One-way repeated measures ANOVA was used to analyze the differences in the changes of body temperature in the active anaphylaxis model. $p < 0.05$ was considered statistically significant.

Results

OVA Sensitization and Symptoms of FA

Sensitization to OVA was confirmed by the production of OVA-sIgE in the FA group compared with the control group, as determined in 4 independent experiments (Fig. 1b, 2a, 3a, 4a). Decreased body temperature (Fig. 2b) and increased rate and score of diarrhea (Fig. 1d, e, 2c, d, 3c, d, 4c, d) induced by oral administration of 50 mg of OVA were detected in the FA group but not in the control group, in agreement with previous studies [17].

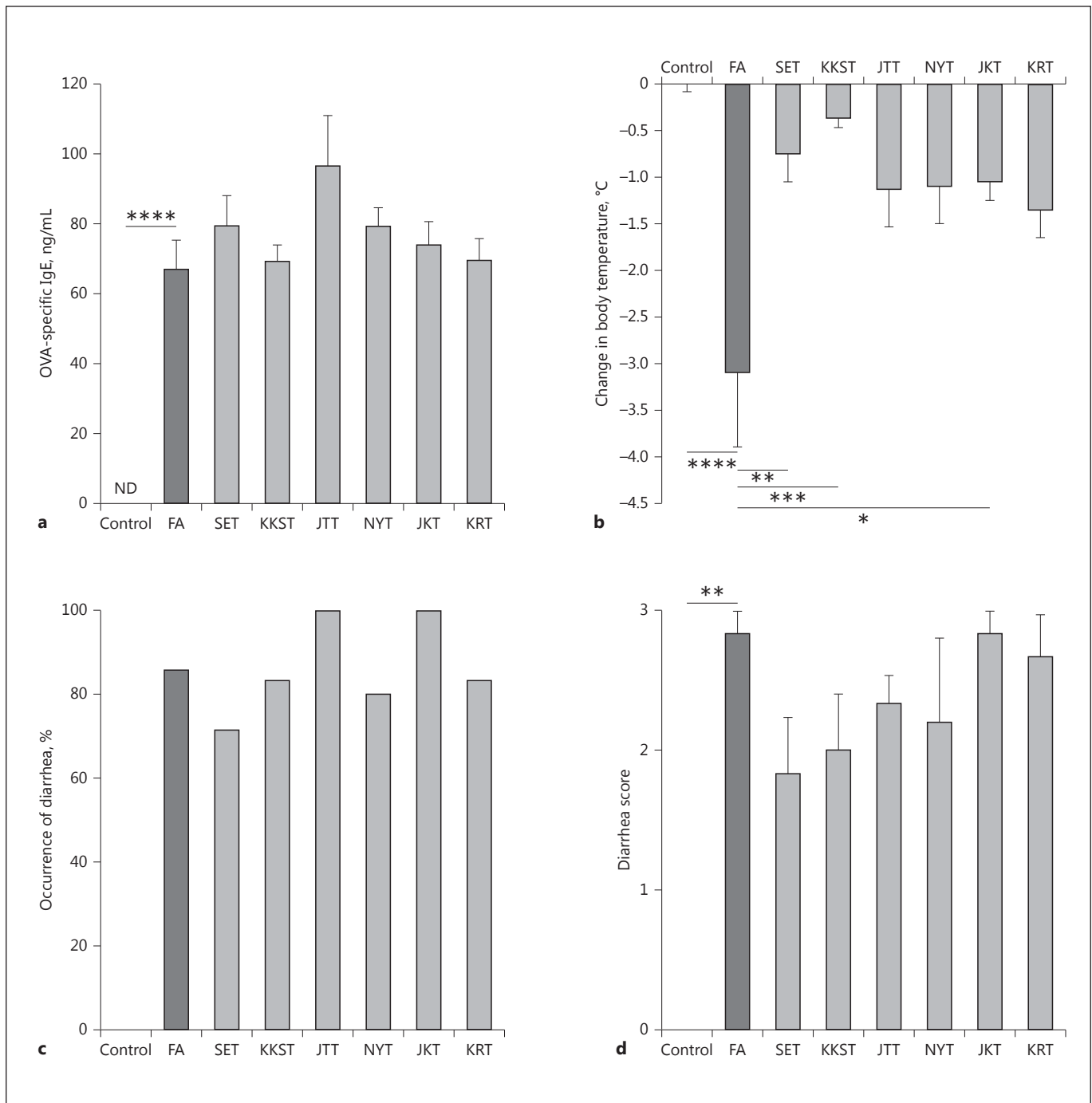


Fig. 2. Evaluation of the therapeutic effects of TJKM in FA. OVA-specific IgE on day 27 (**a**), change in body temperature (**b**), occurrence of diarrhea (**c**), and diarrhea score (**d**) were compared between the FA and TJKM-treated groups. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). SET, seishoekkito; KKST, keishikashakuyakuto; JTT, juzentaihoto; NYT, ninjinyoeito; JKT, jiinkokato; KRT, keigairengyoto; ND, not detected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

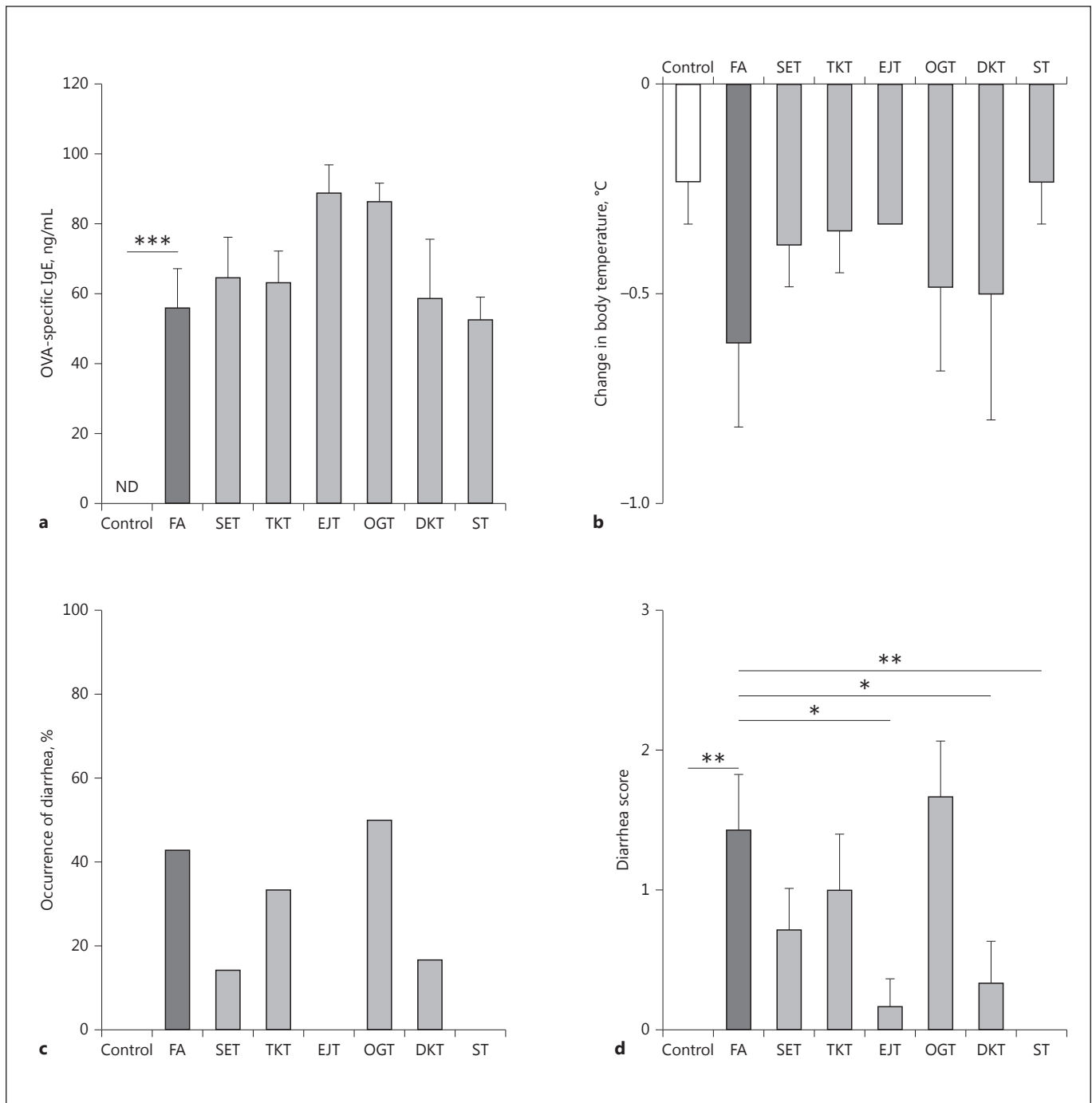


Fig. 3. Evaluation of the therapeutic effects of TJKMs in FA. OVA-specific IgE on day 27 (**a**), change in body temperature (**b**), occurrence of diarrhea (**c**), and diarrhea score (**d**) were compared between the FA and TJKM-treated groups. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). SET, seishoekkito; TKT, tokikenchuto; OGT, orengedokuto; DKT, daikenchuto; ST, shikunshito; ND, not detected. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

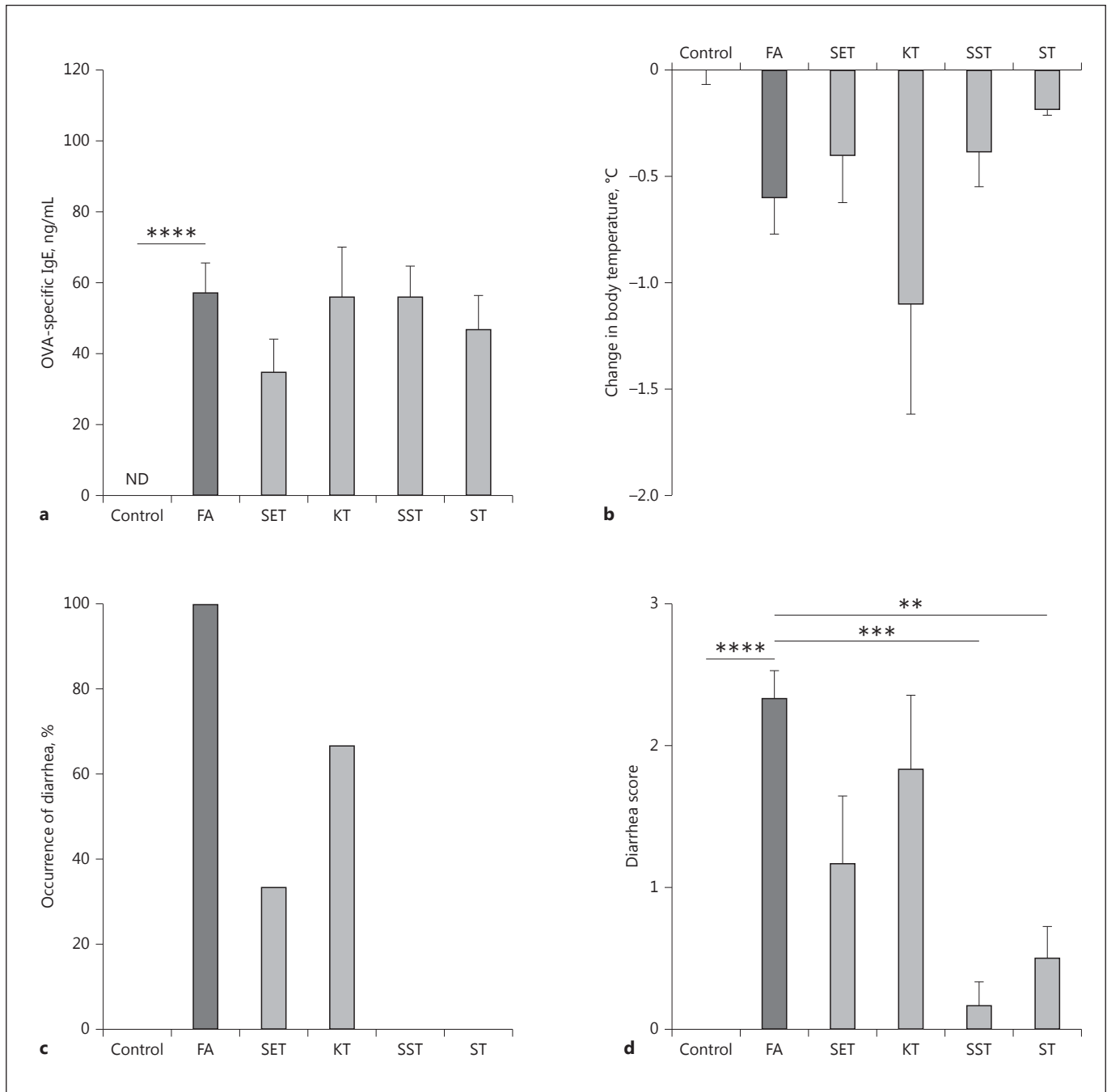
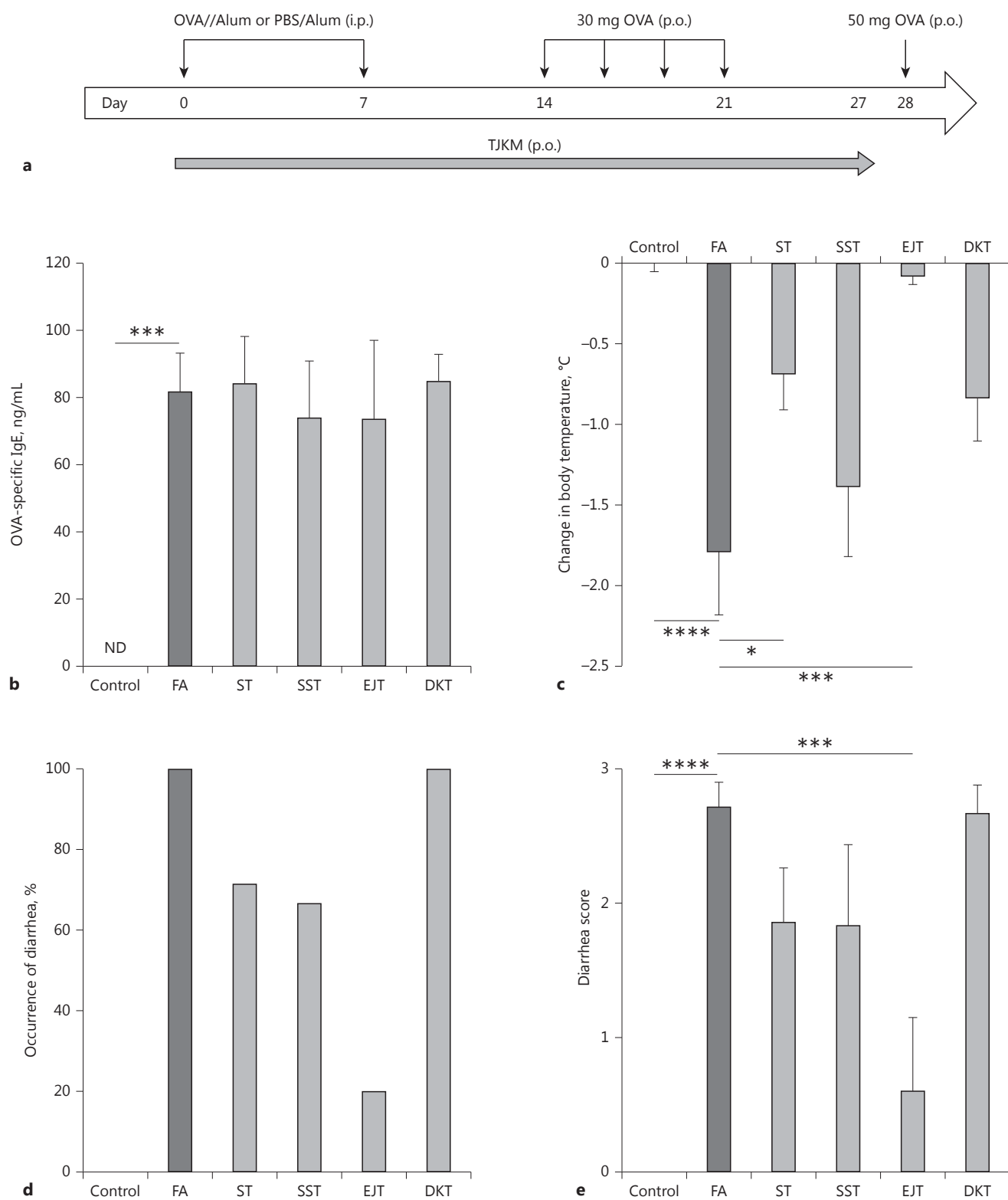


Fig. 4. Evaluation of the therapeutic effects of TJKM in FA. OVA-specific IgE on day 27 (**a**), change in body temperature (**b**), occurrence of diarrhea (**c**), and diarrhea score (**d**) were compared between the FA and TJKM-treated groups. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). SET, seishoekkito; KT, keihito; SST, saikoseikanto; ST, shikunshito; ND, not detected. ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.



(For legend see next page.)

Thus, we defined these outcomes as OVA-induced FA symptoms.

Screening of Kampo Medicine for the Treatment of FA

We evaluated 18 TJKMs in 4 independent experiments to identify candidate therapeutic medications for FA, as shown in Figures 1–4. Comparison of TJKM-treated FA mice with FA mice that did not receive individual TJKMs revealed that seishoekkito reduced the mean OVA-sIgE level from 87.7 ± 7.8 to 39.7 ± 11.7 ng/mL in the FA group in one experiment (Fig. 1b); however, this effect was not reproducible in other experiments (Fig. 2a, 3a, 4a). Seishoekkito also reduced the change in body temperature from $-3.1 \pm 0.8^\circ\text{C}$ to $-0.8 \pm 0.3^\circ\text{C}$ in the FA group in one experiment (Fig. 2b); however, this effect was not reproducible in other experiments (Fig. 1c, 3b, 4b). Conversely, keishikashakuyakuto reduced the change in body temperature from $-3.1 \pm 0.8^\circ\text{C}$ to $-0.4 \pm 0.1^\circ\text{C}$ (Fig. 2c) in the FA group. Shikunshito, saikoseikanto, and EJT completely blocked OVA-induced diarrhea (Fig. 3c and 4c), whereas daikenchuto-treated FA mice were partially protected from allergic diarrhea (Fig. 3c). These results provided support for several of the TJKMs tested as potential therapeutic agents against FA.

EJT Is Effective against FA Symptoms

OVA-induced symptoms in the FA group were weaker than expected, as the decrease in temperature was observed only in one experiment (Fig. 2b), and the percentage of animals with diarrhea was less than 50% in another experiment (Fig. 3d). Thus, we further evaluated the treatment effects of shikunshito, saikoseikanto, EJT, and daikenchuto in a group of mice with increased susceptibility to FA symptoms after administration of 30 mg of OVA (Fig. 5a). FA symptoms were effectively induced in mice in this modified experimental protocol (Fig. 5c–e).

We found that the change in body temperature was reduced from $-1.8 \pm 0.4^\circ\text{C}$ in the FA group to $-0.1 \pm 0.1^\circ\text{C}$ in the EJT-treated group (Fig. 5c). The percentage

of animals with diarrhea was also reduced from 100 to 20% (Fig. 5d) after EJT treatment of the FA mice. Finally, the mean diarrhea score was reduced from 2.7 ± 0.2 in the FA group to 0.6 ± 0.5 (Fig. 5e) in the EJT-treated group.

EJT Suppresses IL-4 Levels

mRNA expression levels of *Ifn- γ* , *Il-10*, and *Tgf- β* in MLNs were not different between the control, FA, and EJT-treated groups (Fig. 6a). However, *Il-4* mRNA levels in MLNs were higher in the FA group than in the control group and were lower in the EJT-treated group than in the FA group (Fig. 6a). Not only the *Il-4* mRNA levels, but also the plasma levels of IL-4 were lower in the EJT-treated group than in the FA group (Fig. 6b).

EJT Suppresses Degranulation of Mucosal Mast Cells

Mast cell counts in both the small and large intestines were higher in the FA group than in the control group but were not different between the FA and EJT-treated groups (Fig. 7a and b). Representative esterase staining of the small intestines is shown in online supplementary Figure 2. Furthermore, mean mMcp1 levels in plasma measured after determination of FA symptoms were below the level of detection in control mice and elevated in FA mice (280 ± 77 ng/mL). Importantly, plasma mMcp1 levels were significantly lower in the EJT-treated group (52 ± 17 ng/mL) than those in the FA group (Fig. 7c), although mast cell numbers were not different. We further evaluated whether EJT treatment could suppress mast cell degranulation in the active anaphylaxis model (Fig. 7d) as well as the passive cutaneous anaphylaxis model (Fig. 7e). Active anaphylaxis monitored by the chronological change in body temperature was alleviated by the oral administration of EJT ($p = 0.030$, Fig. 7d). Similarly, passive cutaneous anaphylaxis, as determined by the Evans blue extravasation, was relatively alleviated by the oral administration of EJT ($p = 0.064$, Fig. 7e).

Discussion

In this study, we found that EJT, a TJKM, was effective in preventing FA symptoms by alleviating the decrease in body temperature and diarrhea. We also found that OVA-mediated increases in mRNA levels of *Il-4*, a major Th2 cytokine in MLNs, were reduced by EJT treatment, with no changes in mRNA levels of *Ifn- γ* , a major Th1 type cytokine, and 2 suppressive cytokines, *Il-10* and *Tgf- β* . EJT treatment reduced not only the mRNA levels, but also the

Fig. 5. Modified experimental protocol and evaluation of the therapeutic effects of TJKMs for FA. **a** The sensitization OVA dose was increased to 30 mg. OVA-specific IgE on day 27 (**b**), change in body temperature (**c**), occurrence of diarrhea (**d**), and diarrhea score (**e**) were compared between the FA and TJKM-treated groups. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). ST, shikunshito; SST, saikoseikanto; DKT, daikenchuto; ND, not detected. * $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$.

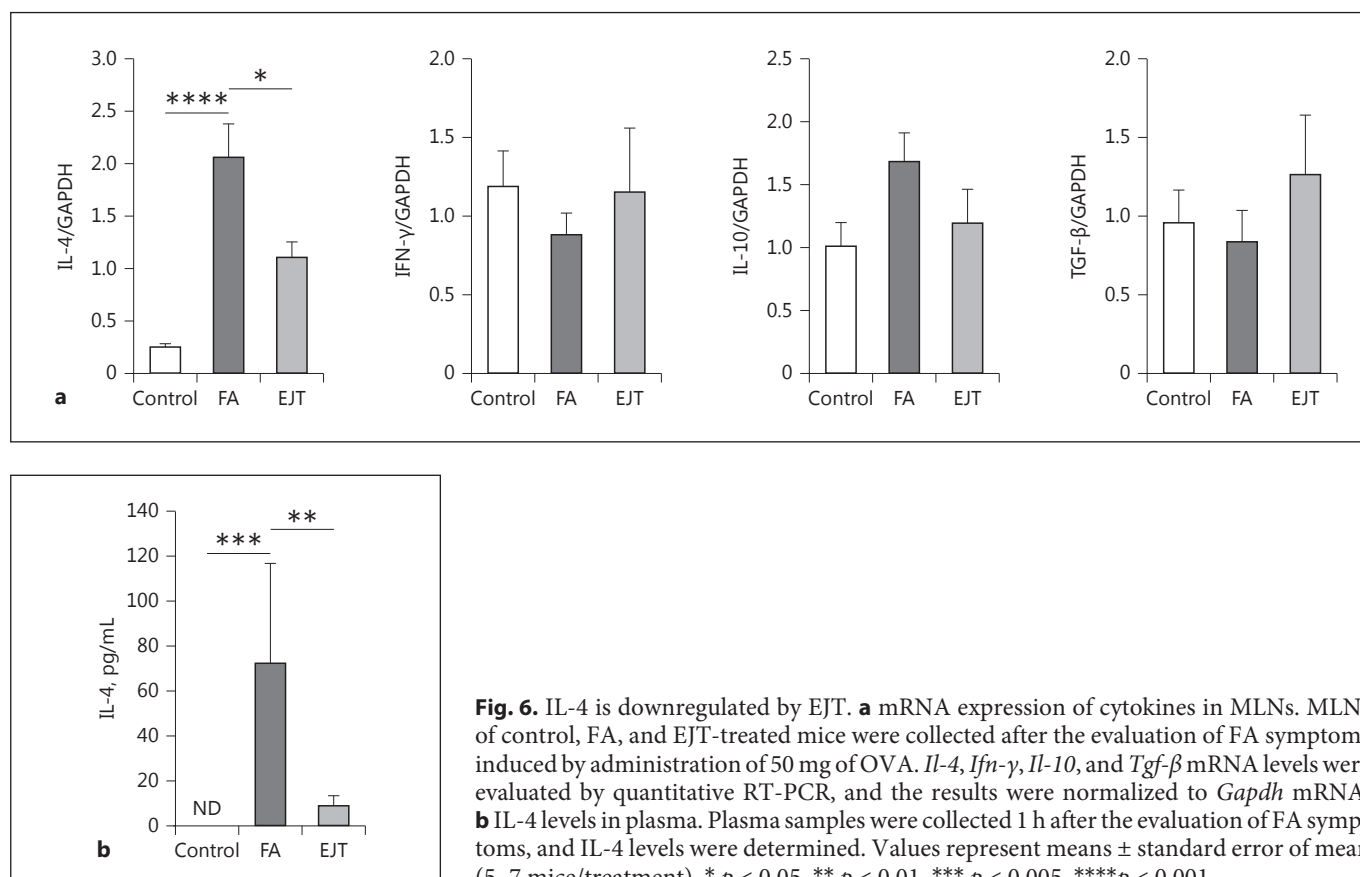


Fig. 6. IL-4 is downregulated by EJT. **a** mRNA expression of cytokines in MLNs. MLNs of control, FA, and EJT-treated mice were collected after the evaluation of FA symptoms induced by administration of 50 mg of OVA. *Il-4*, *Ifn- γ* , *Il-10*, and *Tgf- β* mRNA levels were evaluated by quantitative RT-PCR, and the results were normalized to *Gapdh* mRNA. **b** IL-4 levels in plasma. Plasma samples were collected 1 h after the evaluation of FA symptoms, and IL-4 levels were determined. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

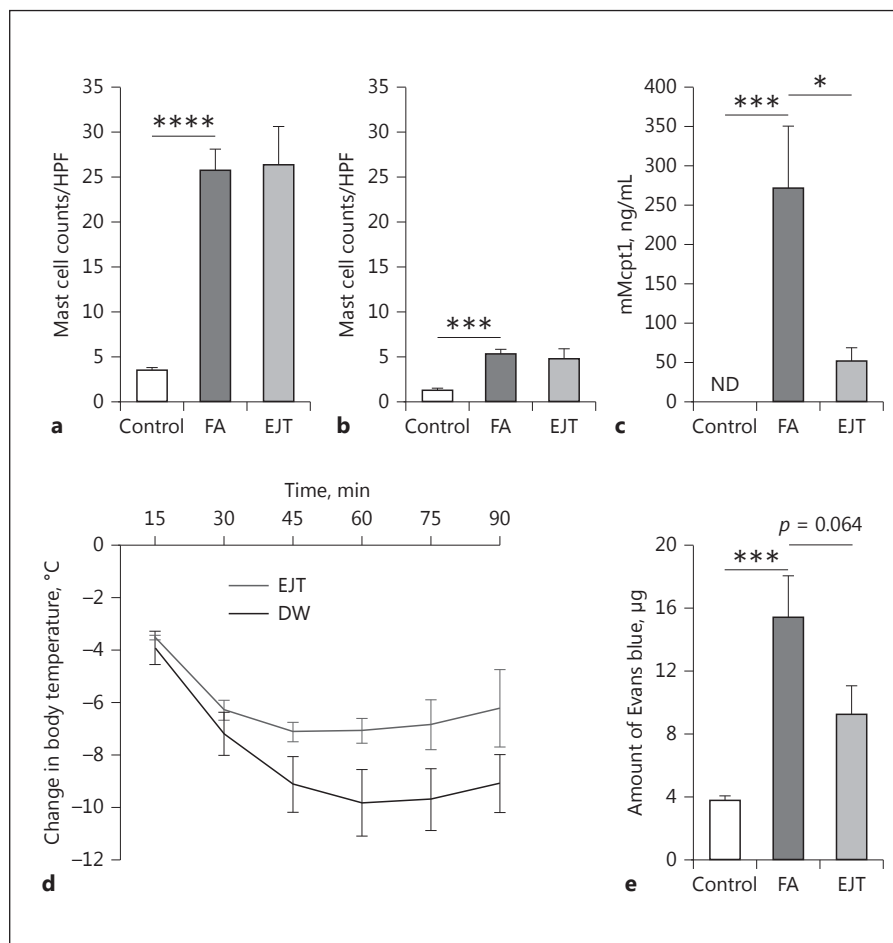
protein levels of IL-4 in plasma. These results suggest that EJT suppressed *Il-4* mRNA levels directly, independent from its potential effects on *Ifn- γ* , *Il-10*, and *Tgf- β* . Of note, the mRNA levels of all 4 cytokines in the spleen that might suggest systemic immunological reaction were not different between the FA and EJT-treated groups (data not shown).

IL-4 is essential in Th2-type responses during FA [23, 24]. It prompts B-cell activation and interacts with antigen-specific B cells, leading to isotype switching to generate food antigen-specific IgE [25]. IL-4 is also involved in the development of Th2 cells [25] and eosinophilic infiltration of gut mucosa [26]. Moreover, IL-4 was shown to enhance the release of histamine as well as leukotrienes from mast cells triggered by IgE receptor crosslinking [27]. IL-4 deficiency was shown to limit FA development [24]. Inversely, a gain-of-function mutation in the IL-4 receptor α -chain was shown to increase susceptibility to anaphylaxis [28]. These findings altogether highlight the pivotal role of IL-4 in inducing FA symptoms. In the present study, we showed that not only *Il-4* mRNA levels in

MLNs but also plasma IL-4 protein levels were decreased in EJT-treated mice compared with those in untreated mice, although OVA-sIgE levels were not suppressed. These results suggest that the suppression of mast cell degranulation due to suppressed IL-4 might be one of the mechanisms of alleviation of FA symptoms by EJT.

FA symptoms are mainly mediated by antigen cross-linking of antigen-specific IgE bound to the high-affinity IgE receptor (Fc ϵ RI) on mast cells and subsequent release of mediators such as histamine and leukotrienes [4, 29]. An increased number of mast cells in the small intestine of FA mice was previously reported [30]. Additionally, allergic diarrhea was completely blocked by mast cell depletion and in Fc ϵ RI-deficient mice [30]. Thus, degranulation of mast cells is critical in the development of FA symptoms. We also found that the number of mucosal mast cells in the small intestine was higher in mice with FA than those in control mice. Both FAHF-2 [6] and kak-konto [9] were previously shown to reduce the number of mast cells in the intestines. Thus, we expected EJT-treated mice to be protected from infiltrating mast cells in the in-

Fig. 7. EJT suppresses mast cell degranulation. The number of mast cells in the intestines (**a, b**) and the levels of mMcp1 in plasma (**c**) of control, FA, and EJT-treated FA mice. Samples were collected after the evaluation of FA symptoms induced by administration of 50 mg of OVA. Mast cells were identified by naphthol AS-D chloroacetate esterase staining, and the number of mast cells per 1 high-power field (HPF, 1,000×) was determined in the small intestine (**a**) and large intestine (**b**). **c** mMcp1 levels in plasma collected after the evaluation of FA symptoms were determined. Values represent means $\pm$ standard error of mean (5–7 mice/treatment). ND, not detected. **d** Changes in the body temperature in an active anaphylaxis model. EJT in distilled water (DW) or DW alone as control was orally administered 1 h before the induction of anaphylaxis. Values represent means $\pm$ standard error of mean (4 mice/group). **e** Level of Evans blue dye extravasation in passive cutaneous anaphylaxis (PCA) model. EJT in DW for the EJT group and only DW for the control and PCA groups were orally administered before DNP-IgE and DNP-BSA injections. The extravasation of Evans blue from both sides of the ears was determined. Values represent means $\pm$ standard error of mean (5 mice/group). * $p < 0.05$, *** $p < 0.005$, **** $p < 0.001$.



testines; however, the number of mast cells in the intestines was not different between FA and EJT-treated mice. In contrast, EJT treatment led to a decrease in plasma mMcp1 levels in EJT-treated mice. mMcp1 is one of the mast cell proteases found in mucosal mast cells, and previous studies showed that plasma mMcp1 levels were significantly increased in mice with FA [9, 30]. These findings raise the possibility that EJT might suppress plasma mMcp1 levels without affecting mucosal mast cell numbers, thus controlling their function and suppressing degranulation. Therefore, we further evaluated the treatment effect of EJT in both an active anaphylaxis model and a passive cutaneous anaphylaxis model by determining whether EJT could suppress mast cell degranulation, and confirmed that EJT suppressed degranulation in both models. These data suggest that EJT suppresses mucosal mast cell degranulation without affecting infiltration into intestines of those.

EJT is extracted from 6 species of medical herbs, including *Zingiberis rhizoma*, *Zizyphi fructus*, *Glycyrrhizae radix*, *Atractylodis lanceae rhizoma*, *Gypsum fibrosum*, and *Ephedrae herba*. Some medical herbs have components that alleviate allergic symptoms [31, 32]; thus, it is important to evaluate the effect of each anti-inflammatory herb and component to determine their efficacy. Conversely, TJKMs are considered to exert their effects via the integrated and synergistic actions of these components. Therefore, we first investigated individual TJKMs in detail. *Gypsum fibrosum* and *Ephedrae herba* were the only herbs containing EJT among 18 TJKMs included in this study; therefore, these herbs might be important for the efficacy of EJT. *Zingiberis rhizoma* is also included in FAHF-2 that is extracted from 9 species of medical herbs, whereas *Z. rhizoma*, *Z. fructus*, *G. radix*, and *E. herba* are found in kakkonto. Importantly, *Z. rhizoma* is found in FAHF-2, kakkonto, and EJT. While no sole medical herb was as effective as FAHF-2 for the treatment of peanut-

induced anaphylaxis, *Z. rhizoma* was previously reported as one of the medical herbs that reduced Th2 cytokine production in splenocytes restimulated by crude peanut extract [33]. Therefore, *Z. rhizoma* might be the key medical herb in EJT that reduces the expression of IL-4. Among 6 medical herbs in EJT, *A. lanceae rhizoma* was shown to inhibit β -hexosaminidase release from mast cell-like rat basophil leukemia cells [34]. Thus, *A. lanceae rhizoma* might be the major medical herb to suppress plasma mMcpt1 levels in our study.

In summary, we herein showed that administration of EJT for 28 days led to alleviation of FA symptoms in mice. Our findings also suggest that EJT exerted these effects through the suppression of IL-4 and inhibition of mucosal mast cell degranulation. There are currently no preventative or curative treatments for FA. Our findings suggest that EJT, a TJKM, should be considered as a new therapeutic to control FA symptoms.

Acknowledgements

This study was supported by a Grant-in-Aid for the Cooperative Research Project from Joint Usage/Research Center (Joint Usage/Research Center for Science-Based Natural Medicine), Institute of Natural Medicine, University of Toyama, in 2015. TJKMs and HPLC data were provided by Tsumura & Co.

Author Contributions

M.M. participated in the design of the study and performed the experiments. H.Y., H.T., and N.I. participated in the design of the study and helped in writing the manuscript. T.M. participated in the design of the study, performed the experiments and statistical analysis, and wrote the manuscript. All authors read and approved the final version of the manuscript.

Disclosure Statement

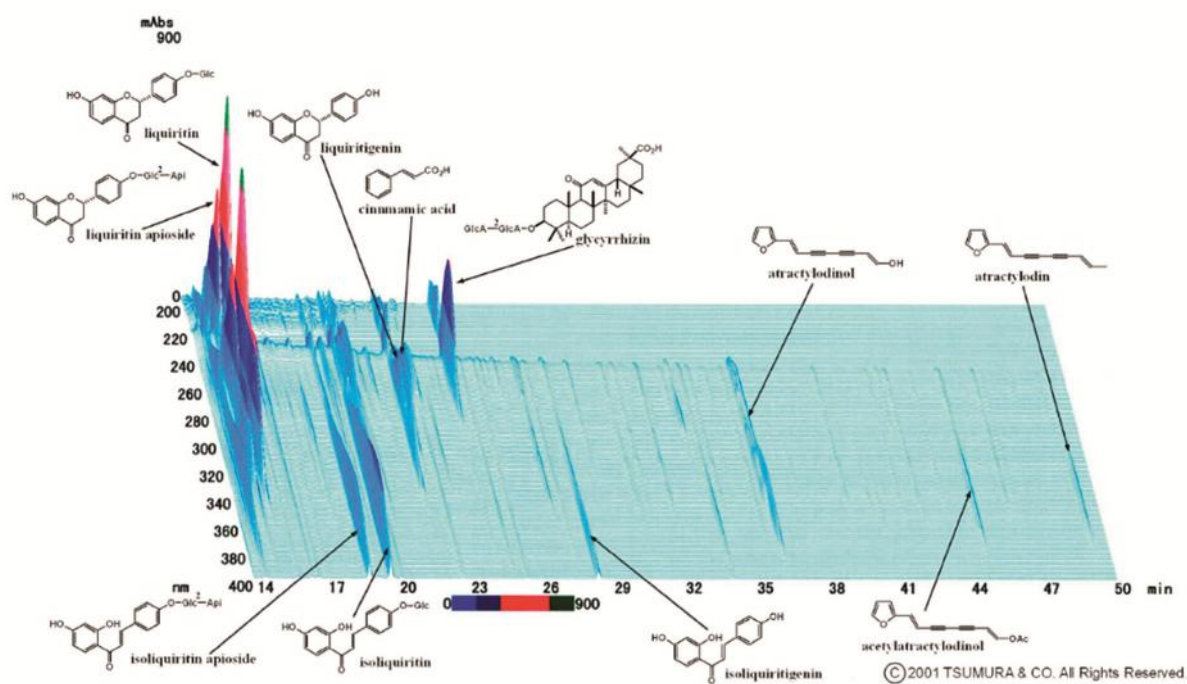
All traditional Japanese Kampo medications were provided free of charge by Tsumura Co. (Tokyo, Japan).

References

- ▶ 1 du Toit G, Tsakok T, Lack S, Lack G: Prevention of food allergy. *J Allergy Clin Immunol* 2016;137:998–1010.
- ▶ 2 Warren CM, Otto AK, Walkner MM, Gupta RS: Quality of life among food allergic patients and their caregivers. *Curr Allergy Asthma Rep* 2016;16:1–8.
- ▶ 3 Sicherer SH, Sampson HA: Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* 2014;133:291–307.
- ▶ 4 Iweala OI, Burks AW: Food allergy: our evolving understanding of its pathogenesis, prevention, and treatment. *Curr Allergy Asthma Rep* 2016;16:1–10.
- ▶ 5 Keet CA, Wood RA: Emerging therapies for food allergy. *J Clin Invest* 2014;124:1880–1886.
- ▶ 6 Song Y, Qu C, Srivastava K, Yang N, Busse P, Zhao W, Li XM: Food allergy herbal formula 2 protection against peanut anaphylactic is via inhibition of mast cells and basophils. *J Allergy Clin Immunol* 2010;126:1208–1217.
- ▶ 7 Wang J, Jones SM, Pongracic JA, Song Y, Yang N, Sicherer SH, Makhija MM, Robison RG, Moshier E, Godbold J, Sampson HA, Li XM: Safety, clinical, and immunologic efficacy of a Chinese herbal medicine (food allergy herbal formula-2) for food allergy. *J Allergy Clin Immunol* 2015;136:962–970.
- ▶ 8 Yamamoto T, Fujiwara K, Tsubota Y, Kageyama-Yahara N, Hayashi S, Kadowaki M: Induction of regulatory T cells as a novel mechanism underlying the therapeutic action of kakkonto, a traditional Japanese herbal medicine, in a murine food allergy model. *Int Arch Allergy Immunol* 2016;169:146–156.
- ▶ 9 Yamamoto T, Fujiwara K, Yoshida M, Kageyama-Yahara N, Kuramoto H, Shibahara N, Kadowaki M: Therapeutic effect of kakkonto in a mouse model of food allergy with gastrointestinal symptoms. *Int Arch Allergy Immunol* 2009;148:175–185.
- ▶ 10 Yamashita H, Makino T, Inagaki N, Nose M, Mizukami H: Assessment of relief from pruritus due to Kampo medicines by using murine models of atopic dermatitis. *J Trad Med* 2013;30:114–123.
- ▶ 11 Yamashita H, Tanaka H, Inagaki N: Treatment of the chronic itch of atopic dermatitis using standard drugs and Kampo medicines. *Biol Pharm Bull* 2013;36:1253–1257.
- ▶ 12 Kobayashi H, Ishii M, Takeuchi S, Tanaka Y, Shintani T, Yamatodani A, Kusunoki T, Furue M: Efficacy and safety of a traditional herbal medicine, hochu-ekki-to in the long-term management of kikyō (delicate constitution) patients with atopic dermatitis: a 6-month, multicenter, double-blind, randomized, placebo-controlled study. *Evid Based Complement Alternat Med* 2010;7:367–373.
- ▶ 13 Nagai T, Nakao M, Shimizu Y, Kodera Y, Oh-Ishi M, Maeda T, Yamada H: Proteomic analysis of anti-inflammatory effects of a kampo (Japanese herbal) medicine “Shoseiryuto (Xiao-Qing-Long-Tang)” on airway inflammation in a mouse model. *Evid Based Complement Alternat Med* 2011;2011:604196.
- ▶ 14 Urata Y, Yoshida S, Irie Y, Tanigawa T, Amayasu H, Nakabayashi M, Akahori K: Treatment of asthma patients with herbal medicine TJ-96: a randomized controlled trial. *Respir Med* 2002;96:469–474.
- ▶ 15 Yu F, Takahashi T, Moriya J, Kawaura K, Yamakawa J, Kusaka K, Itoh T, Morimoto S, Yamaguchi N, Kanda T: Traditional Chinese medicine and Kampo: a review from the distant past for the future. *J Int Med Res* 2006;34:231–239.
- ▶ 16 Matsui T, Tanaka K, Nakagawa T, Sasaki K, Nakata J, Sugiura S, Kando N, Ito K: Sun exposure inversely related to food sensitization during infancy. *Pediatr Allergy Immunol* 2015;26:628–633.
- ▶ 17 Yamashita H, Takahashi K, Tanaka H, Nagai H, Inagaki N: Overcoming food allergy through acquired tolerance conferred by transfer of Tregs in a murine model. *Allergy* 2012;67:201–209.

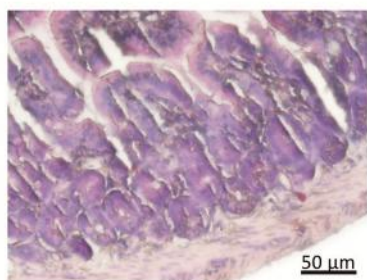
- ▶ 18 Nagai H, Maeda Y, Tanaka H: The effect of anti-IL-4 monoclonal antibody, rapamycin and interferon- γ on airway hypersensitivity to acetylcholine in mice. *Clin Exp Allergy* 1997;27:218–224.
- ▶ 19 Tanaka H, Masuda T, Tokuoka S, Komai M, Nagao K, Takahashi Y, Nagai H: The effect of allergen-induced airway inflammation on airway remodeling in a murine model of allergic asthma. *Inflamm Res* 2001;50:616–624.
- ▶ 20 Friend DS, Ghildyal N, Austen KF, Gurish MF, Matsumoto R, Stevens RL: Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J Cell Biol* 1996; 135:279–290.
- ▶ 21 Beckstead JH, Halverson PS, Ries CA, Bainton DF: Enzyme histochemistry and immunohistochemistry on biopsy specimens of pathologic human bone marrow. *Blood* 1981; 57:1088–1098.
- ▶ 22 Kanda Y: Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant* 2013;48:452–458.
- ▶ 23 Pulendran B, Artis D: New paradigms in type 2 immunity. *Science* 2012;337:431–435.
- ▶ 24 Dourado LP, da Silva Saldanha JC, Gargiulo DL, Noviello MD, Brant CC, Reis ML, de Souza RM, Faria AM, de Souza DD, Cara DC: Role of IL-4 in aversion induced by food allergy in mice. *Cell Immunol* 2010;262:62–68.
- ▶ 25 Paul WE, Zhu J: How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 2010;10:225–235.
- ▶ 26 Bischoff S, Crowe SE: Gastrointestinal food allergy: new insights into pathophysiology and clinical perspectives. *Gastroenterology* 2005;128:1089–1113.
- ▶ 27 Bischoff SC, Sellge G, Lorentz A, Sebald W, Raab R, Manns MP: IL-4 enhances proliferation and mediator release in mature human mast cells. *Proc Natl Acad Sci USA* 1999;96: 8080–8085.
- ▶ 28 Mathias CB, Hobson SA, Garcia-Lloret M, Lawson G, Poddighe D, Freyschmidt EJ, Xing W, Gurish MF, Chatila TA, Oettgen HC: IgE-mediated systemic anaphylaxis and impaired tolerance to food antigens in mice with enhanced IL-4 receptor signaling. *J Allergy Clin Immunol* 2011;127:795–805.
- ▶ 29 Strait RT, Morris SC, Yang M, Qu XW, Finkelman FD: Pathways of anaphylaxis in the mouse. *J Allergy Clin Immunol* 2002;109: 658–668.
- ▶ 30 Brandt EB, Strait RT, Hershko D, Wang Q, Muntel EE, Scribner TA, Zimmermann N, Finkelman FD, Rothenberg ME: Mast cells are required for experimental oral allergen-induced diarrhea. *J Clin Invest* 2003;112: 1666–1677.
- ▶ 31 Liu X, Yu D, Wang T: Sappanone A attenuates allergic airway inflammation in ovalbumin-induced asthma. *Int Arch Allergy Immunol* 2016;170:180–186.
- ▶ 32 Ci X, Zhong W, Ren H, Wen Z, Li D, Peng L: Esculentoside A attenuates allergic airway inflammation via activation of the Nrf-2 pathway. *Int Arch Allergy Immunol* 2015;167: 280–290.
- ▶ 33 Kattan JD, Srivastava KD, Zou ZM, Goldfarb J, Sampson HA, Li XM: Pharmacological and immunological effects of individual herbs in the food allergy herbal formula-2 (FAHF-2) on peanut allergy. *Phytother Res* 2008;22: 651–659.
- ▶ 34 Yamamura K, Kato S, Kato TA, Mizoguchi Y, Monji A, Kanba S, Furue M, Takeuchi S: Anti-allergic mechanisms of Japanese herbal medicine, yokukansan on mast cells. *J Dermatol* 2014;41:808–814.

Supplemental figure 1

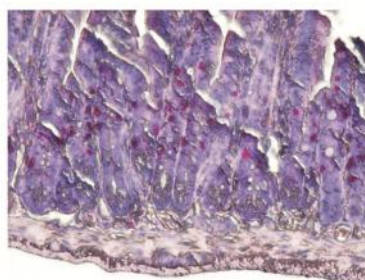


Supplemental figure 2

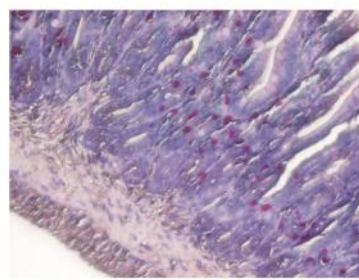
A



B



C



Supplemental figure 1

Eppikajutsuto (1.0 g) was extracted with methanol (20 mL) under ultrasonication and was centrifuged. The supernatant was filtered and then analyzed by high-performance liquid chromatography.

Supplemental figure 2

Small and large intestines of control, food allergy (FA), and eppikajutsuto (EJT)-treated mice were collected after the evaluation of FA symptoms elicited by the administration of 50 mg ovalbumin (OVA). Mast cells were identified by naphthol AS-D chloroacetate esterase staining. Representative histology of small intestinal specimens from control (A), FA (B), and EJT-treated FA (C) mice are shown. Scale bar represents 50 μm .