ORIGINAL ARTICLE

Assessment of relief from pruritus due to Kampo medicines by using murine models of atopic dermatitis

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ABSTRACT

Aim: Atopic dermatitis is a representative intractable chronic eczematous skin disease. Because the symptoms vary between individuals, several therapies are required for atopic dermatitis. Since kampo medicines should be used according to the particular physical and mental conditions, the use of distinctive animal models is necessary for the experimental assessment of the efficacy of kampo medicines. In this study, 7 kampo medicines were assessed for their anti-itching effect in 2 types of atopic dermatitis-like pruritus models for characterizing.

Methods: Long-lasting scratching behavior was induced by multiple treatments with 2,4,6-trinitrochlorobenzene (TNCB) for 4 weeks in NC mice. Kampo medicines were administered orally for 11 d from the following day of the last TNCB-treatment to assess curing pruritus. In the other model, TNCB was applied to ears of BALB/c mice for 10 d every other day to induce scratching behavior. Kampo medicines were administered orally everyday for the same term of the induction of scratching behavior to assess the prevention of dermatitis.

Results: Shofusan (SFS) had an instant effect, even on using a single dose, on scratching behavior in the model of NC mice; it ameliorated chronic itching on oral administration for 11 d, by preventing mast cell differentiation and degranulation. In the model of BALB/c mice, another kampo medicine, hochuekkito (HET) inhibited the induction of scratching behavior.

Conclusion: Kampo medicines such as SFS and HET may improve chronic itching sensation in atopic dermatitis to the same extent as or better than existing antiallergic medicines.

KEY WORDS: alternative medicine, mast cell degranulation, scratching behavior

INTRODUCTION

Atopic dermatitis was defined as a relapsing inflammatory skin disease caused by the intervention of plural unspecific stimulation or specific allergens accompanied with cutaneous physiological dysfunction [1]. It is caused by an imbalance of immunologic responses [2], skin-barrier disruption [3,4], and the excessive response of peripheral nerves to stimuli [5].The numbers of eosinophils and mast cells increase in the peripheral blood and lesioned skin of atopic dermatitis patients [6,7]. The extension of peripheral nerves to the surface layer of the skin causes itchy skin, or "alloknesis" [5,8]. Scratching behavior aggravates the skin symptoms in the patients [9]; the disruption of the skin barrier by scratching exacerbates inflammation, and this inflammation promotes nerve growth. This cycle has been called the vicious itch-scratch cycle, and interruption of the cycle is an important step for the treatment of atopic dermatitis.

The Japanese guidelines for atopic dermatitis suggest that topical steroids be used as primary therapy [10]. Antihistamines and antiallergics should be added to therapy as required. The guidelines also mention that herbal medicines should be used as adjunctive therapy. However, detailed descriptions of the symptoms that are appropriate for treatment by Kampo medicine have not been mentioned in the guideline. Therefore, we attempted to characterize the efficacy of 7 Kampo medicines, juzentaihoto (JTT), hochuekkito (HET), orengedokuto (OGT), shofusan (SFS), yokukansan (YKS), jizusoippo (JZI), and seijyobofuto (SBT), according to the symptoms of atopic dermatitis. JTT and HET are treated to complex syndrome, containing chronic allergy, caused by mental fatigue [11,12]. OGT has effects for erythema due to inflammation, and SFS is used for severe pruritus [13]. Because atopic dermatitis is aggravated by psychological stress [14]. YKS alleviates the development of allergic eczema by psychological

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DOI:10.1111/tkm2.12XXX
Received 17 January 2013; accepted 30 April 2013

stress [15]. JZI and SBT are often used for cephalic and facial dermatitis.

We previously established a murine dermatitis model by repeated application of 2,4,6-trinitrochlorobenzene (TNCB) to both ears in BALB/c mice [16]. Eosinophilic infiltration, acanthosis, hyperkeratosis, and dermal papilla were observed in the entire ear of mice to which TNCB was applied. In addition, the mice exhibited continuous scratching behavior. On the basis of our results with T cell–deficient BALB/c-nu/nu mice and mast cell–deficient WBB6F1-W/W^{v} and Sl/Sl^{d} mice, we suggested that the differentiation and degranulation of mast cells play a pivotal role in inducing chronic scratching behavior. We also reported that suppressing mast cell degranulation by treatment with anti-allergic medications reduced scratching behavior in mice painted repeatedly with TNCB [17]. These data indicate that the inhibition of mast cell activity can help control the serial itch-scratch cycle in atopic dermatitis.

In the present study, we applied Kampo medicines to 2 hapten-treated mouse models. First model involved using NC mice that were painted with 1% TNCB on alternate days for 4 weeks. Efficacies of Kampo medicines on persisting scratching were evaluated by two approaches in NC mice model. One was instant antipruritic by single oral administration, and the other was curing pruritus by serial administrations for 11 d. Second model involved BALB/c mice that were painted with 1% TNCB on alternate days for 10 d. We attempted to evaluate the prevention of dermatitis induction to administrate Kampo medicines at the same time with hapten-treatment.

As results, SFS and HET were found to ameliorate dermatitis with scratching behavior.

MATERIALS AND METHODS

Reagents

TNCB, olive oil, acetone and HPLC-grade methanol were purchased from Nacalai Tesque (Kyoto). Azelastine hydrochloride (AZT) was supplied by Eizai (Tokyo). AZT at a dose of 10 mg/kg body weight was dissolved in distilled water, and administered orally as a positive control. Toluidine Blue O was purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Home-reared female NC/Jic mice (6–9 weeks) were used for the experiments (original pair was obtained from Clea Japan). Female BALB/c mice (7 weeks) were purchased from Charles River Laboratories Japan, Inc. (Yokohama). The mice were kept under conditions of controlled temperature (23°C ± 2°C), humidity (50% ± 10%), and light (lights switched on from 6:00 to 18:00). The mice had ad libitum access to food (CE-2; Clea) and water. The experimental procedures were approved by the Animal Care Committee of the Graduate School of Pharmaceutical Sciences, Nagoya City University, and were in accordance with the guidelines of the Japanese Council on Animal Care.

Preparation of kampo medicines

Seven kampo medicines were extracted according to the formula shown in Table 1: juzentaihoto, JTT; hochuekkito, HET; orengedokuto, OGT; shofusan, SFS; yokukansan, YKS; jizusoippo, JZI; seijyobofuto, SBT. The formula follows daily dose in TSUMURA KAMPO MEDICINE FOR ETHICAL USE. The medicinal components for kampo medicines were provided by Tsumura (Tokyo). The mixture of the components weighed at grams in table 1 was extracted with 600 ml water at 100°C for 1 h. The extract was freeze-dried, and calculated roughly equivalent to 10 times the daily human dose, as follows: JTT, 1.8 g/kg body weight; HET, 1.8 g/kg body weight; OGT, 0.6 g/kg body weight; SFS, 1.5 g/kg body weight; YKS, 1.1 g/kg body weight; JZI, 0.8 g/kg body weight; and SBT, 1.5 g/kg body weight. The freeze-dried extracts were dissolved in distilled water just before each oral administration.

Component profiles were assessed by HPLC. Each kampo extract at a weight of 0.1 g was dissolved in 2 ml methanol followed by ultrasonic extraction for 30 min. The extracts were centrifuged and filtered for HPLC analyses. HPLC was run with an LC-10AD$_{vp}$ pump and an SPD-M10A$_{vp}$ detector (Shimadzu, Kyoto) with the following conditions: column, TOSOH TSK-GEL ODS-80$_{TS}$ (4.6 × 250 mm); flow rate, 1.0 ml/min; column temperature, 40°C; detector, UV 200–400 nm; and eluant, 50% methanol to 100% methanol for 40 min (linear gradient). HPLC profiles of HET, OGT, and SFS are shown in Fig. 1 (JTT, YKS, JZI, and SBT are not shown).

Assessment for antipruritic and curing pruritus

NC mice were painted with 20 μl TNCB dissolved in acetone:olive oil (4:1) as a 1% (w/v) solution on the face of both ears. Six days later, the mice were challenged by painting with 20 μl of 1% TNCB solution repeatedly every 48 h for 4 weeks (Fig. 2A). In the vehicle-treated group, acetone:olive oil (4:1) was applied instead of TNCB. Two days later, Kampo medicines or AZT were administered orally after conformation of a lasting scratching for 4 h. Instant antipruritic effect was evaluated for 8 h after the administration.

Subsequently, we consecutively administered the Kampo medicines orally everyday for 11 d to assess curing pruritus (Fig. 3A). Scratching behavior was then measured again for 8 h, one day after the last oral administration. Ear thickness was measured as an indicator of inflammation by a dial thickness gauge (G-1A, Ozaki MFG. Co., Ltd, Tokyo) before the scratching measurement. Bloods for measurement of IgE levels and ear tissues for histological analysis were collected after the evaluation of scratching.

Assessment for prevention

BALB/c mice were painted with 1% TNCB on alternate days for 10 d after the sensitization by TNCB painting (Fig. 4A). To assess the prevention of dermatitis, Kampo medicines were administered at the same time with hapten-treatment. Efficacy of kampo medicines was evaluated similar as the first model.

Table 1 | Formulations and compositions of Kampo medicines

Component	Juzentaihoto (JTT)	Hochuekkito (HET)	Orengedokuto (OGT)	Shofusan (SFS)	Jizusoippo (JZI)	Seijyobofuto (SBT)	Yokukansan (YKS)
Astragali Radix	3.0 g	4.0 g					
Cinnamomi Cortex	3.0 g						
Rehmanniae Radix	3.0 g			3.0 g			
Paeoniae Radix	3.0 g						
Cnidii Rhizoma	3.0 g				3.0 g	2.5 g	3.0 g
Atractylodis Lanceae Rhizoma	3.0 g	4.0 g		2.0 g	3.0 g		4.0 g
Angelicae Radix	3.0 g	3.0 g		3.0 g			3.0 g
Ginseng Radix	3.0 g	4.0 g					
Poria	3.0 g						4.0 g
Glycyrrhizae Radix	1.5 g	1.5 g		1.0 g	1.0 g	1.0 g	1.5 g
Bupleruli Radix		2.0 g					2.0 g
Zizyphi Fructus		2.0 g					
Aurantii Nobilis Pericarpium		2.0 g					
Cimicifugae Rhizoma		1.0 g					
Zingiberis Rhizoma		0.5 g					
Scutellariae Radix			3.0 g			2.5 g	
Coptidis Rhizoma			2.0 g			1.0 g	
Gardeniae Fructus			2.0 g			2.5 g	
Phellodendri Cortex			1.5 g				
Gypsum Fibrosum				3.0 g			
Bardanae Fructus				2.0 g			
Saposhnikoviae Radix				2.0 g	2.0 g		
Akebiae Caulis				2.0 g			
Anemarrhenae Rhizoma				1.5 g			
Sophorae Radiix				1.0 g			
Schizonepetae Spica				1.0 g	1.0 g	1.0 g	
Sesami Semen				1.5 g			
Cicadae Periostracum				1.0 g			
Carthami Flos					1.0 g		
Rhei Rhizoma					0.5 g		
Lonicerae Folium cum Caulis					2.0 g		
Platycodi Radix						2.5 g	
Glrhniae Radix cum Rhizoma						2.5 g	
Angelicae Dahuricae Radix						2.5 g	
Forsythiae Fructus						2.5 g	
Aurantii Fructus Immaturus						1.0 g	
Menthae Herba						1.0 g	
Uncariae Uncus et Ramulus							3.0 g

Evaluation of scratching behavior

Scratching behavior was measured using the MicroAct (Neuroscience, Tokyo) in which scratching activity could be detected automatically and analyzed objectively [18,19]. Briefly, a ring-type magnet (1.0-mm inner diameter, 2.5-mm outer diameter, 2.0-mm height, and 130–145 mT/cm^2) was attached to both ankles with steel wire (No. 34, TOHO, Hiroshima). Ten to 15 h after magnet placement, each mouse was placed in a plastic chamber (11 cm in diameter and 18 cm in height) surrounded by round coils, and current induced in the coils by movement of the magnets was amplified and recorded. Characteristic signals were identified as scratching behavior using

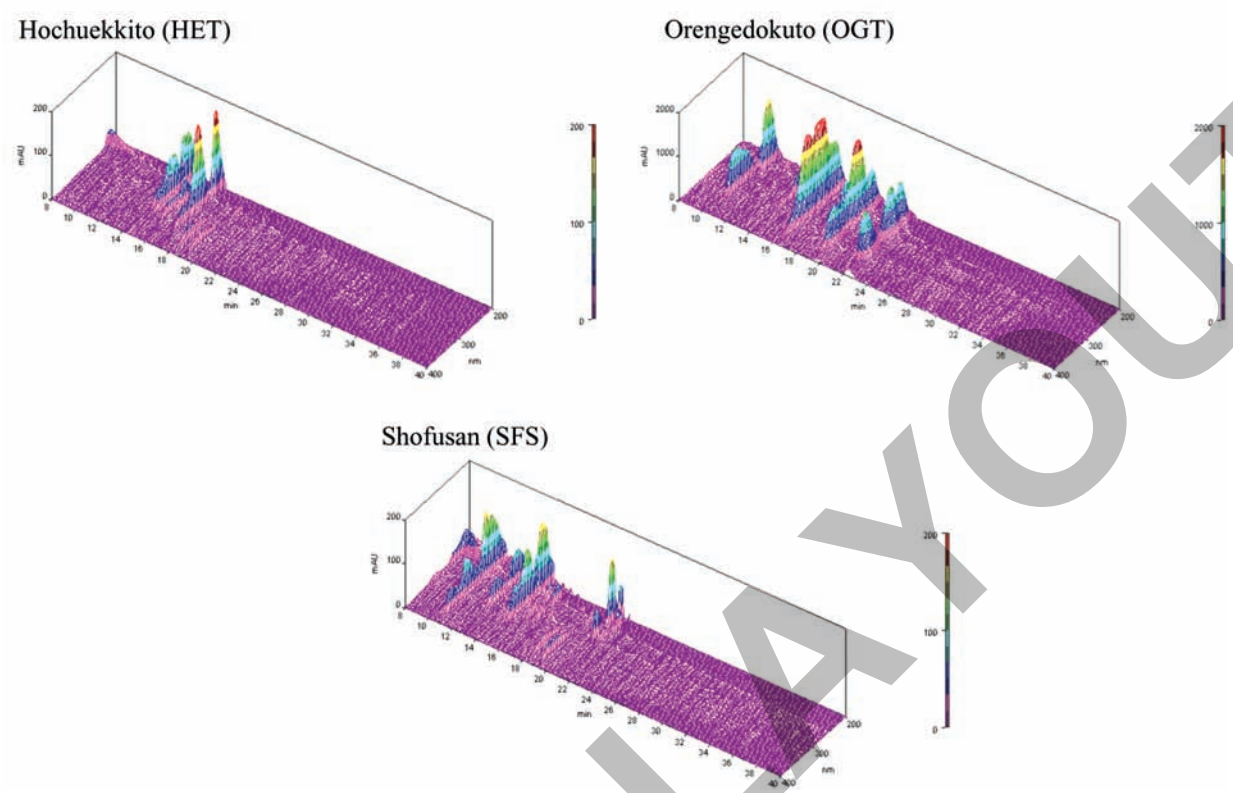

Figure 1 | HPLC profiles of Kampo medicines. HPLC was run according to described in Materials and Methods.

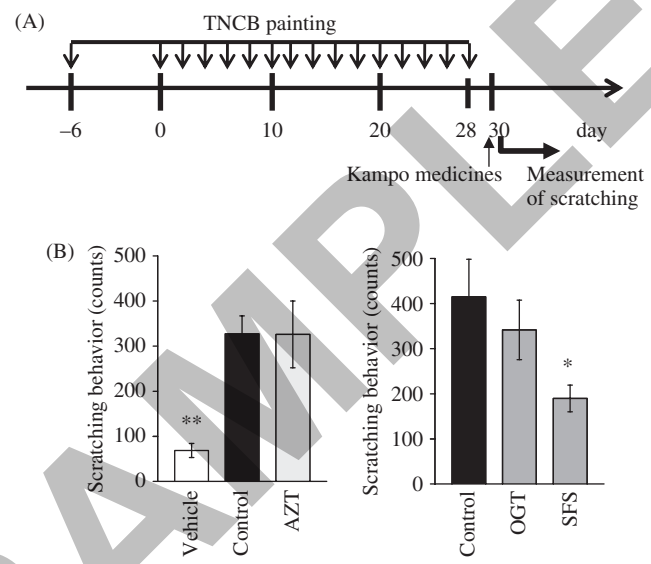

Figure 2 | Effect of Kampo medicines by a single treatment in an atopic dermatitis-like pruritus model. A) Atopic dermatitis-like pruritus was induced by repeated application of TNCB to both ears of NC mice every other day for 4 weeks. Scratching behavior was measured 48 h after the last application. B) Scratching was measured for 8 h after the oral administration of 0.6 g/kg body weight of orengedokuto (OGT), 1.5 g/kg body weight of shofusan (SFS), and 10 mg/kg body weight of azelastine hydrochloride (AZT). Each column represents the mean ± S.E.M. of 6 mice. *,**$p < 0.05$, 0.01 *vs.* control.

the following parameters: threshold, 0.12 V; event gap, 0.02 s; minimum duration, 0.40 s; maximum frequency, 18.0 Hz; and minimum frequency, 5.0 Hz. The measurement of scratching behavior was initiated 48 h after the final application of TNCB, since non-specific stimuli induce unfavorable scratching episodes[16]. The mice were kept in the device for 2 h to acclimatize to the measurement environment. Scratching behavior was recorded for 4 h without the administration of medicine, and then for an additional 8 h following the administration.

Histological observation

After recording the scratching behavior, the mice were sacrificed, and the right ears were excised. The ears were fixed with 10% phosphate-buffered formalin (pH 7.2) and embedded in paraffin. Sections (3 μm) were stained with acidic toluidine blue (pH 4.1). The number of mast cells per square millimeter of dermis in 4 sites chosen at random was counted. The phenotype of the mast cells was classified as degranulate (>10% of the cytoplasmic granules exhibiting fusion or discharge) or normal, and the proportion of degranulated cells was calculated [20,21].

Determination of total immunoglobulin E (IgE) titer in serum

Blood was collected from the scarified mice and serum was stored at −20°C until use. The concentration of total

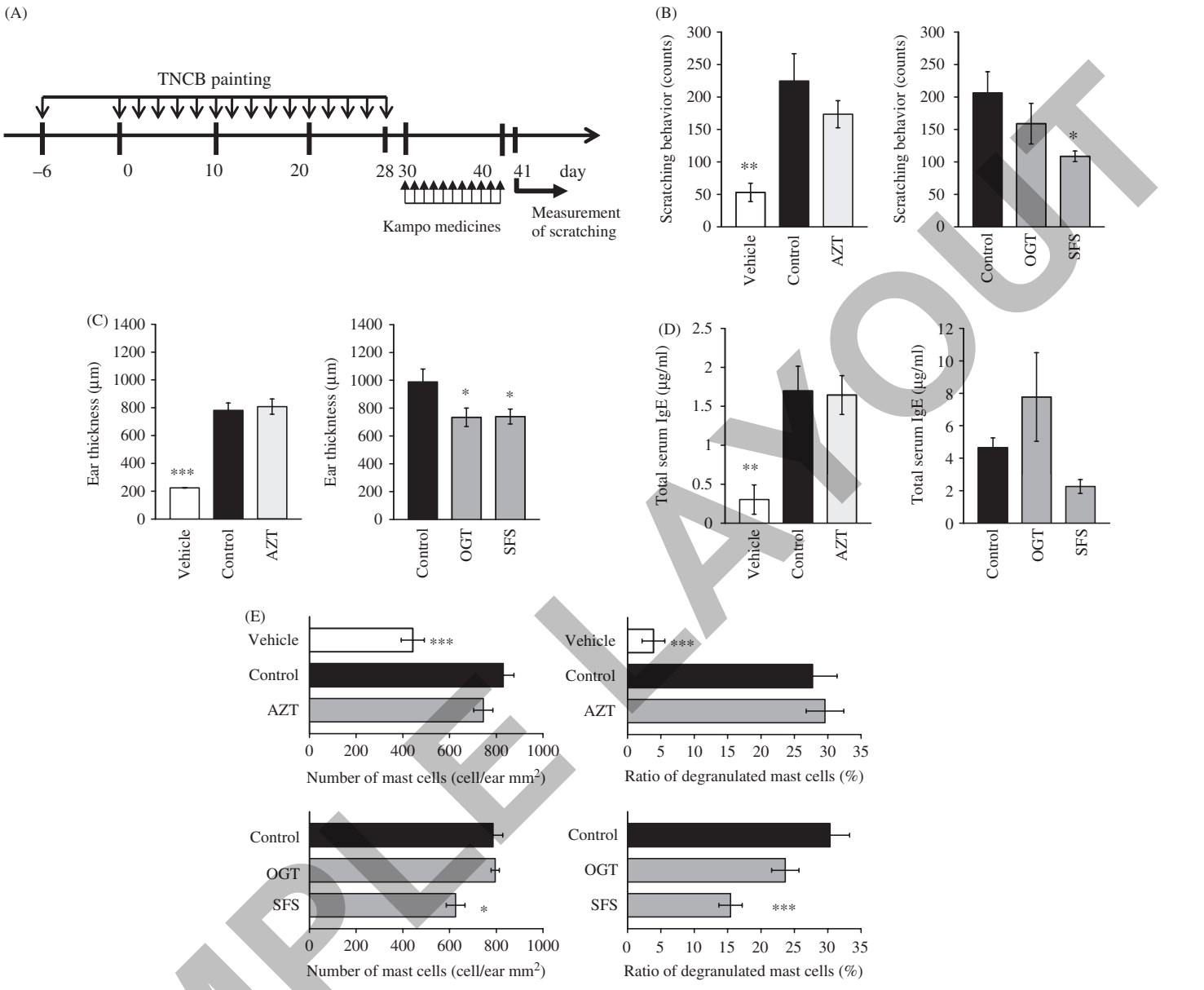

Figure 3 | Assessment of Kampo medicine efficacy for curing of chronic scratching behavior. A) Kampo medicines (0.6 g/kg body weight of orengedokuto, OGT; 1.5 g/kg body weight of shofusan, SFS) and 10 mg/kg body weight of azelastine hydrochloride (AZT) were administered orally to NC mice which have induced dermatitis with pruritus by painting with TNCB continuously for 11 d. The efficacy of each kampo medicine was estimated by scratching behavior, ear thickness, IgE level, and histological analysis. B) Scratching behavior was measured for 8 h starting at 24 h after the last oral administration of medicine. C) Ear thickness was measured as an indicator of inflammation before the scratching measurement. D) Blood was collected after scratching measurement, and IgE was measured by ELISA. E) Ear tissues were collected after the scratching measurement. Ear sections of 3-μm thickness were stained by toluidine blue. Mast cell number and the proportion of mast cell degranulation were estimated by microscopy. Each column represents the mean ± S.E.M. of 6 mice. *,**,***$p < 0.05$, 0.01, 0.001 *vs.* control.

IgE in the serum was measured by a sandwich enzyme-linked immunosorbent assay (ELISA), as previously described [22].

Statistics

All results are presented as mean ± S.E.M. values. Statistical analyses were conducted by Dunnett's multiple *t*-test.

RESULTS

NC mice were painted with 1% TNCB every 48 h for 4 weeks. Scratching behavior was measured 48 h after the last TNCB challenge (Fig. 2A). The efficacy of each kampo medicine on scratching behavior was estimated for 8 h after its oral

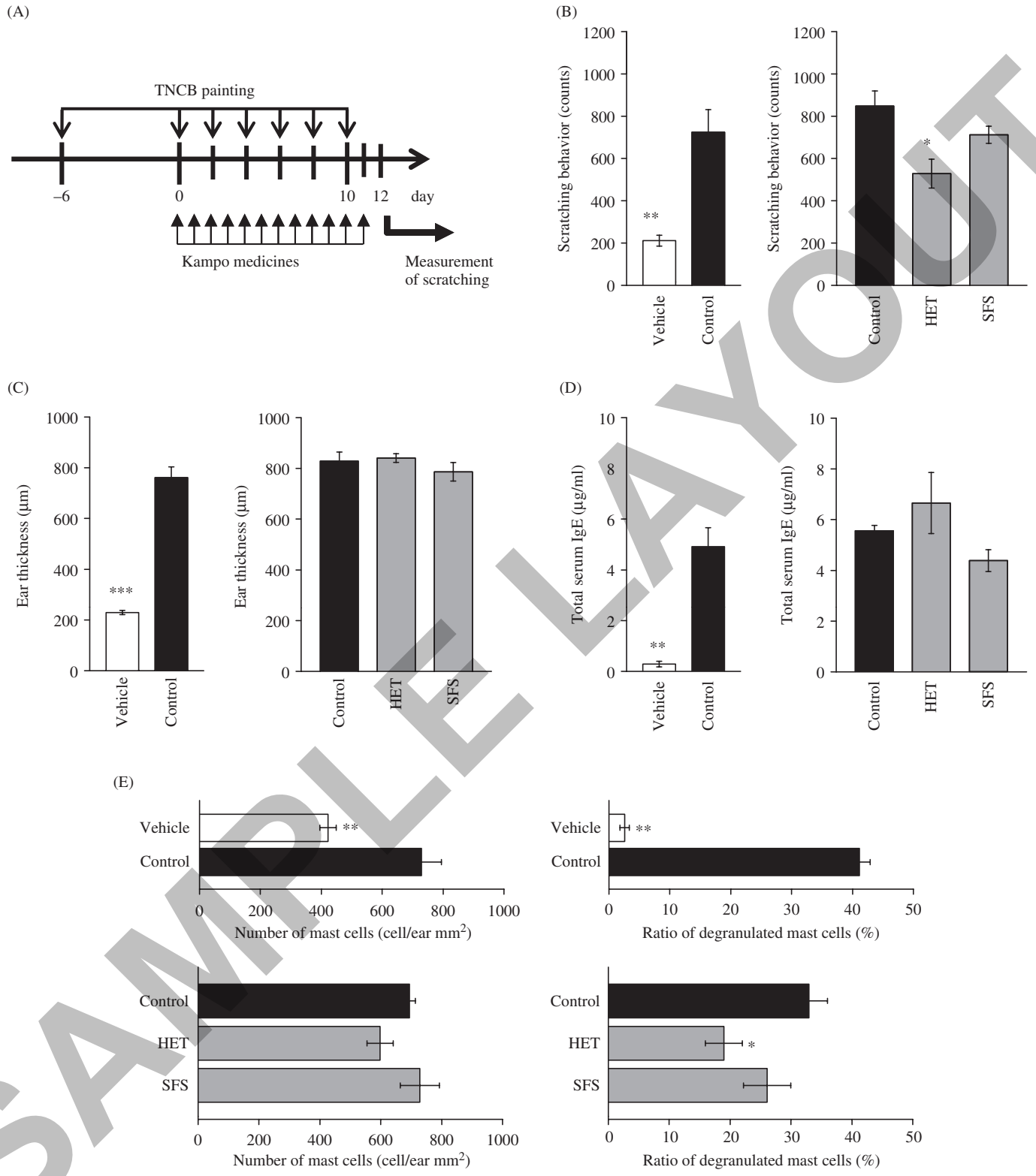

Figure 4 | Assessment of the preventive effect of Kampo medicines on the induction of chronic scratching behavior. A) Kampo medicines (1.8 g/kg body weight of hochuekkito, HET; 1.5 g/kg body weight of shofusan, SFS) were administered orally to BALB/c mice in the same time period as painting with TNCB. B) Scratching was counted for 8 h beginning 24 h after the last administration of medicines. C) Ear thickness and D) IgE levels in the blood were measured. E) Mast cell number and the proportion of mast cell degranulation were estimated by toluidine blue–stained ear sections. Each column represents the mean ± S.E.M. of 5–6 mice. *,**,***$p < 0.05$, 0.01, 0.001 *vs.* control.

administration followed by confirmation of a lasting scratching for 4 h. There were no differences of the scratching for 4 h between control and each Kampo treated group (date not shown). The results are shown in Fig. 2B. SFS significantly reduced the number of scratching behaviors in a single treatment. We expect the suppression by AZT with antiallergic effects, but we could not get the result (Fig. 2B). There were no differences in ear thickness or IgE levels in blood between control and Kampo-treated groups (data not shown). The other Kampo medicines (JTT, HET, JZI, SBT, and YKS) could not attenuate the number of scratching behavior (data not shown). We consecutively administered the Kampo medicines everyday for 11 d (Fig. 3A). Scratching behavior was then measured again for 8 h, one day after the last treatment. SFS also suppressed scratching behavior in this measurement, in which we wished to demonstrate the curing of pruritus (Fig. 3B). OGT and SFS ameliorated the ear thickness (Fig. 3C). Swelling of epidermis and dermis in ear of the mice treated with OGT or SFS had a tendency to be thinner than control in histopathological analysis (data not shown). In the mice treated with SFS, the number of mast cells and the rate of degranulation (Fig. 3E), and the IgE level in serum was apt to be reduced (Fig. 3D). SFS may therefore have an effect on mast cell activity. AZT could not ameliorate the scratching behavior and ear swelling, not improve IgE production, and not suppress the degranulation of mast cells. The other Kampo medicines (JTT, HET, JZI, SBT, and YKS) did not have antipruritic efficacy in this model (data not shown).

We next tried to determine the efficacies of kampo medicines on the induction of dermatitis and scratching. In this study, Kampo medicines were administered during the same time period as hapten painting (Fig. 4A). HET inhibited the degranulation of mast cells and decreased the extent of scratching (Fig. 4B and E). HET could not suppress changes in ear thickness and IgE production (Fig. 4C and D). The other kampo medicines (JTT, OGT, JZI, SBT, and YKS) did not effect on induction of chronic scratching in this model (data not shown.)

DISCUSSION

Atopic dermatitis is a disease comprising skin dysfunction and immune imbalance. Atopic dermatitis depends on genetic background [23–25], as well as environmental factors [26]. Atopic dermatitis comprising various phenotypes is a syndrome; the degree of rash and itching depends on the patient's age and season. Although the primary therapy for atopic dermatitis is topical steroids, additional remedies, such as antihistamines or antiallergics and humectants, are used as needed. In some cases, alternative complementary medicines are applied to atopic dermatitis to reduce the use of steroids.

A multicenter, double-blind, randomized, placebo-controlled clinical study was conducted for the treatment of atopic dermatitis with HET [12]. In that study, HET was used as an additional therapy to topical steroids or immunosuppressants. The study demonstrated that HET was a useful adjunctive treatment for atopic dermatitis. The use of HET significantly reduced the dose of topical steroids and tacrolimus without aggravating dermatitis. These data indicate that Kampo medicines are useful for the therapy of atopic dermatitis.

In this study, we assessed 7 kampo medicines JTT, HET, OGT, SFS, YKS, JZI, and SBT—which have been used on atopic dermatitis patients. JTT and HET composed of Ginseng Radix, Astragali Radix, and other components, enhance physical and mental strength, and regulate basal immune competence. OGT is used for dermatitis with a burning sensation, and SFS is used for eczema madidans with persistent itching. YKS is used for dermatitis that is exacerbated by mental stress. YKS was reported to suppress the exacerbation of eczema by psychosocial stress in mouse models [15,27,28]. JZI and SBT are used for cephalic and facial eczema. We previously assessed the efficacy of JTT, HET, OGT, and SFS using another atopic dermatitis model in which dermatitis was induced by mite antigens [29]. These Kampo medicines corrected the imbalance of Th1/Th2 and inhibited the development of dermatitis.

We used AZT as a positive control, because AZT have suppressed scratching behavior evoked by injection of compound 48/80 which is an degranulation-inducer in mast cells, or substance-P and leukotriene B4 which are mediators from mast cells related with itch [30–32]. Although scratching behavior in our model depends on mast cells degranulation [16], we could not confirm the suppression by AZT. Scratching with chronic inflammation might be not only mast cells' mediators, also other indirect factors, such as T cells and nerve related factors [33,34]. We should have used immunosuppressor such as tacrolimus.

Atopic dermatitis is chronically relapsing complex syndromes containing pruritus and typical eczematous dermatitis such as scales and crusts [1]. Because we can't represent all syndromes, we made the murine model expressing a part of atopic dermatitis. In the studies, we evaluated the medicines using 2 murine models of atopic dermatitis. Characteristic, long-lasting scratching action for more than 48 h was induced by multiple treatments with TNCB on alternate days [16,17].

In the first model, we estimated the single dose effect on chronic pruritus and relief of inflammation and scratching. Chronic scratching behavior was suppressed by a single treatment of SFS, though the blood IgE levels were equivalent among the groups at the time of the first administration of medicine. There were also no differences in scratching frequency and ear thickness before kampo administration (data not shown). Continuous treatment with SFS ameliorated changes in ear thickness, scratching behavior, and IgE production. Additionally, SFS inhibited mast cell degranulation and reduced mast cell number. We propose that the inhibition of IgE production and the suppression of mast cell degranulation and proliferation by SFS effects the amelioration of itching and the suppression of inflammation.

SFS is composed of 13 crude components (Table 1). Seven components, Gypsum Fibrosum, Bardanae Fructus, Akebiae Caulis, Anemarrhenae Rhizoma, Sophorae Radix, Sesame Semen, and Cicadae Periostracum, are not included in the other kampo medicines used in this study. We expect that among these components is the effective ingredient for pruritus.

Byakkokaninjinto is the representative kampo formula containing Gypsum Fibrosum. Byakkokaninjinto suppressed IgE-dependent eczema [35], and itching by cooling the skin temperature [36]. It was also reported that Sophorae Radix suppressed acute scratching in mice that was induced by the injection of serotonin [37]. We believe that the antipruritic effect of SFS may be caused by a combination of these components.

SFS is used to treat eczema with persistent itching in clinical practice. This symptom was expressed in our murine model. We now show evidence of the efficacy of SFS treatment.

In the second model, we evaluated the preventive effect of Kampo medicines on the induction of inflammation and pruritus. HET suppressed scratching behavior with inhibition of mast cell degranulation. SFS could not reduce scratching in the second model. SFS appeared to have little efficacy for elicitation with pruritus. It was reported that HET inhibited inflammatory dermatitis with increases in IgE level in chronic contact hypersensitive model by painting with hapten [38]. This report demonstrated increasing in interleukin 4 level in inflamed ear tissue was suppressed by oral administration of HET. Our laboratory also has shown HET inhibit elevation of serum IgE level via regulation of Th1/Th2 balance in mite antigen-induced dermatitis model [29]. Because the term of administration of HET was shorter than in the reported models, we might be unable to detect suppression of IgE production.

Atopic dermatitis comprises many possible symptoms, and the symptoms change with seasons and age. We believe that Kampo medicines composed of multiple crude medicines can adapt to complex syndromes. We could demonstrate one part of efficacies in this study. However, we need to show more evidences that kampo medicines regulate immune system, such as T cells response and dendritic cells' migration. To use Kampo medicines more often in a clinical setting, we need to provide further evidence of the efficacy of Kampo medicines using basal experiments.

CONFLICT OF INTEREST

Authors declare no conflict of interests for this article.

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