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Oral administration of Yokukansan inhibits the development of atopic dermatitis-like lesions in isolated NC/Nga mice

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ABSTRACT

Background: Increasing evidence suggests that stress can trigger and exacerbate atopic dermatitis (AD). Psychotherapy is becoming more important in the treatment of AD patients. Yokukansan (YKS, Yi-Gan San in Chinese), a traditional Japanese medicine, has been widely utilized in the treatment of neurosis, insomnia and anxiety especially in Asian countries. Furthermore, it was reported that YYS inhibited skin lesions in socially isolated mice but not in group-housed mice. Therefore, in the present study it was investigated whether or not YYS was effective in the treatment of AD using socially isolated NC/Nga mice.

Objective: The present study was designed to assess the effect of YYS on the development of AD-like lesions in socially isolated NC/Nga mice to obtain information about its usefulness in the treatment of AD.

Methods: Ten-week-old male NC/Nga mice were socially isolated under conventional conditions. YYS was administered orally to mice at the dose of 0.5% or 1.0% together with diet. The efficacy of YYS was evaluated by assessing skin lesion severity, scratching behaviors, skin hydration, and infiltration of inflammatory cells in the skin. Grooming behaviors evoked by social isolation stress and serum corticosterone levels were also measured.

Results: Oral administration of YYS to socially isolated NC/Nga mice resulted in the inhibition of exacerbation of AD-like skin lesions. It seemed that the inhibition of exacerbation of AD-like skin lesions observed in NC/Nga mice might be due to suppression of the scratching and grooming behaviors, inhibition of the infiltration of mast cells and eosinophils, and retention of humidity in the skin. Serum corticosterone levels were also significantly inhibited in the 1%-YYS-treated mice as compared with those of the control mice. There were no significant differences in the levels of serum total IgE and nerve growth factor (NGF) between the YYS-treated mice and the non-treated control mice.

Conclusion: YYS inhibited the development of AD-like skin lesions in socially isolated NC/Nga mice by suppressing scratching and infiltration of inflammatory cells in the skin. These results indicate that YYS possesses an anti-itching property, and its anti-itching may be partly through attenuation on social isolation stress. It is expected that YYS might provide an effective alternative therapy for AD in human patients.

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Abbreviations: YYS, Yokukansan; AD, atopic dermatitis.

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1. Introduction

Atopic dermatitis (AD) is a chronic pruritic skin disease associated with increased anxiety. Psychological stress constitutes an increased risk for AD in human [1–3]. Increasing evidence suggests that stress induces or exacerbates AD in human patients [4]. Psychological treatment documents a positive effect on the anxiety level as well as on the course and management of AD [5,6]. Itching is also a serious problem in AD patients because scratching

worsens dermatitis itself. AD patients tend to judge the severity of their eczema more by itchiness than by the feature of the skin lesions. Prevention of itch-associated scratching behaviors has been considered as an effective treatment for AD [7]. Nevertheless, conventional therapies often fail to alleviate severe types of chronic itching. In the previous research we reported that paroxetine (a selective serotonin reuptake inhibitor), an antidepressant, possesses an anti-itching property and inhibits the development of AD-like skin lesions in NC/Nga mice, indicating that centrally acting therapeutic drugs are useful for anti-itching treatment [8].

YKS, a serotonin modulator, traditionally utilized in Japan based on traditional Chinese medicine, has been used as an anti-anxiety agent in treating neurosis, insomnia, and children with night cry [9,10]. Interestingly, it was also reported that YYS possessed anti-allergic effects on IgE mediated triphasic cutaneous reaction in socially isolated mice but not in group-housed mice [11]. However, it has never been reported whether or not YYS is effective in the treatment of AD.

The NC/Nga mouse has been regarded as the most appropriate animal model for evaluating potential therapeutic drugs for human AD [12–14]. NC/Nga mice spontaneously develop dermatitis under conventional conditions, such as erythema, edema, bleeding, hair loss, skin dryness, and scratching behaviors similar to those seen in human AD patients. Social isolation is a model of psychosocial stress that deprives social interaction of animals, and is considered to be relatively comparable to the situation of humans who feel isolated [15]. Social isolation stress enhances grooming, jumping, and aggressiveness in mice with high levels of plasma corticosterone and corticotropin releasing hormone [16]. Furthermore, several studies demonstrated that stress triggered AD in NC/Nga mice due to the secretion of corticotropin releasing hormone and induction of mast cell degranulation in the skin [17–19]. Grooming behaviors could also exacerbate dermatitis in compulsive mice [20]. In the present study, we evaluated serum corticosterone levels and grooming behaviors as parameters of stress levels caused by social isolation in NC/Nga mice. We also assessed the inhibitory effect of YYS on the development of dermatitis to obtain basic information about the usefulness of YYS in the treatment of AD in human.

2. Materials and methods

2.1. Animals

Ten-week-old male NC/Nga mice with mild skin lesions were obtained from SLC Inc. (Shizuoka, Japan). NC/Nga mice were individually housed (cage size: 9(w) cm × 13(h) cm × 20(d) cm) in an animal room maintained at 24 ± °C room temperature, 55 ± 10% relative humidity, and 12–12 h light–dark cycle under conventional conditions. The mice with the skin lesion severity score of about 2 and with the scratching frequency of greater than 15/30 min were divided to each group with the number of mice being 6/group. The mice were allowed to have water and diet *ad libitum*. All the procedures performed on animals were approved by the institutional animal care and use committee (IACUC) of Juntendo University, with the approval number of 200108.

2.2. Drug

YKS, supplied by Tsumura & Co. (Tokyo, Japan), is a vacuum-concentrated extract of seven herbs in the following ratio: 4.0 g of *Attractylodes lancea* Rhizome, 4.0 g of Hoelen, 3.0 g of *Cnidium* Rhizome, 3.0 g of Japanese Angelica Root, 2.0 g of *Bupleurum* Root, 1.5 g of *Glycyrrhiza* Root, and 3.0 g of *Uncaria* Thorn. Each plant material was authenticated by identification of external morphol-

ogy and marker compounds of plants specimens, according to the methods of the Japanese Pharmacopoeia and the Tsumura's standard. The seven medical herbs were extracted with purified water at 95 °C for 1 h, and the extraction solution was separated from the insoluble waste and concentrated by removing water under reduced pressure. The spray-drying technique was used to produce dried extract powder. The mice were given the diet mixed with YYS (Lot No. 2060054020) at the dosage of 0.5% or 1.0% (i.e., 0.6 or 1.2 g/kg body weight). The control mice were given the standard mouse diet (CRF-1, Oriental Yeast Co., Ltd., Japan).

2.3. Evaluation of the severity of dermatitis and observation of scratching and grooming behaviors

Clinical skin lesion severity scores and scratching behaviors were evaluated once a week for 12 weeks. Skin lesions such as erythema, edema, bleeding, erosion, hair loss, and dryness were expressed as follows: no lesion: 0; mild: 1; moderate: 2; severe: 3. The mice were placed in transparent acrylic cylinders (1 mouse/cylinder) for 15 min for acclimatization. The frequency of scratching behaviors such as scratching of the nose, ears and the dorsal skin with hind paws was measured in a 30-min period. Grooming behaviors were defined as grooming of the face and ears with the front paws and licking behaviors. The mice were placed in transparent acrylic cylinders (1 mouse/cylinder), and the frequency of grooming behaviors was counted in a 10 min period once in every 3 weeks. After 12 weeks, the non-treated control mice were treated with 1% YYS for additional 6 weeks in order to examine the “therapeutic effect” rather than the “preventive effect” of YYS on dermatitis.

2.4. Measurement of serum corticosterone, total IgE and nerve growth factor (NGF)

At 0 and 12 weeks after the start of treatment, blood was taken from the retro-orbital plexus of the mice, and the sera were obtained by centrifugation at 1000 × g for 20 min at 4 °C and stored at –80 °C until use. The serum corticosterone, total IgE and NGF levels were measured by the technique of enzyme-linked immunosorbent assay (ELISA) using the mouse corticosterone enzyme immunoassay kit (Assay Designs, Michigan, USA), the mouse IgE kit (Morinaga Institute of Biological Science Inc., Yokohama, Japan), and the NGF kit (Chemicon International, Inc., California, USA), respectively.

2.5. Measurement of the hydration in the dorsal skin

The hydration of the dorsal skin was measured using the Skicon-200 conductivity meter (IBS Co., Ltd., Hamamatsu, Japan) at 0 and 12 weeks after the start of treatment. The values of skin conductivity were recorded when the values stabilized at approximately 10 s after the probe had been placed on the skin.

2.6. Assessment of the infiltrated cells in the dorsal skin

The biopsy samples were obtained from the dorsal skin, small pieces of which were fixed in 20% formalin, embedded in paraffin, and the tissue sections were stained with congo red and toluidine blue for enumerating eosinophils and mast cells, respectively, using a light microscope at a magnification of 400×. Randomly selected five fields on the microscope were assessed for each sample.

2.7. Statistical analyses

All the data are presented as means ± SE. The data were analyzed by the Wilcoxon's test, using the software for testing the differences

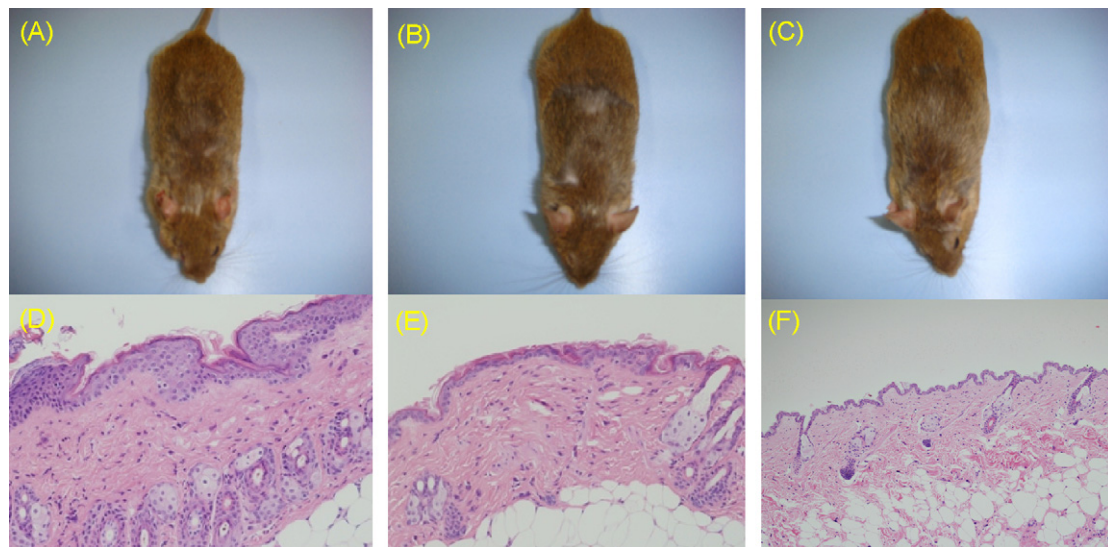


Fig. 1. Clinical skin features and histological features of the dorsal skin in the control mice and in the mice given oral administration of Yokukansan (YKS) for 12 weeks after the start of treatment. A and D: control; B and E: 0.5% YKS; C and F: 1% YKS. D, E, and F: haematoxylin and eosin staining; magnification 10 $\times$.

between the YKS-treated groups and the control groups. *P* values of <0.05 were defined as statistically significant.

3. Results

3.1. Clinical and histopathological features of the skin

Twelve weeks after the start of treatment, clinical analyses showed that the skin lesions such as erythema, erosion, hair loss, and skin dryness were more severe in the non-treated control mice than in the YKS-treated mice (Fig. 1A). Histopathological examinations revealed an amelioration of hypertrophy and hyperkeratosis of the epidermis, and a decrease in inflammatory cell infiltration, together with a decrease in mast cell degranulation in the 1%-YKS-treated mice (Fig. 1F).

3.2. Time courses of the skin lesion severity scores and the numbers of scratching and grooming behaviors

The individually housed NC/Nga mice gradually developed dermatitis over time in the conventional environment. The skin

lesion severity scores in each group were similar (the average skin lesion scores were 2–3) before the initiation of the treatment, while 4 weeks after the start of treatment, 0.5% YKS slightly inhibited the aggravation of skin symptoms. In contrast, administration of 1% YKS resulted in significant inhibition of the development of skin lesions 4 weeks after the start of treatment (Fig. 2A). Most of the non-treated control mice developed dermatitis 10 weeks after the start of experiments. The skin lesion severity score of the control mice was 6.2 ± 0.5 , while the score was 2.7 ± 0.2 ($P < 0.01$) in 1%-YKS-treated mice 10 weeks after the start of treatment. 0.5% YKS also inhibited the development of skin lesions ($P < 0.05$) 7 weeks after the start of YKS administration. In addition, after the 12 weeks, the non-treated control mice were treated with 1% YKS for additional 6 weeks in order to examine the “therapeutic effect” rather than the “preventive effect” of YKS on dermatitis. When the control mice were given 1% YKS for 6 weeks beginning at 12 weeks after the start of experiments, the skin lesion severity score of the non-treated control mice decreased from 6.2 ± 0.5 to 4.0 ± 0.4 after the 6-week administration of 1% YKS. In contrast, when the YKS administration was ceased in the 1%-YKS-treated mice at 12 weeks after the start of experiments, the skin lesion severity score of the 1%-

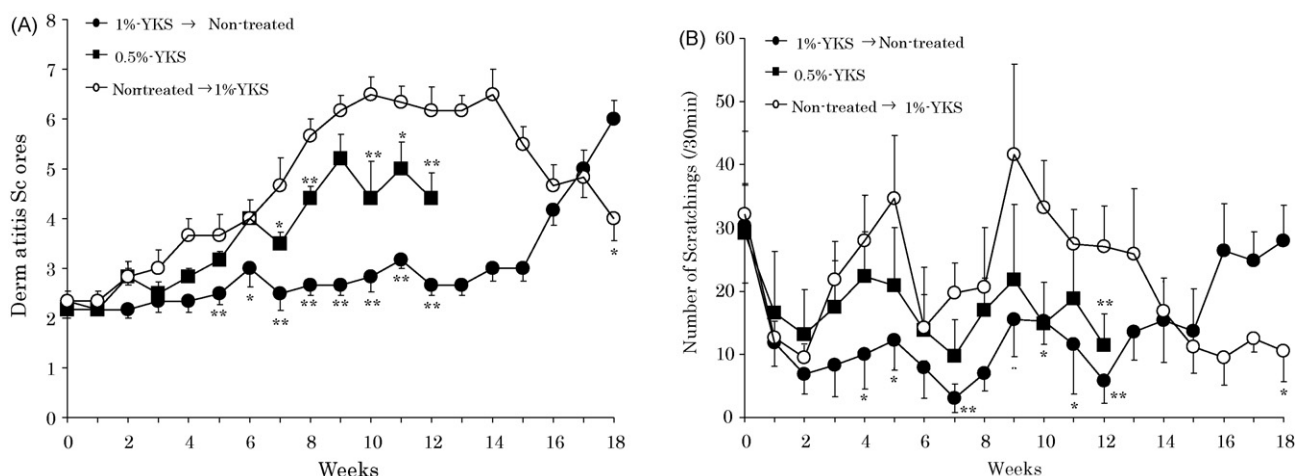


Fig. 2. The time course of dermatitis severity scores (A) and the frequency of scratching behaviors (B) in the NC/Nga mice. The mice were observed once a week as described in Section 2. Each point represents the mean $\pm$ SE of five to six animals. * $P < 0.05$, ** $P < 0.01$ as compared with the control mice by the Wilcoxon's test. Control: $\circ$ –, 0.5% YKS: $\blacksquare$ –, 1% YKS: $\bullet$ –.

YKS-treated mice increased from 2.7 ± 0.2 to 6.0 ± 0.4 after the cessation of YYS administration (Fig. 2A).

At the beginning, scratching and grooming behaviors were not significantly different between the YYS-treated and the control mice. However, 3 weeks after the start of treatment, the administration of 1% YYS resulted in significant suppression of the scratching behaviors compared with that of the control mice. The frequency of scratching behaviors of the 1%-YKS-treated mice was $8.3 \pm 5.0/30$ min as compared with that ($21.8 \pm 6.0/30$ min) of the control mice ($P < 0.05$) (Fig. 2B). Administration of 0.5% YYS decreased the frequency of scratching behaviors, but the efficacy was not statistically different from that in the control group. In addition, the “therapeutic effect” of 1% YYS on scratching behaviors was also observed when the non-treated control mice were treated with 1% YYS for additional 6 weeks beginning at 12 weeks after the start of experiments: the frequency of scratching behaviors of the control mice decreased from 27.0 ± 6.5 to 10.5 ± 4.8 after the 6-week administration of 1% YYS. In contrast, when the YYS administration was ceased in the 1%-YKS-treated mice, the frequency of scratching behaviors of the 1%-YKS-treated mice increased from 5.8 ± 3.6 to 28.0 ± 5.6 after the cessation of YYS administration (Fig. 2B).

Six weeks after the start of treatment, grooming behaviors increased markedly in the control mice. Administration of 1% YYS resulted in significant inhibition of the grooming behaviors compared with the control mice (2.2 ± 0.7 , 2.3 ± 0.6 vs. 8.2 ± 1.5 , 7.8 ± 1.9 ; $P < 0.01$, $P < 0.05$) 6 and 12 weeks after the start of treatment. 0.5% YYS also slightly decreased the frequency of grooming behaviors, but the efficacy was not significantly different from that in the control mice (Fig. 3).

3.3. Serum corticosterone, total IgE and nerve growth factor (NGF) levels

Twelve weeks after the start of experiments, serum corticosterone levels were increased in the control mice, while serum corticosterone levels were decreased in YYS-treated mice. 1% YYS significantly decreased serum corticosterone levels as compared with the non-treated control mice (38.7 ± 16.6 $\mu\text{g/ml}$ vs. 160.2 ± 21.7 $\mu\text{g/ml}$; $P < 0.01$) 12 weeks after the treatment (Fig. 4). Serum total IgE and nerve growth factor (NGF) levels in the mice increased over time after the start of experiments. However, no differences in the total IgE and NGF levels were observed between the YYS-treated mice and the non-treated control mice (data not shown).

3.4. Effect of YYS on the hydration in the dorsal skin

Decrease in the dorsal skin hydration was observed in the control mice after the start of experiments. Administration of YYS inhibited the decrease in the dorsal skin hydration. 12 weeks after

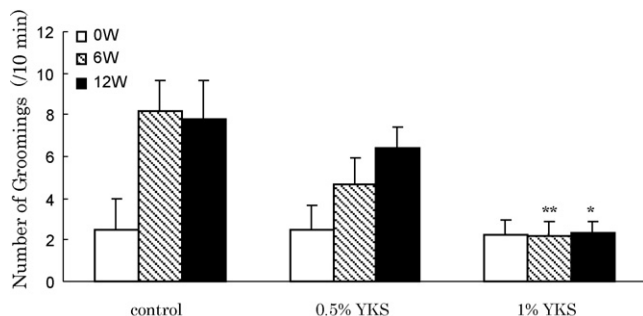


Fig. 3. The effect of YYS on grooming behaviors in the NC/Nga mice. The frequencies of grooming behaviors were observed at 0 weeks (□), 6 weeks (▨) and 12 weeks (■) after the start of treatment. Each point represents the mean $\pm$ SE of five to six animals. * $P < 0.05$, ** $P < 0.01$ as compared with the control mice by the Wilcoxon's test.

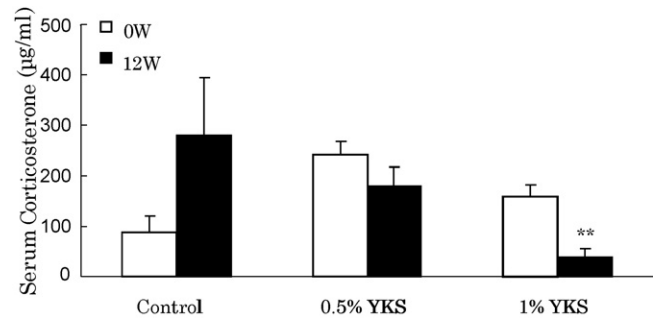


Fig. 4. The effect of YYS on serum corticosterone levels in the NC/Nga mice. Serum corticosterone levels were measured at 0 weeks (□) and 12 weeks (■) after the start of experiments. The values are the mean $\pm$ SE of five to six animals. * $P < 0.05$ as compared with the control mice by the Wilcoxon's test.

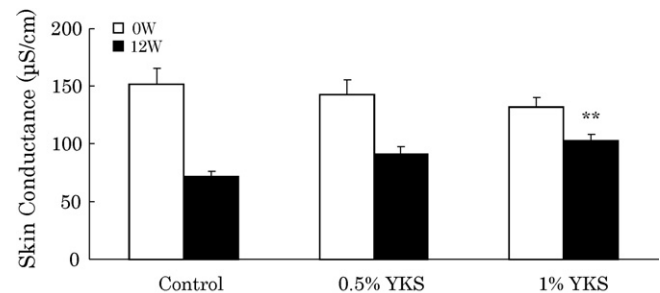


Fig. 5. The effect of YYS on skin hydration in the dorsal skin of the NC/Nga mice. Skin conductance was measured at 0 weeks (□) and 12 weeks (■) after the start of experiments. The values are the mean $\pm$ SE of five to six animals. * $P < 0.05$ as compared with the control mice by the Wilcoxon's test.

the start of treatment with 1% YYS, the skin conductance in the treated mice was 103.1 ± 5.1 $\mu\text{S/cm}$ as compared with that (71.9 ± 4.1 $\mu\text{S/cm}$) in the control mice ($P < 0.05$) (Fig. 5).

3.5. Assessment of the infiltrated cells in the dorsal skin

In histological examination, the numbers of mast cells in the skin of YYS-treated mice were $6.1 \pm 0.56/0.0625$ and $3.4 \pm 0.4/0.0625$ mm^2 in the 0.5%-YKS and 1%-YKS-treated mice, respectively, 12 weeks after the start of treatment (Fig. 6). The decrease in the number of mast cells in the 1%-YKS-treated mice was significant as compared with that in the control mice ($7.6 \pm 0.5/0.0625$ mm^2 ; $P < 0.01$). 1% YYS also significantly decreased the number of eosinophils infiltrated in the skin. The number of eosinophils in the skin of 1%-YKS-treated mice was $1.0 \pm 0.3/0.0625$ mm^2 as compared with that ($2.2 \pm 0.3/0.0625$ mm^2) of the control mice ($P < 0.01$) (Fig. 6).

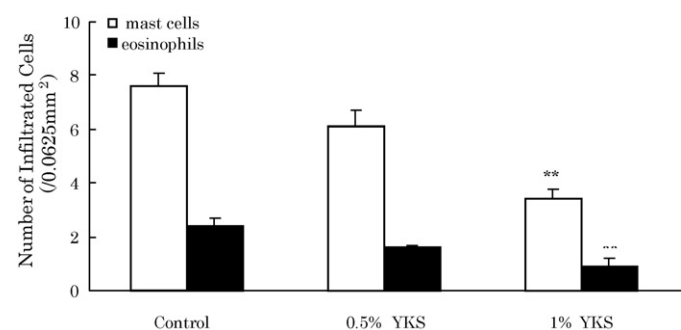


Fig. 6. The numbers of infiltrated mast cells and eosinophils in the skin of the NC/Nga mice. Infiltrated cells were enumerated at 12 weeks after the start of experiments. □: Mast cells; ■: eosinophils. The values are the mean $\pm$ SE of five to six animals. * $P < 0.05$, ** $P < 0.01$ as compared with the control mice by the Wilcoxon's test.

4. Discussion

The NC/Nga mouse was originally established as an inbred strain in Japan [21]. When NC/Nga mice are kept in the conventional environment, they spontaneously develop AD-like dermatitis [12,13]. Social isolation of laboratory animals is regarded as a model of stress and induces behavioral disturbances in the animals [15]. In the present study, the mice utilized successfully showed exacerbation of skin symptoms, together with increasing behavioral disturbances after socially isolated in a conventional animal housing room. Oral administration of YKS suppressed scratching behaviors, infiltration of inflammatory cells in the skin, and improved the skin hydration in a dose-dependent manner, which resulted in the inhibition of the aggravation of AD-like skin lesions in NC/Nga mice. Although oral administration of 0.5% YKS significantly improved dermatitis scores, 0.5% YKS oral administration did not significantly inhibit numbers of scratchings. This discrepancy might be possibly due to the different responses among individual animals, i.e., some mice in the 0.5%-YKS-treated group showed slightly decreased scratching behaviors as indicated by the tendency of decreased scratchings in the 0.5%-YKS-treated mice, although the difference was not significant (Fig. 2B). Interestingly, when the non-treated control mice were treated with 1% YKS for additional 6 weeks, beginning at 12 weeks after the start of experiments, in order to examine the “therapeutic effect” rather than the “preventive effect” of YKS on dermatitis, the skin lesion severity score of the non-treated control mice was significantly decreased at 18 weeks after the start of experiments (Fig. 2A). These data clearly indicated that YKS possessed the “therapeutic effect” on dermatitis as well as the “preventive effect”.

Itching is one of the most common symptoms in dermatological diseases and it is widely accepted that itching can be initiated by inflammation, cancer, metabolic diseases, infection, psychiatric diseases, drug application, stress and others [22]. It has been recognized that stress perception triggers or enhances itching. Stress responses such as the production of nerve growth factor (NGF) and substance P (SP), and the activation of mast cells aggravate chronic skin diseases like AD in human [23,24]. The inflammatory skin environment lowers the threshold for itch stimuli and thus causes itch sensitization. Cutaneous sensory neurons transmit the itch signals via dorsal root ganglia and the spinal cord to the central nervous system, which will result in the perpetuation of stress perception. Itch-provoked scratching thus enhances the “vicious cycle” of itch-scratching behaviors by causing additional skin inflammation. Therefore, obstruction of the “vicious cycle” is important in the treatment of AD. Since the conventional therapies often fail to alleviate these chronic itching, current progress in the elucidation of the neuropathic and/or psychogenic causes of itching would greatly contribute to the development of new anti-itching therapeutic strategies [25].

Oral administration of YKS alleviated social isolation stress as indicated by the significantly decreased serum corticosterone levels and grooming behaviors in a dose-dependent manner as compared with the control mice. It was reported that stress could aggravate AD in both human and NC/Nga mice [5,26]. Stress stimulates the hypothalamic–pituitary–adrenal (HPA) axis, sympatho-adrenomedullary system and sympathetic nervous system, excreting corticosterone into the blood. Stress also activates corticotropin releasing hormone (CRH), which induces mast cell degranulation and increases vascular permeability. Therefore, CRH might play a key role in the development of inflammatory reaction in the skin in socially isolated mice [27–29]. In addition, stress-induced facilitation of the HPA axis is associated with the release of serotonin (5-HT), and the subsequent activation of 5-HT receptors might be one of the mechanisms mediating stress-induced skin

reaction. Neuronal 5-HT interacts with other neurotransmitters that involve in the mediation and regulation of responses at all levels of the HPA axis, including the regulation of receptors, gene expression and the release of hormones [30,31].

YKS possesses anxiolytic and neuroprotective properties, including preservation of emotional balance [10,32]. Furthermore, a recent study showed that YKS possesses the anti-allergic efficacy and ameliorate behavior disturbances in mice by changing the expression of 5-HT_{2A} receptors in the prefrontal cortex [11,33]. In addition, isolated NC/Nga mice showed increased grooming and scratching behaviors as compared with group-housed NC/Nga mice under conventional conditions (unpublished data). In the present study, the control mice showed more scratching and grooming behaviors than the YKS-treated mice. 1% YKS significantly decreased serum corticosterone levels, and suppressed scratching and grooming behaviors without showing sedative action. The present study suggested that YKS alleviated stress by acting on the 5-HT nervous system, which resulted in the decrease of the serum corticosterone levels. The alleviation of stress reduced the scratching behaviors, and thus reduced the number of infiltrating inflammatory cells in the skin. The anti-itching effect of YKS is thought to be due to the down regulation of serotonin receptors and the attenuation of activation of the HPA axis. Further study is necessary to elucidate the mechanism(s) of anti-itching effect of YKS in AD patients as well as in patients with anxiety.

YKS most likely exerts its clinical effects not through a single constituent but as a mixture of several herbal drug ingredients. Oral administration of YKS suppressed the aggravation of AD-like dermatitis in isolated NC/Nga mice without any apparent side effects. Our results also suggested that YKS alleviated the aggravation of AD-like skin lesions in isolated NC/Nga mice due to the suppression of scratching behaviors which are known as one of the most critical exacerbating factors for AD. It is expected that YKS might be one of the alternative or complementary therapeutic options for the treatment of patients with AD.

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