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Inhibitory Effects and Potential Mechanism of Yokukansankachimpihange on SDS-induced Itch-associated Responses in Mice

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Abstract: Yokukansankachimpihange (YKSCH) is a Chinese formula proven in Japan to be an effective treatment for pruritus in patients with atopic dermatitis or chronic urticaria and dry eczema with psychoneurotic symptoms, such as insomnia. Sodium dodecyl sulfate (SDS) may cause skin irritation in both humans and animals. Adverse effects, such as skin dryness, barrier destruction, dermatitis, and pruritus, developed following the repeated application of 10% SDS to a local site. Therefore, the present study investigated the antipruritic effects of YKSCH on skin irritation induced by a repeated SDS stimulation in mice and attempted to elucidate the underlying mechanism of action. ICR mice were stimulated with 10% SDS and co-treated with oral YKSCH extract for four days. Only oral YKSCH extract was administered on the fifth day. Scratching behavior was observed 24 h after the last application of SDS. Intraepidermal nerve growth was investigated by an immunofluorescence analysis. NGF concentrations in the epidermis were measured using an enzyme-linked immunosorbent assay. The expression of amphiregulin (Areg) and semaphorin 3A (Sema3A) was assessed by quantitative real-time PCR. The oral administration of YKSCH (200-400 mg/kg) dose-dependently suppressed pruritus, significantly reduced intraepidermal nerve growth, and down-regulated epidermal Areg mRNA expression in SDS-treated mice. These results suggest that YKSCH exerts antipruritic effects against SDS-induced pruritus in mice. The mechanism of action of YKSCH may involve reductions in intraepidermal nerve density due to the down-regulated expression of Areg.

Keywords: Yokukansankachimpihange, Itch, Intraepidermal Nerve Density, Nerve Growth Factor, Semaphorin 3A, Amphiregulin

1. Introduction

Itching, an unpleasant sensation that provokes the urge to scratch, is a frequent complaint in patients with pruritic diseases. Severe pruritus has a negative impact on quality of life. Pathological itching in pruritic skin diseases, such as eczema, atopic dermatitis (AD), urticaria, psoriasis, and xerosis, may exacerbate skin symptoms through scratching behavior and interfere with treatment [1, 2]. Although H1 histamine-receptor antagonists are the first-line treatment for itching, poor responses have been reported for many pruritic diseases, except acute urticaria [3]. Therefore, the development of novel drugs that are effective against antihistamine-resistant pruritus is awaited.

Yokukansankachimpihange (YKSCH) is a Kampo formula that is used to treat neurosis, insomnia, children crying at night, irritability, and menopause [4]. It is composed of the following ingredients: Atractylodes or Atractylodes lanceae rhizome, Uncaria hook, Cnidium rhizome, Poria sclerotium, Pinellia tuber, Glycyrrhiza, Citrus unshiu peel, Bupleurum root, and Angelica dahurica root. It was recently shown to be effective against pruritus in Japanese patients with AD [5, 6] as well as those with xerotic eczema and chronic urticaria [7] with psychoneurotic symptoms. However, its pharmacological mechanism of action remains unknown.

YKSCH and some of its ingredients were found to exert

inhibitory effects against NGF-induced neurite growth in cultured rat dorsal ganglion (DRG) neurons [8]. A previous study demonstrated that local NGF concentrations were lower in normal skin than in the lesional skin of AD patients; therefore, it may be one of the main mediators of the density of cutaneous innervation [9]. Semaphorin 3A (Sema3A) is an axonal repulsion factor that inhibits the NGF-induced sprouting of nociceptive afferents in the adult rat spinal cord [10]. Amphiregulin (Areg), a member of the epidermal growth factor family, has been shown to promote axonal outgrowth in mouse DRG neurons [11]. Itching in pruritic skin diseases is caused by an increase in the sensitivity of C fibers to external stimuli due to the invasion and proliferation of sensory nerve fibers into the epidermis [12]. The inhibition of nerve growth into the epidermis is an important strategy in the treatment of pruritic skin diseases. Therefore, we have investigated the effects of YKSCH on epidermal nerve density in "itch-scratch" animal models.

Sodium dodecyl sulfate (SDS) is an anionic surfactant that is used in many cleaning and hygiene products. It may cause skin irritation in both humans and animals [13, 14]. Previous studies reported that adverse effects, such as skin dryness, barrier destruction, dermatitis, and itch-scratching, developed in mice following the repeated topical application of 10% SDS [15, 16]. Therefore, the present study was performed to investigate the effects of YKSCH on scratching behavior induced in mice by the repeated topical application of 10% SDS. Its effects on nerve elongation after the SDS treatment and the potential underlying mechanism of action were also examined.

2. Materials and Methods

2.1. Drugs and Reagents

YKSCH comprises the following nine dried medical herbs: Atractylodes lanceae rhizome, Uncaria hook, Cnidium rhizome, Poria sclerotium, Pinellia tuber, Glycyrrhiza, Citrus unshiu peel, Bupleurum root, and Angelica dahurica root (Table 1). YKSCH extract (Lot.MS-219, Ominedo Pharmaceutical Industry Co., Ltd., Nara, Japan) was suspended in 0.5% carboxymethyl cellulose (CMC) before use. SDS (Nacalai Tesque, Inc., Kyoto, Japan) was dissolved in distilled water (10% w/v, pH 6.5). Rabbit polyclonal anti-protein gene product 9.5 (PGP9.5, Ultraclone Ltd., Wellow, Isle of Wight, UK), an Alexa Fluor 488 donkey anti-rabbit IgG antibody (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the NGF enzyme immunoassay kit (Cloud-Clone Corp., Katy, TX77494, USA) were used. The following RNA sequences were used:

Sema3A Forward: 5′-GGCTGGTTCACTGGGATTG- 3′,
Sema3A Reverse: 5′-CCGTTTGCATAGTTTGCTCTGG- 3′,
Areg Forward: 5′-TCACAGTGCACCTTTGGAAAC- 3′,
Areg Reverse: 5′-CCCGTTTTCTTGTCGAAGCC- 3′
GAPDH_Forward: 5′-ATTCAACGGCACAGTCAAGG- 3′,
GAPDH_Reverse: 5′-TCACCCCATTTGATGTTAGTGG- 3′.

Table 1. *Medicinal herb composition of Yokukansankachimpihange (YKSCH).*

English name	Latin name	Weight (g)
Pinellia tuber	*Pinelliae tuber*	5.0
Atractylodes rhizome	*Atractylodis lanceae rhizoma*	4.0
Poria sclerotium	*Hoelen (Poria)*	4.0
Cnidium rhizome	*Cnidii rhizoma*	3.0
Citrus unshiu peel	*Aurantii nobilis pericarpium*	3.0
Japanese Angelica root	*Angelicae radix*	3.0
Uncaria hook	*Uncariae hook*	3.0
Bupleurum root	*Bupleuri radix*	2.0
Glycyrrhiza	*Glycyrrhizae radix*	1.5

2.2. High-performance Liquid Chromatography Analysis of YKSCH

YKSCH extract was mixed with 50% MeOH and centrifuged. The supernatant was filtered through a membrane filter (PFTE, 0.22 mm) and subjected to high-performance liquid chromatography (HPLC). The three-dimensional (3D) HPLC profile of YKSCH was obtained using the Shimadzu Nexera X2 system (analysis system software: LabSolutions, Shimadzu Co., Kyoto) with a SPD-M30A detector over a scan range of 190–700 nm using a reversed-phase column (Kinetex Biphenyl, 50 × 2.1 mm I. D., 1.7 mm, Lot No. H18-058749, temperature 40°C). The solvent system consisted of solvent A (5 mM CH_3COONH_4 containing water) and solvent B (5 mM CH_3COONH_4 containing methanol), and the ratio of solvent A in mixed solvents was initially 95%, reduced to 45% over 7 min, and maintained at 45% for 11 min. Thereafter, solvent A was reduced to 2% over 5 min, maintained at 2% for 2 min, increased to 95%, and maintained at 95% for 8 min with a flow rate of 0.3 mL/min. The analytical results obtained are shown in Figure 1.

2.3. Animal Model for the Application of SDS

Seven- to nine-week-old male ICR mice (Japan SLC, Ltd., Shizuoka, Japan) were housed in sterilized polypropylene cages under controlled temperature (23°C±2°C) and humidity (55%±10%) conditions with a 12-h light-dark cycle (lights on from 8:00-20:00). The procedures for animal experiments were approved by the Experimental Animal Care Committee of Kracie Pharma, Ltd. (Takaoka, Japan) and were conducted in accordance with the guidelines of the Japanese Pharmacological Society. Mice were given *ad libitum* access to laboratory pellet chow (CE-2, Clea Japan Inc., Tokyo, Japan) and water. After acclimatization for seven days, experimental procedures were initiated. The SDS model was

developed according to the method reported by Inami et al. [16]. All mice were intraperitoneally anesthetized with a combined anesthetic (medetomidine, 0.3 mg/kg; midazolam, 4.0 mg/kg; butorphanol 5.0 mg/kg). The rostral part of the back was shaved at least 3 days prior to the start of the experiment and 50 μL of 10% SDS was applied. The topical application of this solution was repeated at 24-h intervals for 4 days.

2.4. Experimental Design

Experiment 1 Forty mice without wounds from shaving were divided into five groups (eight mice per group). In the first group, DW was topically applied to shaved skin and CMC water was orally administered. In the second group, 10% SDS was applied topically to shaved skin and CMC water was orally administered. In the groups treated with 10% SDS, oral YKSCH extract was administered at a dose of 200, 400, or 800 mg/kg (800 mg/kg based on the daily therapeutic dose of YKSCH for humans). All doses were administered 30 min before the application of SDS on four days, and only oral YKSCH extract was given on the fifth day. On the day before the first application of SDS and 22-24 h after each application, the level of hydration in the stratum corneum (SC) was assessed under isoflurane anesthesia with a moisture checker (MY-808s; Scalar Corp., Tokyo, Japan).

Experiment 2 The same procedure was used as that in Experiment 1, except for YKSCH extract being administered at 200 or 400 mg/kg.

2.5. Evaluation of Scratching Behavior (Experiment 1)

Mice were placed into an acrylic cage containing four cells (34×10×18 cm) for at least 1 h for acclimatization. Scratching behavior was assessed as previously described [17]. Hind-paw scratching behavior directed towards the surfactant-treated site was observed for one hour 24 h after the last application of SDS.

2.6. Immunofluorescence (Experiment 1)

Mice were transcardially perfused with phosphate-buffered saline (pH 7.4) followed by 4% paraformaldehyde phosphate buffer solution under a mixture of isoflurane (2%) and O_2 (2 l/min) anesthesia. The skin was removed from the rostral part of the back and post-fixed in the same fixative for 48 h. Tissue cryoprotection was performed in PBS containing 30% sucrose at 4°C for 48 h. Frozen skin tissues were cut on a freezing microtome (YAMATO REM0700, Tokyo, Japan) to a thickness of 30 μm.

After washing with PBS containing 0.1% Tween 20 (PBS-T), sections were incubated at 4°C for 48 hours with the primary antibody PGP9.5 (1:1000) diluted in PBS-T containing 1.5% fetal bovine serum. After washing with PBS-T, sections were incubated at room temperature for 2 hours with the secondary antibody, Alexa Fluor 488 donkey anti-rabbit IgG antibody (1:200) diluted in Can Get Signal solution A. Slides were mounted with Mount-Quick mounting medium. Immunofluorescence was visualized using a confocal fluorescence microscope (KEYENCE BZ-9000) and 3 to 8 images (field size per image: 745.8×561.6 μm) were captured.

On bright-field images, the epidermis and dermis were discriminated by the basement membrane, and the expression of PGP9.5 was observed using ImageJ software (NIH, Bethesda, MD, USA). We binarize nerve fibers grayscale images, and the number of nerves and total number of nerve fibers in the epidermis were counted and divided by the epidermal area.

2.7. Enzyme-linked Immunosorbent Assay (Experiment 2)

To measure cutaneous NGF concentrations, mice were transcardially perfused with PBS under anesthesia 26 hours after the last application of SDS and treated skin was removed. The epidermis and dermis were separated by heating at 60°C for 30 seconds and then subjected to measurements of NGF concentrations.

Samples of the epidermis were homogenized in cell lysis buffer with protease inhibitor (T-PER Tissue Protein Extraction Reagent, # 78510, Thermo Scientific). Samples were centrifuged at 10,000×*g* at 4°C for 10 minutes. The concentration of NGF in the supernatant was measured using an NGF enzyme immunoassay kit.

2.8. Quantitative Real-time PCR (qRT-PCR) (Experiment 2)

Samples of the epidermis were homogenized in Trizol and total RNA was isolated. The relative gene expression of Sema3A and Areg was measured by qPCR using SYBR green. Twenty-five nanograms of RNA was reverse-transcribed to cDNA using the ReverTra Ace qRT-PCR kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Gene expression was measured by RT-PCR using cDNA and the SYBR Green RT-PCR Master Mix (Toyobo, Osaka, Japan). Gene expression values are given as relative expression levels to that of GAPDH.

2.9. Statistical Analysis

Data are shown as the mean ± the standard error of the mean (S.E.M). The significance of differences was examined using a one-way analysis of variance followed by Dunnett's test. Comparisons of mean values between normal and control groups were performed using the Student's *t*-test. P-values of less than 0.05 were considered to be significant. All statistical analyses were conducted using StaCel3 software.

3. Results

3.1. Experiment 1

3.1.1. HPLC Analysis of YKSCH

The 3D-HPLC profile of YKSCH and the results of a chemical analysis are shown in Figure 1. The chemical markers liquiritin, hesperidin, tangeretin, narirutin, rhynchophylline, glycyrrhizic acid, and isoliquiritigenin were used for quality control.

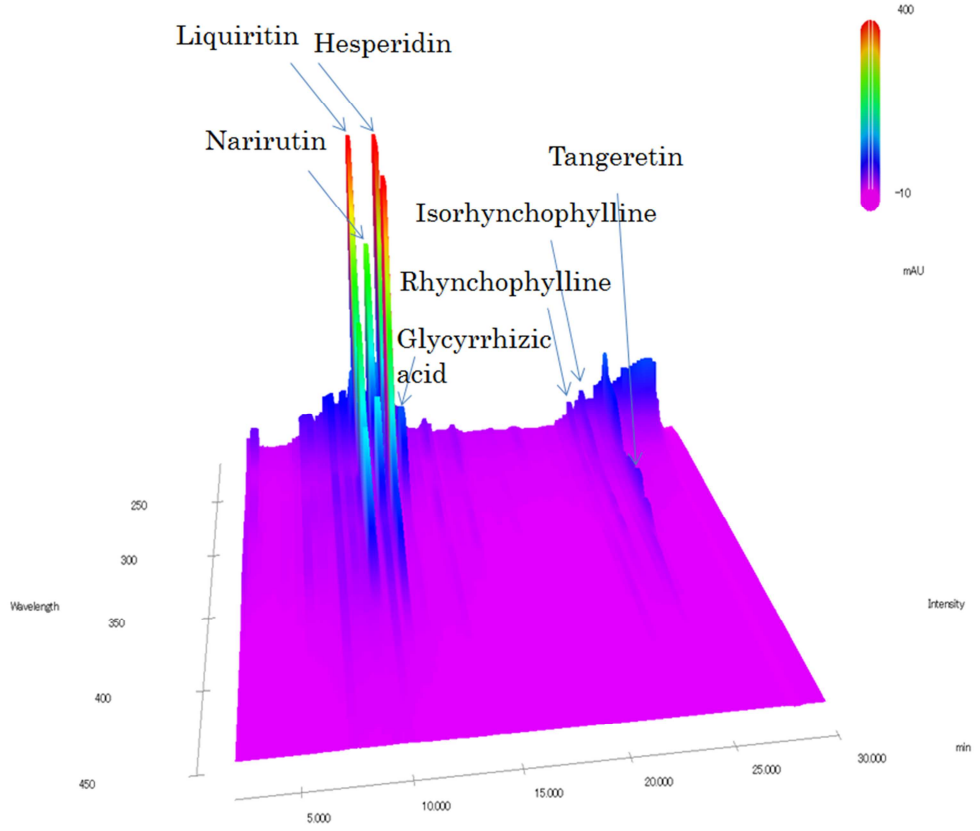

Figure 1. *3D-HPLC profile of yokukansankachimpihange (YKSCH). Chemical markers (liquiritin, hesperidin, tangeretin, narirutin, rhynchophylline, glycyrrhizic acid, and isoliquiritigenin) in the HPLC profile were identified based on comparisons with the retention times and UV spectra (210-450 nm) of their reference standards.*

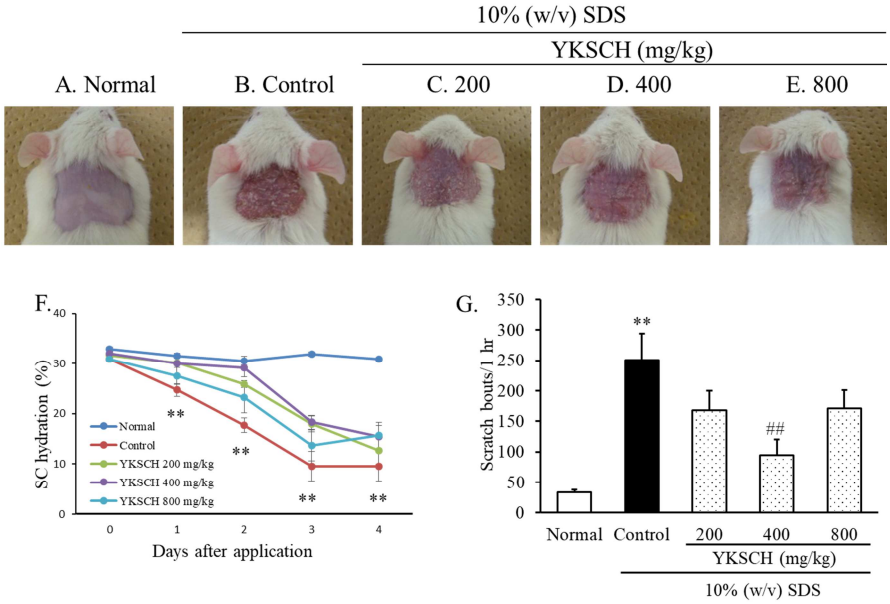

Figure 2. *Effects of YKSCH on dryness in the SDS-induced dry skin mouse model. (A) Normal group; (B) Control group; (C) YKSCH 200 mg/kg group; (D) YKSCH 400 mg/kg group; (E) YKSCH 800 mg/kg group; (F) Stratum corneum (SC) hydration; (G) Mean scratching frequency in 1 hour. Data are expressed as the mean±S.E.M. (n=8 per group). **p<0.01 v.s. Normal by the Student's* t*-test. ##p<0.01 v.s. Control by Dunnett's test.*

3.1.2. Effects of YKSCH on Dry Skin Induced by the Repeated Application of 10% SDS

The repeated topical application of 10% SDS caused dermatitis (Figures 2A–E) and reduced the level of hydration in SC (Figure 2F). The level of hydration in SC time-dependently decreased during the 4-day treatment, with a significant reduction being observed from the first day after

the initiation of treatment. YKSCH did not improve SC hydration levels.

3.1.3. Effects of YKSCH on Scratching Induced by the Repeated Application of 10% SDS

The daily application of 10% SDS for 4 days significantly increased the frequency of hind-paw scratching and this was significantly reduced by the oral administration of YKSCH at a dose of 400 mg/kg (Figure 2G). Therefore, YKSCH exerted inhibitory effects on itching.

3.1.4. Effects of YKSCH on Intra-epidermal Nerve Density

The density of neurons in the epidermis was significantly higher in mice treated with the daily application of 10% SDS than in the controls. YKSCH significantly inhibited this increase in a dose-dependent manner at 200 and 400 mg/kg (Figure 3).

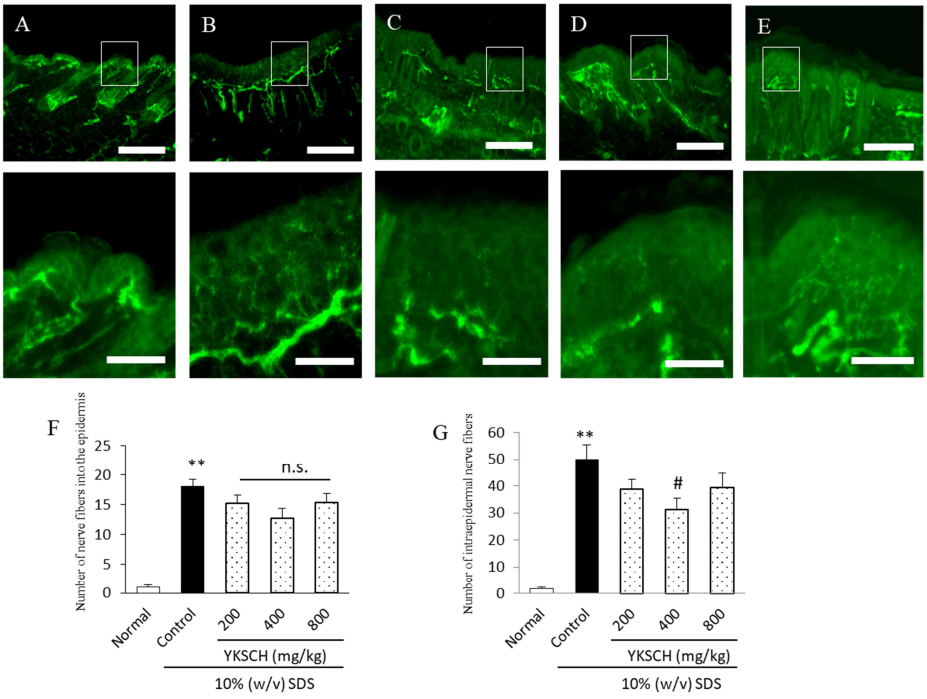

Figure 3. *Effects of YKSCH on nerve fiber elongation in the SDS-induced dry skin mouse model. Typical images of the immunohistochemical staining of PGP9.5 (green) in the skin (scale bar=100 μm) (upper panel). Higher magnification of the image delineated by the rectangles (scale bar=20 μm) (lower panel). (A) Normal group; (B) Control group; (C) YKSCH 200 mg/kg group; (D) YKSCH 400 mg/kg group; (E) YKSCH 800 mg/kg group. Quantitative analysis of immunoreactivity in the epidermis. (F) Number of nerve fibers in the epidermis; (G) Number of intraepidermal nerve fibers. Data are expressed as the mean±S.E.M. (n=8 per group). **p<0.001 v.s. Normal by the Student's* t*-test. #p<0.05 v.s. Control by Dunnett's test.*

3.2. Experiment 2

Effects of YKSCH on Nerve Elongation Factors and Nerve Repulsion Factors

The topical application of 10% SDS and oral YKSCH did not affect NGF concentrations in the epidermis (Figure 4A). The mRNA expression of the nerve repulsion factor *Sema3A* was unchanged (Figure 4B). The topical application of 10% SDS significantly up-regulated the mRNA expression of *Areg* in the epidermis 26 hours after the fourth application; however, this was reduced by the oral administration of YKSCH (Figure 4C).

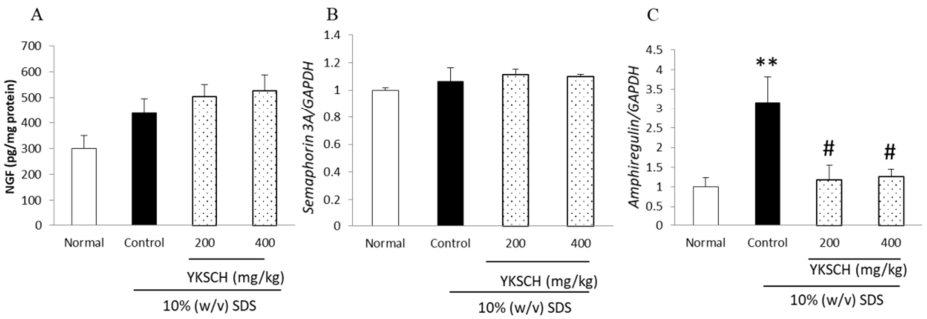

Figure 4. *Effects of YKSCH on mRNA and protein expression levels of nerve elongation and repulsion factors in the SDS-induced dry skin mouse model. (A) NGF protein expression; (B) Sema3A mRNA expression; (C) Areg mRNA expression. Data are expressed as the mean±S.E.M. (n=8 per group). **p<0.01 v.s. Normal by the Student's* t*-test. #p<0.05 v.s. Control by Dunnett's test.*

4. Discussion

In the present study, we used a dry skin model in comparison with "itch-scratch", a standard method for animal skin, which is dependent on the frequency and continuation of SDS application [15]. Scratching behavior was strongly induced by the repeated topical application of SDS, and was significantly suppressed by YKSCH orally administered at a dose of 400 mg/kg. The mechanisms by which the application of SDS induces pruritus currently remain unclear; however, neurite outgrowth stained by PGP9.5 revealed a significant increase in nerve density in the epidermis [18]. Epidermal nerve density has been shown to partially contribute to itch sensitization in pruritic skin diseases, such as AD [19, 20]. YKSCH suppressed SDS-induced scratching frequency and nerve density in the epidermis similarly in treated and control mice. Therefore, YKSCH inhibited scratching by reducing intraepidermal nerve density in the epidermis.

We previously reported that YKSCH inhibited NGF-induced reductions in neurite growth in cultured rat DRG neurons [8]. However, increases were observed in NGF concentrations after the repeated application of SDS [18]. In the present study, NGF protein concentrations were assessed using ELISA. NGF protein concentrations increased in the SDS model and were not affected by YKSCH. Therefore, YKSCH did not inhibit neurite growth through the direct inhibition of NGF. A previous study suggested that YKSCH affected NGF receptors through the phosphorylation of tyrosine kinase [8, 21]. In the present study, the expression of NGF in the SDS model differed from that reported previously [18], and this discrepancy may have been due to the different methods used.

Epidermal innervation is considered to be modulated by the balance between nerve elongation factors and nerve repulsion factors [12]. Areg is a nerve elongation factor that affects neurite outgrowth [11, 22]. Areg expression was shown to be significantly higher in patients with AD or psoriasis than in controls [20, 23]. It may also affect cell–cell junctions, resulting in barrier disruption and epidermal hyperinnervation [20]. Furthermore, Areg expression was found to be up-regulated in the dry skin of acetone-treated mice and associated with intraepidermal nerve growth [24]. Previous studies demonstrated that the expression of Sema3A, a nerve repulsion factor, was down-regulated in the epidermis of AD and psoriasis patients [20, 23].

In the present study, *Areg* mRNA expression levels were significantly increased in the epidermis after the topical application of 10% SDS and reduced by the oral administration of YKSCH. The mRNA expression of *Sema3A* was unaffected by the application of SDS or the oral administration of YKSCH. Therefore, YKSCH does not affect NGF or *Sema3A*. The inhibition of scratching behavior induced by the repeated application of SDS may have been due to the down-regulated expression of *Areg*, which reduced intraepidermal nerve density in the epidermis.

The Kampo medicine YKSCH consists of 9 natural drugs. A previous study reported that hirustin isolated from Uncaria hook and saikosaponins a and d isolated from Bupleurum root inhibited NGF-induced neurite growth in cultured rat DRG neurons [8]. Although the present study did not investigate the crude or chemical constituents of YKSCH, Uncaria hook was previously confirmed to suppress the development of AD-like dermatitis in 2, 4-dinitrofluorobenzene (DNFB)-treated NC/Nga mice by reducing the production of IFN-γ [25]. Furthermore, saikosaponins exerted inhibitory effects on matrix metalloproteinase (MMP)-2 activity in mice with picryl chloride-induced ear contact sensitivity [26]. MMP-2 localized on the growth cone is involved in the mechanism of penetration of nerve fibers into the basement membrane [27]. Therefore, the inhibitory effects of YKSCH on intraepidermal nerves may partly be through MMP2 activity.

5. Conclusions

YKSCH exerted antipruritic effects against SDS-induced scratching in mice. Further studies are needed to identify the active compounds, such as hirustin or saikosaponins a and d, causing these responses in SDS-induced itch and DFNB-treated NC/Nga mouse models. The inhibitory effects of YKSCH on SDS-induced scratching may be partly attributed to a decrease in intraepidermal nerve density due to the down-regulated expression of Areg.

Conflicts of Interest

All the authors do not have any possible conflicts of interest.

Data Availability

The data used to support the results of the present study are available from the corresponding author upon request.

Acknowledgements

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