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Topical Application of Tenshino-Softgel™ Reduces Epidermal Nerve Fiber Density in a Chronic Dry Skin Model Mouse

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Abstract

Background: Dry skin induces antihistamine-resistant itch, as well as epidermal hyperinnervation, which is partly responsible for peripheral itch sensitization. In acute dry skin, topical application of emollients prevents the penetration of nerve fibers into the epidermis. However, the effects of emollients on itch and epidermal hyperinnervation in individuals with chronic dry skin are poorly understood. Objective: This study examined the effects of Tenshino-softgel™ (TSG) on itch-related behavior, epidermal hyperinnervation and skin barrier function in a chronic dry skin model mouse. Methods: Chronic dry skin was induced by application of acetone/ether (1:1) mixture and water (AEW) to the rostral parts of the back of hairless mice twice daily for six consecutive days. As treatment, TSG or, as control, Vaseline (V) was applied to the same areas twice daily. Skin barrier function was evaluated by measuring transepidermal water loss (TEWL) before each treatment. Scratching behavior was recorded and analyzed using a SCLABA®-real system, and skin samples were collected for immunohistochemical assays. Results: TEWL tended to be lower and scratching bouts fewer in AEW + TSG- than in AEW-treated mice. The numbers of protein gene product 9.5-immunoreactive fibers and substance P-immunoreactive fibers were each significantly lower in the epidermis of AEW + TSG- than of AEW-treated mice, but the expression of nerve growth factor in epidermis was similar in the three groups. Semaphorin 3A expression was significantly higher in the epidermis of AEW + TSG- than of AEW- and AEW + V-treated mice. Conclusion: Topical application of TSG may attenuate itch induced by chronic dry skin through a mechanism involving the inhibition of epidermal hyperinnervation.

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Keywords

Dry Skin, Emollient, Epidermal Nerve Fiber, Itch, Skin Barrier

1. Introduction

Dry skin, as observed in patients with senile xerosis and atopic dermatitis, is a very common dermatologic problem frequently presenting with pruritus, defined as an unpleasant sensation and a desire to scratch frequently [1] [2]. Dry skin is induced by decreased water-holding capacity, which is controlled by cutaneous barrier function in the stratum corneum (SC). Skin dryness, as characterized by reduction of SC hydration and transepidermal water loss (TEWL), has been found to induce pruritus [3] [4], as well as epidermal hyperinnervation, which is partly responsible for peripheral itch sensitization [5]. Increased density of nerve fiber in the epidermis is mainly caused by an imbalance between nerve elongation factors such as nerve growth factor (NGF) and nerve repulsion factor such as semaphorin 3A (Sema3A) [6]. These factors may also affect keratinocytes, immune cells and vascular endothelial cells, possibly relating to the modulation of itch [7].

Tenshino-softgel^{TM} (TSG) is a gel-like moisturizing lotion made by Ina Food Industry Co., Ltd. TSG contains water, glycerin, urea, methyl paraben, propyl paraben and agar. Agar is widely used as a food and gelling agent in Asian countries. Solutions of glycerol and/or urea in water are not sufficiently viscous, but the addition of agar was found to enhance the viscosity of TSG. Although agar may have moisturizing and/or anti-inflammatory actions [8], the effectiveness of TSG in treating pruritus due to dry skin is currently unclear. This study therefore evaluated the effects of TSG on itch-related behavior, skin barrier function, epidermal hyperinnervation, and epidermal expression level of axon guidance molecules in mice with chronic dry skin.

2. Materials & Methods

2.1. Animals

Male HR-1 hairless mice (Hoshino Laboratory Animal Inc., Ibaragi, Japan), aged 10 weeks, were maintained under clean conditions, with a 12 h light: 12 h dark cycle at 22˚C - 24˚C and food and tap water provided *ad libitum*. Care and handling of these mice conformed to the NIH guidelines for animal research. All animal procedures were approved by the Institutional Animal Care and Use Committee at Juntendo University Graduate School of Medicine.

2.2. Reagents

TSG was obtained from Ina Food Industry Co., Ltd. (Nagano, Japan), hydrophilic petrolatum (Vaseline, ointment base) from Maruishi Seiyaku Inc. (Osaka, Japan), optimal cutting temperature (O.C.T.) compound from Sakura Finetechnical Co., Ltd. (Tokyo, Japan), normal donkey serum (NDS) from Merck Millipore (Darmstadt, Germany), bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO, USA), Vectashield mounting medium with DAPI from Vector Laboratories Ltd. (Peterborough, UK), and sevoflurane from Abbott Japan (Osaka, Japan).

2.3. Antibodies

Primary antibodies used in this study included rabbit anti-protein gene product 9.5 (PGP 9.5, 1:400 dilution; Enzo Life Sciences Inc., Farmingdale, NY, USA), rat anti-substance P (SP, 1:100 dilution; Merck Millipore), rabbit anti-NGF (1:500 dilution; Merck Millipore), and rabbit anti-Sema3A (1:200 dilution; Abcam Inc., Cambridge, MA, UK). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR, USA).

2.4. Induction of Dry Skin and Application of Emollients

Acetone/ether (1:1) mixture (AE), followed by water (AEW) was applied cutaneously as described [9]. Briefly, cotton (2 × 2 cm) soaked with AE was placed on the rostral parts of the back of mice for 15 seconds under

sevoflurane anesthesia, followed within 5 seconds by placement on the same areas of cotton soaked with distilled water for 30 seconds. Within five seconds after each AEW treatment, TSG or Vaseline (V) was applied to the same area as a therapy or control, respectively. These procedures were performed twice daily for six consecutive days. The number of mice used in this study is as follows: untreated (n = 8), AE (n = 8), AEW (n = 7), AEW + V (n = 7), AEW + TSG (n = 7).

2.5. Measurement of TEWL

Before each of the AEW treatment, TEWL of the treated area (the rostral parts of the back of mice) was measured under sevoflurane anesthesia using a Tewameter®TM210 (Courage & Khazawa, Cologne, Germany), as described [5].

2.6. Measurement of Scratching Behavior

Following the second treatment on the sixth day, the behavior of the mice was recorded using a SCLABA®-Real system (Noveltec Inc., Kobe, Japan), as described [10]. After an acclimation period of at least one hour, the behavior of animals was recorded for two hours with no experimenters present in the observation room. The number of scratching bouts was defined as that of periodical lower limb movements lasting more than 150 millisecond search [11].

2.7. Immunohistochemistry

Skin from the dorsal neck of each mouse was taken under sevoflurane anesthesia on the seventh day. Half of each skin sample was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 hours. After washing with phosphate-buffered saline (PBS, pH7.4), small pieces of the skin were immersed in PBS containing 20% sucrose overnight at 4˚C. The skin specimens were embedded in O.C.T. compound, frozen on dry ice, and cut into cryosections (20 μm thick for PGP9.5 and SP staining or 8 μm thick for NGF) using a CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were mounted onto silane-coated glass slides. After blocking in PBS containing 5% NDS, 2% BSA and 0.2% TritonX-100 (blocking solution), the sections were incubated with primary antibodies overnight at 4˚C. The next day, the sections were washed with PBS containing 0.05% Tween 20(PBS-T) and incubated with secondary antibodies for one hour at room temperature. After washing with PBS-T, the sections were mounted in Vectashield mounting medium with DAPI.

For immunofluorescence staining of Sema3A, the other half of each skin sample was embedded in O.C.T. compound without fixation, and cut into cryosections (8 μm thick) using a CM1850 cryostat, mounted onto silane-coated glass slides. The sections were fixed in ice-cold acetone for 10 minutes at −20˚C, rehydrated in PBS-T, blocked in blocking solution, and then incubated with anti-Sema3A antibody overnight at 4˚C. The next day, the sections were washed with PBS-T, incubated with secondary antibody for one hour at room temperature. After washing with PBS-T, the sections were mounted in Vectashield mounting medium with DAPI.

2.8. Semi-Quantification of Nerve Fibers in Epidermis

Six random fields per mouse were viewed with a confocal laser-scanning microscope (DMIRE2; Leica Microsystems), with optical sections 0.9 μm thick scanned through the z-plane of the stained specimens (thickness 20 μm). Three-dimensional images were reconstructed using Leica Confocal Software (Leica Microsystems). The numbers of nerve fibers penetrating into the epidermis and intraepidermal nerve fibers were hand-counted separately. The average number of six observed fields per mouse was calculated and used for statistical analysis.

2.9. Quantitative Measurements of the Fluorescence Intensities of NGF and Sema3A

Six random fields per mouse were observed with a confocal laser-scanning microscope, with exposure and acquisition settings such that no signal saturation occurred. The sum of the fluorescence intensity of the epidermis and the area of the epidermis in each observed field was measured using Leica Confocal Software. Fluorescence intensity per unit area was calculated and used for statistical analysis.

2.10. Statistical Analysis

Data were analyzed using Prism 5 (Graph Pad software Inc., La Jolla, CA, USA). Statistical analyses were per-

formed by analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test, with $P < 0.05$ regarded as statistically significant.

3. Results

3.1. Evaluation of a Chronic Dry Skin Model Mouse Induced by AEW

Repeated AEW treatment induced the symptoms of dry skin, such as scaling and deep wrinkles (**Figure 1(a)-(c)**). Beginning on the second day of the treatment, TEWL was significantly higher in AEW-treated than in untreated mice, whereas the difference between AE-treated and untreated mice was not significant (**Figure 1(d)**). AEW treatment also significantly increased scratching behavior compared with untreated and AE-treated mice (**Figure 1 (e)**).

3.2. Effects of Tenshino-Softgel™ on TEWL and Scratching Behavior in AEW-Treated Mice

TEWL tended to be lower in AEW + TSG-treated mice, becoming significantly lower in AEW + TSG-treated than in AEW-treated mice on the fourth day (**Figure 2(a)**). The number of scratching bouts was also lower in AEW + TSG-treated mice, but not significantly when compared with the other groups (**Figure 2(b)**).

3.3. Effect of Tenshino-Softgel™ on Epidermal Nerve Fibers in AEW-Treated Mice

The density of PGP9.5-immunoreactive ($PGP9.5^+$) fibers in epidermis was examined immunohistochemically in each group using confocal microscopy (**Figure 3(a)**). The $PGP9.5^+$ fiber density was significantly lower in the AEW + TSG group than in the AEW and AEW + V group (**Figure 3(b)**, **Figure 3(c)**). Assessment of the density

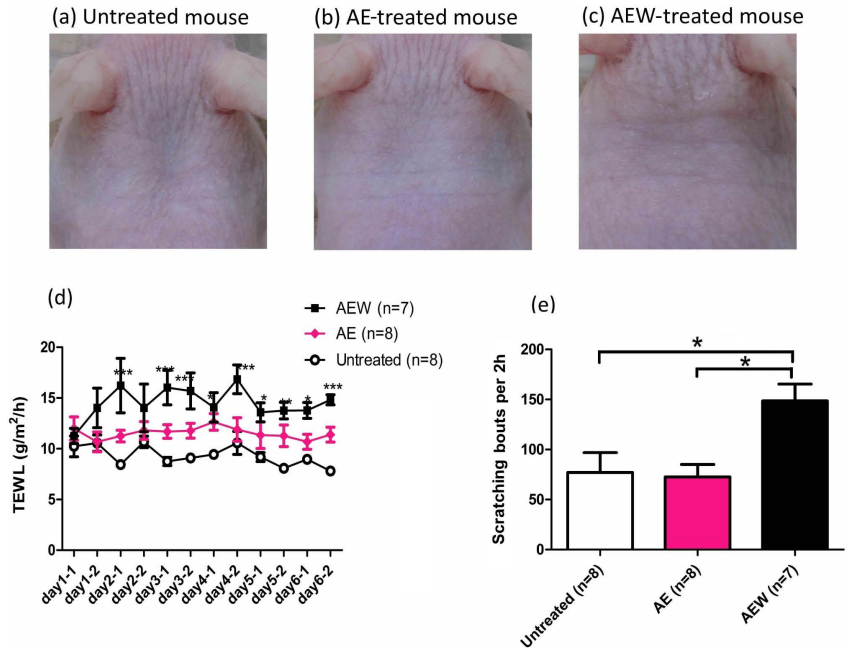

Figure 1. Evaluation of a mouse model of chronic dry skin induced by repeated applications of AEW. (a) Untreated mice showed no abnormalities. (b) Acetone/ether (1:1) mixture (AE)-treated mice showed slight scaling and wrinkling. (c) Mice treated with AE followed by water (AEW) showed scaling and deep wrinkles. (d) Transepidermal water loss (TEWL) was significantly higher in mice receiving repeated AEW treatment than in untreated mice. AE treatment slightly increased TEWL. Results are shown as means ± SEM (standard error of the mean) and compared by two-way ANOVA with Bonferroni's multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001. (e) The number of scratching bouts for two hours was significantly higher in AEW-treated mice than in untreated and AE-treated mice. Results are shown as means ± SEM and compared by one-way ANOVA with Bonferroni's multiple comparison test. *P < 0.05.

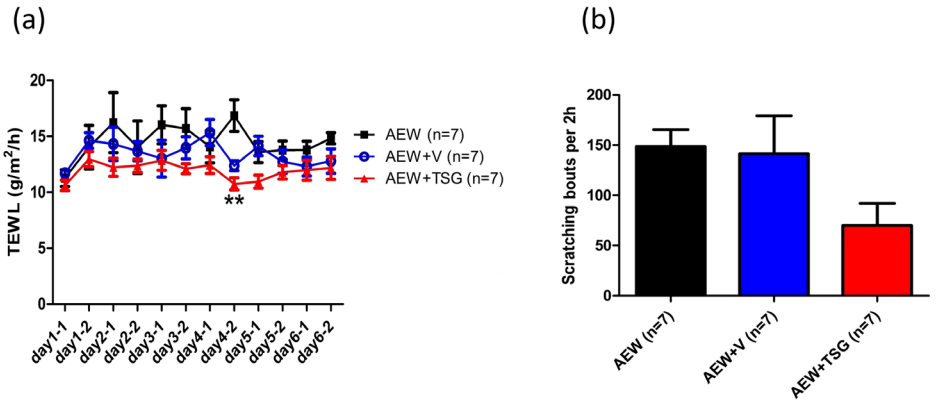

Figure 2. Effects of Tenshino-softgel™ on TEWL and scratching behavior in AEW-treated mice. (a) Compared with acetone/ether (1:1) mixture and water (AEW)-treated mice, AEW + Tenshinosoftgel™-treated mice showed slight improvements in transepidermal water loss (TEWL). Results are shown as means ± SEM and compared by two-way ANOVA with Bonferroni's multiple comparison test. **P < 0.01. (b) Scratching behavior was attenuated in AEW + TSG-treated mice compared with AEW- and AEW + Vaseline-treated mice, but the differences were not significant. Resultsare shown as means ± SEM and compared by one-way ANOVA with Bonferroni's multiple comparison test.

of SP-immunoreactive (SP^+) fibers in the epidermis (**Figure 3(d)**) also showed that the SP^+ fiber density was significantly lower in the AEW + TSG group than in the AEW group (**Figure 3(e)**, **Figure 3(f)**).

3.4. Effects of Tenshino-Softgel™ on the Expression of NGF and Sema3A in the Epidermis of AEW-Treated Mice.

Immunohistochemical examination for the effects of TSG on epidermal NGF and Sema3A expression showed that the expression level of NGF in the epidermis was similar in mice treated with AEW, AEW + V, and AEW + TSG (**Figure 4(b)**). In contrast, the expression level of Sema3A in the epidermis was higher in AEW + TSG-treated mice than in AEW- and AEW + V-treated mice (**Figure 4(d)**).

4. Discussion

This study showed that topical application of TSG significantly reduced the densities of $PGP9.5^+$- and SP^+-epidermal nerve fibers in a mouse model of chronic dry skin (**Figure 3**). In addition, our data showed that topical application of TSG tended to suppress scratching behavior (**Figure 2(b)**). According to the previous study, skin barrier disruption alters epidermal innervation and increases nerve density in the skin [5]. This hyperinnervation is indicative of increases in sensory receptive areas responsive to exogenous triggers of itch, suggesting that hyperinnervation is at least partly responsible for peripheral itch sensitization [12]. Thus, TSG may be therapeutically effective for pruritus in dry skin through inhibiting the epidermal hyperinnervation associated with skin barrier dysfunction. This inhibition in the AEW + TSG group may be due to the increased expression level of Sema3A in the epidermis (**Figure 4(d)**), although the mechanisms underlying the regulation of Sema3A expression in skin remain unclear.

In this study, emollients were applied immediately after skin barrier disruption by AEW treatment. Application of emollients such as heparinoid cream to mice with acute dry skin resulted in greater improvements in epidermal nerve fiber density and epidermal NGF levels, but had no effect on epidermal Sema3A levels [13]. In addition, the increased number of epidermal nerve fibers was lowered more by immediate than delayed application of emollients to dry skin, suggesting that prompt application of emollients is more effective in normalizing epidermal hyperinnervation and epidermal expression of axon guidance molecules [13].

This study showed that repeated AEW treatment elicited spontaneous scratching (**Figure 1(e)**), concomitant with an increase in TEWL (**Figure 1(d)**). Repeated application of AEW to the cheek skin of mice was found to generate itch without pain [14]. Moreover, AEW treatment induced spontaneous scratching in mast cell-deficient mice, indicating that mast cells may not be involved in AEW-induced scratching behavior [9]. Although

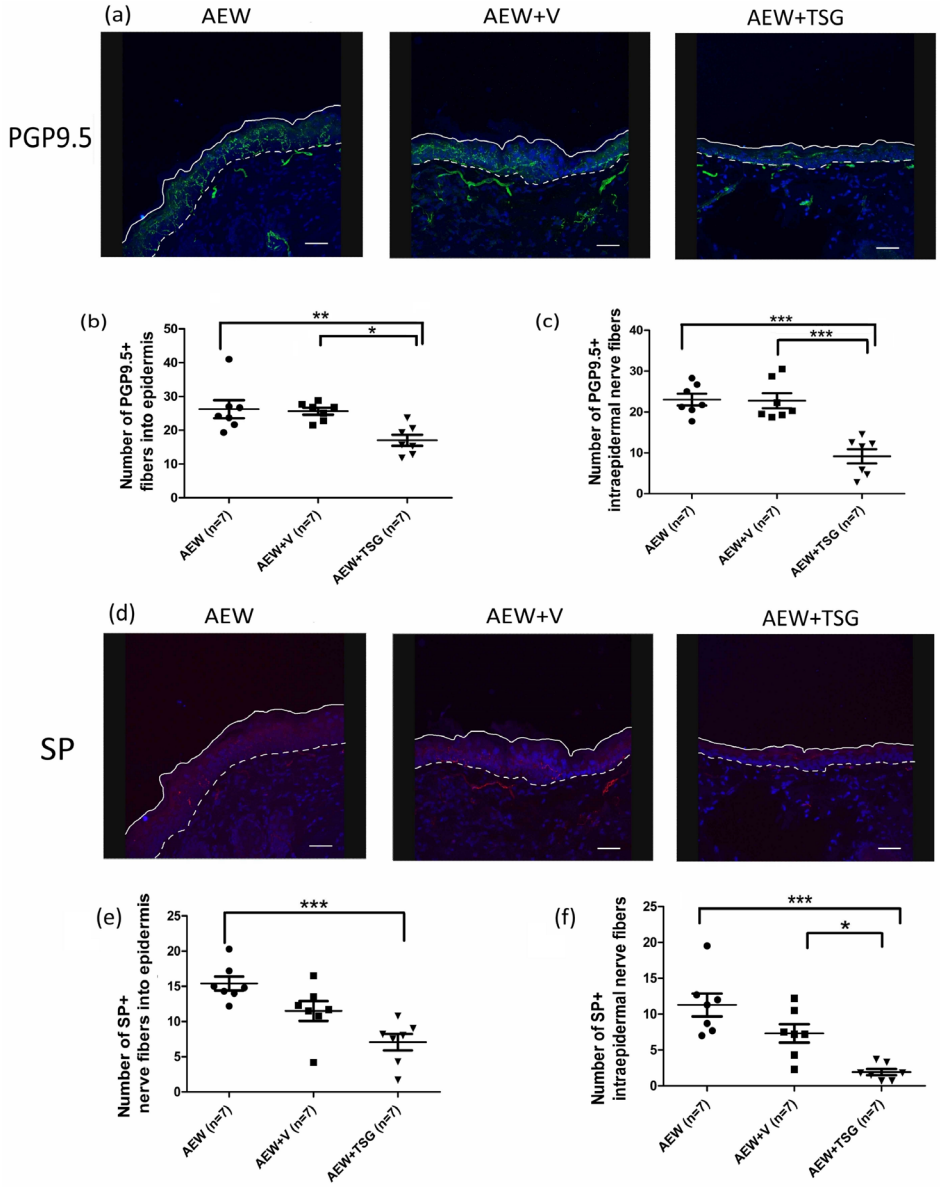

Figure 3. Effect of Tenshino-softgel™ on epidermal nerve fibers in AEW-treated mice. (a) Immunolabeling with anti-protein gene product 9.5 (PGP9.5) antibody (green) of epidermal nerve fibers on lesional skin after application of acetone/ether (1:1) mixture and water (AEW), AEW + Vaseline (V), and AEW + Tenshino-softgel™ (TSG). Images of nerve fibers are superimposed on differential interference microscopic images. White and broken lines indicate the skin surface and the border between the epidermis and dermis (basement membrane), respectively. Scale bars, 100 μm. (b, c) Numbers of PGP9.5-immunoreactive (PGP9.5$^+$) fibers (b) penetrating into and (c) within the epidermis were lower in AEW + TSG-treated mice than in AEW- and AEW + V-treated mice. (d) Immunolabeling with anti-substance P (SP) antibody (red) of epidermal nerve fibers on lesional skin after application of AEW, AEW + V, and AEW + TSG. Images of nerve fibers are superimposed on differential interference microscopic images. White and broken lines indicate the skin surface and the border between the epidermis and dermis (basement membrane), respectively.Scale bars, 100 μm. (e) Numbers of SP-immunoreatctive (SP$^+$) fibers penetrating into the epidermis were lower in AEW + TSG-treated mice than in AEW-treated mice. (f) Numbers of SP$^+$fibers within the epidermis were lower in AEW + TSG-treated mice than in AEW-and AEW + V-treated mice. Results are shown as means ± SEM (n = 7 per group) and compared by one-way ANOVA with Bonferroni's multiple comparison tests. *P < 0.05, **P < 0.01, ***P < 0.001.

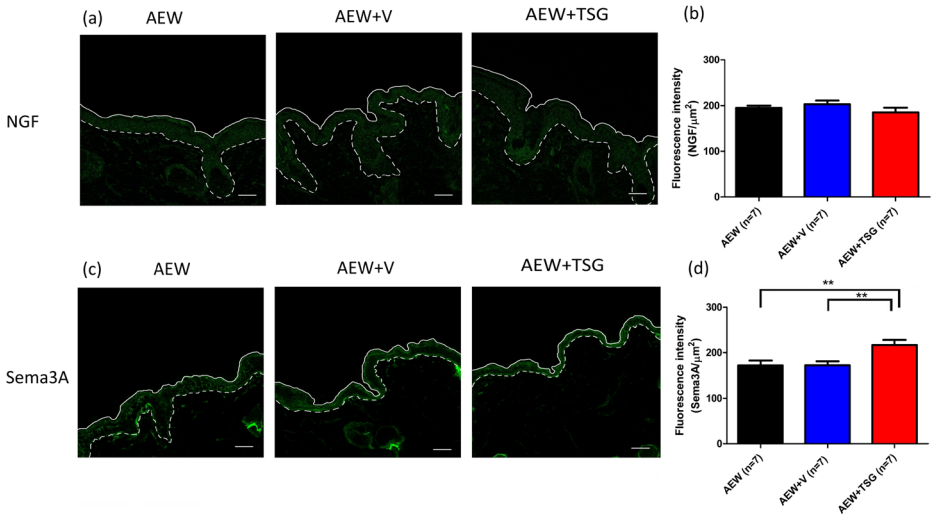

Figure 4. Effects of Tenshino-softgel™ on the expression of NGF and Sema3A in epidermis. (a) Immunolabeling with anti-nerve growth factor (NGF) antibody (green) ofmouse skin after application of acetone/ether (1:1) mixture and water (AEW), alone or plus Vaseline (V) or Tenshino-softgel ™ (TSG). (b) Epidermal NGF expression levels were similar in the three groups. (c) Immunolabeling with anti-Sema3A antibody (green) of mouse skin after application of AEW, alone or plus Vaseline or TSG. (d) Epidermal Sema3A expression levels were significantly higher in the AEW + TSG group than in the AEW- and AEW + V-treated groups. White and broken lines in each panel indicate the skin surface and the border between the epidermis and dermis (basement membrane), respectively. Scale bars, 100 μm. Results are shown as means ± SEM (n = 7 per group) and compared by one-way ANOVA with Bonferroni's multiple comparison tests. **P < 0.01.

the mechanisms are unclear, scratching behavior in mast cell-deficient mice may be caused, at least in part, by increases in epidermal nerve fibers or pruritogens secreted by other dermal cells and/or keratinocytes [15]. Alternatively, spontaneous scratching may be induced by water treatment following AE, but not by organic solvents alone. Water can remove natural moisturizing factors important for skin hydration, impairing SC hydration and flexibility [16]. Water may also induce transient swelling of the SC followed by a drying out of the surface layers. Physical swelling and shrinking of SC may act as mechanical stimuli of C-fibers in upper epidermis and evoke itch. This idea may be supported by the finding that mechanical stimuli induced the sprouting of cutaneous sensory nerve fibers [17].

5. Conclusion

In conclusion, these findings suggest that topical application of TSG restrains the progression of barrier disruption and improves the imbalance of axon guidance molecule by increasing the expression of Sema3A, possibly resulting in a decrease in epidermal nerve fiber density. TSG may therefore be useful as antipruritic therapy in patients with dry skin-based skin diseases.

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Conflict of Interest

The authors state no conflict of interest.

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