Effect of Yupingfeng granules on the skin barrier in atopic dermatitis mice models

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Abstract

OBJECTIVE: To investigate effect of Yupingfeng granules, prepared with Chinese Medicines, on the wound healing and on the expression of aquaporin 3 (AQP3) and the skin barrier in the animal models of atopic dermatitis (AD).

METHODS: Acute skin lesions of AD models were prepared using 2,4-dinitrochlorobenzo (DNCB) in mice and animals were treated with either Yupingfeng granules or placebo for two weeks. Skin wound healing outcome was assessed by measuring skin thickness, weight (quality) of the skin, and trans-epidermal water loss (TEWL). Expression of AQP3 mRNA and protein was assessed by reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting, respectively.

RESULTS: Yupingfeng granule treatment resulted in significant acceleration of wound healing with 63.64% efficiency, which was significantly higher than that of placebo granule treatment (31.82%, P < 0.01 by Wilcoxon Rank-sum test). Skin thickness, weight of the wounded skin, and TEWL were significantly higher in the AD models compared to that of normal animals. Treatment with Yupingfeng granules resulted in significant decrease in skin thickness ([937 ± 31] vs [360 ± 21] μm, P < 0.01), weight of the wounded skin ([42 ± 4] vs [24 ± 5] mg, P < 0.01), and TEWL ([30 ± 4] vs [13 ± 4] g·h⁻¹·m⁻², P < 0.01). Yupingfeng granules also significantly down-regulated mRNA and protein expression of AQP3 in the animal models.

CONCLUSION: Our findings suggested that Yupingfeng granules could be used in AD treatment.

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Keywords: Dermatitis, atopic; Aquaporin 3; Wound healing; Models, animal; Yupingfeng granules

INTRODUCTION

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. The prevalence of human AD is approximately 10%-20% of population in the developed countries, and it has a severe impact on quality of life. Studies indicated that spongiosis is one of the histological features in acute AD skin lesion, while epidermal hyperplasia is the predominant feature in chronic phase of AD. Infiltration of T cells, predominantly...
CD4+ T, and dendritic cells along with an accumulation of mast cells and eosinophils in the area of wounded skin are immunological features of the AD.\(^1\) In this context, acute skin lesions are dominated by Th2-associated cytokines (IL-4, IL-13, and IL-31) as well as IL-22 and IL-17, especially in Asian populations.\(^2\) In the phase of chronic AD, expression of Th1-associated cytokines (IFN-γ and IL-12) was increased in addition to the continuous increase of Th2-associated cytokines.\(^3\)

Aquaporins (AQPs) are expressed in plasma membranes in a variety of cell types including keratinocytes, where they function as pore-like passive transporters in response to alteration of osmotic gradients (for water transport) or glycerol gradients (for glycerol transport). There are at least 13 mammalian AQPs (AQP0-AOP-12). AQP1, 2, 4, 5, and 8 function primarily as water-selective transporters; while AQP3, 7, 9, and 10 transport both water and glycerol.\(^4\) Of them, AQP3 is the most studied and well-validated AQP in skin, and phenotype analysis of transgenic mice lacking AQP3 has revealed that AQP3 is involved in skin hydration, wound healing and tumorigenesis of skin carcinoma.\(^5\)

Yupingfeng granules, which are made from Huangqi (Radix Astragali Mongolici), Baizhu (Rhizoma Atractylodis Macrocephalae), and Fangfeng (Radix Saposhnikoviae), have been reported to intensify immunity, as well as to have anti-virus and anti-bacterial effect, and anti-oxidant effect.\(^6,\)\(^7\) In addition, Yupingfeng granules can also modulate the balance of CD4+ and CD8+ T cell number.\(^8\) In this study, effect of Yupingfeng granules on the wound healing of atopic skin damage, and its effect on the expression of AQP3 were investigated in AD animal models.

**MATERIALS AND METHODS**

**Animals**

A total of 72 BALB/c mice, specific pathogen free grade and 5 weeks old, weighed (22 ± 2) g, were purchased from Beijing WeiTongLiHua Animal Facility (Beijing, China). Animals were kept and cared in the Animal Care Facility, Dongfang Hospital, Beijing University of Traditional Chinese Medicine following the ICAUC approved by the Committee of Beijing University of Traditional Chinese Medicine.

**Drugs and reagents**

Therapeutic drug (Yupingfeng granules, Z10930036) and placebo (placebo granules) were manufactured by Guangdong HuanQiu Pharmaceutical Company (Guangdong, China). Sodium sulfate (NaS), 2,4-dinitrochlorobenzo, DNBC, acetone, starch, and liquid nitrogen were purchased from Shanghai Maclin biochemical technology Co., Ltd., (Shanghai, China).

**Preparation and treatment of the animal models**

AD models were prepared as described previously with minus modification.\(^1\) Briefly, back (area A: 1 cm × 2 cm) and abdomen (area B: 2 cm × 2 cm) were shaved. Area A was then treated with 7% DNBC in acetone. Two weeks later, area B was treated with 0.1% DNBC, once a week for 4 weeks.

Before preparation of the animal models, normal skin tissues (1 cm² size) on the back of 24 randomly selected mice were obtained and stored at liquid nitrogen tank. These 24 normal mice were used as normal control. The rest of 48 mice were used for AD preparation. Of them, 2 mice died during the preparation of AD. The rest 46 AD models were randomly assigned to the treatment group or placebo group. Treatment group (23 mice) was given Yupingfeng granules through gavage, 0.05 g dissolved in 2 mL normal saline, twice a day for two weeks. Placebo group (23 mice) was given placebo granule through gavage, 0.05 g dissolved in 2 mL normal saline, twice a day for two weeks. One animal from treatment group and one from placebo group died from accident intra-tracheal choke during the two-week treatment, which was proved by autopsy of the animals.

Before and after the treatment with Yupingfeng or placebo, trans-epidermal water loss (TEWL) and skin thickness were assessed, and then a size of 0.5 cm² skin tissue was cut from each animal under local anesthesia. The biopsy tissue was weighed and the wound was sutured.

**Criteria for evaluating animal model and its treatment**

Criteria for the AD: AD was determined by the following manifestations: erythema, pimples, scab, exudation, epidermal stripping off, and scales.

AD assessment: severity of the skin damage was evaluated with Eczema Area and Severity Index (EASI) as previously reported.\(^1\) General appearances of the skin lesions were described as following: erythema, swelling or hard swollen, pimples, epidermal stripping off, and mossy. The severity of each manifestation of skin lesion was scored following the Berth-Jones method\(^1\) at six areas with six signs: 0 = none; 1 = mild; 2 = moderate; 3 = severe; 0.5 was scored if it was between none, mild, moderate and severe grade.

Criteria for evaluating therapeutic effect: following the criteria of "Instruction of Clinical Study on New and Herbal Drug", therapeutic results of AD in the animal models were assessed with total score of the following formula: [(Score before treatment − score after treatment) / score before treatment] × 100%. (a) Clinically cure: skin damage was fully or nearly repaired, score was decreased at least 95%. (b) Significantly effective: majority of the wounded skin was repaired, score was decreased over 70% but less than 95%. (c) Effective: wounded skin was partially repaired, score was decreased over 50% but less than 70%. (d) No effect:
there was no recovery in the damaged skin and decrease of the score was less than 50%. Total effective rate = (Number of cured cases + number of significantly effective cases)/total number × 100%.

**Immunoblotting**

Protein level of AQP-3 in the skin tissues was semi-quantitatively analyzed by immunoblotting. Briefly, total protein was extracted from minced skin tissues and protein amount was measured with BCA method. Total 20 µL of mixture of samples and 2 x loading buffer was loaded and subjected for electrophoresis. The proteins were transferred to PVDF membrane at 20 V for 30 min. After one hour blocking at room temperature, monoclonal sheep anti-mouse AQP-3 antibody (1:1000, Beijing OuBei Biotech, Beijing, China) was applied at 4 ºC overnight. After washing, rabbit anti-sheep 2nd antibody (1:3000, Beijing OuBei Biotech, Beijing, China) was applied at room temperature for 1 h. Targeted band was visualized with ECL. β-actin was used as loading control and images were analyzed with Image J software.

**Real time RT-PCR**

Total RNA was extracted using a commercially available RNA extraction kit (Takara Code D9108B, Dalian, China). Reverse transcription was performed using 5 µL total RNA and a commercially available kit Revert AidTM kit (Takara Code DRR047A) following the manufacturer’s instruction. Real time PCR was conducted by mixing 12.5 µL 2x Taq buffer, 0.5 µL forward-primers and backward-primers, 2 µL cDNA, and 4.5 µL ddH2O. Sequences of the primers were as following: AQP3 forward: 5'-CATAGGCCACAGCAGCCCTTA-3'; backward: 5'-CCCCATGACCAGGACACAA-3', with total length of 115bp; GAPDH forward: 5'-ACTCTGTGGATTGGTGGC-3'; backward: 5'-AGAAAGGGTGTAAAACGCAGC-3', with total length of 155 bp. Reaction was performed at 95 ºC for 5 s, 60 ºC for 34 s for each cycle and total 40 cycles with ABI 7500 PCR equipment (Applied Biosystems).

**Trans-epidermal water loss (TEWL), skin thickness, and weight of the wounded skin biopsy assessment**

Skin thickness measurement: skin thickness was measured with a micrometer at 3 locations on the back of each normal animal (marked as A, B, and C). Take the half of the average number as the skin thickness (µm).

(b) TWEL measurement: TEWL was measured at 3 different locations on the back of each animal and average was calculated. Skin quality assessment: before and after the treatment, three pieces of skin were obtained with a punching device (5 mm size) at 3 different locations on the back of each animal. Each biopsy tissue was weighed on a digital balance after the fat underneath the skin was carefully removed. Average weight of the 3 pieces was calculated.

**Statistical analysis**

Data was analyzed with SPSS 15.0 software (SPSS Inc, SPSS for Windows, Version 16.0. Chicago, IL, USA). Measurement data are expressed by mean ± standard deviation (x ± s). Difference between treatment and placebo groups was analyzed with Student t test. One-way analysis of variance was used to compare skin thickness and TEWL. Therapeutic effect of the two groups was compared and analyzed by Wilcoxon Rank-sum test, and P < 0.05 was considered as statistically significant.

**RESULTS**

Atopic skin lesions were nearly healed after two-week treatment with Yupingfeng granules with 63.64% efficiency, which was significantly higher than that treated with placebo (31.82%, P < 0.01 by Wilcoxon Rank-sum test) (Figure 1, Table 1).

**Figure 1 Skin lesions of atopic dermatitis before and after treatment**

A: before treatment of the placebo group; B: after treatment of the placebo group; C: before treatment of the Yupingfeng group; D: after treatment of the Yupingfeng group. Animal models of AD were prepared as described in the methods. The animals were then treated with either Yupingfeng granules or placebo granule for two weeks. Data presented was one representative animal from each group.

Therapeutic effect was assessed by skin thickness of the wound, weight (quality) of the skin and trans-epidermal water loss (TEWL). Before the treatment, skin
thick-ness of the AD model animals (923 ± 22) µm was significantly higher than that of normal control group [(343 ± 25) µm, P < 0.01]. After 2 weeks treatment, it was significantly decreased in the animals treated with Yupingfeng granules (360 ± 21) µm compared to that treated with placebo granule (591 ± 32) µm (P < 0.05) (Table 2). Similarly, weight of wounded skin from the AD model was significantly higher than that of normal control [(40 ± 5) g (19 ± 3) mg, P < 0.01] before the treatment. After 2 weeks treatment with Yupingfeng granules, skin weight (quality) was significantly reduced to nearly normal weight [(24 ± 5) g (21 ± 3) mg, P > 0.05], while the skin weight of the animals treated with placebo was not significantly reduced and significantly higher than that of control skin [(30 ± 4) g (21 ± 3) mg, P < 0.05] (Table 3).

To further confirm the therapeutic effect, TEWL was assessed. As shown in Table 4, TEWL was significantly higher in the AD model before the treatment compared to that of normal control [(27.4 ± 3.1) µm, (4.9 ± 0.5) g·h⁻¹·m⁻², P < 0.01)]. After 2 weeks treatment with Yupingfeng granules, TEWL was significantly reduced compared to that of the animals treated with placebo [(13.3 ± 4.0) µm, (19.0 ± 2.0) g·h⁻¹·m⁻², P < 0.05]. However, it was still higher than that of normal control animals [(5.5 ± 0.1) g·h⁻¹·m⁻², P < 0.05].

Next, AQP3 protein level was examined by immunoblotting. As shown in Figure 2A and Table 5, AQP-2 was detectable in normal skin, which was significantly up-regulated in the animals with atopic skin damage (1.44 ± 0.23 or 1.46 ± 0.22 vs 0.57 ± 0.07 of normal skin). After one-week treatment, AQP3 protein was significantly reduced in the animals treated with Yupingfeng granules and nearly back to the level of normal skin (Figure 2B and Table 5, 0.65 ± 0.07 vs 0.53 ± 0.09). In contrast, it was not significantly reduced in the animals treated with placebo (Figure 2B and Table 5, 1.35 ± 0.31 vs 0.53 ± 0.09). AQP3 protein level was significantly different between the Yupingfeng granules treated animals (0.65 ± 0.07) and placebo treated groups (1.35 ± 0.31, P < 0.01).

In the study, we found that Yupingfeng granule treat-
Immunologically, skin lesions of AD are characterized by infiltration of T cells, predominantly CD4+ T, and dendritic cells as well as dermal mast cells and eosinophils. In Chinese Medicine, AD is also called "SiWan-Feng", which is considered as being caused by insufficiency of internal defense and invasion of external moist and it is believed that abnormal skin barrier function contributes to the prolonged healing period of AD. Yupingfeng granules are prepared with Huangqi (Radix Astragali Mongolici), Baihu (Rhizoma Atractylodis Macrocephalae), and Fangfeng (Radix Sa-podnikovitae) and is known to intensify immunity, anti-virus and anti-bacterial effect, and anti-oxidant effect. In addition, Yupingfeng granules can also modulate the balance of CD4+ and CD8+ T cell number. Here, we report that Yupingfeng granule treatment resulted in significant decrease in skin thickness, weight and trans-epidermal water loss of the wounded skin, suggesting Yupingfeng granules accelerated wound healing of atopic skin damage. Furthermore, Yupingfeng granules significantly inhibited gene expression and protein synthesis of AQP3.

The AQP3s are expressed in a variety of cell types, where they function as pore-like passive transporters responding to transmembrane osmotic gradients (for water transport) or glycerol gradients (for glycerol transport). There are at least 13 mammalian AQP3s (AQP0-AQP-12), which have been divided into two groups on the basis of their permeability. AQP3s 1, 2, 4, 5, and 8 function primarily as water-selective transporters; AQP3s 3, 7, 9, and 10 termed "aquaglyceroporins", transport water as well as glycerol and possibly other small slutes. AQP3, the most studied and well-validated AQP in skin, was first reported in keratinocytes of rat epidermis. AQP3 is a membrane transporter of water and glycerol expressed in plasma membranes in the basal layer keratinocytes of epidermis in normal skin. It has been reported that AQP3 expression in human skin is increased in the milieu of chronic skin diseases including AD as well as in response to variety kinds of stimulants such as retinoic acid. AQP3 plays an important role in the regulation of moisturization and water balance of the skin. Its up-regulation in the wounded skin, which was significantly reduced after two-weeks Yupingfeng granule treatment, not with placebo.

AD is a common chronic inflammatory skin disease characterized by epidermal barrier dysfunction and immunological alterations. Its prevalence has been doubled in industrialized countries during the past decades. It is estimated that 15%-30% of children and 2%-10% of adults are affected by AD. Histologically, spongiosis is the classic feature of skin lesions in acute phase of AD and epidermal hyperplasia is the predominant characteristic in the developing phase of chronic AD as evidenced by increased expression of the proliferation-associated markers keratin 16 (K16) and Ki67.

Figure 2 Protein levels of AQP3 in the AD models
Total proteins were extracted from the normal or wounded skin, and subjected to immunoblotting of AQP3 as described in the methods. A: before the treatment; B: after the treatment. Data presented was one representative of 20 times separated experiments with similar results. 1: placebo group; 2: normal group; 3: treatment group. AQP3: aquaporin 3; AD: atopic dermatitis.

Table 5 Comparison of AQP3 protein levels in normal and wounded skin before and after treatment ( x ± s )

<table>
<thead>
<tr>
<th>Items</th>
<th>Normal skin (n = 24)</th>
<th>Wounded skin placebo (n = 22)</th>
<th>Wounded skin Yupingfeng granule (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>0.57±0.07</td>
<td>1.44±0.23</td>
<td>1.46±0.22†</td>
</tr>
<tr>
<td>After treatment</td>
<td>0.53±0.09</td>
<td>1.35±0.31†</td>
<td>0.65±0.07†</td>
</tr>
</tbody>
</table>

Notes: wounded animals were treated with either placebo granule or Yupingfeng granule, 0.05 g/2 mL normal saline, twice a day for 2 weeks. AQP3: aquaporin 3. P < 0.01 by comparing “a” vs “c”, or “b” vs “c”.

Table 6 Comparison of AQP3 mRNA expression in normal and wounded skin before and after treatment ( x ± s )

<table>
<thead>
<tr>
<th>Items</th>
<th>Normal skin (n = 24)</th>
<th>Wounded skin placebo (n = 22)</th>
<th>Wounded skin Yupingfeng granule (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>1.05±0.13</td>
<td>2.01±0.31</td>
<td>2.05±0.35†</td>
</tr>
<tr>
<td>After treatment</td>
<td>1.10±0.16</td>
<td>1.99±0.24†</td>
<td>1.17±0.18†</td>
</tr>
</tbody>
</table>

Notes: wounded animals were treated with either placebo granule or Yupingfeng granule, 0.05 g/2 mL normal saline, twice a day for 2 weeks. AQP3: aquaporin 3. P < 0.01 by comparing “a” vs “c”, or “b” vs “c”.
portant in skin hydration, wound healing and tumoriogenesis of skin carcinomas. Multiple transcriptome data analysis revealed that AQP3 expression was altered in AD and AQP3 expression in human keratinocyte cultures was increased in response to hyperosmolarity (over 2 fold) retinoic acid (2-fold after 2 h in culture), and phorbol ester application to mouse epidermis in vivo (10 fold by 4 h). Here, we report that AQP3 mRNA and protein was significantly increased in the skin of AD animal models and Yupingfeng granules significantly blocked the up-regulation of AQP3 in the AD animal models.

Ideally, spontaneously inducible models of AD are generally preferred, which possess as many characteristics of the human AD as possible. However, currently, most models of AD will use some procedures to better reproduce the changes seen in the human disease and to enhance the absorption of AD-causing substances. These procedures include genetically engineered models, allergen-induced models, and chemical reagents, such as hapten and 2,4-dinitrochlorobenzene (DNCB), treated models. In the current study, chemical reagent, DNCB, was used to establish the mice model of AD. There were limitations in the current study. First, histological and immunological characteristics of this model were not explored before and after treatment with Yupingfeng granules, which remains to be investigated in the future. Second, only one concentration of Yupingfeng granules was examined in the current study, and thus, the concentration-effect relationship between the treatment and outcome remains to be investigated in the future study. Third, blood biochemical parameters including liver and kidney functional indicators were not examined in the animals.

In conclusion, our findings revealed that Yupingfeng granules significantly stimulated skin tissue repair and healing of the wounded skin. AQP3 protein and mRNA was significantly up-regulated in the wounded skin, which was significantly reduced after two weeks of Yupingfeng granule treatment, which suggests that the granules may be used as an alternative to AD treatment.

REFERENCES

