

Original Article

Effect of *Insamyangyoung-tang* on the Skin Barrier Function of Hairless Mice

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Objective : To study the effect of the *Insamyangyoung-tang* (ISYT) extract on the skin barrier function, the skin pH, skin humidity and transepidermal water loss (TEWL) were measured and histological changes were observed in DNCB (2,4-dinitrochloro-benzen)-induced contact dermatitis (CD) hairless mice.

Methods : The male hairless mice were divided into three groups. Each group consisted of 15 mice. The normal group which had acetone- olive oil applied. The control group which had intentionally induced CD by DNCB and it was fed normal saline orally. The ISYT group which had intentionally induced CD by DNCB and it was fed ISYT extract orally for 7 days. The three groups were checked 24h, 48h and 72h later after inducing CD, and the skin pH, skin humidity and TEWL were observed. Tissue samples were taken, and damage to the epithelial cell was observed. Statistical analysis was performed by using one way-ANOVA: significance was set at p values less than 5% ($p < 0.05$).

Results : ISYTexttract efficiently maintained the pH balance, it kept the skin humidity at a normal level, and it inhibited TEWL of the DNCB-induced CD hairless mouse. The damage to the epithelium was decreased and the regeneration power of the skin was increased in the ISYT group.

Conclusion : *Insamyangyoung-tang* has a good effect on the skin barrier function of DNCB induced contact dermatitis hairless mice.

Key Words : *Insamyangyoung-tang*, Skin barrier function, Skin pH, Skin humidity, Transepidermal Water Loss

Introduction

The skin has evolved as a primary defense mechanism against the external environment, bacterial infections, viral invasion and physical damage. Especially, the highly organized structure of the stratum corneum forms an effective barrier to protect the epidermal water and it contributes to the maintenance of the skin's

humidity and health. So, healthy skin can deal with different types of external simulation and recovers quickly from severe damage¹⁻².

Recently, hypersensitive skin diseases like allergic contact dermatitis or atopic eczema have apparently increased in South Korea³⁻⁴. On one hand, the increasing environmental pollution caused by industrial development has become one main cause of this situation, but on the other hand, the failure of the skin barrier to function correctly is a more basic reason. If the function of the skin barrier is the major key to hypersensitive skin diseases, then a medicine that has an effect on the skin barrier function is needed for treating such diseases.

The skin barrier function is very similar to the

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function of defense-*qi* (衛氣) in Oriental medical theory. The 《Yellow Emperor's Inner Canon: Magic Pivot》 (黃帝內經靈樞) said "Defense-*qi* can make skin and muscle warm and moist. Defense-*qi* also controls the sweating system of the skin pole"⁵⁾.

Insamyangyoung-tang (ISYT) is a representative medicine to treat a deficiency of defense-*qi*⁶⁾. Based on the similarity between skin barrier function and defense-*qi*, we hypothesized that ISYT has an effect on the function of the skin barrier.

2,4-dinitrochlorobenzene (DNCB) has been frequently used *in vivo* studies that are concerned with contact dermatitis (CD) or skin dermatitis⁷⁻⁹⁾. The hairless mouse has been used as an experimental animal model of skin barrier function because of its special skin condition¹⁰⁾.

In this study, we made ISYT extract and we examined how ISYT extract affected the skin barrier function of hairless mice by calculating the skin pH, skin humidity and transepidermal water loss (TEWL). We also observed the histological changes of the epithelial layer of hairless mice.

Materials and Methods

1. Quantitative analysis of marker compounds

HPLC analysis of marker compounds in ISYT, *Paeonia lactiflora* was performed. Chromatographic separation was carried out on a Nucleosil C18 column (4.0 mm × 250 mm); the chromatographic system consisted of Waters Alliance 2690 Separation Module, with column heater, Waters 996 Photodiode Array Detector, and Waters Millennium³² Workstation. Water-methanol-acetonitrile (700:100:50) was used as the mobile phase. Detection of the peaks was

made at 230nm and the sensitivity was set of 0.50 AU. The injection volume was 10 μ l and the flow rate was 1.2ml/min. A standard solution was prepared by dissolving in distilled water (10mg/20ml). The solution was filtered through 0.45 μ m membrane filter (Fig.1).

2. Animals

Healthy male hairless mice (SLC, Japan) weighting 20 grams each were used for the experiment. The animals were individually kept in animal cages in the laboratory (temperature: 23 ± 2°C, relative humidity: 55 ± 5 %, and a 12/12 light/dark cycle) and they had access to feed and water *ad libitum* throughout the experimental period.

3. Medicinal prescription and extract supplementation

All the herbs were obtained from Kyung Hee Oriental Medical Center. This hospital has been authorized by the Ministry of Health and Welfare of the Republic of Korea to use herbs as medicine for clinical research and treatment. All the herbs used this experiment met the standard requirements. The composition of the medicinal herb is listed in Table 1. All the herbs were dried and weighed. To obtain the 1st decocted solution, 165 g of ISYT were put into a glass bottle; 1000cc distilled water were added and the herbs were submerged for 2 hours and then boiled for 3 hours at 100°C in a reflex condenser. Then, the same method was used with an extra 800cc of distilled water to obtain the 2nd decocted solution. Each solution was mixed, filtered (filter paper No.2 Toyo Rashi Kaisha, Ltd. Japan) and condensed by using a rotary vacuum evaporator (EYELA, Japan). The solutions were then powdered by the vacuum freeze

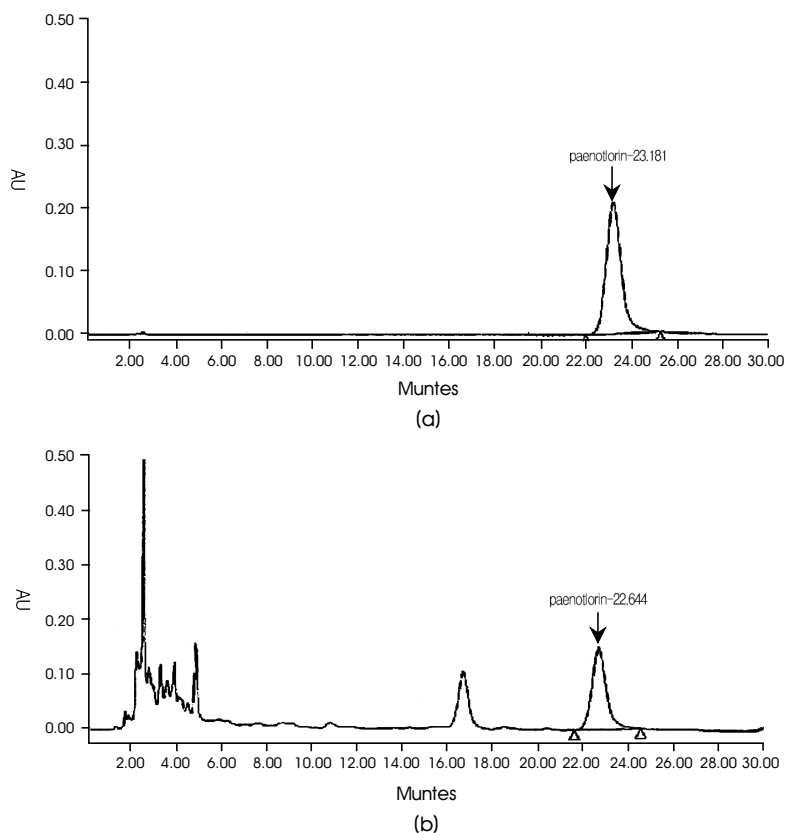


Fig. 1. HPLC chromatogram of *Paeonia lactiflora Pallas* in ISYT. (a) showed paenoflorin standard solution in *Paeonia lactiflora Pallas* and (b) showed paenoflorin standard solution in ISYT.

drying method. A total of 30.03 g (yield: 18.2 %) of powder was obtained from each solution. We next mixed the powder with distilled water at a concentration of 10g powder/ 100cc distilled water and so made the ISYT herbal solution.

4. Treatments

5 % and 2.5 % diluted 2,4-dinitrochlorobenzene (DNCB: Sigma, USA) oil solutions were diluted with a mixed acetone-olive oil solution (mixed at a 4:1 ratio). The hairless mice were divided into three groups 7 days after their stay in the laboratory: each group consisted of 15 mice. The first group had only acetone-olive oil applied to

their skin and were fed normal saline for 14 days (Normal group). The second group was sensitized with 80 μl of 5 % DNCB on the lower part of the dorsum ($2 \times 2\text{cm}^2$) and were fed normal saline for 14 days before the 2nd DNCB application (Control group), and the third group was sensitized with 80 μl of 5 % DNCB on the lower part of the dorsum ($2 \times 2\text{cm}^2$) and were fed ISYT extract solution for 14 days before the 2nd DNCB application (ISYT group). All the solutions were administered orally at a volume of 1 g/ kg / day per mouse. 14 days later after the first application of 80 μl of 5 % DNCB, Control group and ISYT group were again sensitized 30

Table 1. Composition of *Insamyangyoung-tang*

Medicinal plants	Species	Amounts(g)
Paeoniae Radix	<i>Paeonia lactiflora</i> Pallas	24
Angelicae Gigantis Radix	<i>Angelica gigas</i> Nakai	12
Ginseng Radix Alba	<i>Panax ginseng</i> C. A. Meyer	12
Atractylodis Rhizoma Alba	<i>Atractylodes japonica</i> Koidzumi	12
Astragali Radix	Honey roasted <i>Astragalus membranaceus</i> Bunge	12
Cinnamomi Cortex Spissus	<i>Cinnamomum cassia</i> Blume	12
Citri Unshii Pericarpium	<i>Citrus unshiu</i> Markovich	12
Glycyrrhizae Radix	Broiled root of <i>Glycyrrhiza uralensis</i> Fischer	12
Rehmanniae Radix Preparata	<i>Rehmannia glutinosa</i> Libosch var. <i>purpurea</i> Makino	9
Schisandrae Fructus	<i>Schisandra chinensis</i> Baillon	9
Saposhnikoviae Radix	<i>Saposhnikovia divaricata</i> Schisikin	9
Polygalae Radix	<i>Polygala tenuifolia</i> Willd.	6
Zingiberis Rhizoma Crudus	<i>Zingiber officinale</i> Rosco	12
Zizyphi Fructus	<i>Ziziphus jujuba</i> Mill.	12

μl of 2.5 % DNCB on the upper part of the dorsum. Each group of mice was divided into subgroups of five mice each and they were anesthetized by an IM injection of Zoletil (0.1 cc /100g): they were observed 24 hours, 48 hours and 72 hours after the 2nd DNCB application.

5. Measurement of skin pH

The skin pH of each group was measured with a Skin-pH-meter PH 900 PC (Courage+Khazaka electronic GmbH, Germany) at 24 hours, 48 hours and 72 hours after the 2nd application of 2.5 % DNCB.

6. Measurement of skin humidity

The skin humidity of each group was measured with a Corneometer CM 825 PC (Courage+Khazaka electronic GmbH, Germany) at 24 hours, 48 hours and 72 hours after the 2nd application

of 2.5 % DNCB.

7. Measurement of transepidermal water loss

The TEWL of each group was measured with using a Vapometer (Delfin Technology, Finland) at 24 hours, 48 hours and 72 hours after the 2nd application of 2.5 % DNCB.

8. Preparation for histological changes

At 24 hours, 48 hours and 72 hours after the 2nd application of 2.5 % DNCB, the three groups of hairless mice were anesthetized by an IM injection of Zoletil (0.1 cc / 100g). To examine for the histological changes, the skin tissue of the DNCB-applied area was obtained from each hairless mouse and then fixed in 10 % neutral formalin for 24 hours. The paraffin sections (5 μm) were next stained with hematoxyline-eosin (HE) and the general histological changes were

then observed on the HE-stained sections ($\times 100$).

9. Statistical analysis

The data are expressed as means \pm standard deviations. One-way ANOVA analysis of variance followed by Scheffe's test was used for multigroup comparisons. Statistical significance was set at p values less than 5 % ($p < 0.05$).

Results

1. Skin pH

24 hours after the 2nd DNCB application, the Control group showed statistically significant lower skin surface pH levels as compared with the Normal ($p < 0.05$) and ISYT groups ($p < 0.05$) (Control group: 6.08 ± 0.42 , Normal group: 7.04 ± 0.28 , ISYT group: 7.00 ± 0.58). After 48 hours, the Control group showed a very significant lower pH level as compared with the Normal ($p < 0.001$) and ISYT groups ($p < 0.01$) (Control group: 5.76 ± 0.31 , Normal group: 6.94 ± 0.29 , ISYT group: 6.52 ± 0.25). At 72 hours,

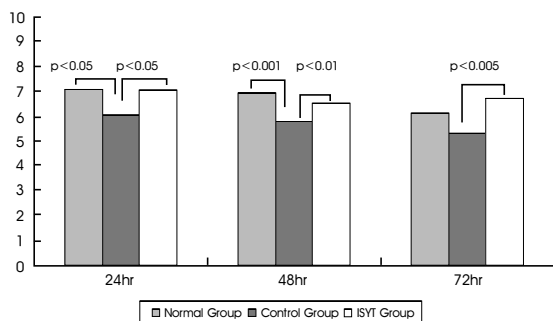


Fig. 2. Skin surface pH change of the three groups of hairless mice at 24h, 48h and 72h after the 2nd DNCB application. Control group showed significantly lower pH level through 24-72hs as compared with the other groups. On the contrary, ISYT group showed minimal change as compared with Normal group (with using one-way ANOVA and statistical significance was set at less than 5 %).

the Control group showed a significant lower pH level as compared with the ISYT group (Control group: 5.28 ± 0.66 , ISYT group: 6.66 ± 0.27 , $p < 0.005$) (Fig. 2).

2. Skin humidity

24 hours after the 2nd DNCB application, the skin humidity of the Control group was significantly diminished as compared with the Normal ($p < 0.05$) and ISYT groups ($p < 0.05$) (Control group: 17.60 ± 11.67 , Normal group: 47.40 ± 7.82 , ISYT group: 47.80 ± 7.98). There was no significant difference among the three groups at 48 hours (Control group: 28.20 ± 8.87 , Normal group: 42.20 ± 8.43 , ISYT group: 41.80 ± 9.98) and 72 hours (Control group: 38.40 ± 0.54 , Normal group: 38.00 ± 17.11 , ISYT group: 43.00 ± 8.03) after the 2nd DNCB application (Fig. 3).

3. Transepidermal water loss

24 hours after the 2nd DNCB application, there was a significant increase of TEWL in the

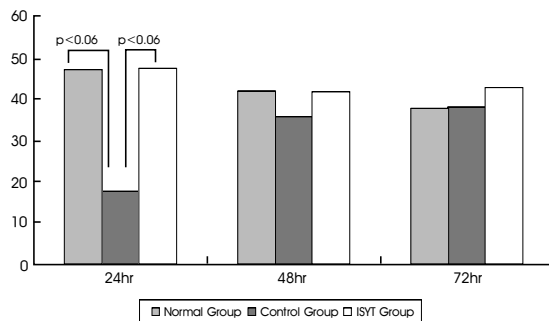


Fig. 3. Skin humidity change of the three groups of hairless mice at 24h, 48h and 72h after the 2nd DNCB application. Control group showed significantly lower skin humidity at 24h as compared with the other groups. There was no statistical difference among three groups at 48 and 72h (with using one-way ANOVA and statistical significance was set at less than 5 %).

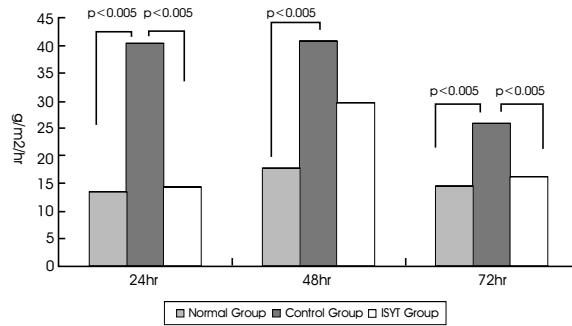


Fig. 4. TEWL change of the three groups of hairless mice at 24h, 48h and 72h after the 2nd DNCB application. Control group showed significantly higher TEWL through 24-72hs compared with the other groups. On the contrary, ISYT group showed some change of TEWL at 48h but there was no statistical difference as compared with Normal group.

Control group as compared with the Normal ($p < 0.005$) and ISYT groups ($p < 0.005$) (Control group: 40.52 ± 12.57 , Normal group: 13.74 ± 7.22 , ISYT group: 14.46 ± 2.68). At 48 hours, the Control group showed significantly increased TEWL as compared with the Normal group ($p < 0.05$) and mildly increased TEWL as compared with the ISYT group (Control group: 40.96 ± 10.10 , Normal group: 17.86 ± 6.36 , RSYRT

group: 34.42 ± 13.13). At 72 hours, the Control group showed significantly increased TEWL as compared with the Normal ($p < 0.005$) and ISYT groups ($p < 0.005$) (Control group: 26.84 ± 3.18 , Normal group: 14.72 ± 5.06 , ISYT group: 16.32 ± 3.06) (Fig. 4).

4. Histopathology

24 hours after the 2nd DNCB application,

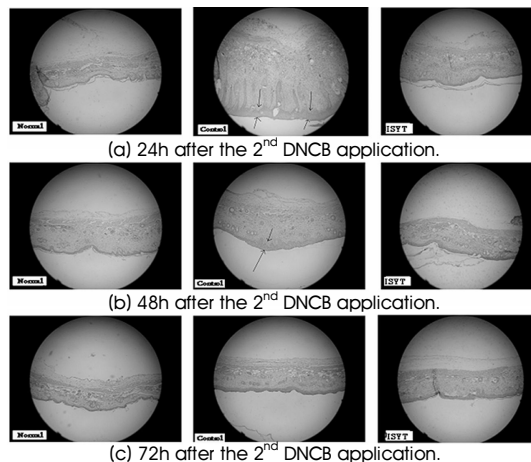


Fig. 5. (a) Control group showed much thick epidermis and a greater infiltration of inflammatory cells than the other groups. Damage of the stratum corneum was seen in both Control group and ISYT group, but the former showed much more severe damage than the latter. (b) Showed the same results as (a). (c) Control group showed thicker epidermis than the other groups.

there was a change of thickness of the dermis and epidermis of the Control group. An infiltration of inflammatory cells in the dermis and damage to the epithelium were also observed in the Control group. In contrast, the ISYT group showed little difference for every aspect of the histopathology as compared with the Normal group. At 48 hours, the results were almost the same as those at 24 hours. At 72 hours, both the Control and ISYT groups showed a slight thickness change of the epidermis as compared with the Normal group, and there were no differences concerning the infiltration of inflammatory cells among the three groups (Fig. 5).

Discussion

ISYT is a representative medicine that intensifies the function of defense-*qi*, and many clinicians have generally used this medicine for fatigue, general weakness, asthma, shortness of breath, acute abdominal pain and palpitation¹¹⁾.

Early studies about ISYT have revealed that this medicine could increase the population of CD4+ cells among the thymocytes, and Thy1+ cells and CD4+ cells among the splenocytes; it could also increase the level of *r*-interferon and interleukin-2 from the thymocytes, and *r*-interferon from the splenocytes¹²⁻¹³⁾.

Skin surface pH is an important index for healthy skin, and this correlates with the severity of experimentally induced irritant dermatitis¹⁴⁻¹⁵⁾. If a medicine could help the skin to maintain its normal pH against external stimulus, then the medicine could be regarded as an effective medicine for treating the skin barrier function. The hairless mouse has a different normal skin pH than that of human. For a human, the skin pH is acidic and it has to be kept acidic

for maintaining healthy skin¹⁶⁾. In this study, the skin damage to the Control group made the skin pH drop lower (more acidic). On the other hand, there was seldom a difference of skin pH between the Normal and ISYT groups at 24 hours, 48 hours and 72 hours. So, it could be hypothesized that ISYT helped the skin of the hairless mouse to continuously keep its correct skin pH for a period of time.

The stratum corneum works with the epithelium as a functional and anatomical unit that controls one of the main purposes of the skin: maintaining homeostasis. By maintaining this balance, these skin layers prevent skin dryness and protect the skin from several external harmful factors. Additionally, the lipid barrier that surrounds the stratum corneum plays an important role for controlling the skin's humidity. The stratum corneum is essentially impermeable to water except for a small yet vital flux that serves to maintain its hydration, and thereby its flexibility¹⁷⁾. The skin barrier in the stratum corneum is explained by a 'bricks and mortar' model, and this model is composed of keratinocytes (the blocks) and intercellular lipids (the mortar) like free fatty acid, sphingolipid and cholesterol^{2,17)}. The gloss and elasticity of skin are determined by the amount of water in the stratum corneum: water diminution in the stratum corneum is probably the major cause of dehydrated and dry skin, which is commonly found in chronic dermatitis patients¹⁸⁻²⁰⁾. Consequently, the ability of the stratum corneum to keep its organized structure against external stimulation is a key to healthy skin¹⁷⁾.

The corneometer is helpful for estimating the water content of the skin in the chronic disease stage²¹⁾. Perhaps the values for the skin that are measured via a corneometer represent the water

storage capability of the stratum corneum, so this furnishes beneficial data about its integrity. In this study, the Control group showed significantly lower amount of water in the skin as compared with the Normal and ISYT groups at 24 hours. On the other hand, there was seldom a difference of skin humidity between the Normal and ISYT groups at 24 hours.

It could be assumed that ISYT maintained the skin humidity of the hairless mice despite the application of DNCB. The hairless mice probably recovered their skin humidity from the damage done to their skin within 72 hours, and that would be the reason why there was no statistical difference among the three groups at 48 and 72 hours after the 2nd DNCB application.

Water loss of the skin occurs by two pathways: sweating and evaporation through the epidermis. Evaporation through the epidermis is passive diffusion, and the rate is determined by external temperature, relative humidity, skin temperature and integrity of the stratum corneum: this is called TEWL. TEWL is regarded as an index to evaluate the skin's condition and especially the condition of the stratum corneum²²⁾. So, the measurement of TEWL is widely used for evaluating function of the stratum corneum^{15,22-23)}.

In this study, the Control group showed continuously increased TEWL. In contrast, the ISYT group showed no statistical difference compared with the Normal group, especially at 24 and 72 hours. Moreover, ISYT showed significant effect on TEWL at 72 hours after the 2nd application of DNCB. From these results, we hypothesized that ISYT could prevent TEWL and keep the skin moist for a comparatively long time.

The application of DNCB evoked histological

changes in this hairless mouse model and ISYT showed the capacity to maintain the epithelium.

Conclusion

In conclusion, this study demonstrated that ISYT had an effect on maintaining the function of the skin barrier of the hairless mouse.

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