

Therapeutic Effects of Atobeauty Cream in Atopic Dermatitis Model Mice Induced with DNCB

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Abstract: This study has been performed to assess the anti-atopic efficiency in DNCB induced BALB/c to examine a therapeutic effect of Atobeauty cream which was provided by NJY Life Science Co., Ltd. on atopic dermatitis. Such BALB/c mice were provided by NJY Life Science Co., Ltd. The randomized complete block design was done by dividing 20 male mice with BALB/c into two groups of which the one group had 10 mice receiving no treatment as a control group and the other group had 10 mice receiving treatment with Atobeauty cream as a treatment group. When Atobeauty cream was applied to the mouse with severe atopic dermatitis 2 times a day for 6 days, it could be observed that its skin recovered to normal condition with the skin surface being clean and smooth without any horny tissue. It was found that each level of IgE and histamine in blood was significantly decreased by 57.80 and 59.41%, respectively in the Atobeauty cream treatment group (here in after, the treatment group), compared with the DNCB induced atopy control group (here in after, the control group) ($p < 0.05$). It was also identified that a mouse with atopic dermatitis to which Atobeauty cream was applied, recovered on the order of a normal mouse as its epidermis got to be softly formed with its surface thickness being even and its connective tissue got to be formed more uniformly.

Key words: Atopic dermatitis, BALB/c mice, IgE, histamine, epidermis

INTRODUCTION

In recent years, the number of juvenile patients with atopic dermatitis has been sharply increasing in Korea. Results of the research on national health and nutrition which was conducted in 2008 showed that 19.2% of 1-5 years old children had atopic dermatitis that is one of 5 children of 1-5 years age had atopic dermatitis (NHNS, 2009). Atopic dermatitis is a chronic inflammatory skin disease featured with itching and inflammation and its onset mechanism is known as follows if allergen, a causative agent bringing about allergy, enters a human body from outside, IgE is produced in blood B-cells and then it moves to the skin mast cells and then it combines with basophil leukocytes to produce and reserve histamine and thereafter if the skin is repetitively exposed to allergen, histamine gets to be discharged to outside to cause allergy symptoms.

Any cause to induce atopic dermatitis has not been clearly known but it is known that genetic predisposition, immunological factors and environmental factors work complexly to develop atopic dermatitis.

Treatment of itching related to the scratching behavior has been considered as an efficient method for treating atopic dermatitis. Anti-histamine treatment is not enough to suppress pruritus in atopic dermatitis patients and application of steroid cannot be used on the long-term basis due to its frequent side effects (Jiang *et al.*, 2009; Yun *et al.*, 2008; Koblenzer, 1999; Mihara *et al.*, 2004).

Studies for developing anti-atopic substances from natural substances are under way and it has been reported that an extract from *Scutellariabai calensis* (by hot water extraction) and extract from Eucommiae, an extract from *Opuntia humifusa*, an extract from Kimchi and extract from *Eriobotrya japonica* and *Saururus chinensis* has an anti-atopic efficacy (Kwon *et al.*, 2011; Choi *et al.*, 2010; Lee *et al.*, 2008a, b; Shon and Nam, 2007; Kim *et al.*, 2007; Kim and Park, 2006). It has been known that Korean traditional medicinal plants including *Houttuynia cordata*, *Acorus gramineus* Soland, *Saposhnikovia divaricata* Schisck, *Astragalus membranaceus*, *Eriobotrya japonica* L., *Sophora flavescens* Ait. and *Saururus chinensis* Baill, etc. have various pharmacological efficacies including an anti-

inflammatory effect. Quercetin from *Houttuynia cordata*, asarone from *Acorus gramineus*, coumarin from *Saposhnikovia divaricata*, formononetin and astragaloside from *Astragalus membranaceus*, terpenoid, amygdalin and saponin from *Eriobotrya japonica*, quercetin and quercitrin from *Saururus chinensis* and flavonoid from *Sophora flavescens* are known to be significant pharmacological components (Jung and Cho, 2010; Ferreres *et al.*, 2009; Jeong *et al.*, 2009; Kwon and Shin, 2009; Kim *et al.*, 2005; Ro *et al.*, 1998) and NJY Life Science Co., Ltd. Republic of Korea has manufactured Atobeauty cream by compounding such plant extracts. This study has been performed to assess the anti-atopic efficiency in DNCB induced BALB/c to examine a therapeutic effect of Atobeauty cream which was provided by NJY Life Science Co., Ltd. on atopic dermatitis.

MATERIALS AND METHODS

Material for experiment: Atobeauty cream used in this experiment was supplied by NJY Life Science Co., Ltd. Republic of Korea and this cream was composed of various extracts from *Houttuynia cordata*, *Acorus gramineus*, *Astragalus membranaceus*, *Eriobotrya japonica*, *Sophora flavescens* and *Saururus chinensis*.

Experimental animals: All the scientific procedures including those applicable to animals were based on European Laboratory Animal Treatment License (SCT-w94058) and performed under the approval by Institutional Animal Care and Use Committees, Kangwon National University. Total 20 male mice with BALB/c which were 5 weeks old, free of specific pathogen and purchased from Daehan Bio Link Co., Ltd. were bred with chow pellet diet and adapted to new circumstances for 1 week and then experiments were made on them. These experimental mice were divided into two groups, the control group and the treatment group on the basis of the randomized complete block design and 10 mice randomized into each group were individually bred on the iterative basis.

The breeding room was kept at the temperature of $20\pm 2^{\circ}\text{C}$ and the relative humidity of $60\pm 5\%$ and its illumination was cyclically regulated at intervals of 12 h. In particular, it was made to be dark for the day time cycle (09:00-21:00) so as to minimize the stress suffered by white mice due to the experimental procedures, considering physiological characteristics of white mice as a nocturnal animal. A purified diet based on AIN-76 was prepared and then pelleted so that it might be used as an experimental diet. The purified diet was composed of casein 20.0%,

corn starch 15.0%, sugar 50.0%, α -cellulose 5.0%, corn oil 5.0%, mineral mixture 3.5%, vitamin mixture 1.0%, choline bitrate 0.2% and DL-methionine 0.3% and its calorie and protein content were adjusted so that they might be maintained at the same level. Experimental feed and water were freely supplied and other control items for breeding were performed in accordance with the practices established at Kangwon National University.

Inducement of dermatitis and treatment of specimens:

Hairs on the back of a mouse with BALB/c were clearly removed and then it was left as it was for 24 h so that tiny scars on the skin could be cured. About 200 μL of 2.5% DNCB solution (2,4-dinitrochloro benzene, Sigma, St. Louis, MO, USA) as an immunity disrupting chemical which was prepared by mixing acetone with olive oil at a ratio of 3:1 ratio was applied to the back to induce the immunization reaction.

After 3 days passed there from atopic dermatitis was induced by applying 1500 μL of 1.0% DNBC solution to the back once every 3 days. After DNCB solution was applied to the back 2 times, crusts were formed and the mouse's behavior of scratching itchy spots got severe and crusts began to peel and thereby atopic dermatitis was induced. After DNCB solution was applied to the back 4 times such induced atopic dermatitis got severe. From then, Atobeauty cream was sufficiently applied to the dermatitis spoits. Atobeauty cream was applied 2 times (11:00, 17:00) a day. While Atobeauty cream was applied to the back, 150 μL of 1.0% DNBC solution was also applied to the back once (10:00) every 3 days to prevent an accidental error due to natural healing.

Collection of blood and skin tissue: On the day when the experiment was completed, the mouse was slightly anesthetized with ethyl ether and 1.0 mL of blood was collected from their heart through a Serum Separate Tube (SST) and then it was left as it was for 20 min at the room temperature so that it might be coagulated. Serum was separated from the coagulated blood by centrifuging it for 10 min at 3,000 rpm. Then, it was kept at -20°C after it was quickly freezed with liquefied nitrogen at -196°C . Euthanasia was administered to the mouse by having it suffering cervical dislocation at once and the skin tissue was collected by cutting it off to a size of $1\times 2\text{ cm}^2$.

Measurement of IgE in blood: Mouse IgE ELISA kit (Shibayagi Co., Ltd. Gunma, Japan) was used to let the collected blood react by following the procedures as described below and then the immunoglobulin E level in the blood was calculated by comparing its absorbance with the absorbance of the standard solution. Well plates

and reagents were all kept at the room temperature and the concentrated washing buffer was diluted 10 times with distilled water. Each standard solution having the standard IgE level (0, 1.0, 10, 25, 50, 75 and 100 ng mL⁻¹) was prepared by using the standard IgE substance and the buffer solution. About 96 wells were filled with the washing buffer and then the antibody-coated well plate was washed 3 times. The diluted specimen and the standard solution were put into all wells of each well plate by 50 µL, respectively and then it was placed on the plate shaker and shaken slowly (800 rpm for 10 sec x 3 times). After they were cultured at the room temperature for 2 h, the resultant mixture was discarded.

After the plate was washed with the washing buffer 3 times, 50 µL of biotin-conjugated anti-IgE antibody was put into all wells and the well plate was shaken in the same manner as described. After they were cultured at the room temperature for 2 h, the resultant mixture was discarded and the well plate was handled in the same manner as described. After 50 µL of HRP-conjugated avidin was put into all wells, the well plate was shaken in the same manner as described above and then they were cultured at the room temperature for 1 h. The resultant mixture was discarded and the well plate was washed 3 times and then 50 µL of chromogenic substrate reagent was added into all wells. Then, the well plate was shaken in the same manner as described above and they were cultured at the room temperature for 20 min. About 50 µL of reaction stopper was added into all wells and the plate was shaken in the same manner as described above. Within 30 min there from each well's absorbance was measured by the precision microplate reader (Molecular Devices Inc, New York, USA). IgE was measured 2 times, repetitively.

Measurement of histamine in blood: ELISA kit for mouse histamine (IBL-America, Inc., Minneapolis, MN, USA) was used to measure histamine in blood as follows: 100 µL of serum was put into a tube for acylation and then 50 µL of a buffer solution for acylation was added to it to let the former react on the latter. Then, it was left as it was at 18°C for 30 min. About 50 µL of the acylated serum was put into 96 wells which was coated with histamine antibody and 50 µL of enzyme conjugate was added to it. Then, it was cultured 4°C for 18 h. The contents of the test wells were sucked and then, they were washed 3 times with the washing buffer. Then, 20 µL of chromogenic substrate reagent was put into the wells to let it react in the dark at the normal temperature and 50 µL of reaction stopper was used to stop the enzyme reaction. Then, its absorbance was measured at 450 nm by the precision microplate reader (Molecular Devices Inc, New

York, USA) to calculate the histamine level. Measurement of histamine was repetitively done 2 times. Then, measurement of histamine of the standard solution was also done in the same manner as described above and then, its absorbance was calculated to be compared with the specimen's absorbance.

Method for observing skin tissue cells: The skin tissue of a mouse which was cut off from the affected parts on the back by 1.5×1.5 cm² was biopsied. Then, it was fixed in 10% formaldehyde solution and kept at 4°C just until it would get to be stained. The specimen was fixed in 4% glutaraldehyde solution (0.1 M cacodylate buffer, pH 7.4) and then dehydration, substitution, infiltration and embedding, polymerization and semi-thin sectioning processes were performed and then it was photographed by the energy-filtering transmission electron microscopy (EF-TEM, Leo 912AB, Carl Zeiss Inc., Germany). The skin tissue was observed, centering around overall state of the skin, infiltration and degranulation of mast cells and infiltration of eosinocytes.

Statistical analysis: SAS program was used for statistical analysis of the collected data. The mean and the standard error for each group were obtained and analysis of variance was done. Then, its significance test was conducted at the 95% confidence level by the Duncan's multiple range test (SAS Institute, 2000).

RESULTS AND DISCUSSION

Figure 1 shows the mouse with atopic DNCB-induced dermatitis. It can be known that since the DNCB solution was applied to the hairs-removed skin of the mouse (Fig. 1a), crusts were formed (Fig. 1b) and the mouse which could not bear the itch due to the crust, scratched the itchy spots heavily and thereby crusts got to peel off and various symptoms of atopic dermatitis including skin spots, erythema, skin dryness, lichenification, edema and bleeding were developed to cause severe atopic dermatitis (Fig. 1c). The mouse with severe atopic dermatitis showed skin damages such as erythema, erosion, hair loss, skin dryness, bleeding and inflammation, compared with the treatment group to which Ato beauty cream was applied. It can be observed that when Ato beauty cream was applied to such mouse 2 times a day for 3 days, dermatitis of the lesion got to improve quickly and when it was applied for 6 days, the skin recovered to normal condition with the skin surface being clean and smooth without any horny tissue (Fig. 1d). Itching which is one of the most common symptoms incidental to a skin disease could be caused by inflammation, cancer, metabolic disease,

Table 1: Effects of Atobeauty cream on serum IgE and histamine levels in DNCB induced atopic dermatitis BALB/c mouse (ng mL⁻¹)

Item	Atopic control	Atobeauty cream
IgE	56.28±5.36*	23.75±2.48
Histamine	40.08±3.51*	16.27±4.12

Values are mean±SE (n = 10) *p<0.05

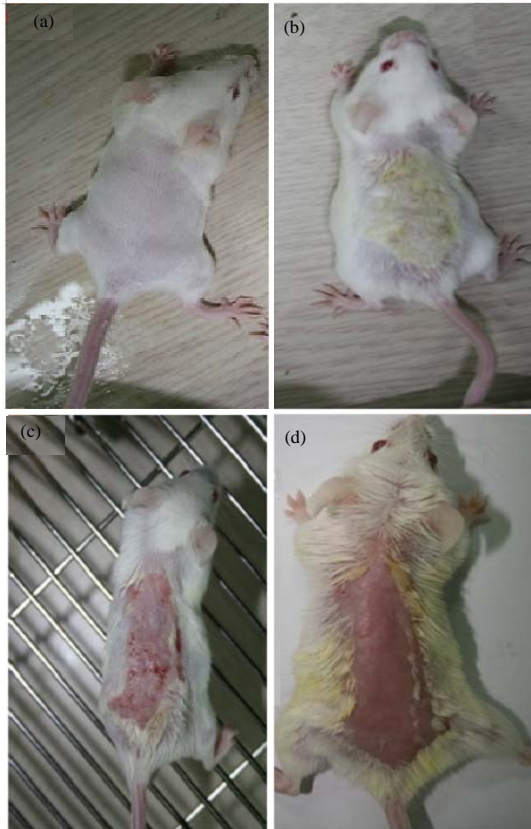


Fig. 1: The therapeutic effects of Atobeauty cream in DNCB induced atopic dermatitis mice; a) groups removed hair; b) groups treated with DNCB for 7 days; c) severe atopic dermatitis groups treated with DNCB for 14 days and d) recovered groups after the application of Atobeauty cream from c for 6 days

infection, psychiatric diseases, drug application, stress and other factors and activation of mast cells causes atopic dermatitis of a human to get worse (Elenkov, 2008; Paus *et al.*, 2006; Stander *et al.*, 2003). This is supported by the fact that when Atobeauty cream was applied to the mouse with atopic dermatitis, it had an effect that IgE in blood decreased and histamine secretion was suppressed (Table 1). The IgE level in blood measured in the treatment group was found to be lower than that in the control group by 32.53 ng mL⁻¹ showing a significant decreasing rate of 57.80% ($p<0.05$) (Table 1). The histamine level in blood measured in the treatment group was found to be

lower than that in the control group by 23.81 ng mL⁻¹ showing a significant decreasing rate of 59.41% ($p<0.05$) (Table 1). This result suggests that if Atobeauty cream is applied to the mouse with atopic dermatitis, it has an effect to treat atopic dermatitis by decreasing production of IgE and suppressing secretion of histamine in blood (Fig. 1). An increase of IgE level in serum is known to be an immunological indicator of atopic dermatitis and particularly IgE is known to be in proportion to clinical severity in a patient with atopic dermatitis. It has been reported that IgE combines with leukocytes of skin mast cells to produce histamine and cause allergic reaction by reacting with infiltrated antigen (Sung *et al.*, 2006; Latvala *et al.*, 2005; Ban and Hetich, 2001; Matsuda *et al.*, 1997; Metcalfe *et al.*, 1981). The skin allergic reaction triggers expansion of blood vessel, accentuation of capillary permeability, increase of mucus and edema and inflammation of mucous membrane by various chemicals produced by histamine, chemical transmitter or T lymphocytes which are discharged as Immunoglobulin (IgE) reacts with antigen (Church and Levi-Schaffer, 1997; Ishizaka, 1984).

In atopic dermatitis, histamine has been reported to be more produced as a major cause of itchy sense in a patient with atopic dermatitis than in a normal person. The skin has a number of opportunities to be in contact with many antigens, forming a border between outside environment and the inside of the body. If atopic dermatitis develops, hypersensitiveness of the skin and the decreased threshold against itch causes the patient to scratch the itchy skin and then the scratching stimulus and inflammatory reaction causes secretion of cytokine in horny cells of the skin, thereby causing inflammatory reaction to be severe so that deformation of horny layer is induced and immunocytes are activated. If immunocytes are activated, an increase of IgE production and an increase of antibody reaction are induced and the activity of IgE-dependent histamine vitreous body is increased to promote secretion of histamine.

Histamin induces infiltration of eosinophils and triggers acute hypersensitive reactions and itchy sense (Budde *et al.*, 2002; White, 1990; Ennis *et al.*, 1980). As a result of observing the skin surface of mice with atopic dermatitis to which Atobeauty cream was applied, it was identified that compared with the control group having mice with severe atopic dermatitis induced by DNCB (Fig. 2b), mice of the treatment group recovered on the order of a normal mouse (Fig. 2a) as its epidermis got to be softly formed with its surface thickness being even and its connective tissue got to be formed more uniformly (Fig. 2c).

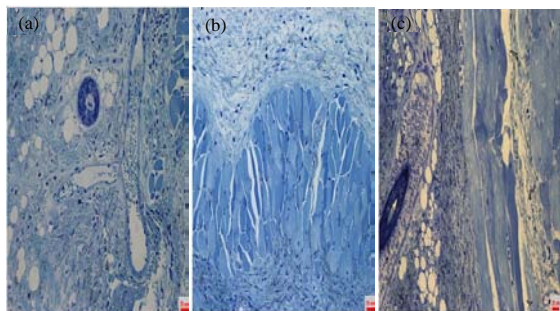


Fig. 2: The effects of Atobeauty cream on DNCB induced atopic dermatitis in mice by microscopic comparison; a) control groups; b) severe atopic dermatitis groups treated with DNCB for 14 days and c) recovered groups after the application of Atobeauty cream from b for 6 days

It can be also known that the treatment group, showed that hair follicles and the sebaceous gland were actively reformed in the dermis, compared with the control group and regeneration of the epithelial tissue and formation of the stratum corneum in the skin and proliferation of the reticular layer and formation of adnexa the basal layer was clearly activated and the histogenesis layer was formed so that it was thick and dense with it being evenly in the uniform direction. In the treatment group, the skin surface was found to be clean and smooth but as bleeding of the skin surface and hypotrophy of the epidermis was histologically noticeable, the skin tissue was found to be considerably thicker than that of the mouse with atopic dermatitis in the control group (Fig. 2c).

However, in contrast, it was identified that in the control group, the skin surface was very coarse due to numerous crusts having edema topically. In the control group, apoptosis of the basal layer was found to be noticeable and cells related to inflammation such as lymphocyte, mast cell, neutrophilic leukocyte, acidophilic leukocyte was observed in the epidermis and the dermis (Fig. 2b). However, in the treatment group, it was observed that such cells were scarcely shown and the thickness of the epidermis was gradually recovering similarly to that in the normal mouse (Fig. 2c). In the skin tissue with allergic contact dermatitis, mast cells and eosinocytes where degranulation takes place, infiltrate into the skin tissue and hypercornification takes place (Sator *et al.*, 2003; Budde *et al.*, 2002; Vestergaard *et al.*, 1999). It is known that if allergic contact dermatitis is induced water gets to be vaporized from the skin surface and thereby, infiltration of antigens is facilitated so that the hypersensitive reaction of the skin gets to be severer and thereby, the skin barrier which is formed by matrix structural protein combining with ceramide, gets to be weakened and as a result, the susceptibility of the skin to

infection and inflammation gets to be higher. Therefore, the best method for treating dermatitis caused by any immunological reaction is to remove the antigen causing the stimulus but it has been reported that in many cases, any specific antigen cannot be known so that antibiotics are generally used and steroid agents are topically used to treat it, involving a number of safety problems due to long-term use of them (Stander *et al.*, 2003).

CONCLUSION

From the study results as describe above, researchers could identify the new fact that if Atobeauty cream is applied to the mouse with DNCB induced atopic dermatitis, it decreases the IgE level in blood while suppressing histamine which is produced in mast cells from being discharged and thereby it has an effect to treat atopic dermatitis.

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