# Histopathologic Features in Animal Model of Atopic Dermatitis Induced by Topical Application of Oxazolone

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Animal models of atopic dermatitis (AD) are widely used to investigate therapeutic effects of candidates for AD. However, the characteristics of each model are not fully understood. This study was designed to compare the animal models of dermatitis induced by dinitrofluorobenzene (DNFB) and oxazolone (Ox). We investigated the effects of DNFB and Ox on skin thicknesses and weights as well as skin lesions associated with AD such as scale, crust and erythematous eruption, and histopathological changes such as hyperkeratosis, dermal and epidermal hyperplasia and immune cell infiltration in inflamed tissues. Multiple application of 0.5% Ox onto the skin increased skin thickness and weight compared to those of DNFB treated mice, as well as those of normal mice. In addition, topical application of DNFB induced marked scale, crust and erythematous eruption, while Ox induced erythematous eruption and mild scale and crust. Histopathological examination revealed that 0.5% Ox induced marked hyperplasia in the dermis and epidermis, large vesicles, spongiotic changes, mild hyperkeratosis and immune cell infiltration in balb/c mice. These data suggest that multiple applications of Ox can induce chronic AD like dermatitis in balb/c mice.

keywords : Animal model, Atopic dermatitis, Oxazolone, Dinitrofluorobenzene

## Introduction

Atopic dermatitis (AD) is characterized by pruritic inflammatory skin disorder accompanied by skin erythematous plaques, eruption and lichenification. A hallmark of AD is dry and itchy skin due to defects in skin barrier function that are related, at least in part, to impairment of skin genes such as filaggrin, loricrin, and involucrin.[1)]

Various animal models of AD have been developed to date, including: (1) AD models induced by topical application of sensitizers; (2) transgenic mice; and (3) AD models that spontaneously develop AD-like skin inflammation.[2)] Among them, the most common and economical model is to induce AD in experimental animals by topical application of sensitizers such as ovalbumin, house dust mite allergen and haptens. Haptens such as oxazolone (Ox), trinitrochlorobenzene (DNCB) and dinitrofluorobenzene (DNFB) are frequently used to induce contact dermatitis (CD), which is characterized by Th1 skewing reactions such as elevated levels of Th1 skewing cytokines such as TNF-α and IFN-γ, and neutrophilic infiltration.[3)] Multiple applications of Ox onto the skin was recently reported to cause AD like skin inflammation and chronic Th2 skewing inflammatory responses, including mast cell and eosinophilic infiltration and elevated IgE level in serum and IL-4 level in the dermis. In addition, repeated application of Ox led to decreased expression of several proteins related to skin barrier function, including filaggrin, loricrin, and involucrin.[4)]

In this study, we compared animal models of dermatitis induced by DNFB and Ox. Specifically, the effects of DNFB and Ox on skin thicknesses, weights and histopathological changes in inflamed tissues were assessed *in vivo*.

## Materials and Methods

1. Animals

Male 6-week-old Balb/c mice were purchased from Samtaco (Incheon, Korea) and housed under specific pathogen-free conditions with a 12-hour light/dark cycle and free access to standard rodent food and water. All animal experiments were approved by our Animal Care and Use Committee and conducted according to institutional

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guidelines (PNU-2012-0140 and PNU-2015-0979).

2. Induction of dermatitis

Dermatitis induction by Ox was performed using a slightly modified version of the method reported by Man et al.[4] Briefly, mice were sensitized by painting 30 ㎕ of Ox (5%, w/v) in AOO onto the dorsum of each ear on day 1. Four days after sensitization, each mouse was challenged by painting 50 ㎕ of Ox (0.1 or 0.5%, w/v) in AOO onto the shaved dorsum every two days (7 times). Dermatitis induction by DNFB was performed as previously described.[5] Briefly, mice were sensitized by painting 30 ㎕ of DNFB (0.1%, v/v) in acetone: olive oil (AOO, 4:1) onto the dorsum of each ear for three consecutive days. Four days after sensitization, each mouse was challenged by painting 50 ㎕ of DNFB (0.2%, v/v) in AOO onto the dorsum of shaved backs every two days (4 times). Naïve animals (NOR) were treated with vehicles (n=6), while DNFB animals (DNFB) were sensitized and challenged with DNFB in AOO (n=6). Ox treated animals (Ox0.1 and Ox0.5) were sensitized with 5% Ox and challenged with 0.1% or 0.5% Ox (n=6) in AOO. The experimental design is summarized in Fig. 1.

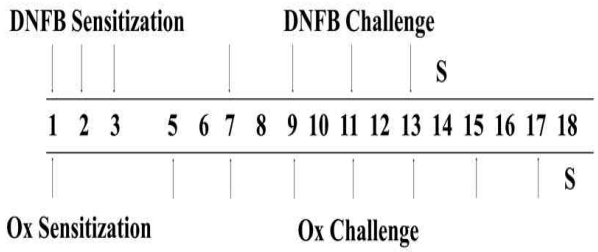


Fig. 1. Experimental design. The Ox group was sensitized by painting with Ox on day 1 and challenged on days 5, 7, 9, 11, 13, 15 and 17. The DNFB group was sensitized by painting with DNFB on days 1, 2, and 3 and challenged on days 7, 9, 11, and 13. The naïve group was treated with vehicle (AOO) in the same way. All animals in DNFB group were sacrificed on day 15, while animals in the Ox group were sacrificed on day 18. Ox and S mean oxazolone and sacrifice respectively.

3. Observation of the skin lesion

At the end of experiment, mice were anesthetized with 30 mg/kg of zoletil (Virbac, Carros, France) and sacrificed with CO2 gas. To observe the overall degree of dermatitis, dorsum skins of mice were observed using a digital camera (Olympus, Tokyo, Japan).

4. Measurement of skin thicknesses and weights

After observing skin lesions of mice, skin tissues were resected and the thicknesses were measured using vernier calipers (Mitutoyo, Tokyo, Japan). The weights of ear pieces (5 mm in diameter) obtained via dermal punch were also weighed using a microbalance (Sartorius AG, Göttingen, Germany).

5. Histopathological examination

At the end of experiment, tissues (4 μm) were resected, formalin-fixed and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin to observe histopathological changes such as dermal and epidermal hyperplasia, spongiosis, hyperkeratosis and immune cell infiltration. Stained tissue sections were observed using a light microscope at 100× magnification (Carl Zeiss AG, Oberkochen, Germany).

6. Evaluation of epidermal hyperplasia and immune cell infiltration

To evaluate the epidermal hyperplasia and immune cell infiltration, five non-overlapping fields per slide were selected at random and observed by light microscopy at 100× magnification (Carl Zeiss AG, Oberkochen, Germany). The vertical length between the basal lamina and top of the outermost stratum granulosum was quantified to measure the epithelial thickness. Total immune cell numbers were quantified by counting immune cells in the same size counting grid.

7. Statistical analysis

The Mann-Whitney U test was used to compare obtained results from each group and Prism 5 for window version 5.01 (GraphPad Software Inc, CA, USA) was used for all analyses. All data are presented as the means ± standard deviations. A $P < 0.05$ was considered significant.

## Results

1. The effects of DNFB and Ox on skin thickness and weights

Repeated application of DNFB increased skin thickness and weight compared to normal mice (P=0.0095). In the 0.5% Ox treated group, marked increases in thickness and weight were observed. Moreover, as shown in Fig. 2, these levels were significantly higher than those in the DNFB groups (thickness, P=0.0139; weight, P=0.0159).

2. Effects of DNFB and Ox on skin lesions in mice

In the DNFB group, marked scale, crust and erythematous eruption were observed. Topical application of 0.1% Ox only induced mild scale in the skin, while 0.5% of Ox induced marked erythematous eruption as well as scale

and crust(Fig. 3).

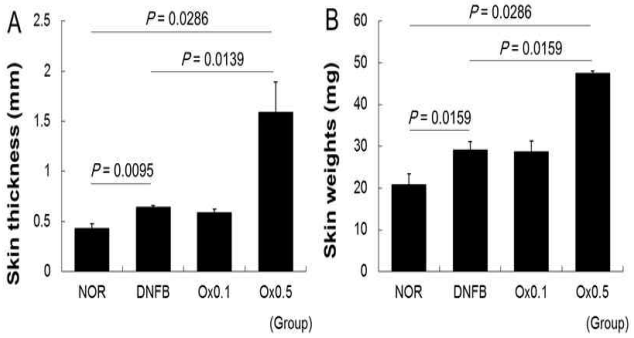


Fig. 2. Effects of Ox and DNFB on skin thickness and weights. The effects of Ox and DNFB on skin thickness and weights were analysed using vernier calipers and a microbalance on day 14 and 18. NOR, non-treated naïve mice; DNFB, challenged with 0.2% DNFB; Ox0.1, challenged with 0.1% Ox; Ox0.5, challenged with 0.5% Ox. (A) Skin thicknesses; (B) skin weights. All values are presented as the means ± SD.

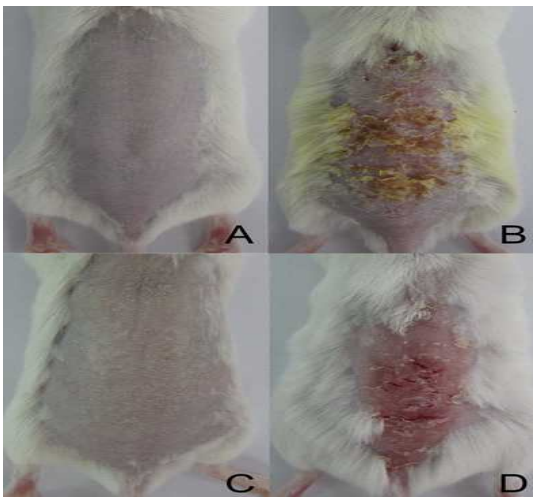


Fig. 3. Effects of DNFB and Ox on skin lesions in mice. The effects of DNFB and Ox on skin lesions were observed using a digital camera at the end of the experiment. (A), NOR; (B), DNFB; (C), 0.1% Ox; (D), 0.5% Ox.

### 3. Effects of DNFB and Ox on histopathological changes

Repeated painting with DNFB induced severe hyperkeratosis (filled arrow) and tissue injury, while treatment with 0.5% Ox induced mild hyperkeratosis and mild tissue injury. Both DNFB and 0.5% Ox induced epithelial hyperplasia (yellow bars). In the DNFB group, marked infiltrations of immune cells were observed. Topical application of 0.5% Ox induced vesicles and mild immune cell infiltration; however, marked infiltrations of immune cells were not observed in the Ox group (Fig. 4). The epithelial thickness in the 0.5% Ox group was significantly thicker than that in the DNFB group (P=0.0260), while the number of infiltrated immune cells in the DNFB group was significantly higher than that in the 0.5% Ox group (P=0.0095) (Fig. 4).

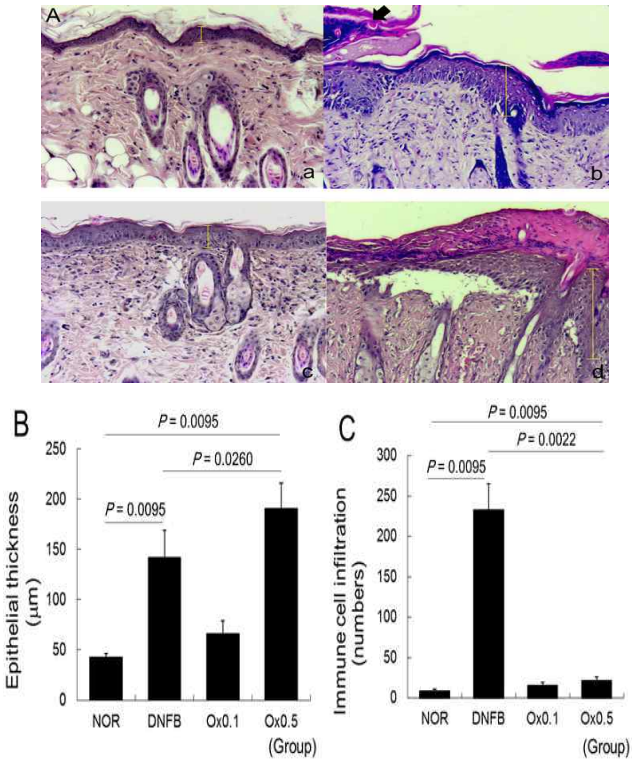


Fig. 4. Effects of DNFB and Ox on histopathological changes in mice. Skin tissues were stained with H&E and observed using a light microscope. (a), NOR; (b), DNFB; (c), 0.1% Ox; (d), 0.5% Ox. The observations were made at a magnifications of 100× (A). In addition, the levels of epithelial hyperplasia (B) and immune cell infiltration (C) in each group of mice were evaluated using quantitative methods. NOR, non-treated naïve mice; DNFB, challenged with 0.2% DNFB; Ox0.1, challenged with 0.1% Ox; Ox0.5, challenged with 0.5% Ox. All values are presented as the means ± SD (C).

## Discussion

AD is a complicated syndrome with various types of pathogenesis that is not yet fully understood. Therefore, it is important to select appropriate animal models of AD that are suitable for their research needs. AD or CD animal models that employ epicutaneous application of sensitizers are frequently used to investigate anti-inflammatory effects of drugs in vivo because of their low cost and ease of induction and maintenance.

Topical application of super antigens such as DNFB and DNCB can induce CD in experimental animals and humans. CD is characterized by skin lesions with clear borders, large vesicles and pustules, erythematous eruptions and elevated Th1 skewing cytokine profiles and neutrophilic infiltration.[6] Additionally, the IL-4 levels in the dermis are not changed by DNFB and DNCB in animal models (Balb/c mice).[7] Moreover, elevated IgE levels in serum induced by DNFB are relatively lower than those caused by Th2 skewing sensitizers such as ovalbumin and trimellitic anhydride.[8] Ox has been used to induce colitis in

experimental animals, and high concentrations of Ox can induce CD.[4,9] Recently, Man et al. reported that multiple applications of low dose Ox could induce AD in mice. In their study, topical application of Ox damaged skin barrier function as well as several proteins related to skin barrier function, including filaggrin, loricrin, and involucrin.[4] It is also well known that the production levels of Th2 skewing cytokines such as IL-4, IL-6 and IL-13 can be elevated by multiple sensitizations of Ox.[10,11]

In this study, we compared animal models of dermatitis induced by DNFB and Ox. As shown in Fig. 2, repeated painting of 0.5% Ox markedly increased skin thickness and weight, and these increases were significantly larger than those in the DNFB group (Fig. 2). These increases in thickness and weight by Ox appear to be due to hyperplasia in both dermis and epidermis. In the DNFB and Ox group, dermal and epidermal hyperplasia were observed, and Ox induced more severe hyperplasia in the dermis than DNFB (Fig. 4).

Hyperkeratosis is one of hallmarks of acute AD and CD. In addition, immune cell infiltration tends to be diminished in the chronic phase of skin inflammation.[6] In this study, DNFB markedly induced hyperkeratosis compared to Ox, and more than 5 times the immune cells were infiltrated by DNFB compared to those by Ox (Fig. 4). The infiltrated immune cells consisted primarily of neutrophils, although a few monocytes and macrophages were seen (data not shown). In addition, the spleen body weight ratio, which is a hallmark of systemic inflammation that can be diminished by immune-suppressive agents such as corticosteroids,[12] was higher in the DNFB group than the Ox group (data not shown). As shown in Fig. 3, skin lesions in the DNFB group were more severe than in the Ox group (Fig. 3). In addition, large vesicles and spongiotic changes, which are recognized as evidence of inflammation, were observed in 0.5% Ox group (Fig. 4A). These results indicate that dermatitis induced by Ox is more similar to chronic dermatitis than that induced by DNFB.

We believe that 0.1% Ox can induce enlargement of skin thickness and weight via hyperplasia in the dermis (Fig. 2). In addition, only mild micaceous scale, which is frequently shown in patients with psoriasis, was observed on the surface of the skin (Fig. 3). These results may be due to the relatively low toxicity of Ox compared to other super antigens such as DNFB and DNCB and low sensitivity of mouse strain (balb/c) compared to transgenic mice that either overexpress or lack selective molecules. Koeper et al. reported that the lowest observed effect level of DNFB in mice is more than 100 times lower than that of Ox.[13] Taken together, these findings indicate that DNFB is more toxic than Ox with respect to the ability to induce skin inflammation. In general, topical application of 0.1-0.2% DNFB can induce skin inflammation, regardless of mouse strain; however, Ox cannot. Moreover, hairless mice have frequently been used as AD animal models of the effects of Ox. In previous reports, 0.1% Ox sufficiently induced AD in hairless mice;[4,14] however, based on the results of our study, 0.1% Ox was insufficient to induce AD in balb/c mice. This discordance may be due to the use of different mouse strains, specifically, the balb/c strain used in this experiment has low sensitivity to sensitizers; however, it is easier to maintain and less expensive than hairless mice.

When combined with the results of previous studies obtained using AD models, especially cytokine profiles induced by Ox, our results indicate that multiple applications of 0.5% Ox can induce chronic AD like dermatitis in balb/c mice.

## Conclusion

In the present study, we compared animal models of dermatitis induced by DNFB and Ox. DNFB and Ox were topically applied onto the shaved dorsum. The results revealed that multiple applications of 0.5% Ox markedly enlarged skin thickness and weights compared to those in the DNFB group, as well as in the non-treated normal group. Topical application of 0.5% Ox induced erythematous eruption, scale and crust, as well as marked hyperplasia in the dermis and epidermis, large vesicles, spongiotic changes, mild hyperkeratosis and immune cell infiltration. These results indicate that Ox can induce chronic AD like dermatitis in balb/c mice.

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