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RESEARCH ARTICLE



External application of NF- κ B inhibitor DHMEQ suppresses development of atopic dermatitis-like lesions induced with DNCB/OX in BALB/c mice

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ABSTRACT

Context: Dehydroxymethylepoxyquinomicin (DHMEQ) which is originally developed as an analog of antibiotic epoxyquinomicin C is a specific and potent inhibitor of NF- κ B and has been shown to possess promising potential as an anti-inflammatory and anti-tumor agent.

Objective: This study examines DHMEQ's effect on therapeutic potential for atopic dermatitis (AD)-like lesions.

Materials and methods: AD lesions were chronically induced by the repetitive and alternative application of 2,4-dinitrochlorobenzene (DNCB) and oxazolone (OX) on ears in BALB/c mice. The mice were then externally treated with DHMEQ ointment. Macroscopic and microscopic changes of the skin lesions were observed and recorded.

Results: DHMEQ inhibited ear swelling and relieved clinical symptoms of the AD-like lesions induced by DNCB/OX in BALB/c mice. Histopathology examination illustrated that it significantly decreased DNCB/OX-induced epidermal thickness, the infiltration of inflammatory cells, and the count of mast cell. The elevated level of immunoglobulin E (IgE) in serum and the mRNA levels of interferon γ (IFN- γ), interleukin 4 (IL-4) and IL-13 in the ear tissues, were also suppressed by DHMEQ.

Discussion and conclusion: This study indicated that DHMEQ would be useful for the treatment of AD.

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Dehydroxymethylepoxyquinomicin; 2,4-dinitrochlorobenzene; oxazolone; atopic dermatitis; BALB/c mice; immunoglobulin E

Introduction

Atopic dermatitis is a serious skin disorder and an inflammatory reaction easily relapsing. Its clinical manifestations include severe pruritus, dryness, erythematous, edema, and lichenification, which can transiently be controlled with steroidal agents. Long-term application of steroids, however, may produce side effects such as skin thinning and atrophy and immune-suppression^{1,2}. Calcineurin inhibitors like pimecrolimus are also used clinically to relieve the symptoms, but serious safety concerns have been raised³. For example, although tacrolimus ointment is commonly used for the treatment of atopic dermatitis, recent studies showed that it might induce malignant lymphoma⁴. It is clear that new safe and effective drugs are urgently needed for the treatment of AD.

NF- κ B is a key transcription factor to regulate the expression of genes in immune and inflammatory response inducing the transcription of various pro-inflammatory genes^{5,6}. It promotes the transcription of the immunoglobulin kappa chain and cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-8, and IFN- γ ^{7,8}. NF- κ B is often activated in tissues in autoimmune diseases including atopic dermatitis⁹. In 2000, Umezawa et al. designed and synthesized a novel and potent inhibitor of NF- κ B, DHMEQ^{10,11}. DHMEQ is now known to

bind to the NF- κ B components directly to inhibit their DNA binding^{12,13}. The compound has been reported to be effective for the treatment of glomerulonephritis, keloid fibroblasts, arthritis, breast cancer, prostate cancer, thyroid cancer, adult T cell leukemia, and myeloma *in vitro* and *in vivo*^{14,15}, with very less side effects^{16,17}. It also inhibited the growth of human hepatic cancer cell lines, HuH-6 and HA22T/VGH by decreasing the DNA-binding capacity of NF- κ B p65 subunit^{18,19}. Moreover, the topical application of DHMEQ ointment was found to suppress allergic inflammation via its NF- κ B inhibition on AD-like lesions in NC/Nga mice²⁰. However, evidence that it is effective for the treatment of AD is not far enough yet. It was recently reported that an NF- κ B inhibitor was a promising agent for the treatment of ocular inflammation and some autoimmune disorders²¹. Collectively, DHMEQ ointment is expected to be a candidate for anti-inflammatory drug against atopic dermatitis and other skin inflammations.

Materials and methods

Animals

Six-week-old female BALB/c mice were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical

University. The animals were housed with 5 mice per cage in the room maintained at a temperature of $23 \pm 3^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$ throughout the study. Animal treatment and maintenance complied with guidelines issued by the ethics committee for animal welfare at Shenyang Pharmaceutical University (SYPU-IACUC-S20160309-06).

Chemicals and reagents

DNCB was purchased from Xiya reagent Inc. (Chengdu, China) and OX from Sigma (St. Louis, MO). RayBio® Mouse IgE ELISA Kit is the product of RayBiotech, Inc. (Atlanta, GA). Trizol® reagent was from Ambion Inc. (Carlsbad, CA). PrimeScript™ reagent Kit with gDNA Eraser (Perfect Real Time) and SYBR® premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara BIO, Inc. (Dalian, China). Synthesis of DHMEQ and preparation of its ointment were carried out by Shenzhen Wanhe Pharmaceutical Co., Ltd, Guangdong, China. The vehicle ointment is plastibase. One gram of the plastibase contains 950 mg of liquid paraffin and 50 mg of polyethylene. It was obtained from Taisho Pharmaceutical Co. Ltd (Tokyo, Japan).

The induction of AD-like lesions on the ears of mice and DHMEQ administration

To induce AD-like lesions, 1.0% DNCB in acetone and 1.0% OX in acetone were repetitively and alternatively applied according to the experimental scheme described in Figure 1. Briefly, mice ($n=5$) were randomly divided into four groups which were DNCB/OX(−) group, DNCB/OX(+) group, and DNCB/OX(+) plus DHMEQ (0.1% and 0.3%) groups. The surfaces of both ear lobes were sheared with medical scissors ahead of time. Twenty microliter of the DNCB was painted on each ear and paw as well for sensitization. Thereafter, 20 μL of the DNCB or the OX was painted on each ear seven days later for challenge. The two agents were alternatively applied to both ears twice a week in the entire process. On day 18, DHMEQ was administrated to the ears of each mice in the group of DNCB/OX (+) plus DHMEQ (0.1% and 0.3%) twice a day (b.i.d.) till day 45.

The macroscopical measurement of ear thickness

Ear thickness was determined with a dial thickness gauge (Shanghai, China) 24 h after every time of the DNCB or OX application in the entire process.

Histopathology examination

On days 31, 38 and 45, ear tissues were used for histopathological analysis. They were fixed with 10% formaldehyde and embedded in paraffin. Five micrometer slice was stained with hematoxylin and eosin. The inflammatory cells and epidermal thickness were examined under a microscope (20 $\times$). The thickness of epidermis was measured based on three randomly selected fields from each slice. As for the measurement of mast cells, the slice was stained with toluidine blue

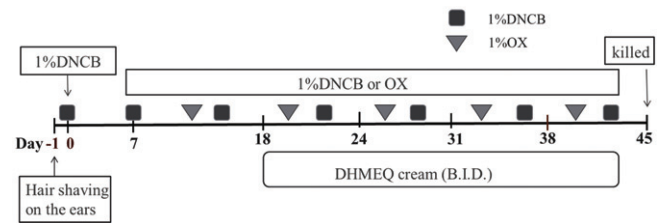


Figure 1. Experimental regime. Mice were sensitized by being painted with 1.0% DNCB in acetone on two ears and four paws on day 0. Thereafter, they were challenged by being alternatively painted with 1.0% DNCB and 1.0% OX on the two ears from day 7 to day 44. DHMEQ and vehicle were painted twice a day (b.i.d.) from days 18 to day 44. The mice were sacrificed on day 45 and blood and ear tissues were used to determine the indices.

Table 1. Primer sequences used for real-time PCR.

Gene	Primers
IL-4	Forward 5'-ACA GGA GAA GGG ACG CCA T Reverse 5'-GAA GCC GTA CAG ACG AGC TCA
IL-13	Forward 5'-GCA ACA TCA CAC AGG ACC AGA Reverse 5'-GTC AGG GAA TCC AGG GCT AC
IFN- γ	Forward 5'-GAG CCA GAT TAT CTC TTT CTA CC Reverse 5'-GTT GTT GAC CTC AAA CTT GG
β -Actin	Forward 5'-GCT TCT TTG CAG CTC CTT CGT Reverse 5'-AGC GCA GCG ATA TCG TCA TC

and the number of mast cells of each group is the average value of five mice. Five sites were chosen at random of each slice was counted.

The determination of serum IgE by ELISA

Blood samples were collected 24 h after the DNCB/OX application on day 31. Serum was prepared by centrifugation and it was stored at -80°C until use. The level of IgE in serum was determined following the instructions from the manufacturer of ELISA kit. Briefly, this assay employs an antibody specific for mouse IgE coated on a 96-well plate. Standards and samples are pipetted into the wells and IgE present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-mouse IgE antibody is added. After washing unbound biotinylated antibody away, HRP-conjugated streptavidin is pipetted to the wells. A TMB substrate solution is added to the wells after they are rinsed again. Color develops in proportion to the amount of IgE bound. The stop solution changes the color from blue to yellow. The intensity of the color is determined at 450 nm. IgE level was indicated by the O.D. value.

RNA isolation, complementary DNA synthesis, and real-time PCR

Total RNA in ear tissue was isolated using Trizol® reagent according to the protocol from the manufacturer on day 45. The equal amount of RNA was reversely transcribed into cDNA using Prime Script™ RT reagent Kit with gDNA Eraser®. Quantitative real-time PCR was performed using Agilent Technologies Stratagene M $\times$ 3000 P (Agilent Technologies Inc., Waldbronn, Germany) with SYBR® premix Ex Taq™ and specific primers of IFN- γ , IL-4, IL-13, and β -actin (Table 1). The amplification program is consisted of 1 cycle of 95°C for 30 s, 40

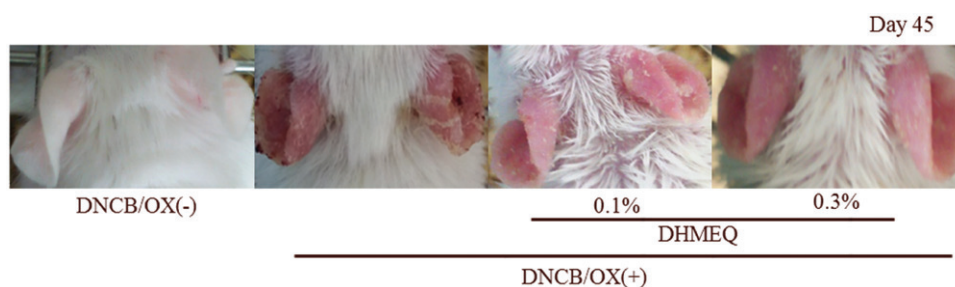


Figure 2. Representative photographs of ear skin. They were taken 24 h after the DNCB/OX application on day 45. They showed AD-like skin lesions with and without DHMEQ ($n = 5$ in each group).

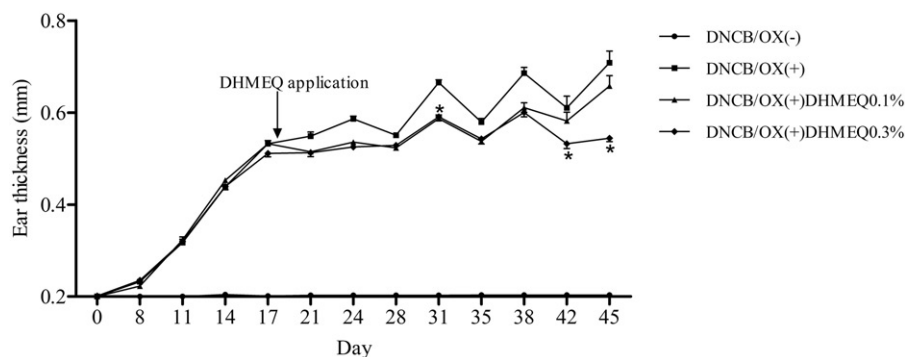


Figure 3. Ear swelling daily measured with a thickness gauge. DHMEQ (0.1% and 0.3%) was externally painted twice a day from days 18 to 44. Five mice were put into each of four groups. Data are expressed with mean $\pm$ SD of five determinations. * $p < .05$, compared with the DNCB/OX (+) group.

cycles of 95 °C for 5 s, and 60 °C for 31 s, and the last cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. Fluorescence intensity was measured in real time using the optical module. Melt curves were used to determine product specificity. Results were normalized to the level of β -actin gene expression.

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical analyses were performed using Version 5 of GraphPad Prism (GraphPad Inc., La Jolla, CA). Therapeutic effects of the tested drug were analyzed using one-way analysis of variance followed by Dunnett's test. A value of $p < .05$ was used to indicate statistically significant difference.

Results

DHMEQ ameliorates clinical manifestations of DNCB/OX-induced AD-like lesions

As shown in Figure 2, the repetitive application of DNCB/OX to the ears of BALB/c mice induced AD-like clinical symptoms. Skin edema became severe with the increase of days (Figure 3). The DNCB/OX-evoked ear edema is closely correlated with relapsing edema and oozing. The continual and repeated application of the two agents to BALB/c mice induced AD-like lesions with severe clinical manifestations including erythema/haemorrhage, edema, excoriation/erosion, and dryness/scaling (Figure 2).

To determine the therapeutic effect of DHMEQ on the AD-like lesions, it was b.i.d. painted on the ears of mice with

AD-like lesions induced by DNCB/OX from days 18 to 45 (Figure 1). The daily administration of DHMEQ relieved the skin lesions (Figure 2). The ear thickness of mice treated with DHMEQ (0.1%, 0.3%) was decreased significantly (Figure 3).

DHMEQ inhibits inflammation observed under a microscope: infiltration of inflammatory cells, epidermal thickness, and the count of mast cells

The therapeutic effects of DHMEQ were investigated in mice with DNCB/OX-induced AD-like lesions by the evaluation of histopathology. Histological analysis of the ear of mice in DNCB/OX (–) group revealed that the tissue was normal, whereas that of mice in DNCB/OX (+) group exhibited epidermal hypertrophy, the hyperkeratosis of epidermis and the infiltration of inflammatory cells (Figure 4). The administration of DHMEQ ameliorated the histopathological alterations as compared with those in DNCB/OX (+) group. The drug suppressed DNCB/OX-induced epidermis thickening and the infiltration of inflammatory cells (Figure 4). Mast cells were counted in the present study considering that they are important effector cells when AD occurs. It is shown in Figure 5 that DHMEQ significantly lowered the number of mast cells.

DHMEQ suppresses the DNCB/OX-induced increase of IgE in serum

It was investigated whether the effects of DHMEQ were involved in the alteration of IgE production, since IgE is

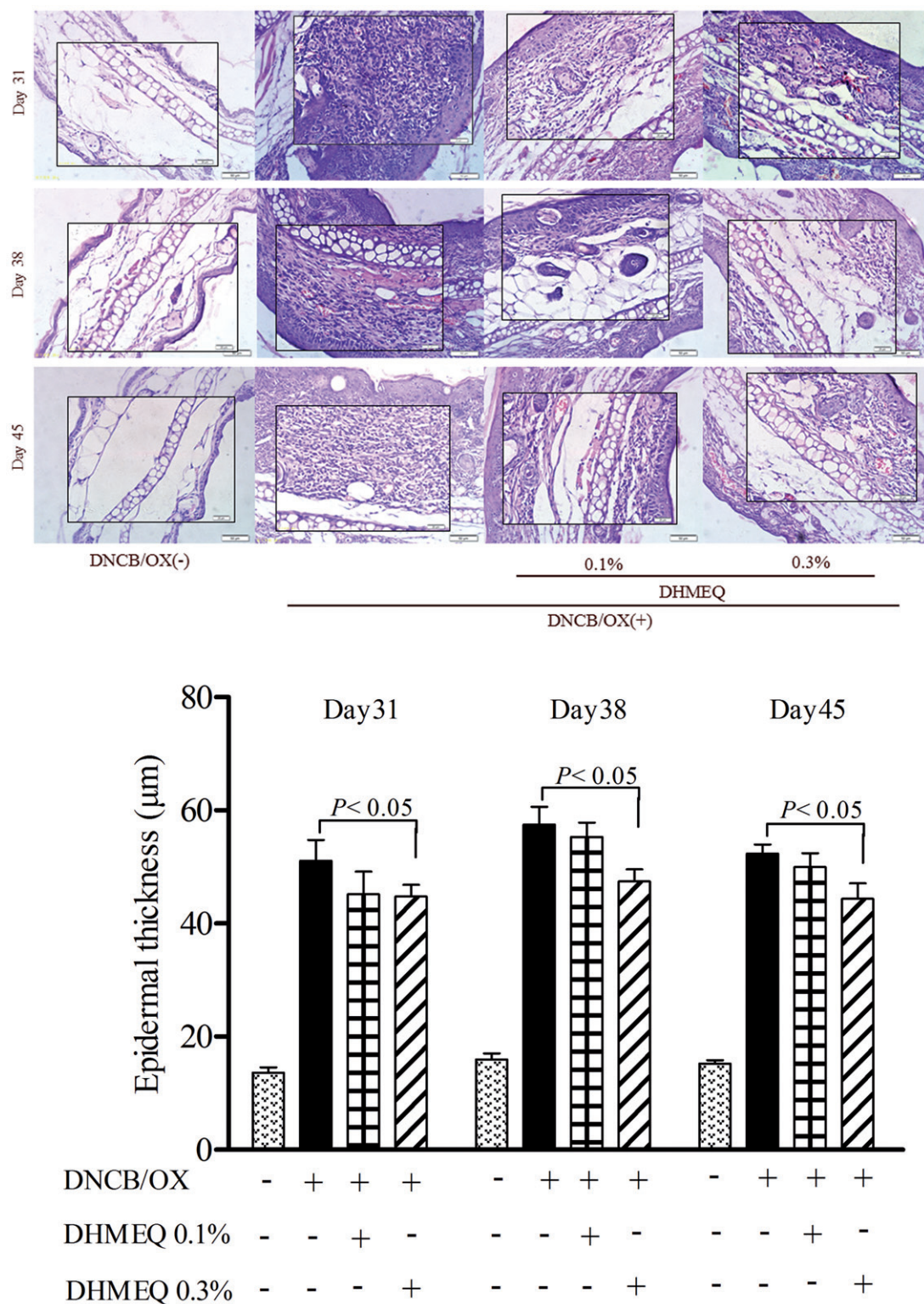


Figure 4. Histopathological analysis of inflammation in ear skin stained with hematoxylin and eosin. The upper figure showed data obtained from ear sections which were stained with hematoxylin and eosin (bar = 50 μm). It showed the infiltration of inflammatory cells and epidermal thickness. Information in the small box was magnified to show the details in the corresponding site (bar = 20 μm). The calculated data in the column figure were from three representative areas of each slice. They are expressed with mean ± SD of five slices. $p < .05$, compared with the DNCB/OX (+) group.

known acting as an important pathogenic factor in AD. The serum levels of IgE in DNCB/OX (+) group were markedly upregulated, compared with those in DNCB/OX (-) group. DHMEQ (0.1%) was observed to effectively reduce the elevated levels of IgE (Figure 6).

DHMEQ suppresses the DNCB/OX-induced expression of cytokines in ear tissues

AD is characterized by an immunological imbalance, leading to the predominance of T helper type 2 (Th2) cells in acute skin lesions. The immunologic response, however, switches

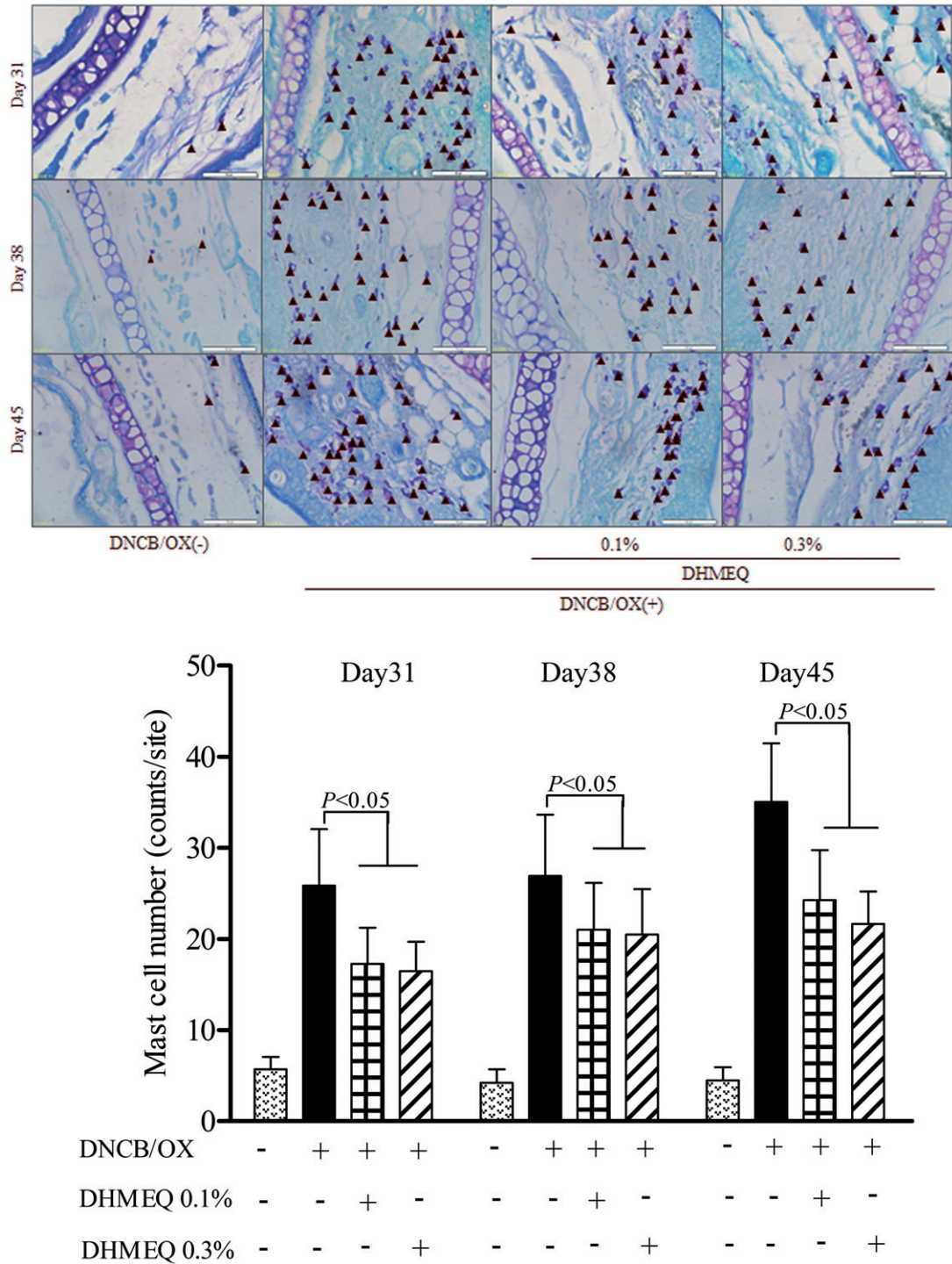


Figure 5. Histopathological analysis of mast cells in ear skin stained with toluidine blue. The upper figure showed data obtained with ear sections which were stained with toluidine blue (bar = 50 μ m). It showed the infiltration of mast cells indicated with arrowheads. Data in the column figure were obtained from the histopathological data. Mast cells in toluidine blue-stained sections were counted under a microscope. Total numbers of mast cells in three slices from one mouse, each of which included five representative areas, were counted. They are expressed with mean $\pm$ SD of five mice. $p < .05$, compared with the DNCB/OX (+) group.

to the Th1 type cells during the chronification of the atopic dermatitis. Therefore, the acute phase of AD is predominantly a Th2 response, while the chronic phase is characterized by Th1 response. The effect of DHMEQ on the expression of AD-related inflammatory cytokines like IFN- γ , IL-4, and IL-13 in ear tissues was examined by real-time PCR in the present study. All the cytokines in AD-like lesion tissues were upregulated and DHMEQ suppressed the enhanced expression of both Th1 (IFN- γ) and Th2 (IL-4, IL-13) cytokines (Figure 7).

Discussion

The pathogenesis of AD includes genetic, immunologic, and environmental factors¹. Therefore, it appears difficult to find generally effective drugs with few side effects for AD. DHMEQ is a new NF- κ B inhibitor that exhibited strong anti-inflammatory and anticancer activities *in vivo* without apparent toxicity so far²². Studies showed that it inhibited the secretion of inflammatory cytokines²³ and showed no

harmful effect in mice or rats²⁴. In the current study, DHMEQ did not influence the mouse body weight of mice, while tacrolimus and mometasone furoate decreased the weight when they were externally applied (data not shown).

Hamasaka et al.²⁰ found that DHMEQ inhibited the contact hypersensitivity response of Balb/c mice via suppressing

inflammatory cytokines and it improved AD manifestation in mouse model. In the present study, beneficial effect of DHMEQ on BALB/c mice with severe AD-like lesions induced by DNCB/OX was observed. The AD-like model in mice was induced by the alternative application of 1.0% DNCB and 1.0% OX, which is considered to be more severe than the models commonly used²⁵. DNCB/OX-induced ear thickness was significantly lowered by DHMEQ (Figure 3). Moreover, it also relieved other clinical manifestations including erythema, edema, excoriation and hyperkeratosis (Figure 2).

It is well known that skin lesions in AD patients are characterized by the proliferation and infiltration of inflammatory cells such as mast cells, basophils, eosinophils, and T cells²⁶. Sehra et al.²⁷ found that the homeostatic expression of epidermal differentiation complex was regulated by mast cells. The repetitive application of DNCB/OX disturbs the physical barrier of skin and induces the activity of inflammatory cells like eosinophils and mast cells. The secretion of various immune mediators induces cytokine cascade and promotes the accumulation of collagen, which preludes dermal thickening and increases the tissue damage¹. It is illustrated in this study that the consecutive application of DNCB/OX led to increased epidermal thickness and the infiltration of

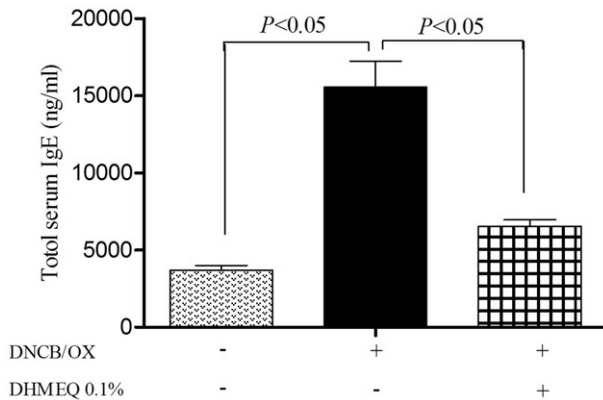


Figure 6. The level of total IgE in serum of mice with AD-like lesions. Blood samples were collected on day 31 and IgE level in serum was determined with ELISA kit. Data are expressed as mean $\pm$ SD. $p < .05$, compared with the DNCB/OX (+) group.

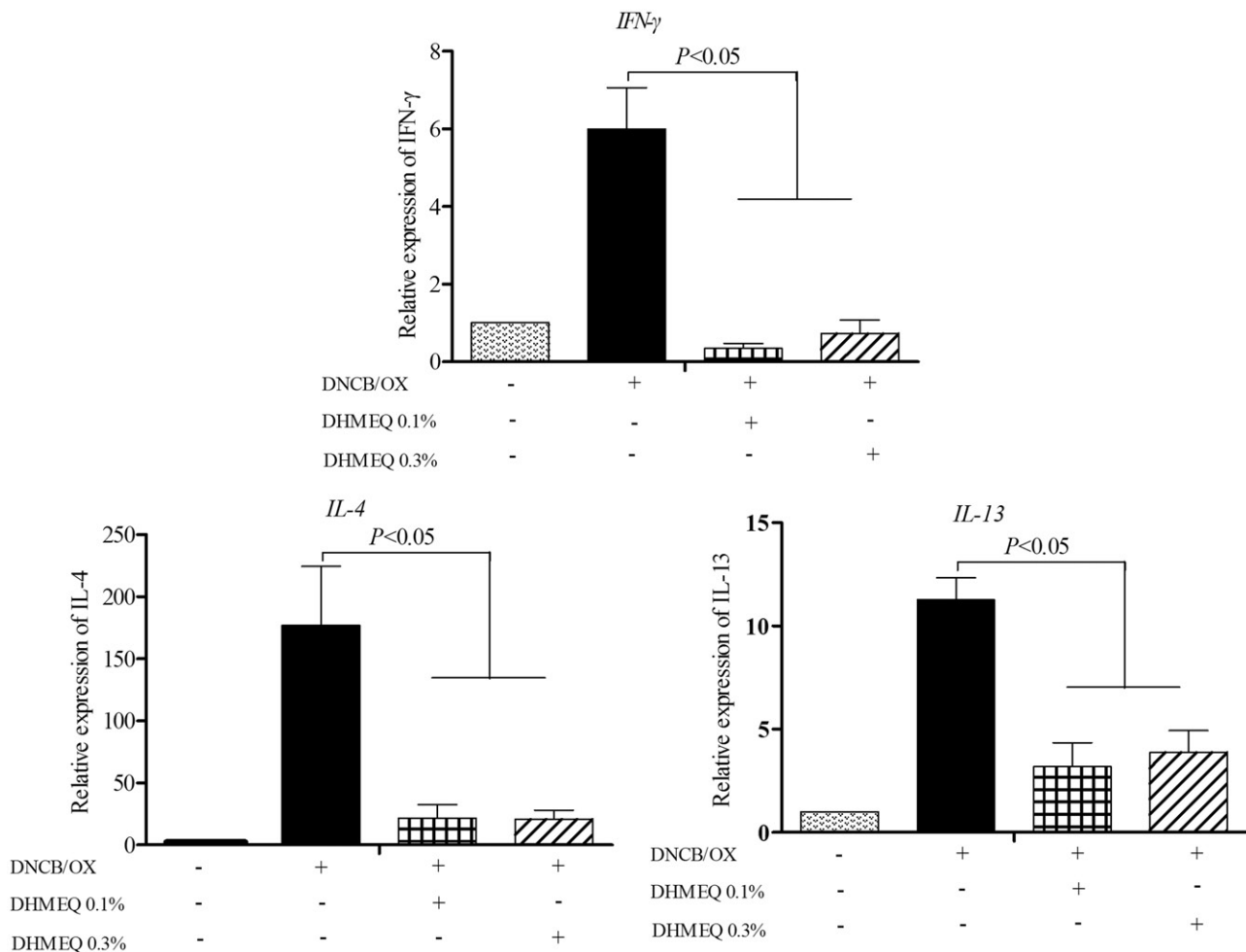


Figure 7. The suppression of DHMEQ on the increased expression of cytokines in ear tissues. The ears were excised and total RNA in it was extracted. Quantitative real-time PCR was performed following the description in method. Data are presented as mean $\pm$ SD of triplicate determinations. $p < .05$, compared with the DNCB/OX (+) group.

eosinophils in ear skin (Figure 4). DHMEQ treatment suppressed the increased thickness and the infiltration of eosinophils and mast cells (Figures 4 and 5). The agent was previously found to reduce eosinophilic inflammation in airway significantly²¹ and suppressed mast cell migration via the inhibition of NF- κ B²⁸.

Allergen-sensitized IgE activates mast cells, leading to the development of Th2 cells and the secretion of cytokines, which induce the development of AD skin lesions²⁹. In contrast, the infiltration of the activated mast cells is related to either the production of inflammatory cytokines or the increase of IgE synthesis³⁰. In the present study, it was found that the level of total IgE in serum was significantly elevated after the sustained application of DNCB/OX in BALB/c mice, while DHMEQ (0.1%) could significantly lower the serum level of IgE (Figure 6). However, DHMEQ at 0.3% was not effective to reduce the elevated levels of IgE (data not shown). DHMEQ may not penetrate enough to the skin in the present formulation with platiabase. Whether the basecream affects the release of DHMEQ or not is needed to be further investigated.

It has been reported that Th2/Th1 ratio is imbalance in AD patient³¹, where Th2-cells play an important role in the initial phase of inflammatory reactions, whereas Th1-cells can be detected in greater numbers in later stages³². Repeated treatment with DNCB/OX induces both Th1 and Th2 cytokines to release by the activation of helper T cells^{33–35}. DHMEQ treatment significantly decreased IL-4 and IL-13 which is thought to be as one of the Th2 cytokines and IFN- γ which is thought to be as one of the Th1 cytokines³⁶. The mRNA expression of IFN- γ , IL-4, and IL-13 was found to be significantly elevated after the 4-week's alternative use of DNCB/OX in the current study (Figure 7), indicating that leukocyte infiltration might be one of the mechanisms in the development of the AD skin lesion. DHMEQ significantly suppressed the elevation of the inflammatory factors (Figure 7). It is very likely that the topical application of the drug is helpful to attenuate Th1 and Th2 responses. Shimizu et al.²¹ found that DHMEQ significantly suppressed the level of Th2 cytokines IL-5 and IL-13 in broncho-alveolar lavage fluid in acute asthma model.

Conclusions

DHMEQ ointment inhibited ear thickness and relieves some clinical manifestations including erythema, edema, excoriation, and hyperkeratosis. DHMEQ treatment reduced the increased epidermal thickness and the infiltration of eosinophils and mast cells. It inhibited the synthesis of IgE and the mRNA expression of IFN- γ , IL-4, and IL-13 in the lesion tissues. However, molecular mechanisms in detail for the therapeutic effects of DHMEQ in AD-like models have not been fully elucidated. Further studies are evidently necessary to investigate the mechanisms whereby DHMEQ regulates inflammatory mediator that correlates with NF- κ B.

Disclosure statement

The authors declare no conflicts of interest.

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