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ORIGINAL ARTICLE



Dehydroxymethylepoxyquinomicin suppresses atopic dermatitis-like lesions in a stratum corneum-removed murine model through NF- κ B inhibition

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ABSTRACT

Background: Dehydroxymethylepoxyquinomicin (DHMEQ) is a specific and potent inhibitor of nuclear factor-kappa B (NF- κ B) and has been shown to possess promising potential as an anti-inflammation including anti-atopic dermatitis (AD)-like skin lesions.

Objective: To further evaluate the activity of DHMEQ *in vivo* modified AD-like lesion model in BALB/c mice and *in vitro* AD-like lesion cell model in human keratinocytes.

Materials and methods: In this study, *in vivo* modified AD-like lesion model in BALB/c mice was chronically induced by the repetitive and alternative application of 2,4-dinitrochlorobenzene (DNCB) and oxazolone (OX) on ears, and stratum corneum of the ear skin was additionally stripped off with surgical tapes before each challenge with DNCB/OX. Moreover, *in vitro* AD-like lesion cell model in human keratinocytes (HaCaT) achieved by stimulating HaCaT cells with tumor necrosis factor (TNF)- α plus interferon (IFN)- γ was used to investigate mechanisms of the action.

Results: The lesions derived from the stratum corneum-removed AD-like lesion model reaches to peak as well as DHMEQ arrives to its efficacy a week earlier than the data previously obtained from the common AD-like lesion model. Results showed that the drug reduced the ear thickness, epidermal thickness, mast cell infiltration, and gene expressions of interleukin (IL)-4, IL-13, and interferon (IFN)- γ in ear tissues. It significantly inhibited the expression of cytokines IL-6 and IL-1 β , chemokines thymus and activation-regulated chemokine (TARC)/CCL17, and macrophage-derived chemokine (MDC)/CCL22 in the stimulated HaCaT cells.

Discussion and conclusion: This study indicated that the action of DHMEQ's anti-AD like lesions might be related to its inhibition on NF- κ B.

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Introduction

Atopic dermatitis (AD) is among frequently occurred and easily relapsing allergic skin diseases in clinical settings [1]. It is shown that some pro-inflammatory cytokines are responsible for the initiation and maintenance of the skin inflammation in the patients [2], therefore, leading to development of AD. The cytokines such as IL-4, IL-13, IFN- γ , IL-1 β , and IL-6 [3] as well as chemokine TARC/CCL17 and MDC/CCL22 are generally regarded as typical inflammatory markers in the investigation of AD [4]. Furthermore, those cytokines were also demonstrated to be involved in the NF- κ B signaling pathway [5–8].

Repeated application of OX or dust mite extract and DNCB to mice can induce AD-like skin lesions which therefore are used as an animal pathological model for investigation of the efficacy of new compounds and mechanisms of the action [9,10]. A surgical tape was additionally used to moderately strip off stratum corneum of the skin on basis of immunological changes induced by the application of the

reagents [11,12] which might be helpful for some of extrinsic substances such as drugs to participate into the superficial skin layer [11,13]. Moreover, this modified AD-like lesion model has more similar characters with AD occurred in clinic because mechanical and artificial injuries to skin led by patients' scratching happened frequently once they cannot being endured pruritic eczema, the most common and severe symptom of patients with AD [14].

DHMEQ, a specific potent nuclear factor-kappa B (NF- κ B) inhibitor [15], was elicited to have promising potential as an anti-tumor or/and anti-inflammatory agent [16]. Both *in vitro* and *in vivo* studies of DHMEQ's anti-tumor effect have validated the potential, including the efficacy in breast cancer, prostate cancer, thyroid cancer, adult T cell leukemia and myeloma, etc. [17]. Topical application of DHMEQ ointment was found to suppress allergic skin inflammation in both BALB/c mice and NC/Nga mice. Molecular mechanism of the action is not much clear yet, although it was considered, in Hamasaka's study in NC/Nga mice [18,19], that the mechanism was partly involved in the drug's anti-NF- κ B activity. The

present study is attempted to further explore the efficacy of DHMEQ's anti-AD lesion after its percutaneously increased concentration via the injured skin in the modified AD murine model and the mechanisms of the action on cytokines and chemokines through its inhibition of NF- κ B.

Materials and methods

Animals

Six-week-old female BALB/c mice were provided by the Laboratory Animal Center of Shenyang Pharmaceutical University. The mice randomly divided into Normal, Vehicle, DHMEQ, DHMEQ, DHMEQ. All experimental procedures involving animals and their care conformed to the guide for the care and use of laboratory animals in Shenyang Pharmaceutical University (SYPU-IACUC-S2016.09.05-101).

Chemicals and reagents

DNCB was purchased from Xiya reagent Inc. (Chengdu, China) and OX from Sigma (St. Louis, MO, USA). RayBio[®] Human MDC Enzyme-linked immunosorbent assay (ELISA) Kit, RayBio[®] Human TARC ELISA Kit and RayBio[®] Mouse TNF- α ELISA Kit were products of RayBiotech, Inc. (Atlanta, Georgia, USA). Recombinant Human TNF- α and Recombinant Human IFN- γ were from PeproTech Inc. Trizol[®] reagent was from Ambion Inc. (Carlsbad, CA, USA). 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). DHMEQ synthesis and the preparation of its ointment were performed in Shenzhen Wanhe Pharmaceutical Co., Ltd. Vehicle ointment is plastibase, one gram of which contains 950 mg liquid paraffin and 50 mg polyethylene. It was obtained from Taisho Pharmaceutical Co. Ltd (Tokyo, Japan).

Induction of stratum corneum-removed AD-like skin lesions on mouse ears, DHMEQ treatment and determination of indices

The murine AD-like lesion model was induced according to the method reported in the literature with minor modification [11,19]. Briefly, the surfaces of both ear lobes were shearing with medical scissors ahead of time. About 20 μ L of DNCB (1.0%) in acetone was painted to the ears and paws for sensitization. Subsequently, AD-like skin lesions were alternately induced by challenge with the topical application of 20 μ L of 1.0% DNCB in acetone and 1.0% OX in acetone to the ears of mice every 3–4 days. Besides, the stratum corneum layer of the ear skin was stripped off with surgical tape before each time of the challenge (Figure 1). The normal group was applied with nothing.

All mice were randomly divided into five groups ($n = 5$ for each group), that is, normal group, vehicle, 0.1% DHMEQ, 0.3% DHMEQ, and 0.7% DHMEQ. The vehicle and drug treatment groups were then topically applied, respectively, with vehicle ointment (plastibase), 0.1%, 0.3%, and 0.7% of

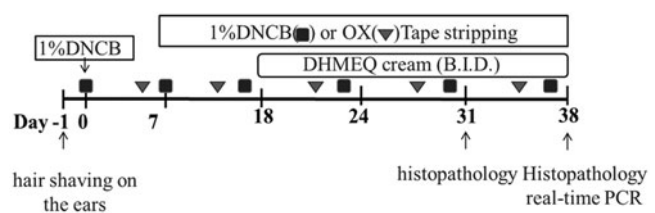


Figure 1. Experimental procedure. Mice were sensitized by being painted with 1.0% DNCB in acetone on two ears and four paws on day 0. Thereafter, 20 μ L of the DNCB or the OX was alternatively painted every 3–4 days for challenge on each ear from day 7 on. Besides, the horny layer of the ear skin was stripped off with adhesive tape before each time of the challenge. DHMEQ and vehicle were respectively painted twice a day (b.i.d.) from day 18 to day 38. The mice were sacrificed on day 31 and 38, respectively, and the ear tissue samples were kept for the examination of histopathology and those obtained on day 38 were used for the determination of cytokine IFN- γ , IL-4, and IL-13.

DHMEQ ointment (micronized DHMEQ were evenly mixed into the plastibase), to the ear area of mice twice per day (b.i.d.) from day 18 till day 38 (Figure 1). Ear thickness was measured every 3–4 day with a dial thickness gauge (Shanghai, China) 24 h after each application of DNCB or OX from day 7. The mice were respectively sacrificed on day 31 and 38, and their ear tissues were kept for the examination of histopathology and those obtained on day 38 were used for the determination of cytokine IFN- γ , IL-4, and IL-13.

Histopathological examination

Histopathological examination of inflammatory status or mast cell infiltration was performed according to the description in the previous study [19]. Briefly, the ear tissues were fixed in 10% formaldehyde once they were excised. Then they were embedded in paraffin and 5 μ m slices were prepared. One part of the slices was stained with hematoxylin and eosin (H&E, HE). Infiltrated lymphocytes, thickening of the epidermis, and fibrosis in dermis were observed under microscope. Epidermis thickness in the H&E-stained slices was blindly analyzed basing on five randomly selected fields, which were observed under 200 times' magnification. The other part of the slices was stained with toluidine blue for examination of the infiltration of mast cell. The number of mast cells in five sites chosen at random under 400 times' magnification of microscope was counted blindly by a person who did not know the treatment.

HaCaT cell culture

HaCaT cell culture was performed according to the method described in literature [4]. Briefly, HaCaT cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified 5% CO₂ atmosphere at 37 °C. They were plated in 96-well culture plates in density of 10⁶ cells/ml culture medium and allowed to attach to bottom of the plates for 24 h. Thereafter, they were treated with DHMEQ (in PBS) for 6 h and then stimulated with TNF- α /IFN- γ (10 μ g/ml) for 24 h. The supernatant of the cells was collected after the 24 h-stimulation to be used for the determination of

TARC/CCL17 and MDC/CCL22 with ELISA kits. The cell pellets were used for the determination of IL-1 β and IL-6 with real-time PCR as described below.

Determination of cytokines and chemokines

The ear tissues were excised at the end of *in vivo* experimental period and total RNA in the tissues were isolated with Trizol[®] reagent according to the manufacturer's protocol.

HaCaT cells were pretreated with DHMEQ (in PBS) for 6 h and then stimulated with TNF- α (10 ng/mL) plus IFN- γ (10 ng/mL) for 24 h. Total RNA in the cell pellets was isolated as described in literature [4].

Quantitative real-time PCR was used to determine the levels of the cytokines in the ear tissues and the cell pellets, respectively, with SYBR[®] premix Ex TaqTM according to the manufacturer's protocol on Agilent Technologies Stratagene M $\times$ 3000P (Agilent Technologies Inc., Germany). The amplification program is consisted of 1 cycle of 95 $^{\circ}$ C for 30 s, 40 cycles of 95 $^{\circ}$ C for 5 s, and 60 $^{\circ}$ C for 31 s, and the last cycle of 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 1 min, and 95 $^{\circ}$ C for 15 s. Mouse-derived specific primers of β -actin, IFN- γ , IL-4, and IL-13 (Table 1) were used to determine the cytokines in the ear tissues obtained on day 38 and human-derived specific primers of β -actin, IL-6, and IL-1 β (Table 2) were used to determine the cytokines in the cell pellets. Fluorescence intensity was measured in real time with optical module. Melt curves were used to determine product specificity. Results were normalized to level of the β -actin gene expression.

On the other hand, levels of TARC/CCL17 and MDC/CCL22 in the supernatant of HaCaT cell culture were measured by

RayBio[®] Human TARC ELISA Kit and RayBio[®] Human MDC ELISA Kit according to the manufacturer's instructions, respectively.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD). Statistical analysis was performed with one-way analysis of variance followed by Dunnett's test, which were analyzed with Graphpad prism software (Version 5). The $p < .05$ was considered statistically significant.

Results

Ameliorate the severe clinical manifestations

Stratum corneum-removed AD-like skin lesions were induced in Balb/c mice with DNCB and OX plus tape. The topical application of DHMEQ to the mice twice a day remarkably inhibited the severe AD-like skin lesions. It ameliorated the severe clinical manifestations such as erythema/hemorrhage, edema, excoriation/erosion, and dryness/scaling (Figure 2). The ear thickness was significantly decreased by DHMEQ, especially the low concentration (0.1%) of the drug (Figure 3).

Inhibit hyperplasia of epidermis and infiltration of inflammatory cells

Ear tissue samples excised on day 31 and 38 were exerted histopathological examination after their slices being stained with hematoxylin/eosin or toluidine, respectively. Upper figures in Figures 4 and 5 are representative photos of the

Table 1. Mouse-derived specific primers of β -actin, IFN- γ , IL-4, and IL-13 for quantitative real-time polymerase chain reaction.

Gene	Primers
IL-4	Forward 5'-ACAGGAGAAGGGACGCCAT Reverse 5'-GAAGCCGTACAGACGAGCTCA
IL-13	Forward 5'-GCAACATCACACAGGACCAGA Reverse 5'-GTCAGGGAATCCAGGGCTAC
IFN- γ	Forward 5'-GAGCCAGATTATCTCTTTCTACC Reverse 5'-GTTGTTGACCTCAAACCTGG
β -Actin	Forward 5'-GCTTCTTTGCAGCTCCTTCGT Reverse 5'-AGCGCAGCGATATCGTCATC

Table 2. Human-derived specific primers of β -actin, IL-6, and IL-1 β for quantitative real-time polymerase chain reaction.

Gene	Primers
IL-1 β	Forward 5'-GCAATGAGGATGACTTGTTT Reverse 5'-TAGTGGTGGTCGGAGATTC
IL-6	Forward 5'-TGTTGTGAAAGCAGCAAAGAG Reverse 5'-ACCTCAAACCTCAAAAGACC
β -Actin	Forward 5'-GATTCCTATGTGGGCGAC Reverse 5'-TTGTAGAAGGTGTGGTGCC

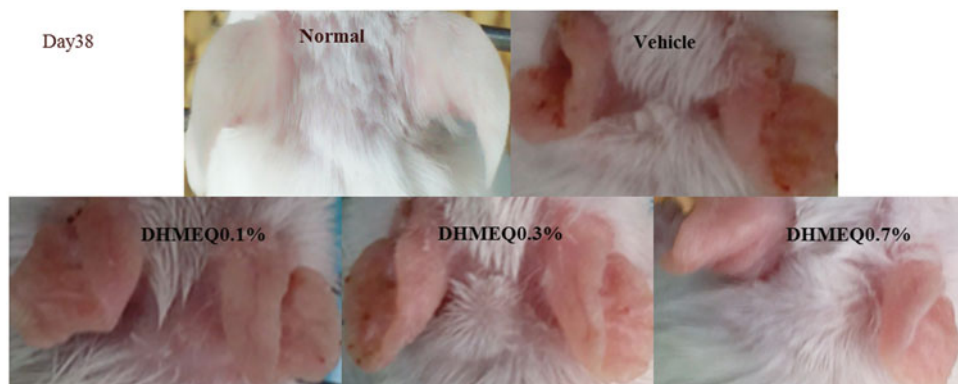


Figure 2. Representative photographs of the mouse ears. They were taken 24 h after tape-DNCB/OX application on day 38, depicting AD-like skin lesions with and without DHMEQ.

histological analysis of the slices. Hyperplasia and thickening of epidermis could obviously be seen in the tissue samples of the mice with stratum corneum-removed AD-like lesions and inflammatory cell infiltration was remarkably observed as well. DHMEQ significantly inhibited the skin inflammation. The mast cell infiltration was also observed in the samples

after being stained with toluidine blue and the infiltration was shown to be remarkably decreased by DHMEQ on both day 31 and 38, but high concentration of the drug did not show significant action on day 38 (Figure 5). All concentrations of the drug suppressed the number of mast cells on day 31 although the concentration-effect relationship was not very clear, while the mid-concentration exerted most significant effect on day 38 (Figure 5).

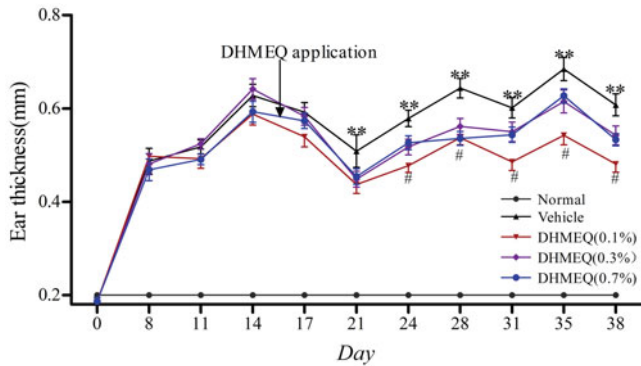


Figure 3. Ear thickness which was measured every 3–4 day with a dial thickness gauge 24 h after each challenge with DNCB or OX from day 7 till 38. DHMEQ was externally painted twice a day from day 18 to 38. Five mice were randomly allocated to each of the groups. Data were expressed as mean $\pm$ SD of five determinations. ** $p < .01$, compared with the normal group; # $p < .05$, compared with the vehicle group.

Suppress cytokines and chemokines

Data in Figure 6 showed that all concentrations of DHMEQ suppressed significantly the increased mRNA levels of IL-4, IL-13, and IFN- γ in the ear tissues subjected to the AD-like skin lesions.

Considering that cytokines and chemokines are involved in AD-like skin inflammation [4], effects of the DHMEQ on the production or expression of some cytokines and chemokines in HaCaT cells stimulated by the combination of TNF- α and IFN- γ was investigated. Results showed that mRNA levels of both IL-1 β and IL-6 in the cells increased after the cells being stimulated by TNF- α and IFN- γ (Figure 7). DHMEQ (10 μ g/ml) remarkably suppressed the increased mRNA levels of the two cytokines (Figure 7). In addition, the secreted level

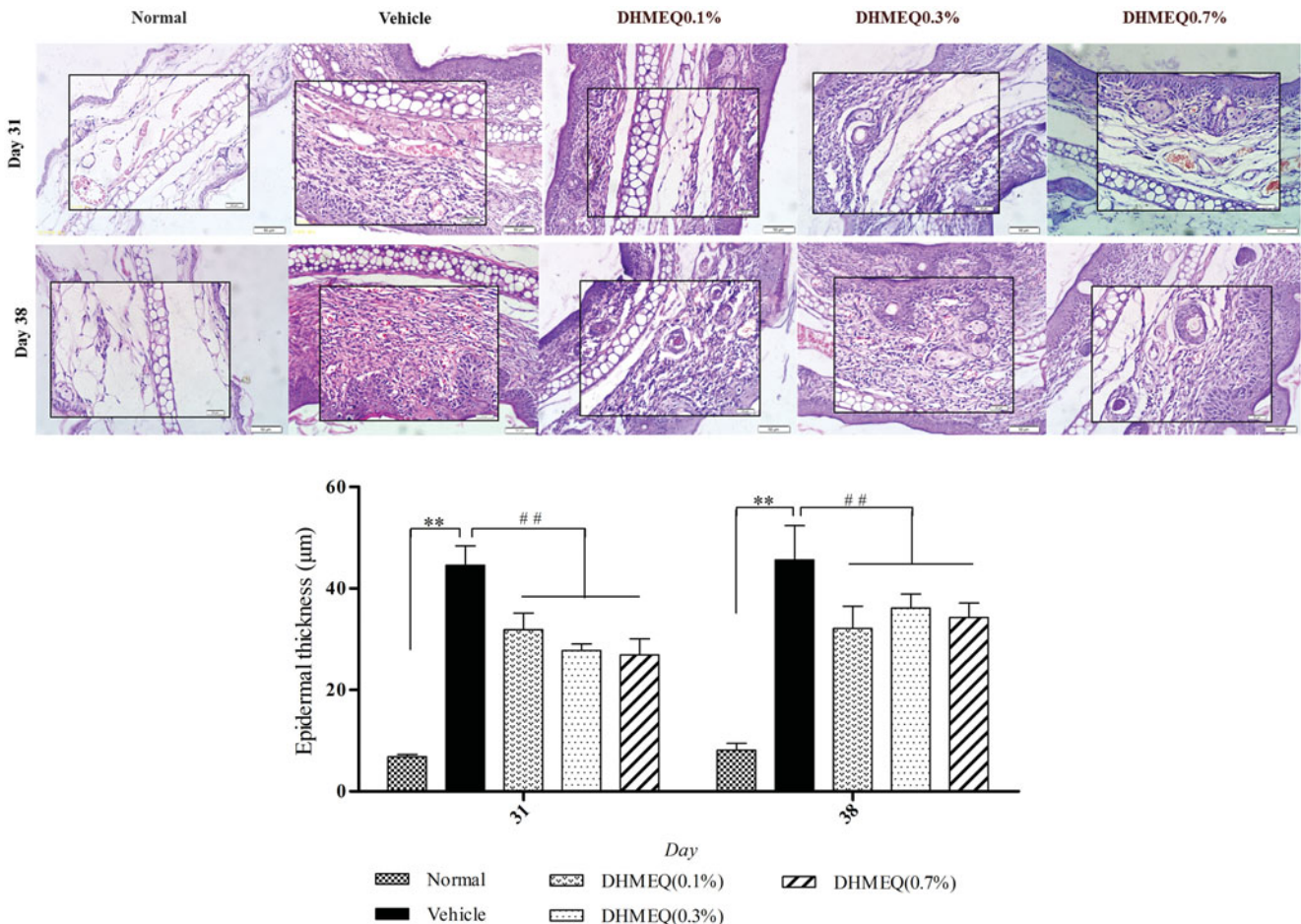


Figure 4. Histopathological analysis of the hyperplasia and inflammation of epidermis. Upper figures showed data (bar = 50 μ m, 200 $\times$) that were obtained from ear tissue slices which were exerted HE staining. Inset information shows the details (bar = 20 μ m, 400 $\times$) in the corresponding site. Data in bottom were calculated from three representative areas of each slice. They were expressed with mean $\pm$ SD of five slices. ** $p < .01$, compared with the normal group; ## $p < .01$, compared with the vehicle group.

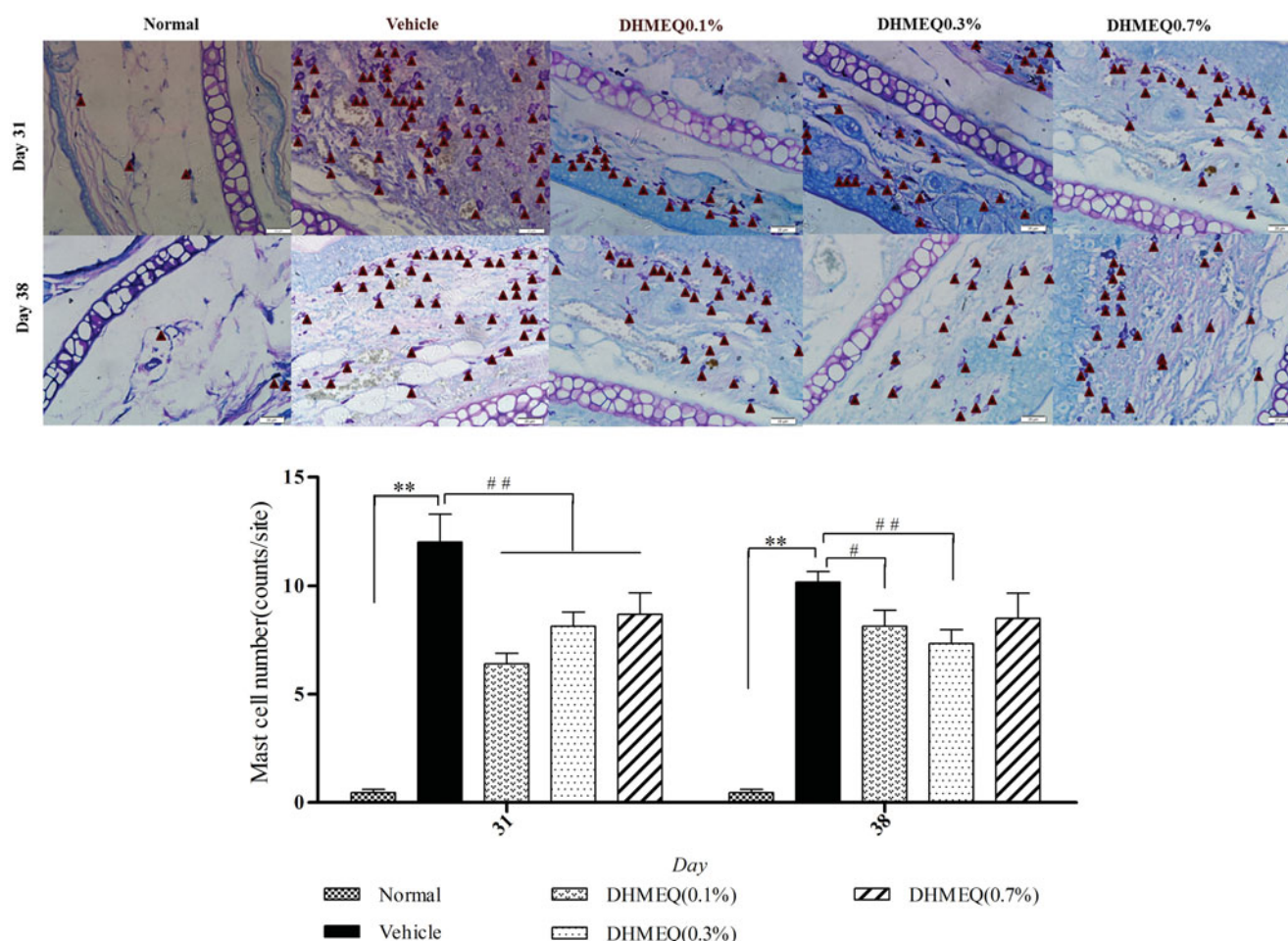


Figure 5. Histopathological analysis of the infiltration of mast cells. Upper figures (bar = 20 μ m, 400 $\times$) were obtained from ear tissue slices which were exerted toluidine blue staining, showing the infiltration of mast cells which were indicated with arrowheads. Data in bottom were calculated from the histopathological data. Mast cells in the toluidine blue-stained slices were counted under a microscope. Total numbers of mast cells in three slices from one mouse, each of which included five representative fields, were counted. They were expressed as mean $\pm$ SD of five mice. ** p < .01, compared with the normal group; # p < .05 and ## p < .01, compared with the vehicle group.

of TARC/CCL17 and MDC/CCL22 in the culture supernatant was also determined by ELISA. Results showed that both TARC/CCL17 and MDC/CCL22 were remarkably increased by the stimulation of TNF- α /IFN- γ . The increased release of the chemokines was significantly suppressed by DHMEQ in a concentration-dependent manner (Figure 8).

Discussion

It has been found in the previous study that the increased level of inflammatory cytokines in ear tissues came to a climax on day 45 in the murine AD-like lesion model induced with DNCB and OX. One of the advantages of the DNCB/OX-induced model is that it elicits good reproducibility [19]. Besides, accumulating evidences support the notion that skin barrier dysfunction contributes to the progressive development of inflammatory skin disorders such as AD [20]. Defective skin barrier is a prominent feature of successively built AD-like lesion model [21]. Therefore, in the current study, the stratum corneum layer of the ear skin was additionally stripped off with surgical tape before each time of the AD-like inflammation being induced with DNCB/OX. Results showed that the application of the adhesive tape

quickened the formation of AD-like skin lesions and the inflammation reaches its peak on day 38, which might be related to the barrier being damaged. DHMEQ's efficacy indicated that it had good therapy on this skin damage. It is indicated that the remove of the stratum corneum is helpful for the drug to arrive better and faster at its local target and well exert its efficacy. The severe AD-like skin lesions were achieved in mice by making the stratum corneum of ear skin stripped off with surgical tape basing on DNCB/OX-induced dermatitis. The reason why this severe AD-like lesion model was used in the study is that the effect of DHMEQ against AD-like skin lesions needs further to be evaluated on one hand on the basis of the previous study. On the other hand, the stratum corneum-removed skin might be helpful for the active drug in the ointment formulation penetrating easily the outer skin barrier and arriving at its target [13,22], since previous study found that DHMEQ's pharmacological efficacy was partially limited by its getting at the target before it was partly inactivate in niche of the body fluid.

AD is characterized by lichenification accompanied with epidermis hyperplasia [23]. The complex chronic progress in the skin disorder was initiated and promoted by immunological and inflammatory reactions [24], among which mast

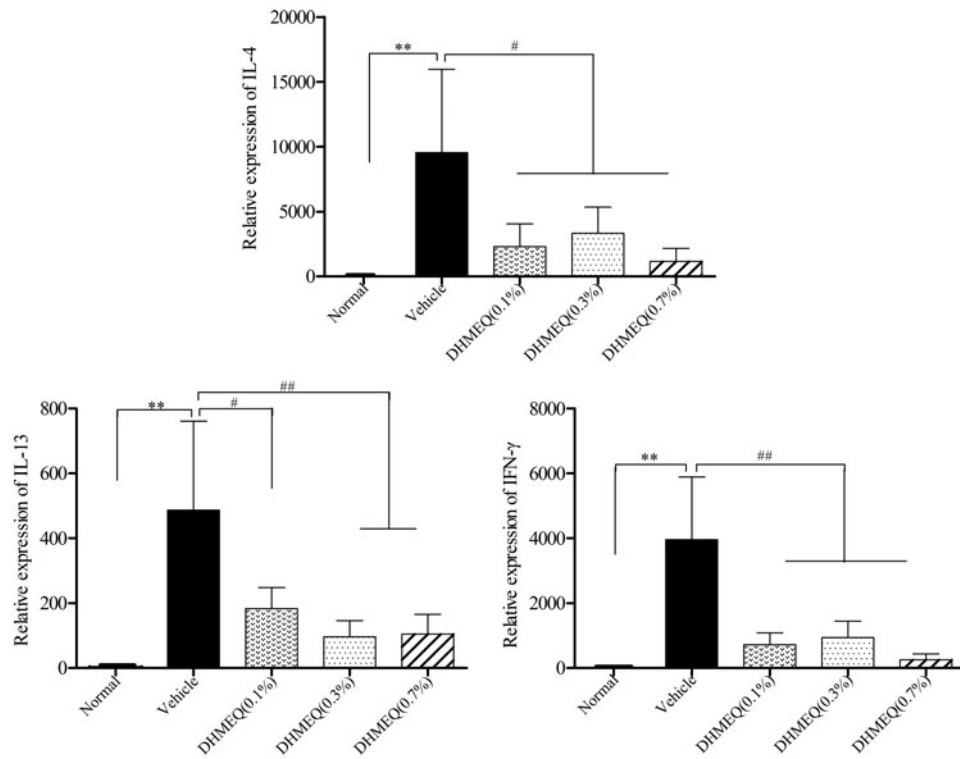


Figure 6. Suppressive effect of DHMEQ on the increased mRNA expression of IL-4, IL-13, and IFN- γ in ear tissues triggered by the modified AD-like lesion model. The ears were excised and total RNA in them was isolated on day 38. Quantitative real-time PCR was performed according to the description in the section of method. The figures showed in sequence the level of IL-4, IL-13, and IFN- γ . Data were presented as mean $\pm$ SD of triplicate determinations. ** $p < .01$, compared with the normal group; # $p < .05$ and ## $p < .01$, compared with the vehicle group.

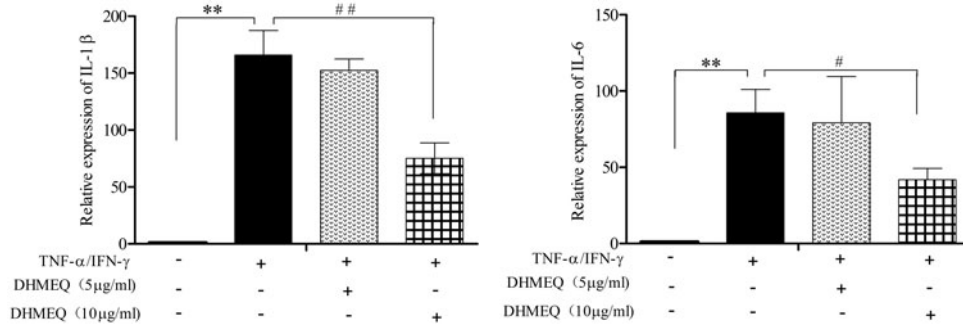


Figure 7. Effects of DHMEQ on mRNA levels of IL-1 β and IL-6 in HaCaT cells stimulated by TNF- α /IFN- γ . The cells were pretreated with DHMEQ for 6 h and then exposed to TNF- α plus IFN- γ for 24 h. mRNA expression of the cytokines in the cells were determined by real-time PCR. Data were expressed as mean $\pm$ SD. ** $p < .01$, compared with the normal group; # $p < .05$ and ## $p < .01$, compared with the vehicle group.

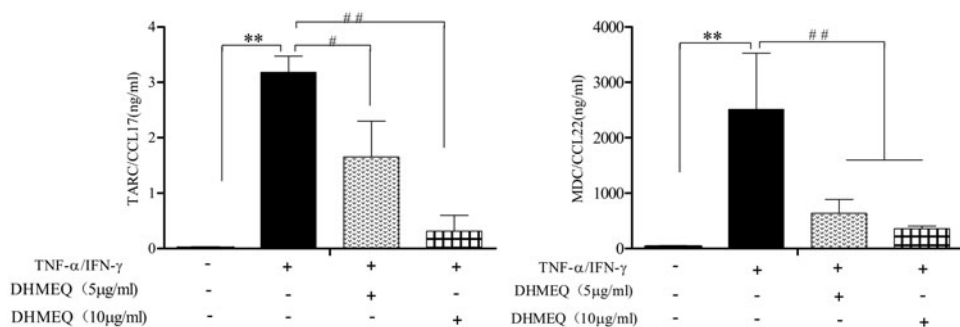


Figure 8. Effects of DHMEQ on the levels of chemokine TARC/CCL17 and MDC/CCL22 in supernatant of the culture of HaCaT cells stimulated by TNF- α /IFN- γ . The cell culture was pretreated with DHMEQ for 6 h and then exposed to TNF- α plus IFN- γ for 24 h. Levels of TARC/CCL17 and MDC/CCL22 in supernatant of the culture were respectively determined by ELISA. Data were presented as mean $\pm$ SD. ** $p < .01$, compared with the normal group; # $p < .05$ and ## $p < .01$, compared with the vehicle group.

cells were considered the main source producing a broad array of pro-inflammatory mediators. Most of these mediators usually are in store in mast cells but they are released by exocytosis when the cells are stimulated [25]. AD-like skin lesions and epidermal hyperplasia were observed in the present mouse AD-like lesion model. DHMEQ treatment significantly suppressed the increased epidermis thickness and the infiltration of eosinophils and mast cells. The infiltration of inflammatory cells is reported to be related to the production of inflammatory cytokine [26]. Deregulated Th1 and Th2 responses characterized by Th2-dominant allergic inflammation are thought to be central events in AD pathogenesis. In particular, Th2 immune responses mediated by IL-4 and IL-13 are very crucial [27]. The level of the cytokines in the current study was showed to be significantly elevated after the sustained application of tape-DNCB/OX in BALB/c mice, which came to a climax also on day 38, 1 week earlier than that in the previous study [19]. DHMEQ remarkably inhibited the expression of IL-4 and IL-13, as well as, IFN- γ , a factor derived from Th1 response, indicating the drug might suppress both Th1 and Th2 responses. However, we noted that some of the indices determined in the present *in vivo* study, for instance the ear thickness (Figure 2) and the count of the mast cells in the ear tissues (Figure 5), did not elicit clearly dose-dependent relationship. The low dose 0.1% DHMEQ elicited better effects than 0.3% and 0.7% DHMEQ. The phenomenon might largely involve the physic-chemical properties of the drug as well as the route patching the drug on the skin. Detail reasons should certainly be further investigated in future.

In order to investigate the mechanism of the effect of DHMEQ on the AD-like skin lesions, an *in vitro* AD-like lesion model in HaCaT cells [4] was also established. Relevant experiments were performed to assess the regulatory effects of DHMEQ, as a NF- κ B inhibitor, on the signaling pathways of NF- κ B in the cells. Results in the HaCaT cells stimulated with TNF- α /IFN- γ showed that the drug significantly suppressed the production and release of MDC/CCL22 and TARC/CCL17 from the cells. The mRNA levels of IL-1 β and IL-6 in the cells were also suppressed significantly. Considering that the chemokines and cytokines are crucial factors in NF- κ B pathway, it suggested that the mechanism underlying the anti-AD like lesions of DHMEQ might be involved in its suppression of NF- κ B pathway. It is reported in literature that the inhibition of production of MDC/CCL22 and TARC/CCL17 induced by TNF- α combined with IFN- γ can be abrogated by suppressing NF- κ B [4,18]. And inhibiting the expression of IL-1 β and IL-6 in HaCaT cells led to down regulation of NF- κ B pathway [28]. Jung et al found that the decreased TARC/CCL17 induced by TNF- α /IFN- γ in the HaCaT cells was achieved partially through inhibiting Raf-1, JAK2, and p38 MAPK, some upstream signaling kinases activating NF- κ B pathway [4]. Also they demonstrated that the suppression of LPS-induced expression of iNOS and cytokine genes such as IL-1 β and IL-6 in murine macrophage cells was largely related with the blocking of NF- κ B pathway [29].

Collectively, the current results demonstrate that DHMEQ suppresses the development of severe AD-like skin lesions,

the stratum corneum-removed AD-like skin lesions, in which upper epidermal layer of the skin does not exist. The severity of the modified AD-like lesion model induced with tape-DNCB/OX reaches its peak in day 38 and this modified AD-like lesion model is more compatible to be used in the investigation of DHMEQ's efficacy. Besides, it is indicated in the *in vitro* HaCaT cell AD-like lesion model that DHMEQ inhibits some key inflammatory cytokines and chemokines, which might be related to action of the drug's inhibiting NF- κ B. This study additionally provides evidences that DHMEQ acts as a potential therapeutic candidate for AD therapy, especially, being suitable for some severe AD-like skin lesions with symptoms such as erythema/hemorrhage, excoriation/erosion, eczematous lesions, and lichenification.

Disclosure statement

No potential conflict of interest was reported by the authors.

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