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Effect of dehydroepiandrosterone on atopic dermatitis-like skin lesions induced by 1-chloro-2,4-dinitrobenzene in mouse

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ABSTRACT

Background: Th2 cells are overexpressed in the skin and serum of atopic dermatitis (AD) patients. Previously, we found that dehydroepiandrosterone (DHEA) decreased eosinophil infiltration in asthmatic mice through the suppression of Th2-associated cytokines. Therefore, we hypothesized that DHEA might improve the symptoms of AD syndrome.

Objective: In this study, we evaluated the symptom improvement and anti-inflammatory response that result from the modulation of immunity by DHEA modulated in AD-like mice.

Methods: Female BALB/c mice were sensitized and challenged with 1-chloro-2,4-dinitrobenzene. On days 14–29 after sensitization, mice were treated with cutaneous (skin smear) or oral administration of DHEA. In addition, human keratinocyte (HaCat) cells were used to evaluate the effect of DHEA on the in vitro production of proinflammatory cytokines and chemokines.

Results: Both cutaneous and oral DHEA were able to decrease ear swelling and skin inflammation in AD-like mice. DHEA also attenuated eosinophil and mast cell infiltration into ear and skin tissue. Additionally, Th2-associated cytokines were inhibited in splenocyte culture, and suppressed the levels of IgE and interleukin 4 in serum. Oral and cutaneous administration of DHEA reduced the inflammatory response, as evidenced by AD-like skin lesions, in a similar manner. DHEA significantly reduced inflammatory cytokines and chemokines through the nuclear factor- κ B and mitogen-activated protein kinases pathways in tumor necrosis factor- α activated HaCat cells.

Conclusion: DHEA ameliorates AD-like mouse skin inflammation and reduces eosinophil and mast cell infiltration by reducing the production of Th2-associated cytokines and chemokines.

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1. Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease. Cutaneous symptoms of AD include erythema, itching, eczematous lesions, excoriation, edema, and thickening of the skin [1,2]. Patients with severe AD often scratch the affected area, which can cause increased fluid extravasation, exacerbate the itching and

bacterial infection [2]. AD is an allergic disease, and environmental allergenic factors such as dust mites, pollen, and animal dander may induce AD skin lesions [3]. Milk, seafood and other foods can also induce AD [4].

Recent studies have found that the overexpression of Th2 cells may cause the skin symptoms associated with AD [5,6]. Those Th2 cells might secrete more interleukin (IL)-4, and IL-5 to increase the production of IgE by B cells, as well as eosinophil and mast cell infiltration into the skin [7–9]. Therefore, reducing Th2 cell activity should ameliorate symptoms of AD.

Steroids are immunosuppressants and reduce all Th cell functions [10,11]. Most patients use a topical steroid cream, and some take oral steroids or anti-histamines to treat symptoms of AD [4,12]. However, chronic steroids use can cause thinning of the

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skin, leading to cracking and bleeding [13]. Hence, researchers are still looking for additional AD treatment alternatives.

Dehydroepiandrosterone (DHEA), a 19-carbon androstenedione, is an endogenous steroid hormone that is produced by the adrenal glands [14]. During cholesterol metabolism, DHEA is synthesized and released into the circulatory system to maintain normal physiological function [15]. In the blood, DHEA can be converted into DHEA sulfate [16]. Both DHEA and DHEA sulfate have anti-aging effects and act to regulate immune function [17,18]. After 30 years of age, serum levels of DHEA reduce gradually in humans [15,19]. Therefore, it has been suggested that DHEA supplementation in the elderly might help maintain physiological function and prevent aging. Previously, researchers found that DHEA also attenuated rheumatoid arthritis, diabetes, and hyperlipidemia in clinical trials [20–24].

Whether DHEA improves the symptoms of AD is unclear. In previous studies, DHEA attenuated asthma syndrome in ovalbumin-sensitized mice by suppressing the levels of Th2 cytokines [25]. The purpose of this research was to determine whether DHEA has a therapeutic effect in AD-like mice. In addition, DHEA was also tested *in vitro* to confirm whether it plays an important role in inhibiting the inflammatory response in keratinocytes.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All animal experiments were undertaken according to the guidelines of the Animal Care Committee of Chang Gung University of Science and Technology and Chang Gung University. All mice were housed at constant temperature and humidity, and maintained on a 12-h light-and-dark cycle at the Animal Center of Chang Gung University.

2.2. Sensitization, challenge, and drug treatment

To induce AD-like skin lesions, BALB/c mice were shaved of dorsal hair and 200 μ l 0.5% 1-chloro-2,4-dinitrobenzene (DNCB; Sigma–Aldrich, St. Louis, MO, USA) in acetone/olive oil (3:1) was applied to the shaved area on days 1–3 (Fig. 1). On days 14, 17, 20, 23, 26, and 29, mice were challenged with 200 μ l 1% DNCB on the dorsal skin and 20 μ l 1% DNCB on each ear. Mice were divided into six groups ($n = 8$ per group): (1) normal control mice (N group) were sensitized and challenged with normal saline; (2) sensitized control mice (S group) were sensitized and challenged with DNCB;

(3) experimental mice were sensitized and challenged with DNCB and topical skin treated with 50 mg/kg or 100 mg/kg DHEA (Sigma–Aldrich) (groups named A50 and A100, respectively); (4) experimental mice were sensitized and challenged with DNCB and fed 50 mg/kg or 100 mg/kg DHEA (groups named B50 and B100, respectively). All described treatments were applied during days 14–29.

2.3. Measurement of ear thickness

Twenty-four hours after the final DNCB challenge, the thicknesses of the left and right ears were measured using a dial thickness gauge (Mitutoyo, Tokyo, Japan).

2.4. Histopathological studies

After mice were sacrificed, the ears and dorsal skin were removed, fixed in 10% formalin, and embedded in paraffin. The tissues were sliced into 6- μ m sections, and slides were stained with hematoxylin and eosin (HE) as previously described [26,27]. Toluidine blue was used to stain mast cells. Each slide was deparaffinized and washed with distilled water, then toluidine blue solution was added for 2–3 min at room temperature. Each slide was washed, dehydrated, and mast cells were observed and measured in five high-power fields (HPFs) using a light microscope at 200 $\times$ magnification.

2.5. Serum collection and splenocyte cultures

Blood was collected and centrifuged at 6000 rpm for 5 min; then the serum was collected and stored at -80°C until needed. Spleen cells were aseptically removed and prepared as previously described [28]. Spleen cells (5×10^6 cells/ml) were cultured in RPMI 1640 medium (Invitrogen–Gibco™, Paisley, Scotland) containing 10% fetal bovine serum (FBS, Biological Industries, Haemek, Israel), 100 U/ml penicillin/streptomycin, and stimulated with 2 μ g/ml concanavalin A (Con A) for 2 days. Next, the supernatants were collected and frozen at -80°C until use.

2.6. Determination of cytokine, chemokine, and serum antibody levels

Cytokines and chemokines were measured using an enzyme-linked immunosorbent assay (ELISA) according to a previously described method [29]. Supernatants were measured using ELISA kits specific for interferon (IFN)- γ , IL-4, IL-5, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage-derived chemokine (MDC), thymus and activation regulated chemokine (TARC), and

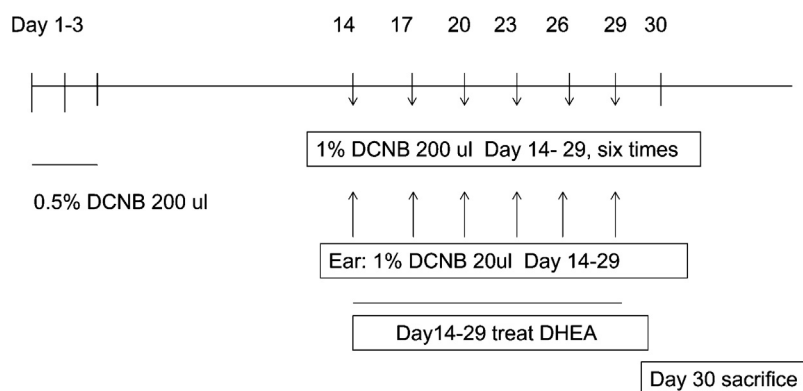


Fig. 1. A summary of the DNCB allergen sensitization and challenge experiment. Mice treated with topical 0.5% DNCB on days 1–3, and then challenged with 1% DNCB on days 14, 17, 20, 23, 26, and 29. DNCB-sensitized mice were treated with topical or oral DHEA on days 14–27. Mice were orally administered normal saline or DHEA, or topically administered DHEA on skin or ears during days 14–29. Finally, mice were sacrificed on day 30 and the skin or spleen cells were processed.

tumor necrosis factor (TNF)- α (R&D Systems, Minneapolis, MN, USA); as well as IgG1, IgG2a, and IgE (BD Biosciences, San Diego, CA, USA).

2.7. Western blotting

Proteins (20 μ g each) were separated using 10% SDS polyacrylamide gels, and electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) as previously described [30]. Membranes were blocked and incubated with primary antibodies, including antibodies raised against p65, proliferating cell nuclear antigen (PCNA), inhibitor of kappa ($\text{I}\kappa\text{B}$)- α , phosphorylated $\text{I}\kappa\text{B}$ - α (Santa Cruz, CA, USA), extracellular signal-regulated kinase 1/2 (ERK1/2), p38, c-Jun N-terminal kinase (JNK), phosphorylated ERK 1/2, phosphorylated p38, phosphorylated JNK (Millipore), intercellular adhesion molecule 1 (ICAM-1), and β -actin (Sigma–Aldrich) overnight at 4 °C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. Specific proteins were immunodetected and quantitated with Luminol/Enhancer Solution (Millipore) using the BioSpectrum 600 system (UVP, Upland, CA, USA).

2.8. Culture and treatment of human keratinocytes with DHEA

Human keratinocyte (HaCat) cells (10^6 cells/ml) were seeded on 24-well plates in serum-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen-Gibco™) overnight. DHEA (concentrations of 3–100 μ M dissolved in $\leq 0.1\%$ DMSO) were added to plates containing DMEM plus 10% FBS for 1 h. Next, 10 ng/ml TNF- α was added for 24 h (IL-8, MDC, MCP-1, and TARC assays) or 48 h (IL-6 assay). Supernatants were collected and stored at -80°C until ELISA.

2.9. Preparation of total and nuclear protein from HaCat cells

For total protein assays, HaCat cells (10^6 cells/ml) were seeded and pre-treated with or without DHEA (0–100 μ M concentration) for 1 h in 6-well plates. Cells were cultured with or without 10 ng/ml TNF- α for 24 h (to assay total protein) or 30 min (to detect protein phosphorylation) according to a previously described method [30]. Cells were lysed in 300 μ l protein lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1 mM EDTA, 0.5% NP40, and 0.1% SDS) containing protein inhibitor cocktail (Sigma–Aldrich). For nuclear protein assays, the cells were treated with DHEA and stimulated with 10 ng/ml TNF- α for 1 h. Proteins were extracted using NE-PER® nuclear and cytoplasmic extraction reagent kits (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. All protein concentrations were quantified using the BCA protein assay kit (Pierce).

2.10. Statistical analysis

All statistical analyses were determined using one-way ANOVA followed by post hoc Dunnett's test. Experimental data are presented as mean $\pm$ standard deviation (SD); $P < 0.05$ was considered statistically significant.

3. Results

3.1. DHEA inhibits DNCB-induced ear swelling and inflammatory reactions of skin

To understand the effect of DHEA on AD, we used DNCB to sensitize and induce AD-like skin inflammation in BALB/c mice. Pruritus, edema, erythema, scarring, and excoriation were clearly

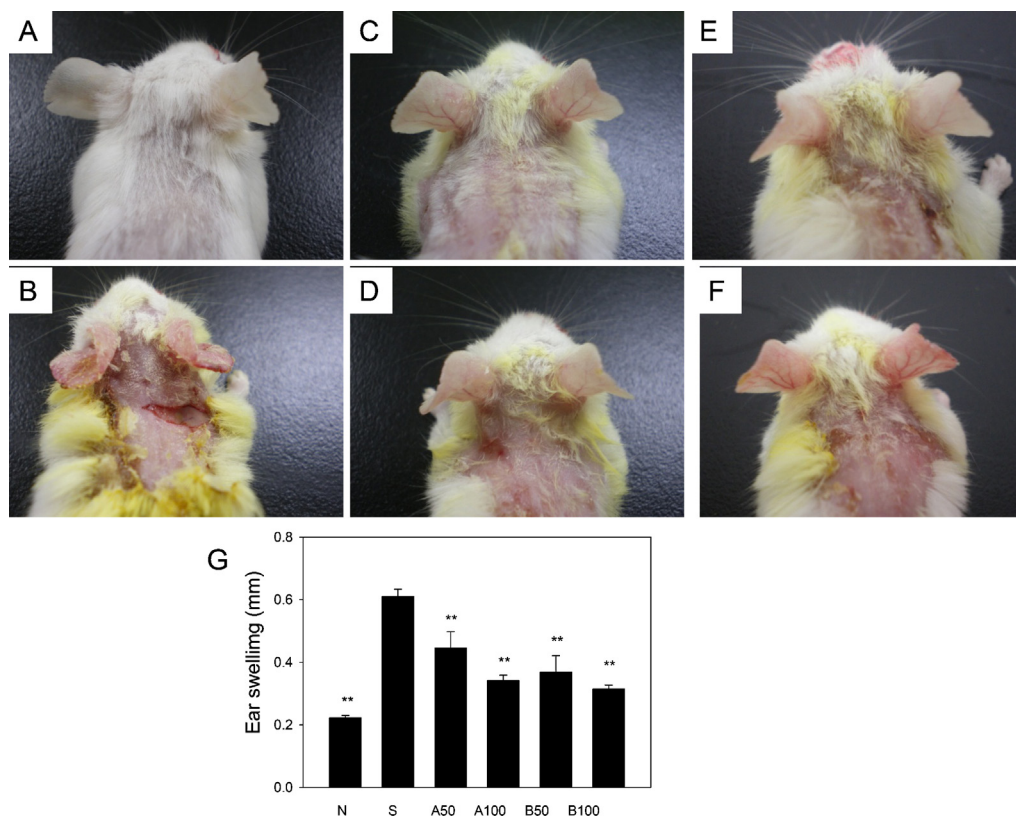


Fig. 2. Clinical features of AD-like skin lesions treated topically with DHEA in normal control mice (A); sensitized control mice (B); sensitized mice topically treated with 50 mg/kg DHEA (A50, C) or 100 mg/kg DHEA (A100, D); and mice orally treated with 50 mg/kg DHEA (B50, E) or 100 mg/kg DHEA (B100, F); $n = 8$ mice per group for all groups. Ear thicknesses were also calculated (G). Data are presented as mean $\pm$ SD. * $P < 0.05$ versus the sensitized control mice. ** $P < 0.01$ versus sensitized control mice.

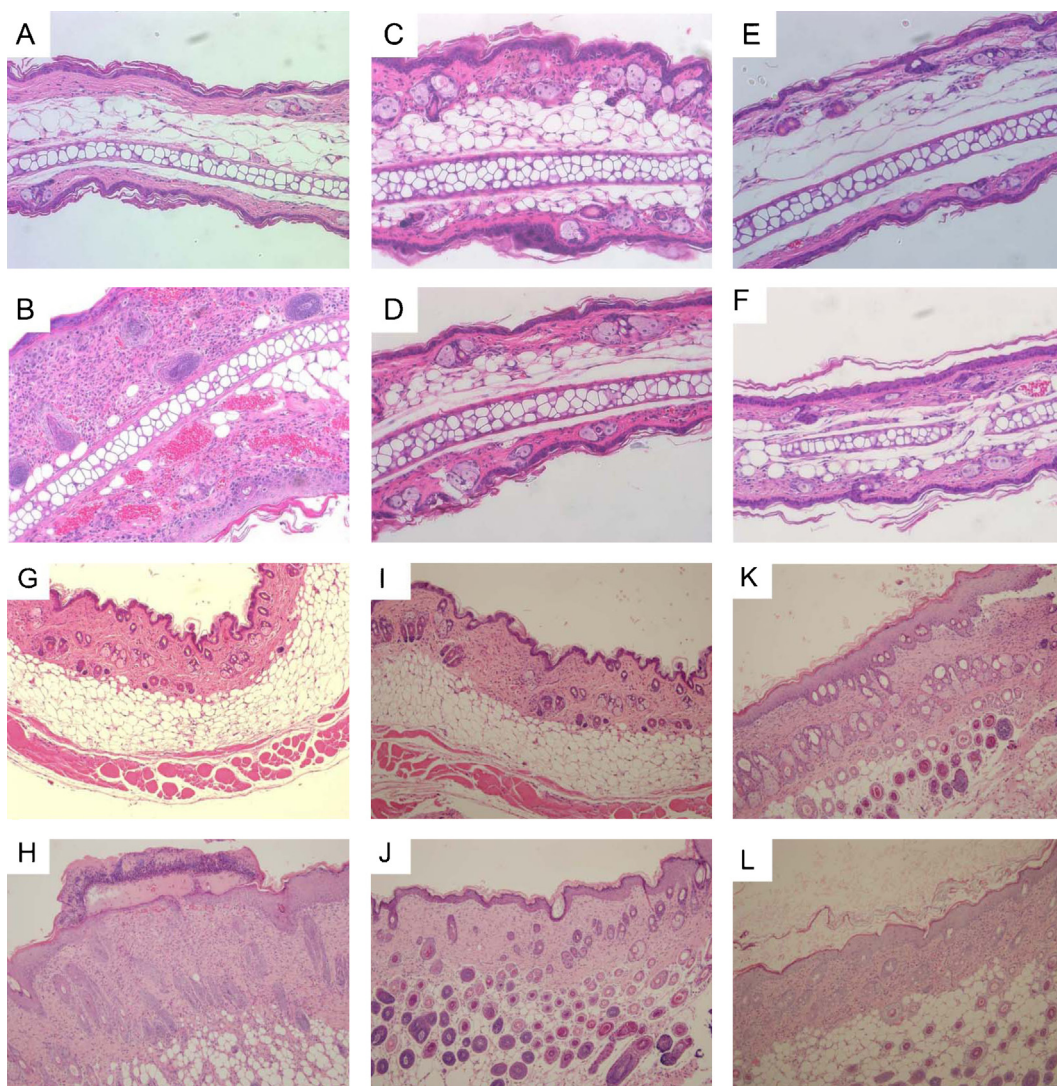


Fig. 3. The effects of DHEA on eosinophil infiltration in ears and skin. Ear sections were stained with HE and analyzed for eosinophil infiltration in normal control mice (A); sensitized control mice (B); sensitized mice topically treated with 50 mg/kg DHEA (A50, C) or 100 mg/kg DHEA (A100, D); and mice orally treated with 50 mg/kg DHEA (B50, E) or 100 mg/kg DHEA (B100, F); $n = 8$ mice per group for all groups. Skin sections were also stained with HE: normal control mice (G), sensitized control mice (H), A50 (I), A100 (J), B50 (K), and B100 (L) groups.

apparent on the skin of DNCB-sensitized mice compared with normal mice (Fig. 2A and B). Mice shaved hair twice a week, and shed their fur about days 18–24. However, the new dorsal hair and skin were relatively hard, and it was not easy to shave the hair using a knife in DNCB-sensitized mice. DNCB-sensitized mice treated with topical or oral DHEA reduced the severity of skin and ear dermatitis (Fig. 2C–F).

Ear thickness was measured the next day after the final DNCB challenge (Fig. 2G). DNCB-sensitized mice treated with different topical doses of DHEA exhibited significantly decreased ear thickness compared with DNCB-sensitized control mice (A50: 0.40 ± 0.08 mm, $P < 0.01$; A100: 0.36 ± 0.05 mm, $P < 0.01$; each vs. the S group: 0.61 ± 0.09 mm). Oral administration of DHEA also suppressed ear thickness in treated vs. sensitized control mice (B50: 0.30 ± 0.08 mm, $P < 0.01$; B100: 0.31 ± 0.06 mm, $P < 0.01$; each vs. the S group: 0.61 ± 0.09 mm). Therefore, DHEA improved the ear skin swelling. The extent of improvement did not differ significantly between topical and oral DHEA administration.

3.2. DHEA suppressed infiltration of ears and skin by eosinophils and mast cells

Ears and skin sections were stained with HE to examine eosinophil infiltration (Fig. 3). Eosinophil infiltration and the epidermal layer were decreased significantly in AD-like mice treated with DHEA, especially the A100 and B100 groups, compared with DNCB-sensitized mice. Next, toluidine blue was used to examine mast cell infiltration (Fig. 4). There was clearly more mast cell infiltration in the ears and skin of AD-like mice. Topical or oral administration of DHEA suppressed mast cell infiltration in both the ears (A50: 34.8 ± 14.8 , $P = 0.26$; A100: 14.5 ± 8.6 , $P < 0.01$; B50: 25.2 ± 12.8 , $P < 0.05$; B100: 16.7 ± 7.1 , $P < 0.01$; all vs. the S group: 48.5 ± 9.3 ; Fig. 4M) and the skin (A50: 34.8 ± 14.8 , $P = 0.26$; A100: 14.5 ± 8.6 , $P < 0.01$; B50: 25.2 ± 12.8 , $P < 0.05$; B100: 16.7 ± 7.1 , $P < 0.01$; all vs. the S group: 48.5 ± 9.3 ; Fig. 4N). Topical and oral administration of DHEA did not differ significantly in the reduction of eosinophil or mast cell infiltration.

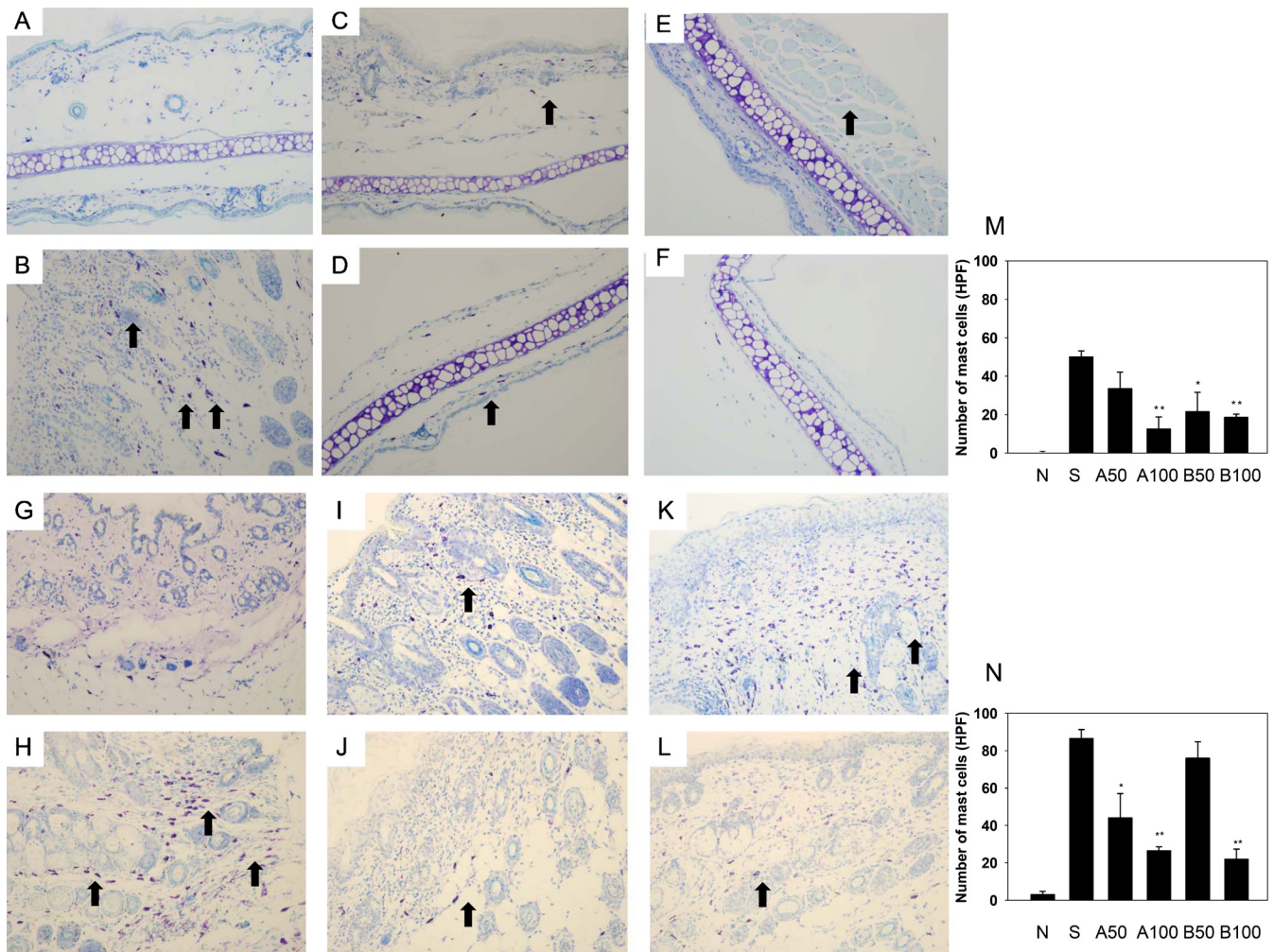


Fig. 4. The effects of DHEA on mast cell infiltration in ears and skin tissue. Ear sections were stained with toluidine blue to analyze mast cell infiltration in normal control mice (A); sensitized control mice (B); sensitized mice topically treated with 50 mg/kg DHEA (A50, C) or 100 mg/kg DHEA (A100, D); and mice orally treated with 50 mg/kg DHEA (B50, E) or 100 mg/kg DHEA (B100, F); $n = 8$ mice per group for all groups. Skin sections were also stained with toluidine blue: normal control mice (G), sensitized control mice (H), A50 (I), A100 (J), B50 (K), and B100 (L) groups. Arrows indicate mast cells. The number of mast cells decreased significantly in ear (M) and skin (N) treated with DHEA. Data are presented as mean $\pm$ SD. * $P < 0.05$ versus sensitized control mice. ** $P < 0.01$ versus sensitized control mice.

3.3. Effects of DHEA on serum IgE, TNF- α , and IL-4

DNCB-sensitized mice exhibited significantly increased IgE, IgG1, and IgG2a levels compared with the normal group (Fig. 5A). DHEA treatment significantly reduced IgE and IgG1 levels, but did not increase the IgG2a level. Treatment with DHEA (A100 and B100 groups) significantly decreased IL-4 and TNF- α levels compared with the S group (Fig. 5B).

3.4. DHEA suppressed IL-4 levels in spleen cell cultures

Spleen cells were cultured and continuously stimulated with Con A for 2 days. IFN- γ levels were increased and IL-4 levels were decreased significantly in DHEA-treated mice compared with DNCB-sensitized mice, regardless of the mode of administration (Fig. 5C).

3.5. DHEA suppressed the expression of proinflammatory cytokines and chemokines in inflammatory HaCat cells

Skin cells release chemokines and proinflammatory cytokines to induce an inflammatory response, eosinophil migration, and mast cell migration in AD patients [7]. HaCat cells were stimulated

with TNF- α to evaluate whether DHEA was able to suppress the levels of inflammatory mediators. Although DHEA suppressed levels of IL-6, IL-8, MCP-1, TARC, and MDC (Fig. 6), eotaxin was not detected (data not shown). Pre-treatment with DHEA significantly decreased ICAM-1 protein expression in TNF- α -activated HaCat cells (Fig. 7).

3.6. Inhibition of NF- κ B and MAPK pathways in TNF- α -activated HaCat cells by DHEA

TNF- α stimulated HaCat cells promoted expression of nuclear factor- κ B (NF- κ B) transcription factor in the nucleus and phosphorylation of mitogen-activated protein kinases (MAPK). Pretreatment with DHEA significantly inhibited I κ B- α phosphorylation and decreased p65 nuclear translocation. Subsequently, we evaluated whether MAPK signaling pathways were suppressed by DHEA. We observed that DHEA suppressed the TNF- α stimulated phosphorylation of ERK1/2, p38, and JNK in a significant and concentration-dependent manner. These results indicated that DHEA also significantly inhibited inflammation-associated signaling pathways, including I κ B- α phosphorylation, the nuclear translocation of NF- κ B p65, and MAPK phosphorylation (Figs. 8 and 9).

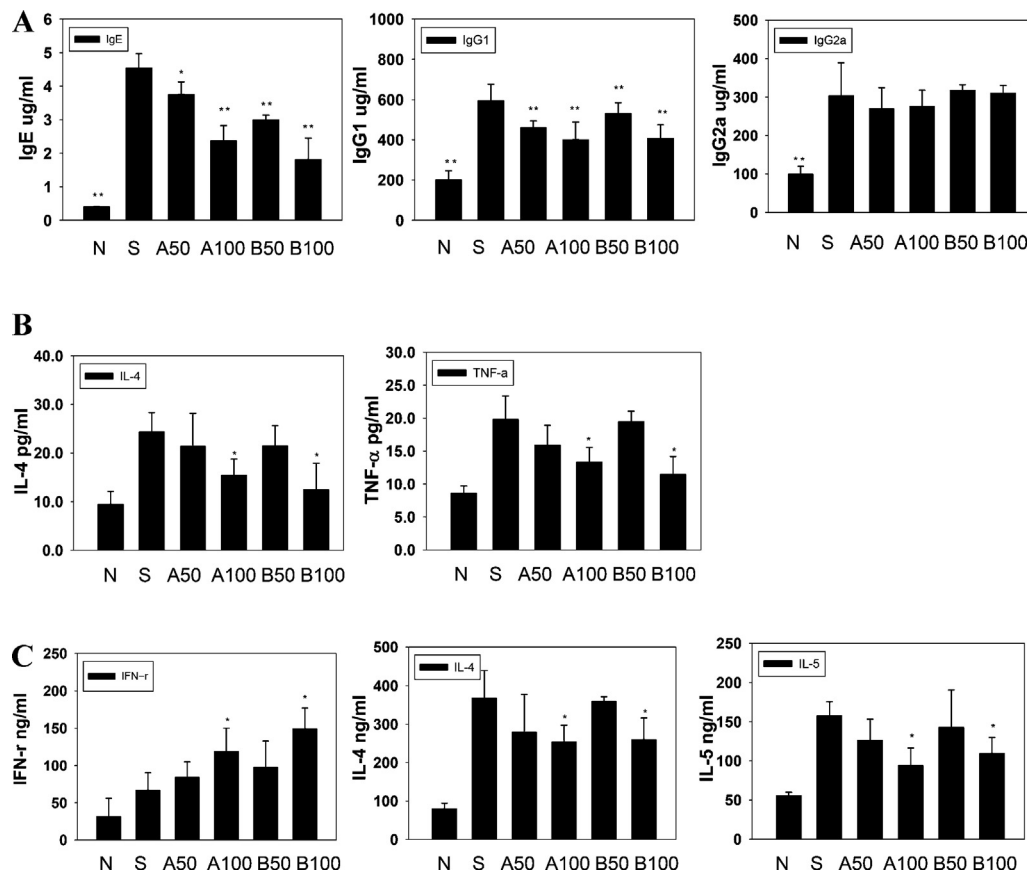


Fig. 5. Effect of DHEA on levels of serum antibodies (A), serum cytokines (B), and supernatant from Con A-activated spleen cells (C). Data are presented as mean $\pm$ SD. * $P < 0.05$ versus sensitized control mice. ** $P < 0.01$ versus sensitized control mice.

4. Discussion

AD is a chronic inflammatory allergic skin disease [4,31,32]. In most patients, AD symptoms begin before 5 years of age and continue into adulthood [33]. Sudden itchy skin can interfere with the patient's sleep, and even work [5,11]. Skin disorder of AD skin lesions includes itching, eczema, erythema, remodeling of the skin surface and thickening and hardening of the skin due to repeated inflammation. The skin also becomes dry and rough, even to the point of edema and hemorrhage [34,35].

Many researchers are searching for alternative treatments to replace steroids for AD therapy [36–39]. In the present study, we evaluated whether DHEA attenuated AD-like skin lesions in mice. The results demonstrated that oral or topical DHEA decreased skin erythema and scaling in AD-like mice, and also suppressed ear swelling. DHEA also reduced eosinophil and mast cell infiltration in the skin and ears. In addition, DHEA suppressed levels of Th2-associated cytokines and IgE. We expected that DHEA would improve AD-like skin lesions by blocking Th2 cell activity. However, topical and oral administration of DHEA did not differ significantly in their ability to suppress the dorsal skin erythema and edema, as well as other AD markers. Therefore, we suggest that both oral and topical DHEA can effectively ameliorate the AD-like skin lesions in mice.

We observed that the dermis of the ear was significantly thicker in DNCB-sensitized mice, and there was a high degree of eosinophil infiltration in ear and skin tissue. However, topical and oral DHEA can each suppress the thickening of the epidermal layer and reduce eosinophil and mast cell infiltration. IL-5 induces increased differentiation of eosinophils from bone marrow, and eotaxin increases the migration of eosinophils into the inflamed tissue [40]. We observed that DHEA was able to suppress IL-5 levels in

cultured splenocytes. However, DHEA did not inhibit serum eotaxin levels, and eotaxin was not detected in the supernatant from splenocytes or TNF- α -stimulated HaCat cells (data not shown). Therefore, we did not confirm the effect of the eotaxin in this experimental model.

ICAM-1 is also expressed in inflammatory tissue [41]. Our results demonstrated that DHEA can decrease ICAM-1 protein expression in TNF- α active HaCat cells. We hypothesized that DHEA would decrease the differentiation of eosinophils by suppressing IL-5 levels and inhibit eosinophil infiltration by suppressing ICAM-1 expression.

IL-4 is the primary cytokine produced by Th2 cells [42]. Asthma and AD can cause IL-4 over-secretion to induce B cell activity and affect a class switch from IgM to IgE [7]. Next, IgE binds with allergens and mast cells to induce mast cell degranulation and the release of inflammatory mediators [42]. Mast cell infiltration was observed in the ears and skin of sensitized AD-like mice and was reduced by treatment with DHEA. We also observed that DHEA inhibited serum IgE levels and decreased IL-4 levels in serum and spleocyte culture. These results suggest that DHEA decreases mast cell infiltration by suppressing the secretion of IL-4 by Th2 cells and the production of IgE by B cells.

The skin and serum of AD patients are known to contain proinflammatory cytokines [6,31]. Previous studies have reported high levels of TARC and MDC in the serum of AD patients [43–45]. Other studies found that proinflammatory cytokines induce TARC and MDC production in keratinocytes [46,47]. We found that DHEA suppresses the levels of TARC and MDC in TNF- α -stimulated HaCat cells. TARC and MDC are chemokines through which Th2 cell migration is related to tissue inflammation [7,44]. We believe that DHEA both suppresses Th2-associated cytokines production and reduces the levels of the chemokines TARC and MDC for improving

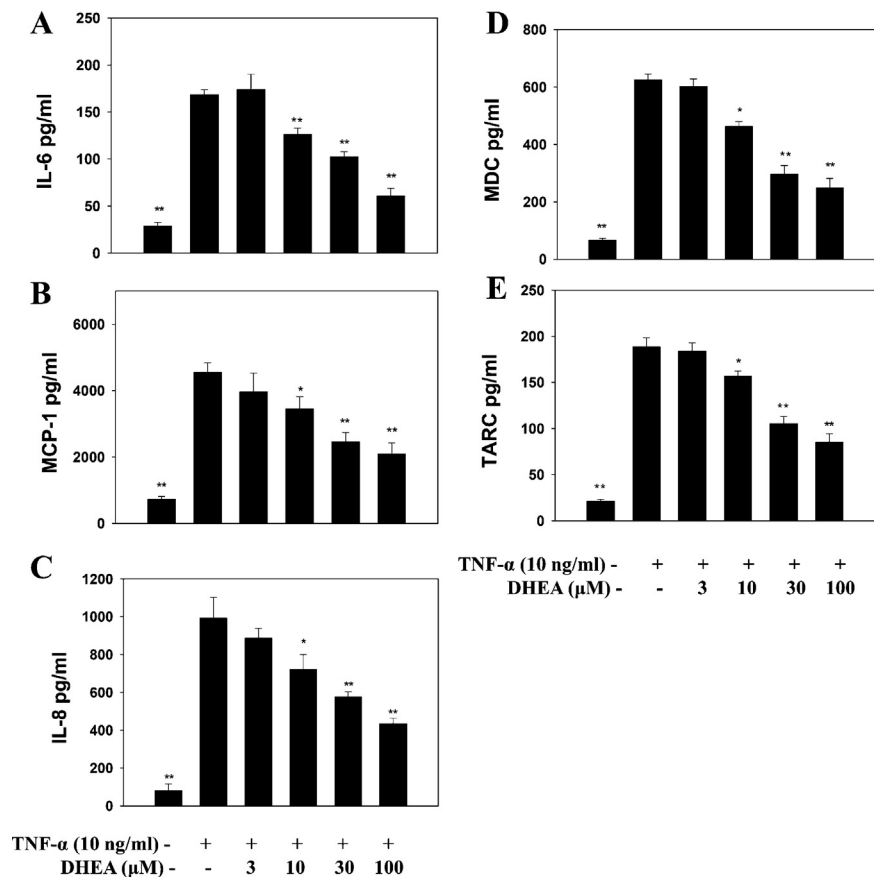


Fig. 6. DHEA reduced cytokine and chemokine levels in HaCat cells. The concentrations of IL-6 (A), MCP-1 (B), IL-8 (C), MDC (D), and TARC (E) were measured by ELISA. Data are presented as mean $\pm$ SD. * $P < 0.05$ compared to the TNF- α treated group. ** $P < 0.01$ compared to the TNF- α treated group.

the development of AD. Additionally, DHEA significantly inhibits the IL-6, IL-8, and MCP-1 levels in inflammatory HaCat cells. Previously studies have reported that NF- κ B and MAPK pathways also are activated when inflammatory cells secrete proinflammatory cytokines or chemokines [48,49]. Therefore, we suggested that DHEA can significantly inhibit the inflammatory response by blocking NF- κ B and MAPKs pathways in TNF- α -stimulated HaCat cells.

In conclusion, DHEA reduces inflammatory symptoms in AD-like mice by suppressing the production of Th2-associated and pro-inflammatory cytokines to suppress eosinophil and mast cell infiltration. However, our experimental results did not indicate whether oral or topical DHEA is more an effective treatment for AD-like mice.

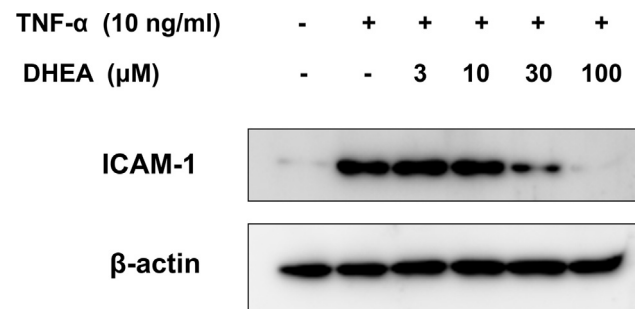


Fig. 7. Effects of DHEA on ICAM-1 protein expression in HaCat cells (10^6 cells/ml) cultured with TNF- α for 24 h. ICAM-1 protein in DHEA-treated cells was detected by Western blotting ($n = 3$ per group). β -Actin expression was used as an internal control.

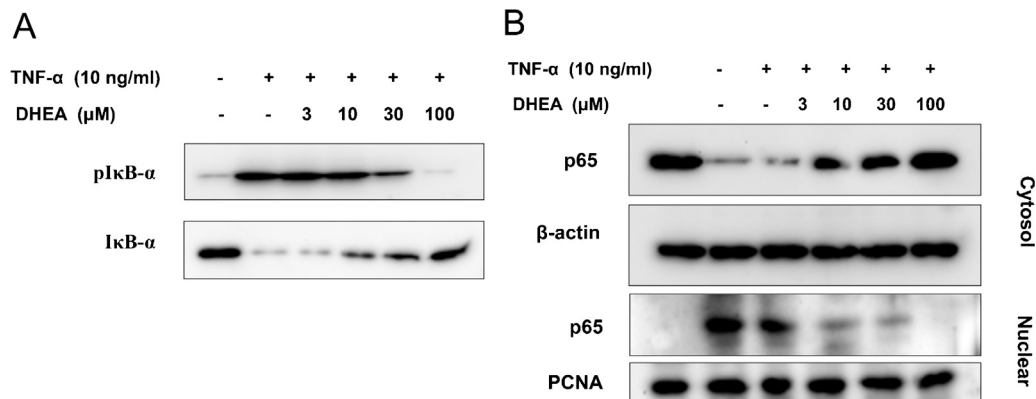


Fig. 8. Inhibitory effects of DHEA on phosphorylated I κ B- α (A) and the nuclear translocation of NF- κ B in HaCat cells (B). Cells (10^6 cells/ml) were treated with different concentrations of DHEA (3–100 μ M) for 1 h and then incubated in the presence or absence of TNF- α (10 ng/ml). Nuclear PCNA and cytosolic β -actin were used as internal controls.

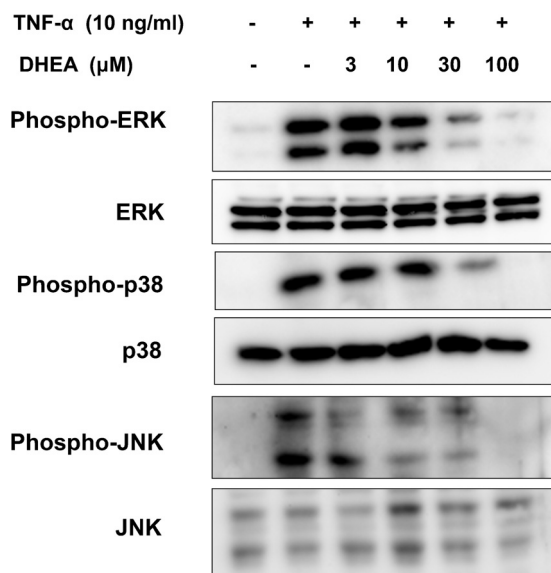


Fig. 9. Inhibitory effects of DHEA on the phosphorylation of MAPK in HaCat cells. Cells (10^6 cells/ml) were pretreated with varying concentrations of DHEA (3–100 μ M) for 1 h and then incubated in the presence or absence of TNF- α (10 ng/ml) for 30 min. Protein samples were analyzed using Western blotting with phospho-specific antibodies. Total MAPK levels were used as internal controls.

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