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Therapeutic potential of topically administered γ -AlOOH on 2,4-dinitrochlorobenzene-induced atopic dermatitis-like lesions in Balb/c mice

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Running Title: Boehmite prevent atopic dermatitis-like lesion

Abstract

Boehmite (γ -AlOOH) has a wide range of applications in a variety of industrial and biological fields. However, little is known about its potential roles in skin diseases. The current study investigated its effect on atopic dermatitis (AD). Following characterization, cytotoxicity, pro-inflammatory response, and oxidative stress associated with boehmite were assessed, using TNF- α -induced keratinocytes and mast cells. In addition, therapeutic effects of boehmite, topically administered to Balb/c mice induced by 2,4-dinitrochlorobenzene (DNCB), were evaluated. Expression of cytokines (TLSP, IL-25, and IL-33) and the generation of ROS from keratinocytes induced by TNF- α , were significantly inhibited by boehmite without affecting cell viability. MAPKs (ERK, JNK, and p38) required for cytokine expression were suppressed by boehmite treatment. Up-regulation of cytokines (TSLP, IL-4, IL-5, IL-13, RANTES) in human mast cells treated with phorbol 12-myristate 13-acetate and calcium ionophore (PMACI) was also suppressed by boehmite. Boehmite improved the AD-severity score, epidermal hyperplasia, and transepidermal water loss in DNCB-induced atopic dermatitis-like lesions. Moreover, Th2-mediated cytokine expression, mast cell

hyperplasia, and destruction of the skin barrier were improved by boehmite treatment. Overall, we demonstrated that boehmite may potentially protect against atopic dermatitis.

Keywords: Boehmite, γ -AlOOH, atopic dermatitis, skin barrier, immune response

1. Introduction

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases. Typical symptoms of AD include itching and erythema [1]. However, the main symptoms of atopic dermatitis are dry skin and pruritus, the causes of which are known to be abnormal vascular responses or immunologic abnormalities, which easily respond to external stimuli or antigens due to impairment of the barrier function of the keratin layer [2]. Allergic inflammatory diseases, such as AD, allergic rhinitis, anaphylaxis and allergic asthma, are caused by a type 2 immune response, characterized by the role of immunoglobulin E (Ig E) and T helper 2 (Th2) cells [3]. Reportedly, oxidative stress due to active oxygen is a factor which accelerates AD [4]. Oxidative stress increases superoxide radicals [O₂-] [5], production of IgE [6], and degranulation mast cells bound to IgE [7]. Histamine, which is secreted during this process, causes itching (pruritus) [8]. Patients with AD are injured by repeated scratching due to pruritus [7], which damages normal skin structure and reduces skin barrier function, thus facilitating invasion by pathogens. Increased scratching behavior of AD patients results in changes in epidermal structure, leading to lichenification, which is a thickening of the epidermis.

Currently, antihistamines, steroids, FK506, and cyclosporin are mainly used as clinical therapeutic agents for AD. However, the long-term use of these agents may not only cause serious side effects due to increased tolerance, but also make AD fundamentally impossible to treat. Therefore, the development of safe and effective treatments for AD is felt to be necessary [2].

Alumina particles are metal oxides, which are widely used in catalysts, adsorbents, ceramics, the food industry, and medicines [9-13]. Boehmite (aluminum oxide hydroxide, γ -AlOOH) is known for its enormous potential for a wide range of applications, such as pharmaceuticals, dyes, and agrochemicals among others. Many other studies are currently in progress. It exhibits low toxicity to humans and animals, stable electro-optic properties, strong adsorption capacity and high catalytic efficiency [14, 15].

Although boehmite has been widely studied for usefulness in various biological fields, its effectiveness in alleviating skin diseases has not been researched as of yet. Therefore, the objective of the current study was to investigate potential therapeutic effects of boehmite on atopic dermatitis *in vitro* as well as *in vivo*.

2. Materials and Methods

2.1. Preparation of boehmite

In order to produce monovalent aluminum hydroxide particles, aluminum particles and ultrapure water were mixed at a weight ratio of 1:3 to 1:2 to produce a suspension. The aluminum particles used had an average size of 2 μm and a purity of 99.9 % or higher. The electrical conductivity of the ultrapure water was lower than 10 $\mu\text{S}/\text{cm}$ in the pH range of 5 to 6. The suspension was autoclaved at a temperature of 190 to 260°C to initiate the hydration reaction. Following hydration, the reaction product was dried at a temperature of 150°C for approximately 3 h. Drying effectively removed residue molecules on the surface and inside of monohydric aluminum hydroxide particles, which were hydration reaction products. Drying time was reduced as the temperature increased. The monovalent aluminum hydroxide particles obtained were of an average particle size ranging from approximately 1 to 6 μm .

2.2. Characterization of boehmite

The particle size and morphology were analyzed using transmission electron microscopy (TEM; JEM-2000, JEOL, Tokyo, Japan) and scanning electron microscopy (FE-SEM; JSM-7610,

JEOL). Thermogravimetric analysis/differential scanning calorimetry (TGA/DSC) was performed at 5°C/min, air atmosphere, and 25–1000°C in a thermogravimetric analyzer (TGA/DSC-1. Mettler-Toledo corp., Zurich, Switzerland).

2.3. Cell culture and animals

HaCaT keratinocytes and human mast cells (HMC-1) were incubated in a 37 °C, 5 % CO₂ incubator with 10 % FBS (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) and Dulbecco's Modified Eagle's Medium (DMEM, Gibco; Thermo Fisher Scientific Inc.) containing 1 % penicillin/streptomycin (Gibco; Thermo Fisher Scientific Inc.).

Six-week-old male BALB/c mice were supplied by RaonBio, Inc. (Yongin, Korea). Solid feed (no antibiotics added) and water were supplied until the day of the experiment, and the animals were used for experiments following a 1-week adaptation period at temperatures of 23±2°C, humidity of 55±10 %, and a 12-12 h light-dark cycle. All animal experiments were conducted in accordance with the principles of laboratory animal care of the National Institutes of Health (NIH, Bethesda, Maryland, USA) and with the approval of the Ethics Committee for Laboratory Animals at Chung-Ang University (201800048).

2.4. Induction of atopic dermatitis model

Repeated application of 2,4-dinitrochlorobenzene (DNCB, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) to a local site causes AD-like skin lesions. In the first inflammation induction process, 100 µL of 1 % DNCB solution (acetone:olive oil = 4:1) was applied to the same dorsal area twice for 3 days in all groups, except in the normal group (NOR). The vehicle (acetone:olive oil = 4:1) was used to treat the NOR group. Following a latent period of 4 days, 100 µL of 0.4 % DNCB was used for a second sensitization. Meanwhile, 4 h before the DNCB treatment, 0 and 5 % cream (200 µg) were applied to the boehmite group, and 100 µM dexamethasone (DEX, Sigma-Aldrich) (200 µL) was applied to the DEX group. Boehmite used in this study was manufactured by Osang Jaiel Co., Ltd. (Incheon, Korea)

2.5. Measurement of ROS production

Levels of intracellular hydrogen peroxide and mitochondrial ROS were determined using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and MitoSOX™ (Invitrogen), respectively. Cells were treated with H₂O₂ and boehmite and incubated with H₂DCFDA and MitoSOX™ for 30 min at 37°C in 5 % CO₂. Cells were washed twice with PBS and analyzed using a Spectra Max i3x Multi-Mode Detection Platform (Molecular Devices). Fluorescence was evaluated prior to the addition of each fluorescent probe, since autofluorescence at near-UV wavelengths may be intense.

2.6. Measurement of AD severity score and epidermal thickness

AD severity and epidermal thickness were measured as described previously [16]. Clinical symptoms were photographed using a digital camera (Canon 3000D, Canon, Tokyo, Japan). Epidermal thickness of 10 randomly selected areas in 3 sections of each mouse was measured using a microscope aided by hematoxylin and eosin (H&E) staining and ImageJ version 1.51 (NIH) (DM750, Leica, Wetzlar, Germany). Assessments were performed in a blinded manner.

2.7. Measurement of total serum IgE and inflammatory cytokines

Blood collected from mice was placed in an e-tube and centrifuged at 4°C and 3000 rpm for 30 min. Following centrifugation, supernatant was stored at -70°C until measurement. Serum IgE was measured using an enzyme-linked immunosorbent assay (ELISA). Each sample was added onto a 96-well plate treated with the primary antibody using a mouse IgE ELISA Kit (Invitrogen), and washed 4 times with the working solution. After washing, the samples were treated with the secondary antibody, HRP-conjugated goat anti mouse IgE (1:10,000), for 1 hour after which a color development reagent was used. Following color development, the reaction was stopped using a stop solution, and absorbance was measured at 450 nm via a Max i3x Multi-Mode Detection Platform (Molecular Devices). Serum levels of the cytokines interleukin (IL)-1 β , IL-4, IL-5 and, CCL5 (regulated on activation, normal T-cell expressed and secreted, RANTES) were also measured using ELISA kits (R&D Systems, Minneapolis, MN, USA).

2.8. Histological examination

Each tissue slide was prepared as described previously [16]. Staining was carried out via H&E and toluidine blue, followed by immunohistochemical staining with anti-PCNA (1: 1000, ab15497, Abcam), anti-filaggrin (1:500, b24584, Abcam) and anti-loricrin (1:500, ab85679, Abcam) antibodies. Staining was performed using an Ultravision Quanto Detection System (TL-060-QHD; Thermo Fisher Scientific). After staining, tissues were dehydrated and sealed in Permount (SP15-100, Thermo Fisher Scientific), and observed via optical microscopy (DM750, Leica, Wetzlar, Germany).

2.9. RNA extraction and real-time PCR

Total RNA was extracted from isolated skin tissue and cultured cells using Tri-RNA reagent (Favorgen Biotech, Taiwan). Single strand cDNA synthesis from whole RNA templates was performed with PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). Resulting cDNA was subjected to real-time PCR with the CFX96 (Bio-Rad, Hercules, CA, USA) and qPCR 2× PreMIX SYBR (Enzynomics, Seoul, Korea). PCR used to amplify all genes was performed for 40 cycles (95°C for 10 min; 60°C for 15 s; and 72°C for 30 s) following the denaturation process at 95°C for 10 min. Expression data were calculated as a cycle threshold (Ct) value using the Δ Ct quantification method normalized to GAPDH. Oligonucleotides used for real-time PCR are listed (Supplementary Table 1).

2.10. Western blot analysis

Cells and dorsal tissues were collected and lysed in PRO-PREP (iNtRON, Seongnam, Korea) containing a protease inhibitor cocktail (Complete™; Roche, Mannheim, Germany). Amounts of protein in the lysates were quantitated using a BCA kit (Thermo Fisher Scientific). Following quantitation, equal amounts of protein were resolved on 10 % SDS-PAGE gels and electrotransferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5 % skim milk in Tris-buffered saline containing 0.5 % Tween-20 (TBST), membranes were probed with anti-filaggrin (1:2500, ab24584; Abcam, Cambridge, UK), anti-involucrin (1:2500, ab68; Abcam), anti-loricrin (1:2500, ab85679; Abcam), anti-phospho-extracellular signal-regulated kinases (ERK)

(1:2500, #9101; Cell Signaling Technology, Inc., Denver, MA, USA), anti-ERK (1:2500, #9102; Cell Signaling), anti-phospho-p38 (1:2500, #4511; Cell Signaling), p38 (1:2500, #9212; Cell Signaling), anti-c-Jun NH2-terminal kinases (JNK) (1:2500, #9252; Cell Signaling), phospho-JNK (1:2500, #9251; Cell Signaling) and anti- β -actin (1:1000, sc-47778; Santa Cruz Biotechnology) antibodies at 4°C overnight. After washing, membranes were incubated with HRP-conjugated anti-mouse (1:10,000, PI-2000; Vector Labs Inc., Burlingame, CA, USA) or anti-rabbit (1:10,000, PI-1000; Vector Labs Inc.) secondary antibodies. Immunoreactive signals were detected using enhanced chemiluminescence (ECL) reagents (EzWestLumi plus; ATTO, Tokyo, Japan)

2.11. Statistical Analysis

Results were expressed as mean $\pm$ standard error of the mean (SEM), and mean differences were analyzed using one-way analysis of variance (ANOVA). Tukey's HSD was used for post-hoc tests. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Boehmite suppresses up-regulation of AD-like inflammation gene expression and ROS in keratinocytes.

Boehmites were agglomerated into a spherical particle of 3 to 5 μm , a crystal size of approximately 40 nm as observed via SEM and TEM images (Figs. 1A and B). TGA and DSC curves of boehmite prepared under hydrothermal conditions are shown (Fig. 1C). Water adsorbed on the surfaces of particles was 1 %, primary crystal water condensation was 13 %, the secondary crystal water condensation was 1.4 % and total primary and secondary crystal water was 14.4 %.

The role of boehmite in the regulation of TNF-derived cytokine expression in HaCaT keratinocytes was investigated first. We found that boehmite was not toxic to HaCaT keratinocyte following a 72 h exposure at concentrations ranging from 0.01 to 100 $\mu\text{g/mL}$ (Fig. 1D). The expression of AD-related inflammatory cytokines, TSLP, IL-25, and IL-33, under inflammatory condition induced by TNF- α

increased more than twice. However, the expression of TSLP, IL-25 and IL-33 decreased following administration of DEX or boehmite. (Fig. 1E). To clarify mechanisms underlying boehmite-induced reduction of cytokine expression, phosphorylation of MAPKs, which are typical inflammatory signaling mechanisms, was examined in HaCaT keratinocytes treated with TNF- α . Consistent with previous reports [17, 18], phosphorylation of JNK, ERK and p38 by TNF- α was increased. However, boehmite treatment inhibited phosphorylation of JNK, ERK, and p38 (Fig. 1F). Thus, boehmite inhibited the expression of inflammatory cytokines in HaCaT cells, and exhibited anti-inflammatory effects by inhibiting the activities of JNK, ERK, and p38 under inflammatory conditions induced by TNF- α .

To assess the possible role of boehmite in TNF- α -induced ROS generation, we evaluated fluorescence of H2DCFDA and MitoSOX as a measure of cytosolic and mitochondrial ROS. Production of cytosolic and mitochondrial ROS was significantly increased by 93 % and 25 %, respectively, in the TNF- α -treated group compared to the normal group (Figs. 1G and H). However, enhanced cytosolic and mitochondrial ROS production by TNF- α was reduced by 37 – 56 and 47 – 93 %, respectively, by boehmite compared to the TNF- α -treated group (Figs. 2A and B). Boehmite significantly inhibited the production of TNF- α -induced cytokines and ROS, thereby suggesting that the use of an appropriate concentration of boehmite may have excellent anti-inflammatory and antioxidant effects on human skin.

3.2. Boehmite leads to reduction of cytokine expression in human mast cells

Next, the effect of boehmite in human mast cells (HMC-1) which are involved in allergic inflammatory reactions, was analyzed [19]. Inflammation was induced in HMC-1 cells using PMACI (PMA 25 nM + A23187 1 μ M) and the expression of several cytokines and chemokines was evaluated. Expression of TSLP, IL-5, IL-13, and RANTES (CCL5) was significantly reduced by administration of boehmite in a concentration-dependent manner (Fig. 2A). In addition, enhanced secretion of TSLP, IL-5, IL-13 and TNF- α by PMACI was also reduced by DEX- and boehmite administration (Fig. 2B). We also investigated phosphorylation of MAPKs which are important mediators of mast cell response to extracellular signals [20, 21]. Boehmite markedly inhibited PMACI-induced phosphorylation of all

three MAPKs, ERK, JNK and p38, in a concentration-dependent manner (Fig. 2C). These results suggest that boehmite may inhibit degranulation as well as cytokines production in mast cells by inhibiting PMACI-induced phosphorylation of JNK, ERK and p38.

3.3. Boehmite exerts a protective effect against DNCB-induced atopic dermatitis-like lesions.

Clinical characteristics of AD, such as skin lesion shape and skin thickness, improved following boehmite administration (Fig. 3A). Dermatological symptoms, such as dryness, erythema, erosion, edema/excoriation, and lichenification, accompanied by skin barrier destruction, inflammation and epidermal thickness, became severe in mice induced by DNCB compared to normal mice. However, these symptoms improved in the 5 % boehmite-treated mice (Fig. 3A). Dermal histological characteristics were also improved, compared to DNCB-treated mice (Fig. 3B). In general, hyperkeratosis and parakeratosis were reduced and epidermal hyperplasia (PCNA staining, Fig. 3C) and spongiosis were also reduced, as epidermal thickness decreased (Figs. 3B and G). In addition, scratching was relatively decreased in 5% boehmite-administered mice, compared to the DNCB and 0 % boehmite groups, indicating that dermatitis was significantly decreased (Fig. 3H).

Next, we evaluated skin barrier function including the expression of skin barrier proteins and transepidermal water loss (TEWL). The expression of filaggrin, involucrin, and loricrin markedly increased as DNCB-induced atopic dermatitis-like lesions, but significantly improved following treatment with 5 % boehmite (Figs. 3D, E, J, and K). Moreover, treatment with 5 % boehmite as well as DEX improved dryness (Fig. 3I). Collectively, these results indicate that boehmite may be effective in protecting the skin barrier.

3.4. Boehmite inhibits atopic dermatitis-like skin inflammation

To determine whether boehmite reduces skin inflammation, we investigated mast cell infiltration and cytokines expression. Toluidine blue staining indicated that mast cells around the dermis were heavily infiltrated by DNCB treatment. However, 5 % boehmite-administered mice showed a decrease in mast cell infiltration, as compared to the DNCB and 0 % boehmite groups (Figs. 4A and B). The serum total IgE level (322.9 ng/mL) induced by DNCB was significantly reduced to

175.9 and 154.2 ng/mL by treatment with DEX and 5 % boehmite, respectively (Fig. 4C). In addition, enhanced expression of IL-4, IL-13, and Th2 differentiation-inducing cytokines, such as TSLP and IL-33, was decreased by approximately 46.8, 47.3, 53.1, and 49.2 % respectively, where these decreases were all significant. The DEX group, which was the positive control group, also showed a decrease of 33.6, 35.4, 45.8 and 57.9 % for IL-4, IL-13, TSLP and IL-33, respectively (Fig. 4D). Furthermore, inflammatory cytokines present in the serum, IL-1 β , IL-4, IL-5 and RANTES, were significantly reduced by 5 % boehmite (Fig. 4E). Therefore, 5% boehmite may inhibit the expression of Th2 cell-associated cytokines and mitigate symptoms of AD via the inhibition of mast cell infiltration and IgE production.

4. Discussion

Nanosized boehmite particles are most important nanomaterials that are much in demand for use in a wide range of industrial applications including pharmaceuticals, biotechnology and adjuvants in human vaccines [22, 23]. However, the potential toxicity of boehmite nanoparticles is a concern, both at the workplace as well as for product users. Boehmite nanoparticles may induce cytotoxicity and inflammatory responses in Raw 264.7 and A549 cell lines [24]. Moreover, boehmite cytoplasmic inclusions were present in macrophages of lesions associated with an inflammatory myopathic disease related to a boehmite-containing vaccine [25]. Recent reports have indicated that alumina microparticles are much less toxic than nanoparticles [26], and that an increase in specific surface area involves increase in toxicity [27]. Therefore, we produced micro-sized boehmite particles with small specific surface areas. As a result, no toxicity was observed in keratinocytes and human mast cells treated with boehmite microparticles. Furthermore, the expression of inflammatory cytokines induced by TNF- α or PMACI was inhibited in groups treated with boehmite in keratinocytes or mast cells, via suppression of phosphorylation of MAPKs, such as ERK, JNK, and p38. TSLP is an initial stimulant of skin immune response occurring in keratinocytes or mast cells, and recent studies have suggested that TSLP may directly stimulate nerve cells which modulate itching or scratching [28]. TSLP receptors are present in the C-fibers of primary sensory afferent neurons of the skin, and TSLP,

secreted from keratinocytes, sensitizes C-fibers, enabling recognition of pathological itching [28, 29]. IL-33 activates mast cells and basophils, and interacts with IL-25 to activate mucosal innate lymphoid cells. In addition, previous studies have shown that MAPK signaling is activated by IL-33 exposure [30]. Regulation of TSLP expression was mediated by MAPK signaling [31]. Inflammatory gene expression by TSLP in the human airway smooth muscle was induced by MAPKs and STAT3 activation [32]. Moreover, the amount of ROS produced following treatment with TNF- α in HaCaT cells was significantly decreased by boehmite treatment in a concentration-dependent manner. ROS induces inflammation via activation of various signaling mechanisms [33, 34]. Boehmite may have excellent anti-inflammatory and antioxidant effects on human skin.

Transdermal delivery efficiency of FITC-conjugated boehmite (boehmite-FITC) in isolated human skin tissue was determined. We applied 200 μ g of 5 % boehmite-FITC on human skin tissue for 12 h using FranzTM diffusion cell. Boehmite-FITC signals were observed in the human epidermis as well as in the dermis. However, the FITC signal without boehmite was observed almost at the skin surface (Fig. 4F). It is expected that boehmite in microparticles will form a skin barrier as opposed to skin penetration and thereby protect skin damage. Nonetheless, these results demonstrate the possibility that boehmite may partially penetrate the epidermis of mice and humans and thus affect immune cells. In this study, boehmite was administered to mouse AD models as a skin application in order to evaluate its effect. In comparison with the groups treated only with DNCB, the boehmite groups showed low AD severity scores and IgE levels for AD-like lesions. It has been reported that an increase in serum IgE levels occurred in up to 70–80% of patients with AD. IgE not only promotes the release of acute and chronic inflammatory mediators by binding to the Fc ϵ receptor I (Fc ϵ RI) present on the surface of mast cells and basophils, but also promotes the activation of memory Th2 cells in AD by binding to the surface of dendritic cells and capturing allergens [35, 36]. Moreover, serum levels of IL-1 β , IL-4, IL-5 and RANTES significantly decreased. Th2 differentiation plays an important role in the inflammatory response, and IL-4 overexpressed transgenic mice exhibited dry skin, inflammatory skin lesions, scratching behavior, skin infection and elevated IgE levels [37]. IL-5

modulates the viability and activity of eosinophils, and increases eosinophilia and invasion into the skin [38]. In AD, RANTES was involved in the migration and activation of eosinophils and T cells, and caused inflammation by inducing lymphocyte adhesion to the surface of endothelial cells [39].

In addition, the expression levels of filaggrin, involucrin, and loricrin were significantly increased by boehmite. Eventually, a decrease in filaggrin and an increase in cytokines worsens AD. By contrast, cytokines cause a decrease in filaggrin in allergic diseases. Reportedly, TSLP downregulates filaggrin expression in keratinocytes via STAT3- and ERK-dependent pathways [40]. Consequently changes in the expression of skin barrier proteins in AD may have important implications, and boehmite may be a most important and effective agents for restoring and maintaining the skin barrier, which may aid in treating and managing AD patients.

In conclusion, this study investigated the effect of boehmite on AD *in vitro* and *in vivo*. Boehmite is not toxic to keratinocytes and mast cells responsible for skin immunity, and exerts anti-inflammatory and antioxidant effects by inhibiting cytokine production, MAPKs phosphorylation and ROS production. Boehmite may play a significant role in preventing or alleviating the inflammatory reactions of AD-like lesions in a pathological pattern due to internal or external causes. In addition, boehmite was found to be effective in the treatment of AD-like lesions. Therefore, the potential of boehmite as a therapeutic agent for treatment of AD patient should be further investigated.

Acknowledgments

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Conflict of interest

The authors declare no competing interests.

Author contribution

B.J.K., J.N., and D.H.B designed research; D.H.B., B.C.L., E.L., and J.N. conducted research; B.J.K., J.N., D.H.B., M.J.C., T.R.K., J.H., S.K.M., K.L., and S.K. analyzed data; B.J.K., J.N., and D.H.B. prepared figures; B.J.K. and J.N. wrote the paper. B.J.K. and J.N. had primary responsibility for final content. All authors read and approved the final manuscript.

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Figure legends

Figure 1. Boehmite suppresses inflammation and ROS by TNF- α in HaCaT keratinocytes.

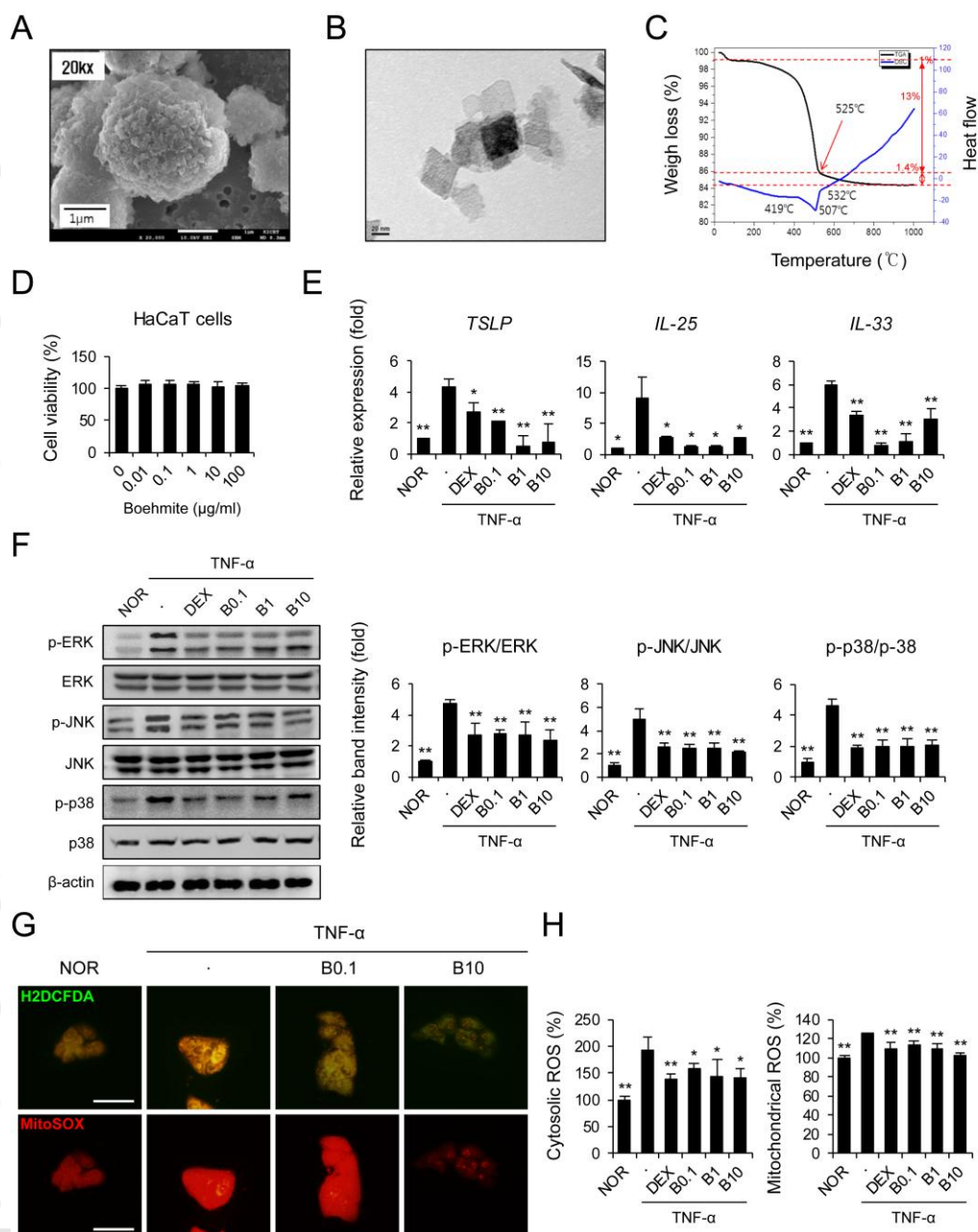
Morphology of boehmite powder observed by scanning electron microscopy (A) and transmission electron microscopy (B). (C) Thermogravimetric analysis/differential scanning calorimetry curves of boehmite powder were measured. (D) Boehmite was not cytotoxic to HaCaT keratinocytes during a 72 h period at concentrations ranging from 0.01 $\mu\text{g/ml}$ to 100 $\mu\text{g/mL}$. (E-H) Following pretreatment with dexamethasone (DEX, 10 μM) and boehmite (B0.1, B1, and B10 indicate 0.1, 1, and 10 $\mu\text{g/mL}$, respectively) for 2 h, the cells were treated with TNF- α (50 ng/mL) for 4 h or 24 h. (E) Transcripts of TSLP, IL-25, and IL-33 were quantified using real-time PCR. (F) Boehmite treatment inhibited TNF- α -induced phosphorylation of JNK, ERK and p38 in HaCaT keratinocytes. The lysates were immunoblotted with anti-p-JNK, anti-JNK, anti-p-ERK, anti-ERK, anti-p-p38 or -p38 antibodies. Western blots were analyzed quantitatively. The expression levels of H2DCFDA and MitoSOX Red were compared via fluorescence microscopy and microplate assays. (G) Typical images of cytosolic ROS (green fluorescence) and mitochondrial ROS (red fluorescence) were captured by fluorescence microscope. scale bar, 50 μm , (H) Cytosolic and mitochondrial ROS generation were examined using a microplate reader. Data are presented as mean $\pm$ SEM; * = $p < 0.05$, ** = $p < 0.01$ vs. negative control, TNF- α).

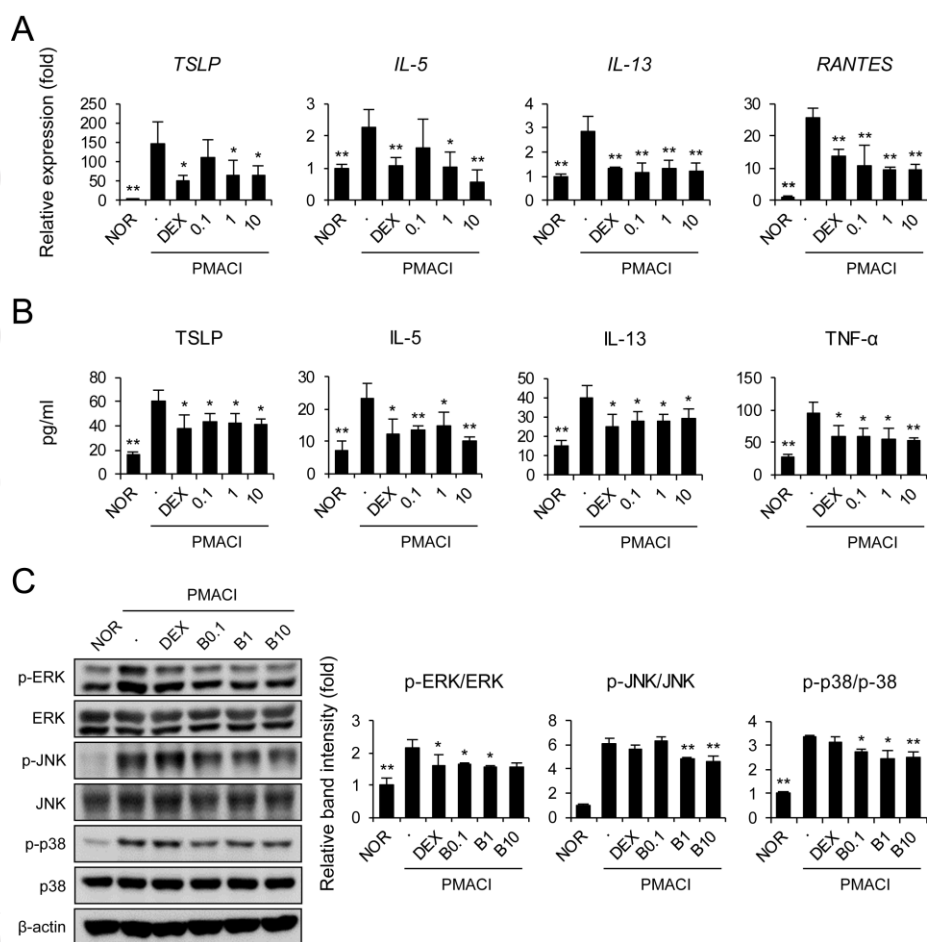
Figure 2. Boehmite attenuates inflammation by PMA and calcium ionophore in human mast cells.

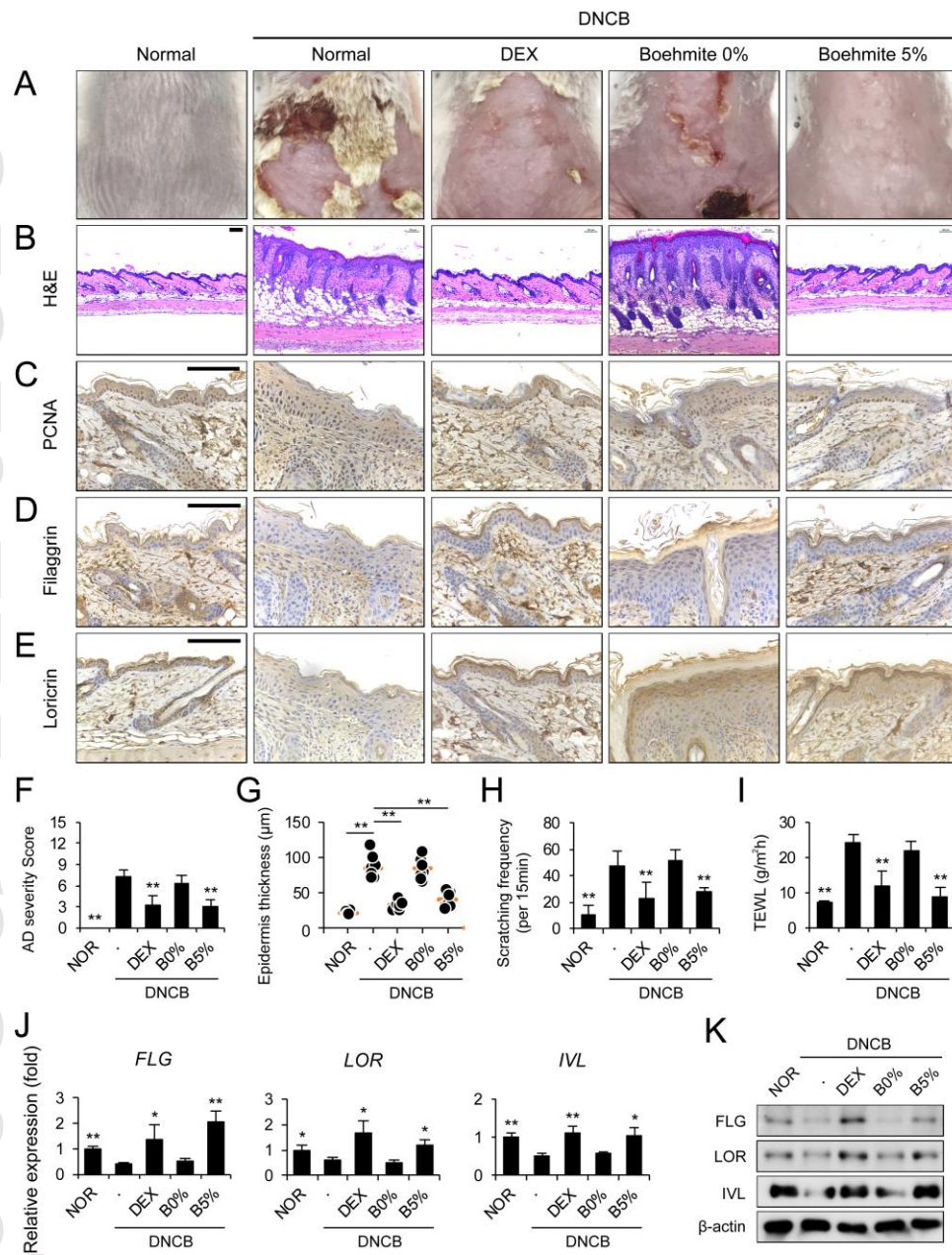
Following pretreatment by dexamethasone (10 μM) and boehmite (0.1, 1, and 10 $\mu\text{g/mL}$) for 2 h, the cells were treated with PMACI (PMA: 25 nM; A23187: 1 μM) for 4 h or 12 h. (A) Transcripts of TSLP, IL-5, IL-13, and RANTES were quantified using real-time PCR. (B) Cytokine secretion of TSLP, IL-5, IL-13, and TNF- α were determined using ELISA in HMC-1 cells. (C) Boehmite treatment inhibited PMACI-induced phosphorylation of JNK, ERK and p38 in HMC-1 cells. Lysates were immunoblotted with anti-p-JNK, anti-JNK, anti-p-ERK, anti-ERK, anti-p-p38 or anti-p38 antibodies. Western blots were analyzed quantitatively. Data are presented as mean $\pm$ SEM; * = $p < 0.05$, ** = $p < 0.01$ vs. negative control, PMACI.

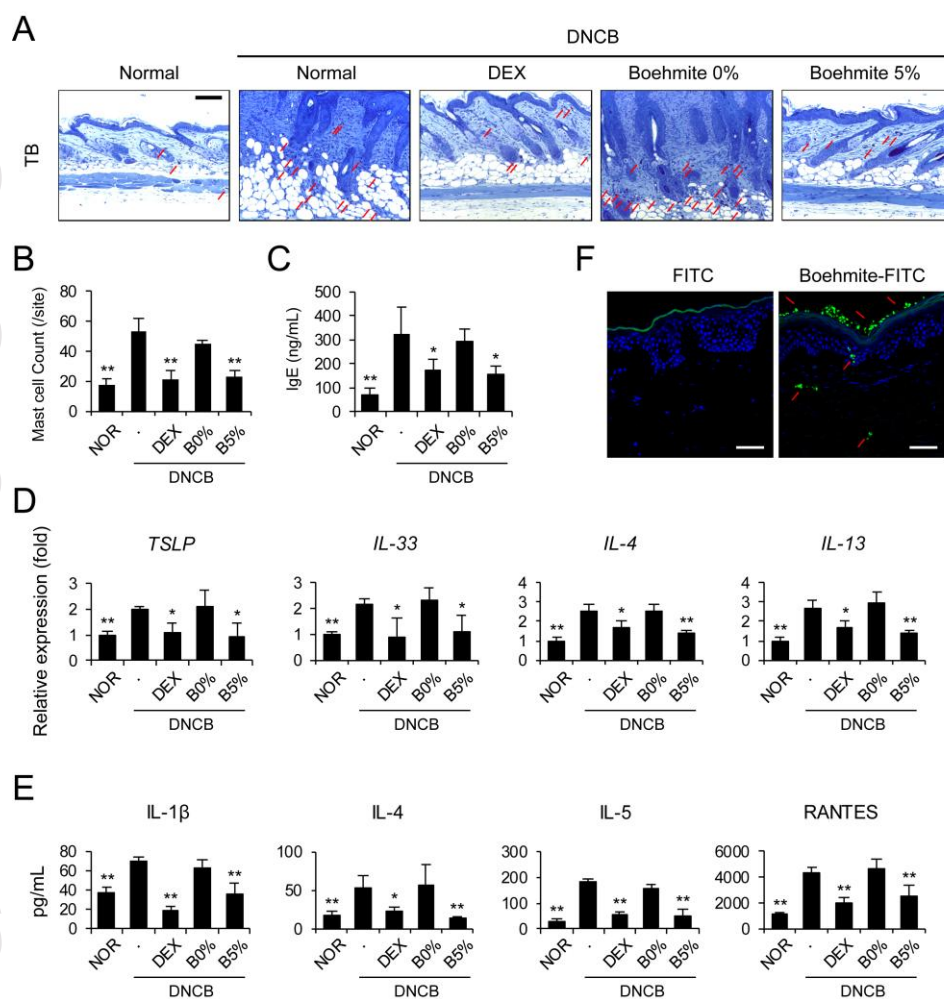
Figure 3. Boehmite alleviates dermatitis and enhances skin barrier repair in 2,4-dinitrochlorobenzene (DNCB)-induced atopic dermatitis-like skin lesions in mice. Boehmite administration alleviated dermatitis induced by DNCB treatment. While DNCB was administered twice weekly for 3 weeks on the dorsal area of the mouse, dexamethasone (10 μ M) and 5% boehmite was topically administered daily. (A) Photographs of skin lesions on the dorsal area of the mouse. (B) Histopathological variation due to DNCB induction was evaluated using hematoxylin and eosin staining; scale bar, 100 μ m. Anti-PCNA (C), anti-filaggrin (D), and anti-loricrin (E) antibodies were stained. scale bar, 100 μ m. (F) Clinical evaluation was determined by a scoring index of atopic dermatitis. Epidermis thickness (G), Scratching frequency (H), and transepidermal water loss (I) were measured. (J) Transcripts of filaggrin, loricrin, and involucrin were quantified using real-time PCR. (K) Lysates were immunoblotted with anti-filaggrin, anti-loricrin, and anti-involucrin antibodies. Data are presented as mean $\pm$ SEM; * = $p < 0.05$, ** = $p < 0.01$ vs. negative control, DNCB.

Figure 4. Boehmite inhibits mast cell infiltration and cytokine expression in DNCB-induced atopic dermatitis-like skin lesions in mice. Boehmite administration alleviated dermatitis induced by DNCB treatment. While DNCB was administered twice weekly for 3 weeks on the dorsal area of the mouse, dexamethasone (10 μ M) and 5% boehmite were administered daily in order to observe the degree of AD mitigation (A) Histopathological variation by DNCB induction was evaluated via toluidine blue staining; red arrows indicate mast cells, scale bar, 100 μ m. (B) The number of mast cell of the skin from toluidine-stained sites was counted from five randomly selected fields. (C) Total plasma IgE level was determined using ELISA. (D) Transcripts of TSLP, IL-33, IL-4 and IL-13 were quantified using real-time PCR. (E) The lysates were immunoblotted with anti-IL-1 β , anti-IL-4, anti-IL-5 and anti-RANTES antibodies. (F) Human skin tissues were treated with boehmite-FITC for 12 h. Nuclei were identified using DAPI staining. red arrows indicate boehmite, Scale bar, 100 μ m. Data are presented as mean $\pm$ SEM; * = $p < 0.05$, ** = $p < 0.01$ vs. negative control, DNCB.









Supporting Information

Table S1. Primers used for RT-PCR

Primers	Direction	Sequence 5'→3'
Human TSLP	forward	TAT GAG TGG GAC CAA AAG TAC CG
	reverse	GGG ATT GAA GGT TAG GCT CTG G
Human IL-25	forward	CTC CTG TCT CTT CCT CTT TTC C
	reverse	TGC ACC CAA AGT AGA TGC TC
Human IL-33	forward	CAA GTA TCA CAG GAA TTT CAC C
	reverse	TCA AGT CTT CAA CAT ATA TCT CA
Human GAPDH	forward	CAC CCA CTC CTC CAC CTT TGA C
	reverse	GTC CAC CAC CCT GTT GCT GTA G
Mouse IL-4	forward	GGT CTC AAC CCC CAG CTA GT
	reverse	GCC GAT GAT CTC TCT CAA GTG AT
Mouse IL-13	forward	CCT GGC TCT TGC TTG CCT T
	reverse	AGG TTT GAT TCA GGC AGA TGT T
Mouse TSLP	forward	AGC TTG TCT CCT GAA AAT CGA G
	reverse	AGG TTT GAT TCA GGC AGA TGT T
Mouse IL-33	forward	TCC AAC TCC AAG ATT TCC CCG
	reverse	CAT GCA GTA GAC ATG GCA GAA
Mouse filaggrin	forward	ATG TCC GCT CTC CTG GAA AG
	reverse	TGG ATT CTT CAA GAC TGC CTG TA
Mouse loricrin	forward	CTC CTG TGG GTT GTG GAA AGA
	reverse	TGG AAC CAC CTC CAT AGG AAC
Mouse involucrin	forward	ATG TCC CAT CAA CAC ACA CTG
	reverse	TGG AGT TGG TTG CTT TGC TTG
Mouse GAPDH	forward	AGG TCG GTG TGA ACG GAT TTG
	reverse	AGG TTT GAT TCA GGC AGA TGT T