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# Chelidonic acid ameliorates atopic dermatitis symptoms through suppression the inflammatory mediators in in vivo and in vitro

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## Abstract

Chelidonic acid (CA), a γ-pyrone compound, exerts various pharmacological functions, including anti-allergic and anti-colitis activities. However, the anti-atopic effect of CA and the mechanisms involved therein are not completely understood. The aim of the present study was to elucidate whether CA modulates atopic dermatitis (AD) in vitro and in vivo. We examined the pharmacological effects of CA on compound 48/80- or histamine-induced scratching behaviors and 2, 4-dinitrochlrobenzene-induced AD-like skin lesions in mice. Additionally, we evaluated the regulatory effects of CA on the expression of tumor necrosis factor -α, interleukin-6, cyclooxygenase -2 and inducible nitric oxide synthase and activation of nuclear factor-kappa B (NF-κB) in vivo and in vitro. The results showed that CA inhibited the symptoms of AD such as itching, eczema, erythema and dryness, and decreased the serum levels of IgE and histamine in mice. The inhibition rates of IgE and histamine levels by CA (2 mg/kg) were approximately 36.21 ± 4.19% and 28.93 ± 6.16%, respectively. Moreover, CA significantly attenuated the expression of inflammatory-related genes and NF-κB activation in AD-like skin lesions and mouse peritoneal macrophages. The maximal inhibition rates of NF-κB activation by CA were approximately 42.05 ± 2.12% (in AD-like skin lesions) and 37.17 ± 6.12% (in LPS-stimulated peritoneal macrophages), respectively. These results suggest that CA may be a useful therapeutic agent for skin inflammatory condition such as AD.

**Keywords** Chelidonic acid, Atopic dermatitis, Inflammatory mediators

## Introduction

Atopic dermatitis (AD) is a chronic and incurable inflammatory skin disease and its incidence has increased steadily over recent years. Clinical symptoms of AD include skin erythema and eruption accompanied by intense pruritus and cutaneous hypersensitivity [1]. The pathogenesis and progression of AD are influenced by genetic, environmental and immunological factors [2, 3]. To date, steroid therapy is crucial in the treatment of AD. However, steroids cannot be applied for long periods of time, because of their deleterious side-effects [4]. Researchers have thus attempted to find a novel and effective drug for AD treatment.

Skin inflammation is a process that involves the action of multiple factors within a complex network. Activation of macrophages is a hallmark of inflammation, especially in AD, where macrophages mediate chronic inflammation by producing cytokines. Macrophage accumulation

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was reportedly observed in AD skin lesions [5]. In response to various stimuli, macrophages generate a variety of inflammatory mediators, including interleukin (IL)-6 and tumor necrosis factor (TNF)-α [6]. The release of these factors may be of critical importance in the development of inflammatory skin disorders [7]. Therefore, the inhibition of inflammatory mediators can contribute to the development of useful therapeutic strategy against allergic inflammatory diseases such as AD. Nuclear factor-kappa B (NF-κB) exerts a crucial function by affecting the expression of various genes involved in skin inflammation [8]. In the nucleus, NF-κB activates gene transcription and is critically involved in the regulation of inflammatory responses, by controlling the transcription of inflammatory genes [9]. Increased NF-κB activity associated with the secretion of high levels of IL-6 and TNF-α was shown to be involved in skin inflammation [10]. Previous studies have demonstrated that NF-κB activation and the subsequent increase in inflammatory mediator expression are important in AD pathology.

As natural products are safe and free of toxicity, they have been the subject of increasing interest for the development of new drugs against a wide range of diseases such as cancers and inflammatory diseases. Researchers have been investigating alternative treatments to replace steroids for AD therapy. Chelidonic acid (CA) is a γ-pyrone compound that exhibits various biological functions such as regulating neurotransmitters, and reducing allergic rhinitis and ulcerative colitis [11, 12]. However, the anti-atopic effect and mechanism of CA are not completely understood.

The aim of the present study is to elucidate whether CA modulates AD in vivo and in vitro. To provide experimental evidence supporting that CA may be a useful therapeutic drug for AD, we examined the pharmacological effects of CA on scratching behavior induced by compound 48/80 (well-known histamine releaser) or histamine and AD symptoms induced by 2,4-dinitrochlrobenzene (DNCB) in mice. Additionally, we evaluated the effect of CA on the expression of inflammatory mediators in AD-like skin lesions and lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages.

## Materials and methods

### Reagents

The following chemicals were obtained from Sigma (St. Louis, MO, USA): 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazoliumbromide (MTT), bicinchoninic acid (BCA), CA, compound 48/80, DNCB, dexamethasone (DEX) and LPS. Anti-mouse TNF-α/IL-6, recombinant TNF-α/IL-6, biotinylated TNF-α/IL-6, anti-mouse IgE, recombinant IgE and biotinylated IgE were procured from Pharmingen (San Diego, CA, USA). Dulbecco's modified Eagles medium (DMEM) and thioglycollate (TG) were purchased from Difco Laboratories (Detroit, MI, USA). Specific antibodies against cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and α-tubulin were purchased from Santa Cruz Biotechnology (CA, USA).

### Animals

Male ICR mice (5 weeks old) and C57BL/6 mice (7 weeks old) were purchased from Da-Mool Science (Daejeon, Republic of Korea). Animals were housed at a constant temperature (24 ± 1 °C) and humidity (60 ± 10%) under a 12 h light/dark cycle (light on 08:00–20:00) and were given access to water and food ad libitum. All animal procedures and experiments were approved by the Wonkwang University of Animal Ethics Committee (WKU15-50).

### Assessment of scratching behavior

Before the experiment, the ICR mice (n = 6) were housed in acrylic cages (22 cm × 22 cm × 24 cm) for approximately 30 min for acclimation. The behavioral experiments were performed as previously described [13]. The rostral part of the skin on the back of mice was clipped, and compound 48/80 (50 μg/kg) or histamine (100 μg/kg) was intradermally injected. The scratching agents were dissolved in tween 80 before use. Immediately following intradermal injection, the mice were put back into the same cage for observation of scratching. Scratching of the injection site by the hind paws was counted and compared with scratching of other sites, such as the ears. The mice generally exhibited several scratches per second, and a series of these behaviors was counted as one incident of scratching over 30 min. CA (2 mg/kg) and terfenadine (10 mg/kg) were orally administered one hour prior to the injection of the scratching agents.

### Induction of AD-like dermatitis

To induce AD-like dermatitis, DNCB sensitization and challenge were performed following a procedure described previously [14]. DNCB was dissolved in a solution of acetone and olive oil at a ratio of 3:1. The dorsal skin of BALB/c mice was shaved using a depilatory and gauzed one day before sensitization. Mice were randomized divided into 4 groups (n = 6 per group): normal, DNCB, DNCB plus CA (2 mg/kg) and DNCB plus DEX (1 mg/kg). Exposed skin was treated with vehicle or 200 μL of 1% DNCB. Four days after sensitization, the dorsal skin was challenged with 200 μL of 0.5% DNCB solution three times per week for 2 weeks. The normal group was treated with vehicle. CA and DEX were orally

administered every day for two weeks. After sacrifice, blood samples and dorsal skin were collected for molecular and histological analysis.

#### Evaluation of skin dermatitis severity

The severity of dermatitis was assessed macroscopically according to the Eczema Area and Severity Index scoring system as follows: 0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms. The severity of dermatitis was evaluated by the naked eye of three examiners who were blinded to the groupings. The sum of the individual scores was defined as the dermatitis score for erythema/hemorrhage, edema, excoriation/erosion and scaling/dryness [15].

#### Histopathological analysis

The dorsal skin of the mice was removed and fixed in 10% phosphate-buffered formalin. The skin Sections (4 μm in thickness) were stained with hematoxylin and eosin to evaluate epidermal hyperplasia using light microscopy.

#### Histamine assay

The level of histamine in the serum isolated from the sacrificed mice was measured with a specific enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction (Abnova, CA, USA).

#### Peritoneal macrophage culture

C57BL/6 mice were administered an intraperitoneal injection of 2.5 mL TG, and TG-elicited macrophages were harvested after 3–4 days, as reported previously [16]. Peritoneal lavage was performed using 8 mL Hank's balanced salt solution (HBSS) containing 10 U/mL heparin. The cells were then transferred to 24-well tissue culture plates ($3 \times 10^5$ cell/well) containing DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), and incubated for 4 h at 37 °C in an atmosphere of 5% $CO_2$. The plates were washed three times with HBSS to remove non-adherent cells and equilibrated with DMEM containing 10% FBS before treatment.

#### MTT assay

Cell viability was determined by an MTT assay. Briefly, suspensions (500 μL) of peritoneal macrophages treated with different concentrations of CA were cultured in 24-well plates ($3 \times 10^5$ cell/well) for 24 h. MTT solution (50 μL of 5 mg/mL) was added, and the cells were incubated for 4 h at 37 °C. After washing, the insoluble formazan crystals were dissolved in dimethylsulfoxide, and transferred to 96-well plates. The absorbance was measured at 540 nm using an ELISA reader. The absorbance of formazan in the untreated control cells represented 100% viability.

#### Cytokine assay

Cytokine assays were performed using modified ELISA, as described previously [17]. In this method, the wells of 96-well plates were coated with specific mouse monoclonal antibodies against TNF-α, IL-6 and IgE. The coated plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) before proceeding to the subsequent steps. All reagents used in this assay were incubated for 2 h at 37 °C. Recombinant TNF-α, IL-6 and IgE antibodies were diluted and

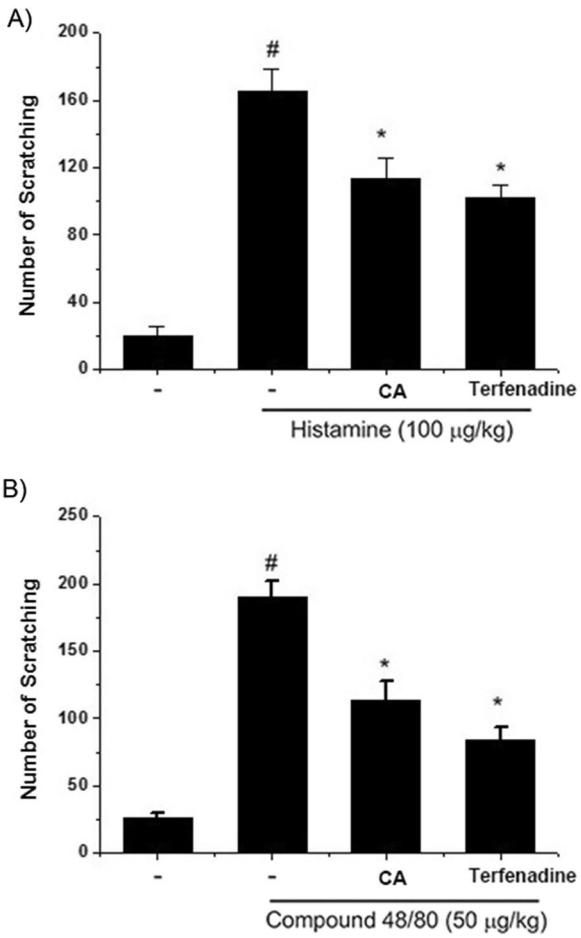


**Fig. 1** Effect of CA on the scratching behavior in mice (n = 6/group). CA was orally administered 1 h before the intradermal injection of histamine (**A**) or compound 48/80 (**B**). Scratching behavior was counted as one incident of scratching for 30 min. Terfenadine (10 mg/kg) was used as a positive control. The data represents the mean ± S.D. of three independent experiments ($^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. compound 48/80 or histamine-treated group)

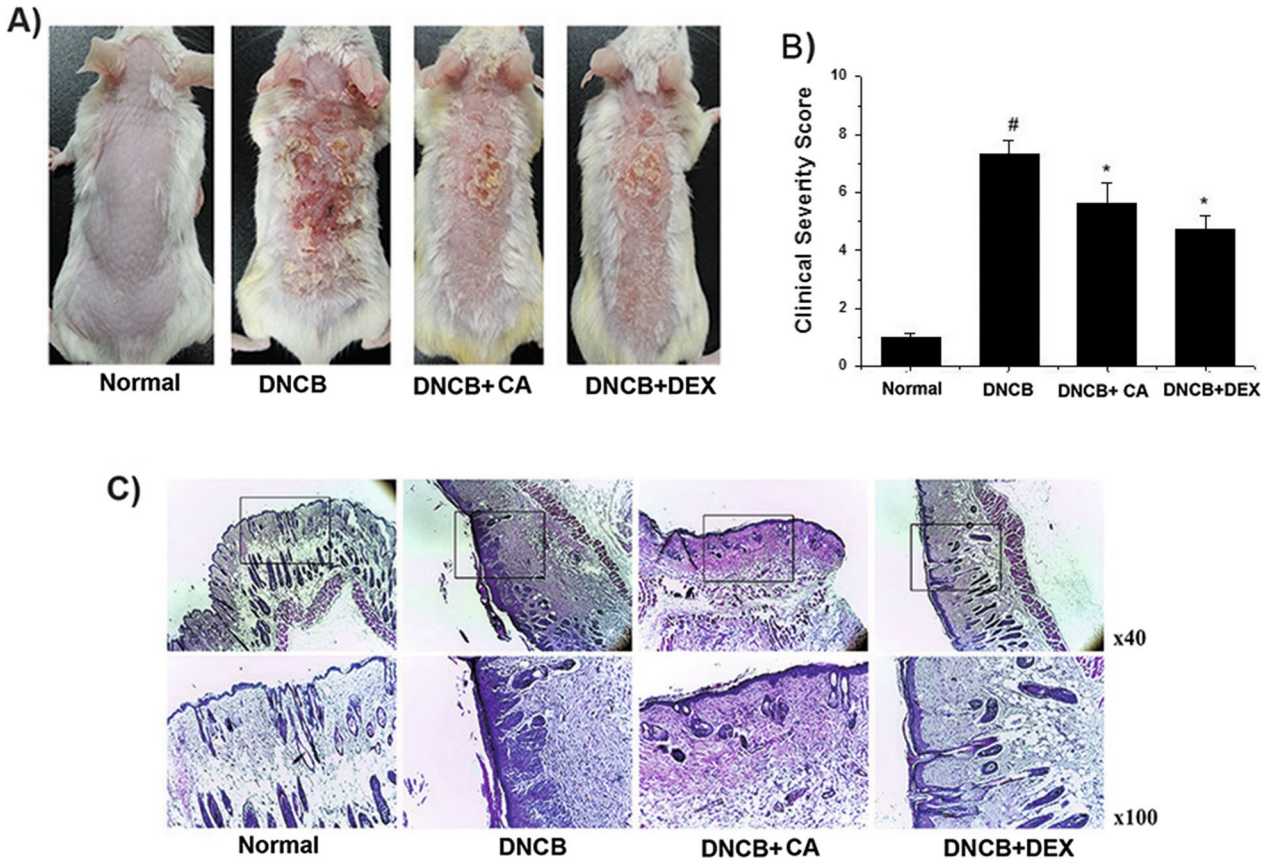


**Fig. 2** Effect of CA on DNCB-induced AD in mice. **A** Clinical feature of AD-like skin lesions in mice. **B** The score of skin severity is represented. **C** Epidermal thickness in skin was analyzed in the H&E-stained sections (magnification × 100). Dexamethasone was used as a positive control. The data represents the mean ± S.D. of three independent experiments ($^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. DNCB-treated group)

used as standards. Serial dilutions from 10 ng/mL were used to establish the standard curve. The assay plates were sequentially exposed to biotinylated mouse antibodies against TNF-α, IL-6 and IgE avidin peroxidase, and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution containing 30% distilled water. The absorbance of the plates was measured at 405 nm.

### Western blot

Cells were rinsed with PBS and nuclear extracts were prepared by Nuclear Extraction Reagents (Pierce Thermo Scientific, Rockford, IL, USA). After bicinchoninic acid protein quantification, the supernatant was mixed with a sample buffer, separated by gel electrophoresis, and transferred to membranes. Thereafter, the membranes were blocked with 5% skim milk and incubated with primary antibodies. After washing, the membranes were then incubated with secondary antibodies for 1 h. After washing with 0.1% PBST, protein bands were visualized using an enhanced chemiluminescence detection system (Pierce Thermo Scientific).

### Statistical analysis

Results are expressed as the mean ± S.D. of independent experiments and statistical analyses were performed using a one-way ANOVA, offered by Tukey's multiple tests. A value of $P < 0.05$ was considered statistically significant.

## Results

### Effect of CA on scratching behaviors in mice

The anti-scratching effects of CA were investigated in mice induced by histamine or compound 48/80. When CA was orally administered one hour prior to the injection of histamine or compound 48/80, scratching

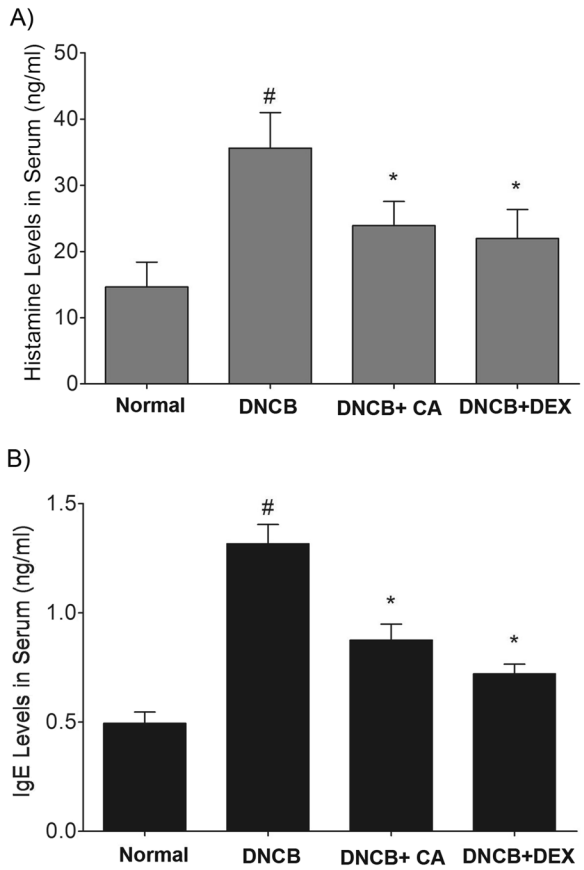


**Fig. 3** Effect of CA on the histamine and IgE levels in the DNCB-induced AD in mice. Blood samples were collected and the levels of serum histamine (**A**) and IgE (**B**) in the indicated groups were measured using ELISA. The data represents the mean ± S.D. of three independent experiments ($^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. DNCB-treated group)

was reduced. The inhibition rate of CA (2 mg/kg) was approximately 31.51 ± 3.17% and 33.43 ± 4.27%, respectively (Fig. 1). Terfenadine (anti-histamine drug) was used as a positive control.

#### Effect of CA on DNCB-induced AD like dermatitis in mice

To evaluate the regulatory effects of CA in an in vivo AD model, mice were treated with DNCB. We examined whether CA suppressed the signs of AD-like skin lesions and showed that treatment with CA for two weeks relieved eczema, erythema and dryness (Fig. 2A). The skin severity scores of mice treated with CA and DEX were significantly lowered than those of DNCB-treated mice (Fig. 2B). Additionally, we examined the histopathological changes in the back skin of the experimental mice. The skin of the DNCB-treated mice showed marked epidermal hyperplasia compared to that of normal mice. However, this hyperplasia was reduced by CA (Fig. 2C).

#### Effect of CA on serum levels of histamine and IgE in mice

An important feature of AD is the pathological secretion of IgE and histamine. Thus, we evaluated the effect of CA on the serum levels of histamine and IgE in mice. As shown in Fig. 3A, DNCB resulted in increased release of serum histamine, whereas CA-treatment led to a considerable reduction in serum histamine level. We also observed that the plasma concentration of IgE was significantly lower in CA-treated mice than that in DNCB-treated mice (Fig. 3B).

#### Effect of CA on the expression of skin inflammation-related genes in AD-like skin lesion

To elucidate the regulatory effect of CA on skin inflammation in vivo, the levels of inflammatory cytokines in skin tissue were measured using ELISA. DNCB resulted in increased levels of TNF-α and IL-6, but CA-treatment induced a considerable decrease in the levels of TNF-α and IL-6 in skin tissue (Fig. 4A). The expression of COX-2 an iNOS in skin tissue of CA-treated mice were significantly lower than that in DNCB-treated mice (Fig. 4B). The relative levels of COX-2 and iNOS are shown in Fig. 4C.

#### Effect of CA on inflammatory cytokines in LPS-stimulated mouse peritoneal macrophages

Peritoneal macrophages were treated with CA at various concentrations (2, 20 and 200 μM) prior to LPS-stimulation. CA did not affect cell viability (Fig. 5A). We investigated the anti-inflammatory effect of CA on the production of inflammatory cytokines in LPS-stimulated peritoneal macrophages using ELISA. As shown in Fig. 5B and C, TNF-α and IL-6 production increased dramatically by 24 h after LPS-stimulation, but was inhibited by CA in a concentration-dependent manner. The inhibition rates of TNF-α and IL-6 at 200 μM CA were approximately 62.76 ± 2.78% and 53.75 ± 13.04%, respectively.

#### Effect of CA on COX-2 and iNOS expression in LPS-stimulated mouse peritoneal macrophages

Western blot was conducted to determine the effect of CA on LPS-induced COX-2 and iNOS expression. The cells were pretreated for 1 h with CA at various concentrations (2, 20, and 200 μM) and subsequently treated

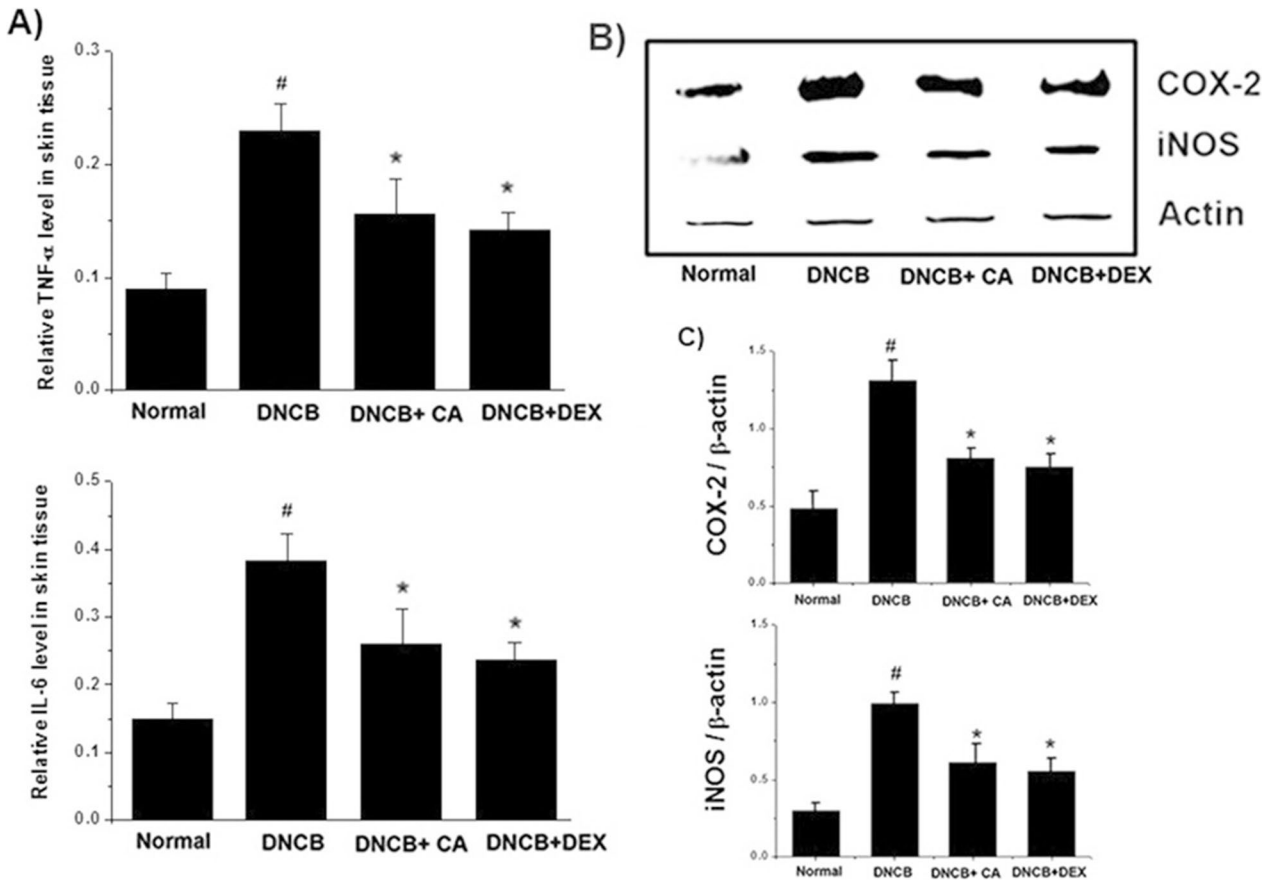


**Fig. 4** Effect of CA on skin inflammation-related gene levels in AD-like skin lesion. **A** At the end of experiment, the skin tissues were cut out and homogenized. The level of TNF-α and IL-6 in the indicated groups was measured via ELISA. **B** The levels of COX-2 and iNOS in the indicated groups were determined by Western blot analysis. **C** The relative COX-2 and iNOS levels were determined by densitometry. The data represents the mean ± S.D. of three independent experiments ($^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. DNCB-treated group)

with LPS. As shown in Fig. 6A and C, LPS significantly increased the expression of COX-2 and iNOS, which was markedly attenuated by CA in a concentration-dependent manner. The relative levels of COX-2 and iNOS are represented in Fig. 6B and D.

### Effect of CA on NF-κB activation in AD-like skin lesion and LPS-stimulated mouse peritoneal macrophages

NF-κB activation is associated with inflammatory response and require the translocation of the NF-κB into nucleus. We predicted that the role of CA is mediated by the suppression of NF-κB activation, and thus we evaluated the effect of CA on the nuclear pool of NF-κB (RelA/p65) by western blot. In DNCB-induced AD-like skin lesions, the levels of NF-κB in the nucleus were increased, but CA reduced these enhanced nuclear levels of NF-κB (Fig. 7A). The relative levels of NF-κB are shown in Fig. 7B. In LPS-stimulated peritoneal macrophages, the levels of NF-κB (RelA/p65) were increased in the nucleus, but subsequently reduced by CA (Fig. 7C). The relative levels of Rel/p65 are shown in Fig. 7D.

## Discussion

Natural products have been a subject of growing interest because of their potential in the treatment of AD. CA is known to exhibit beneficial effects on human health including analgesic, anti-inflammatory and antimicrobial activities. However, the regulatory effect and mechanism of CA in AD-like dermatitis is not completely understood. The findings of this study showed that CA attenuated compound 48/80- or histamine-induced scratching and alleviated DNCB-induced AD symptoms in mice. Additionally, we showed that the anti-atopic effect of CA was mediated by the suppression of inflammatory factors and NF-κB activation in AD-like skin lesion and LPS-simulated mouse peritoneal macrophages.

AD is a common chronic inflammatory skin disease that results in intense itching, edema, erythema, severe

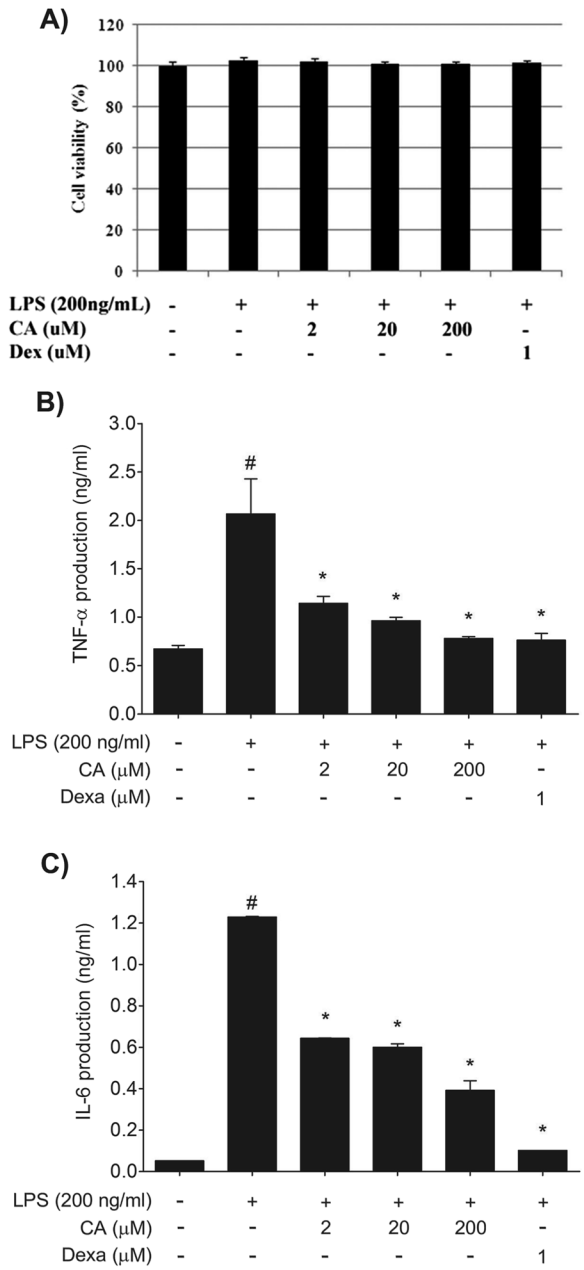


**Fig. 5** Effect of CA on IL-6 and TNF-α production in LPS-stimulated peritoneal macrophages. Cells ($3 \times 10^5$ cell/well) were pretreated with various concentrations of CA for 1 h and subsequently stimulated with LPS (200 ng/ml) for 24 h. Dexa (1 μM) was used as a positive control. **A** Cell viability was determined by an MTT assay. **B** and **C** The levels of TNF-α and IL-6 in cell supernatants was measured using ELISA. Values are the mean ± S.D. of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. un-stimulated cells, $^{*}p < 0.05$ vs. LPS- stimulated cells)

pruritus, and eczematous skin lesions [3]. One particular feature of AD is dry skin and continuous itching, the latter of which reduces the quality of life of the patients. Generally, steroid-based therapy has been applied for the treatment of AD, but clinical use of steroid has been restricted as long-term administration leads to deleterious side effects such as hypopigmentation, skin thinning, and atrophy [18]. Therefore, natural compounds with lower toxicity and high effectiveness are being sought as new potential anti-AD agents. In pathological skin conditions, histamine is involved in the induction of itching and edema [19]. This study focused on the manner in which CA regulates the scratching behaviors in mice. We showed that CA inhibited scratching induced by compound 48/80- or histamine in mice. Additionally, AD is characterized by potent skin inflammation associated with elevated levels of IgE against many types of allergens. IgE dysregulation has been implicated in the pathogenesis of AD and serum IgE concentration was reportedly elevated in AD patients [20, 21]. On the basis of these studies, we evaluated the effects of CA on IgE levels in the DNCB-induced AD model. The findings revealed that CA significantly relieved the AD symptoms such as eczema, erythema and dryness. CA also reduced the serum of IgE and histamine induced by DNCB. These results demonstrated the potential effect of CA on anti-atopic responses via the regulation of IgE and histamine.

Recently, AD was revealed to be associated with abnormal immunological responses. Macrophages are important effecter cells in the pathogenesis of skin inflammation, and inflammatory mediators derived from macrophages play important role in the development of AD [22]. High expression of TNF-α and IL-6 may be associated with the infiltration of macrophages into the dermis [23]. During an inflammatory reaction, COX-2 and iNOS are also induced in activated macrophages, and are pivotal in provoking inflammatory response in dermatitis. The levels of COX-2 and iNOS are higher in AD like skin lesions compared to those in normal skin lesions [24, 25]. Moreover, NOS inhibition was shown to attenuate inflammation in AD lesions [26]. On the basis of these observations, the suppression of inflammatory mediators may be an effective therapeutic strategy for preventing AD. Our results reveal that CA inhibited the secretion of TNF-α and IL-6 in LPS-simulated macrophages. The maximal inhibition rates of TNF-α and IL-6 production by CA were approximately 62.76 ± 2.78% and 53.75 ± 13.04%, respectively. Likewise, CA inhibited

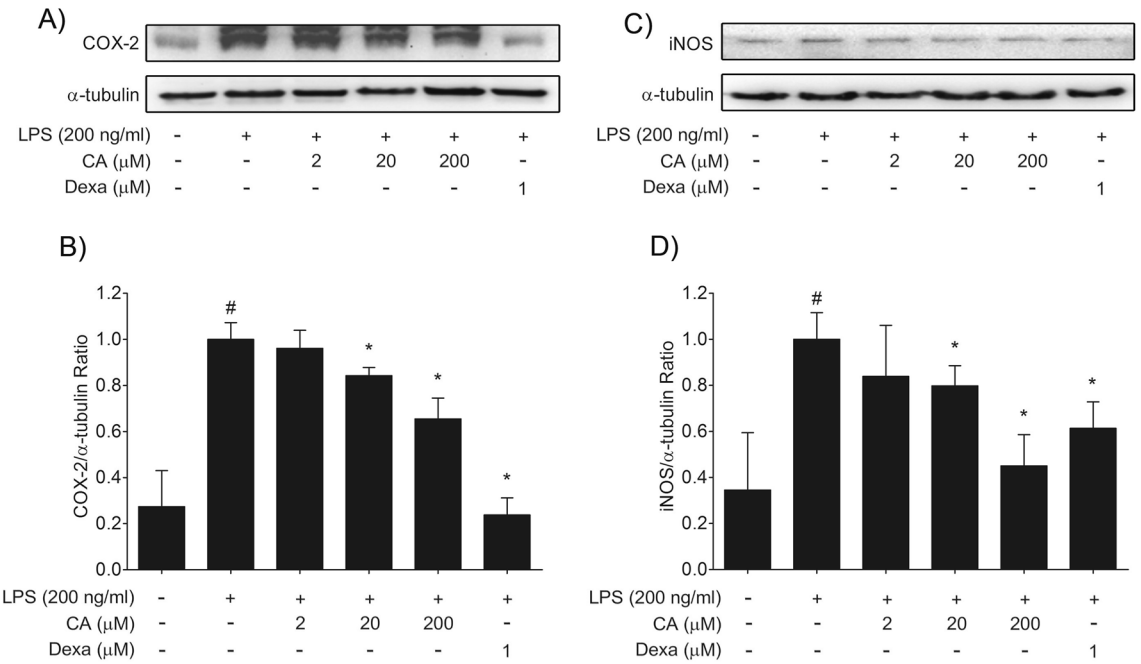


**Fig. 6** Effect of CA on COX-2 and iNOS expression in LPS-stimulated peritoneal macrophages. Cells ($5 \times 10^6$ cell/well) were pretreated with various concentrations of CA for 1 h and subsequently stimulated with LPS (200 ng/mL) for 24 h. **A** and **C** COX-2 and iNOS expression was determined by western blotting analysis. **B** and **D** The relative COX-2 and iNOS levels were determined by densitometry. Values are the mean ± S.D. of duplicate determinations from three separate experiments ($^{\#}p < 0.05$ vs. un-stimulated cells, $^{*}p < 0.05$ vs. LPS-stimulated cells)

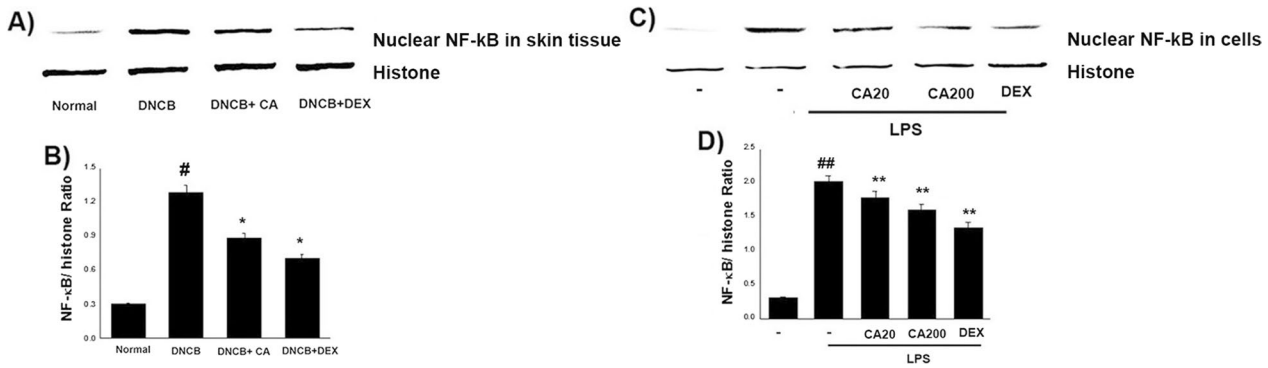


**Fig. 7** Effect of CA on NF-κB activation in AD-like skin lesion and LPS-stimulated mouse peritoneal macrophages. **A** The nuclear extract of skin tissue was isolated by Nuclear Extraction Reagents. The levels of NF-κB p65 in nucleus were evaluated by western blot analysis. **B** The relative expression level of NF-κB p65 was measured using an image analyzer. **C** Cells ($6 \times 10^6$) were pretreated for 1 h with CA and then treated for 1 h with LPS. The levels of NF-κB p65 in cell nucleus were evaluated by Western blot analysis. **D** The relative expression level of NF-κB p65 was measured using an image analyzer. The data represents the mean ± S.D. of three independent experiments ($^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. DNCB-treated group, $^{**}p < 0.05$ vs. LPS-stimulated cells)

the expression of COX-2 and iNOS in LPS-stimulated mouse peritoneal macrophages. Additionally, we showed that CA regulated the levels of cytokines and attenuated the expression of COX-2 and iNOS in DNCB-induced AD-like skin lesions. These results suggested that CA exerts anti-atopic effects by suppressing the production of inflammatory mediators.

The increase in inflammatory mediators is associated with activation of the transcription regulator NF-κB [10]. In the inflammatory process, the IκB kinase complex is phosphorylated and degrades the IκB protein. NF-κB is thus translocated into the nucleus where it can combine with the promoter of target genes to activate gene expression. Based on this phenomenon, suppression of NF-κB

activation has been identified as an anti-inflammatory strategy. We examined whether the anti-atopic action of CA occurs through the regulation of NF-κB activation. The results demonstrated that CA inhibited NF-κB translocation into the nucleus in AD-like skin lesions and LPS-simulated mouse peritoneal macrophages. We thus hypothesized that CA exerts anti-atopic effects via the regulation of NF-κB activation. Although CA attenuated NF-κB activation, the effect of CA on pathways involving NF-κB (phosphorylation of IκB-α and IKK activation) has not been determined. Further studies are necessary to clarify the role of CA on the NF-κB pathway.

In conclusion, this study suggested that CA regulate allergic response in vivo, by affecting scratching induced by compound 48/80- or histamine and alleviating DNCB-induced symptoms of AD in mice. Additionally, we demonstrated that the anti-atopic effect of CA may be attributed, at least in part, to the inhibition of inflammatory-related gene expression and NF-κB activation in AD-like skin lesions and LPS-simulated mouse peritoneal macrophages. Our study may provide a basis for the therapeutic use of CA in inflammatory skin diseases such as AD.

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**Author contributions**
SJK and DSK contributed to the study conception, design, and material preparation. Data collection and analysis were performed by SHL, EMA, JYK, and SHH. The first draft of the manuscript was written by SJK and DSK commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
The data that support the findings of this study are available from the first author, [S.-J. Kim], upon reasonable request.

## Declarations

**Competing interests**
The authors have no competing interests.

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