Original Article

Inhibitory effects of calophyllum inophyllum extract on atopic dermatitis induced by DNCB in mouse

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ABSTRACT

Objective: Atopic dermatitis, or eczema, is a common skin disease that isoften associated with other atopic disorders, such as allergic rhinitis and asthma. One hypotheses concerning the mechanism of atopic dermatitis that the primary defect resides in an immunologic disturbance thatcauses IgE-mediated sensitization, with epithelial-barrier dysfunction regarded as aconsequence of the local inflammation. Calophylluminophyllumextract(CIE) has been used in oriental medicines and many previous studies reported its anti-microbial and anti-inflammatory effect via cyclooxygenase inhibitions. This study examined whether the CIE exerting anti-inflammationcould alleviate the symptoms of atopicdermatitis (AD) induced with 2,4-dinitrochlorobenzene(DNCB) in mice.

Methods: Mice were sensitized and challenged on the skin of their backs with DNCB. At 28-54 days aftersensitization, mice were treated with topical application of CIE as skin ointment. Skin thickness, collagen density, serum cytokine analysis and scratching behavior test were used to evaluate the effects of CIE on DNCB-induced mice by ELISA, histological analysis.

Results: Topical application of CIE attenuated AD-like skin inflammatory symptoms. CIE decreased scratching frequencies, the epidermal thickness, fibrotic tissue changeof dorsal skinand the level of inflammatory cytokines-IgE and histamine.

Conclusion: CIE properly improved AD-like skin inflammatory symptoms on the dorsal skin of DNCB-inducedmice, partly by suppressing production of histamine and IgE, fibrotic changes in inflamed skin. Therefore, CIE is a potential therapeutic agent for skin inflammatory diseases such as AD.

Keywords: Atopic dermatitis, calophylluminophyllum, DNCB induced mouse model, itching, anti-inflammation.

INTRODUCTION

Atopic dermatitis (AD) is a chronic relapsing inflammatory skin disease with a worldwide prevalence, and causes itch-scratch cycles that injured skin and exacerbate problems[1].The pathogenesis of AD has been attributed to complex interactions between multiple factors such as genetic and environmental factors, altered skin barrier function, and the immune system and extrinsic AD caused by environmental factors or allergens induces Th2 cells[1,2]. The diagnosticcriteria in the pathophysiology of many atopic skin diseases havebeen established, and include, the hyperproduction of immunoglobulin E (IgE)and infiltration of mast cells[3]. In addition, AD is characterized by poorly definederythema with edema, vesicles, and weeping in the acute stage andby skin thickening in the chronic stage[4]. However, its pathologyhas not been fully clarified.A number of pharmacological controls for AD involve topical orsystemic administration of steroids, antihistamines[5]. However, prolongeduse of steroid has side effects such as thinning of the skin, leadingto cracking and bleeding[6].Thus, thedevelopment of effective and safe therapies for AD is required.

Natural compounds from herbs andplants are potential sources of therapeutic agents for preventingand treating inflammatory skin diseases[7-9]. Also, the canophyllal and canophyllol of calophylluminophyllum extract is strongly anti-inflammatory andinhibits lipooxygenase and cyclooxygenase activities[10].

Recently, the efficacy of natural extract as a therapeutics for2,4-dinitrochlorobenzene (DNCB)-induced model of AD in rats has been proved[9]. Natural extracts highlight on various un-cured disease treatment. Some studies show the immunemodulatory effect includes autoimmune disease of natural extracts[11]. Also, antimicrobial effects and anti-inflammatory effects of natural extracts were well-known[12,13]. Thus, in the study, we investigated inhibitory effects of calophylluminophyllum extracton DNCB-induced AD mouse models. We found that topical applications of calophylluminophyllum extractinhibited AD related skin lesions andreduced epidermal thickness, accumulation of inflammation related collagens, and itching behavior.

MATERIALS AND METHODS

Extraction methods

The fruits of C. inophyllum purchased from Alexandrian laurel farm (Hawaii, USA). The raw samples were separated into nuts and endocarps before finely cut and dried in oven at55 ℃ for 7 days. Then, those were grinded to fine powder using Laboratory Mill. Solvent extraction where sample was consecutively extracted with $CHCl_3$and methanol, followed by method where sample was extracted directly by boiling in water, filtered, cooled andextracted with $CHCl_3$ then evaporated.

Animals and maintenance

To induce AD related mice model, we followed a previously report.[9]Male C57BL/6 mice were from Orient Bio. Inc. (Gyeonggi-do, Korea) and were 8 weeks old at initiation ofexperiments. All mice were maintained at the animal facility of Seoul National University (Seoul, Korea) and were housed in anenvironmentally controlled room with a 12 h light/dark cycle andallowed free access towater. Room temperaturewas maintained at22 ℃ with a relative humidity of 50%. Animals were fedwith a sterilized pelleted diet (Orient Bio. Inc.). DNCB (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in vehicle (3:1

acetone:olive oil) and used as a sensitizer for inducing AD-like skin lesionsin mice. Backs of mice were shaved with a clipper and depilatorycream, washed with sterilized PBS and gauzed a day beforesensitization. Mice were divided into 4 groups with 5 mice pergroup: vehicle, DNCB, and DNCB plus topical treatment of 10 wt% or 30 wt% of calophylluminophyllum extract. Sensitization schedule was as shown in Fig.1bExposed skinwas treated with vehicle alone or with 0.1ml of 1% ofDNCB in vehicle. On day 4 after sensitization, skin was resensitizedwith 0.1% of DNCB solution daily for about two months.On day 30 after initiation of sensitization, a cream containing of calophylluminophyllum extract was applied to skin lesions with DNCB dailyfor a month.

Measurement of serum IgE and histamine level

We collected blood from mice after sacrifice. Serum sampleswere obtained after centrifugation. Using mouse IgE ELISA kits(Shibayagi, Gunma, Japan), total IgE serum levels were measured.For serum histamine levels, mouse histamine ELISA kits (OxfordBiomedical Research Inc., Oxford, MI, USA) were used.

Histological analysis

Skin samples of mice were shaved and fixed in 3.7% formalin,embedded in paraffin and serially sectioned at 5 μm Sectionedsamples were stained with a hematoxylin/eosin solution orMasson's trichrome solutionaccording to the manufacturer's instructions (Sigma–Aldrich, St. Louis, MO, USA) for detecting epidermalthickness or composition of connective tissues, especially collagen fiber on AD-like skin lesions induced by2,4-dinitrochlorobenzene (DNCB) in mice. Histopathologicalchanges were examined by light microscopy. The epidermal thickness and connective tissues were measured in four random fields (X20) and presented asmeans of five independent numbers.

Behavioral studies

The fur on the dorsal skin was shaved and mice were habituated to a Plexiglas recording arena with a transparent cover one week prior to testing. On a specific day after CIE treatment, mice were placed into a clear glass arena containing mirrors set at angles to allow multiple views of the animal that were captured with a high-definition camera. Investigators left the room during recording. The camera was set at high definition and slow motion capture modes to facilitate the assessment of biting and licking behaviors in a frame-by-frame video playback conducted offline by two investigators blinded as to the experimental treatment. The numbers of scratching episodes were recorded for 60 min. To identified normal grooming behavior, a scratching is defined as direct contact of the hindlimb with dorsal skin.

Statistical analysis

Unless stated otherwise, all experiments were performed withtriplicate samples and repeated at least three times. Data are presented as mean $\pm$SD and statistical comparisons betweengroups were performed using Student's t-test.

RESULTS

Calophylluminophyllumextractattenuated DNCB-induced skin inflammation

In previous studies, more than 12 metabolites were isolated from the fruits of C. inophyllum and Isoprenylcoumarins isolated from leaves of C. inophyllum were reported to be the mostactive substances in inhibiting thecyclooxygenase activity in in vitro experiments. Fig. 1A shows well-known isolated compound from C. inophyllum;inopyllum and calophylloides[10].

To investigate the effects of Calophylluminophyllumextract(CIE) on AD-like skin lesions *in vivo*, we sensitized C57BL/6 mice with DNCB allergen to induce AD-likecontact skin inflammation. Mice were challenged withmultiple applications of the indicated doses of DNCB to dorsal skin(Fig.1B). Animals in Mock groupwere not treated with DNCB. On alternate days from day 28to 56, animals in the DNCB + CIE group wereadministeredCalophylluminophyllum extract(CIE), whereas animals in the positive control group, named as DNCB + PBS group, were administered PBS rather than CIE.Edema, erythema excoriation and scarring were apparenton the skin of DNCB-sensitized mice compared with untreatedcontrol mice. Severity of lesions was reduced in DNCB-sensitizedmice treated with topical CIE and these results significantly increase upon CIE concentration.(Fig. 1C).

Dermatitis score, represents the accumulated effects ofscratchingprovides a more objective evaluationcriterion than skin changes. Severities of dermatitiswere evaluated weekly. Cutaneous lesions were inspected andassessed by scoring as described previously[14]. Summedscores of three different regions (middle, upper, and lower dorsal skin) arereferred to asfinal dermatitis scores. On day 28, mild dermatitisappeared in the all DNCB treatment groups, and became moresevere with time, and peaked at day49. Dermatitis scores were consistently lower from day 35 to 56 in the DNCB + CIE groupthan in the DNCB + PBS or DNCB group (Fig. 1D).

Calophylluminophyllum extractreducedinflammation-mediated skin phenotype changes.

DNCB induced hyperkeratosis in mice. Increased epidermalthickness was probably due to increased epidermal proliferationcaused by altered differentiation at the inflamed skin tissues. Ourresults showed that the thickness of the epidermal layer and fibrotic changes wassubstantially reduced by treatment of CIE (Fig. 2A and B).

Skinsections were stained with H&E to examine hyperkeratosis layer thickness by micrometer. On day 56, that is, immediately after CIE treatment, skin tissues and bloodsamples were obtained from mouse per group under anesthesia.Histopathologic changes of skin were assessed using hematoxylinand eosin(HE)staining. The DNCB andDNCB + PBS groups showed significant histopathological changes,such as, epidermal hyperplasia, hyperkeratosis (Fig. 2C).

Studies in various animal models of wound healing or fibrotic diseases have suggested a causal link between fibrocyte accumulation and ongoing tissue fibrogenesis or vascular remodeling in response to tissue inflammation[15]. Masson's Trichrome stain for detection of collagen depositionfor quantification of collagen in the skin were performed to compare the levels of collagen in AD-like lesion from the three groups of DNCB treated mouse. The skin samples from DNCB + PBS showed intense and extensive Masson's Trichrome staining and higher skin fibrosis density. Conversely, mouse treated with CIE had markedly reduced collagen deposition and significantly lower skin fibrosis density and these tendency showed gradually increase in higher CIE concentration (Fig. 2D)

Calophylluminophyllum extract inhibited overproduction of inflammatory cytokines and relief related scratch behavior

Mast cells are sources of histamine, which is a symptom-inducing substance in AD[16]. Thus, the serum levels of histamine were examined. CIE inhibited the serum levels of histamine in DNCB-sensitized

mice. (Fig. 3A) IgE antibody production isassociated with Th2 immune response[17]. To examine the effectof CIE on Th2 response, we measured serum levels of IgE.Compared with AD-induced control mice, total IgE was significantlyreduced by topical administration of CIE. (Fig. 3B)

Consistentwith observed clinical features, the frequencies of scratchingwere markedly reduced in the DNCB + CIE group, compared to control ($p<0.05$). Once a week from the day28 to 56, scratchingbehavior was evaluated by video-recording for 1 h. Scratching behaviorsfollowed similar patterns, but a significant difference between the DNCB + CIE and DNCB groups was only observed at day 49, that is, after 3 weeks of CIE treatment, suggesting CIE had a cumulative effect. (Fig. 3C)

DISCUSSION

In previous study, many metabolites were isolated from the C. inophyllum. Based on our previous experiences, solvent extraction gave a gummy extract which complicate separation of metabolites. However, boiling the nuts in hot water produces oil which separated out on the top layer. After cooling, the layer contains isoprenylatedcoumarins that can be easily separated by phase-seperation. This isthe traditional method of producing the oil used for medicinal ointment[10].

In this study, we demonstrated that topical C. inophyllumextract reduced inflammatory signs-edema, hemorrhage, excoriation, scaling, dryness and behavior symptoms- itching, scratchingin the skin of DNCB-induced mice. Mast cells release immunemediators such as histamine, which has potent pro-inflammatoryactivities. CIE suppressed serum histamine and IgE level. Furthermore,we showed that application of CIE inhibited skin hyperkeratosis and fibrotic infiltration in AD-like skin inflammation model. Our results suggested that CIE might attenuate AD-like symptoms.

Topicalapplication of DNCB on the backs of mice induced many of thecutaneous histopathological symptoms of AD within a month. AD-likeskin lesions in mice were similar to those in AD patients. Weobserved a high degree of histamine and IgE level in the serum of DNCB-sensitized mice.Symptoms were significantly improved after 5 weeks of treatment with CIE, as observed in AD-likeskin lesions on mice. Epidermal thickening, fibrosis of skin layer and an increase of serum inflammatory cytokines are the main featuresof AD lesions. Topical application of CIE effectively anddose dependently attenuated epidermal thickening and fibrosis of skin layer. Clinically, 80% of ADpatients have increased serum IgE, which is closely related todisease severity. IgE induced activation of mast cells, whichresulted in the release of inflammatory mediators such ashistamine. Histamine, amajor excreted cytokine of mast cells, is involvedin induction of itching and edema[9]. Our results showed that CIE significantly inhibited serum histamine levels in AD-inducedmice. We propose that topical application of CIE mitigatedAD-like symptoms partly by inhibiting the elevation of serumhistamine via reduction in IgE.

Inophyllums and calophylloideswere the major component in C. inophyllumextracts.In-vitro assays demonstrated that the crude extract at concentrationof 50 ug/ml inhibited 77% and 88% cyclooxygenase and lipooxygenase activities[10], respectively, indicating its potential as anti-inflamatoryagentand Our findings suggested that CIE also potently inhibited ADand could be developed as a therapeutic agent.

CONCLUSION

In conclusion, C. inophyllum extract efficiently inhibit inflammatory lesion of DNCB induced mouse skin and show superior redundant of itching behavior and inflammation related signal molecules of disease with low toxicity. C. inophyllum, as a natural product, should be adopted as a skin applicants for chronic use. The obtained data highlight the potential of using C. inophyllum extract as an alternative treatment option for atopic dermatitis.

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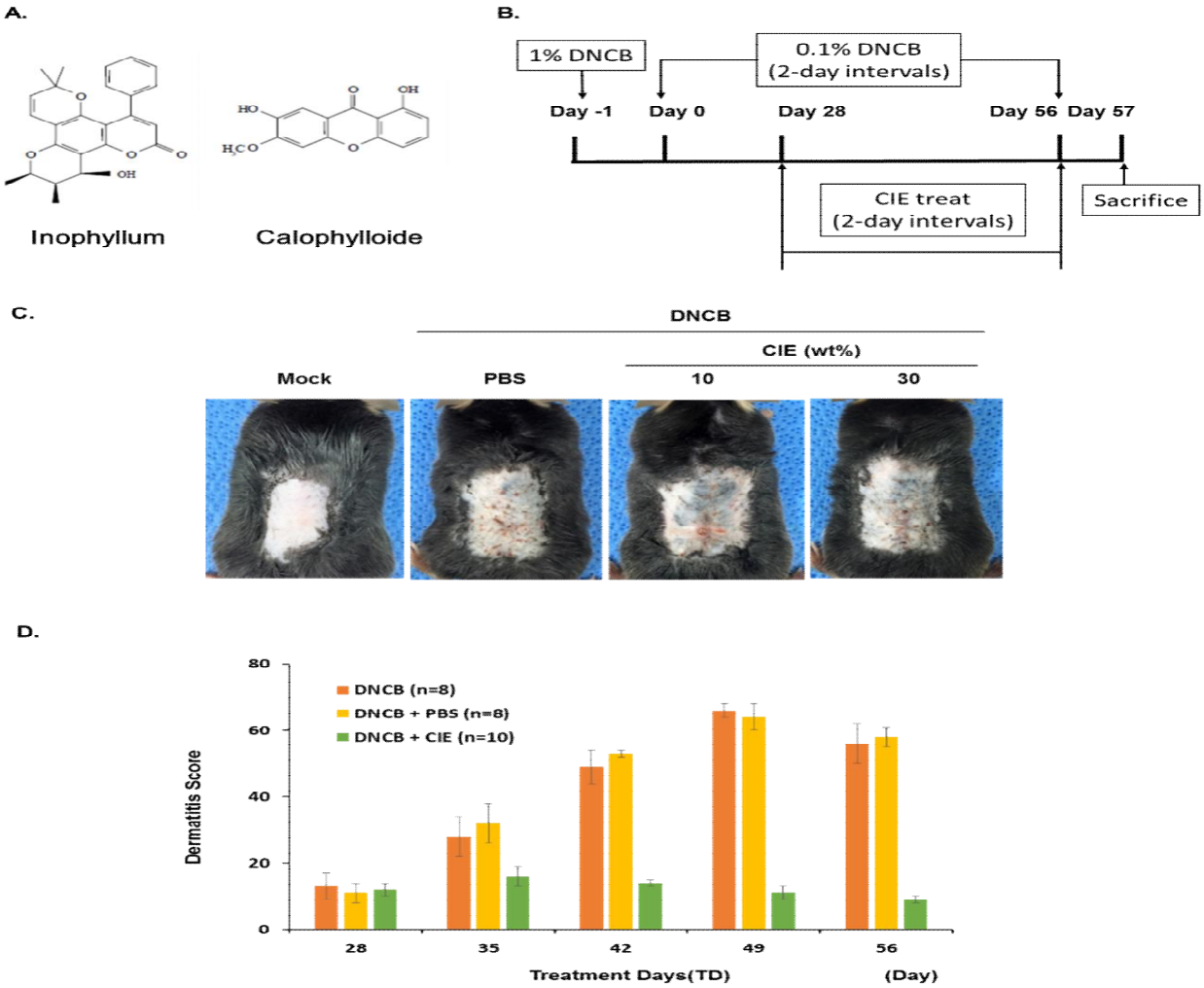

Fig. 1: Effects of CIE on DNCB-induced AD skin severity.
(a) Major metabolites of C. inophyllum extracts; Inophyllums and Calophylloides (b) Sensitization of DNCB mice (n = 8~10): stimulation was with DNCB for 28 days, and after 28 days, with cream containing 10 or 30 wt% of CIE. Mice were sacrificed on day 57. (c, d) Clinical severity of inflammatory skin lesions.Atopic dermatitis-like skin lesions evaluations and photographs were taken on the day before mice were sacrificed.

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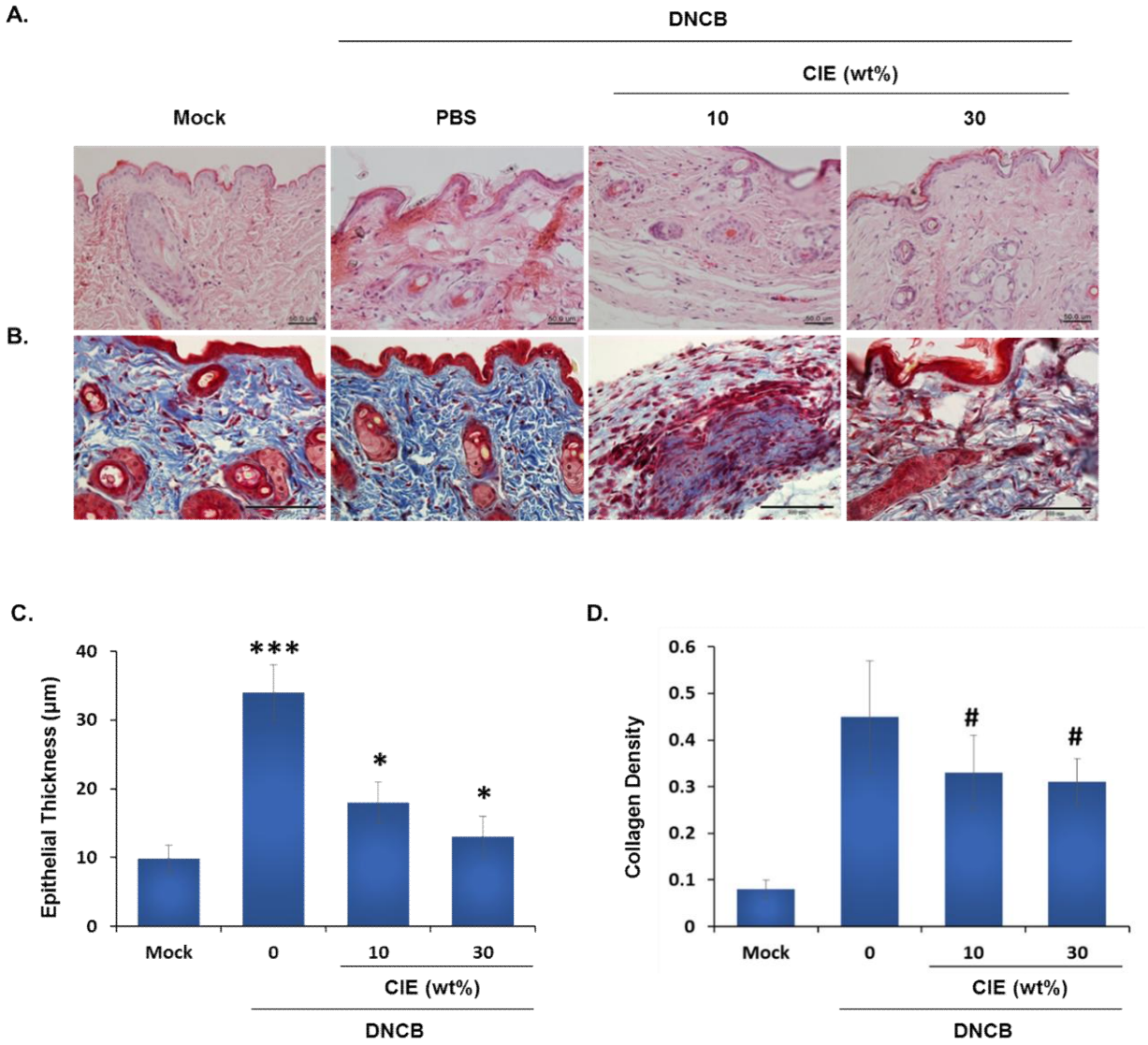

Fig. 2: Histological analysis of DNCB-induced skin lesion. Mouse back skin lesions were fixed in 3.7% formaldehyde, sectioned at 5.5mm and stained with hematoxylin and eosin (a) and Masson's trichrome (b). Epidermal thickness was measured with a micrometer for each groups (c). significant difference in the skin collagen deposition between the two groups of mice by Masson's Trichrome staining(d). (original magnification X 20) Data shown are the average of six samples per group and are shown as the mean values ± SD. *p<0.05, ***p<0.001 vs. control and #p<0.05 vs. NEGATIVE control.

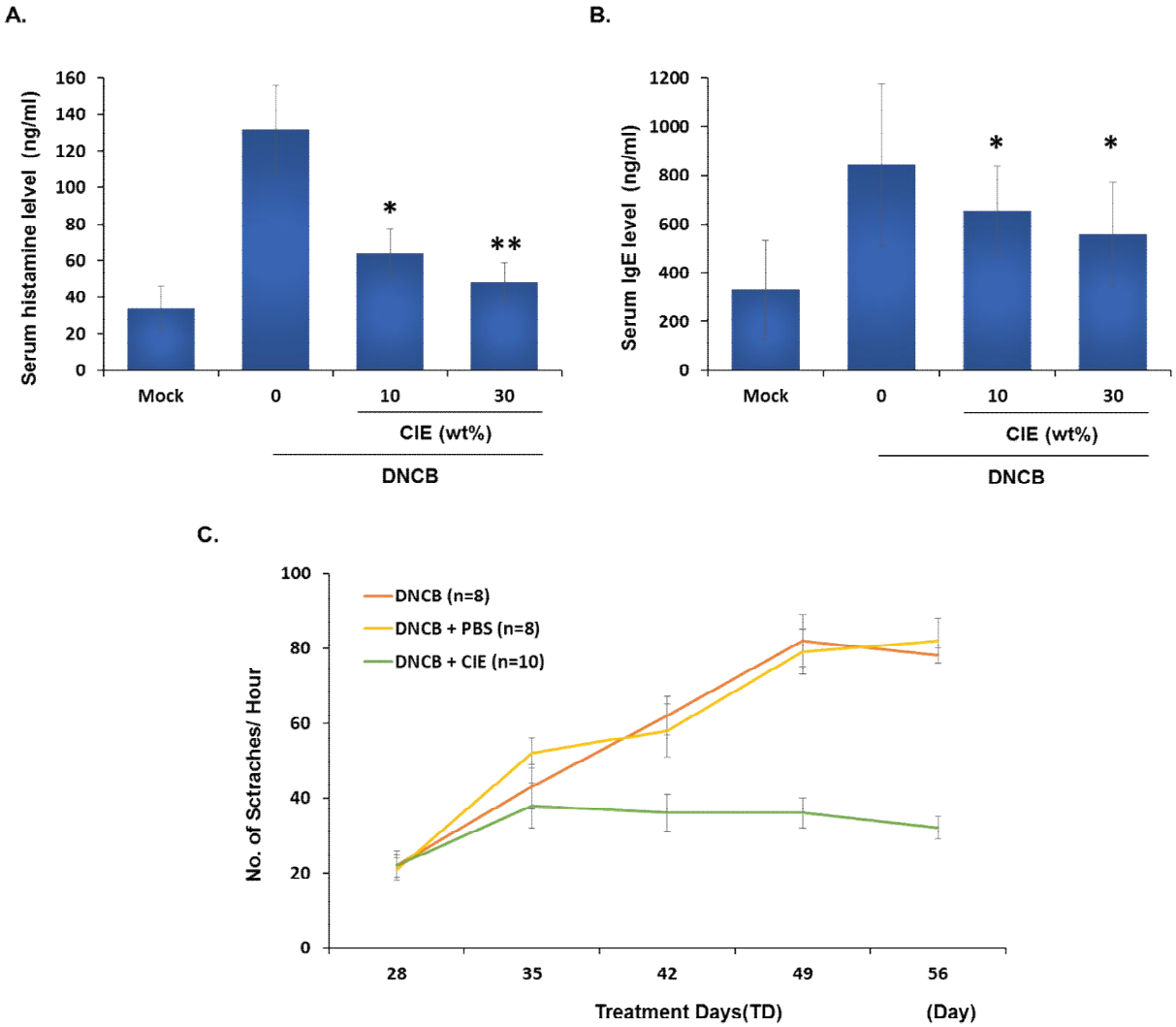

Fig. 3: Effects of ICE on IgE and serum histamine levels and immune cell recruitment and symptom related scratching. Serum was collected 24h after final CIE and/or DNCB treatment. DNCB + 0 group means the group treated vehicle(PBS) for negative control. Serum histamine (a) and IgE(b) were analyzed by ELISA. The effect of CIE on scratching behavior for DNCB-induced mouse (c) Data shown are the average of six samples per group and are shown as the mean values ± SD. *p<0.05, **p<0.01 vs. NEGATIVE control(DNCB + PBS group).