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

Yu Lim Um*1 and Young Wook Jo2

1North London Collegiate School Jeju, Korea
2Department of bioengineering, Yonsei University and Biostandard, Korea

ABSTRACT

Objective: Atopic dermatitis, or eczema, is a common skin disease that is often associated with other atopic disorders, such as allergic rhinitis and asthma. One hypothesis concerning the mechanism of atopic dermatitis is that the primary defect resides in an immunologic disturbance that causes IgE-mediated sensitization, with epithelial-barrier dysfunction regarded as a consequence of the local inflammation. Calophylluminophyllum extract (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-inflammation could alleviate the symptoms of atopic dermatitis (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 after sensitization, 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 change of dorsal skin and the level of inflammatory cytokines IgE and histamine.

Conclusion: CIE properly improved AD-like skin inflammatory symptoms on the dorsal skin of DNCB-induced mice, 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. 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. The diagnostic criteria in the pathophysiology of many atopic skin diseases have been established, and include, the hyperproduction of immunoglobulin E (IgE) and infiltration of mast cells. In addition, AD is characterized by poorly defined erythema with edema, vesicles, and weeping in the acute stage and by skin thickening in the chronic stage. However, its pathology has not been fully clarified. A number of pharmacological controls for AD involve topical or systemic administration of steroids, antihistamines. However, prolonged use of steroids has side effects such as thinning of the skin, leading to cracking and bleeding. Thus, the development of effective and safe therapies for AD is required.

Natural compounds from herbs and plants are potential sources of therapeutic agents for preventing and treating inflammatory skin diseases. Also, the canophyllal and canophyllol of calophylluminophyllum extract is strongly anti-inflammatory and inhibits lipooxygenase and cyclooxygenase activities.

Recently, the efficacy of natural extract as a therapeutics for 2,4-dinitrochlorobenzene (DNCB)-induced model of AD in rats has been proved. Natural extracts highlight on various uncured disease treatment. Some studies show the immunemodulatory effect includes autoimmune disease of natural extracts. Also, antimicrobial effects and anti-inflammatory effects of natural extracts were well-known. Thus, in the study, we investigated inhibitory effects of calophylluminophyllum extracton DNCB-induced AD mouse models. We found that topical applications of calophylluminophyllum extract inhibited AD related skin lesions and reduced 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 at 55 °C for 7 days. Then, those were grinded to fine powder using Laboratory Mill. Solvent extraction where sample was consecutively extracted with CHCl₃ and methanol, followed by method where sample was extracted directly by boiling in water, filtered, cooled and extracted with CHCl₃ then evaporated.

Animals and maintenance

To induce AD related mice model, we followed a previously report. Male C57BL/6 mice were from Orient Bio. Inc. (Gyeonggi-do, Korea) and were 8 weeks old at initiation of experiments. All mice were maintained at the animal facility of Seoul National University (Seoul, Korea) and were housed in an environmentally controlled room with a 12 h light/dark cycle and allowed free access to water. Room temperature was maintained at 22 °C with a relative humidity of 50%. Animals were fed with a sterilized pelleted diet (Orient Bio. Inc.). DNBC (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 lesions in mice. Backs
of mice were shaved with a clipper and
depilatory cream, washed with sterilized PBS
and gauzed a day before sensitization. Mice
were divided into 4 groups with 5 mice per
group: vehicle, DNCB, and DNCB plus
topical treatment of 10 wt% or 30 wt% of
calophyllinophyllum extract. Sensitization schedule was as shown in
Fig.1bExposed skin was treated with vehicle
alone or with 0.1 ml of 1% of DNCB in
vehicle. On day 4 after sensitization, skin
was resensitized with 0.1% of DNCB
solution daily for about two months. On
day 30 after initiation of sensitization, a
cream containing of calophyllinophyllum
extract was applied to skin lesions with
DNCB daily for a month.

Measurement of serum IgE and histamine
level

We collected blood from mice after
sacrifice. Serum samples were 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
Sectioned samples were stained with a
hematoxylin/eosin solution or Masson’s
trichrome solution according to the
manufacturer’s instructions (Sigma–Aldrich,
St. Louis, MO, USA) for detecting
epidermal thickness or composition of
connective tissues, especially collagen fiber
on AD-like skin lesions induced by 2,4-
dinitrochlorobenzene (DNCB) in mice.
Histopathological changes were examined by
light microscopy. The epidermal thickness
and connective tissues were measured in
four random fields (X20) and presented
as means 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 with triplicate
samples and repeated at least three times.
Data are presented as mean ± SD and
statistical comparisons between groups were
performed using Student’s t-test.

RESULTS

Calophyllinophyllum extract attenuated
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 most active substances in
inhibiting the cyclooxygenase activity in vitro experiments. Fig. 1A shows well-
known isolated compound from C.
inophyllum; inophyllum and calophyloides

10.,
To investigate the effects of Calophylluminophyllum extract (CIE) on AD-like skin lesions in vivo, we sensitized C57BL/6 mice with DNCB allergen to induce AD-like contact skin inflammation. Mice were challenged with multiple applications of the indicated doses of DNCB to dorsal skin (Fig. 1B). Animals in Mock group were not treated with DNCB. On alternate days from day 28 to 56, animals in the DNCB + CIE group were administered Calophylluminophyllum 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 apparent on the skin of DNCB-sensitized mice compared with untreated control mice. Severity of lesions was reduced in DNCB-sensitized mice compared with untreated control mice. Severity of lesions was reduced in DNCB-sensitized mice treated with topical CIE and these results significantly increase upon CIE concentration (Fig. 1C).

Dermatitis score, represents the accumulated effects of scratching provides a more objective evaluation criterion than skin changes. Severities of dermatitis were evaluated weekly. Cutaneous lesions were inspected and assessed by scoring as described previously. Summed scores of three different regions (middle, upper, and lower dorsal skin) are referred to as final dermatitis scores. On day 28, mild dermatitis appeared in all DNCB treatment groups, and became more severe with time, and peaked at day 49. Dermatitis scores were consistently lower from day 35 to 56 in the DNCB + CIE group than in the DNCB + PBS or DNCB group (Fig. 1D).

Calophylluminophyllum extract reduced inflammation-mediated skin phenotype changes.

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

Skin sections were stained with H&E to examine hyperkeratosis layer thickness by micrometer. On day 56, that is, immediately after CIE treatment, skin tissues and blood samples were obtained from mouse per group under anesthesia. Histopathologic changes of skin were assessed using hematoxylin and eosin (HE) staining. The DNCB and DNCB + 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. Masson’s Trichrome stain for detection of collagen deposition for 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. Thus, the serum levels of histamine were examined. CIE inhibited the serum levels of histamine in DNCB-sensitized
mice. (Fig. 3A) IgE antibody production is associated with Th2 immune response. To examine the effect of CIE on Th2 response, we measured serum levels of IgE. Compared with AD-induced control mice, total IgE was significantly reduced by topical administration of CIE. (Fig. 3B)

Consistent with observed clinical features, the frequencies of scratching were markedly reduced in the DNCB + CIE group, compared to control (p<0.05). Once a week from the day 28 to 56, scratching behavior was evaluated by video-recording for 1 h. Scratching behaviors followed 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 isoprenylated coumarins that can be easily separated by phase-separation. This is the traditional method of producing the oil used for medicinal ointment.

In this study, we demonstrated that topical C. inophyllum extract reduced inflammatory signs—edema, hemorrhage, excoriation, scaling, dryness and behavior symptoms—itching, scratching in the skin of DNCB-induced mice. Mast cells release immunomediators such as histamine, which can be easily separated by phase-separation. This is the traditional method of producing the oil used for medicinal ointment.

Inophyllums and calophylloides were the major component in C. inophyllum extracts. In-vitro assays demonstrated that the crude extract at concentration of 50 μg/ml inhibited 77% and 88% cyclooxygenase and lipooxygenase activities, respectively, indicating its potential as anti-inflammatory agent. Our findings suggested that CIE also potently inhibited AD and 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.

ACKNOWLEDGMENT

We wish to express our appreciation to the laboratory of Yonseiuniversity and Biostandard, Seoul, KOREA and chief scientist YOUNG-WOOK JO and all staff of the department for their support.

REFERENCES


15. Bellini A, Mattoli S. The role of the fibrocyte, a bone marrow-derived mesenchymal progenitor, in reactive and reparative fibrosis. Lab Invest. 2007;87(9):858-870.


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.
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).