Topical application of Corchorus olitorius leaf extract ameliorates atopic dermatitis in NC/Nga mice

Satoshi Yokoyama1*, Keiichi Hiramoto1, Takahiko Fujikawa1, Hiroya Kondo2, Nobuyuki Konishi2, Shu Sudo1, Makoto Iwashima1 and Kazuya Ooi1

*Correspondence: s-yoko@suzuka-u.ac.jp

1Faculty of Pharmaceutical Sciences, Suzuka University of Medical Science, Mie, Japan.
2Mie Prefecture Agricultural Research Institute, Matsuzaka, Mie, Japan.
3Research and Development Department, Fundamental Research Division, Mikimoto Pharmaceutical & CO. Ltd., Ise, Mie, Japan.

Abstract

Background: Corchorus olitorius leaves are rich in antioxidants, fatty acids, minerals, vitamins and mucilaginous polysaccharides, and have been used as traditional folk medicine. In a previous study, we found that Corchorus olitorius extract reduced transepidermal water loss, and increased skin hydration in atopic dermatitis (AD)-like lesions in NC/Nga mice. The aim of this study was to investigate the effects of topical application of Corchorus olitorius leaf extract on atopic dermatitis (AD), and to elucidate the mechanism underlying the ameliorating effect of COEE on AD-like skin lesions.

Methods: NC/Nga mice housed under specific pathogen-free (SPF) and conventional conditions were each divided into three groups (control, COEE, base cream). At the start of the experiment, the AD scores on the rostral skin of SPF mice and conventional mice were 0 and 8, respectively. We prepared the COEE cream, and applied it on the rostral skin in NC/Nga mice, and then performed a macroscopic evaluation, an enzyme-linked immunosorbent assay to analyze the plasma levels of immunoglobulin E (IgE) and histamine and immunohistochemical staining for tryptase, matrix metalloproteinase-9 (MMP-9) and collagen type IV.

Results: After 14 days of treatment with the COEE cream under conventional conditions, the AD scores and plasma IgE concentrations in the COEE group were significantly lower than those in the other groups. Compared to the control and BC groups, the expression levels of tryptase and MMP-9 were lower, and the degradation of collagen type IV at the basement membrane area was not observed in the COEE group by immunohistochemistry. The mice housed under the SPF conditions were not affected by the test creams.

Conclusions: Our results indicate that COEE may therefore be a useful therapeutic candidate for AD due to its suppression of the plasma IgE level and degranulation of mast cells.

Keywords: Atopic dermatitis, Corchorus olitorius, immunoglobulin E, histamine, tryptase

Introduction

The epidermis serves as the first line of defense against invading pathogens and allergens [1]. In atopic dermatitis (AD)-like skin lesions, this epidermal skin barrier is disrupted, which is reflected by increased transepidermal water loss (TEWL), as well as reduced hydration of the stratum corneum. Further, the penetration of pathogens and allergens through the skin is facilitated, which can trigger an inflammatory response via synergistic immunological mechanisms [2]. The inflammatory response increases the plasma immunoglobulin E (IgE) levels, and under inflammatory conditions, an increased number of mast cells can be found in the dermis, which play a crucial role in the pathophysiology of AD. In AD-like skin lesions, allergen induced cross-linking of IgE-binding sites on mast cells is followed by an explosive release of granular mediators. Thus, mast cells release histamine and tryptase into the extracellular matrix during the degranulation process [3]. Excess histamine causes an inflammatory response and pruritus. Pruritus is the most disturbing symptom of AD. Severe pruritus often leads to an “itch-scratch” cycle that may compromise the epidermal barrier, resulting in TEWL elevation and reduced skin hydration [4,5]. One of the reasons for the TEWL elevation is the disruption of the basement membrane [6]. The basement membrane is mainly composed of collagen type IV. Disruption of this collagen is related to the tryptase activity. Tryptase is also released from mast cells, and activates matrix metalloproteinase-9 (MMP-9). MMP-9 also degrades collagen type IV [7]. The degradation of collagen type IV induces the breakdown of the basement membrane, and may increase the TEWL. Thus, it presumed that suppressing the degranulation of mast cells would prevent the disruption of the skin barrier function.

Corchorus olitorius is a plant from the Tiliaceae family from the Mediterranean region, the leaves of which are rich in antioxidants, such as vitamin C, vitamin E, β-carotene, α-tocopherol, glutathione and phenols [8]. The leaves also contain fatty acids, minerals, other vitamins and mucilaginous

© 2014 Yokoyama et al; licensee Herbert Publications Ltd. This is an Open Access article distributed under the terms of Creative Commons Attribution License (http://creativecommons.org/licenses/by/3.0). This permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
polysaccharides, and have been used as traditional folk medicine. We previously developed a cream formulation containing Corchorus olitorius leaf Extract Excluding high molecular weight compounds (COEE) like polysaccharides, and investigated the skin hydration effect of this cream.

NC/Nga mice were established as an inbred strain from Japanese fancy mice in 1957, and have recently been shown to spontaneously develop AD-like dermatitis with IgE hyper-production under air-uncontrolled, conventional circumstances [9]. Our previous study showed that the COEE cream increased the skin hydration and reduced the TEWL on the AD-like skin lesions in NC/Nga mice, and protected the skin barrier function in these mice. Further, the COEE cream improved the AD symptoms, and suppressed the increases in the AD score [10]. We hypothesized that COEE might be effective against AD. This study was performed to evaluate the effects of COEE on the development of AD-like skin lesions in NC/Nga mice by measuring the symptom severity, plasma IgE and histamine concentrations and by performing an immunohistochemical examination. The effect of COEE might be associated with its suppression of the immunological and inflammatory response in AD.

Materials and methods

Experimental animals
Six-week-old male NC/Nga mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were divided into two groups: specific pathogen-free (SPF; n=9) and conventional (n=15) group. In each group, the mice were further divided into three groups: control, COEE and base cream (BC) (n=3 in each SPF group, n=5 in each conventional group). The mice in the conventional group were housed under conventional conditions, while those in the SPF group were housed under SPF conditions. All mice were individually housed throughout the experiment (therefore, under social isolation stress) with access to water and food ad libitum. The experimental protocol for this study was approved by the animal care regulations of Osaka City University Medical School.

Preparation of the COEE
Dry powdered Corchorus olitorius leaves were purchased from Mie Prefecture Agricultural Research Institute. This sample was extracted three times for 48 h at room temperature using acetone. To isolate the highly polar fraction, the residue was further extracted with methanol:H_2O (2:1) at 85°C under reflux conditions for three hours. After filtration and freeze-drying, this extract was used for the formulation of the COEE cream. The COEE produced by our extraction methods described above contains few compounds over 1,000 molecular weight. However, various phenols and fatty acids are contained in the COEE, which was confirmed by thin-layer chromatography.

Formulation of the COEE cream
The COEE cream contained 0.2% extract of Corchorus olitorius leaf in the base cream, with the pH adjusted to approximately 6.0. The other ingredients, in order of decreasing concentration, were water, glyceryl tri(2-ethylhexanoate), acrylic acid, alkyl methacrylate copolymer, 1,3-butandiol, L-arginine, 1,2-dihydroxypentane, 2-hydroxyethyl phenyl ether and hydrogenated egg yolk phospholipids (Table 1).

Table 1. The ingredients in COEE cream and base cream.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>COEE cream (%)</th>
<th>Base cream (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COEE</td>
<td>0.2</td>
<td>--</td>
</tr>
<tr>
<td>Water</td>
<td>52.1</td>
<td>52.3</td>
</tr>
<tr>
<td>Glyceryl tri(2-ethylhexanoate)</td>
<td>18.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Acrylic acid-alkyl methacrylate copolymer</td>
<td>16.0</td>
<td>16.0</td>
</tr>
<tr>
<td>1,3-butandiol</td>
<td>6.8</td>
<td>6.8</td>
</tr>
<tr>
<td>L-arginine</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>1,2-dihydroxypentane</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>2-hydroxyethyl phenyl ether</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Hydrogenated egg yolk phospholipids</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

COEE: Corchorus olitorius leaf extract excluding the high molecular weight compounds

Application of formulations to the rostral skin of mice
The skin on the back of each mouse was shaved with electric clippers and used as a sensitizing area. The rostral skin of the COEE group mice was treated with 0.1 g of COEE cream once a day. This treatment was continued for 14 days. The rostral skin of the BC group mice was treated with 0.1 g of the base cream without COEE.

Evaluation of the severity of AD
The lesions on the rostral skin were assessed macroscopically according to the following four symptoms: erythema, edema, erosion and dryness, and the sum was considered to be the individual’s score (0: no symptoms; 1, mild; 2, moderate; 3: severe) [9,11]. At the start of this experiment, the AD score in the SPF and conventional groups were 0 and 8, respectively. Then, the skin lesions were examined and the severities were scored 14 days after the application of each formulation. These visual assessments were performed by at least two independent investigators. The changes in the rostral skin symptoms of the NC/Nga mice were evaluated by viewing photographs of the mice.

Isolation of rostral skin samples and blood
Mice were sacrificed 14 days after initiating the application of
the test creams. Approximately 1 mL of blood was withdrawn from the heart. Skin biopsies obtained from the rostral skin were fixed in phosphate-buffered saline containing 4% paraformaldehyde.

Analysis of the plasma IgE and histamine concentrations
The plasma was fractionated from collected blood samples by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was used for the IgE and histamine assays. The plasma IgE and histamine concentrations were measured using an enzyme linked immunosorbent assay kit (YamasaShoyu Co., Ltd., Chiba, Japan and Bertin Pharma, Montigny le Bretonneux, France, respectively) according to the manufacturers’ instructions. The optical density was measured with a microplate reader (Molecular Devices, Sunnyvale, CA).

Histopathological and immunohistochemical analysis
Fixed skin specimens were embedded in frozen Tissue Tek OCT compound, and cut into 5 µm thick sections. Thin sections were stained with hematoxylin-eosin and analyzed histologically to evaluate the degree of inflammation. Other thin sections, after being washed with phosphate-buffered saline (PBS), were incubated with a goat anti-mast cell tryptase (1:50) polyclonal antibody (Santa Crus Biotechnology Inc., Santa Cruz, CA), rabbit anti-MMP-9 (1:50) polyclonal antibody (Abnova, Taipei, Taiwan) or a rabbit anti-collagen type IV (1:100) polyclonal antibody (Abcam, Tokyo, Japan) overnight at 4°C. The specimens were then washed in PBS, and were incubated at room temperature for 2 h with TRITC conjugated anti-goat immunoglobulin or FITC-conjugated anti-rabbit immunoglobulin (1:30; DakoCytomation, Denmark). The expression of tryptase, MMP-9 and collagen type IV was evaluated immunohistochemically under fluorescence microscopy using Image J software program.

Statistical analysis
All data are presented as the means±SD. The comparison of the AD scores was performed using the Wilcoxon rank-sum test. Comparisons among three groups were performed using a one-way analysis of variance (ANOVA), with Tukey’s test for multiple comparisons used for the post-hoc analysis. Values of p<0.05 were defined as statistically significant.

Results
Evaluation of the severity of AD
There were no symptoms associated with AD after the 14 days of application on the skin of the SPF mice. On the other hand, the conventional mice in the control group after 14 days exhibited marked symptoms characteristic of AD, including edema, erythema and hemorrhage of their rostral skin. The dermal symptoms were ameliorated in the COEE group after the 14 day application (Figure 1).

At the start of this experiment, the AD scores of SPF group and conventional group were 0 and 8, respectively. After the 14 days of application of the cream, the AD scores of the SPF groups did not change. In contrast, the symptoms or dermatitis in the conventional control mice significantly increased in severity (p<0.05), while the AD score in the conventional COEE group significantly decreased (p<0.05). There was no significant change in the AD score in the BC group after the application compared to the value at baseline (Figure 2).

Histopathological changes after 14 days of applications
The morphological findings are shown in Figure 3. Histopathological changes were not detected between the control (Figure 3a), COEE (Figure 3b) and BC (Figure 3c) groups under the SPF condition. In the conventional control group (Figure 3d), notable acanthosis, hyperplasia of the epidermis, ulceration and infiltration of large numbers of lymphocytes to the dermis were evident. In the conventional BC group (Figure 3f), the severity of dermatitis was also exacerbated after 14 days. On the other hand, the hyperplasia and thickening of the epidermis were obviously suppressed, and there was minimal inflammatory cell infiltration in the conventional COEE group (Figure 3e).

Plasma IgE and histamine concentrations
No plasma samples showed evidence of hemolysis, as assessed by visually examining the color. There were no significant differences in the plasma IgE concentrations among the control, COEE and BC groups under the SPF condition. On the
**Figure 2.** The effects of COEE on the atopic dermatitis score for the rostral skin of NC/Nga mice under conventional conditions. The scoring was performed at the start of the experiment and after 14 days of application. The values represent the means±S.D. (n=3 in each SPF group, n=5 in each conventional group). Control: not exposed to any topical preparation, COEE: treated with a cream containing Corchorus olitorius leaf extract excluding high molecular weight compounds, BC: treated with the base cream, *: p<0.05 compared with the values at the start of the experiment (Wilcoxon rank-sum test).

**Figure 3.** The effects of COEE on the histopathological changes in the skin lesions in NC/Nga mice under SPF and conventional conditions. The rostral skin samples from the mice were collected on the final day of the protocol, and the sections were subjected to H&E staining. Control: not exposed to any topical preparation, COEE: treated with a cream containing Corchorus olitorius leaf extract excluding high molecular weight compounds, BC: treated with the base cream.

On the other hand, the plasma IgE concentrations of the conventional control and BC groups were markedly increased. However, the plasma IgE concentration in the conventional COEE group was significantly lower compared to that in the conventional control or BC group (Figure 4).

**Figure 4.** The effects of COEE on the plasma IgE concentrations in NC/Nga mice housed under conventional condition. The topical application of COEE significantly reduced the IgE concentration under conventional conditions. The data are presented as the means±S.D. (n=3 in each SPF group, n=5 in each conventional group). *: p<0.05, **: p<0.01 (one-way ANOVA followed by Tukey’s test). Control: not exposed to any topical preparation, COEE: treated with a cream containing Corchorus olitorius leaf extract excluding high molecular weight compounds, BC: treated with the base cream.

**Figure 5** shows the plasma histamine concentrations in the conventional groups. The plasma histamine concentrations of the SPF group were equal among the control, COEE and BC groups. Under the conventional condition, the plasma histamine concentrations in the control, COEE and BC groups were all higher than those under the SPF condition. However, the plasma histamine concentration in the conventional COEE group was significantly lower than that in the conventional control group, and tended to be lower than that in the BC group.

Immunohistochemical examination of the tryptase, MMP-9 and collagen type IV expression in the skin

**Figures 6 and 7** show photographs of the rostral skin sections stained for tryptase and MMP-9 in the epidermis of the conventional and SPF mice. No expression of tryptase or MMP-9 in the SPF group was observed. Under the conventional condition, the expression levels of tryptase and MMP-9 in the control group were clearly increased. In the conventional BC group, the expression levels of tryptase and MMP-9 were lower than those in the conventional control group, but were still elevated compared to the SPF group. The expression
levels of tryptase and MMP-9 were markedly reduced in the conventional COEE group, and were similar to those of the SPF group.

Under the SPF condition, the expression of collagen type IV was observed in all of the mice. On the other hand, the expression level of the collagen type IV in the conventional control group and conventional BC group was lower than that in the SPF groups. In the conventional COEE group, the expression level of the collagen type IV was similar to that in the SPF group (Figure 8).

Discussion
In this study, we investigated whether COEE could ameliorate the severity of AD-like skin lesions in NC/Nga mice. Our results showed that topical treatment of the rostral AD-like skin of NC/Nga mice with the COEE cream improved the AD score and symptoms characteristic of AD (hyperplasia and a thickened epidermis). It seemed that topical application of the base cream prevented the aggravation of AD, and when COEE was added to the base cream, it improved the AD symptoms. The plasma IgE levels are generally elevated in AD. IgE can bind the high-affinity IgE receptor, and leads to mast cells degranulation and their secretion of histamine and tryptase [12]. The COEE cream led to a reduction of the plasma IgE level and suppressed the histamine and tryptase secretion from mast cells, and also inhibited the disruption of collagen type IV by MMP-9.

IgE production involves the type 2 helper T cell (Th2)-biased response. Th2 cells play a central role in the allergic inflammatory response. The Th2 cytokines, such as interleukin (IL)-4 and IL-13, stimulate the B cells and induce the production of allergen-specific IgE [13]. It is accepted that some natural dietary substances have the ability to suppress the elevation of the IgE level during the inflammatory response. Previous studies demonstrated that Konjac glucomannnann was able to prevent the increase in the plasma IgE and epsilon germline transcript levels due to its inhibition of the class-switching of B cells [14]. Fucoidan is also a natural dietary substance,
and reduces the IgE production by preventing the activation of the nuclear factor kappa B-mediated pathways by CD40 [15]. Our findings implied that there might be some natural substance(s) contained in the COEE that suppress the class switching of B cells. Konjacglucamannann and fucoidan were administered orally, but COEE was administered by topical application. However, the oral administration of COEE may also have the ability to attenuate AD symptoms, and this should be evaluated in future studies.

Corchoionoside A, B and (6S, 9R) roseoside are ionone glucosides contained in the leaves of Corchorus olitorius. These compounds were previously shown to inhibit the histamine release from rat peritoneal exudate cells induced by the antigen-antibody reaction [16]. The same effect was found in our in vivo study. Histamine, a potent mediator of inflammatory responses, induces pruritus, leading to the “itch-scratch” cycle [17], and upregulates MMP-9 production [18]. Tryptase, which is also an inflammatory marker released from mast cells, either alone or in conjunction with the activation of MMPs, can participate in extracellular matrix damage and possibly in the destruction of the basement membrane [7]. Our investigation revealed that the COEE suppressed the release of histamine and tryptase from mast cells and prevented the degradation of skin tissue.

Another possible mechanism was suggested by the fact that the extract from C. Olitorius leaf has antioxidant properties [19]. Corchorus olitorius leaf was found to contain high quantities of antioxidant molecules such as phenols, flavonoids, ascorbic acid and carotenoids [8]. Reactive oxygen species (ROS) are generally released from neutrophils, macrophages and eosinophils during the inflammatory response. Although ROS play a role in excluding allergens, greatly increased levels of ROS contribute to the development of skin tissue disruption, and increase the severity of AD [20]. Mast cells stimulated by IgE produce endogenous ROS, and these ROS have important functions in regulating various mast cell

Figure 7. The results of the immunohistochemical analysis of the MMP-9 expression in the skin of NC/Nga mice.
(a) The MMP-9 expression in the conventional COEE group was decreased compared with that in the conventional control and BC groups. Control: not exposed to any topical preparation, COEE: treated with a cream containing Corchorus olitorius leaf extract excluding high molecular weight compounds, BC: treated with the base cream. The scale bar represents 100 µm.
(b) Comparison of MMP-9 positive cell counts among three groups in conventional conditions. *: p<0.05, **: p<0.01 (one-way ANOVA followed by Tukey’s test).

Figure 8. The results of the immunohistochemical analysis of the collagen type IV expression in the skin of NC/Nga mice.
(a) The collagen type IV expression in the conventional control and BC groups was decreased compared with that in the conventional COEE group. Control: not exposed to any topical preparation, COEE: treated with a cream containing Corchorus olitorius leaf extract excluding high molecular weight compounds, BC: treated with the base cream. The scale bar represents 100 µm.
(b) Comparison of fluorescent intensity of collagen type IV among three groups in conventional conditions. a.u.: arbitrary unit, *: p<0.05, **: p<0.01 (one-way ANOVA followed by Tukey’s test).
responses, including degranulation, leukotriene secretion and cytokine production, which lead to the disruption of the skin’s structure [21]. Thus, the antioxidants contained in COEE might lead to the suppression of AD. Moreover, COEE contains fatty acids. Fatty acids have the ability to exert anti-inflammatory effects [22,23]. Therefore, the phenols and fatty acids included in COEE may have ameliorated the aggravation of AD in NC/Nga mice.

The mechanism by which the COEE suppresses IgE production remains unclear, and further studies are needed on this point, but our study revealed that COEE suppressed the IgE production and attenuated the aggravation of AD. The limitations of this study are related to the fact that it was an animal study with low numbers, and because it is directly translate the conclusions drawn in mice to humans. However, with regard to the animal numbers, we performed the same experiments three times, and confirmed the reproducibility. Therefore, we believe that our results have high reliability. Further, the COEE cream can be feasibly and safely administered topically, because there was no influence of the COEE cream on the AD score or plasma inflammatory marker levels in SPF mice.

Conclusions
These results suggest that COEE may be a useful therapeutic candidate for AD. However, the effects of the COEE on human AD still remain to be clarified and should be investigated in further studies. We are hoping to elucidate and clarify this issue in the future.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

<table>
<thead>
<tr>
<th>Author’s contributions</th>
<th>SY</th>
<th>KH</th>
<th>TF</th>
<th>HK</th>
<th>NK</th>
<th>SS</th>
<th>MI</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Research concept and design</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Collection and/or assembly of data</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Data analysis and interpretation</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Writing the article</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Critical revision of the article</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Final approval of article</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>✓</td>
<td>✓</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>✓</td>
</tr>
<tr>
<td>Contributed essential reagents and tools</td>
<td>--</td>
<td>--</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

Publication history
EIC: Katalin Csiszar, University of Hawaii, USA.
Received: 14-Jan-2014 Revised: 19-Feb-2014
Accepted: 17-Mar-2014 Published: 21-Mar-2014

References


Citation: