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Momordica charantia Ameliorates Atopic Dermatitis by Inhibiting the Expression of Inducible Nitric Oxidase Synthase in NC/Nga Mice

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Abstract

Introduction: *Momordica charantia* (MC) has been reported to possess various beneficial effects. Improvement in natural aging of the skin has been observed with the use of MC. However, few studies have detailed the effects of MC on atopic dermatitis (AD). Therefore, in this study, we investigated the effects of MC on the skin symptoms of AD. **Methods:** Specific pathogen-free and conventional NC/Nga mice were orally administered a 50 mg/kg/day dose of MC every day for 2 weeks. **Results:** The expression levels of lipopolysaccharide (LPS), inducible nitric oxidase synthase (iNOS), and prostaglandin E2 (PGE2) remarkably increased in AD, but were suppressed by MC administration. As a result, the degradation of filaggrin by PGE2 was suppressed. Furthermore, in AD, iNOS induced macrophage type 1 and increased NO levels. In contrast, due to suppression of iNOS with MC administration, macrophages shifted to type 2 and an increase in L-ornithine was observed, which subsequently promoted filaggrin synthesis. **Conclusions:** These findings indicate that the AD-like skin symptoms were decelerated by MC via the regulation of the LPS/iNOS/PGE2/filaggrin and LPS/iNOS/Arginase 1/L-ornithine/filaggrin signaling pathways.

Keywords

Atopic Dermatitis (AD), *Momordica charantia* (MC), LPS, iNOS, Filaggrin

1. Introduction

Common symptoms of atopic dermatitis (AD) include dry, itchy skin, and red

rashes, particularly in infants and children. AD is either acute or chronic. Both forms involve thickened skin with an acidic pH, reduced stratum corneum hydration, and elevated transepidermal water loss and erythema index due to changes at the tissue and cellular levels [1]. In 2006, variation in the filaggrin gene was reported to be an important factor for the onset of AD [2]. Filaggrin is a protein of the horny layer that plays an important role in the skin barrier function. Filaggrin is produced as profilaggrin with a 10 - 12 filaggrin repeat structure. It has a molecular weight of approximately 400 kDa and becomes a 37 kDa filaggrin by the action of various proteases [3] [4]. Meanwhile, filaggrin plays a role in maintaining the strength and plasticity of the horny layer. In the absence of filaggrin, there is an increase in transepidermal water loss (TWEL), and horny cells separate easily [5]. In the crust region of the horny layer, filaggrin is decomposed further and becomes a natural moisturizing factor, such as an amino acid and urocanic acid. In an AD mouse model, inhibition of filaggrin gene expression was observed, which led to a decrease in skin barrier function. However, skin barrier function can be improved by accelerating the expression of filaggrin [6].

Momordica charantia (MC) is a plant that belongs to the Cucurbitaceae family, which exerts several beneficial effects. It is commonly known as bitter gourd, balsam pear, bitter melon, kugua, or karela [7]. The plant lives up to its common name "bitter melon" or "bitter gourd," as the fruits of the plant have a bitter taste [8] [9]. MC is a traditional herbal medicine that possesses various pharmacological functions, including antidiabetic, abortifacient, anthelmintic, contraceptive, antimalarial, and laxative functions. It is used to treat dysmenorrhea, eczema, gout, jaundice, leprosy, poles, pneumonia, psoriasis, rheumatism, and scabies [10] [11] [12]. Several medicinal properties of MC, including its hypoglycemic, anti-bacterial, anti-viral, anti-tumor, immunomodulatory, anti-oxidant, anti-diabetic, anthelmintic, antimutagenic, antilipolytic, antifertility, hepatoprotective, and anti-inflammatory activities, as well as anti-ulcerogenic, anti-oxidative, and immune-modulatory activities [13] [14] [15], have been studied. We previously reported that natural aging of the skin is ameliorated in MC-administered mice [16]. This effect due to MC administration is induced via the regulation of 17β-estradiol/mast cell/matrix metalloprotease (MMP)-1/hyaluronidase (HYAL) 2 and testosterone/mast cell/interleukin (IL)-33 signaling pathways. Many studies have demonstrated that MC is a good natural source of antioxidants under experimental conditions; it possesses antioxidant activity *in vitro* and *in vivo* [17] [18] [19]. In addition, MC signifies depressed macrophage infiltrators in epicardial adipose tissues (EAT) and brown adipose tissues (BAT) of rats fed a high-fat diet, and downregulated the expression of the pro-inflammatory cytokine, monocyte chemotactic protein-1, tumor necrosis factor (TNF)-α and IL-6 in EAT [20]. Moreover, MC attenuated inflammatory stress in mice with sepsis by reducing the secretion of pro-inflammatory cytokines and the expression of cyclooxygenase (COX)-2, inducible nitric oxide synthase [iNOS], and nuclear factor [NF]-κB associated with inflammation [21].

Thus, MC is closely related to inflammation. In AD, inflammatory cytokines, prostaglandin (PGE) 2, COX-2, and reactive oxygen species (ROS) build up the tangle symptoms intricately. However, the influence which exert on the AD of MC is unclear. Furthermore, the relationship with filaggrin, which is an important onset factor of AD, is unknown. In this study, we orally administered MC to an AD mouse model and investigated the effect of MC on AD.

2. Materials and Methods

2.1. Animal Experiments

Conventional and specific-pathogen-free (SPF) 8-week-old male NC/Nga mice (SLC, Hamamatsu, Shizuoka, Japan) were used. Mice were individually maintained in cages in an air-conditioned room at 23˚C ± 1˚C with a 12-h light/12-h dark cycle. SPF mice were maintained under SPF conditions during the experimental procedures. Conventionally maintained mice spontaneously started to exhibit symptoms characteristic of AD at 7 weeks of age (under uncontrolled, normal maintenance conditions). As expected, all of the conventionally maintained NC/Nga mice used during the experiment exhibited AD-like symptoms that were scored based on severity, including edema, erythema, and hemorrhage (0, none; 1, slight; 2, moderate; 3, severe), as described previously [22]. Mice were divided into the following groups (n = 6/group): SPF mice, solvent-administered SPF mice, MC-administered SPF mice, conventional mice with AD, solvent-administered conventional mice with AD, and MC administered conventional mice with AD. All mice were examined simultaneously. After administering MC to the mice for 2 weeks, samples were collected on the final day of examination. This study was approved by the Suzuka University of Medical Science Animal Experiment Ethics Committee on September 25, 2014, and was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Suzuka University of Medical Science (Approval number: 34). All surgeries were performed under pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

2.2. MC Treatment

The MC fruit extract was provided by ChromaDex, Inc. (Irvine, CA, USA). Approximately 50 mg/kg body weight of MC in distilled water (DW) was orally administered to mice every day for 2 weeks. The solvent administration group was given distilled water [23].

2.3. L-NAME Treatment

Mice were intraperitoneally injected with 20 mg/kg L-nitro-arginine-methyl ester (L-NAME; antagonist of NOS; Sigma, St Louis, MO, USA) dissolved in 0.1 M phosphate buffer (pH 7.2) every day for 2 weeks [24]. The same volume of phosphate buffer was administered to the control group.

2.4. Arginase Inhibitor 1 Treatment

Mice were orally administered 10 mg/kg arginase inhibitor 1 (Med Chem Express, Monmouth Junction, NJ, USA) dissolved in 0.9% saline every day for 2 weeks [25]. The same volume of saline was administered to the control group.

2.5. Preparation and Staining of the Dorsal Skin

For histological studies, mice were sacrificed 2 weeks after the start of the experiment. Skin specimens were fixed in 4% phosphate-buffered paraformaldehyde, embedded in frozen Tissue Tek, OCT compound (Sakura Finetek, Tokyo, Japan), and cut into 5-μm-thick sections. The presence of filaggrin was immunohistochemically evaluated by staining the specimens with rabbit polyclonal anti-filaggrin (1:100; Covance, Emeryville, CA, USA) primary antibody, as previously described [26]. The specimens were subsequently incubated with a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody (1:30; Dako Cytomation, Glostrup, Denmark).

2.6. Western Blotting

Western blotting was performed as previously described [27]. Briefly, the samples were separated by electrophoresis. Further, the membranes were incubated at 25˚C for 1 h with primary antibodies against iNOS (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA), Arginase 1 (1:1000, GeneTex Inc., Hsinchu City, Taiwan), ionized calcium binding adapter protein 1 (Iba1) (marker of macrophage: 1:1000, Wako Pure Chemical Institutes, Ltd., Osaka, Japan), chemokine receptor 7 (CCR7) (marker of M1 macrophage; 1:1000, Abcam, Cambridge, MA, USA), CD163 (M2 macrophage marker; 1:1000, Abcam), or β-actin (1:5000; Sigma-Aldrich Corp., St. Louis, MO, USA). Immune complexes on the membranes were visualized by incubation with horseradish peroxidase-conjugated secondary antibody (1:1000; Norvex, Frederick, MD, USA) and ImmunoStar Zeta reagent (Wako Pure Chemical Institutes, Ltd.). Images were acquired using the Multi-Gauge Software program (Fujifilm, Greenwood, SC, USA).

2.7. Measurement of IgE Level in Plasma and LPS, PGE_2, COX2, and Nitric Oxide (NO) Levels in the Skin

The IgE plasma level and PGE_2 and COX2 skin levels were determined using a commercial enzyme-linked immunosorbent assay kit (IgE: Yamase Shoyu Co., Chiba, Japan; PGE_2: Enzo Life Sciences Inc., Farmingdale, NY, USA; COX2: Abcam). The levels of LPS and NO in the skin were determined using a commercial assay kit (LPS: GenScript, Piscataway, NJ, USA; NO: BioAssay Systems, Hayward, CA, USA), in accordance with the manufacturer's instructions.

2.8. L-Ornithine Analysis

The preparation of amino acid samples for determination in free fractions was performed as described by Muramatsu *et al.* [28], except that 2% (w/v) perchloric acid was used for deproteinization instead of 10 % (w/v) trichloroacetic acid,

and the supernatant solution was neutralized to pH 7.0 with 5 M KOH. Amino acids in each sample were derivatized according to the method described by Gehrke *et al.* [29]. The levels of the derivatized amino acids were analyzed using a computer-controlled selected ion monitoring gas-chromatograph mass spectrometer (Shimadzu QP-1000, Shimadzu Co. Ltd., Kyoto, Japan).

2.9. Statistical Analysis

All data are presented as the mean ± standard deviation (SD). The results were analyzed using Microsoft Excel 2010 software (Microsoft Corp., Redmond, WA, USA). Differences between groups were evaluated by one-way ANOVA, followed by Tukey's post-hoc test, using the SPSS version 20 software (SPSS, Inc., Chicago, IL, USA). The results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of MC Treatment on the Severity of AD in NC/Nga Mice

We first established that AD-like symptoms (edema, erythema, and hemorrhaging) developed in the skin of 9-week-old conventional NC/Nga mice. These AD-like symptoms were improved in conventional NC/Nga mice treated with MC (**Figure 1(A)** and **Figure 1(B)**). Blood IgE levels, which are indicators of AD, increased in conventional NC/Nga mice but decreased with MC administration (**Figure 1(C)**). In SPF mice, no skin symptoms were observed in any group, and no increase in IgE was observed.

3.2. Effect of MC Treatment on the Skin Levels of LPS, iNOS, PGE_2, and COX2 in NC/Nga Mice

LPS, iNOS, PGE_2, and COX2 levels in the skin were increased in conventional NC/Nga mice; however a decrease was observed with MC administration (**Figures 2(A)-(D)**). These levels did not change in the skin of SPF mice.

3.3. Effect of MC Treatment on the Expression of NO, Ornithine, and Arginase I in NC/Nga Mice

The expression of NO, ornithine, and arginase I was affected by iNOS in the skin. The expression of NO in the skin was increased in conventional NC/Nga mice and a decrease was observed following MC administration (**Figure 3(A)**). In contrast, the expression of ornithine and arginase I in the skin did not increase in untreated NC/Nga mice; however, an increase was observed upon MC administration (**Figure 3(B)** and **Figure 3(C)**). No changes were observed in the expression of ornithine and arginase I in the skin of SPF mice.

3.4. Effect of MC Treatment on the Expression of Filaggrin in NC/Nga Mice

The expression of filaggrin in the dorsal skin was remarkably reduced in conventional NC/Nga mice, but it improved by MC administration (**Figure 4**). There was no change in the expression of filaggrin in the skin of SPF mice.

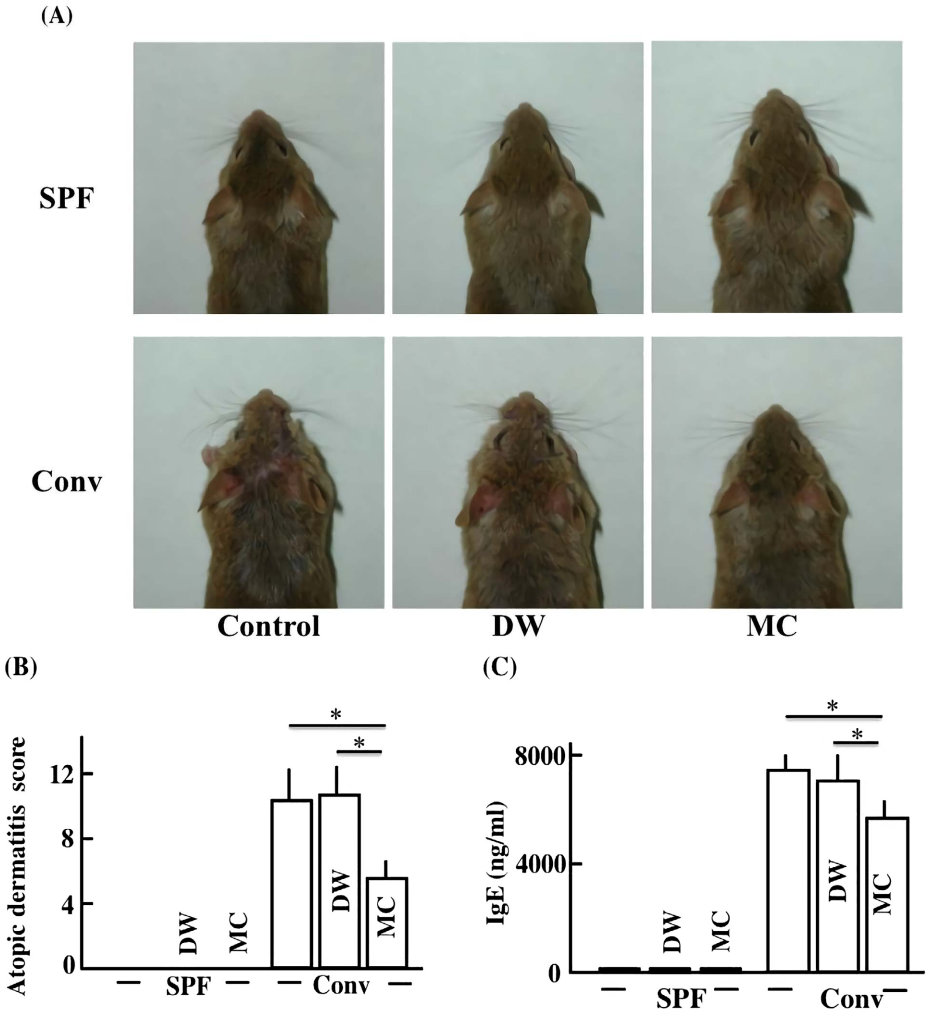
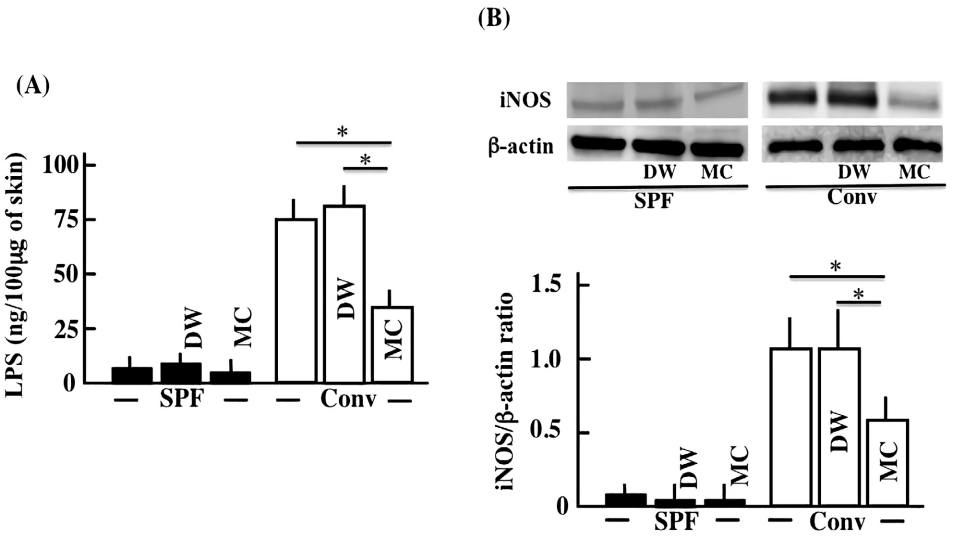


Figure 1. Effects of MC treatment on the skin of NC/Nga mice. (A) Assessment of AD-related skin symptoms, (B) AD score, and (C) plasma level of IgE. MC: *Momordica charantia.* DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05.



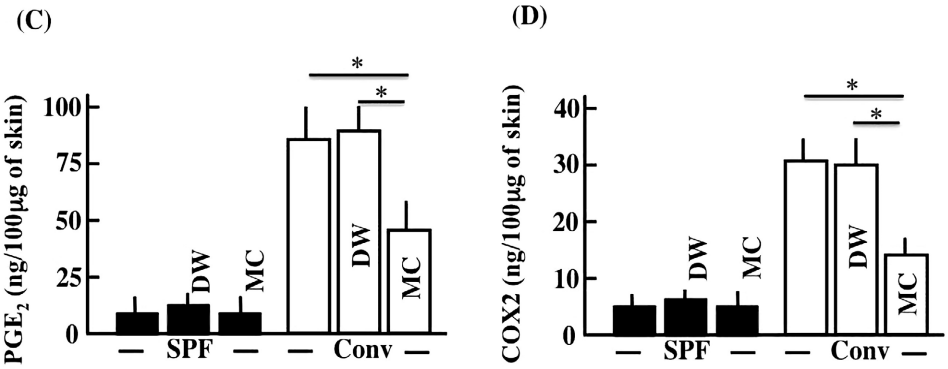


Figure 2. Effects of MC treatment on the skin levels of LPS (A), iNOS (B), PGE2 (C), and COX2 (D) in NC/Nga mice. MC: *Momordica charantia*. DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05.

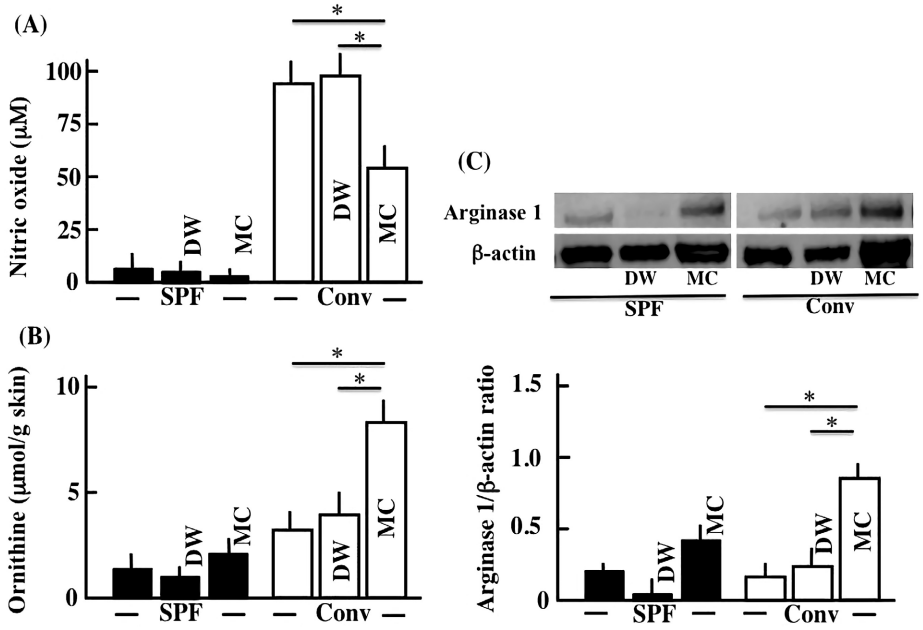


Figure 3. Effects of MC treatment on the skin levels of NO (A), L-ornitine (B), and arginase 1 (C) in NC/Nga mice. MC: *Momordica charantia*. DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05.

3.5. Effect of MC Treatment on the Expression of Iba1, CCR7, and CD163 in NC/Nga Mice

The expression of Iba1 (total macrophages) did not differ between the treatment groups (Figure 5(A)). The expression of CCR7 (M1 macrophages) was significantly reduced with the administration of MC (Figure 5(B)). However, a significant increase in CD163 (M2 macrophages) was observed upon MC administration (Figure 5(C)).

3.6. Effect of L-NAME Treatment on the Severity of AD and the Expression of Filaggrin in NC/Nga Mice

AD-like symptoms were improved in L-NAME-treated conventional NC/Nga

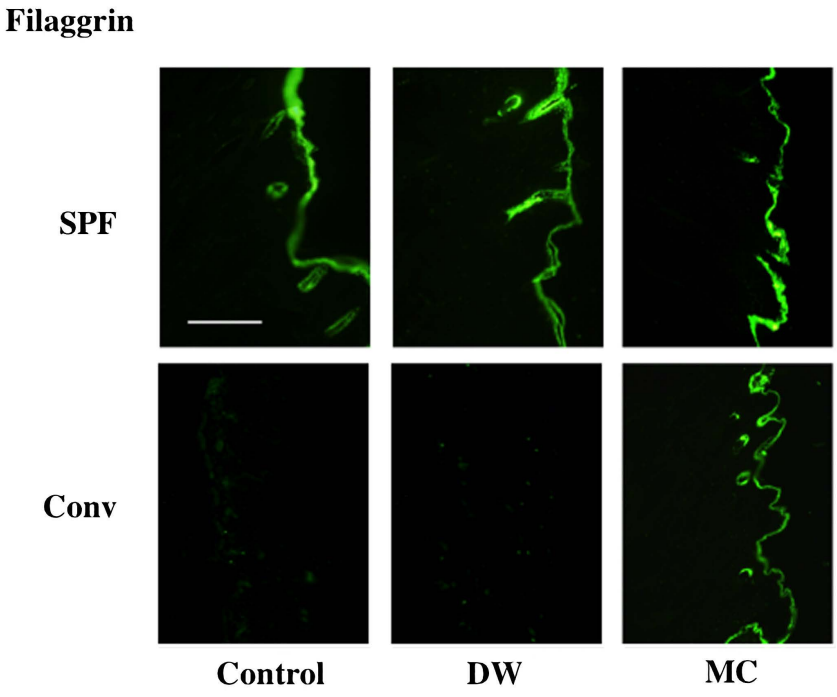


Figure 4. Effects of MC treatment on filaggrin expression in NC/Nga mice. MC: *Momordica charantia*. DW: distilled water. The data are from one representative experiment conducted with six mice. Scale bar = 100 μm.

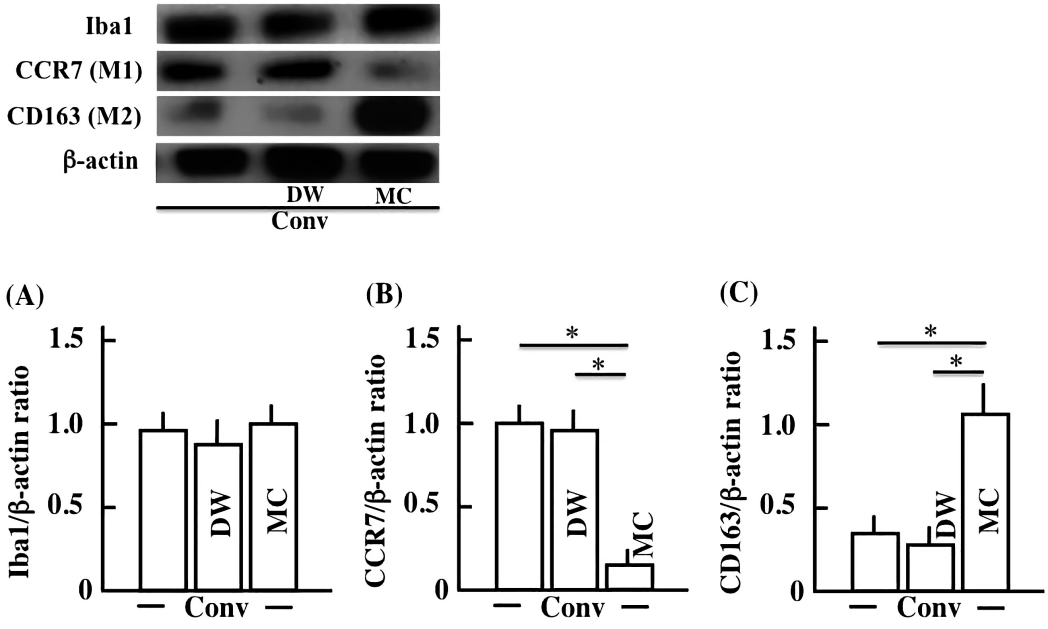


Figure 5. Effects of MC treatment on the expression of Iba1 (A), CCR7 (B), and CD163 (C) in NC/Nga mice. MC: *Momordica charantia*. DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05.

mice (Figure 6(A)). Further, the expression of filaggrin in the dorsal skin was remarkably reduced in conventional NC/Nga mice; however, an improvement in filaggrin expression was observed upon L-NAME administration (Figure 6(B)).

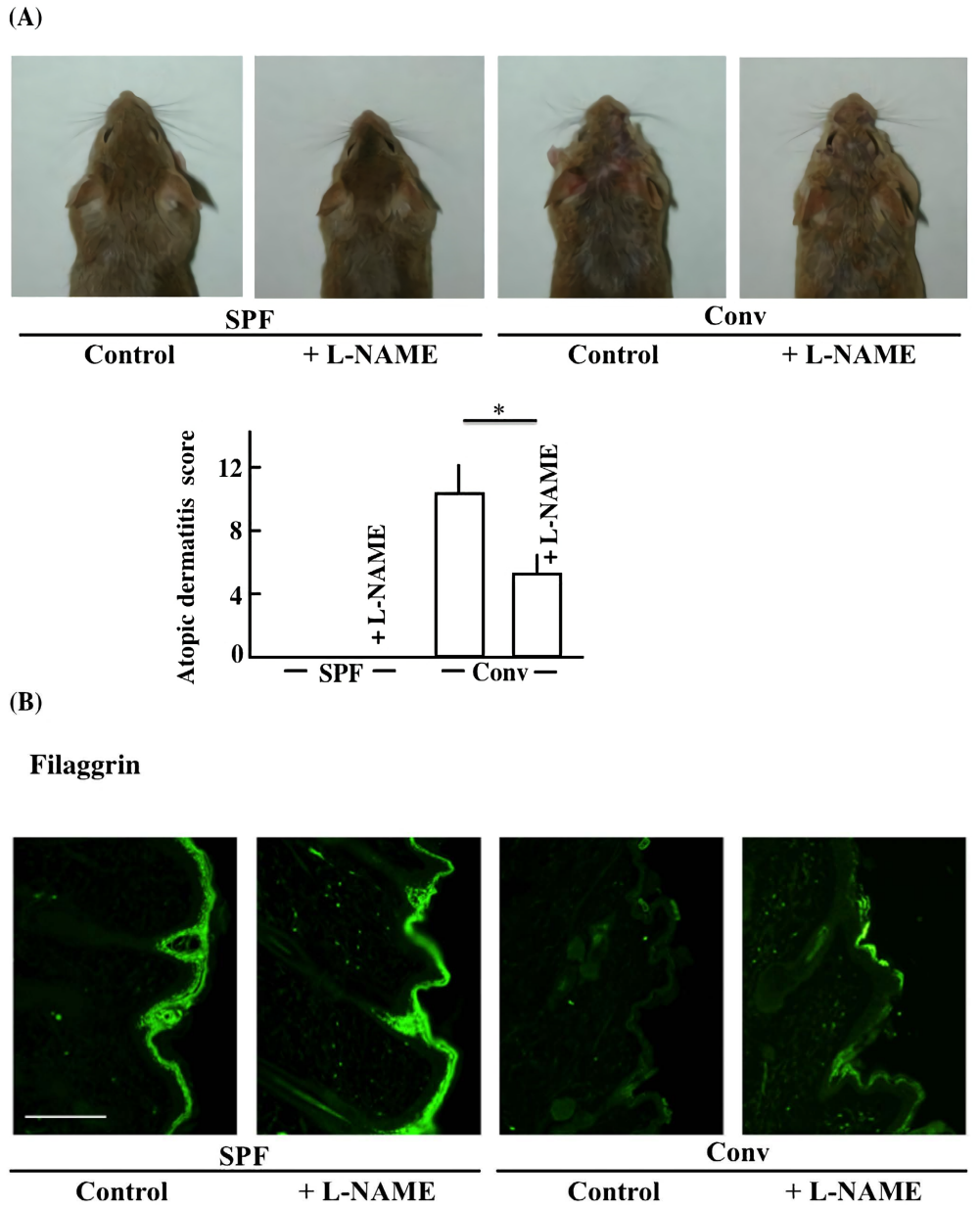


Figure 6. Effects of L-NAME (antagonist of NOS) treatment on skin symptoms in NC/Nga mice. (A) skin symptoms and AD score and (B) filaggrin expression in the skin. MC: *Momordica charantia*. DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05. The data are from one representative experiment conducted with six mice. Scale bar = 100 μm.

3.7. Effect of Arginase Inhibitor 1 Treatment on the Severity of AD and the Expression of Filaggrin in NC/Nga Mice

AD-like symptoms were improved with MC treatment; however, a decrease in the improvement effect was observed with the administration of arginase inhibitor 1 (**Figure 7(A)**). The expression of filaggrin in the dorsal skin was increased with MC treatment in conventional NC/Nga mice compared to that in untreated conventional NC/Nga mice; however, a decrease in filaggrin expression was observed upon administration of arginase inhibitor 1 (**Figure 7(B)**).

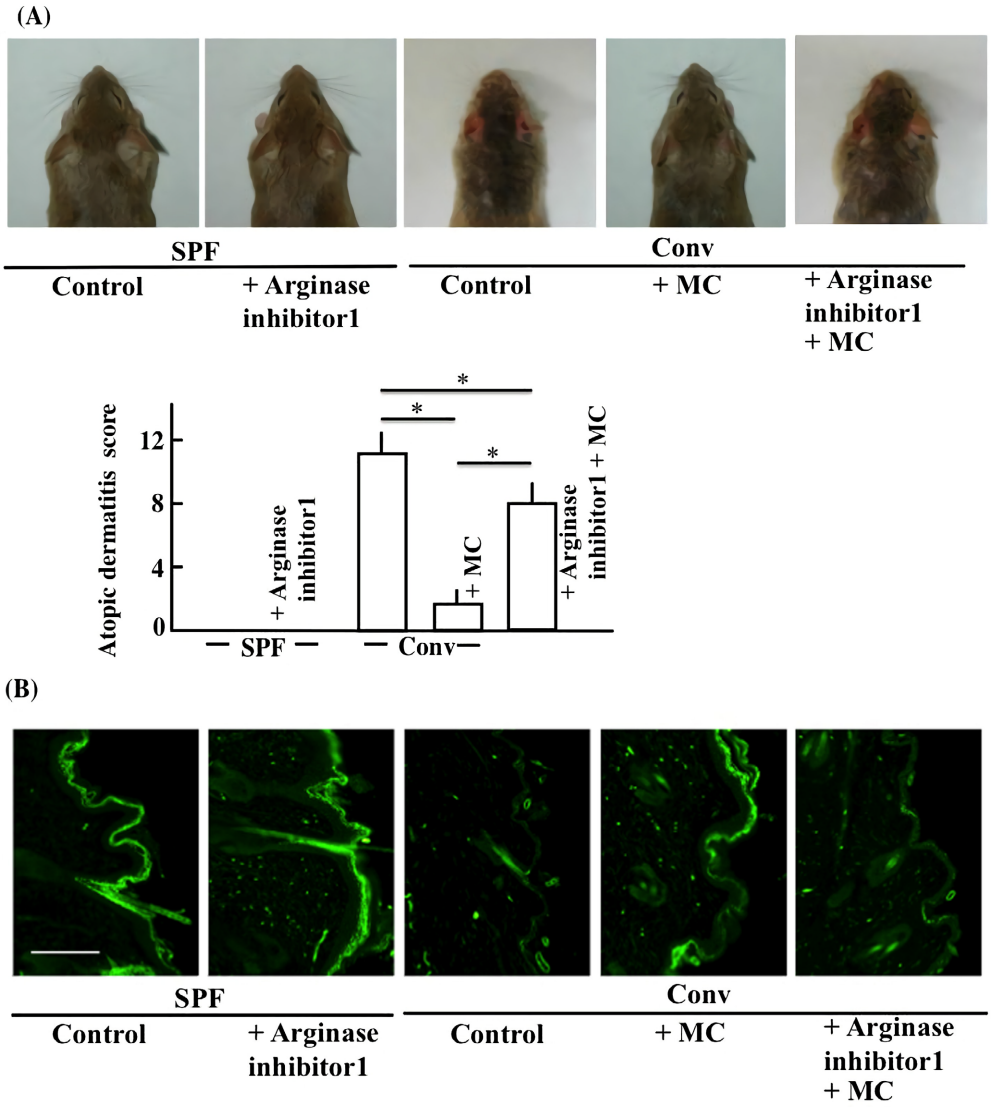


Figure 7. Effects of arginase inhibitor 1 treatment on skin symptoms in NC/Nga mice. (A) skin symptoms and AD score, and (B) filaggrin expression in the skin. MC: *Momordica charantia*. DW: distilled water. The values are expressed as mean ± SD derived from six animals. *P < 0.05. The data are from one representative experiment conducted with six mice. Scale bar = 100 μm.

4. Discussion

In this study, MC administration was found to improve AD-like symptoms. An increase was observed in the skin levels of LPS, iNOS, and PGE2 due to AD, which decreased upon MC administration. Furthermore, MC administration decreased NO level in macrophages and conversely increased arginase 1 levels, resulting in an increase in L-ornithine.

AD is caused by many factors, and recent studies suggest that an allergen-induced immune response is a major cause of AD [30] [31]. In AD, T cells excessively differentiate into Th2 cells and induce both IgE synthesis and mediate mast cell differentiation through Th2 cytokines [32]. Histamine secreted by mast cells causes itching [33]. Excessive scratching breaks the skin barrier and exposes

it to external microbes, such as bacteria [34]. LPS present on the outer membrane of gram-negative bacteria acts as an endotoxin and induces the expression of iNOS [35]. INOS increases COX2 expression [36] [37] and PGE2 production [38]. A variety of pro-inflammatory cytokines, including COX2 and PGE2, exert their biological effects through signaling cascades, leading to skin inflammation [39] [40]. Many studies have demonstrated the activation of COX2/PGE2/nuclear factor kappa B (NFκB) signaling and the downregulation of filaggrin in the skin of patients with AD [41] [42] [43]. The present study indicates that MC administration may suppress filaggrin degradation by suppressing the expression of LPS, iNOS, and COX2/PGE2.

Allergens that invade the body are captured by myeloid cells, such as macrophages and dendritic cells. There are two types of macrophages, M1 and M2. M1 type macrophages cause inflammatory cytokine production, tissue damage, and bactericidal action. In contrast, M2-type macrophages contribute to allergic responses, fat metabolism, wound healing, and cancer metastasis [44] [45]. It has been reported that in AD, Ly6c-positive inflammatory monocytes are converted into M2-type macrophages by IL-4 produced by basophils and suppress allergic inflammation [46]. Macrophages activated by allergens release inflammatory cytokines, such as TNF-α and IL-1β, as well as inflammatory mediators, such as NO. NO is induced by iNOS when the amino acid, L-arginine, is used as a substrate. M1 macrophages carry out this reaction. In M2-type macrophages, the enzyme, arginase 1, using L-arginine as a substrate, is expressed. INOS and arginase 1 compete for common substrate (L-arginine) utilization [47] [48]. In this study, it was considered that macrophages were converted to the M2 type with

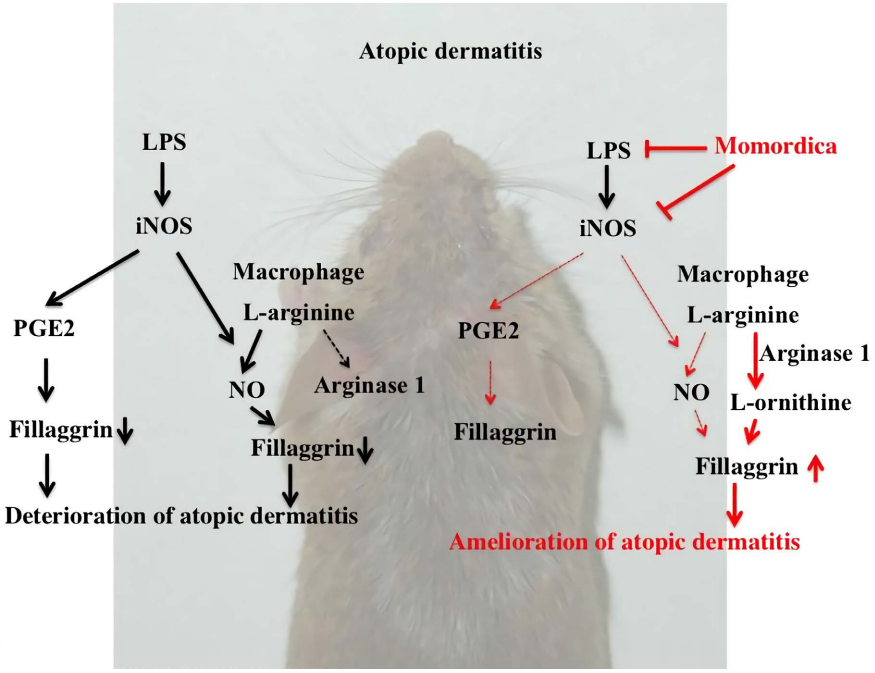


Figure 8. Mechanism of the effect of *Momordica charantia* on the symptoms of atopic dermatitis.

the administration of MC, and L-arginine was converted to urea and L-ornithine by the action of arginase 1. L-ornithine has been reported to promote wound healing and has been known to promote growth hormone secretion and collagen synthesis [49] [50]. In addition, L-ornithine leads to an increase in filaggrin and Natural Moisturizing Factor (NMF) [51] [52]. Thus, the effect of MC induces a decrease in NO synthesis from L-arginine and an increase in arginase 1 by suppressing the increase in iNOS. Arginase 1 was considered to improve AD-like skin symptoms by increasing L-ornithine levels as well as filaggrin synthesis.

5. Conclusion

MC improved the skin-related symptoms of AD in mice by suppressing the degradation of filaggrin and promoting its synthesis. Filaggrin degradation was suppressed by downregulating the LPS/iNOS/PGE2 pathway, which is increased by AD. The decrease in iNOS caused macrophages to shift to type 2, thereby increasing filaggrin expression (**Figure 8**). However, the mechanism by which MC reduces LPS and iNOS expression is unclear. Of note, this study detailed the role of MC in AD model mice. Therefore, it is necessary to further investigate of the role of MCs in AD and carry out clinical studies with patients.

Statement of Ethics

All experimental procedures described in the present study were conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Suzuka University of Medical Science (Approval number: 34). All surgeries were performed under pentobarbital anesthesia. Efforts were taken to minimize animal suffering.

Fund

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Conflicts of Interest

The authors declare no conflicts of interest associated with this study.

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