

DR. NORIKO TAKAHASHI (Orcid ID : 0000-0003-0630-6468)

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## Effects of $\beta$ -carotene on oxazolone-induced atopic dermatitis in hairless mice

Takamichi Kake, Masahiko Imai, and Noriko Takahashi\*

Laboratory of Physiological Chemistry, Institute of Medicinal Chemistry, Hoshi University,  
2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

**RUNNING TITLE:** Inhibitory effects of  $\beta$ -carotene on atopic dermatitis

\*To whom requests for reprints should be addressed:

Noriko Takahashi, Ph.D.

Laboratory of Physiological Chemistry,

Institute of Medicinal Chemistry,

Hoshi University,

2-4-41 Ebara,

Shinagawa, Tokyo 142-8501,

Japan

TEL/FAX: +81-3-5498-5950

E-mail: t-noriko@hoshi.ac.jp

### Abstract

Skin acts as a barrier, which protects internal tissues and promotes moisture retention. Atopic dermatitis (AD) is an inflammatory skin disease associated with a variety of genetic and environmental factors that involve helper T cells.  $\beta$ -Carotene (provitamin A) exhibits antioxidant activity and activates the immune system. However, it is not clear whether

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inflammation in AD skin is improved by post-treatment with  $\beta$ -carotene. In the current study, we investigated the effects of  $\beta$ -carotene on the skin of hairless mice with oxazolone-induced inflammation/edema (Ox-AD mice). We found that skin inflammation was significantly reduced by oral administration of  $\beta$ -carotene. In addition, treatment with  $\beta$ -carotene suppressed protein levels of TNF- $\alpha$ , IL-1 $\beta$ , and MCP-1, as well as mRNA expression associated with IL-1 $\beta$ , IL-6, IL-4, and Par-2 in skin tissues. Furthermore, the mRNA and protein levels of filaggrin, a structural protein in the epidermal stratum corneum, were elevated by  $\beta$ -carotene administration as compared with Ox-AD mice.  $\beta$ -Carotene significantly reduced the activity of proMMP-9, but not proMMP-2. These results suggest that in Ox-AD mice,  $\beta$ -carotene improves skin inflammation by suppressing the expression of inflammatory factors, promoting filaggrin expression, and reducing MMP-9 activity.  $\beta$ -Carotene is a potent anti-inflammatory agent that improves the barrier functions of AD-skin.

**KEYWORDS:**  $\beta$ -carotene, atopy, atopic dermatitis, oxazolone

## 1. INTRODUCTION

Skin is the largest organ in the human body, and it plays a vital role in a human's overall health. Skin is divided into the epidermis and dermis. The epidermis is composed of keratinocyte layers at distinct differentiation stages. The epidermis exhibits a barrier function to protect the body from external influences, including physical stimuli and ultraviolet light, and it promotes the retention of water in body tissues.<sup>[1-3]</sup>

Atopic dermatitis (AD) is an inflammatory skin disease associated with a variety of factors (immunological abnormalities and allergen exposures). It is manifested by itching, eczema, and pathogenic infections. In addition, enhanced oxidative stress (exposure to oxygen and

reactive oxygen species) and attenuation of antioxidant activities are found in AD skin damage. In AD-associated inflammation, T helper (Th) cells play a central role and are classified as being Th1 or Th2 cells, depending on the type of cytokine produced.<sup>[4]</sup> Th1 cells, which induce cell-mediated immunity and phagocyte-dependent inflammation, produce cytokines, including interferon (IFN)- $\gamma$ , tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-2, IL-6, and IL-8. Th2 cells induce humoral immunity with antibody production and eosinophil accumulation that neutralize the Th1 response. They also produce cytokines including IL-1 $\alpha$ , IL-4, IL-5 and IL-10.<sup>[4,5]</sup> Thymic stromal lymphopoietin (TSLP) is a main regulator of Th2 inflammatory responses in allergic diseases. It is known that Th2 cells play an important role in early inflammation and allergic symptoms, which occur when Th2 cells become dominant. Excessive pro-inflammatory responses can lead to tissue damage, fever, and inflammation, and an excessive Th2 response is associated with allergies and atopy. The balance between Th1 and Th2 is important for good health.<sup>[4,6-8]</sup>

$\beta$ -Carotene (Table 1) is a naturally-occurring vitamin A precursor contained in certain fruits and vegetables. Among carotene isomers ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ ),  $\beta$ -carotene is the most abundant in food and it the most effective. It is known that  $\beta$ -carotene is an antioxidant that inhibits free-radical damage to lipids, proteins, and DNA by eliminating singlet oxygen.  $\beta$ -Carotene also activates the immune system by stimulating the release of natural killer cells, lymphocytes, and monocytes.<sup>[9]</sup> Furthermore, previous studies have shown that serum concentrations of carotenoids and lycopene in children with AD are significantly lower as compared with normal children.<sup>[10]</sup> The intake of antioxidant nutrients, including  $\beta$ -carotene reduces the risk of AD<sup>[11]</sup> and functional food factors that include  $\beta$ -carotene and polyphenols might prevent allergic diseases.<sup>[12,13]</sup> Recently, it was reported that a diet containing  $\beta$ -carotene or lycopene prevents AD-like symptoms in association with a suppression of chemokines in murine model.<sup>[14]</sup> However, it is not clear whether  $\beta$ -carotene

improves AD-like skin in distinct AD-model mice induced by oxazolone (Ox). Nor is it clear whether cytokines and factors other than chemokines in AD-like skin tissues are affected by  $\beta$ -carotene. In the current study, we investigated the influence of oral administration of  $\beta$ -carotene on the expression of factors involved in inflammation (inflammatory factors), filaggrin (a structural protein in stratum corneum of the epidermis) and matrix metalloproteinases (MMPs) in skin tissues of AD-model mice. AD-like inflammation in mouse skin induced by topical treatment of Ox was significantly improved by oral  $\beta$ -carotene treatment.

## **2. MATERIALS AND METHODS**

### **2.1. Chemicals**

$\beta$ -Carotene and oxazolone (Ox) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade.

### **2.2. Animals**

Specific pathogen-free hairless mice (HR-1, 7 weeks of age, male) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). To generate mice with atopic dermatitis, mice were divided into three experimental groups (n=6), that received the following treatments; vehicle (control; Con), 0.1% Ox (Ox) or 0.1% Ox +  $\beta$ -carotene (Ox/ $\beta$ C) (Table 1). Ox- and Ox/ $\beta$ C-mice were sensitized by a single topical treatment of 5% Ox in acetone (40  $\mu$ l, 2 cm<sup>2</sup>), while Con-mice were treated with acetone only (vehicle).<sup>[15]</sup> After 1 week, Con-mice or Ox-mice were treated topically with acetone or 0.1% Ox daily for 8 weeks, respectively. Ox/ $\beta$ C-mice were treated topically with 0.1% Ox and administered  $\beta$ -carotene (20 mg/kg, 0.6 mg/0.24 ml/day) in corn oil orally daily for 8 weeks, while Con- and Ox-mice were treated with corn oil only (vehicle). Animal experiments were conducted under ethical approval from the University Institutional Animal Care and Use Committee.

### 2.3. Preparation of skin extracts

Skin tissues (0.1 g, wet weight) from each animal were sectioned, suspended in 0.5 ml of protein-solubilizing solution (9.5 M urea, 2% NP-40, 0.1 M dithiothreitol and 1/100 volume protease inhibitor cocktail), and homogenized using a polytron homogenizer (ULTRA-TURAX T8, IKA-WERKE GmbH & Co., Stanfen, Germany) on ice for 40 seconds (three times). Skin homogenates were centrifuged (10,000 x g, 30 minutes), and resultant supernatants were used as skin extract. Protein concentrations were measured using the procedure of Bradford<sup>[16]</sup> with bovine serum albumin as a standard (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### 2.4. Western blotting

Proteins (20 µg) were separated by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore Co., Bedford, MA, USA) using an electro blotter (Bio Craft, Tokyo, Japan). Immunoreactivity with specific antibodies against TNF-α (Peprotech Inc., Rocky Hill, NJ, USA), IL-1β (Peprotech), monocyte chemoattractant protein 1 (MCP-1, Peprotech), Filaggrin (Biolegend, Dedham, MA, USA), and β-actin (Santa Cruz Biotechnol. Inc., Santa Cruz, CA, USA) were visualized by chemiluminescence staining using a Western Blotting Substrate Plus (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions.

### 2.5. RNA isolation and quantitative RT-PCR (qPCR)

Total RNA (1 µg) was prepared from mouse skin tissues treated with and without compounds using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. For reverse transcriptase (RT) reactions, RNA were reverse-transcribed using a superscript<sup>®</sup> VILO<sup>™</sup> cDNA synthesis kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instruction. The RT reactions were then amplified with specific primers by Step One Plus (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Primer sequences were as follows: IL-1 $\beta$  (forward 5'- GCA ACT GTT CCT GAA CTC AAC T -3', reverse 5'- ATC TTT TGG GGT CCG TCA ACT -3'), IL-4 (forward 5'- CTT CCA AGG TGC TTC GCA TA -3', reverse 5'- CTT ATC GAT GAA TCC AGG CAT -3'), IL-6 (forward 5'- AGT TGC CTT CTT GGG ACT GA -3', reverse 5'- CAG AAT TGC CAT TGC ACA AC -3'), protease-activated receptor-2 (Par-2)(forward 5'- TCT CTG CAC CAA TCA CAA GC -3', reverse 5'- CTT AGC CTT CTT GCC AGG TG -3'), Filaggrin (forward 5'- AGA CTG GGA GGC AAG CTA CA -3', reverse 5'- CCT GCC TCC TTC AGA GTC AC -3'), MMP-2 (forward 5'- ACC TGA ACA CTT TCT ATG GCT G -3', reverse 5'- CTT CCG CAT GGT CTC GAT G -3'), MMP-9 (forward 5'- GGA CCC GAA GCG GAC ATT G -3', reverse 5'- CGT CGT CGA AAT GGG CAT CT -3'), and  $\beta$ -actin (forward 5'- AGC CAT GTA CGT AGC CAT CC -3', reverse 5'- TGT GGT GGT GAA GCT GTA GC -3').

## **2.6. Preparation of skin extract for assays of extracellular matrix metalloproteinase**

Skin tissues (0.1 g, wet weight) from each animal were cut into pieces and suspended in 1 ml of 0.05 M Tris-HCl buffer (pH 7.5) containing 0.2 M NaCl, 5 mM CaCl<sub>2</sub> and 0.1% Triton X-100, and then homogenized by a polytron homogenizer (ULTRA-TURAX T8) on ice for 40 seconds (three times). Skin homogenates were centrifuged (10,000 x g, 30 min), and the resultant supernatants were assayed for extracellular matrix (ECM) metalloproteases, including MMPs. Protein content was measured using the Bicinchoninic Acid Protein Assay (Pierce, Rockford, IL, USA).

## **2.7. Gelatin zymography assay for MMP activity**

Skin extract proteins (10  $\mu$ g) were applied to gelatin-impregnated gel (10% polyacrylamide gel containing 1 mg/ml gelatin) according to the procedure of Lateef et al.<sup>[17]</sup> After electrophoresis, gels were incubated for enzyme reactions in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl<sub>2</sub>, 0.2 M NaCl and 1 mM ZnCl<sub>2</sub> for 16 hours at 37 °C. After incubation,

gels were stained with Coomassie Brilliant Blue R-250 (CBB, Sigma, St. Louis, MO, USA) and destained. MMP activity was quantitated in clear bands using Chemi Stage CC-16H (KURABO Ind. Ltd., Osaka, Japan).

## 2.8. Statistical analysis

Data were analyzed using Prism version 6. The statistical significance of the data was evaluated by Student's *t*-test (Figure 4) or by one-way ANOVA followed by Bonferroni's multiple comparisons test (Figures 1~3). \**p*<0.05, \*\**p*<0.01, and \*\*\**p*<0.001 vs. Ox were considered significant.

## 3. RESULTS

### 3.1. Changes in the appearance of skin in AD-model mice following orally administered $\beta$ -carotene

We investigated whether the skin of AD-model mice treated orally with  $\beta$ -carotene (Table 1) differed from those not treated with  $\beta$ -carotene. Mouse skin was sensitized by a single topical treatment of 5% Ox in acetone (AD-model mice, Ox-mice), while control mice were treated with vehicle only (Con-mice). After 1 week, Con-mice, Ox-mice, or Ox-mice orally administered with  $\beta$ -carotene (Ox/ $\beta$ C-mice) were treated topically with vehicle, 0.1% Ox or 0.1% Ox daily for 8 weeks, respectively (Table 1). In Ox-mice, wrinkle-like, xerotic eczema and irregular skin were seen as compared with skin in Con-mice (Figure 1A). In contrast, these symptoms were not observed in Ox/ $\beta$ C-mice (Figure 1A).  $\beta$ -Carotene showed drastic effects on AD-like skin.  $\beta$ -Carotene did not induce adverse symptoms, *e.g.* desquamation and inflammation etc., by long-term administration (2 months). These results suggest that inflammatory appearance associated with AD skin is ameliorated by  $\beta$ -carotene administration.

### **3.2. Reduction of protein expression of inflammatory factors in AD-model mouse skin by $\beta$ -carotene administration**

We investigated the protein levels of inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) and chemokine (MCP-1) by western blot using specific antibodies. As shown in Figure 1B, protein expression of TNF- $\alpha$  was increased by Ox-treatment (approximately 2-fold) and significantly decreased (approximately 80%) by the administration of  $\beta$ -carotene. While Ox-treatment enhanced the expression of IL-1 $\beta$  protein (approximately 3.6-fold) and MCP-1 (approximately 3-fold),  $\beta$ -carotene administration significantly decreased these proteins (approximately 77% and 46% as compared to those in Ox-mice, respectively). These data suggest that  $\beta$ -carotene reduced protein levels of inflammatory factors in AD-skin tissues induced by Ox treatment.

### **3.3. Effect of $\beta$ -carotene administration on the expression of inflammatory factor genes in AD-model mouse skin**

Since we found that the protein levels of inflammatory factors in the skin of Ox-mice were decreased by treatment with  $\beta$ -carotene, we examined the effects on the levels of inflammatory factor genes. mRNAs were extracted from skin tissues in Con-, Ox-, and Ox/ $\beta$ -mice, and the mRNA expression of inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-4) and Par-2 were quantified using qPCR with specific primers.

Topical Ox treatment significantly increased the mRNA expression of IL-1 $\beta$ , IL-6, and IL-4, approximately 1.3-fold, 1.7-fold, and 3.4-fold as compared with those in Con-mice, respectively (Figure 2). The level of Par-2 mRNA expression was increased by approximately 1.2-fold by Ox treatment. Treatment of Ox-mice with  $\beta$ -carotene decreased the expression of IL-1 $\beta$  and IL-6 genes to approximately the same levels as found in Con-mice skin. The expression levels of IL-4 and Par-2 mRNA in Ox-mice skin was significantly reduced by

$\beta$ -carotene treatment (approximately 27% and 48% as compared with those in Ox-mice, respectively) (Figure 2). These results indicate that inflammatory factor mRNA expression induced by topical Ox-treatment is suppressed by  $\beta$ -carotene administration and that inflammatory effects in Ox-mice skin might be due to alterations in protein levels associated with increased mRNA expression.

### **3.4. Alteration of filaggrin expression in AD-model mouse skins incurred by $\beta$ -carotene administration**

Filaggrin is a major protein in the stratum corneum of the epidermis. Filaggrin interacts with intermediate filaments (particularly keratins), contributes to the structure, function, and hydration of skin tissues, and affects skin barrier function.<sup>[18]</sup> In addition, it is known that filaggrin mutations are associated with skin allergic diseases<sup>[19,20]</sup> and that filaggrin deficiencies can cause eczema and dry skin.<sup>[18]</sup> We examined the effects of  $\beta$ -carotene on protein and gene expression of filaggrin in skin tissues by western blotting and qPCR, respectively.

Topical Ox treatment decreased filaggrin protein levels approximately 53% as compared to those in Con-mice (Figure 3A). In contrast,  $\beta$ -carotene administration increased filaggrin protein levels by approximately 2.4-fold as compared to those in Con-mice, and by approximately 5-fold compared to those in Ox-mice. Filaggrin gene levels in skin tissues of Ox-mice did not significantly change as compared with those in Con-mice (Figure 3B), while  $\beta$ -carotene administration increased filaggrin gene levels by approximately 1.6-fold and 1.3-fold as compared to those in Con- and Ox-mice, respectively. These results indicate that treatment with  $\beta$ -carotene increased filaggrin protein levels by transcriptional regulation.

### **3.5. Changes in the expression and activity of MMPs in AD-model mouse skins following $\beta$ -carotene administration**

Skin inflammation in Ox-mice was ameliorated through  $\beta$ -carotene administration by reducing the expression of various Th cell-produced factors. The appearance of AD-like skin in Ox-mice changed to what was observed with normal skin in Con-mice (Figures 1-3). These results led us to investigate whether  $\beta$ -carotene alters the gene expression and the activity of MMPs, which degrade dermal ECM proteins in skin tissues. As compared with skin tissues of Con-mice, the mRNA expression of MMP-2 and MMP-9 increased, but not significantly (Figure 4A, dashed line).  $\beta$ -Carotene decreased the mRNA expression of MMP-9, but not MMP-2 in skin tissues of Ox-mice (Figure 4A). We performed gelatin zymography to measure the activities of MMPs in the skin tissues of Con-, Ox-, and Ox/ $\beta$ C-mice. The activities of proMMP-2 and proMMP-9 were also quantified. The activities of proMMP-2 and proMMP-9 in the skin tissues of Ox-mice trended to increase as compared to those of Con-mice (Figure 4B, dashed line).  $\beta$ -Carotene administration decreased the activity of proMMP-9, not of proMMP-2. The proMMP-9 activity in Ox/ $\beta$ C-mice skin tissues was approximately 60% of that in Ox-mice. These results indicate that  $\beta$ -carotene suppressed proMMP-9 activity significantly and inhibited ECM degradation in skin tissues of AD-model mice.

## **4. DISCUSSION**

In the current study, we found that administration of  $\beta$ -carotene (Table 1) improved the appearance of AD-like skin of mice induced by topical Ox treatment (Figure 1A). Ox treatment significantly enhanced protein and mRNA expression of inflammatory cytokines and factors in skin tissues, while  $\beta$ -carotene administration significantly suppressed these increases (Figures 1B and 2). In addition, both the protein and mRNA expression of filaggrin

were increased by  $\beta$ -carotene administration as compared to Ox-mice (Figure 3). Furthermore,  $\beta$ -carotene treatment significantly reduced proMMP-9 activity (Figure 4). These results indicate that  $\beta$ -carotene suppressed skin inflammation in AD-like skin of Ox-mice by decreasing the expression of inflammatory factors and the activity of proMMP-9 activity, while increasing filaggrin expression (Figures 1-4).  $\beta$ -Carotene is a potent anti-inflammatory agent that improves AD and enhances the barrier function of skin.

Previous studies have shown that Ox-treated mice can be used as an experimental model for AD, based on skin dryness, impaired barrier function, epidermal thickening, and changes in immunological parameters, which arise as a consequence of skin barrier dysfunction associated with the development of AD-like pruritus.<sup>[15,21-26]</sup> Yoon et al. reported that in skin tissues, the expression of mRNAs associated with Th1 cytokines (IL-1 $\alpha$ , TNF- $\alpha$ ) and Th2 factors (Par-2 and TSLP) were increased in hairless mice treated with Ox and decreased by glucocorticoid treatment.<sup>[15]</sup> In addition, Jegal et al. have shown that Ox treatment increases serum IL-4 levels.<sup>[26]</sup> These results agree with our finding that Ox treatment enhances the mRNA levels of inflammatory factors, Th1 cytokines (IL-1 $\beta$ , IL-6) and Th2 factors (IL-4, Par-2) as shown in Figure 2. We also found that the protein levels of TNF- $\alpha$  and IL-1 $\beta$  (cytokines) and MCP-1 (chemokine) were increased by Ox treatment (Figure 1B).  $\beta$ -Carotene inhibited skin inflammation arising from the production of inflammatory factors, including cytokine, induced by Ox treatment, and improved the skin condition significantly. It would be interesting to ascertain whether  $\beta$ -carotene affects transcriptional regulation of these factors by serving as an antioxidant.

Recently, it has been reported that a diet containing  $\beta$ -carotene or lycopene prevents AD-like symptoms in mice fed with a low zinc/magnesium diet (HR-AD diet).<sup>[14]</sup> Normal mice were fed with 1) a standard diet, 2) a HR-AD diet, 3) a HR-AD diet containing

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$\beta$ -carotene, and 4) a HR-AD diet containing lycopene for 8 weeks. For the HR-AD diet containing  $\beta$ -carotene, an estimated 3~5 mg/day dose of  $\beta$ -carotene was used and the administration of  $\beta$ -carotene and the feeding of the HR-AD diet were performed simultaneously. The above study reported the appearance or histopathological observations in the skin as well as levels of serum IgE or skin chemokines (TARC and CCL27). The levels of CCL27 were significantly lower in the HR-AD diet containing  $\beta$ -carotene group than in the HR-AD diet group. In our current study, we used a distinct AD-mouse model induced by Ox and an experimental design that differs from the above study, in that a specific quantity of  $\beta$ -carotene was sequentially administered to the AD-like mice. In addition, we assessed the effects of  $\beta$ -carotene on the levels of cytokines and inflammatory factors other than chemokines in AD-like skin tissues. Oral administration of  $\beta$ -carotene (0.6 mg/day) for 8 weeks to the AD-like mice induced by treatment with 5% Ox for one week, was accomplished by a feeding-needle (Table 1). The quantity of  $\beta$ -carotene (0.6 mg/day, 8 weeks) was approximately one seventh of the estimated amount of  $\beta$ -carotene (3~5 mg/day, 8 weeks) and the administration schedule was the same period (8 weeks) as those used in the previous study.<sup>[14]</sup> We did not observe an obvious orange pigmentation of the skin, most probably because we used less  $\beta$ -carotene than in the previous study.<sup>[14]</sup> AD-symptoms were remarkably improved by  $\beta$ -carotene administration as evidenced by the reduction of both the expression of inflammatory factors and MMP activity and an increase in the expression of filaggrin in the skin tissues (Figures 1-4). Our current report is the first to show that post-administration of  $\beta$ -carotene can be extremely effective to ameliorate AD symptoms in AD-model mice induced by Ox treatments.

Previous studies have shown that  $\beta$ -carotene suppresses the degranulation of mast cells, which plays a pivotal role in inflammation and allergic reactions by secreting biologically active substances, including histamine, cytokines, chemokines, and proteolytic enzymes.<sup>[27,28]</sup>

This action of  $\beta$ -carotene is mediated by inhibiting the aggregation of the high affinity IgE receptor (Fc $\epsilon$ RI) by decreasing antigen-induced translocation of Fc $\epsilon$ RI to lipid rafts. In AD-like skin, the actions of  $\beta$ -carotene might involve multiple targets, including Fc $\epsilon$ RI and the activation of the immune system.

The endopeptidase MMPs are associated with a variety of physiological and pathological processes. Degradation of the ECM by MMPs is associated with cancer invasion and metastasis.<sup>[29,30]</sup> In addition, MMP degradation of the ECM (collagen, elastin, hyaluronic acid etc.) increases both photoaging and chronological aging of skin tissues. Consequently, reduction of the ECM induces wrinkles.<sup>[17,31]</sup> Among MMPs, the gelatinases MMP-2 and MMP-9 degrade the basement membrane and collagen fibers.<sup>[32,33]</sup> MMP-2 is constitutively expressed in healthy tissues and is generally not responsive to stimuli. In contrast, MMP-9 is inducible and it is thought to be an inflammation marker.<sup>[34]</sup> In the current study,  $\beta$ -carotene significantly decreased the activity of proMMP-9, but not proMMP-2, in the skin tissues of Ox-mice (Figure 4). These results indicate that  $\beta$ -carotene suppressed skin inflammation by inhibiting MMP-9 activity, which is a cause of inflammation.

$\beta$ -Carotene is a scavenger of free radicals and it is known to be a potent quencher of singlet oxygen,  $^1\text{O}_2$ .<sup>[35-37]</sup>  $\beta$ -Carotene has been intensively studied for its potential to prevent skin photodamage<sup>[38-40]</sup> and skin damage by ozone.<sup>[41]</sup>  $\beta$ -Carotene also has prooxidant properties in skin exposed to ultraviolet A light.<sup>[42,43]</sup> Thus, there is a debate regarding the protective or potentially harmful role of  $\beta$ -carotene in human skin. In the current study,  $\beta$ -carotene protected and improved AD-like skin by reducing inflammation and ECM degradation and by increasing filaggrin expression, which plays a pivotal role in skin barrier function.  $\beta$ -Carotene might be useful for the prevention and treatment of skin disorders, including AD.

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## CONFLICTS OF INTEREST

The authors have declared no conflicting of interests.

## AUTHOR'S CONTRIBUTIONS

TK, MI, and NT performed the research. NT designed the research study. TK and MI analyzed the data and NT wrote the paper.

## 5. REFERENCES

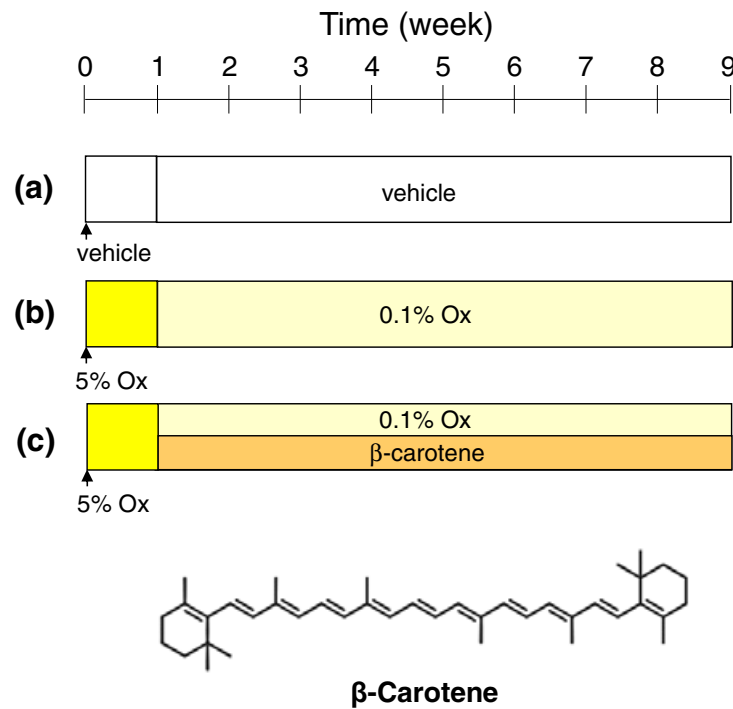
- [1] R. Eichner, M. Kahn, *J. Cell Biol.* **1990**, 110, 1149.
- [2] E. Fuchs, *Princess Takamatsu Symp.* **1994**, 24, 290.
- [3] E. Fuchs, C. Byrne, *Curr. Opin. Genet. Dev.* **1994**, 4, 725.
- [4] J. Y. Chen, L. S. Fu, J. J. Chu, H. C. Chen, C. S. Chi, *J. Microbiol. Immunol. Infect.* **2007**, 40, 168.
- [5] K. Shinkai, M. Mohrs, R. M. Locksley, *Nature.* **2002**, 420, 825.
- [6] F. S. Dhabhar, *Immunol. Res.* **2014**, 58, 193.
- [7] P. Kidd, *Altern. Med. Rev.* **2003**, 8, 223.
- [8] Y. K. Kim, M. Maes, *Acta Neuropsychiatr.* **2003**, 15, 148.
- [9] D. A. Hughes, *Nutrition.* **2001**, 17, 823.
- [10] R. Ruhl, C. Taner, F. J. Schweigert, U. Wahn, C. Gruber, *Pediatr. Allergy Immunol.* **2010**, 21, 1072.
- [11] S. Y. Oh, J. Chung, M. K. Kim, S. O. Kwon, B. H. Cho, *Eur. J. Clin. Nutr.* **2010**, 64, 245.
- [12] H. Akiyama, S. Katayama, T. Kanda, M. Maeda-Yamamoto, M. Totsuka, S. Takahashi,

- T. Shoji, T. Inakuma, S. Nakamura, *Curr. Pharm. Des.* **2014**, 20, 892.
- [13] H. Rosenlund, J. Magnusson, I. Kull, N. Hakansson, A. Wolk, G. Pershagen, M. Wickman, A. Bergstrom, *Clin. Exp. Allergy.* **2012**, 42, 1491.
- [14] M. Hiragun, T. Hiragun, I. Oseto, K. Uchida, Y. Yanase, A. Tanaka, T. Okame, S. Ishikawa, S. Mihara, M. Hide, *J. Dermatol.* **2016**, 43, 1188.
- [15] N. Y. Yoon, M. Jung, D. H. Kim, H. J. Lee, E. H. Choi, *Arch Dermatol Res.* **2015**, 307, 569.
- [16] M. M. Bradford, *Anal. Biochem.* **1976**, 72, 248.
- [17] H. Lateef, M. J. Stevens, J. Varani, *Am. J. Pathol.* **2004**, 165, 167.
- [18] S. Kezic, I. Jakasa, *Curr. Probl. Dermatol.* **2016**, 49, 1.
- [19] K. Horimukai, K. Morita, M. Narita, M. Kondo, S. Kabashima, E. Inoue, T. Sasaki, H. Niizeki, H. Saito, K. Matsumoto, Y. Ohya, *Allergol. Int.* **2016**, 65, 103.
- [20] N. Novak, H. Baurecht, T. Schafer, E. Rodriguez, S. Wagenpfeil, N. Klopp, J. Heinrich, H. Behrendt, J. Ring, E. Wichmann, T. Illig, S. Weidinger, *J. Invest. Dermatol.* **2008**, 128, 1430.
- [21] T. Tamura, M. Matsubara, K. Hasegawa, K. Ohmori, A. Karasawa, *Clin. Exp. Allergy.* **2005**, 35, 97.
- [22] M. Q. Man, Y. Hatano, S. H. Lee, M. Man, S. Chang, K. R. Feingold, D. Y. Leung, W. Holleran, Y. Uchida, P. M. Elias, *J. Invest. Dermatol.* **2008**, 128, 79.
- [23] T. A. Le, T. Takai, A. T. Vu, H. Kinoshita, X. Chen, S. Ikeda, H. Ogawa, K. Okumura, *Int. Arch. Allergy Immunol.* **2011**, 155, 31.
- [24] H. J. Kim, B. Kim, B. M. Park, J. E. Jeon, S. H. Lee, S. Mann, S. K. Ahn, S. P. Hong, S. K. Jeong, *Int. J. Dermatol.* **2015**, 54, e401.
- [25] G. Nam, S. K. Jeong, B. M. Park, S. H. Lee, H. J. Kim, S. P. Hong, B. Kim, B. W. Kim, *Ann. Dermatol.* **2016**, 28, 22.
- [26] J. Jegal, N. J. Park, S. K. Bong, H. Jegal, S. N. Kim, M. H. Yang, *Nutrients.* **2017**, 9.
- [27] S. Sakai, T. Sugawara, T. Hirata, *Biosci. Biotechnol. Biochem.* **2011**, 75, 1013.
- [28] S. Sakai, T. Sugawara, K. Matsubara, T. Hirata, *J. Biol. Chem.* **2009**, 284, 28172.
- [29] T. E. Cawston, A. J. Wilson, *Best Pract. Res. Clin. Rheumatol.* **2006**, 20, 983.
- [30] K. Kupai, G. Szucs, S. Cseh, I. Hajdu, C. Csonka, T. Csont, P. Ferdinandy, *J. Pharmacol. Toxicol. Methods.* **2010**, 61, 205.
- [31] J. H. Chung, H. C. Eun, *J. Dermatol.* **2007**, 34, 593.
- [32] H. Nagase, J. F. Woessner, Jr., *J. Biol. Chem.* **1999**, 274, 21491.
- [33] F. R. Gutierrez, M. M. Lalu, F. S. Mariano, C. M. Milanezi, J. Cena, R. F. Gerlach, J. E. Santos, D. Torres-Duenas, F. Q. Cunha, R. Schulz, J. S. Silva, *J. Infect. Dis.* **2008**, 197, 1468.
- [34] G. Opdenakker, P. E. Van Den Steen, B. Dubois, I. Nelissen, E. Van Coillie, S. Masure, P. Proost, J. Van Damme, *J. Leukoc. Biol.* **2001**, 69, 851.

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- [35] A. Mortensen, L. H. Skibsted, T. G. Truscott, *Arch. Biochem. Biophys.* **2001**, 385, 13.
  - [36] G. W. Burton, K. U. Ingold, *Science*. **1984**, 224, 569.
  - [37] W. Stahl, S. Nicolai, K. Briviba, M. Hanusch, G. Broszeit, M. Peters, H. D. Martin, H. Sies, *Carcinogenesis*. **1997**, 18, 89.
  - [38] H. K. Biesalski, U. C. Obermuller-Jevic, *Arch. Biochem. Biophys.* **2001**, 389, 1.
  - [39] F. Dreher, H. Maibach, *Curr. Probl. Dermatol.* **2001**, 29, 157.
  - [40] J. Fuchs, *Free Radic. Biol. Med.* **1998**, 25, 848.
  - [41] G. Valacchi, A. Pecorelli, M. Mencarelli, E. Maioli, P. A. Davis, *Toxicol. Ind. Health*. **2009**, 25, 241.
  - [42] U. C. Obermuller-Jevic, P. I. Francz, J. Frank, A. Flaccus, H. K. Biesalski, *FEBS Lett.* **1999**, 460, 212.
  - [43] U. C. Obermuller-Jevic, B. Schlegel, A. Flaccus, H. K. Biesalski, *FEBS Lett.* **2001**, 509, 186.

**TABLE 1. Chemical structures of  $\beta$ -carotene and its administration schedule.**

Mouse skin was sensitized by a single topical treatment of vehicle (a) and 5% Ox (b, c) in acetone. After 1 week, Con-mice (a), Ox-mice (b), or Ox-mice orally administered with  $\beta$ -carotene (Ox/ $\beta$ C-mice, c) were treated topically with vehicle, 0.1% Ox or 0.1% Ox daily for 8 weeks, respectively.



## FIGURE LEGENDS

### **FIGURE 1. Effects of $\beta$ -carotene administration on skin appearance and protein levels of inflammatory factors in Ox-mice skin.**

Mouse skin was treated with vehicle (Con), 0.1% Ox (Ox) and 0.1% Ox+ $\beta$ -carotene (Ox/ $\beta$ C) daily for 8 weeks as described under “MATERIALS AND METHODS”. **A**, Changes in mouse skin were observed macroscopically. **B**, Protein (20  $\mu$ g) prepared from mouse skin was subjected to separation by 10% 1D-PAGE. Inflammatory factor levels were analyzed by immunoblotting with specific antibodies against TNF- $\alpha$ , IL-1 $\beta$ , MCP-1, and  $\beta$ -actin as described under “MATERIALS AND METHODS”. Results were analyzed by scanning densitometry. Relative expression levels were estimated by normalization with  $\beta$ -actin. The protein expression in Con was defined as 1.0. Results represent the mean  $\pm$  SE of each group ( $n=6$ ). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  versus Ox treatment compared by Bonferroni’s multiple comparisons test.

### **FIGURE 2. Expression of inflammatory factor genes in skin tissues of Ox-mice administered $\beta$ -carotene.**

Mouse skins were treated with vehicle (Con), 0.1% Ox (Ox) and 0.1% Ox+ $\beta$ -carotene (Ox/ $\beta$ C) daily for 8 weeks. Total RNA (1  $\mu$ g) was prepared from skin tissues, and qPCR was performed using specific primers against IL-1 $\beta$ , IL-6, IL-4, Par-2, and  $\beta$ -actin as described under “MATERIALS AND METHODS”. Relative expression levels were estimated by normalization with  $\beta$ -actin. The expression of mRNA in Con was defined as 1.0. Results represent the mean  $\pm$  SE of each group ( $n=6$ ). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  versus Ox treatment compared by Bonferroni’s multiple comparisons test.

**FIGURE 3. Protein and gene levels of filaggrin in skin tissues of Ox-mice administered  $\beta$ -carotene.**

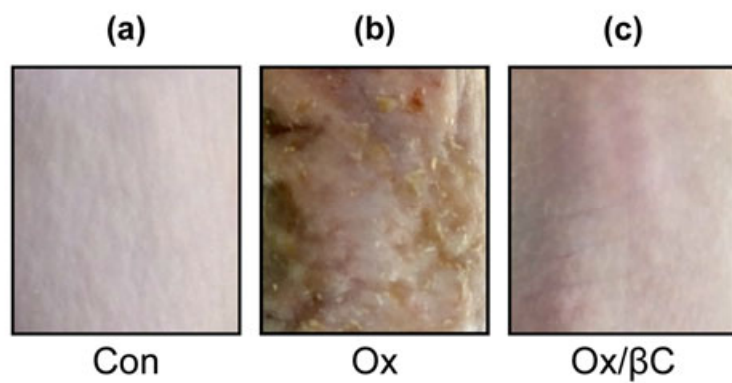
Mouse skins were treated with vehicle (Con), 0.1% Ox (Ox) and 0.1% Ox+ $\beta$ -carotene (Ox/ $\beta$ C) daily for 8 weeks. **A**, Protein (20  $\mu$ g) prepared from mouse skin was subjected to separation by 1D-PAGE. Protein levels were analyzed by immunoblotting specific antibodies against filaggrin and  $\beta$ -actin as described under “MATERIALS AND METHODS”. Results were analyzed by scanning densitometry. Relative expression levels were estimated by normalization with  $\beta$ -actin. The protein expression in Con was defined as 1.0. Results represent the mean  $\pm$  SE of each group ( $n=6$ ). **B**, Total RNA (1  $\mu$ g) was prepared, and qPCR was performed using specific primers against filaggrin and  $\beta$ -actin as described under “MATERIALS AND METHODS”. Relative expression levels were estimated by normalization with  $\beta$ -actin. The expression of mRNA in Con was defined as 1.0. Results represent the mean  $\pm$  SE of each group ( $n=6$ ). \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  versus Ox treatment compared by Bonferroni’s multiple comparisons test.

**FIGURE 4. MMP gene expression and gelatin zymography of MMPs in the skin of Ox-mice administered  $\beta$ -carotene**

Mouse skins were treated with vehicle (Con), 0.1% Ox (Ox) and 0.1% Ox+ $\beta$ -carotene (Ox/ $\beta$ C) daily for 8 weeks. **A**, Total RNA (1  $\mu$ g) was prepared, and qPCR was performed using specific primers against MMP-2, MMP-9, and  $\beta$ -actin as described under “MATERIALS AND METHODS”. Relative expression levels were estimated by normalization with  $\beta$ -actin. The expression of mRNA in Ox was defined as 1.0. Results represent the mean  $\pm$  SE of each group ( $n=6$ ). **B**, Protein (10  $\mu$ g) from skin extracts was applied to gelatin-impregnated gel. After electrophoresis, gels were incubated for 16 h at 37 °C in 50 mM Tris-HCl (pH 7.5) containing 5 mM  $\text{CaCl}_2$ , 0.2 M NaCl, and 1  $\mu$ M  $\text{ZnCl}_2$  as described under “MATERIALS AND METHODS”. Each MMP band was densitometrically quantified by computer image analysis. The activity in Ox was defined as 1.0. Dashed line showed control level (Con). Each bar represents the mean  $\pm$  SE ( $n=6$ ). \* $p<0.05$  versus Ox treatment compared by Student’s *t*-test.

**Figure 1**

**(A)**



**(B)**

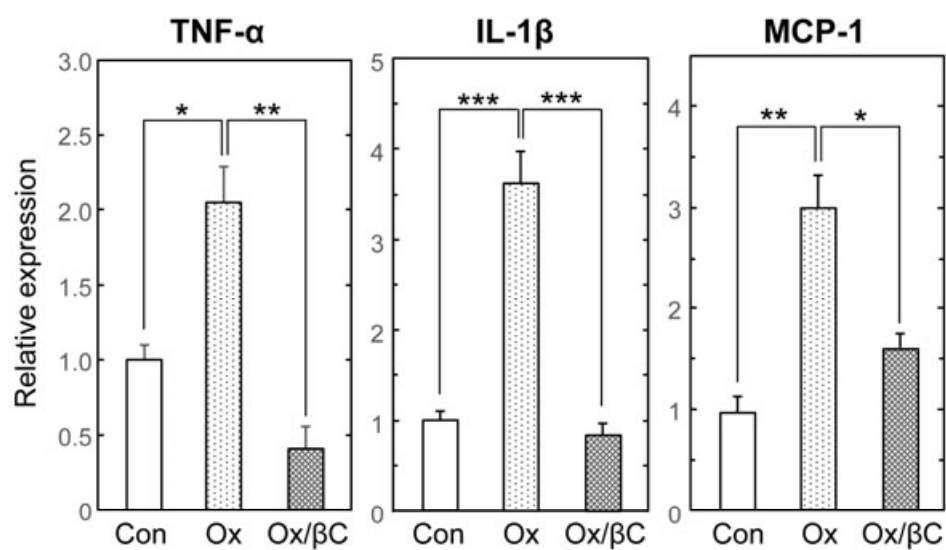


**Figure 2**

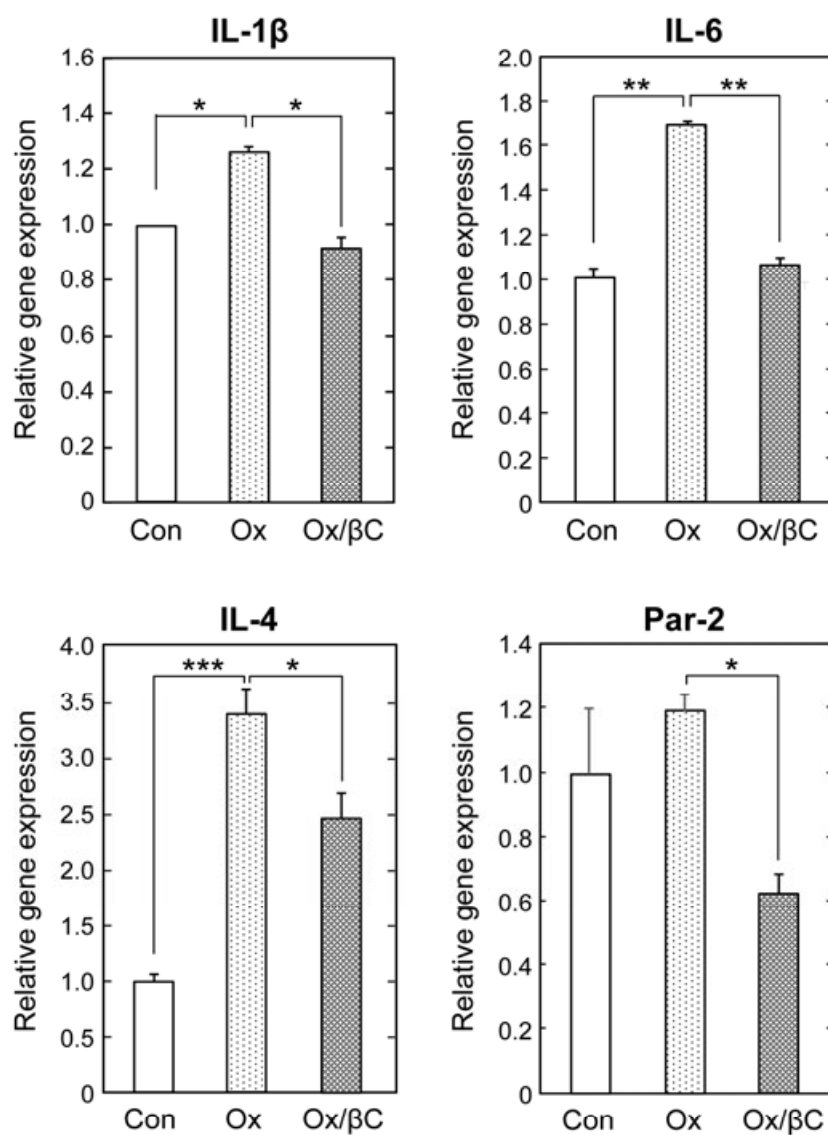


**Figure 3**

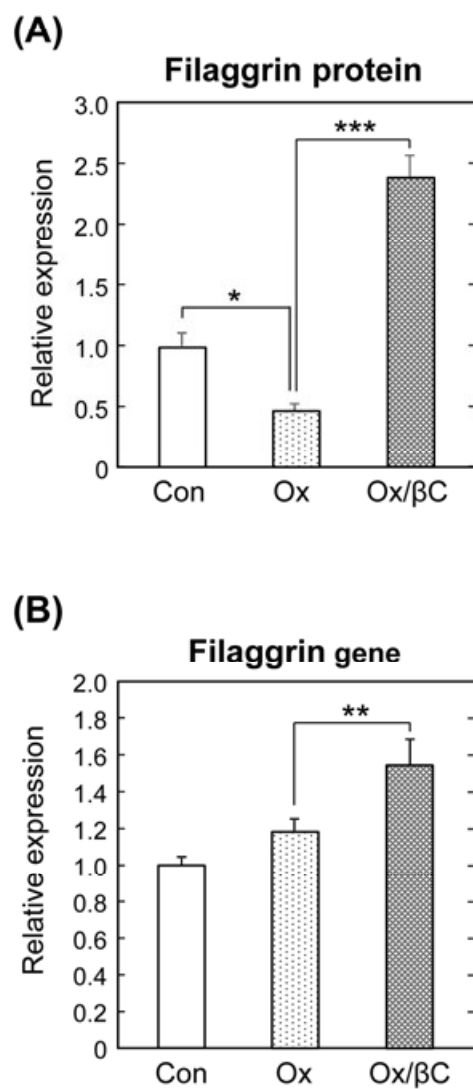


**Figure 4**

