Effects of Post-administration of β-Carotene on Diet-induced Atopic Dermatitis in Hairless Mice

Noriko Takahashi*, Takamichi Kake, Shinya Hasegawa, and Masahiko Imai

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

Abstract: Atopic dermatitis (AD) is a cutaneous condition characterized by itchy, swollen, and dry skin, which is mediated by T helper cell-related cytokines. β-Carotene, a natural red pigment found in plants, exhibits antioxidant activity that has been shown to promote an inflammatory response. Because it is not clear whether β-carotene suppresses inflammation in AD skin tissues, we examined the effects of oral administration of β-carotene in mice induced by a low zinc/magnesium diet (HR-AD diet). Our studies found that AD-like inflammation was remarkably reduced by β-carotene. In addition, β-carotene significantly suppressed protein expression of TNF-α, IL-1β, and MCP-1 and mRNA expression of TSLP, IL-6, IL-1β, IL-4, IL-5, and Par-2 in AD-like skin tissues. It was also found that mRNA and protein expression of filaggrin (a major structural protein in epidermis) in AD-like skin was significantly elevated by β-carotene administration. Furthermore, β-carotene treatment significantly reduced the activity and/or mRNA expression of matrix metalloproteinases (MMPs), degradation of the extracellular matrix and regulation of chemokines. These results suggest that β-carotene reduces skin inflammation through the suppressed expression of inflammatory factors or the activity of MMPs as well as the promotion of filaggrin expression in AD-like skin.

β-Carotene is a potent anti-inflammatory agent, which improves AD-like skin by enhancing the skin barrier function.

Key words: β-carotene, atopic dermatitis, atopy

1 Introduction

Skin is the largest organ in the body, and it is composed of the epidermis and the dermis. Skin has a barrier function that prevents an invasion of foreign substances and conserves moisture in the body. In addition, skin plays an important role in maintaining homeostasis between the body and the environment. Atopic dermatitis (AD) is an inflammatory skin disease that results in an allergic response including dry skin, itching, and thickened scaly skin. People with AD exhibit a weakened skin barrier, which allows foreign substances to enter through the skin. While healthy people are protected by antioxidants that eliminate reactive oxygen species (ROS), AD patients appear to have a lessened ability to activate antioxidants. An accumulation of ROS has been found in sites of inflammation present in AD patients.

Cytokines play a role in cell interaction and helper T cells participate in the production and regulation of cytokines. There are two subtypes of cells: helper T cells-type 1 (Th1) and helper T cells-type 2 (Th2). Their production depends on the type of cytokine generated. Th1 cells can induce cell-mediated immunity and phagocyte-dependent inflammation. Th1 cells can also exacerbate disease states by producing pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-2, IL-6, IL-8, and IFN-γ. In contrast, Th2 cells induce strong humoral immunity with associated antibody production and eosinophil accumulation, which neutralize the Th1 response. Excessive pro-inflammatory responses can lead to tissue damage, fever, and inflammation. An excessive Th2 response can also be associated with allergic reactions. Because allergic symptoms in AD occur when the numbers...
of Th2 cells in early inflammation stages become excessive, Th1 and Th2 cells are thought to play important roles in total inflammation. A balance between the numbers of Th1 and Th2 cells is critical for maintaining good health\(^1\-^4\).

AD also causes an imbalance in the number of Th1/Th2 cells in the immune response in skin lesions\(^5\). A vicious cycle involving stress and inflammation exacerbates the symptoms of AD. Stress triggers the release of corticotrophin-releasing factor (CRF) and substance P (SP) in the nervous system, causing the release of histamine and pro-inflammatory cytokines, e.g. TNF-α, IL-6, and IL-4\(^6\-^7\). In particular, the Th2 response is thought to be critical in allergic inflammation associated with a variety of diseases, including AD. IL-4 is important for the pathogenesis of atopic disorders because up-regulation of immunoglobulin E (IgE), a major cause of atopic inflammation, is regulated by IL-4, a representative Th2 cytokine\(^8\). IL-4 stimulates the degranulation of mast cells, as well as the release of SP (related to itching) and activation of matrix metalloproteinase-9 (MMP-9) (edema induction), thereby promoting skin inflammatory and damage\(^9\). In addition, MMP-9 knockout in mice results in an increased level of IL-4 and IL-13 in lung tissues\(^10\). Involvement of other MMPs in the pathogenesis of AD has also been reported\(^11\). On the other hand, filaggrin has a crucial structural and functional role in the skin by promoting homeostasis\(^12\) and maintaining hydration\(^13\). Filaggrin has been considered to be a major factor in atopic disorders\(^14\). In particular, there is a strong association between filaggrin mutations and the development of AD. Filaggrin deficiency provides a fundamental contribution to the pathogenesis of AD\(^7\-^12\). Thus, inflammatory cytokines, filaggrin, and MMPs are intimately related to the development of AD.

β-Carotene (Fig. 1) is a natural red pigment found in plants and bacteria. It exhibits antioxidative activity that inhibits free-radical (singlet oxygen) damage to lipids, proteins, and DNA. In addition, β-carotene activates the immune system by stimulating the release of natural killer cells, lymphocytes, and monocytes\(^15\). Previous studies have shown that carotenoids might prevent allergic conditions including AD\(^16\,17\). These studies found that serum concentrations of carotenoids and lycopene are significantly lower in children with AD as compared with normal children\(^18\). It has also been shown that the intake of antioxidant nutrients, including β-carotene, reduce the risk of AD\(^19\). Recently, AD-like symptoms induced in hairless mice fed with a low zinc/magnesium diet (HR-AD diet) containing β-carotene or lycopene were ameliorated as compared to those in mice fed with HR-AD diet\(^20\). An HR-AD diet containing β-carotene or lycopene (estimated dose, 3–5 mg/day) resulted in the absence of AD-like symptoms, which would be associated with the suppression of chemokine CCL27 in skin tissues of AD-model mice. However, it is not clear whether β-carotene improves AD-like symptoms in AD-model mouse skin or if it affects factors involved in inflammation (inflammatory factors) including cytokines in AD-like skin. It is also not clear whether a lower dose of β-carotene would be effective. In the current study, we examined the effects on skin inflammatory factors of post-administering a fixed amount of β-carotene through a feeding needle to AD-model mice (dose, 0.6 mg/day) and maintained AD-model mice. We found that β-carotene remarkably decreased skin inflammation.

### 2 Experimental

#### 2.1 Chemicals

β-Carotene was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of reagent grade.

#### 2.2 Animals

Pathogen-free hairless mice (HR-1, 5 weeks of age, male) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). In order to study the induction of atopic dermatitis, mice were divided into three experimental groups (n = 8), which received the following treatments: a normal diet (control group), a HR-AD diet (HR group), or a HR-AD diet + β-carotene (HR/βC group). Mice in the Con group (Con-mice) were fed with a normal diet (F-2; Funabashi Farm, Chiba, Japan) for 15 weeks. Mice in the...
HR group (HR-mice) were fed with a special diet (HR-AD diet; Norsan Corp., Yokohama, Japan) for 15 weeks. Mice in the HR/βC group (HR/βC-mice) were fed with the HR-AD diet for 11 weeks, and then fed with a combination of the HR-AD diet and administered β-carotene (dose, 0.6 mg/day) orally for 4 weeks. 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 diithiothreitol and 1/100 volume protease inhibitor cocktail), and homogenized using a polytron homogenizer (ULTRA-TURAX T8, IKA-WERKE GmbH & Co., Staufen, Germany) on ice for 40 s (3 x). Skin homogenates were centrifuged (10,000 x g, 30 min), and resultant supernatants were used as skin extract. Protein concentrations were measured using the procedure of Bradford with bovine serum albumin (BSA) as a standard (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.4 Western blotting
Proteins (20 µg) were separated by 1D-PAGE and transferred to polyvinylidene difluoride membranes (PVDF, Millipore Co., Bedford, MA USA) using an electroblotter (Bio Craft, Tokyo, Japan). Immunoreactivity with specific antibodies against TNF-α (Peprotech Inc., Rocky Hill, NJ, USA), IL-6 (Peprotech), IL-1β (Peprotech), 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 using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions. For reverse transcriptase (RT) reactions, RNA were reverse-transcribed using a superscript™ VILO™ 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: TSLP (forward 5’- CGA CAG CAT GGT TCT TCT CA -3’, reverse 5’- CGA TTT GCT CGA ACT TAG CC -3’), Par-2 (forward 5’- TCT CTG CAC CAC TCA CAA GC -3’, reverse 5’- CTT AGC CTT CCT GCC AGG TG -3’), IL-1β (forward 5’- GCA ACT GTT CCT GAA CTC AAC T -3’, reverse 5’- ATC TTT TGG GCG 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-5 (forward 5’- GCT GAA GGC CAG CGC TGA AGA -3’, reverse 5’- ACA GAG TCT CTT GAT GCG ACA -3’), IL-6 (forward 5’- AGT TGC CTT CTT GGG ACT GA -3’, reverse 5’- CAG AAT TGC CAT TGC ACA AC -3’), Filaggrin (forward 5’- AGA CTG GGA GCC 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 GGC GAC ATT G -3’, reverse 5’- CGT GTG CGA AAT GGG CAT CT -3’), and β-actin (forward 5’- AGC CAT GTG CGT AGC CAT CC -3’, reverse 5’- TGT GGT GGT GAA GCT GTA GC -3’).

2.6 Preparation of skin extract for assay 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 CaCl2 and 0.1% Triton X-100, and then homogenized by a polytron homogenizer (ULTRA-TURAX T8) on ice for 40 s (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 (5 µg) were applied to gelatin-impregnated gel (10% polyacrylamide gel containing 1 mg/mL gelatin) according to the procedure of Lateef et al. Gels were then incubated for enzyme reactions in 50 mM Tris-HCl (pH 7.5) containing 5 mM CaCl2, 0.2 M NaCl and 1 mM ZnCl2 for 16 h at 37°C. After incubation, gels were stained with Comassie Brilliant Blue R-250 (CBB, Sigma, St. Louis, MO, USA) and then de-stained. 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 one- or two-way ANOVA followed by Bonferroni’s multiple comparisons test. *p < 0.05, **p < 0.01, and ***p < 0.001 were considered significant.

3 Results
3.1 Appearance of mice skin after oral administration of β-carotene
We investigated whether the skin of AD-model mice would change when administered β-carotene orally. As shown in Fig. 1, hairless mice were given a normal diet (control) or an HR-AD diet (AD-model) for 11 weeks.
Control mice were given a normal diet and orally administered vehicle (corn oil) (Con), while AD-model mice were given an HR-AD diet and orally administered vehicle (corn oil) (HR). In addition, AD-model mice were given an HR-AD diet and orally administered β-carotene (HR/βC). After 4 weeks, the skin appearance in each group of mice was observed macroscopically.

We observed that HR-mice had AD symptoms, including wrinkled and dry skin (Fig. 2b), which were not found in Con-mice (Fig. 2a). However, these symptoms were not seen following the administration of β-carotene to HR-mice (Fig. 2c). We did not observe symptoms, e.g. desquamation and inflammation, etc. induced by long-term administration (2 months) of β-carotene. These results suggest that post-administration of β-carotene improved skin appearance and inflammation associated with AD and that it seemed to cure AD.

3.2 Effect of β-carotene administration on protein expression of inflammatory cytokines and monocyte chemoattractant protein 1 (MCP-1) in AD-like mouse skin

We investigated protein expression levels of inflammatory factor cytokines (TNF-α, IL-1β) and monocyte chemoattractant protein 1 (MCP-1) in mouse skin tissues by western blot using specific antibodies. As shown in Fig. 3, TNF-α protein expression increased approximately 1.28-fold in HR-mice and it was decreased by approximately 50% by the administration of β-carotene, as compared to HR-mice. In addition, protein expression levels of IL-1β and MCP-1 significantly increased by 1.87-fold and 3.79-fold in HR-mice, as compared to that in Con-mice. Protein expression

Fig. 2 Skin appearance in HR-mice administered β-carotene. Changes in skin of Con-mice fed with normal diet (a), HR-mice fed with HR-AD diet (b), and HR/βC-mice fed with HR-AD diet and administrated with β-carotene (c), were observed macroscopically.

Fig. 3 Effect of β-carotene administration on cytokine and MCP-1 protein levels in the skin of HR-mice. Mice were fed a normal diet (Con) or a HR-AD diet without (HR) and with (HR/βC) p.o. administration of β-carotene daily for 4 weeks. Protein (20 μg) prepared from mouse skin was separated using 10% 1D-PAGE. Cytokine levels were analyzed by immunoblotting specific antibodies against TNF-α, IL-1β, MCP-1, and β-actin as described under "Experimental". Results were analyzed by scanning densitometry. Relative expression levels were estimated by normalization with β-actin. The protein expression in Con was defined as 1.0. Results represent the mean ±SE of each group (n = 8). *p < 0.05 versus HR group compared by Bonferroni’s multiple comparisons test.
levels were decreased by approximately 72% to 36% by the administration of β-carotene, as compared to HR-mice. These results suggest that β-carotene suppressed the expression of inflammatory cytokines and MCP-1 proteins in AD-like skin tissues.

3.3 Changes of gene expression of inflammatory cytokines, thymic stromal lymphopoietin (TSLP), and protease-activated receptor-2 (Par-2) in AD-like mouse skin by β-carotene administration

Next, we analyzed the genetic expression of the inflammatory factor cytokines, thymic stromal lymphopoietin (TSLP), and protease-activated receptor-2 (Par-2). Extracted mRNA from mouse skin and mRNA expression of TSLP, cytokines (IL-6, IL-1β, IL-4, IL-5), and Par-2 were quantified using qPCR with specific primers.

The genetic expression of TSLP, IL-6, IL-1β, IL-4, IL-5, and Par-2 in the skin tissues of HR-mice increased significantly, as compared with those in Con-mice (Fig. 4). In particular, the HR-AD diet markedly elevated the expression of TSLP (approximately 3.3-fold) and IL-6 (approximately 2.3-fold). In contrast, the genetic expression levels of IL-1β, IL-4, IL-5, and Par-2 slightly increased by approximately 1.25~1.39-fold, significantly. The administration of β-carotene suppressed gene expression of inflammatory factors increased by HR-AD diet to the same as or less than

Fig. 4  Expression of cytokine, TSLP, and Par-2 genes in the skin of HR-mice administered β-carotene.
Mice were fed a normal diet (Con) or a HR-AD diet without (HR) and with (HR/βC) p.o. administration of β-carotene daily for 4 weeks. Total RNA (1 µg) was prepared from skin tissues, and qPCR was performed using specific primers against TSLP, IL-6, IL-1β, IL-4, IL-5, Par-2, and β-actin as described under “Experimental”. Relative expression levels were estimated by normalization with β-actin. The expression of mRNA in Con was defined as 1.0. Results represent the mean of ± SE of each group (n = 8). *p < 0.05, **p < 0.01 and ***p < 0.001 versus HR treatment compared by Bonferroni’s multiple comparisons test.
Filaggrin is a major protein in the stratum corneum of the epidermis, contributing to the structure, function, and moisture of skin. Filaggrin mutations are also associated with skin allergic diseases\(^{23,24}\), and filaggrin deficiencies can cause eczema and dry skin\(^{25}\). Based on this, we examined the effects of β-carotene on the expression of filaggrin protein and gene by western blotting and qPCR, respectively. As shown in Fig. 5a, mice fed with the HR-AD diet showed decreased filaggrin protein levels by approximately 50\%, as compared to those fed a normal diet. However, β-carotene administration increased filaggrin protein levels in skin tissues, by approximately 1.75-fold as compared to those in Con-mice fed a normal diet, and approximately 3.5-fold as compared to those in HR-mice fed with HR-AD diet (Fig. 5a). In contrast, while filaggrin gene levels were not significantly changed by feeding with an HR-AD diet, filaggrin gene expression in mice administrated β-carotene increased by approximately 3-fold as compared to that in Con- and HR-mice (Fig. 5b). These results indicate that β-carotene treatment enhanced filaggrin protein levels at the transcriptional level.

3.4 Effect of β-carotene on filaggrin expression

These results led us to investigate whether β-carotene changes the expression and activity of matrix metalloproteinases (MMPs), which are involved both with degrading ECM and with regulating chemokines in mouse skin. The mRNA expression of MMP-2 and MMP-9 in the skin tissues of HR-mice did not change as significantly as that of Con-mice (Fig. 6a). In contrast, β-carotene significantly decreased mRNA expression of MMP-9, but not MMP-2 in skin tissues (approximately 19\%). These results indicate that β-carotene administration suppresses MMP-9 gene expression, while the feeding of HR-AD diet in Con-mice did not induce genetic expression of MMPs in skin tissues.

Next, gelatin zymography was performed to measure the activities of MMPs in the skin tissues of each group of mice. In the application of gelatin zymography against hairless mouse skin, proMMP-2, MMP-2, and proMMP-9 can be detected, however it is difficult to detect MMP-9\(^{26}\). The changes observed in the activity of mature MMP-2 on...
gelatin zymography using skin tissues are positively correlated with the activities of proMMP-2, because the inhibition of proMMP-2 found in vivo has been removed under the conditions of the assay. For this reason, only the levels of proMMP-2 and proMMP-9 were quantified. As shown in Fig. 6b, proMMP-2 and proMMP-9 activities increase in the skin tissues of HR-mice. β-Carotene administration significantly decreased these activities, approximately 25% for proMMP-2 and 57% for proMMP-9 as compared to those in HR-mice. These results indicate that β-carotene suppressed the activities of both proMMP-2 and proMMP-9.

4 Discussion

In the current study, we found that administration of...
β-carotene (Fig. 1) markedly improved the appearance of AD-like skin of mice fed with an HR-AD diet (Fig. 2). The protein and mRNA expression of inflammatory factors in the skin tissues of HR-mice, were significantly increased as compared to those of Con-mice and were suppressed by treatment with β-carotene (Figs. 3, 4). In addition, β-carotene administration increased the mRNA and protein expression of the structural protein filaggrin in the stratum corneum of the epidermis (Fig. 5), while significantly reducing the activity and mRNA expression of MMP-9 (Fig. 6). Overall, β-carotene was shown to be a potent anti-inflammatory agent, which reduced inflammation caused by AD and enhanced the barrier function of the skin (Fig. 7).

Previous studies have shown that the mice fed with an HR-AD diet for 56 days (8 weeks) could be used as a dry skin-based experimental model for AD. This is based on skin dryness, impaired barrier function, epidermal thickening, and through immunological parameter changes. In addition, it has been reported that symptoms induced by feeding an HR-AD diet for 8 weeks, 8~10 weeks, or 12~13 weeks, are related to deficiencies of magnesium, unsaturated fatty acids, or certain starches. In our current study, we have shown that AD-like skin inflammation in mice fed with HR-AD diet for 11 weeks was completely resolved by the intake of β-carotene (Figs. 2-6). Since normal and HR-AD diet contains vitamin A, but not β-carotene, the function of β-carotene in AD-model mice might not be mediated by vitamin A. These results suggest that a dietary deficiency of β-carotene might be a key cause of AD-like symptoms and that β-carotene administration influences various physiological processes in skin tissues, including the enhancement of cell communication and the inhibition of metabolic activation. The mechanism of action of β-carotene on AD-like skin is currently under investigation.

Recently, it has been reported that the changes in the skin of mice with AD that have been fed with the HR-AD diet containing β-carotene or lycopene were less than in those of mice fed with the HR-AD diet. Normal mice were fed with (1) a standard diet (Co group), (2) HR-AD diet (low zinc/magnesium diet) (HR group), (3) HR-AD diet containing β-carotene (HR-C group), and (4) HR-AD diet containing lycopene (HR-L group) for 8 weeks. The administration of β-carotene and the feeding of HR-AD diet was performed simultaneously, since an HR-AD diet containing β-carotene was used. The dose of β-carotene in this study was estimated to be 3~5 mg/day. The appearance or histopathological and hematological observations in the skin were assessed, and the levels of serum IgE or skin TARC (thymus and activation regulated chemokine) and CCL27 were measured. CCL27 in the HR-C group or TARC in the HR-L group were significantly lower than in the HR group.

In the current study, we used an experimental design that differs from a previous study, in which a specific amount of β-carotene was sequentially administered into AD-like mice. Oral administration of β-carotene (0.6 mg/day) for 4 weeks to AD-like mice fed with an HR-AD diet for 11 weeks was accomplished by a feeding-needle (Fig. 1c). The amount and administration schedule of β-carotene (0.6 mg/day, 4 weeks) was approximately one seventh of the estimated amount of β-carotene (3~5 mg/day, 8 weeks) and one half of the period (8 weeks) used in the previous study. Since we used less β-carotene than the previous study, we did not see the obvious orange pigmentation of skin in that report. As shown in Fig. 2, β-carotene improved AD-symptoms remarkably through reduction of both the expression of inflammatory factors and MMP levels and an increase of filaggrin expression in skin tissues (Figs. 2-6). This report is the first to show that post-administration of β-carotene can be extremely effective to ameliorate AD symptoms in HR-mice. It would be interesting to investigate the effects of β-carotene treatment on processing enzymes such as MT-MMP-1, thrombin, and factor Xa as well as tissue inhibitor of metalloproteinase (TIMP) in skin tissues.

β-Carotene is a scavenger of free radicals such as peroxyl radicals, superoxide anions, hydroxyl radicals, and in par-
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In particular, it is known to be a potent quencher of $^{1}\text{O}_2^{\bullet\bullet}$, $^{3}\text{O}_2$ and $^{3}\text{O}_2$. β-Carotene has been intensively studied for its potential to treat skin that is photo damaged$^{33-35}$ or skin damaged by ozone$^{36}$. β-Carotene also has prooxidant properties in skin exposed ultraviolet A light$^{37,38}$. It is not clear whether β-carotene acts as a quencher of $^{1}\text{O}_2$ (antioxidant), since it exhibits both protective as well as potentially harmful roles in human skin.

5 Conclusion

In the current study, β-carotene improved AD-like skin by reducing inflammation and degrading ECM and by increasing filaggrin expression, which plays a pivotal role in skin barrier function. β-Carotene might be a useful therapeutic and preventive agent against skin disorders, including AD.

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Conflict of interest statement

The authors declare no conflict of interest.

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