Journal of Acupuncture Research

Journal homepage: http://www.e-jar.org

Original Article

Astaxanthin Ameliorates Atopic Dermatitis by Inhibiting the Expression of Signal Molecule NF-kB and Inflammatory Genes in Mice

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Article history:
Submitted: October 05, 2022
Accepted: October 25, 2022

Keywords:
astaxanthin, atopic dermatitis, inflammation, NF-kB

https://doi.org/10.13045/jar.2022.00255
pISSN 2586-288X eISSN 2586-2898

ABSTRACT

Background: This study was conducted to determine the anti-inflammatory effect of astaxanthin, on atopic dermatitis.
Methods: Changes in mouse body weight, lymph node weight, and the degree of improvement in symptoms were measured to determine the inflammatory response. Real-time reverse transcription-polymerase chain reaction tests were performed to determine the degree of expression of inflammation-related cytokines (IL-31 and IL-33 and chemokines such as CCL17 and CCL22), and western blot analysis was performed to evaluate the expression of inflammation-related factors (iNOS, COX-2, and NF-kB signaling molecules p-IkBα, p50, p-65 and pSTAT3).
Results: The degree of symptoms significantly improved in the PA+AX group. Lymph node weight in the PA+AX group was lower than the PA group. Inflammatory cytokines (IL-31, IL-33, and inflammatory chemokines such as CCL17 and CCL22) were significantly reduced in the PA+AX group compared with the PA group. The expression of inflammatory genes (iNOS, COX-2, NF-kB and signaling molecules (p-IkBα, p50, p65, and p-STAT 3) was lower in the PA+AX group compared with the PA group.
Conclusion: Astaxanthin may modulate the inflammatory response in a mouse model of atopic dermatitis and has an anti-inflammatory effect.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin condition/disease associated with various factors, including immunological abnormalities and exposure to allergens that contribute to the pathogenesis and development of skin lesions [1]. AD is a recurrent skin condition/disease with various symptoms such as dryness, edema, erythema, psoriasis, epidermal hypertrophy, and scab [2-6]. AD is considered challenging for patients because of the physical and mental burdens it places on the patient [7]. Corticosteroids are commonly used as an effective treatment for treating AD. However, long-term use corticosteroids are not recommended because of the associated side effects. Common symptoms are skin atrophy and symptomatic rebound after stopping treatment, especially with topical steroids such as betamethasone valerate [8,9].

Recently, bioactive natural products have been in the spotlight as a potential medication for numerous conditions/diseases [10]. Astaxanthin [3,3'-dihydroxy-β, β-carotene-4,4'-dione (AX)] is a xanthophyll carotenoid which can be found in microalgae, crustaceans, and complex plants [11]. It has been reported that AX has anti-inflammatory effects in adipose tissues, and has significant protection against skin damage [12-15]. It has also been reported

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to be a powerful anti-oxidant [16], which suppresses oxidative stress and inflammation [17]. AX has been reported to inhibit the production of inflammatory related factors by inhibiting the NF-kB transcription pathway [18,19].

Several pharmacological effects such as anti-oxidant and anti-inflammatory effects of AX have been studied, however, no study has been dedicated to the potential effect of AX on AD. In general, phosphorylation of STAT3 increases in an inflammatory response or disease state. It is thought that STAT3 plays a major role in controlling inflammation in skin conditions/diseases such as AD and psoriasis as well as in normal keratinocyte metabolism [20]. In addition, activation of the NF-kB pathway leads to the transcription of numerous genes including cytokines, chemokines, and growth factors that are involved in the beginning of the inflammatory response [21].

STAT3 and NF-kB are important therapeutic targets in AD since they play a crucial role in the pathogenesis of this condition/disease [22-25]. Therefore, in this study an animal model of AD was used to investigate whether AX could ameliorate AD related symptoms through an anti-inflammatory effect by controlling the activity of NF-kB transcription factor and STAT3.

Materials and Methods

Materials

AX was purchased by Sigma-Aldrich Korea. RiboEX RNA Extraction Kit was obtained from GeneAll Biotechnology, Seoul, Korea. High-Capacity RNA-to-cDNA kit and StepOnePlus PCR System was obtained from Applied Biosystems, Foster City, CA, USA. Nitrocellulose membrane was obtained from Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA.

Animal treatment

Eight week old male HR1 mice (Saeronbio, Uiwang, Korea) were purchased and managed according to KFDA guidelines of the humane animal care and use, which were all 9 and eight weeks old. There were three randomized experimental groups including PA+vehicle (n = 3). PA+AX (n = 3), and Con (n = 3) in this experiment.

Ethics statement

The protocols used in this experiment were recognized by the Chungbuk National University Institutional Animal Care and Use Committee (Approval no.: CBNUA-1073-17-01) after suitability for ethical and scientific care procedures scrutinized and confirmed.

Administration of AX

Each group was made up of 3 mice. AD was induced by using PA on the dorsum skin. In the first group (PA+vehicle, n = 3), 100 μl (10 μl/cm^2) of 5% PA solution was spread on the dorsum of back skin three times a week for four weeks. The second group (PA+AX, n = 3) were applied with PA, and 3 hours after 100 μl of 1 mg/ml AX (10 μg or /cm^2) were applied. Age-matched HR-1 mice were used as the control group (Con, n = 3).

Weight Measurement of lymph node weight as well as body

In order to measure Body weight changes during the experimental period, an electronic balance (Mettler Toledo, Greifensee, Switzerland) was used once a week for 4 weeks. Lymph nodes was collected from the sacrificed mice and then they were also measured in the same manner.

Evaluation of clinical score

The development of erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), or 3 (severe). The total clinical dermatitis severity score for each mouse was defined as the sum of the individual scores [26].

RNA quantification

mRNA expression levels of IL-31, 33, and CCL-17, 22 were measured in mouse back skin using RT-PCR as previously described [27]. To retain total RNA, the tissue from the skin of the mice back was collected and the RiboEX RNA Extraction Kit was used. The High-Capacity RNA-to-cDNA kit was utilized for cDNA synthesis. RT-qPCR was then performed with specific primers in the StepOnePlus PCR System. Levels of mRNA were normalized to the 18S sequence, which was used as a house-keeping control. The fold change between groups was determined for all targets using the 2ΔΔCt method. Specific primer sequences are described as follows.

Western blot analysis

100 mg of skin or ear tissue, or about 1 × 10^6 cells were harvested and homogenized with lysis buffer [50 mM Tris pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μl/ml aprotinin, 1% igapel 630 (Sigma Chem. Co. St. Louis, MO, USA), 10 mM NaF, 0.5 mM EDTA, 0.1 mM EGTA and 0.5% sodium deoxycholate]. The extracts were centrifuged at 23,000 g for 1 hour. Equal amounts of protein (20 μg) were separated on a sodium dodecyl sulfate (SDS) / 10%-polyacrylamide gel, and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Blots were blocked for 2 hours at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline [10 mM Tris (pH 8.0) and 150 mM NaCl] solution containing 0.05% tween-20. The membrane was incubated for 4 hours at room temperature with specific antibodies: mouse monoclonal antibodies directed against iNOS, COX-2 and STAT-3 (1:500; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), and rabbit polyclonal antibodies against p65 and IkB-α (1:500), and mouse monoclonal antibody against p50 (1:500; Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA) The blot was then incubated with the equivalent conjugated anti-rabbit immunoglobulin G-horseradish peroxidase (Santa Cruz

Biotechnology Inc. Santa Cruz, CA, USA) was then incubated with the blot. the enhanced chemiluminescent Western blotting detection system (Piscataway, NJ, USA) was utilized for finding Immunoreactive proteins.

Statistical analysis

The experimental process was repeated more than three times with similar findings. GraphPad Prism 5 software (Version 5.03; GraphPad software, Inc., San Diego, CA) was utilized during the statistical analysis. One-way ANOVA and Tukey's multiple comparison test were used for to test for statistical significance. All values were given in the form of mean ± SD. Statistical significance was set at $p < 0.05$ for all tests.

Results

Effects of AX treatment on body weight

Body weight during the experimental period did not significantly differ during treatment (Fig. 1).

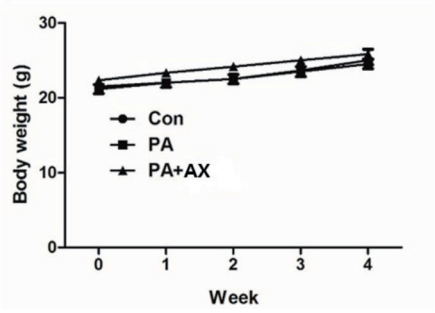


Fig. 1. Differences in body weight of mice. There were three randomized experimental groups including PA+vehicle (n = 3), PA+AX (n = 3), and Con (n = 3) in this experiment. 100 μl (10 μl/cm^2) of 5% PA solution was applied on the back skin of the mice three times a week for four weeks. The PA+AX group (n = 3) were applied with PA, and 3 hours later 100 μl of 1 mg/ml AX (10 μg or /cm^2) was applied. Age-matched HR-1 mice were used as the control group (n = 3).
AX, astaxanthin; con, control; PA, phthalic acid.

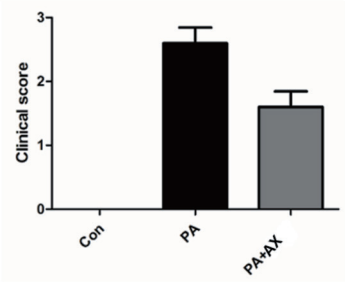


Fig. 2. Differences in clinical score. Compared with PA group, the clinical score of the PA+AX group was significantly less indicating AST treatment improved the symptoms of AD.
AX, astaxanthin; con, control; PA, phthalic acid.

Effects of AX treatment on clinical score

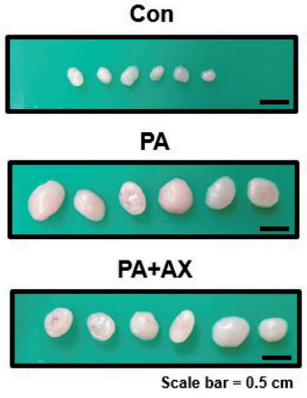
The clinical score was measured during the experimental period. The score of the PA+AX treated group significantly decreased compared with the PA treated group (Fig. 2).

Effects of AX treatment on weight of lymph node

The lymph nodes become enlarged as typical of an inflammatory response or in diseased state. Therefore, the lymph node weight was evaluated to determine whether AX could inhibit the increase in volume of lymph node. Compared with the control group, the lymph node weighed more in the PA treated group. When the weight of the PA group lymph node was compared with lymph node weight in the PA+AX treated group, the weight was significantly lower (Fig. 3).

Effects of AX treatment on inflammation- related cytokine and chemokine expressions

The expression levels of related cytokines(IL-31, IL-33) and chemokines (CCL-17, CCL-22) had significantly increased in the PA treated group compared with control, however, in the PA+AX



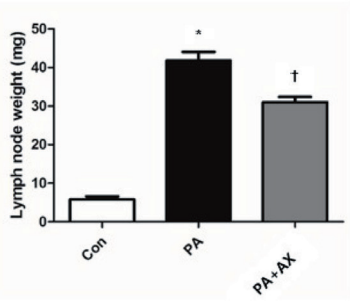


Fig. 3. Differences in weight of lymph node. Lymph node was dramatically enlarged in the PA treated group as compared with the control group. However, the size and weight of lymph node was significantly less in the AX treated group compared with the PA treated group.
AX, * and † represents astaxanthin.
* $p < 0.001$, significant compared with control group.
† $p < 0.001$, significantly decreased compared with PA group respectively.
AX, astaxanthin; con, control; PA, phthalic acid.

treated group it was significantly less compared with the PA treated group (Fig. 4).

Effects of AX treatment on inflammatory genes

The expression level of inflammatory genes including iNOS and COX-2 was significantly higher in the PA group compared with control, while in the PA+AX treated group it was significantly less than the PA treated group (Fig. 5).

Effects of AX treatment on NF-kB signal molecules

The expression level of p-IkBα, p50, p65 was significantly increased in the PA group compared with control. However, the expression level in the PA+AX treated was less compared with the PA group (Fig. 6).

Effects of AST treatment on STAT3 pathways

The expression level of p-STAT3 was significantly increased in the PA group compared with control. On the other hand, the expression level in the PA+AX treated group was significantly less compared with the PA group (Fig. 7).

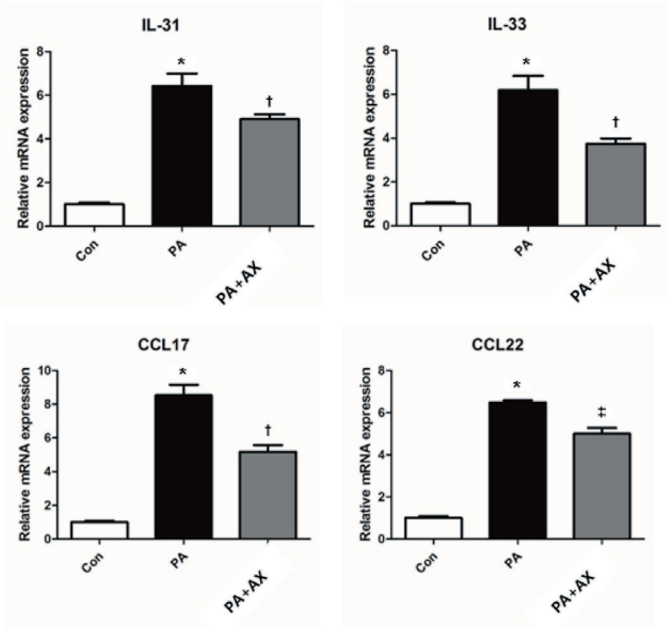


Fig. 4. Effects of AX on IL-31, IL-33, CCL-17, and CCL-22 expression. mRNA expression levels of pro-inflammatory cytokines and chemokines in back skin of mice were measured using quantitative real-time RT-PCR. In the PA treated mice the expression of cytokines and chemokines increased significantly compared with the control, but, compared with the PA+AX treated group it was significantly less.
AX *, † and †† represents astaxanthin.
* $p < 0.001$, significant compared to control group.
† $p < 0.05$, significantly decreased compared to PA group.
‡ $p < 0.001$, significantly decreased compared to PA group respectively.
AX, astaxanthin; con, control; PA, phthalic acid.

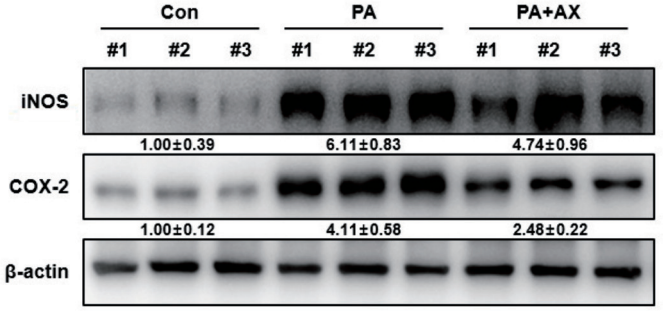


Fig. 5. Effects of AXon iNOS and COX-2 expressions. 100 mg skin or ear tissues or about 1×10^6 cells of mice were harvested and homogenized, then western blot analysis was performed to confirm the expression levels of the inflammatory genes iNOS and COX-2 (β-actin was used as a housekeeping protein).
AX, astaxanthin; con, control; PA, phthalic acid.

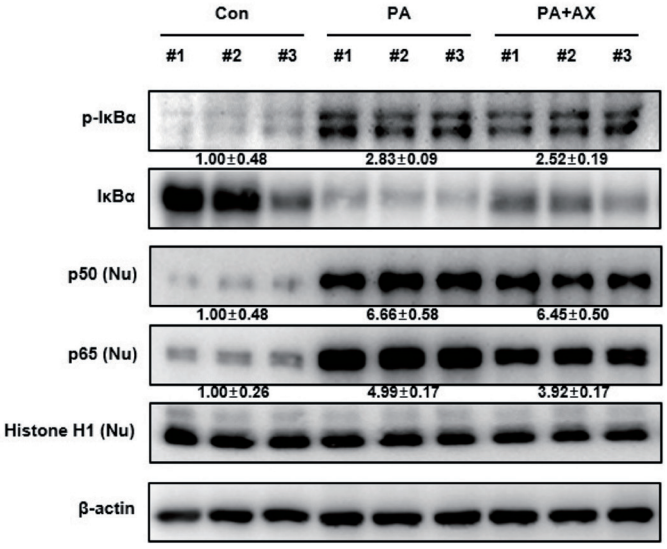


Fig. 6. Effects of AST on p-IkBα, p50 and p65 expressions. 100 mg skin or ear tissues or about 1×10^6 cells of mice were harvested and homogenized, and western blot analysis was performed to confirm the expression levels of the NF-kB signal molecules p-IkBα, IkBα, p50, p65 (histone H1 and β-actin were used as a housekeeping proteins).
AX, astaxanthin; con, control; PA, phthalic acid.

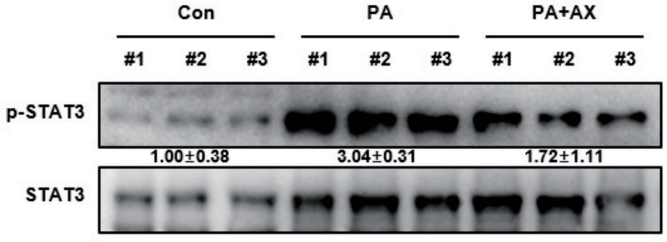


Fig. 7. Effects of AX on p-STAT3 expressions. 100 mg skin or ear tissues or about 1×10^6 cells of mice were harvested and homogenized, western blot analysis was performed to confirm the expression levels of p-STAT3 and STAT3.
AX, astaxanthin; con, control; PA, phthalic acid.

Discussion

AD is a chronic, relapsing inflammatory skin condition/disease which is characterized by itching, pruritus, skin dryness, skin wrinkles, eczema, and elevated serum IgE levels [28]. In addition, AD has a major impact on the patient's lifestyle. Significant problems that patients confront in daily life include secondary infections, severe discomfort, impaired social interactions and sleep disturbances such as insomnia and awakening [29].

The pathogenesis of AD involves various factors including genetic, environmental, and other factors such as related cytokines and immunoglobulin (Ig) E produced by T-cells, B-cells, Langerhans cells, eosinophils, and keratinocytes [30]. Keratinogenesis results in pro-inflammatory cytokines such as interleukin IL-1, IL-6, and TNF-α production through stimulation. This attracts molecules that can be attached to lymphocytes, macrophages, and eosinophils, further aggravating inflammation [31].

Activation of the NF-kB pathway initiates and exacerbates the inflammatory response. In addition, increased phosphorylation of STAT3 has been observed in inflammatory response or disease state in recent studies. Suppressing the activation of STAT3 and NF-kB signal molecules, and may therefore be a mechanism of AD treatment.

Due to its complex pathophysiology, a systematic and continuous treatment for AD is required [32]. Treatment approaches of AD include moisturizing, managing the skin, training to avoid aggravating factors, topical treatment, treatment for symptom relief, and use of immunosuppressants [33]. Since some of these treatments have severe side effects, treatments with fewer side effects are needed, and bioactive natural products may be a potential alternative.

AX, a carotenoid pigment recently reported to have substantial antioxidant effect, is found in a marine environment [34]. Chlorophyte alga Hematococcus pluvialishas contains the most AX in nature, where it synthesizes a large amount of AX during photosynthesis or when exposed to ultraviolet light [35]. There is an abundance of AX available as a natural resource.

Studies have reported that AX exerts its anti-inflammatory effect by suppressing the expression of proinflammatory cytokines through the inhibition of NF-kB activation in neutrophils, macrophages, and lymphocytes [36-38]. AST inhibits ROS and cell protection from NO-induced oxidative stress. The purpose of this study was to determine whether AST treatment ameliorates inflammation of skin lesions such as AD, in an animal model where AD is induced by PA, by inhibiting the activation of NF-kB and STAT3 pathways.

AX treatment had no significant difference in the body weight of mice compared with the PA treated group and the control group. This suggests that AX treatment could be used as a safe therapy for AD, since it does not overburden the body. Moreover, the AX treated group showed a significant decrease in clinical score compared with the PA treated group. This suggests that AX has an obvious therapeutic effect on AD (in the murine model).

PA-induced AD increased lymph node weight in a murine model, but when treated with AX, the weight of lymph node was significantly less in the AX group of mice. To investigate the effect of AX on inflammatory cytokines such as IL-31 and IL-33, and chemokines such as CCL17, CCL22.

Quantitative Real-Time PCR was used to quantify levels. AX treatment resulted in a lower level of AD related cytokine and chemokine expression compared with the PA group. This showed that AX suppressed the inflammatory response, which may lead to the improvement in the symptoms of AD. Through western blot analysis, the expression of inflammatory genes such as iNOS and COX-2 were significantly lower in the AX treated group compared with the PA group. In addition, the expression of NF-kB signal molecules such as p-IkBα, IkBα, p50, p65 were significantly lower in the AX treated group compared with the PA group. Furthermore, the expression level of p-STAT3 was also significantly lower in the AX treated group compared with the PA group. These results suggest that AX may work as an anti-inflammatory agent through the NF-kB and STAT3 pathways, which play a crucial role in the pathogenesis of inflammatory diseases including AD. In Korea, bee venom pharmacopuncture is commonly used as a treatment for AD. Recent studies showed that bee venom (BV) also inhibits the generation of inflammatory factors by suppressing NF-kB activity [39]. As mentioned above, AST works as an anti-inflammatory material through NF-kB and STAT3 pathways, therefore, AST could be used as a substitute for BV in the treatment of AD. However, there are some limitations in this study. Although both AX and BV have an anti-inflammatory effect, the efficacy of these chemicals must be compared. In studies in the future, it would be valuable to determine whether AX is more effective in treating AD than BV. In addition, this was an animal study, therefore, the safety and efficacy of AX treatment for AD needs to be assessed in clinical trials. Moreover, AX can be easily oxidized and reduced in activity by heat and light, so the stability of AX needs to be examined, for example, via AX liposome treatment [40,41].

Conclusion

AX may be a safe and effective treatment for AD due to its anti-inflammatory and antioxidant therapeutic effects and may in the future be an alternative for ameliorating AD related symptoms and inflammatory response.

Author Contributions

Conceptualization: DHK. Methodology: DHK and HSS. Formal investigation: DHK and HSS. Data analysis: DHK, YSK and HSS. Writing original draft: DHK. Writing - review and editing: DHK, YSK and HSS.

Conflicts of Interest

Ho Sueb Song has been the editor in chief of Journal of Acupuncture Research since April 2011, but had no role in the decision to publish this original article. No other potential conflict of interest relevant to this article was reported.

Acknowledgments

This work was supported by the National Research Foundation

of Korea (NRF) grant funded by the Korea government (MSIT) (no.: 2020R1A2C1013956).

Ethical Statement

This research did not involve any human or animal experiment.

Data Availability

All relevant data are included in this manuscript.

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