Astaxanthin Supports Normal Human Dermal Fibroblasts from Neutrophil-Induced Collagen Damage in Co-Culture

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REVIEW

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ABSTRACT

Collagen I and collagen III are the major structural protein in the extracellular matrix of skin produced by fibroblasts. UV exposure as well as other insults results in the infiltration of neutrophils within the epidermis and dermis, inducing collagen damage and contributing to the process of photo-aging. To study the efficiency of Astaxanthin as protection from collagen damage induced by primed neutrophils, we first studied the effect of Astaxanthin on neutrophil activation. Astaxanthin inhibited the production of superoxides and MPO in TNFα-stimulated neutrophils in a dose-dependent manner in a range of 5-50 μM. The addition of neutrophils activated with TNFα to normal human dermal fibroblast cultures, caused significant collagen I and collagen III damage. To study whether Astaxanthin may protect from collagen damage, it was added to neutrophils activated with TNFα for 10 min before their addition to fibroblasts. Astaxanthin prevented both collagen I and collagen III damage induced by activated neutrophils in a dose-dependent manner in the co-cultures in correlation with the inhibition of both NADPH oxidase-producing superoxides and MPO activity-producing halides. The addition of Astaxanthin to fibroblast cultures did not increase their expression. In conclusion, the results suggest that Astaxanthin is effective against collagen damage in fibroblasts induced by neutrophils, thus indicating Astaxanthin's possible potential for enhanced skin health.

Keywords: Dermal fibroblasts, neutrophils, collagen I, collagen III, superoxides, astaxanthin.

ABBREVIATIONS

TNFα - Necrosis factor-alpha

NHDF – Normal human dermal fibroblasts

HRP - Horseradish peroxidase

MPO- Myeloperoxidase

ROS – Reactive oxygen species

INTRODUCTION

Dermal collagen represents the most abundant extracellular matrix (ECM) protein and constitutes the bulk of human skin [1]. The major components of the dermis are fibroblasts that produce, organize, and maintain collagen

and elastic fibers [2]. Although 28 types of collagen have been identified, collagens I and III are the main constituents of the dermal extracellular matrix composing approximately 85-90% and 8-11%, respectively, of the total collagen in the skin and play a major role in skin elasticity [3]. Synthesis of collagen I and III are reduced in aged and photodamaged skin, possibly resulting in the altered organization of fibrillar collagen and, thus, contributing to the wrinkled appearance of skin [4].

Inflammation and accumulation of reactive oxygen species are now believed to be the causative factors in both types of skin aging: intrinsic (or chronological) and extrinsic UV-induced aging (or photoaging) [4, 5]. Several global gene expression profiling studies have linked the immune system and inflammation genes with photoaging, regardless of ethnic type [6]. UV induces an array of events that can lead to inflammation: release of inflammatory cytokines by epidermal keratinocytes and fibroblasts such as tumor necrosis factor-alpha (TNFα) [7], ROS generation [8], production of inflammatory mediators [9], peroxidation of the membrane lipids [10], and skin cell death [11]. Neutrophils have been reported to infiltrate the skin within the epidermis and dermis following exposure to natural sunlight to erythemogenic doses of UVB and solar simulating radiation (SSR), infrared radiation, and heat [12, 13]. These neutrophils are packed with potent proteolytic enzymes for the clearance of UV-induced apoptotic cells and for killing skin cells with oxidized surface lipids, but they are also capable of degrading collagen and elastic fibers [14]. Furthermore, it was reported that infiltrating neutrophils, rather than keratinocytes and fibroblasts, were the major source of proteolytic enzymes and free radicals following exposure to erythemogenic doses of SSR, and thus may be the key players in photoaging [15]. Extracellular matrix molecules, such as collagens, are good targets for oxygen free radicals. Collagen is the only protein susceptible to fragmentation by superoxide anion as demonstrated by the liberation of small 4-hydroxyproline-containing-peptides. The susceptibility of collagen to superoxide anions may be due to its special triple-helical structure. Hydroxyl radicals in the presence of oxygen or hypochlorous acid cleave collagen into small peptides, and the cleavage seems to be specific to proline or 4-hydroxyproline residues [16]. Carotenoids and phenolics are associated with beneficial health effects, which are related at least in part to their antioxidant activity [17, 18]. Astaxanthin (3,3-dihydroxy-beta, beta-carotene-4,4-dione) belongs to the xanthophyll subclass of carotenoids. The powerful chain-breaking antioxidant properties of Astaxanthin have been demonstrated, both in vitro and in vivo, especially as an inhibitor of LDL oxidation [19]. The present study aimed to determine the inhibitory effect of Astaxanthin on neutrophil activation and its potential effect to protect from skin damage induced by primed neutrophils. Prevention of collagen damage induced by neutrophils in the co-cultures with fibroblast by Astaxanthin may have a great significance in protecting and preventing skin aging.

MATERIALS AND METHODS

Neutrophil purification – Forty ml blood with neutrophil count between 3-7 X 10^6/ml was drawn from healthy volunteers with their written contest. Neutrophils at 95% purity were obtained by Ficoll/Histopaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes [20]. Cells were counted and their viability was determined by trypan blue exclusion. The study was approved by the institutional Human Research Committee of the Soroka University Medical Center (No. 0370-16-SOR). Neutrophils were stimulated with TNFα (Peprotech, Rocky Hill, NJ, USA) Lycored Astaxanthin 10% was supplied by LycoRed Natural Products Industries Ltd. (Beer-Sheva, Israel).

Fibroblasts cell culture: Normal human dermal fibroblasts (NHDF) of adult donors (Promocell, Heidelberg, Germany), were cultured in and supplemented with Fibroblast Growth Medium-2 (Promocell) (final supplemental concentration in the medium: fetal calf serum 0.02ml/ml, basic fibroblast recombinant human growth factor 1mg/ml, and recombinant human insulin (5µg/ml), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Beit-Haemek, Israel). The cells were

maintained at 37°C in a humidified atmosphere containing 5% CO_2. When fibroblasts reached more than 80% confluence, the cells were seeded in 24-well plates.

Immunofluorescence analysis: For immunofluorescence detection, fibroblasts or co-cultures of fibroblasts and neutrophils were fixed with methanol at -20°C for 3 min followed by a wash in PBS. Fixed co-cultures were incubated with anti-collagen III antibodies (Santa Cruz Dallas, Texas, USA) 1:50 or with anti-collagen I antibodies (Southern Biotech Birmingham, Alabama, USA) 1:500 in 5% BSA/PBS for 90 min at room temperature. The cells were washed three times in PBS and incubated with Cy3 anti-mouse, (1:100 in 5% BSA/PBS; Jackson Immuno Research Laboratories, Inc., PA, USA) for 60 min at room temperature. The cells were washed three times in PBS, and the nuclei were stained with DAPI. Then a final wash was performed, and the cells were analyzed by fluorescence microscopy (Olympus, BX60, Hamburg, Germany). Fluorescence intensity for collagen I and III was determined using a CellProfiler Program.

Superoxide generation: A. Cytochrome C reduction-The production of the superoxide anion by neutrophils was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c by the microtiter plate technique, as previously described [20]. Cells ($2.5x10^5$/well) were suspended in 100 µl Hanks' Balanced Salt Solution (HBSS) containing ferricytochrome c (150 mM). The reduction of acetyl ferricytochrome c was followed by the change of absorbance at 550 nm at 2-min intervals on a Versamax Microplate Reader (Molecular Devices, Menlo Park, CA). The maximal rates of superoxide generation were determined and expressed as nmoles.

B. Horseradish peroxidase (HRP) - dependent oxidation of the highly sensitive fluorescent biosensor Amplex Red [21]. The oxidation of Amplex Red occurs outside the cells by HRP, which traps H_2O_2 as soon as it is generated by spontaneous dismutation of O_2-, the first product of NADPH oxidase. Resting and activated neutrophils ($2x10^5$/well) were suspended in KRPG buffer (phosphate buffer, 145 mM NaCl, 4.86 mM KCl, 1.22 mM $MgSO_4$, 5.5 mM D-glucose, 0.54 mM $CaCl_2$, pH 7.35) containing HRP (0.1 unit/ml) and Amplex Red (50 µM). Fluorescence was recorded using a microplate reader with 535nm-excitation and 595nm-emission wavelengths. Background fluorescence was measured in the absence of neutrophil cells.

Myeloperoxidase (MPO) activity: 100 µl of 37°C O-dianisidine hydrochloride solution (1 mg O-dianisidine, 10ml phosphate buffer pH 6.0 + 0.0015% H_2O_2) was added to 100 µl supernatant in a 96-well plate immediately before the optical density was followed by the change of absorbance at 450 nm at 2-min intervals on a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA).

Statistical analysis - Data are presented as the mean ± SEM. Significant differences from control conditions were determined using either one- or two-way analysis of variance (ANOVA) followed by a posteriori Bonferroni's test for multiple comparisons provided by GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

RESULTS

The effect of Astaxanthin on the activation of neutrophils was studied. Astaxanthin was added to neutrophils for 15 min at 37°C before activation by TNFα overnight and MPO was determined in the supernatant. The effect of Astaxanthin on the production of superoxide by activated neutrophils was determined immediately by cytochrome C reduction. As shown in Figure 1, the addition of Astaxanthin in a range of 0.1-50 µM caused a dose-dependent inhibition of MPO release and superoxide production.

To study the effect of Astaxanthin on the damage to collagen I and collagen III induced by primed neutrophils, we used the optimal conditions of co-cultures of fibroblasts and neutrophils that were determined in our earlier study [21]. Immunofluorescence staining of collagen I and collagen III were performed by specific antibodies.

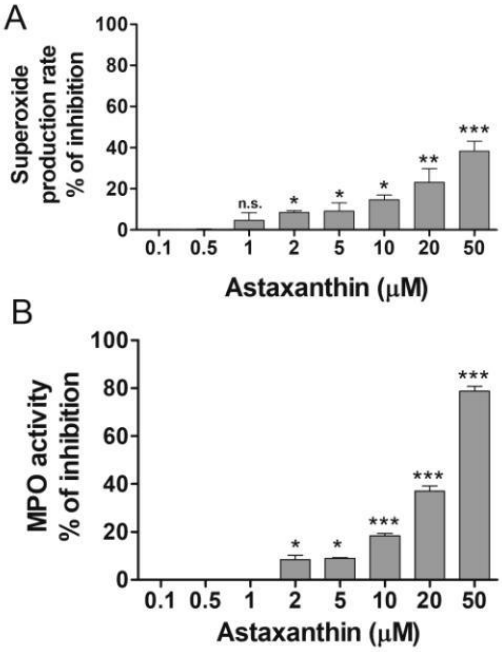

Figure 1: Astaxanthin inhibited neutrophils activation in a dose-dependent manner.

Preincubation of neutrophils with Astaxanthin in a range of 0.1-50 μM for 15 min before theaddition of 100 ng/ml TNFα caused a dose-dependent inhibition of Superoxide production (**A**) and MPO production (**B**) determined at 24 h in the cell supernatant. Superoxide production was measured by cytochrome C reduction immediately after theaddition of TNFα. The results are expressed as % inhibition and are the means ± SEM of 3 independent experiments each in triplicates. * - p<0.05, ** - p<0.01, *** - p<0.001, significant increase from the stimulated neutrophils.

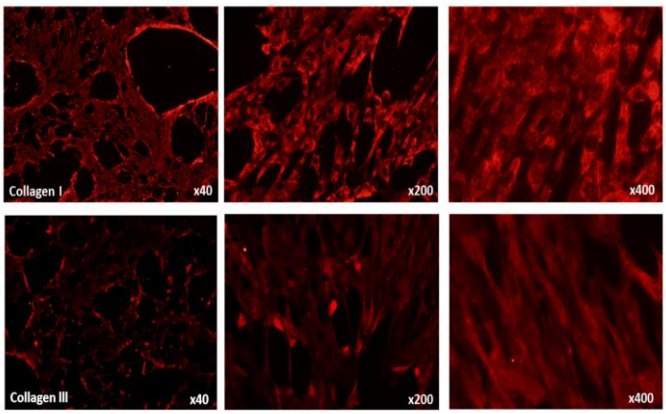

Figure 2: **Different patterns of collagen I and collagen III immunofluorescence staining.**

A representative from three different experiments of collagen staining with 1:500 anti-collagen I (**A**) and with 1:50 anti-collagen III (**B**) in fibroblasts. The pictures were taken with similar exposure.

As can be seen in Figure 2, high magnification shows that there are differences in the pattern of the staining of the two types of collagen, in accordance with others [22], indicating the specificity of the antibodies. For co-cultures, $1X10^5$/ml fibroblasts were plated overnight to obtain confluent cultures. Astaxanthin was added for 15 min to $2X10^5$ neutrophils/ml before their activation with 100 ng/ml TNFα. Activated neutrophils with and without Astaxanthin were added to the fibroblasts overnight. The addition of $2X10^5$ activated neutrophils/ml with TNFα caused significant collagen I damage as shown by immunofluorescence staining (Figure 3A). The addition of Astaxanthin in the range of 5-50 μM causes a dose-dependent inhibition of collagen I damage. The bar graphs (Figure 3B) present the collagen I area staining in the cultures determined by densitometry. The addition of activated neutrophils, significantly ($p< 0.001$) reduced the collagen I area from 65+1% to 42.9±1.2%. Collagen I area was significantly higher ($p<0.05$ or $p<0.01$) in the presence of 20 μM or 50 μM Astaxanthin respectively, in the co-cultures, reaching 48.1±2.5% and 49.1±1.8% respectively. Figure 3C presents the calculated prevention of collagen I loss deduced by the densitometry. As shown 20 μM or 50 μM Astaxanthin prevented collagen I lose by 23.6±11.6% or 28.1±8% respectively. Addition of neutrophils reduced the cell number which had remained unchanged by the addition of Astaxanthin as determined by DAPI staining (Figure 3D), suggesting that it did not induce proliferation. The effect of Astaxanthin on collagen I expression and cell number was also studied in fibroblast cultures. Astaxanthin was added in a range of 5-50 μM to a lower concentration of fibroblasts ($5x10^4$/ml) that resulted in a non-confluent culture, enabling a better evaluation of the effect. The addition of Astaxanthin to the fibroblasts overnight did not affect the expression of collagen I, as shown by representative immunofluorescence analysis (Figure 3E) and collagen I densitometry (Figure 3F). Addition of Astaxanthin to fibroblasts overnight did not affect fibroblast cell number as measured by DAPI (Figure 3G).

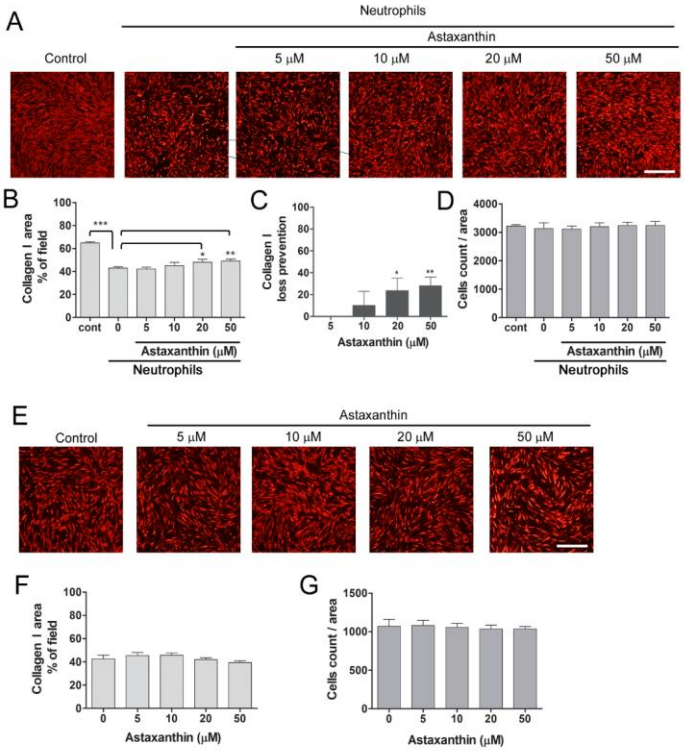

Figure 3: **Prevention of collagen I damage by Astaxanthin.**

The addition of Astaxanthin prevented in a dose-dependent manner collagen I damage induced by neutrophils stimulated with 100ng/ml TNFα. $1X10^5$/ml fibroblasts were plated for 24h to obtain confluent cultures before the addition of neutrophils. Neutrophils $2X10^5$/ml in growth medium were incubated with Astaxanthin for 15 min at 37°C before activation with 100 ng/ml TNFα for 15 min, and then were replaced with the medium of the fibroblast cultures and incubated overnight.

A. Representative immunofluorescence staining of collagen I, magnification X40. **B.** The bar graph presents the densitometry of the immunofluorescence staining of collagen I in A. Shown are the means + SEM of three different experiments. In each experiment, ten fields of each treatment were scanned. **C.** The bar graph presents the calculated % of protection of collagen I damage after scanning the densitometry of the immunofluorescence staining of collagen I. Shown are the mean ± SEM of five different experiments. In each experiment, ten fields of each treatment were scanned. **D.** Cell count by DAPI staining in the cultures shown in A. **E.** Addition of Astaxanthin in the range of 5-50 μM to fibroblasts plated in the concentration of $5x10^4$ cells/ml did not affect Collagen I expression, as shown by the representative immunofluorescence staining, magnification X40. **F.** The bar graphs present the densitometry of the immunofluorescence staining of Collagen I in the average of three different experiments. In each experiment, five fields of each treatment were scanned. **G.** Addition of Astaxanthin did not affect cell number measured by DAPI staining in the fields analyzed for collagen expression presented in E. Shown are the mean ± SEM of four different experiments each in triplicates. Significance: *p<0.05, ** p<0.01.

Similar experiments were performed to study the effect of Astaxanthin in protecting collagen III damage in the co-cultures. The addition of $2x10^5$-primed neutrophils/ml with TNFα caused significant collagen III damage as shown by immunofluorescence staining (Figure 4A). The addition of Astaxanthin in the range of 5-50 μM caused a dose-dependent inhibition of collagen III damage. The bar graphs (Figure 4B) present collagen III area staining in the cultures determined by densitometry. The addition of primed neutrophils significantly ($p<0.001$) reduced the collagen III area from 88.1±1.2% to 58.6±3.9%. The presence of 20 μM caused a significant ($p<0.05$) increase in collagen III area to 65.5±5% and the presence of 50 μM Astaxanthin was more efficient with a significance of $p<0.01$ resulting with 73.2±0.8% collagen III area. Figure 4C presents the calculated prevention of collagen III loss by Astaxanthin deduced by the densitometry. As shown 20 μM and 50 μM Astaxanthin prevented collagen III loss by 23.26±6.7% and 49.4±5.9% respectively. Astaxanthin did not affect cell number as determined by DAPI staining (Figure 4D). The effect of Astaxanthin on collagen III expression and cell number was studied in fibroblast cultures, as was done for collagen I. Addition of Astaxanthin in a range of 5-50 μM a non-confluent culture ($5x10^4$/ml) for overnight did not affect the expression of collagen III, as shown by representative immunofluorescence analysis (Figure 4E) and the densitometry (Figure 4F), and did not affect fibroblast cell number as measured by DAPI (Figure 4G).

The effect of Astaxanthin on the release MPO from neutrophils in the supernatant of the co-culture experiments, described in Figure 4 and 5 was measured overnight. MPO release measured by its activity in the supernatant was inhibited by Astaxanthin in a dose dependent manner (Figure 5A). Superoxides measured by immediately showed a dose-dependent inhibition of superoxides by Astaxanthin (Figure 5B).

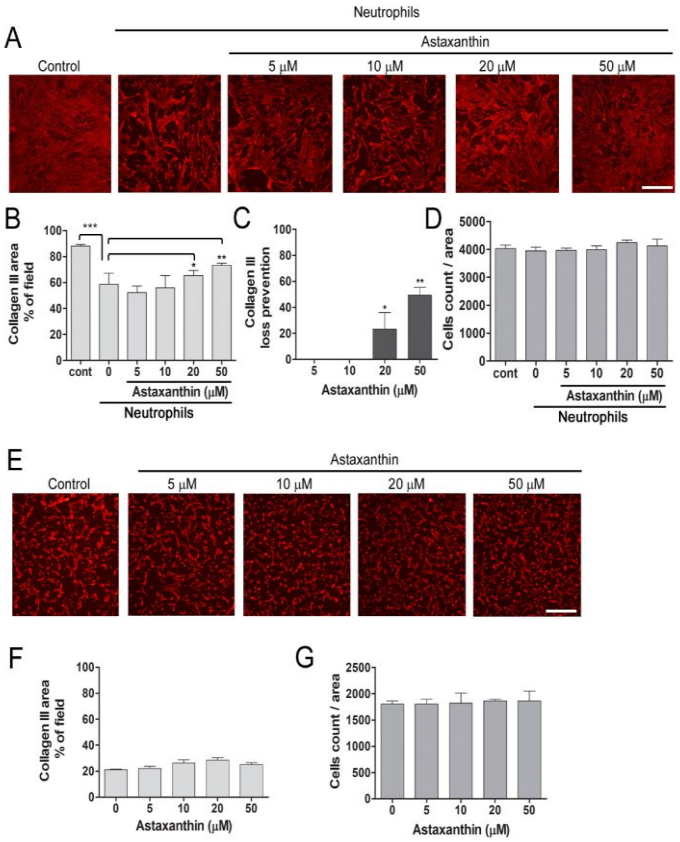

Figure 4: **Prevention of collagen III damage by Astaxanthin.**

The addition of Astaxanthin prevented in a dose-dependent manner collagen III damage induced by stimulated neutrophils as described in Figure 3. **A**. Representative immunofluorescence staining of collagen III, magnification X40. **B**. The bar graph presents the densitometry of the immunofluorescence staining of collagen III in A. Shown are the means ± SEM of three different experiments. In each experiment, ten fields of each treatment were scanned. **C**. The bar graph presents the calculated % of protection of collagen III damage after scanning the densitometry of the immunofluorescence staining of collagen III. Shown are the mean ± SEM of five different experiments. In each experiment, ten fields of each treatment were scanned. **D**. Cell count by DAPI staining in the cultures shown in A. **E.** Addition of Astaxanthin in the range of 5-50 μM to fibroblasts plated in the concentration of $5x10^4$ cells/ml did not affect collagen III expressions, as shown by the representative immunofluorescence staining, magnification X40. **F**. The bar graphs present the densitometry of the immunofluorescence staining of collagen III in the average of three different experiments. In each experiment, five fields of each treatment were scanned. **G**. Addition of Astaxanthin did not affect cell number measured by DAPI staining in the fields analyzed for collagen expression presented in E. Shown are the mean ± SEM of four different experiments each in triplicates. Significance: *p<0.05, ** p<0.01.

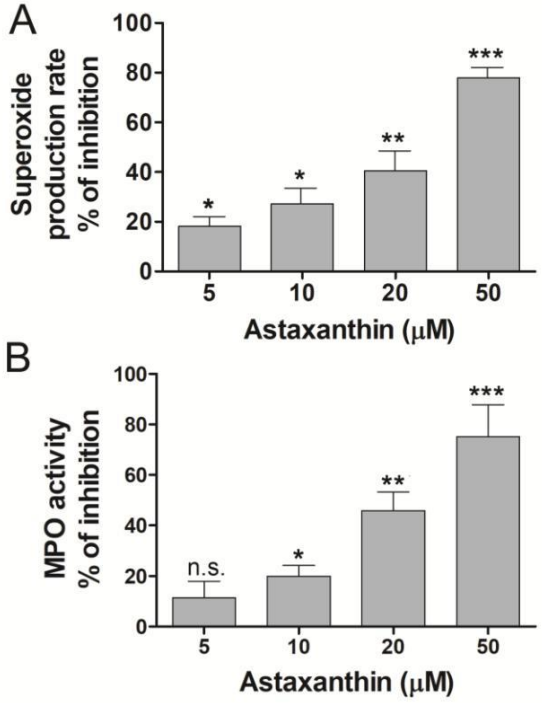

Figure 5: **Inhibition of MPO secretion and superoxide production by Astaxanthin in the co-cultures**.

Dose-dependent inhibition of superoxide production **(A)** and MPO secretion **(B**) by Astaxanthin in the co-cultures. MPO was measured in the supernatant of the co-cultures described in Figures 4 and 5. Superoxide production was measured by Amplex red immediately after the addition of TNFα. Average ± SEM of all experiments described in Figures 4 and 5. Significance: * p<0.05, ** p<0.01, *** p<0.001.

DISCUSSION

In the present study, we show that fibroblast-collagen damage induced by primed neutrophils could be prevented by the addition of Astaxanthin to the co-cultures in a dose-dependent manner, thus, indicating its possible potential for skin protection. Reduction of collagen-I and collagen III may result in the altered organization of fibrillar collagen, thus, contributing to the wrinkled appearance of skin [23]. Collagen I is the most common type of protein and makes up 90% of the skin, [24]. Type-3 collagen is also an important regulator of type-I collagen fibrillogenesis, determining the diameter of the fibrils [25]. Collagen-3 is the first to emerge and acts as a bridge to the wound, after which type-1 collagen appears in conjunction with type-3 during tissue reformation to build a solid bracket and

facilitate wound healing [26]. Addition of Astaxanthin protected against damage of both collagen I and collagen III, indicating its potential in protecting skin damage.

Collagen damage can be induced by different pro-inflammatory and proteolytic enzymes from neutrophils infiltrating the skin following exposure to natural sunlight, erythemogenic dose of UVB, or solar stimulating radiation [12]. Neutrophils have been shown to release various superoxides, cytokines, and proteolytic enzymes including elastase, collagenase, and gelatinase [27]. In our previous study, we showed that the main contributors to collagen damage by neutrophils are superoxides production and hypochlorous acid produced by NOX2 NADPH oxidase and MPO activities, respectively [21]. In the present study we show that both were inhibited by Astaxanthin in a dose dependent manner in correlation to the protection of collagen I and collagen III damage. At physiological concentrations, hypochlorous acid was shown to be a major end-product of the neutrophil respiratory burst [28] with a powerful antimicrobial nature [29]. However, the properties that make it such a useful antimicrobial agent also place the host at considerable risk, and were implicated in the tissue injury associated with various inflammatory diseases [30], and in collagen damage as shown in the present study [21] and accordance with the literature that reported that neutrophils depletion blocked collagen degradation in mouse liver, while addition of neutrophils to corneal fibroblast caused collagen degradation [31, 32].

Our results are in accordance with the reports demonstrating Astaxanthin acts as a scavenger of free radicals and as a potent quencher of reactive oxygen and nitrogen species including singlet oxygen, single and two-electron oxidants [33, 34]. Besides its antioxidant action, it was shown that Astaxanthin also has anti-inflammatory and immunomodulatory activities [35] that result primarily from its ability to scavenge ROS [36]. Its antioxidant and anti-inflammatory effects are synergistic increased when combined with Lyc-O-mato [37]. In addition, it was reported that Astaxanthin interfered with the induction of MMP-1 and neutral endopeptidase elicited by UVA and participate in skin aging and wrinkles [38, 39]. Our results demonstrate the beneficial effect of Astaxanthin in preventing collagen damage in co-culture of fibroblasts and neutrophils, it will be more significant to study also the contribution of keratinocytes in the co-cultures. However, the effect of Astaxanthin supplementation in improving skin texture, appearance (wrinkles), and moisture and in protecting against UV-induced skin was demonstrated in many randomized, controlled trials [40-42]. All of the skin aging characteristics have been shown to be associated with the oxidative metabolism and subsequent ROS production that define this unavoidable phenomenon [43].

In conclusion, activated neutrophils with TNFα-induced collagen I and collagen III damage in fibroblast cultures that is probably mediated by free radicals. The presence of Astaxanthin in the co-cultures prevented collagen I and collagen III damage that correlated with the inhibition of the production of superoxides and halides in a dose-dependent manner, suggesting that Astaxanthin protects against collagen damage by preventing the harmful effect of neutrophils.

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