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Astaxanthin cream alters type I procollagen and Matrix metalloproteinase-1 (MMP-1) gene expression induced by ultraviolet B irradiation in rat skin

Abstract— Anti-oxidant properties of astaxanthin might protect skin from premature aging caused by UVB-rays exposure. In the present study, we explore whether topical astaxanthin cream could decrease MMP-1 and increase type I procollagen gene expression on UVB-exposed skin. Wistar rats were randomly assigned into 3 group: group that only exposed to UVB (P₀) without topical treatment, UVB-exposed groups with base-cream treatment (P₁) and UVB-exposed groups with astaxanthin-cream treatment (P₂). UVB exposure was done three times a week with a dose of 130mJ/cm² for 6 consecutive weeks. Creams were applied daily. Skin biopsy was performed to examine the expression of MMP-1 and type I procollagen using semi-quantitative PCR and Western blot technique. The result showed that the expression of MMP-1 in P₂ group was significantly lower than P₀ group (P<0,05). Antioxidant activity of topical astaxanthin might be involved in decreasing MMP-1 gene expression. Therefore, topical astaxanthin might be considered as a treatment to protect skin from UVB-induced photoaging

Keywords—Astaxanthin cream, photoaging, MMP-1, type I procollagen, UVB rays

1 INTRODUCTION

Skin aging involves intrinsic and extrinsic process [8]. Environmental factors, such as primarily chronic ultraviolet B (UVB) light exposure causes extrinsic skin aging which is then called photoaging [21,17,11]. UVB increases the production of Reactive Oxygen Species (ROS) of the skin [11,25]. ROS could act as a secondary messenger which upregulated activator protein-1 (AP-1), resulted in collagen degradation and inhibition of procollagen synthesis [30,29,24,10].

Among genes involved in the aging process, matrix metalloproteinases (MMPs) and Type I collagen show prominent roles in determining skin aging progression. MMPs are a family of structurally related matrix-degrading enzymes that play important roles in various destructive processes, including skin aging [23,8]. In particular, MMP-1, known as interstitial collagenase, is a main collagenolytic enzyme that contributes to the degradation of collagen in the skin exposed to chronic UVB rays [3].

Among the 28 types of collagen that have been identified in human tissue, type I collagen is the most abundant subtype in dermal extracellular matrix, contributing to 80% -85% of total collagen of dermis [2,16]. Type I collagen is synthesized as procollagen type I, a soluble precursor secreted by fibroblasts when organizing the main ECM (Extra Cellular Matrix) components [28]. The inhibition of the transforming growth factor- β (TGF- β) signalling pathway due to upregulation of AP-1, leads to a decrease in the synthesis of type I procollagen which, in turn, decreases the amount of type I collagen. Disorganization, fragmentation, dispersion and decrease in collagen type I are prominent features of photodamaged human skin [22,2,10].

By countering and balancing the ROS level, we may be able to delay skin aging caused by UVB. Antioxidant agents might be utilized to reduce the tissue damage, caused by overproduction of ROS. There is a new form of antioxidant, astaxanthin, a natural red pigment which is synthesized by the microalgae, *Haematococcus pluvialis* [27,1]. Interestingly, astaxanthin has a strong antioxidant property. Previous studies showed that astaxanthin antioxidant activity was 10, 54, 65, and 100 times stronger than zeaxanthin, β -carotene, vitamin C and alpha-tocopherol, respectively [26,6,19].

Astaxanthin might reduce UVB-induced photoaging process of human skin by balancing oxidant/antioxidant status. Furthermore, ROS-mediated upregulation of MMP-1 and downregulation of procollagen synthesis could be minimized [5]. In the present study, we elaborate the antioxidant effect of topical astaxanthin against UVB-induced skin photoaging in the rat's skin.

2 METHODOLOGY

Fifteen of 8 weeks olds, Wistar male rats were acclimatized for one-week prior study and randomly divided into three groups (n=5 rats per group). P0 group as control (UVB exposure only), P1 group (exposed to UVB and treated with base cream), and P2 group (exposed to UVB and treated with astaxanthin cream). Skin area located at 10 cm distal from ear was exposed by using hair shaver and marked with permanent marker. All procedures approved by Ethical Committee, Faculty of Medicine, Universitas Padjadjaran (Animal Ethics Number: 288/UN6.C.10/PN/2017).

UVB Irradiation

Rats were irradiated thrice a week by UVB lamp (Kernel KN-4003, China) for 6 consecutive weeks at the interscapular area, with size of exposure 3x3 cm. Radiation dosage used was 130 mJ/cm² for 30 minutes. The distance between UVB lamp and rat's skin was 42 cm.

Astaxanthin Cream

The astaxanthin component was extracted from *Haematococcus pluvialis* supplied by Faculty of Pharmacy, Universitas Padjadjaran. The astaxanthin cream vehicle was cocoa oil. The base cream contained cream vehicle only.

On exposure day, astaxanthin cream or base cream was applied twice, 20 minutes before and 4 hours after UVB irradiation. The dose given was 0.3 gram/cm² of irradiated skin. On non-exposure day, creams were applied once in the morning (09.00 am).

Skin sample Collection

Twenty four hours after last UV exposure, under isoflurane anaesthesia, skin sample was excised using sterile surgical blade and collected from interscapular area 3x3 cm square. Skin sample was divided into 3 sections. For western blot sample and RNA purposes, the skin was snap frozen using liquid nitrogen then stored at -80°C. For Haematoxylin-Eosin (HE) staining, the skin was stored in 2% paraformaldehyde in 4°C.

RNA Extraction and Semiquantitative RT-PCR

The total skin RNA was extracted using TRIzol reagent (Thermo Fisher; USA) according to manufacturer's protocol. RNAs were stored at -80 C until use. According to manufacturer's protocol, 150 ng of RNA and 0.2 μ M of forward and reverse specific primers were added to a mixture of One-step RT-PCR kit (MyTaq One-step RT-PCR kit, Biorline, USA). PCR stage conditions are as follows: Denaturation is 94°C for 2 minutes; Annealing is 56°C for 2 min; Amplification/Extension is 72°C for 3 min, all these processes repeated for 34 cycles. Primers used were as listed in table 1. All PCR products were verified by 1% gel stained blue dye (Thermo Scientific, USA) electrophoresis. Gel documentation was performed using darkroom camera and results were analysed by Image J software (NIH Image). The PCR products of each gene of interest were normalized by β -actin level as an internal control. All procedures were repeated three times to confirm the results consistency.

Protein extraction and Western blotting

Skin samples were lysed using RIPA lysis buffer (20 mM Tris-HCl pH 7.5; 150 mM NaCl; 1 mM Na₂EDTA; 1 mM EGTA; 1% NP-40; 1% sodium deoxycholate; 2.5 mM sodium pyrophosphate; 1 mM β -glycerophosphate; 1 mM Na₃VO₄; 1 μ g/mL leupeptin) with 1 mM protease inhibitors cocktail (Sigma, Merck KGA, Darmstadt, Germany) and 1 mM sodium orthovanadate (phosphatase inhibitor, Sigma) The tissue lysates were centrifuged for 15 min at 15,000 x g, 4°C. Protein concentrations were measured by protein assay kit (company). Equal amount of protein samples were resolved by SDS-PAGE gels, transferred to nitrocellulose membranes and immunoblotted with anti-MMP1 antibody (Cat no. orb214254, Biorbyt Ltd., Cambridge, UK) and anti-type I procollagen antibody (Cat no. GTX55675; GeneTex, Irvine, CA, USA). The equality of protein loading was confirmed by probing blots with anti- β -Actin antibody 2A3 (Cat no. sc 517582, Santa Cruz Biotechnology Inc., CA, USA). The secondary antibodies used were Goat anti-mouse IgG Secondary Antibody HRP (sc-2005, Santa Cruz Biotechnology Inc., CA, USA) for MMP1, R&D laboratories for type I procollagen and R&D laboratories for β -actin. The antigen-antibody complexes were detected by Western Sure ECL Substrate (LI-COR Biotechnology, Lincoln, NE, USA), and visualized with LICOR C-Digit Western Blot Scanner (LI-COR Biotechnology, Lincoln, NE, USA). Densitometric analysis was performed using Image J software (NIH Image).

Histology (Hematoxylin and Eosin (H&E) Staining)

Five-micrometre-thick sections were collected from paraffin-embedded skin tissues. The sections were stained with hematoxylin (Cat no. GHS316, Sigma, USA) and eosin solution (Cat no. HT110116, Sigma, USA) for 30 s, washed, stained with eosin solution (Cat no. 230251, Sigma, St. Louis, MO, USA) for 1 min, washed again, dehydrated with an alcohol series, and cleared with xylene. HE staining was performed as follows: hematoxylin staining for 10 minutes, water rinse for 10 minutes, eosin staining for 2 minutes, decoloring in 90% ethanol for 5 minutes, 95 % ethanol for 5 minutes, and clearing in xylene solution for 5 minutes. After mounting, the tissues were observed by light microscopy (Carl Zeiss, German).

Statistical Analysis

Quantitative values were determined in at least three independent experiments and expressed as the means \pm standard deviation (SD). A statistical comparison of different treatment groups was determined by one-way analysis of variance (ANOVA) or Kruskal-Wallis test and further tested with post-hoc LSD or Mann-Whitney analysis using GraphPad Prism 5.01 (GraphPad Software, Le Jolla, CA). *p*-value of less than 0.05 was considered as statistically significant. All statistics were computed using SPSS 17.0 software. Data are expressed as mean \pm Standard Error Minimum (SEM). Statistical significant was considered at *p* < 0.05.

3 RESULTS

Comparison of physical appearance and histology appearance in skin after UVB treatment

Based on macroscopical and histopathological observation of the skin tissues in all groups, UV exposure caused irritation and itchiness proven by visible scratch sign. Big open arrows showed thicker of epidermal layer after UV radiation and thinner after cotreated with astaxanthin cream. Small open arrows pointed to a change at the subdermal area showing an increase of hypergranulation process which was signs of tissue damage and inflammation (Figure 1). Interestingly, subdermal hypergranulation in astaxanthin group was less prominent.

Astaxanthin reduces UVB-induced MMP1 gene upregulation

The mRNA expression of MMP-1 and Type-1 procollagen were measured by semiquantitative RT-PCR. After normalizing with β -actin mRNA, the result showed that the relative ratio of MMP-1 expression was 0.81 ± 0.287 in P0 group, 0.67 ± 0.128 in P1 group and 0.42 ± 0.155 in P2 group (Figure 2). There was a significant difference in MMP-1 expression between group P0 and P2 (*p* < 0.01). Furthermore, the relative ratio of Type-I procollagen expression was 0.90 ± 0.284 , 0.87 ± 0.116 , and 0.89 ± 0.078 in P0, P1, and P2 group, respectively. There was no significant difference in Type-I procollagen expression between groups P0 and P2. The result indicates that astaxanthin cream treatment might affect MMP-1 expression, but not Type-1 procollagen.

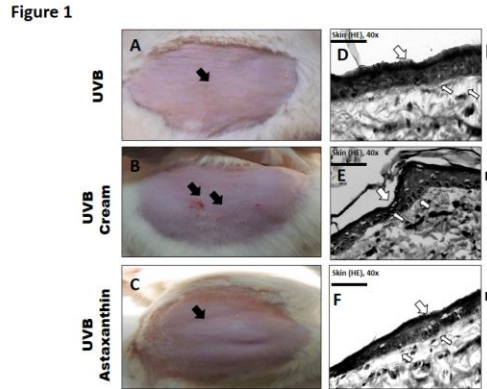


Figure 1. Astaxanthin cream reduces keratinization and hypergranulation in rat skin. UVB increases dryness of the skin and this effect was reduced by astaxanthin cream. In the histological appearance, there are significant hypergranulation by UVB induction and reduced by Astaxanthin cream. (A) Representative photograph of skin surface after UVB; (B) Representative photograph of skin surface after UVB plus cream base; (C) Representative photograph of skin surface after UVB plus Astaxanthin cream. (D) Representative histology photograph of skin surface after UVB; (E) Representative histology photograph of skin surface after UVB plus cream base; (F) Representative histology photograph of skin surface after UVB plus Astaxanthin cream. (Black arrow shows dryness and irritation sign on the skin; Large white arrow shows a granular and small white arrow - keratin layers)

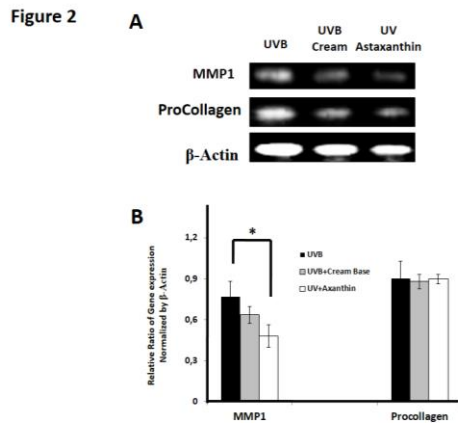


Figure 2. Astaxanthin cream significantly reduces MMP1 mRNA expression and did not change Procollagen I mRNA expression in rat skin. (A) Representative of electrophoresis PCR product. (B) Quantification of ratio (normalized by b-Actin) showing net changes of MMP1 and Procollagen mRNA expression. Bars represent the means of the respective individual densitometry of ratios \pm SEM, (n = 5, *P<0.05).

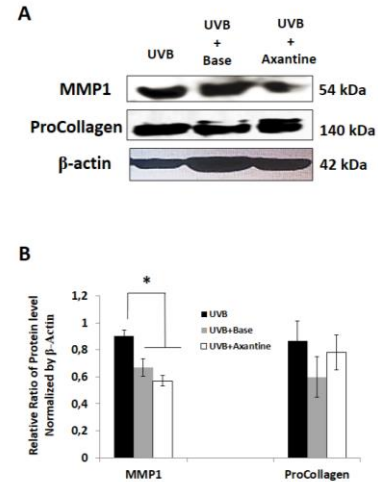


Figure 3. Astaxanthin cream significantly reduces MMP1 protein expression and did not change Procollagen I protein expression. (A) Representative immunoblot and quantitation showing MMP1 and Procollagen protein levels in the skin of rats with or without astaxanthin cream treatment and exposure of UV B (B) Quantification of ratio (normalized by b-Actin) showing net changes of MMP1 and Procollagen protein level ratios. Bar graphs show the means of the respective individual ratios \pm SEM (n=5, *P<0.05).

Table 1. Primer List and Condition of PCR with referral journal

No	Gene Names	Sequence	Reference
1.	MMP-1	F: TGGGATTTCCAAAA GAGG R: CGTGGTTCCTGAG AAGA	Kang et al., 2017
2.	type I procollagen (COL1A2)	F: GCAGTGTGCAATAT GATCCA R: TTGACAATGTCCAC AACAGG	(Liang et al., 2010);
3.	β -actin	F: TGGAGAAGATTTGG CACC R: CCAGAGGCATACAG GGACAA-	(Hyatt et al. 2008).

Astaxanthin significantly decreased MMP1 protein levels by 0,4 folds however there was no change in type I Procollagen mRNA expression against β -actin protein levels, MMP-1; type I Collage - β -actin gene ratio was obtained. The mean of the ratio was 0.941 ± 0.187 in P0, 0.63 ± 0.122 in P1 and 0.48 ± 0.175 in P2 group. From further test results with post-Hoc LSD, significant differences between group P0 and P2 were obtained ($p < 0, 01$).

4 DISCUSSION

One promising strategy for the prevention of photoaging is by harnessing natural phytochemicals which could promote collagen synthesis and suppress collagen degradation. As natural product, these phytochemicals most likely are relatively harmless and possess a variety of beneficial properties. In this study, we evaluated the antioxidant properties of astaxanthin on the expression of MMP-1 enzyme and type I procollagen in UVB-exposed rats' skin.

We found that after 6 weeks of treatment with astaxanthin cream, MMP-1 expression was significantly decreased compared to control group. Interestingly, we did not see any significant changes in Type I procollagen expression between treated and control groups. (Fig.2). This might be caused by inadequate UVB exposure. The type I procollagen is synthesized in fibroblast cell nuclei [16], and the radiation dose that we used (130 mJ/cm² for 6 weeks) could not suppress the synthesis of type I procollagen [14]. In addition, topical astaxanthin administration is less effective in promoting the synthesis of type I procollagen because topical preparation can only penetrate into stratum corneum. On the other hand, the administration of oral astaxanthin can accumulate in the subcutis and then slowly released to the dermis and epidermis, resulted in stimulation of type I procollagen synthesis [4,7]. Furthermore, Yoon et al. (2014) has demonstrated that administration of oral astaxanthin and collagen hydrolysate for 12 weeks could significantly increase mRNA levels of type I procollagen in UVB-exposed human skin.

The synthesis of type I procollagen begins with the transcription of two distinct genes, the *COL1A1* and *COL1A2* genes. The mature mRNAs of *COL1A* gene are transported from the nucleus to the fibroblast cells rough endoplasmic reticulum to be translated into pro α (procollagens) protein [12,16]. To detect up/down regulation of type I procollagen, we used a primer that binds to *COL1A2* gene. The transcription rate of *COL1A2* gene is two times slower than the *COL1A1* gene. In addition, the *COL1A2* gene only produces one $\alpha 2$ procollagen chain while the *COL1A1* gene produces two $\alpha 1$ procollagen chains [16,20]. Therefore, detecting the expression changes in *COL1A2* gene might not be sensitive enough to determine the expression level of type I procollagen.

5 CONCLUSION

Antioxidant activity of topical astaxanthin might be involved in decreasing MMP-1 expression, but not necessarily increasing type I procollagen expression in the skin of Wistar male rats irradiated by UVB.

CONFLICTS OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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