Astaxanthin cream alters type I procollagen and MMP-1 gene expression induced by ultraviolet B irradiation in skin rats

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Abstract

Astaxanthin has a protective effect on premature skin aging due to chronic UVB-rays exposure (photoaging) by its biological activity as a potent antioxidant. In the present study, we explore whether topical astaxanthin cream could decrease MMP-1 and increasing type I procollagen gene expression on skin of rats exposed to UVB rays. Male Wistar, 8 weeks old rats was used and divide into 3 group, dorsal hair of rats had been shaved previously, were randomly assigned into 3 groups: groups only exposed to UVB (P_0), UVB-exposed groups and base-cream administration (P_1) and UVB-exposed groups and astaxanthin-cream administration (P_2). UVB Exposure was done 3 times a week with a dose of 130mJ/cm^2, and cream smearing was done daily on the rats' skin for 6 weeks. On 43th day, skin biopsy was performed to examine the expression of MMP-1 and type I procollagen using semiquanitative PCR and western blot technique. The result showed significantly lower expression of MMP-1 found in group P_2 compared to P_0 ($P<0,05$), however, the significantly higher expression of type I procollagen was not found in P_2 compared to P_0 ($P>0,05$). Antioxidant activity of topical astaxanthin was proven involved in decreasing MMP-1 gene expression and protein levels, but not necessarily altering type I procollagen gene expression and protein expression on Wistar male rats irradiated by UVB.

Introduction

Skin aging involves intrinsic and extrinsic process (Farage et al., 2008). Environmental factors, such as primarily chronic ultraviolet B (UVB) light exposure cause extrinsic skin aging which is then called photoaging (Pittayapruek et al., 2016, Krutmann, 2010, Fisher and Voorhees, 1998). UVB increases the production of ROS of the skin (Fisher and Voorhees, 1998, Rinnerthaler et al., 2015) and it acts as a secondary messenger to activate the molecular signals pathway which is involved in collagen degradation and inhibition of procollagen synthesis by upregulation of a nuclear transcription factor i.e. activator protein-1 (AP-1). (Yaar and Gilchrest, 2012, Xu and Fisher, 2005, Rabe et al., 2006, Fisher et al., 2002).

Among gene involved in the skin aging process, matrix metallo proteinases (MMPs) and Type I collagen are important genes in determining the skin aging process. MMPs are a family of structurally related matrix-degrading enzymes that play important roles in various destructive processes, including skin aging (Quan et al., 2009, Fanjul-Fernández et al., 2010). 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 (Brennan et al., 2003).

There are 28 types of collagen present, especially type I collagen is the most collagen subtype in the dermal extracellular matrix of about 80% -85% of total collagen dermis (Baumman and Saghari, 2009, Krieg et al., 2012). Type I collagen is synthesized as procollagen type I, a soluble precursor secreted by fibroblasts when organizing the main ECM components (Varani et al., 2000). The inhibition of the transforming growth factor-β (TGF-β) signaling pathway due to upregulation of AP-1, leads to a decrease in the synthesis of type I procollagen which is in turn decreases the amount of type I collagen. Disorganization, fragmentation, dispersion and decrease in collagen type I are prominent features of photo damaged human skin (Quan et al., 2004; Baumman and Saghari, 2009; Fisher et al., 2002).

By countering and balancing the ROS level, we may be able to delay skin aging caused by UVB. Utilizing an antioxidant agent is one of the solution approach. There is a new form antioxidant, Astaxanthin, is a natural red pigment of which the best and most important source is green microalgae *Haematococcuspluvialis* (Sharma and Chand, 2014, Ambati et al., 2014). Interestingly, astaxanthin has biological activity as a potent antioxidant, for instant *in vitro* studies have shown that astaxanthin has antioxidant activity 65 times stronger than vitamin C, 54 times compared to β-carotene, 10 times compared with lutein, zeaxanthin, and cantaxanthin, and 100 times stronger when compared with alpha-tocopherol (Shah et al., 2016; Dhankhar *et al*., 2012; and Miki, 1991).

Therefore, utilization of asthaxanthin will be a good solution to counter accumulation of reactive oxygen species (ROS) in the photoaging process of human skin, in which causes upregulation of MMP-1 and decrease of procollagen synthesis (Demeule et al., 2000). In the present study, we elaborate the antioxidant effect of topical astaxanthin against UVB-induced skin photo aging in the rat's skin.

Methods

Fifteen, 8 weeks olds, Wistar male rats were acclimatized for 1 week prior study. Rats were randomly divided into 3 treatment groups, P0 group as control (UVB exposure only), P1 group (UVB exposed andsmeared with base cream), and P2 group (exposed to UVB and smeared with astaxanthin cream). Rat's dorsal hair were shaved to be set as irradiated areas.

UVB Irradiation

Irradiation was done 3 times a week (Monday, Wednesday, Friday), using Kernel-made UVB type KN-4003, with radiation dosage of 130 mJ/cm^2, distance of UVB source with rat's dorsal

skin was approximately 42 cm, and each irradiation was done for 30 minutes per day for 6 weeks.

Astaxanthin Cream

The astaxanthin cream and base cream were applied twice daily, i.e. 20 minutes before irradiation and 4 hours after irradiation with dose of 0.3 gr/cm^{2}of each rats' skin surface area. Cream application was still done on days without irradiation.

RNA Extraction

Total RNA extraction and Semiquantitative polymerase chain reaction (PCR). Rats were anasthesized and 7 cm dorsal at interscapularis line , skin was incized with size 2x2 cm then stored at -80C until used. Total RNA were extracted and isolated from frozen dorsal skin using TRIzol reagent and protocol followed company recomendation.

Semiquantitative RT-PCR

Primers were designed by *Sigma-Aldrich., Pte Ltd*, Singapore for the genes MMP-1, type I procollagenand β-actin as internal control. The sense sequence are as follows: MMP-1, forward: 5'-TGGGATTTCCAAAAGAGG-3',reverse: 5'-ACGTGGTTC CCTGAGAAG A-3'(Kang et al., 2017), type I procollagen (COL1A2), forward: 5'-GCAGTGTGCAATATGATCCA-3', reverse: 5'-TTGACAATGTCCACAACAGG-3'(Liang et al., 2010) and β-actin, forward: 5'-TGGAGAAGATTTGGCACC-3', reverse: 5'-CCAGAGGCATACAGGGACAA-3'(Hyatt et al. 2008). Relative Ratio of RNA expression levels were normalized by internal control gene (β-actin) mRNA levels. The gel electrophoresis signals of the PCR products were quantified using Image J Software.

Western blotting

Tissue samples were lysed using RIPA lysis buffer and immunobloting was performed as per the manufacturer's guidelines (Bio-Rad, Hercules, CA, USA). Photo image was taked using C Digit machine, USA. Western bolt densitometry analysis was performed using Image J software (NIH, Bethesda, MD, USA). Antibody details provided as table (**Supplementary Table 2**).

Histology

The skin was excised for observation and picture was taken. Part of the skin tissue was fixed with 4% paraformaldehyde. The fixed skin tissues were then paraffin embedded and cut. Paraffin sections were stained with hematoxylin and eosin (HE) for routine examination. Hematoxyline was purchased from Sigma Aldrich, code number : GHS316 Hematoxylin Solution, Gill No 3 and HT110116 Eosin Y Solution.

Statistics

Data processing was done by comparing the differences of MMP-1 and type I procollagen expression between P0, P1 and P2 groups with one-way ANOVA or *Kruskal-Wallis* test and further test with post-hoc LSD or *Mann-Whitney* analysis. All statistics were computed using SPSS 17.0 software. Data are expressed as mean±Standard Error Minimum (SEM). Statistical significant was considered at $p<0, 05$.

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Conflict of interest: The authors have declared that no conflict of interest exists.

Result

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

There is a scrath sign in control and base cream group. among UVB and control group and histologically, there are hypergranulation appearance caused by UVB (Black arrow-showing hypergranulation in subdermis area and treatment astaxanthin capable to reduse expression gen (Figure 1).

Astaxanthin reduces UVB-induced MMP1 gene expression and no effect on type I procollagen gene expressions.

After quantified using Image J and normalized against β-actin mRNA levels, MMP-1 – and Type 1 Procollagen mRNA expression ratio were obtained. The mean of the ratio was 0.81 ± 0.287 in P0, 0.67 ± 0.128 in P1 and 0.42 ± 0.155 in P2 group. From further test results with post-Hoc LSD, significant differences between group P0 and P2 were obtained (*p = 0.006*). But unexpectedly, after type I procollagen - β-actin gene ratio were obtained, the mean of the ratio was 0.90 ± 0.284 in P0, 0.87 ± 0.116 in P1 and 0.89 ± 0.078 in P2 group. Further tests with *Mann-Whitney* protein found no statistically significant differences between groups P0 with P2 and type I procollagen expression in the group P2 was lower than P0 (*p = 0,690).* (Figure 2).

Astaxanthin reduces UVB-induced MMP1 in protein levels, but doesnot change of type I procollagen protein level ins skin rats

Astaxanthin significantly decrease of MMP1 protein levels by 0,4 fold however there is no change in type I Procollagen mRNA expression against β-actin protein levels, MMP-1; type I Collage - β-actin gene ratio were 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 ($p = 0.003$).

Discussion

One promising strategy for the prevention of photoaging is suppression of collagen degradation and collagen synthesis decrease using natural phytochemicals. 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, a naturally occurring carotenoid of which the best source is green microalgae *Haematococcus pluvialis*, on the expression of MMP-1 enzyme and type I procollagen on UVB-exposed rats' dorsal skin. We have found that 6 weeks of application with astaxanthin cream can significantly decrease expression of collagen-degrading enzyme, MMP-1 mRNA after UVB radiation.

Astaxanthin reduces UVB-induced MMP1 gene expression and no effect on type I procollagen gene expressions.

There is significantly decrease of MMP1 gene expression 0, 74 folds compared to the control group. However, we did not see any significant change on amongs groups. (Fig.2) expectedly, expression of type I procollagen did not increase. This is due to type I procollagen is a soluble precursor of type I collagen synthesized on fibroblast cell nuclei (Krieg et al., 2012) and radiation for 6 weeks at a dose of 130 mJ / cm^2 (Imokawa and Ishida, 2015) cannot suppress the synthesis of type I procollagen that occurs intracellularly. In addition, topical astaxanthin administration is less effective in promoting the synthesis of type I procollagen because astaxanthin only accumulates in the stratum corneum in a short period of time compared to oral administration in which astaxanthin accumulates in the subcutis and then slowly released to the dermis and epidermis (Darvin et al., 2011,

Draelos, 2010). *Yoon et al*. 2014 had demonstrated that UVB broadband human buttock skin exposure, at dose of 2 MED accompanied by the administration of astaxanthin and oral collagen hydrolysate for 12 weeks, significantly increased mRNA levels of type I procollagen compared with the placebo group ($p = 0.038$).

The synthesis of type I procollagen begins with the transcription of two distinct genes, the COL1A1 and COL1A2 genes into mRNA, where mature mRNAs are transported from the nucleus to the fibroblast cells cytoplasm to be converted into pro α (procollagens) chains in ribosomes from rough endoplasmic reticulum(Gilkes et al., 2014, Krieg et al., 2012).This is related to no increase in expression of type I procollagen caused by the primer used to detect type I procollagen mRNA in this study i.e. primer of COL1A2 gene (Liang et al., 2010), while transcription rate of COL1A2 gene to procollagen mRNA is two times slower than the COL1A1 gene. In addition, the COL1A2 gene only produces one α2 procollagen chain while the COL1A1 gene produces two α1 procollagen chains.(Krieg et al., 2012, Pan et al., 2013).

Taken together, astaxanthin is new antioxidant agent in which we may reduces genes MMP-1 Expression and no change in protein and

Conclusion

Antioxidant activity of topical astaxanthin was proven involved in decreasing MMP-1 expression, but not necessarily increasing type I procollagen expression in the skin of Wistar male rats irradiated by UVB. Alteration of type I procollagen genes expression might be due to the lack of duration of irradiation performed and the primer to detect type I procollagen mRNA should be the primer of the COL1A1 gene.

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Figure Legends

Figure 1. Astaxanthin cream reduces keratinization and hyperganularation in rat skin. UvB increses dryness the skin and reduced by astaxanthine cream. In the histological appereance, there are significant hypergranulation by UVB induction and reduce 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; White arrow shows an granulair and keratin layers)

Figure 2. Astaxanthin cream significantly reduces MMP1 mRNA expression and didnot changes Procollagen I mRNA expression in skin rat. There. Bars represent the mean of the respective individual ratios ± SEM**(D)** Quantification of ratio showing net increaseinLC3II.Bars represent the means of the respective individual densitometry of ratios ±SEM, n = 3,*P<0.05.

Figure 3. Astaxanthin cream significantly reduces MMP1 protein expression and didnot changes Procollagen I protein expression.. (**A**) Representative immunoblot and quantitation showing time-dependent of induction LC3BII and decretion of p62 protein levels in soleus muscle of T_3-treated mice (20 µg T_3/100 gram BW for 10 days). (**B and C)** Bar graphs showthe means of the respective individual ratios±SEM (n=5, *P<0.05). **(D)** Representative electron micrograph showing T_3induction ofautophagosome formation at subsarcolemal area of soleus muscle. (**E**)Bar graph showing percentage of autophagosomes (AVs) in control and T_3-treated soleus muscle based on EM micrograph images. Scoring was done by counting 5 random fields per slide condition. Bars represent the means of the respective individual per slide ±SEM

(n=3, *P<0.05). (**F**) mRNA expression of LC3BII and p62 genes in soleus muscle of T_3-treated mice (20 µg T_3/100 gram BW).