Protective effect of Ethylhexyl Methoxycinnamate and Phytoene & Phytofluene against UV irradation induced hair carbonylation

Kyung, Seoyeon[1]; Chae Da Eun[2], **Kang, Seunghyun[1]**

[1]R&I Center, COSMAX BTI, Seongnam 13486, Republic of Korea; [2]R&I Center, COSMAX, Seongnam 13486, Republic of Korea;

** Kang, Seunghyun, Seongnam 13486, Republic of Korea; shyunk@cosmax.com*

Abstract (Maximum of 250 words)

Background: Humans are normally exposed to various external stimuli such as tempereture change, pressure, pH, pollutant and light. The negative effects of solar ultraviolet (UV) radiation, mostly combination of UVB (290-320nm) and UVA (320-400mm) wavelength, have been extensively studied because they are regarded as the main causes of skin aging and hair damage.

When hair undergoes irreversible oxidative damage by UV, hair shows carbonlyated structural transformation resulting in protein loss, ultimately resulting in discoloration and brittleness. Especially, protein and lipid carbonylation is an important indicator of oxidative stress, and it is well known that carbonylation is induced by UVA and UVB. Also Phytoene (7,8,11,12,70,80,110,120-octahydro-w, w-carotene, PT) and phytofluene (7,8,11,12,70,80-hexahydro-w, w-carotene, PTF) are well known UV light absorbing cartenoid. Although these chemicals have been widely used as a UV screener for skin, its hair protection effect against UV is not known yet.

Methods: We evaluated the effects of ethylhexyl methoxycinnamate and UV-ene, combination of PT and PTF, on hair protection in human hair. The 7-(diethylamino)-coumarin-3-carbohydrazide (CHH) was used to stain carbonylated proteins of hair after treatment with ethylhexyl methoxycinnamate. Carbonylated phospholipid of hair was stained by E06-monoclonalAb-TopFlour™ antibody.

Results: Increased levels of carbonylated protein and phospholipid stained parts of hair by UV were decreased in ethylhexyl methoxycinnamate containg oil compared to control. Furthermore, PT and PTF decreased carbonylated protein and phospholipid.

Conclusion: These results suggest that ethylhexyl methoxycinnamate and PT&PTF complex protect hair texture in human hair through absorbing harmful UV.

Keywords: Ethylhexyl methoxycinnamate, Phytoene, Phytofluene, Ultra Violet Protection, Hair Carbonylation

Introduction.

Human are mostly exposed exclusively to solar ultraviolet (UV) radiation, which contains UVA and UVB that can be harmful to skin and hair. UVA and UVB have different properties and induce various reactions on the skin and hair, and UVB radiation is much more energetic than UVA radiation [1]. There is an increasing need for analysis of molecular mechanisms and biological events induced by UVA and UVB, which will be used as a method of clinical treatment and cosmetic application.

A number of reports indicate that UV radiation significantly disrupt the redox balance in human skin cell by overproducing reactive oxygen species (ROS) [2-4]. Furthermore, ROS derived oxidative stress can be accumulated in macromolecules, which result in oxidatively damaged (carbonylated) proteins and lipids. Oxidative modified protein mostly shows reduced enzymatic activity, reduced stability to heat, increased hydrophobicity, and increased susceptibility to proteolysis degradation [5]. Also, oxidized lipids play an important role in the pathogenesis of oxidative stress-related human disorders such as atherosclerosis, obesity, inflammation and autoimmune diseases. And it has been reported that carbonylated glycerophospholipids can initiate and mediate chronic inflammation [6].

Human hair is mostly made up of keratin, which forms 65-95% of hair with insoluble cystine-containing helix protein complex [7]. Also, Hair lipids have been described as fatty substances loosely attached to the hair surface and can be easily extracted by lipid solvent [8]. Those hair lipids constitute mainly with sebum, oily secretions from the sebaceous glands of the scalp, and with lipids excreted as a by-product of epidermal keratinization [9]. Since most of the constituent materials of the hair surface are proteins and lipids, it was decided to measure carbonylated macromolecules as methods to study that can measure changes in the hair surface by UV.

Octylmethoxycinnamate (OMC, also known by ethyl-hexylmethoxycinnamate or octinoxate) is one of the most common sunscreens on the market in cosmetics to resist UVB from sunlight due to its excellent UV absorption curve, high lipophilicity and good oil solubility [10, 11]. By the way, the photo-instability of UV-B filters is an urgent issue, and therefore, they have received special attention today [12]. Also, OMC is listed as an endocrine disruptor compound (EDC) by the European Union’s database [13] because of its potential risk for DNA damage associated with endocrine disrupting effects. In this sense, it

was necessary to develop new formulations of sunscreens to stabilize or replace OMC and increase their protective effect, increasing their efficiency and safety for human health [14].

Phytoene (7,8,11,12,70,80,110,120-octahydro-Ψ, Ψ-carotene, PT) and phytofluene (7,8,11,12,7',8'-hexahydro-Ψ, Ψ-carotene, PTF) are special carotenoids as they are colorless, and precursors of all other carotenoids. Also, PT and PTF have light absorbing properties. PT absorbs maximally at 286nm, and PTF absorbs maximally at 348nm [15, 16]. As UVB radiations ranges from 290 to 320 nm, PT and PTF have possibilities to be used as sunscreens. Also, some studies suggest that PT and PTF can exhibit antioxidant capacity against 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation [17], which can be help structural stability when present together with structurally unstable substances. In addition, it has been reported that PT and PTF can protect against erythema and DNA damage induced by UV radiation and hydroxyl radicals, and may have anti-inflammatory effects as observed in human peripheral blood lymphocytes, *in vitro*, and mouse ear edema model, *in vivo* [18].

Based on the UV absorption ability of OMC, PT, and PTF, there have been quite a few studies on the skin protection effect, but the hair protection effect from UV has not yet been studied. Therefore, the purpose of our study is to visualize UV induced hair damage through staining of carbonylation, and to try to confirm the UV protective efficacies of OMC, PT and PTF.

Materials and Methods.

Hair Samples Preparation

Hair tresses (Light Black hair from Asian donors) used in this study were free from chemical treatment and significant physical damage. The hair strands were washed for 1 hour with 0.1% of Triton X-100. After washing, hair strands were rinsed with DPBS and dried naturally in ambient conditions. The hairs were stocked in dark condition at room temperature until experiments.

Samples Preparation

To confirm the UV blocking effect of OMC, a formulation sample containing 7.5% OMC in control oil was prepared. Also, for the most suitable method for economical and eco-

friendly industrial production of PT and PTF, bio-synthesis using microbial cell factory has been used to synthesis PT and PTF. The combination of PT and PTF obtained through the cell factory system was provided from Xenofocus, and this was named 'UV-ene'. To evaluate the UV protecting effect of UV-ene, four formulation samples containing 1% UV-ene were prepared, oil type with silicone (Oil with Si), oil type without silicone (Oil without Si), emulsion type with silicone (Emulsion with Si), and emulsion type without silicone (Emulsion without Si).

UV irradiation

Hair strands were gently cut into small pieces (2-3cm) and incubated with control oil or formulation samples treatment for three hour. Control oil and formulation samples were absorbed on paper towel, and dried hair strands were disposed into small petri dish. UVA (365nm) irradiation was perfomed using CL-100L UV crosslinker at 2 doses (Dose 1:56J, Dose 2:112J). And UVB irradiation (254nm) was performed using Vilber Louvert Biolink™ BLX UV Crosslinker at 2 doses (Dose 1: 1.6J, Dose 2:16J).

Immunocytochemical detection of carbonylated protein and phospholipids

Formulation samples treated on hair strands were re-absorbed on paper towel, and hair strands are washed with DPBS for 3 times. Hair strands were incubated in blocking solution (1% BSA in DPBS, 1h, RT). And then the 7-(diethylamino)-coumarin-3-carbohydrazide (CHH), was used to stain carbonylated proteins of hair (0.2mmol/L, 2h, RT). After washing out CHH for 3 times with DPBS, carbonylated phospholipid of hair was stained by E06-monoclonalAb-TopFlour™ antibody (1:100 in DPBS, 1h, RT). After washing 3 times with DPBS, the cover slips were mounted on cover slides, and then images for co-localization were obtained with Inverted phase contrast fluorescent microscope (AXIO OBSERVER Z1) or confocal microscope (LSM 700).

Cell Culture

Human Hair follicle (HF) dermal papilla cells (DPCs) were obtained from PromoCell and cultured in follicle DPC growth medium containing supplement mix (PromoCell) with 100 U/mL penicillin, and 100 μg/mL streptomycin at 37℃ in 5% CO2. When the cells reached

about 80% confluence, cells were subcultured with 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-buffered saline solution, trypsin/ethylenediaminetetraacetic acid (EDTA) solution, and neutralizing solution (PromoCell).

Cell proliferation assay

The proliferation of DPCs was determined using a 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 2×10^4 cells in 96-well tissue culture plates. After 24 h, the cells were treated with UV-ene concentrations ranging from 0.01 μg/mL (0.01ppm) to 1 μg/mL (1ppm), and then incubated for 24 h. After removing the medium, cells were treated with 0.5mg/mL of MTT and incubated for 4 h. After reaction, 100 μL of dimethyl sulfoxide (DMSO) (Sigma Aldrich) was added into the cells. Optical density (OD) values were measured at the wavelength of 590 nm using a VersaMax ELISA microplate reader spectrophotometer.

RNA extraction and real-time RT-PCR analysis

Cells were seeded at a density of 3×10^5 cells in 6-well tissue culture plates. After 24 h, the cells were treated with UV-ene concentrations ranging from 0.1 μg/mL (0.1ppm) to 1 μg/mL (1ppm), and then incubated for 24 h. After removing the medium, total RNA was isolated using TRIzol reagent, and synthesis of cDNA from 2 μg total RNA using Reverse Transcription Premix. Gene expression signals were quantified, and the data were analyzed using the StepOne Plus™ system software. Real-time RT-qPCR amplification reactions were performed in a SYBR Green PCR Master Mix with premixed ROX. Used primer pairs are listed in Table 1. The mRNA expression of β-actin was used as an internal control.

Table 1. Primers used for PCR.

	Sequence 5'→3'
VEGF	GTGCCCACTGAGGAGTTCAAC CCCTATGTGCTGGCCTTGAT
FGF7	CATGAACACCCGGAGCACTAC CACTGTGTTCGACAGAAGAGTCTTC

β-actin	GGCCATCTCTTGCTCGAAGT GACACCTTCAACACCCCAGC

Results

Protein carbonylation induced by both UVA and UVB

Traditionally, to evaluate hair damage at a molecular level, protein expression has been usually determined. The degradation of protein is quantified through tryptophan degradation, which has a limitation in that it is an amino acid unit experimental method. In addition, there is a limit that protein quantification carried out through the Bradford assay is affected by the melanin content, so these experimental methods lack the accuracy or sensitivity of the experiment [19, 20]. For this reason, we selected carbonylation as an oxidative stress indicator of hair, and measured the carbonylation of hair by UV in the hair shaft based on the permanent oxidative damage caused by UV to the hair. To induce protein carbonylation in human hair strands, UVA and UVB crosslinkers were used.

According to the papers studied so far, since the UV exposure concentration was high and the time was long, the irradiation concentrations of UVA and UVB were determined, respectively, referring to the literature that caused significant carbonylation *in vitro* and *in situ* [21, 22]. 56J and 112J of UVA, 1.6J and 16J of UVB irradiations are conducted on hair shaft, and then carbonylated proteins have been visualized *in situ* with inverted phase contrast fluorescent microscope upon labeling with CHH. Carbonylated proteins fluorescence signal (in green) increases with UV irradiation (**Figure 1**).

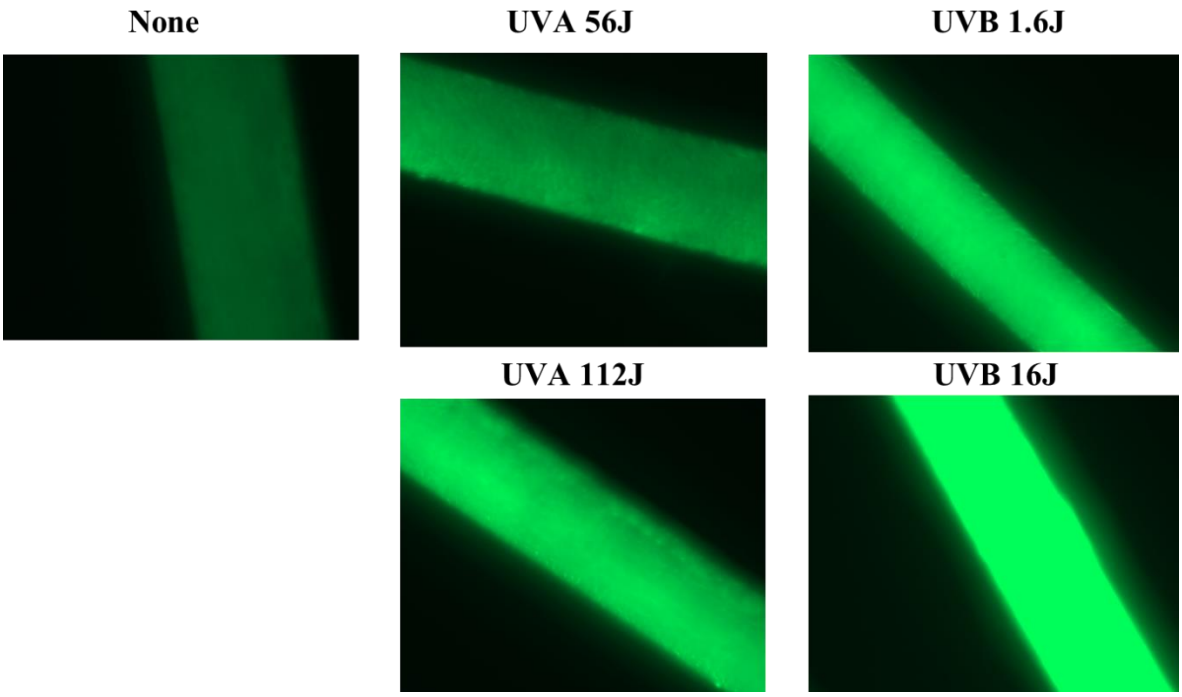


Figure 1. *In situ* visualization of carbonylated proteins induced by UVA and UVB. Carbonylated proteins were labeled *in situ* on hair shaft with CHH. They are represented by the green signal. Left panel : Image of a hair strand without UV irradiation. Middle panel : Image of hair shafts after UVA irradiation (56J, 112J). Right panel : Image of hair shafts after UVB irradiation(1.6J, 16J) Images were taken with a 40X zoom.

Phospholipid carbonylation induced by both UVA and UVB

Since the lipids existing outside the hair are mainly by-products derived from the scalp including sebum [9], peroxidation of these lipids has the potential to act as another ROS-inducing substance and damage the hair. As far as we know, the only immunocytochemical approach to detect oxidized phospholipids is using Ab E06, which recognize oxidized phosphatidylcholines. Therefore, we co-stained carbonylated phospholipids using E06 in addition to CHH by narrowing down the UV irradiation concentration based on the previous UVA and UVB irradiation experiments using CHH. 56J of UVA, 1.6J of UVB irradiations are conducted on hair shaft, and then carbonylated phospholipids have been visualized *in situ* with inverted phase contrast fluorescent microscope upon labeling with CHH and E06. Carbonylated proteins fluorescence signal (in green) increases with UVB irradiation, and

carbonylated phospholipids fluorescence signal (in red) dramatically increases with UVB irradation (**Figure 2**).

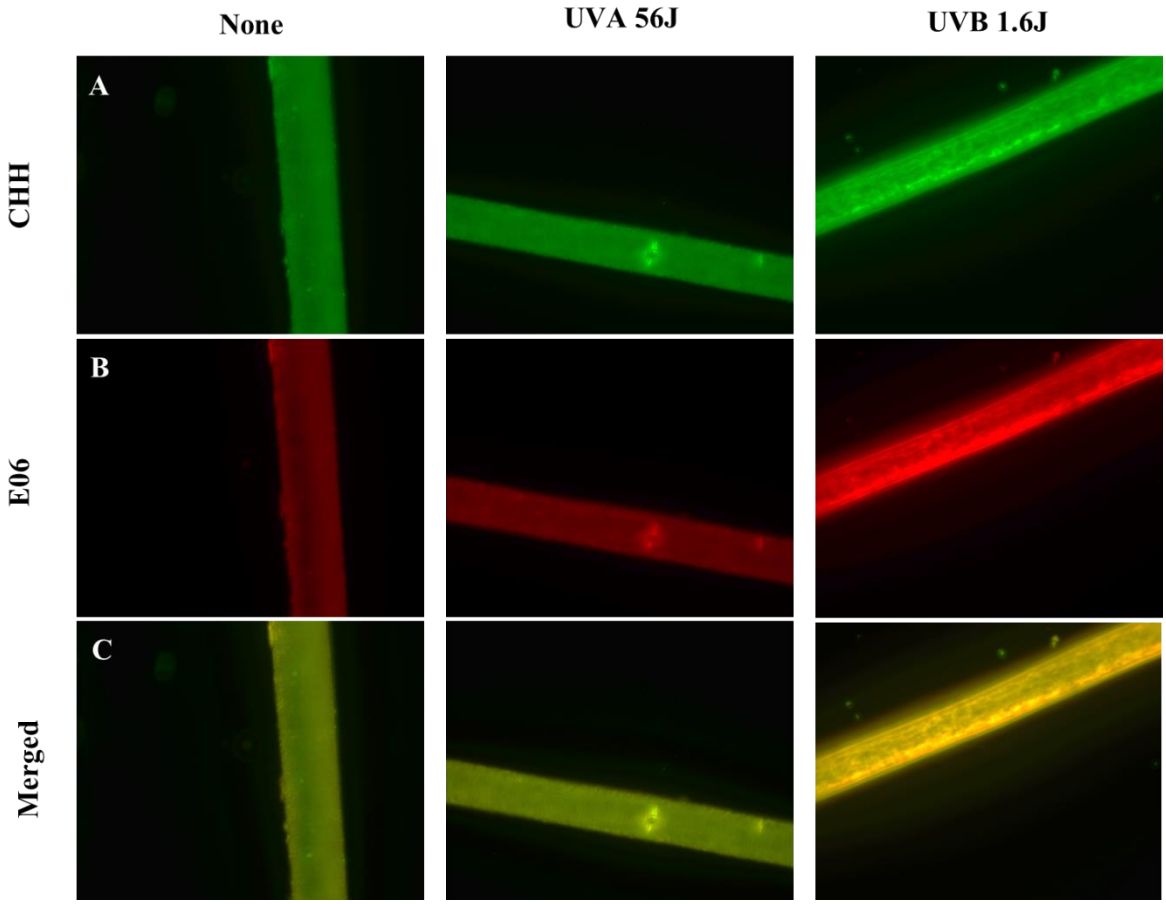


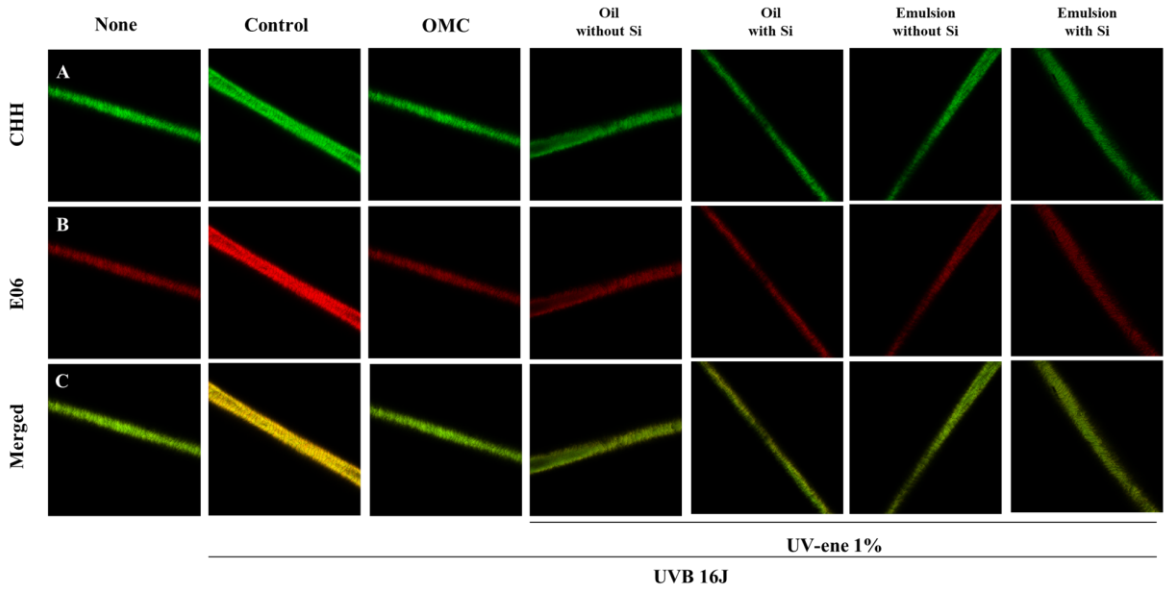
Figure 2. *In situ* visualization of carbonylated protein and lipids induced by UVA and UVB. Carbonylated proteins were labeled *in situ* on hair shaft with CHH. Carbonylated phospholipids were labeled *in situ* on hair shaft with E06. For better representation of co-staining, CHH stain shown in green(A), E06 stain shown in red(B). Merged images (yellow) demonstrate the co-localization of carbonylated phospholipid and carbonylated protein. Left panel: Image of a hair strand without UV irradiation. Middle panel: Image of hair shafts after UVA irradiation (56J). Right panel: Image of hair shafts after UVB irradiation(1.6J) Images were taken with a 40X zoom.

Protein and phospholipid carbonylation induced by UVB are prevented by OMC and UV-ene

Based on the previous two experiments, concerns about UVB irradiation conditions continued. Since both OMC and PT/PTF are known to absorb UVB wavelength band, it is preferred to set the experimental conditions in UVB rather than UVA. Also, in UVB than UVA, the amount of carbonylated phospholipid showed a tendency to increase despite the smaller dose, and carbonylated protein also showed a tendency to increase as the UV irradiation concentration increased. Therefore, UVB of 16J was determined as the dose.

Each sample was irradiated with UV 16J after applying four formulations with and UV-ene, a mixture of PT and PTF, or control oil or OMC. In the same way as in the previous method, the experiment was conducted through co-staining of CHH and E06, and quantitative analysis was attempted to compare the UV damage inhibition efficacy according to each formulation type. In the case of the previously used fluorescence microscope, quantitative analysis is not easy and resolution tends to decrease, so a confocal microscope (LSM 700) was used for imaging **(Figure 3, A-C)**. In the case of quantitative analysis, it is believed that it is more accurate to analyze fluorescence within the same area than the entire area, so a region of interest (ROI) was set, the fluorescence intensity was measured for each channel **(Figure 3, D)**, and the average value was plotted as a graph **(Figure 3, E)**.

As a result, it was confirmed that the carbonylated protein and phospholipid increased by UV were reduced in UV-ene-containing formulations and OMC treated group. Also, as estimated from numerical values, the efficacy of inhibiting carbonylated phospholipids increased by UV was found to be high in the order of Emulsion without Si, Emulsion with Si, Oil with Si, and Oil without Si. Although carbonylated protein increased very weakly in the UV-treated group compared to the untreated group, the carbonylated protein reduction effect was confirmed by the formulations containing OMC and UV-ene. In the order of Oil with Si, Emulsion without Si, Emulsion with Si, and Oil without Si, the efficacy of inhibiting carbonylated protein increased by UV was confirmed.



D

Sample (Channel)	Area	Mean	Min	Max	IntDen	RawIntDen
None Average (Green)	11781	46129.7	19091.7	65535	543,453,671	543,453,671
None Average (Red)	11781	27608.3	11182	49026	325,253,305	325,253,305
UV Average (Green)	11780	46772.1	23141.7	64908.3	550,975,505	550,975,505
UV Average (Red)	11780	56302.3	27632.7	65535	663,240,630	663,240,630
OMC Average (Green)	11783	38904.2	14809.7	63968	458,408,303	458,408,303
OMC Average (Red)	11783	23433.8	7858.33	42675.3	276,120,921	276,120,921
Oil without Si Average (Green)	11780	28040.1	10364	46865.7	330,314,738	330,314,738
Oil without Si Average (Red)	11780	21466.4	7822	42019.7	252,874,722	252,874,722
Oil with Si Average (Green)	11782.7	23605.9	5981.67	52402.3	278,140,682	278,140,682
Oil with Si Average (Red)	11782.7	19501.9	4140	46,338	229,783,490	229,783,490
Emulsion without Si Average (Green)	11779.7	23611.7	6071	47953.3	278,129,581	278,129,581
Emulsion without Si Average (Red)	11779.7	16840.8	3016.67	38645.3	198,374,204	198,374,204
Emulsion with Si Average (Green)	11782	24528.1	7757.33	47384.7	288,989,490	288,989,490
Emulsion with Si Average (Red)	11782	17583.6	5498.33	37112	207,170,490	207,170,490

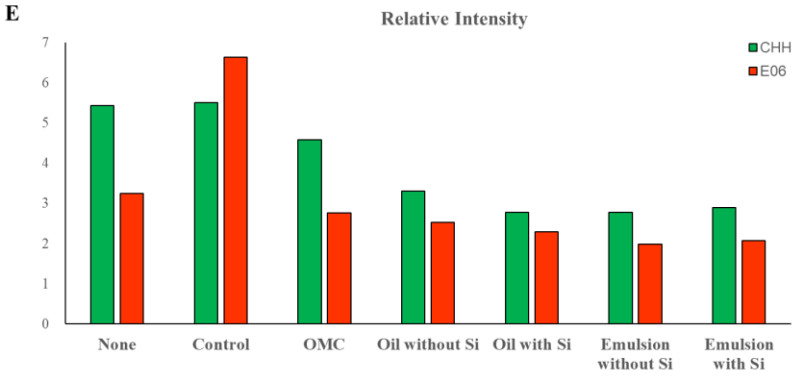


Figure 3. ***In situ*** **visualization of carbonylated proteins and phospholipids induced by UVB in OMC or UV-ene treated hair shaft.** Carbonylated proteins were labeled *in situ* on hair shaft with CHH. Carbonylated phospholipids were labeled *in situ* on hair shaft with E06. For better representation of co-staining, CHH stain shown in green(A), E06 stain shown in red(B). Merged images (C, yellow) demonstrate the co-localization of carbonylated phospholipid and carbonylated protein. Images were taken with a 20X zoom. The average

fluorescence values from three different hair shafts are shown in the table for each channel (D), and the relative intensity is graphed based on this (E).

Effects of UV-ene on cell proliferation and hair growth factor expressions in DPC

In addition to helping hair damage from UVB, cell proliferation and growth factor expression of HFDPCs were measured during 24h of UV-ene treatment to determine the potential to promote hair growth. Upon UV-ene treatment, it was confirmed that cell proliferation occurred in a concentration-dependent manner in HFDPC. In addition, it was confirmed that vascular endothelial growth factor (VEGF) expression was increased in a concentration-dependent manner when UV-ene was treated, and in the case of fibroblast growth factor 7 (FGF7), it was confirmed that it was significantly increased when treated with 1ppm.

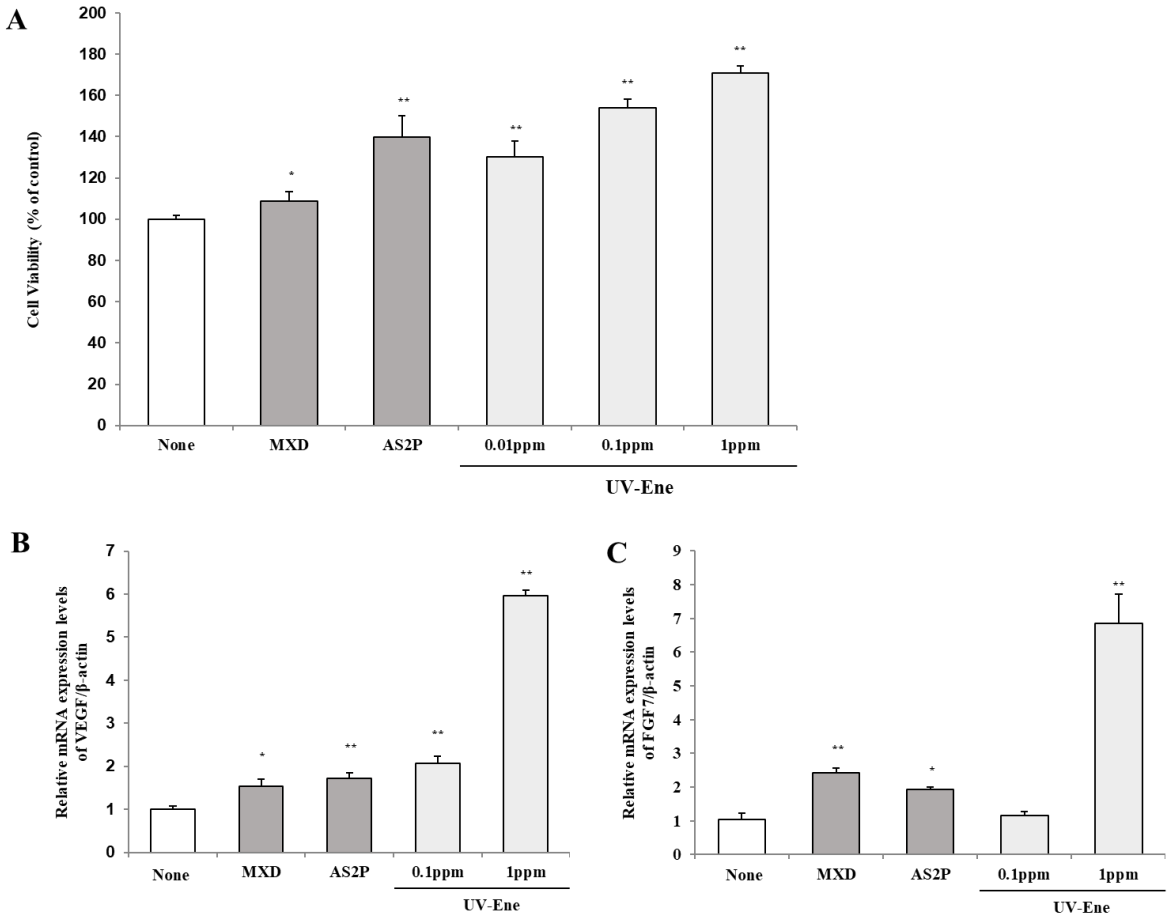


Figure 4. The effects of UV-ene on (a) cell proliferation and (b,c) mRNA expression levels of growth factors in DPCs. (a) DPCs were treated with indicated concentrations of UV-ene for 24 h. Cell proliferation was measured by the MTT assay. Results are expressed as mean values ± SE of three independent experiments. **p<0.01, *p <0.05 compared to the control. For mRNA expression levels, DPCs were treated with of UV-ene for 24 h. Relative mRNA expression levels of (b) VEGF and (c) FGF7 were measured by RT-qPCR. The means ± SEs are the average of three independent experiments. **p<0.01, *p <0.05 compared to the control.

Discussion

Carbonylation plays an important role in many oxidative stress related human disorders and disease progression, and refers to a complex mixture of structurally modified proteins and lipids [23]. In previous studies dealing with UV-induced hair damage, there have been studies that confirmed the change in mass or color change for protein loss after irradiation for more than 220 hours [24]. In reality, irradiating UV for 220 hours is very inefficient, and it is not suitable for quickly confirming the efficacy evaluation of substances. In order to break through this limitation, while searching for a hair damage biomarker that increases by UV, we wanted to confirm that carbonylation can be an oxidative stress marker in hair. In addition, it was attempted to prepare a tool for evaluating the UV damage inhibition efficacy of materials using this.

In this study, we confirmed the possibility of suppressing UVB-induced hair carbonylation by using OMC, a UVB filter, a well-known sunscreen. Similar to the principle of UVB blocking by OMC, it was thought that PT and PTF, which are carotenoids characterized by a wavelength band that absorbs UVB, would also suppress UV damage. In addition, in the modern cosmetics market, as the spread of chemiphobia and preference for naturalistic products increase, the demand for natural raw materials based on vegetable and biomaterials to replace them along with the avoidance of chemical materials is rapidly demanding. Accordingly, in order to overcome the limitations of chemically synthesized UV blocking materials, many companies are interested in the development of UV blocking materials derived from natural substances, and research on the production of materials that are safe and eco-friendly for the human body is being actively conducted. Based on this, we focused on

UV-ene, a material obtained by synthesizing PT and PTF using E. coli as a platform and using synthetic biology technology, which meets the demand for PT and PTF. As UV-ene is the most suitable replacement candidate of chemical sunscreen because of its eco-friendly industrial production, we used UV-ene in our experiments because we thought it would lead the next sunscreen market.

In our study, it was confirmed that UV-induced carbonylation of proteins and lipids was reduced when OMC and UV-ene were treated. Although the value of protein carbonylation by UVB seems to fluctuate somewhat, it was confirmed that it decreased compared to the UV control when the formulation containing OMC and UV-ene was treated, so that protein damage caused by UV could be suppressed. On the other hand, in the case of UV-induced carbonylation of phospholipids, it was confirmed that UV-induced carbonylation was significantly decreased in OMC and UV-ene formulations. Since most of the lipids on the surface of the hair are waste products derived from the human body, the oxidative damage of these wastes will damage the surface of the hair, so it would be easy to accept that these carbonylated phospholipids should be removed and mitigated. In addition, despite UV irradiation after treatment of the formulation containing oil, the lipid oxidation damage caused by UV was significantly reduced with OMC and UV-ene treatment. Considering this point, formulations containing OMC and UV-ene could help protect hair from UV rays without additional lipid oxidation. In addition, cell proliferation and the increase in the expression of hair growth factors of HFDPC were confirmed during UV-ene treatment. Therefore, we would like to propose UV-ene that can help not only hair damage from UV, but also hair root activation.

Conclusion.

The present study demonstrates that UVA and UVB irradiation increase carbonylation of protein and lipid, an indicator of oxidative damage on the hair surface. In addition, by utilizing the principle of OMC, which is a UVB blocker, it suggests the possibility of expanding the use of formulations that can protect not only the skin but also the hair from UV rays. In addition, it was confirmed that UV-ene, a complex of PT and PTF, which are carotenoids similar to OMC, has a hair protection effect. By further confirming the HFDPC activating effects of UV-ene, UV-ene could be widely used in hair care formulations.

Acknowledgments

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Conflict of Interest Statement

The authors declare no conflict of interest

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