



Aqueous Extract of *Chrysanthemum morifolium* (菊花 Jú Huā) Enhances the Antimelanogenic and Antioxidative Activities of the Mixture of Soy Peptide and Collagen Peptide

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ABSTRACT

The possible synergistic effect between the aqueous extract of *Chrysanthemum morifolium* (菊花 Jú Huā) (AECM) and the peptide mixture (PM) containing soy peptide and collagen peptide was investigated in an ultraviolet (UV) irradiation-induced skin damage mouse model. The irradiated mice were treated with the PM or PM + AECM (containing PM and AECM), respectively. Both PM and PM + AECM groups displayed an apparent photoprotective effect on the UV-irradiated skin damage of mice. Histological evaluation demonstrated that the epidermal hyperplasia and melanocytes in the basal epidermal layer of the UV-irradiated skin in mice decreased when treated with either PM or PM + AECM. Further study showed that soy peptide, collagen peptide, and AECM also inhibited the activities of mushroom tyrosinase with IC_{50} values of 82.3, 28.2, and 1.6 $\mu\text{g/ml}$, respectively. Additionally, PM + AECM reduced melanogenesis by 46.2% at the concentration of 10 mg/ml in B16 mouse melanoma cells. Meanwhile, the UV-induced increase of antioxidative indicators, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA), was reduced significantly after treatment with 1.83 g/kg/dbw of PM + AECM. This evidence supported the synergistic antioxidative effect of AECM with PM. These results demonstrated that oral intake of PM and AECM had synergistic antimelanogenic and antioxidative effects in UV-irradiated mice.

Key words: Antimelanogenic, Antioxidant, *Chrysanthemum morifolium*, Collagen peptide, Soy peptide

INTRODUCTION

Ultraviolet (UV) radiation can cause serious skin damage that is generally characterized by wrinkling, roughness, laxity, and pigmentation. UV radiation generates reactive oxygen species (ROS) and, thus, leads to oxidative stress. Oxidative stress induces pro-inflammatory cytokines, which in turn increase the intracellular levels of ROS.^[1] The ROS induced by UV radiation assist melanin biosynthesis and DNA damage, which results in

up-regulation of the gene for tyrosinase, the rate-limiting enzyme in melanin biosynthesis, and subsequently in epidermal hyperpigmentation.^[2-4] Therefore, the inhibitors of tyrosinase or antioxidants may suppress melanogenesis in the epidermal layer of the skin. ROS are also found to activate the cytoplasmic signal transduction pathways in the resident fibroblasts. This activation relates to growth, differentiation, senescence, and connective tissue degradation and also causes permanent genetic changes.^[5] Considering that UV-induced oxidative stress mediates adverse effects in

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the skin and body, regular intake of antioxidants in combination with an antioxidant topical treatment is a useful way to reduce the pigmentation and harmfulness of UV radiation.^[6]

The flowers of *Chrysanthemum morifolium* (菊花 Jǔ Huā) (Compositae) are commonly used in tea and as an herbal drug in China. They have been reported to possess antibacterial, antifungal, anti-spirochetal, anti-inflammatory, and antioxidant activities.^[7] The flavonoids, alkaloids, and sesquiterpene lactones are thought to contribute to the pharmacological activities of *C. morifolium*.^[7-9] A recent report indicated that the flavonoids in the extracts of *C. morifolium* protected the brain, liver, and kidney against lead-induced oxidative damage in mice. Moreover, the extracts provided significant protection against cerebral ischemia and reperfusion injury in rats through their antioxidant effect.^[10,11]

Soy peptide (SP) and collagen peptide (CP) have been used as important active components in medicinal and food industries because of their excellent bioactivity, biocompatibility, good penetrability, and lack of irritation.^[12] Our previous study confirmed that SP had synergistic antioxidant activities with CP. The mixture of SP and CP showed good antioxidant activity in the senescent mouse model.^[13] However, neither the additive nor the synergistic effect of the aqueous extract of *C. morifolium* (AECM) and peptides has been reported yet. By examining the *in vivo* effects of the peptide mixture (PM) in the presence or absence of AECM, we set out to investigate whether AECM has a synergistic effect in a UV-irradiated mouse model and to uncover the possible mechanisms for the antimelanogenesis and antioxidant activities.

MATERIALS AND METHODS

Materials

The SP was obtained from Fuji Oil (Osaka, Japan). The CP was obtained from Haishi (Zhoushan, China). The air-dried and powdered aerial parts of *C. morifolium* were extracted with water. Nuclear fast red, tyrosinase, L-tyrosine, alpha-melanocyte stimulating hormone (α -MSH), and all other chemicals were obtained from the Sigma-Aldrich company (Shanghai, China) and were of analytical grade.

Mice treatment and UV radiation

Four-week-old female Kuming (KM) mice [weighing 25 ± 3 g, SCXK (Xiang) 2009-0004] were obtained from Hunan SJA Lab Animal Ltd. Mice were housed under controlled conditions [SYXK (Yue) 2008-0003] with $55 \pm 5\%$ relative humidity at a temperature of $25 \pm 1^\circ\text{C}$ (12 h light/dark cycle). The experiments were carried out according to the Chinese legislation on the use and care of laboratory animals and were permitted by the Institutional Animal Care and Use Committee of Guangzhou Institute of Pharmaceutical Industry. Mice were given free access to pellets and drinking water during the experiment. After 5 days acclimation, the back of each mouse was denuded using sulfureted sodium over the depilated area of 15 cm^2 , and each mouse was randomly assigned to one of five groups, with 12 mice in each group. The groupings are as follows:

Negative control (NC) group: Normal group, normal saline by oral intake

Vehicle/UV group: Model group, normal saline by oral intake
PM (SP: CP = 1:1) group: A dose of 1.67 g/kg/dbw of the PM by oral intake

PM + AECM (SP: CP: AECM = 1:1:0.2) group: A dose of 1.83 g/kg/dbw of the PM containing AECM by oral intake

Vitamin C (Vit C) group: A dose of 200 mg/kg/dbw by oral intake

All mice were given the substances in the same volume of 0.2 ml. The samples were freshly prepared and given to the mice every day using a stomach sonde needle. All the mice, except the ones in normal group, were irradiated with the same UV source.

The back areas of the mice were irradiated for 30 min every day with 235 mJ/cm^2 of UV each time for 30 consecutive days. The source of light was a UVB-313EL lamp (Q- Panel Lab Products, Westlare, USA). The distance from the lamps to the animals' back was 25 cm. During the period of exposure, the mice were group housed in a stainless steel irradiation chamber and the animals could freely move around in the chamber. A non-irradiated group of animals was included as a control. The animals were anesthetized, sampled, and sacrificed after the final irradiation.

Histological evaluation of mouse skin

Hematoxylin and eosin (H and E) staining was conducted for a routine examination of the tissue and to measure the epidermal thickness, fibrous tissue, and adipose tissue. The pathological status of the skin was evaluated by taking 10 representative sample measurements per tissue section under the microscope.

Measurement of DOPA-positive melanocytes in mice

The mice were sacrificed after the final samples were administered. Biopsies were obtained from the UV-irradiated central dorsal skin, and 3,4-dihydroxyphenylalanine (DOPA) staining of the epidermal sheet was performed.^[14] Briefly, the biopsies were fixed in 10% buffered formalin and then incubated with 0.0056 mol/l of DOPA solution. After being fixed in Bouin's fixative, dehydrated with a sequence of ethanol solution, and embedded in paraffin, 5- μm -thick serial sections were cut. The sections were stained with 0.1% nuclear fast red and then observed for melanocytes by a photomicroscope.

Sample collection and preparation

The whole blood collected was diluted prior to measurement. The dorsal skin and liver were removed and immediately placed in ice-cold 50 mM potassium phosphate buffer (pH 7.4). Tissues were cleaned by rinsing carefully in buffer and then homogenized at 20,000 rpm in 50 mM potassium phosphate buffer (pH 7.4) for 15-25 s in a tissue homogenizer (model T25-S1; IKA Labortechnik, Staufen, West Germany). Tissue sample specimens ranging in size from 25 to 100 mg were further homogenized in 1.0 ml of ice-cold buffer. The homogenates were centrifuged (Beckman High-Speed Refrigerated Centrifuge, model J2-HS) for 10 min at 10,000 g and 4°C . The supernatants were used for immediate enzyme activity assays or stored at -70°C until analysis. All enzymes were assayed within 3 weeks.

Assay for GSH-Px and SOD activity

Glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activities were assayed by using the GSH-Px

and SOD detection kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Assay for the MDA and protein carbonyl content

After determining the total amount of protein in the supernatants, free radical damage was determined by measuring malondialdehyde (MDA) and protein carbonyl content using the detecting kits from Nanjing Jiancheng Bioengineering Institute in China.

Assay for total antioxidant activity

A total antioxidant capacity (T-AOC) detection kit was obtained from Nanjing Jiancheng Bioengineering Institute in China. According to the detection method, the antioxidant defense system, which consists of both enzymatic and non-enzymatic antioxidants, is able to reduce Fe³⁺ to Fe²⁺. T-AOC was measured by the reaction that occurred between phenanthroline and Fe²⁺ at 520 nm using a spectrophotometer.

Tyrosinase inhibition assay using mushroom tyrosinase and melanogenesis assay of cultured B16 mouse melanoma cells

To estimate the inhibitory action of SP, CP, and AECM on tyrosinase, mushroom-isolated tyrosinase was utilized as previously described, with a minor modification.^[15] Briefly, 20 µl of aqueous solution of mushroom tyrosinase (100 units) was added to a 96-well microplate. Each well contained a total volume of 100 µl of assay mixture of 1 mM L-tyrosine solution and 50 mM phosphate buffer (pH 6.5). The assay mixture was incubated at 25°C for 30 min. Following incubation, the amount of dopachrome produced in the reaction mixture was determined spectrophotometrically at 492 nm in a microplate reader. We determined the log-linear curves and their equations based on the inhibition percentages at three doses for each experiment. Then, we calculated individual IC₅₀ values when the y-axis showed 50% of the inhibition percentage.

B16 mouse melanoma cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco, New York, USA) and penicillin/streptomycin (100 IU/50 µg/ml) in a humidified atmosphere containing 5% CO₂ in air at 37°C. B16 cells (3 × 10⁴) were cultured in 12-well plates and incubated in the presence or absence of 100 nM α-MSH for 24 h. The melanin content was then determined after the cells were treated with different concentrations of SP, CP, AECM, PM, and PM + AECM or arbutin for another 72 h. After treatment, the cells were detached by incubation in trypsin/ethylenediaminetetraacetic acid (EDTA). After precipitation, cell pellets containing a known number of cells were solubilized in 1 N NaOH at 60°C for 60 min. The melanin content was assayed by spectrophotometric analysis at an absorbance of 405 nm and calibrated by the protein concentrations. All the experiments were performed in triplicate and repeated three times to ensure reproducibility.

Statistical analysis

Statistical analysis of the experimental data was performed by one-way analysis of variance (ANOVA) Tukey test, which was used for the comparison of measured data with SPSS 20.0 statistical software. Differences were considered statistically significant at P < 0.05.

RESULTS

Histological observations and DOPA-positive melanocyte changes

The body weights of the experimental mice orally administered PM or PM + AECM for 30 days exhibited no significant change. UV irradiation on the dorsal skin of mice induced a significant change in skin histology.^[16] Therefore, the effects of the oral intake of PM or PM + AECM on the epidermis and dermis of mouse skin were studied. After UV irradiation, the epidermis and dermis were significantly thicker in the vehicle-treated group than in the no-irradiation group. As shown in Figure 1, the number of adipocytes in the subcutaneous region was also significantly greater than that in the vehicle/UV group. Conversely, the number of adipocytes was slightly less in the Vit C group than in the vehicle-treated group. The largest decrease was found in the PM + AECM treated group. Furthermore, the incrustation decreased for the PM or PM + AECM group compared with the vehicle/UV group. These results suggest that the oral intake of Vit C, PM, or PM + AECM for 30 days could induce a decrease in the thickness of epidermis and dermis. The number of DOPA-positive melanocytes in the PM + AECM and PM groups, as well as in the Vit C group, tended to decrease in comparison with the control group [Figure 1b].

Measurement of melanogenesis of cultured B16 melanoma cells

The inhibitory effect of AECM, PM (SP:CP = 1:1), and PM + AECM (SP:CP: AECM = 1:1:0.2) on the melanin content in B16 melanoma cells was assayed. The α-MSH-stimulated B16 cells were treated with different concentrations of AECM, PM, and PM + AECM for 72 h. The cell proliferation after treatment of all the samples remained unchanged, indicating no cytotoxic-

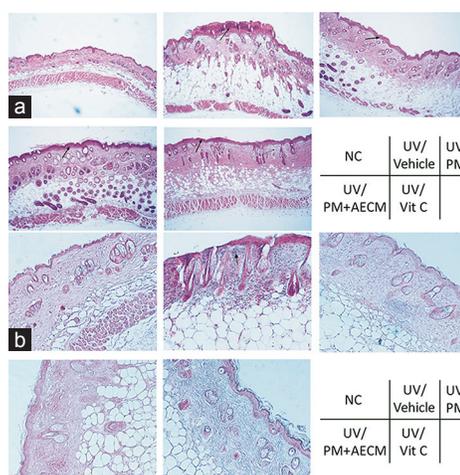


Figure 1. Inhibition of UV-induced histological alteration and changes in DOPA-positive melanocytes by PM and PM + AECM in mice. (a) The histological alteration in dorsal skin of the mice was observed from the epidermis to dermis. Thick arrow: Crust formed by increased epidermal cells; thin arrow: Derangement of the fibrous tissue. (b) The number of melanin-positive cells decreased in the UV-irradiative mice administered PM or PM + AECM as compared with mice administrated with saline (vehicle/UV) or vitamin C (Vit C). PM: Peptide mixture; AECM: Aqueous extract of *C. morifolium*

ity for all other samples at all doses [Table 1]. Meanwhile, the level of melanin production decreased on treatment with AECM, PM, and PM + AECM, respectively. Treatment with 1 mg/ml of AECM showed synergistic inhibitory activity with 10 mg/ml of PM, compared with the treatment of AECM or PM alone [Table 1].

Measurement of the inhibitory effect on mushroom tyrosinase

Table 2 shows the IC_{50} values of SP, CP, and AECM, as well as arbutin on mushroom tyrosinase activity.

Total antioxidant activity in skin

T-AOC is an important integrative index used to reflect the total antioxidant capacity of the skin of mice. Figure 2 shows that both PM + AECM and Vit C (200 mg/kg/d) significantly elevated T-AOC values in the UV-irradiated mouse skin ($P < 0.01$). The T-AOC in the skin of the PM group increased apparently, but not significantly, compared with the model group.

Antioxidant enzyme activities

As shown in Figure 3, UV irradiation resulted in decrease in activity of GSH-Px and SOD in mouse blood and skin compared to the normal mice, with a significant difference. Treatment with PM + AECM could protect GSH-Px and SOD against the damages, as depicted by the significant increase of the enzyme activities in blood ($P < 0.05$) and skin ($P < 0.01$). This effect was even slightly higher than that seen with Vit C at a concentration of 200 g/kg/d. However, PM could only restore the SOD activity in skin ($P < 0.01$). The GSH-Px in both blood and skin and the

Table 1. Effects of PM and PM+AECM on α -MSH stimulated melanogenesis in B16 cells

Samples	Concentration (mg/ml)	Melanin content of control (%)	Cell proliferation (%)
PM	2.5	100.0±0.8	100.0±1.4
	5	75.0±1.5	102.0±2.9
	10	68.7±2.8	107.4±2.3
AECM	0.25	60.9±0.7	101.3±0.8
	0.5	82.0±3.7	96.8±2.5
	1	72.0±1.1	98.1±3.4
PM+AECM	2.5	65.8±2.5	100.5±2.3
	5	73.0±0.8	102.2±30.9
	10	62.0±0.3	102.3±4.2
		53.8±1.2	99.1±1.4

Each value in the melanin content of control percentage and cell proliferation represents the mean±SE of three experiments. PM: Peptide mixture of soy peptide and collagen peptide; AECM: Aqueous extract of *C. morifolium*

Table 2. Effects of SP, CP, AECM, and arbutin on mushroom tyrosinase activity

Component	IC_{50} (mg/ml)
SP	82.3
CP	28.2
AECM	1.6
Arbutin	0.6

SP: Soy peptide; CP: Collagen peptide; AECM: Aqueous extract of *C. morifolium*

SOD in blood showed no significant decrease after the administration of PM.

MDA and protein carbonyl levels

The exposure of animals to UV radiation induced increases in MDA levels in the blood (51%) and in skin (80%) to different extents, compared with the mice in the control group ($P < 0.01$). PM + AECM significantly inhibited MDA formation; in particular, significant differences in skin MDA levels were seen compared with both the model group ($P < 0.01$) and the control group ($P < 0.01$). In contrast, PM treatment moderately depleted the MDA concentrations. Both PM and PM + AECM significantly decreased the protein carbonyl levels in skin ($P < 0.01$) but not in blood [Figure 4].

DISCUSSION

Melasma is the most common pigmented disorder among Asians. It has been reported in 50-70% of pregnant women^[17] and in non-pregnant women who are taking birth control pills.^[18] Sun exposure, coupled with endocrine disorders, genetic factors, medications, nutritional deficiency, and hepatic dysfunction are the risk factors for melasma.^[19] Recently, increasing effort has been devoted to revealing the relationship between food intake and skin condition.^[20] It has been reported that some nutrients, such as vitamins A, E, and C, as well as herbal extracts, such as pycnogenol, exhibited skin lightening effects due to their antioxidant effects.^[21,22] In this respect, the importance of a dietary source for photoprotection has attracted a great interest.

As important raw materials, SP and CP are commonly used in medicine and food industries. It has been reported that the benefits for skin increased markedly when taking SP and CP together in comparison to the intake of CP alone.^[8] In our previous study, we found that the combination of SP and CP showed good antioxidant activities in the senescent mouse model.^[13] Despite many reports linking the benefits of the peptides to their antimelanogenesis and antioxidant activities, the synergistic efficacy of the peptides with herbal extracts, such as AECM, on UV-damaged skin has not been investigated.

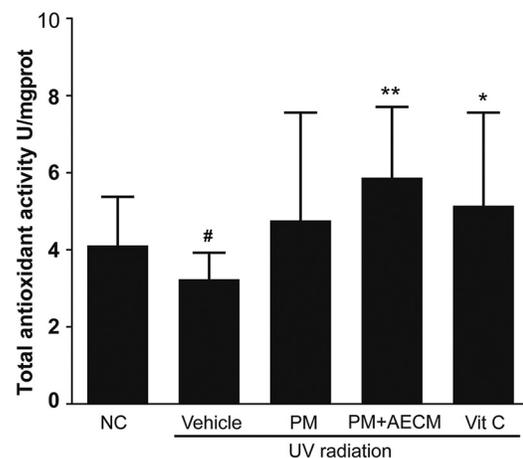


Figure 2. Effect of PM and PM + AECM on the total antioxidant capacity (T-AOC) of the UV-radiated mouse skin. Data are means ± standard deviations ($n = 12$). * $P < 0.05$, ** $P < 0.01$ vs. vehicle, # $P < 0.05$ vs. NC

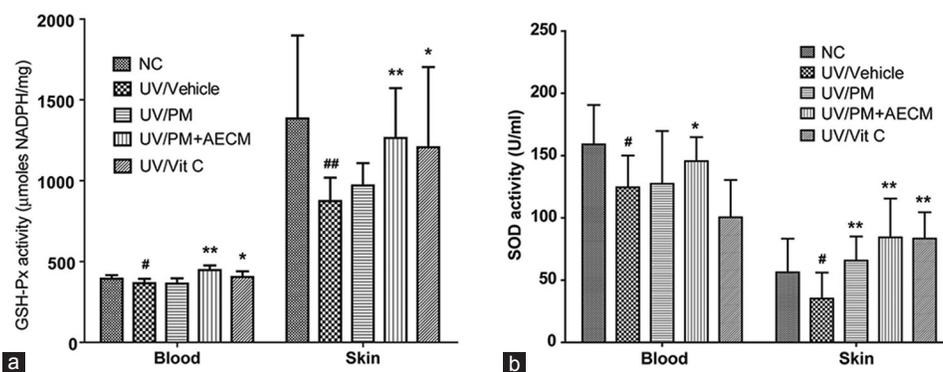


Figure 3. Measures of antioxidant defensive factors of (a) Glutathione peroxidase (GSH-Px) and (b) Superoxide dismutase (SOD) in blood and skin after PM and PM + AECM treatment in UV-irradiated mice. Data are means ± standard deviations ($n = 12$). * $P < 0.05$, ** $P < 0.01$ vs. vehicle; # $P < 0.05$ vs. NC, ## $P < 0.01$ vs. NC. PM: Peptide mixture; AECM: Aqueous extract of *C. morifolium*

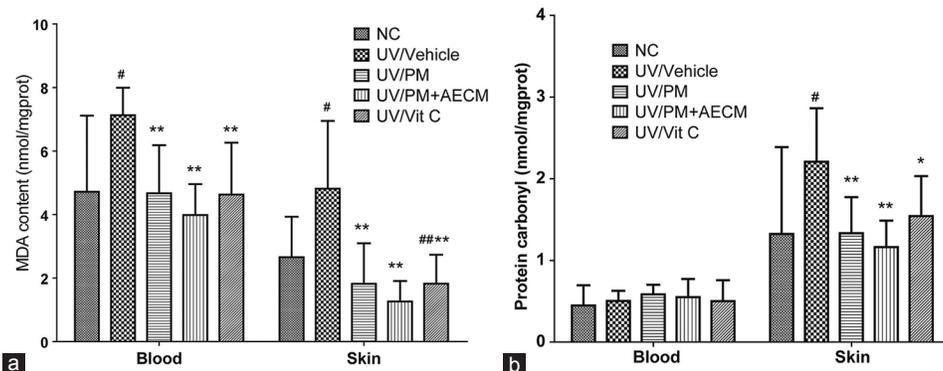


Figure 4. (a) MDA level and (b) Protein carbonyl content in blood and skin after treatment with PM and PM + AECM in UV-irradiated mice. Data are means ± standard deviations ($n = 12$). * $P < 0.05$, ** $P < 0.01$ vs. vehicle; # $P < 0.05$ vs. NC, ## $P < 0.01$ vs. NC. MDA: Malondialdehyde; PM: Peptide mixture; AECM: Aqueous extract of *C. morifolium*

For the first time, we demonstrated that AECM enhanced the photoprotective and antioxidant activities of these peptides both in the UV-induced mouse model and *in vitro*.

Extensive histochemical studies on the effects of UV irradiation were also conducted. As shown in Figure 1a, 4 weeks of UV irradiation induced an increase in the thickness of the mouse epidermis in comparison to the normal mice. However, the mean epidermal thickness in the PM group and that in the PM + AECM group were significantly lesser than that in the UV-irradiated group. The skin protective effects of PM and PM + AECM were accompanied by a decrease in the number of DOPA-positive melanocytes in the basal layer of the epidermis [Figure 1b]. Moreover, Tables 1 and 2 show that SP, CP, AECM and their combination were all capable of inhibiting mushroom tyrosinase activity as well as melanogenesis in B16 mouse melanoma cells.

Many studies have indicated that UV radiation could induce the formation of ROS^[23] and that ROS assists melanin biosynthesis in melanocytes.^[3] Considering the possible side effects of the synthetic antioxidants (e.g. 2,6-bis (1,1-dimethylethyl)-4-methylphenol), natural antioxidants derived from dietary sources have received much attention in recent years.^[24] Protein hydrolysates, or peptides, are being utilized to improve the antioxidant capacity in functional foods and to confer an additional nutritional value, as well as other desired functional properties, e.g. good water solubility, emulsion

and foaming properties.^[25] Our investigation showed that severe oxidative stress induced by UV irradiation in mouse skin was accompanied by a notable decrease in activities of SOD and GSH-Px. However, decrease in the enzyme activities was prevented when treated with PM, showing that PM could exert favorable effects against pathologic alterations induced by UV irradiation. Moreover, the formulation containing AECM showed more significant effects on increasing the enzyme activities than PM, which indicated the synergistic activity of AECM with the peptides.

MDA is a lipid peroxidation product and is usually quantified to estimate lipid peroxidation. The phenomenon that UV exposure of mouse skin caused an obvious increase in MDA levels indicated that UV irradiation might exhaust the endogenous reducing power and catalyze the lipid peroxidation, likely via pathways involving ROS. However, the MDA levels in PM and PM + AECM mice decreased significantly in comparison to UV-irradiated mice. The MDA levels of the PM + AECM group were lower than those in normal mice, which suggests that AECM exerts additive antioxidant activities to the PM.

The most general indicator, and by far the most commonly used marker of protein oxidation, is protein carbonyl content.^[26] Oxidatively modified proteins tend to accumulate in the upper dermis and result in solar elastosis.^[27] We observed that PM and PM + AECM reduced the liver carbonyl contents in UV-irradiated

mice [Figure 4]. PM + AECM could even reduce the liver carbonyl content to a normal level, which indicates AECM enhances the antioxidant activities of the PM.

These actions might also be explained by the antioxidant role of SP, CP, and AECM to suppress ROS formation and enhance free radical scavenging, and they further resulted in the decrease of MDA and protein carbonyl levels. As mentioned earlier, it has been reported that ROS assist in melanin biosynthesis.^[2] Therefore, the ROS scavenging effect may contribute to the reduction of pigmentation by PM and PM + AECM.

Some studies indicated that low-molecular-weight peptides showed protective effects on UV-induced mouse skin damage through the antioxidative mechanism.^[28] Previous studies demonstrated that some nonpeptidic natural antioxidants, such as ascorbic acid, showed synergistic antioxidant effects with peptides.^[11,25] In our studies, peptides from soy protein and collagen both showed high oxygen radical absorbance capacity, and the AECM enhanced the antioxidant properties of the peptides both *in vitro* and *in vivo*. Our results indicated that the formula containing AECM showed stronger protective effects against mouse skin photoaging and stronger antioxidant effects than the PM. Furthermore, the mixture of low-molecular-weight peptides with AECM is water soluble and can thus be easily absorbed by the intestine to reach the plasma and target organs, and have stronger effects.

CONCLUSION

Our findings revealed that an oral intake of PM or PM + AECM showed both antioxidant and antimelanogenic activities in the UV-irradiated mouse model by directly inhibiting tyrosinase activity and simultaneously suppressing oxidative stress. Furthermore, AECM showed synergistic antioxidant properties with peptides, which would benefit human nutrition and health.

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