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ORIGINAL ARTICLE

Melanogenesis-promoting effect of *Cirsium japonicum* flower extract in vitro and ex vivo

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Abstract

Objective: In this study, we examined the effect of *C. japonicum* flower extract (CFE) on melanogenesis and its mechanism in vitro and ex vivo.

Methods: The effect of CFE on melanogenesis was investigated with lightly (HEMn-LP) and moderately (HEMn-MP) pigmented normal human melanocytes, reconstituted three-dimensional skin (3D skin) model and ex vivo human hair follicles. The melanogenesis-inducing effect of CFE was evaluated using melanin content and intracellular tyrosinase activity assay. The amount and type of eumelanin and pheomelanin were analysed by using HPLC method. The mechanism involved in the effect of CFE on hyperpigmentation was explored by cyclic adenosine monophosphate (cAMP) immunoassay and western blot analysis for tyrosinase, microphthalmia-associated transcription factor (MITF) and phosphorylated CRE-binding protein (pCREB) expression. The degree of pigmentation in 3D skin and L-values were measured using a CR-300 chroma meter. The amount of dissolved melanin was measured using a spectrophotometer. The content of melanin in the hair follicles was evaluated by Fontana Masson staining.

Results: *C. japonicum* flower extract significantly increased the melanin content and cellular tyrosinase activity in both HEMn-LP and HEMn-MP cells. The markers of pheomelanin and eumelanin in HEMn-LP and HEMn-MP were also increased by CFE. We observed that CFE treatment on melanocytes increased intracellular cAMP with inducing pCREB and up-regulating the protein levels of TYR and MITF. Furthermore, CFE considerably increased the melanin content in a 3D skin model and ex vivo human hair follicles.

Conclusions: These results suggest that CFE exerts hyperpigmentation activity through cAMP signalling in human melanocytes that it can improve follicular depigmentation and vitiligo by stimulating the melanin synthesis.

KEYWORDS

3D skin, cAMP, *Cirsium japonicum*, hair follicle, hyperpigmentation

Résumé

Objectif: Dans cette étude, nous avons examiné l'effet de l'extrait de fleur de *C. japonicum* (EFC) sur la mélanogenèse et son mécanisme *in vitro* et *ex vivo*.

Méthodes: L'effet du EFC sur la mélanogenèse a été étudié avec des mélanocytes humains normaux légèrement (HEMn-LP) et modérément (HEMn-MP) pigmentés, un modèle de peau reconstituée en 3 dimensions (peau 3D) et des follicules pileux *ex vivo*. L'effet inducteur de la mélanogénèse de la EFC a été évalué en utilisant la teneur en mélanine et le dosage de l'activité de la tyrosinase intracellulaire. La quantité et le type d'eumélanine et de phéomélanine ont été analysés en utilisant la méthode HPLC. Le mécanisme impliqué dans l'effet de la EFC sur l'hyperpigmentation a été exploré par immunoessai à l'adénosine monophosphate cyclique (AMPc) et Western blot pour l'expression de la tyrosinase, du facteur de transcription associé à la microphtalmie (MITF) et l'expression de la protéine CREB phosphorylée. Le degré de pigmentation de la peau 3D, les valeurs L ont été mesurées à l'aide d'un chromamètre CR-300. La quantité de mélanine dissoute a été mesurée à l'aide d'un spectrophotomètre. La teneur en mélanine des follicules pileux a été évaluée par coloration Fontana Masson.

Résultats: EFC a augmenté de manière significative la teneur en mélanine et l'activité de la tyrosinase cellulaire dans les cellules HEMn-LP et HEMn-MP. Les marqueurs de phéomélanine et d'eumélanine dans HEMn-LP et HEMn-MP ont également été augmentés par EFC. Nous avons observé que le traitement EFC sur les mélanocytes augmentait l'AMPc intracellulaire en induisant pCREB et en régulant à la hausse les niveaux de protéines de TYR et MITF. De plus, le EFC a considérablement augmenté la teneur en mélanine dans un modèle de peau 3D et dans les follicules pileux humains ex vivo.

Conclusions: Ces résultats suggèrent que la EFC exerce une activité d'hyperpigmentation via la signalisation de l'AMPc dans les mélanocytes humains qu'elle peut améliorer la dépigmentation folliculaire et le vitiligo en stimulant la synthèse de mélanine.

INTRODUCTION

Exposure to UV radiation (UVR) has been related to photoageing, eye disorders, such as a cataract, damaged-immune system and skin cancer [1–3]. Besides, UVR induces reactive oxygen species (ROS) that can lead to oxidative damage to all components of the cell, including proteins, membrane lipids and DNA [4–6]. Melanin is a natural sunscreen pigment produced in skin, hair, eyes and so on. It prevents UVR from penetrating deeply in skin by absorbing UV. In addition, melanin has function as a cation chelator and a free radical scavenger.

Melanocytes are located in the bottom layer of skin epidermis and hair follicles. Melanocytes produce melanin in special organelles called melanosomes [7]. A process of melanin formation called melanogenesis can be induced by various cytokines, such as α-melanocyte-stimulating hormone (α-MSH), stem cell factor (SCF), endothelin-1 (ET-1) and adrenocorticotropic hormone (ACTH) via several signal transduction pathways. Melanin is synthesized by increasing the expression of microphthalmia transcription factor (MITF) and its downstream melanocyte-specific proteins, such as tyrosinase (TYR), tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2) [7, 8].

There are two types of melanin known as eumelanin and pheomelanin. Eumelanin is a brown-black insoluble polymer with high molecular weight and pheomelanin is a yellow-reddish soluble polymer with low molecular weight. These two pigments vary considerably in structural, physical and chemical properties depend on the biosynthetic process [8] The first step in melanin synthesis is the formation of dihydroxyphenylalanine (DOPA) from tyrosine and then conversion of DOPA to Dopaquinone by tyrosinase which is a critical rate-limiting enzyme for melanin biosynthesis. In the presence of cysteine, pheomelanin is formed after converting Dopaquinone to Cysteinyl-DOPA

which is degraded into thiazole-2,4,5-tricarboxylic acid (TTCA) by H_2O_2 oxidation. However, Eumelanin is formed by converting Dopaquinone to Dopachrome in the absence of cysteine. Dopachrome is then degraded into pyrrole-2,3,5-tricarboxylic acid (PTCA) by H_2O_2 oxidation [9, 10].

Skin type with a major component in eumelanin appears mostly in African and Asian who have brown-black hair, dark skin and superior photoprotection. On the other hand, skin type with a major component in pheomelanin appears mostly in Caucasian who have red or blond hair, freckles, fair skin and high risk of photodegradation by UVR [11, 12]. It has been demonstrated that the amount of UV reaches the upper dermis of fair skin is five times higher than as reaches that of darker skin. It increases skin cancer in light-skinned people compared to dark skin. Skin cancer in the United States represents 35%–45% of Caucasian, 4%–5% of Hispanic, 2%–4% of Asian and 1%–2% of Black [13, 14]. These results indicate that there is a difference in the extent of skin photoprotection in accordance with the content and composition of pigment by ethnic skin types. To examine the effect of *Cirsium japonicum* on the melanin synthesis in different skin types, we used the lightly (HEMn-LP) and moderately (HEMn-MP) pigmented human melanocytes and measured the amount and type of eumelanin and pheomelanin by analysing specific degradation product, such as PTCA and TTCA.

Cirsium japonicum, a perennial plant of *Compositae* family, is known as "Korean thistle". It is listed in Korean and Chinese pharmacopoeias. It has been used as a diuretic, tonic, anti-hepatitis, antihypertensive, anti-haemorrhagic and detoxifying agent. Several studies have reported that *Cirsium japonicum* has anti-cancer, haemostasis, anti-inflammatory, analgesic, antioxidant and anti-obesity effects [15–19]. However, the effect of *Cirsium japonicum* on melanin synthesis has been rarely studied. Thus, we investigated the effect of *Cirsium japonicum* flower extract (CFE) on the melanin synthesis in various conditions, including two pigmentation types of melanocytes, the reconstituted three-dimensional skin (3D skin) model and *ex vivo* hair follicles. In this study, we found that CFE enhanced the melanogenesis of melanocytes via cAMP signalling pathway. Moreover, hyperpigmentation effect of CFE was confirmed in 3D skin and ex vivo hair follicles.

MATERIALS AND METHODS

Materials

Sodium hydroxide (NaOH), 3-(4,5-dimethyl-2-thiazo lyl)-2,5-diphenyltetrazolium bromide (MTT), L-3,4-dihydroxyphenylalanine (L-DOPA), dimethyl sulphoxide (DMSO), potassium carbonate (K_2CO_3), hydrogen peroxide (H_2O_2, 30%), sodium sulphite ($NaSO_3$), hydrochloric acid (HCl), HPLC grade acetonitrile and monopotassium phosphate (KH_2PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against tyrosinase (TYR) and β-actin were obtained from Santa Cruz Biotechnology (CA, USA). Microphthalmia-associated transcription factor (MITF) antibody was purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). Antibody against cAMP response element-binding protein (CREB), phospho-CREB and secondary antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Extraction of *Cirsium japonicum* flower

Cirsium japonicum flower was purchased from the Bisealsan Nongwon (Daegu, South Korea) and identified by comparison with voucher specimen (NIBRVP0000373346), which is already deposited at the National Institute of Biological Resources. *Cirsium japonicum* flower was desiccated using a hot air drier at 50°C overnight and pulverized. The dried powder of *Cirsium japonicum* flower (100 g) was extracted with 70% (v/v) ethanol at room temperature with stirring. After filtration using filter paper, the solvent was removed by a rotary vacuum evaporator (Heidolph, Schwabach, Germany) and CFE was lyophilized. The dried powder of *Cirsium japonicum* flower (10.98 g) was dissolved in DMSO at 1000-fold higher concentration of final concentration in medium. DMSO at 0.1% v/v treated cells was used as control.

Cell culture and viability assay

Human epidermal melanocytes (neonatal, lightly pigmented donor; HEMn-LP and moderately pigmented donor; HEMn-MP) were purchased from Thermo Fisher Scientific, Inc (Pittsburgh, PA, USA). These cells were cultured with M254 culture medium (Life Technologies Corporation, NY, USA) containing HMGS supplement (Life Technologies Corporation, NY, USA) in a humidified incubator, 5% CO_2, at 37°C.

Melanin content and cell viability assay

Human melanocytes were seeded in a six-well plate at a constant cell density. After incubation with CFE for 5 days, cells were washed twice with PBS and harvested. The cell pellets were dissolved in 1 N NaOH for 2 h at 70°C and centrifuged at 13 000× *g* for 10 min. Absorbance of the supernatant at 450 nm was measured using a spectrophotometer (BioTek, VT, USA). The content of melanin

was evaluated using a valid synthetic melanin as standard [20].

Cell cytotoxicity was assessed using MTT assay. Human melanocytes were incubated with CFE for 5 days. Cells were treated with MTT solution for 2 h at 37°C and the medium was discarded. Then, DMSO was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a spectrophotometer (BioTek, VT, USA).

Analysis of eumelanin and pheomelanin by HPLC

Pyrrole-2,3,5-tricarboxylic acid (PTCA) and thiazole-2,4,5-tricarboxylic acid (TTCA) as melanin markers were obtained from Prof. Ito (Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan). Standard solution of PTCA was prepared by dissolving in 10 ml of 20% methanol. Standard solution of TTCA was prepared by dissolving in 10 ml of distilled water. Alkaline H_2O_2 oxidation was performed to measure PTCA and TTCA [22] Samples (1.0×10^6 cells) in 10-ml conical test tubes were suspended by adding 100 µl of water, 375 µl of 1 M K_2CO_3 and 25 µl of 30% H_2O_2. Samples in tubes were well mixed for 20 h at 25°C. Then, 50 µl of 10% $NaSO_3$ was added to decompose residual H_2O_2. The mixture was acidified with 140 µl of 6 M HCl and centrifuged 3500× *g* for 10 min. The supernatant was injected into HPLC system equipped with Waters 2695 Separations module and 996 photodiode array detector to analyse PTCA and TTCA. Synergi Hydro-RP column (80 A 250 × 4.6 mm, 4 µm, Phenomenex, USA) was used at 30°C. The column was eluted with 50 mM of 99% KH_2PO_4 buffer (pH 2.6)/HPLC grade acetonitrile, 99:1 (v/v) for 13 min followed by a linear gradient to 5% acetonitrile in 2 min. After an isocratic period of 10 minutes, a second linear gradient was used to return to initial conditions. A flow rate of 0.7 ml/min was used. UV absorbance at 269 nm was monitored.

Cellular tyrosinase activity

Cells were cultured in the presence of CFE for 5 days and then harvested. Cell pellets were lysed in lysis buffer composed of 0.1 M Potassium phosphate buffer (pH 6.8), 1% Triton X-100, 1 mM PMSF and 5 mM EDTA. After centrifugation at 13 000× *g* for 10 min, the supernatant was collected, and Bradford assay with bovine serum albumin (BSA) was performed to measure protein content. The reaction mixture containing 50 mM of phosphate buffer (pH 6.8), 2 mM of L-DOPA and 200 µg of supernatant protein was incubated at 37°C for 1 h. Absorbance at 450 nm was measured using a spectrophotometer (BioTek, VT, USA).

Cyclic AMP immunoassay

Human melanocytes were treated with CFE for 1 h and lysed with 0.1 M HCl to inhibit phosphodiesterase activity. After incubation at room temperature for 20 min, cells were collected using a cell scraper and centrifuged at 1000× *g* for 10 min. The concentration of cAMP was evaluated using a cAMP immunoassay kit purchased from Cayman Chemical (Ann Arbor, MI, USA) according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed to measure expression levels of melanogenesis-related proteins in human melanocytes. In brief, cells treated with CFE were lysed with RIPA buffer (Thermo scientific, PA, USA) supplemented with protease and phosphatase inhibitors (Thermos scientific, PA, USA). The supernatant obtained by centrifugation (13 000× *g* for 20 min at 4°C) was quantified using Bradford assay kit (Thermo scientific). Cell lysates were separated on 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel and transferred to polyvinylidene difluoride (PVDF) membrane (Thermo scientific, PA, USA) with MOPS [3-(N-morpholino) propanesulfonic acid] buffer. The membrane was blocked with 5% skim milk in TBS-T (Tris-Buffered Saline containing 0.1% Tween-20) for 1 h, incubated overnight with primary antibodies (1:1000) at 4°C and then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000, Cell Signaling) for 1 h at room temperature. Proteins were detected with PicoEPD Western reagent (ELPIS-Biotech, Daejeon, Korea). Bands were quantified using LAS 500 image analyser (GE Healthcare Life Sciences, Korea).

The reconstituted three-dimensional human skin model

The reconstructed three-dimensional human skin model, Neoderm-ME (Tego Science, Seoul, Korea), consisted of normal human epidermal keratinocytes and normal human epidermal melanocytes. 3D skin was incubated with CFE for 14 days in maintenance medium (Tego Science, Seoul, Korea). To measure the degree of pigmentation, L-values were measured using a CR-300 chroma meter (Minolta, Tokyo, Japan). Then, 3D skin was treated with 1 N NaOH and incubated at 70°C for 4 h to dissolve

melanin. The amount of dissolved melanin was measured using a spectrophotometer (BioTek) at 450 nm.

Ex vivo human hair follicles

The pigmented hair follicles were obtained from 65-year-old female donor (a human skin fragment face lift, plastic surgery) in University Regional Hospital Center of Tours (Chambray-Les-Tours, France). All the samples used in this study were signed by the consent of the patient. The study was approved by the ethical committee of the Hospital of Tours, and was performed by Bioalternatives (Gencay, France). Hair follicles were cultured with treatment of CFE for 7 days in E William's medium supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 μg/ml insulin and 0.004 μg/ml hydrocortisone at 37°C. After incubation, hair follicles were washed and fixed with formaldehyde solution. The fixed hair follicles were dehydrated in multiple baths with increased concentration of ethanol and then embedded in paraffin. The transversal sections were performed using a microtome with 5 μm thickness. The sections were deparaffinized and stained by Fontana Masson solution. The sections were observed using a NIKON E400 microscope and digital images were captured by a NIKON DS-Ri1 camera and processed with NIS-Elements 4.13.04 software. The mosaic images (objective lens, ×40) of hair follicles were obtained by using Image J software. The content of melanin in the hair follicles was evaluated by a quantitative measurement of the melanin surface normalized to the hair follicle area.

Statistical analysis

All experiments were independently performed three times. All data are expressed as mean ± standard deviations. The statistical significance of results of treated cells vs. that of control (untreated) was determined by one-way analysis of variance. $*p < 0.05$ and $**p < 0.01$ indicated statistical significance.

RESULTS

Effect of CFE on the melanin content of lightly and moderately pigmented human melanocytes

To investigate effects of CFE on melanin synthesis, we measured the levels of melanin contents in lightly and moderately pigmented melanocytes. As shown in Figure 1a, the melanin content of HEMn-LP treated with 5 μg/ml CFE increased more than 1.8-fold compared with untreated control. Also, CFE increased melanin content of HEMn-MP in a dose-dependent manner. 5 μg/ml CFE-treated HEMn-MP showed 2.2-fold higher level of melanin content than untreated control. To exclude the possibility that such increases of melanin content might be attributed to cell-proliferating effect of CFE, 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed. Treatment with CFE in various concentrations (1, 3, 5 μg/ml) for 5 days did not affect cell viability or proliferation of HEMn-LP and HEMn-MP (Figure 1b). These results suggested that CFE increases the melanin synthesis in both HEMn-LP and HEMn-MP without affecting on cell proliferation.

Analysis on markers of pheomelanin and eumelanin

Eumelanin, the major pigment determining the colour of human skin, is composed of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) moieties. The DHICA moiety in eumelanin is known to give free PTCA during the oxidative degradation induced by alkaline hydrogen peroxide oxidation. The benzothiazole unit from conversion of benzothiazine moiety in Pheomelanin gives TTCA and TDCA during alkaline hydrogen peroxide oxidation [21]. Therefore, PTCA and TTCA were determined as markers of eumelanin and pheomelanin respectively.

The amounts of pheomelanin and eumelanin in lightly and moderately pigmented human melanocytes were measured by using alkaline H_2O_2 oxidation method described by Ito et al. [22]. In Figure 2, TTCA, a degradation product from pheomelanin, is indicated by arrow 1, whereas PTCA, a degradation product from eumelanin, is indicated by arrow 2.

As shown in Table 1, the amounts of both TTCA and PTCA showed higher in HEMn-MP than HEMn-LP, whereas the ratio of TTCA/PTCA was higher in HEMn-LP than HEMn-MP. However, all yields of TTCA and PTCA in HEMn-LP and HEMn-MP after CFE treatment were increased 2-fold compared to those in untreated control. There were no noticeable changes in the ratio of TTCA/PTCA in both melanocytes by CFE. Taken together, CFE increased both eumelanin and pheomelanin in without change in the ratio of them.

Effect of CFE on cellular tyrosinase activity

Tyrosinase is the main enzyme in melanin biosynthesis. Therefore, we observed the effect of CFE on intracellular tyrosinase activity. Cells were cultured in the

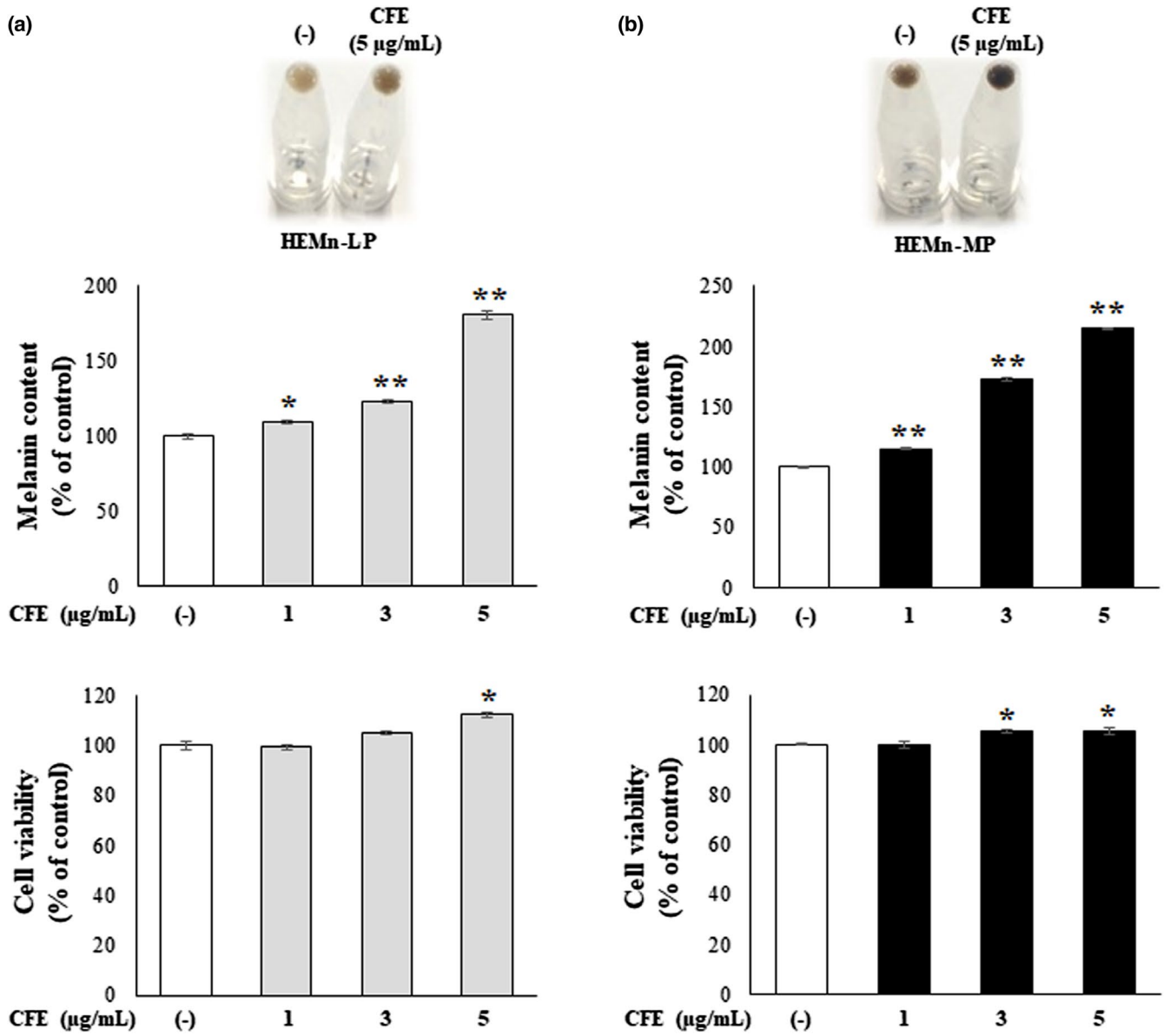

FIGURE 1 Melanin content and cell viability of lightly (a) and moderately (b) pigmented melanocytes treated with CFE. Cells were cultured in the presence of CFE for 5 days. Melanin content was determined under the same condition. Cell viability was determined by MTT assay. All results were expressed as percentages of the untreated control. Data are representatives of three independent experiments and values are expressed in mean ± SD (*$p < 0.05$, **$p < 0.01$ vs. untreated control)

presence of various concentrations of CFE (1, 3, 5 μg/ml) for 5 days and cell lysates were then used to measure cellular tyrosinase activity. L-DOPA was used as a positive regulator of melanogenesis to determine cellular tyrosinase activity. As shown in Figure 3, CFE increased cellular tyrosinase activity of both HEMn-LP and HEMn-MP in a dose-dependent manner. The activities of cellular tyrosinase in HEMn-LP and HEMn-MP treated with 5 μg/ml CFE showed more than 2-fold and 3-fold increase compared with untreated control respectively. Therefore, these results showed that CFE regulates the activities of cellular tyrosinase in both HEMn-LP and HEMn-MP.

Effect of CFE on cAMP signalling pathway

It is well known that cyclic AMP (cAMP), a key factor in the regulation of melanogenesis, up-regulates the expression of MITF by activating protein kinase A (PKA) and phosphorylating cAMP response element-binding protein (CREB) [23]. To investigate the molecular mechanism of melanogenic effect by CFE, cAMP immunoassay was performed using human melanocytes treated with CFE. Results showed that CFE considerably increased intracellular cAMP concentration in HEMn-LP and HEMn-MP (Figure 4a). As shown in Figure 4b, phosphorylated CREB, MITF and tyrosinase

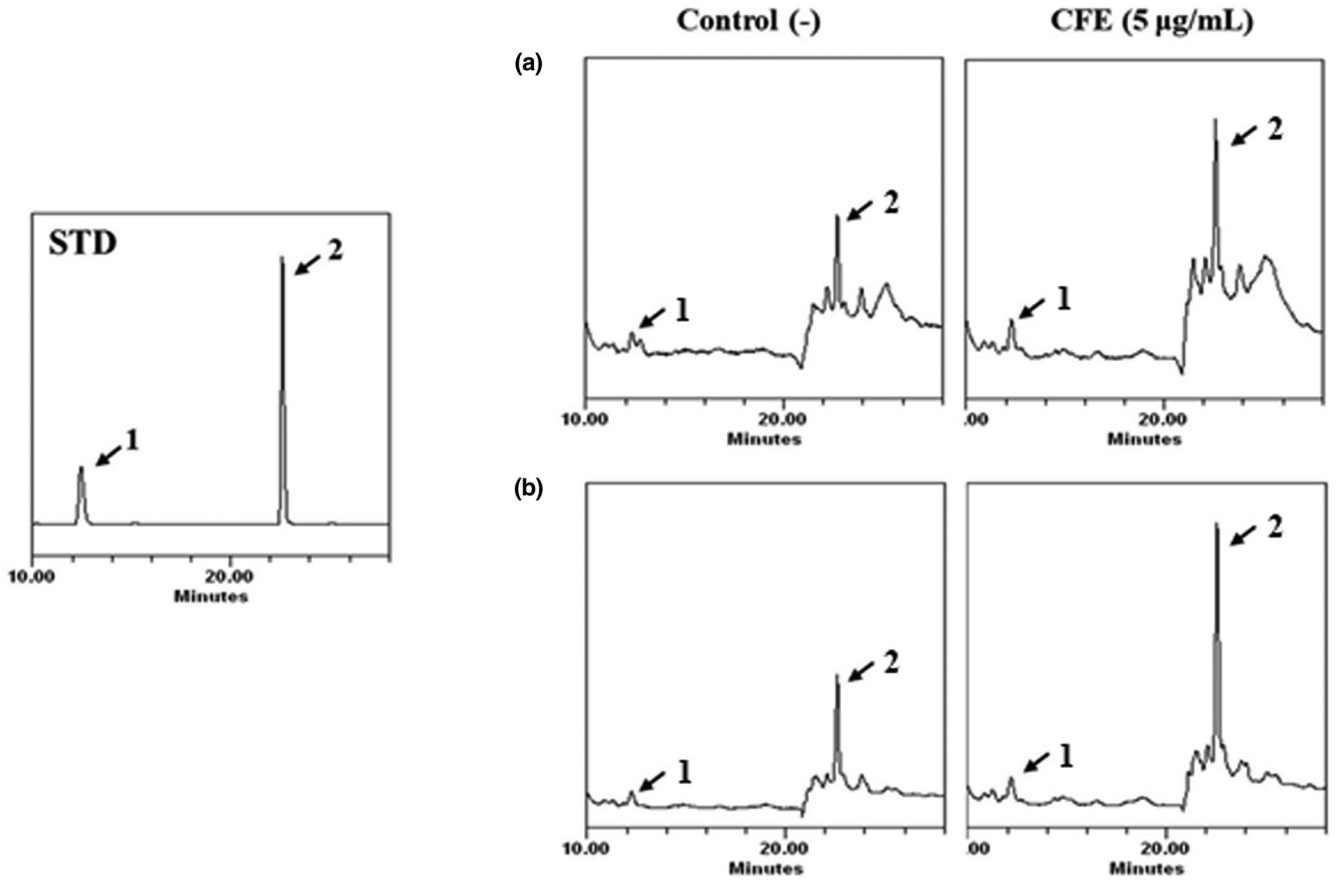

FIGURE 2 HPLC chromatograms of TTCA and PTCA from lightly (a) and moderately (b) pigmented melanocytes. Arrow 1 and arrow 2 indicate thiazole-2,4,5-tricarboxylic acid (TTCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA), respectively. Both cells were treated with 5 μg/ml of CFE for 5 days. HPLC analysis was performed to detect TTCA and PTCA, melanin markers, after degradation of melanin by alkaline H2O2 oxidation

TABLE 1 TTCA and PTCA as specific markers in lightly and moderately pigmented melanocytes

Sample		Alkaline H_2O_2 oxidation (μg/ml)[a]		Percentage (%)[b]		Ratio of TTCA/PTCA
		TTCA	PTCA	TTCA	PTCA	
HEMn-LP	Control	3.9 ± 0.2	26.3 ± 2.8	12.9%	87.1%	0.148
	CFE (5 μg/ml)	8.6 ± 0.8	48.8 ± 1.2	15.0%	85.0%	0.176
HEMn-MP	Control	9.7 ± 0.6	107.3 ± 5.4	8.3%	91.7%	0.090
	CFE (5 μg/ml)	19.6 ± 1.5	237.4 ± 5.4	7.6%	92.4%	0.082

Note: CFE (5 μg/ml) was added into lightly (HEMn-LP) and moderately (HEMn-MP) pigmented melanocytes. TTCA and PTCA obtained after alkaline H_2O_2 oxidation were evaluated via HPLC analysis. Data are representatives of three independent experiments and values are expressed as mean ± SD.

[a]Calculated as $[A_{TEST}/A_{STD} * c * d * p]$. A_{TEST}; Area of analyte from test solution, A_{STD}; Area of analyte from standard solution, c; known concentration of standard solution (μg/ml), d: dilution factor, p; purity of reference.

[b]Percentages (%) of TTCA and PTCA. TTCA; TTCA/(TTCA + PTCA) × 100, PTCA/(TTCA + PTCA) × 100.

(TYR) expression were analysed by western blot assay. Results revealed that the level of phosphorylated CREB was increased by treatment with 5 μg/ml CFE in both pigmented types of melanocytes. In addition, CFE up-regulated expression of MITF, an important transcription factor in melanogenesis. Treatment with 5 μg/ml of CFE also increased the expression of TYR related to melanin synthesis. β-actin was used as a loading control in western blot analysis to normalize the levels of proteins correcting interpretation of the results. Taken together, the results showed that CFE regulates melanogenesis in both of HeMn-LP and HEMn-MP by increasing intracellular cAMP that induces phosphorylation of CREB and increases the expressions of MITF and TYR.

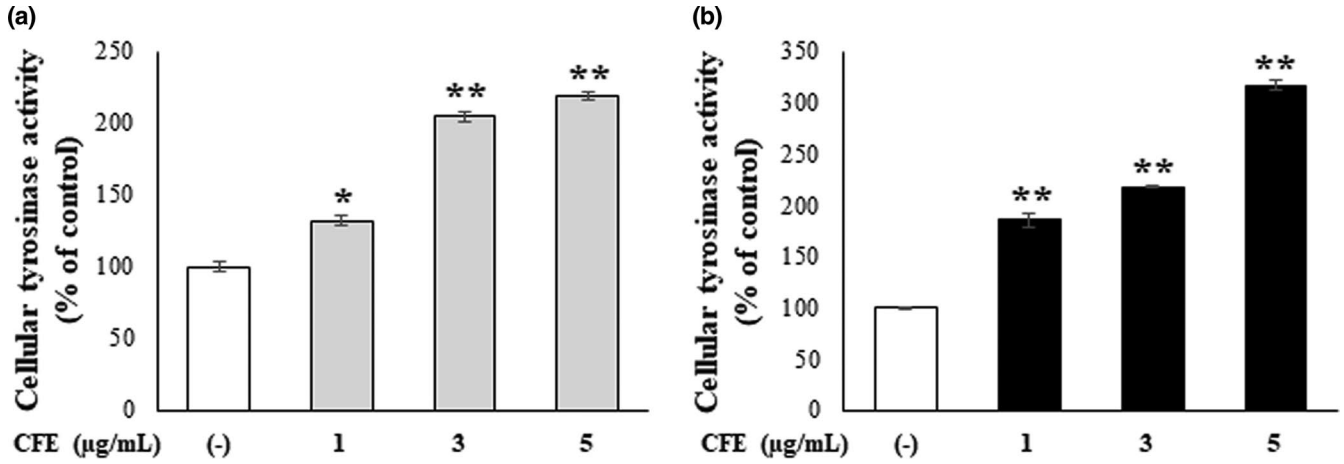

FIGURE 3 Effect of CFE on cellular tyrosinase activity in lightly (a) and moderately (b) pigmented melanocytes. Cells were incubated with CFE (1, 3, 5 μg/ml) for 5 days. Cellular tyrosinase activity was measured as described in "MATERIALS AND METHODS". Results are expressed as percentages of the untreated control. Data are representatives of three independent experiments. Values are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$ vs. untreated control)

Melanogenic effect of CFE on 3D skin model

To investigate the melanogenic effect of CFE in an equivalent condition of human skin, the reconstituted three-dimensional human skin (3D skin) containing normal epidermal keratinocytes and melanocytes was cultured in the presence of CFE (50, 100 μg/mL) for 14 days. As shown in Figures 5a, 3D skin treated with CFE was observed to be distinctly darker than untreated control via visual assessment. In addition, dissolved melanin of 3D skin was increased by CFE in a dose-dependent manner (Figure 5b). We could confirm that CFE induces melanogenesis not only *in vitro* cell condition, but also in an equivalent condition of human skin.

Effect of hyperpigmentation on ex vivo human hair follicles

We further examined whether CFE also affected pigmentation of hair. The melanin content in *ex vivo* human hair follicles was evaluated by Fontana-Masson staining after 7 days of treatment with 5 μg/ml CFE. The quantity of melanin localized in the hair bulb and shaft was measured by normalizing melanin surface to the bulb and shaft area. As a result of microscopic observation, the melanin in the hair bulb of human hair follicles treated with 5 μg/ml CFE was strikingly increased than untreated control (Figure 6a). Similarly, the melanin in the hair shaft of human hair follicles with treatment of 5 μg/ml CFE was observed a significant increase compared with untreated control (Figure 6b). These results indicated that CFE can affect melanogenesis of the hair follicles and regulate pigmentation of hair.

DISCUSSION

Previous studies revealed that α-MSH, 3-isobutyl-1-methylxanthine (IBMX) and forskolin can stimulate melanin synthesis by the cAMP-dependent pathway [23]. cAMP increases the expression of MITF through activation of the CREB phosphorylation. MITF, a basic helix-loop-helix leucine zipper transcription factor, is reported to bind the M-box in the promoter sequence of tyrosinase. When MITF binds to the M-box motif in the promoter region, it transactivates the tyrosinase, which leads to the stimulation of melanogenesis. In this study, we observed that CFE increased the melanin content and tyrosinase activity in lightly and moderately pigmented human melanocytes (Figures 1, 3). Moreover, CFE increased intracellular cyclic AMP concentration stimulating CREB phosphorylation, and MITF expression (Figure 4). These results demonstrate that CFE affects cAMP/CREB/MITF pathway to up-regulate tyrosinase activities, thereby leading to the increase of melanin synthesis in both type of melanocytes. This hyperpigmentation effect of CFE was also taken in an equivalent condition of human skin. CFE applied on reconstituted 3D human skin increased the melanin content as within the melanocytes (Figure 5).

Along with skin colour, the hair colour also is affected by physiological melanogenesis. Melanin synthesis in hair follicles occurs in follicular melanocytes. Synthesized melanin is transferred to the hair shaft composed of keratinocytes by cytoplasmic organelles called melanosome. Ito et al. have proposed that the pH and cysteine level of melanosomes play a critical role in determining the course of mixed melanogenesis leading diversity in hair colour [24]. The cysteine level of melanosomes is regulated by

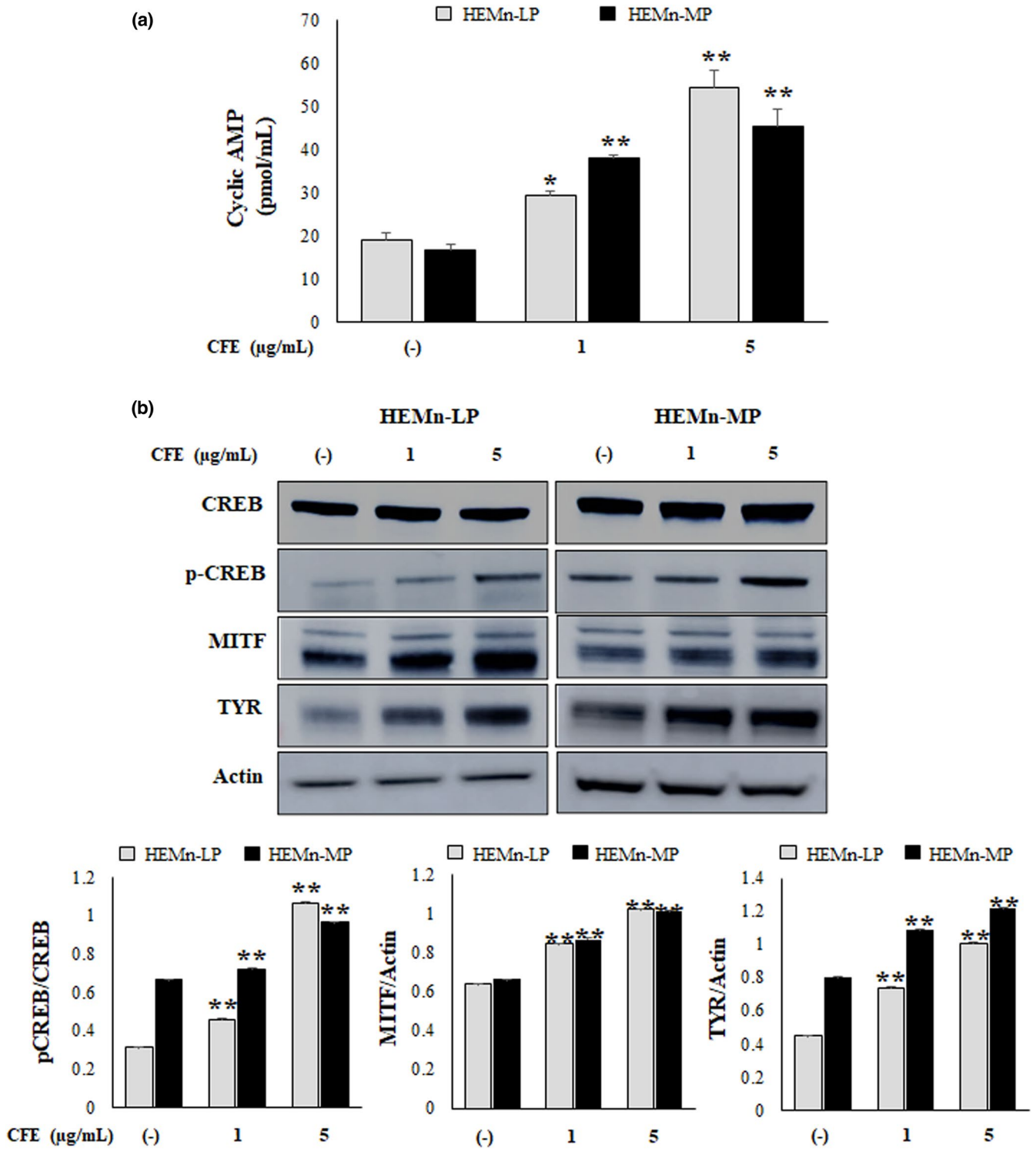

FIGURE 4 Effect of CFE on activation of cAMP signaling pathway in lightly and moderately pigmented melanocytes. Human lightly (HEMn- LP) and moderately (HEMn- MP) pigmented melanocytes were incubated with CFE (1, 5 μg/ml). (a) The level of cAMP was detected with immunoassay. The absorbance was measured at 405 nm. (b) The expression of protein including CREB, p-CREB, MITF and TYR were detected with Western blot assay. The expression level of p-CREB was represented in ratio compared with CREB. The expression levels of MITF and TYR were represented in ratio compared with Actin. Data are representatives of three independent experiments. Values are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$ vs. untreated control). CREB, cAMP-response element-binding protein; MITF, microphthalmia-associated transcription factor; p-CREB, Phosphorylated CREB; TYR, tyrosinase

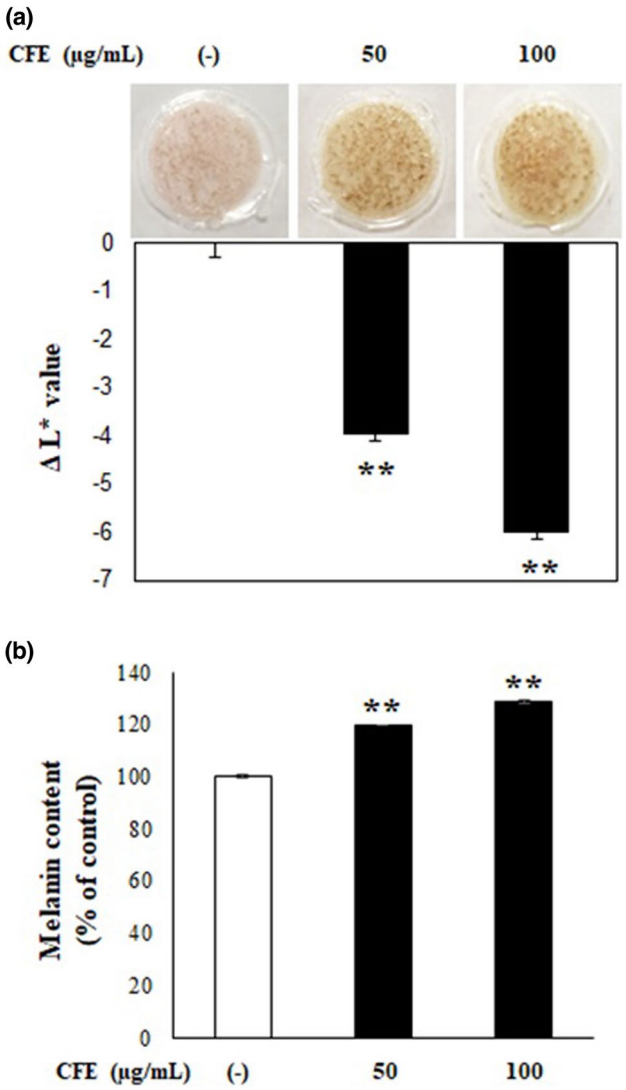

FIGURE 5 Hyperpigmentation effect on reconstituted human skin model treated with *C. japonicum* flower extract (CFE). 3D skin composed of human epidermal keratinocytes and melanocytes was treated with CFE (50, 100 μg/ml) three times for 14 days. (a) L values of cell pigmentation were measured using a chromameter (CR-300, Minolta). (b) Melanin content was measured by dissolving in 1N NaOH at 70°C. Values are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$ vs. untreated control)

melanocortin-1 receptor (MC1R) signalling which activates the melanogenic transcription factor, MITF by raising the level of cAMP. Hair greying is one of the typical signs of human ageing and is associated with a decrease in follicular melanocyte population and a decrease in melanin content [25]. To confirm the effect of CFE on hair follicles, we applied CFE in ex vivo human hair follicles and observed that CFE also increased melanin in hair bulb and hair shaft (Figure 6).

Melanin is a group of complex pigment structure with wide diversity in colour, composition, size and function. There are two chemically distinct types of melanin, eumelanin and pheomelanin. Both eumelanin and pheomelanin are synthesized from dopaquinone. Dopaquinone is converted to dopachrome through auto-oxidation. Dopachrome is decarboxylated to form DHI or tautomerized to form DHICA which are further oxidized to form eumelanin. It also reacts with the cysteine to form benzothiazine derivatives, the basic unit of pheomelanin. Eumelanin plays a photoprotective role through radiation absorption and radical scavenging ability, while pheomelanin is phototoxic through superoxide production [26]. To confirm the effect of CFE on synthesis of eumelanin and pheomelanin in both type of melanocytes, we measured the PTCA, a product of the peroxidation of DHICA in eumelanin, and the TTCA, a degradation product of pheomelanin by using HPLC method (Figure 2). The previous study demonstrated that melanin in human epidermis composed of 75% eumelanin and 25% pheomelanin regardless of the degree of pigmentation [27]. Our results showed that the compositions of pheomelanin/eumelanin were 12.9%/87.1% in HeMn-LP and 8.3%/91.7% in HEMn-MP. According to the previous report, in tyrosine-induced melanogenesis, there is a difference in pheomelanin and eumelanin synthesis between light-pigmented and dark-pigmented melanocytes. Van Nieuwpoort et al. reported that the ratio of pheomelanin/eumelanin increased more in light-pigmented melanocytes than in dark-pigmented melanocytes through activating pheomelanogenesis increasing the risk of oxidative stress [28]. In this study, CFE increased both types of melanin 2-fold more than untreated control regardless of melanocytes pigmentation types; however, there were no changes in the ratios of pheomelanin/eumelanin. This finding suggests that CFE induces melanogenesis without preference for eumelanin or pheomelanin by up-regulation of tyrosinase activities, which play a critical role in eumelanin and pheomelanin synthesis in the rate-limiting step.

Accumulated oxidative stress has been suggested as one of the major factors of vitiligo and hair greying. Increased free radical generation such as H_2O_2 contributes to apoptosis of hair follicle melanocytes and DNA damage [29]. H_2O_2 also induces hypopigmentation via the down-regulation of MITF, which play important roles not only in the control of differentiation, but also in melanocyte survival [30]. Therefore, we investigated the effect of CFE on the H_2O_2-induced inhibition of melanogenesis. As shown in Figure S1, the H_2O_2-induced reduction in melanin content was increased by CFE treatment.

In Summary, *Cirsium japonicum* flower extract (CFE) increased melanin content and cellular tyrosinase activity in human lightly and moderately pigmented melanocytes via cAMP signalling pathway. Moreover, CFE increased the melanin content in a 3D skin model and hair follicles.

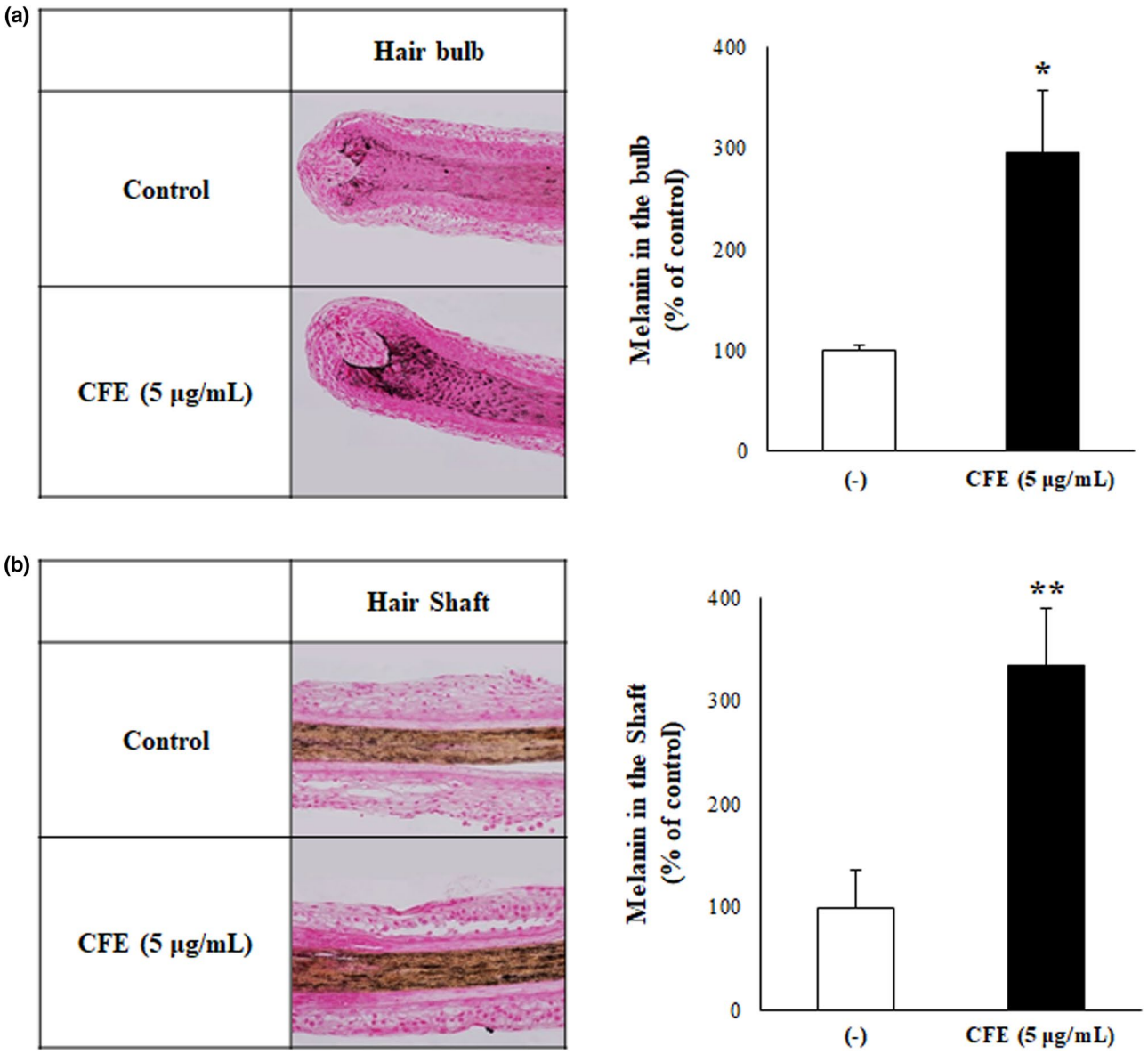

FIGURE 6 Ex vivo hyperpigmentation effect of CFE on human hair follicles. Human hair follicles were treated 5 μg/ml of CFE and incubated for 7 days. The hair bulb (a) and hair shaft (b) was stained by Fontana-Masson solution. The melanin content in the bulb and shaft was measured using ImageJ software. Values are expressed as mean ± SD (*$p < 0.05$, **$p < 0.01$ vs. untreated control)

These results suggest that CFE exerts hyperpigmentation activity through cAMP signalling in human melanocytes that it can be applied as a potential melanogenesis stimulator for hypopigmentation disorders, such as grey hair and vitiligo.

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CONFLICT OF INTEREST

The authors have no conflict of interest regarding the publication of this paper.

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REFERENCES

1. Gruber F, Peharda V, Kastelan M, Brajac I. Occupational skin diseases caused by UV radiation. Acta Dermatovenerol Croat. 2007;15(3):191–8.
2. Morison WL. Effects of ultraviolet radiation on the immune system in humans. Photochem Photobiol. 1989;50(4):515–24.
3. Young AR, Claveau J, Rossi AB. Ultraviolet radiation and the skin: Photobiology and sunscreen photoprotection. J Am Acad Dermatol. 2017;76(3S1):S100–9.
4. de Jager TL, Cockrell AE, Du Plessis SS. Ultraviolet light induced generation of reactive oxygen species. Adv Exp Med Biol. 2017;996:15–23.
5. Mikkelsen RB, Wardman P. Biological chemistry of reactive oxygen and nitrogen and radiation-induced signal transduction mechanisms. Oncogene. 2003;22(37):5734–54.
6. Brenner M, Hearing VJ. The protective role of melanin against UV damage in human skin. Photochem Photobiol. 2008;84(3):539–49.
7. Costin GE, Hearing VJ. Human skin pigmentation: melanocytes modulate skin color in response to stress. FASEB J. 2007;21(4):976–94.
8. Videira IF, Moura DF, Magina S. Mechanisms regulating melanogenesis. An Bras Dermatol. 2013;88(1):76–83.
9. Thody AJ, Higgins EM, Wakamatsu K, Ito S, Burchill SA, Marks JM. Pheomelanin as well as eumelanin is present in human epidermis. J Invest Dermatol. 1991;97(2):340–4.
10. Wakamatsu K, Ohtara K, Ito S. Chemical analysis of late stages of pheomelanogenesis: conversion of dihydrobenzothiazine to a benzothiazole structure. Pigment Cell Melanoma Res. 2009;22(4):474–86.
11. Nasti TH, Timares L. MC1R, eumelanin and pheomelanin: their role in determining the susceptibility to skin cancer. Photochem Photobiol. 2015;91(1):188–200.
12. Borges CR, Roberts JC, Wilkins DG, Rollins DE. Relationship of melanin degradation products to actual melanin content: application to human hair. Anal Biochem. 2001;290(1):116–25.
13. Kaidbey KH, Agin PP, Sayre RM, Kligman AM. Photoprotection by melanin–a comparison of black and Caucasian skin. J Am Acad Dermatol. 1979;1(3):249–60.
14. Gloster HM Jr, Neal K. Skin cancer in skin of color. J Am Acad Dermatol. 2006;55(5):741–64.
15. Mok JY, Kang HJ, Cho JK, Jeon IH, Kim HS, Park JM, et al. Antioxidative and anti-inflammatory effects of extracts from different organs of Cirsium japonicum var. ussuriense. Korean J Herbology. 2011;26(4):39–47.
16. Park JY, Kim HY, Shibamoto T, Jang TS, Lee SC, Shim JS, et al. Beneficial effects of a medicinal herb, Cirsium japonicum var. maackii, extract and its major component, cirsimaritin on breast cancer metastasis in MDA-MB-231 breast cancer cells. Bioorg Med Chem Lett. 2017;27(17):3968–73.
17. Lee JH, Choi SI, Lee YS, Kim GH. Antioxidant and anti-inflammatory activities of ethanol extract from leaves of Cirsium japonicum. Food Sci Biotechnol. 2008;17:38–45.
18. Yin HH, Cho BO, Fang CZ, Shim JS, Jang SI. Anti-obesity effects of water extracts from different organs of *Cirsium japonicum* var. ussuriense. J Physiol Pathol Korean Med. 2015;29(4):322.
19. Park MR, Lee YW, Cho JH, Yoo HS, Son CG, Cho CK. Effects of *Cirsium japonicum* var. ussuriense extract on tumor immunity. Korean J of Oriental Med. 2006;27(4):30–47.
20. Uchida R, Ishikawa S, Tomoda H. Inhibition of tyrosinase activity and melanine pigmentation by 2-hydroxytyrosol. Acta Pharm Sin B. 2014;4(2):141–5.
21. Ito S, Wakamatsu K, Sarna T. Photodegradation of Eumelanin and Pheomelanin and Its Pathophysiological Implications. Photochem Photobiol. 2018;94(3):409–20.
22. Ito S, Nakanishi Y, Valenzuela RK, Brilliant MH, Kolbe L, Wakamatsu K. Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins. Pigment Cell Melanoma Res. 2011;24(4):605–13.
23. Buscà R, Ballotti R. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res. 2000;13(2):60–9.
24. Ito S, Wakamatsu K. Diversity of human hair pigmentation as studied by chemical analysis of eumelanin and pheomelanin. J Eur Acad Dermatol Venereol. 2011;25(12):1369–80.
25. Sarin KY, Artandi SE. Aging, graying and loss of melanocyte stem cells. Stem Cell Rev. 2007;3(3):212–7.
26. Solano F. Melanins: skin pigments and much more—types, structural models, biological functions, and formation routes. N J Sci. 2014;2014:1–28.
27. Del Bino S, Ito S, Sok J, Nakanishi Y, Bastien P, Wakamatsu K, et al. Chemical analysis of constitutive pigmentation of human epidermis reveals constant eumelanin to pheomelanin ratio. Pigment Cell Melanoma Res. 2015;28(6):707–17.
28. van Nieuwpoort F, Smit NP, Kolb R, van der Meulen H, Koerten H, Pavel S. Tyrosine-induced melanogenesis shows differences in morphologic and melanogenic preferences of melanosomes from light and dark skin types. J Invest Dermatol. 2004;122(5):1251–5.
29. Arck PC, Overall R, Spatz K, Liezman C, Handjiski B, Klapp B, et al. Towards a "free radical theory of graying": melanocyte apoptosis in the aging human hair follicle is an indicator of oxidative stress induced tissue damage. FASEB J. 2006;20(9):1567–9.
30. Wood JM, Decker H, Hartmann H, Chavan B, Rokos H, Spencer JD, et al. Senile hair graying: H_2O_2-mediated oxidative stress affects human hair color by blunting methionine sulfoxide repair. FASEB J. 2009;23(7):2065–75.

SUPPORTING INFORMATION

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