The effects of phycocyanin to relieve oxidative damage on mouse dermal papilla cells

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Abstract

The reactive oxygen species (ROS) is one of the main reasons leading to the senescence and apoptosis of dermal papilla cells, whose function is to regulate the hair cycle. Phycocyanin (CPC), a pigment-protein, is a powerful antioxidant that can stimulate fibroblasts' and keratinocytes' proliferation. This study experimented with the ability of CPC to reduce oxidative stress. CPC was extracted from Arthrospira platensis and was purified. Mouse dermal papilla cells (DP cells) were isolated from the vibrissa of C57/BL6 mouse and cultured by explant method. The DP cells were pre-treated with CPC (5, 10 µg/ml) and 150 µM H_2O_2 to test their protective ability.

The results showed that CPC was collected with analytical grade (PI=4.11). The cultured cells expressed some DP cells' main characteristics: fibroblast-like shape, vimentin, α – SMA, formation of cell clusters and spheroid in 3D culture. CPC is non-toxic to DP and 5-10 µg/ml concentration induced DP proliferation. The CPC 5-10 µg/ml reduced H_2O_2-induced damage, decreasing death rate, total ROS and ß-galactosidase expression, preserving cell morphology and inducing cell growth. CPC 10 µg/ml is the optimal concentration that induces PD replication and alleviates H_2O_2-induced oxidative stress on DP.

Keywords: Phycocyanin, mouse dermal papilla cells, hydrogen peroxide.

Introduction

Hair loss, a common heath condition in males and females, is caused by miniaturization symptoms. The explanation for this issue is the continuous damage to dermal papilla cells, which play a crucial role in regulating the hair cycle by secreting paracrine hormones to control the movement and the proliferation of epithelial cells[17,18]. Despite holding the main functions, dermal papilla cells were found to be sensitive to reactive oxygen species (ROS)[37]. ROS affections could be considered as one of the primary reasons for cell senescence, consisting of the broken DNA structure and proteins and induced apoptosis[6]. This phenomenon gives rise to hair miniaturization symptoms: thinner hair shaft, shortened hair cycle for a long time and hair loss[21]. As a result, it is hard to find sufficient treatments for this situation. Several treatment therapies, such as hair transplantation and herbal extracts have been explored to address the issue of hair loss. However, it is challenging to search for a suitable treatment due to the side effects of these methods[25].

Phycocyanin (CPC) from spirulina algae (*Arthrospira platensis*) may be a potential choice for these problems. Phycocyanin, a pigment-protein extracted from Spirulina algae, is a powerful antioxidant. CPC is well-known for its signature blue colour contributed by the bonding between α and β - subunits through the phycocyanobilin chromophore molecular[8]. CPC can absorb light energy and can transfer it to the photosynthetic center[31]. This property has led to its wide application in functional food and as a colouring agent in the food and cosmetics industries. Its high safety profile and ability to stimulate fibroblast and keratinocyte proliferation and wound healing make it a promising candidate for addressing hair loss[10,14].

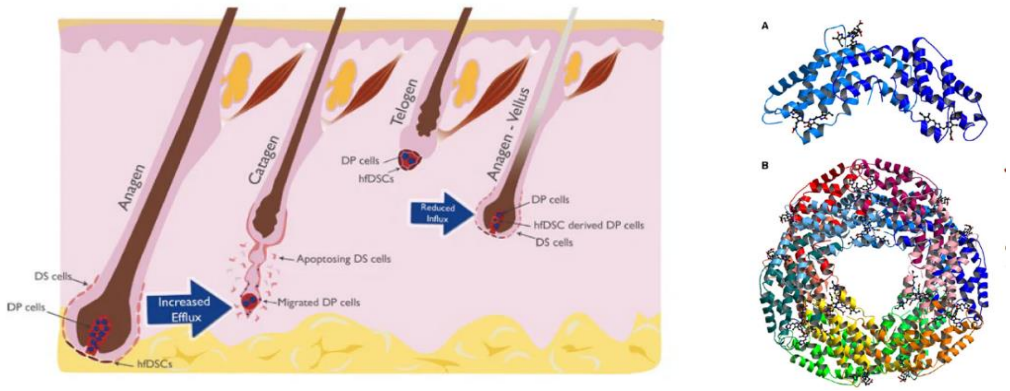

Figure 1: Introduction of follicle miniaturization and phycocyanin. Left. Follicle miniaturiazation[27]. Right: phycocyanin structure. A: monomer (αβ), B: heterohexamer (αβ)6[27]

Furthermore, CPC is known for its potent antioxidant properties, which can be crucial in reducing oxidative stress and potentially reversing the effects of hair loss. Given CPC's properties, this research aims to evaluate its effectiveness in promoting proliferation and reducing oxidative stress induced by H_2O_2 on C57BL/6 dermal papilla cells. This study is initial step in exploring CPC's potential in hair loss treatment.

Material and Methods

Extraction and purification of phycocyanin: Five grams of dry biomass were suspended in 40 ml of 1% $CaCl_2$. The mixture was frozen at -20°C for 12 hours and thawed at 20°C for 4 hours and this cycle was repeated three times[16]. The phycocyanin-containing clear blue supernatant was collected by centrifugation[12]. The extract was purified by incubating in 80 g/L activated charcoal (AC) and the sample was stirred and centrifugated to obtain the blue extract[26]. Next, ammonium sulfate (AS) was added to the blue extract (35% w/v), incubated for 24 hours at 4°C and then centrifugated to obtain the pellet. Next, the pellet was resuspended in 30% AS (w/v), incubated for 24 hours and centrifugated to isolate the pellet.

The pellet was dissolved in sodium phosphate buffer (Na-buffer) and dialyzed by dialysis membrane with MWO 10 kDa in Na-buffer overnight at 4°C. Next, CPC was purified by anion exchange chromatography (AEC) on a HiTrap Capto Q column. The column was equilibrated with 0.01 M Na-buffer (pH = 6.5); then the sample was injected into the column. CPC was eluted by increasing the NaCl concentration in the Na-buffer from 0.05 - 0.25 M at a 10 mL/min flow rate. Each 3 mL of fraction was isolated and OD_{280} and OD_{620} were measured. After the first cycle of AEC, the phycocyanin fractions with purity index (PI) > 3 were obtained and were used for the second cycle. After the second cycle, the phycocyanin fraction with PI > 4 was collected. Amicon™ Ultra-15 Centrifugal Filter was used to concentrate purified CPC[36].

Spectroscopic measurements: The C-phycocyanin concentration (C-PC) was calculated as follows: [C-PC] = (OD_{620} - 0.474 x OD_{650})/5.34. The purify index (PI) = OD_{620}/OD_{280} where OD_{620} is the optical density of the sample at 620 nm and OD_{650} is the optical density of the sample at 650 nm[4,20].

Isolation of the mouse vibrissal dermal papilla cells: The 4 to 6-week-old C57BL/6 mice provided by the Institute of Tropical Biology were euthanized by cervical dislocation. The whisker pad was rinsed with 70° ethanol before dissection. The microdissection technique was applied precisely to separate the individual follicles and to remove surrounding tissues such as adipose and dermal tissue. The dermal papillae (DP), obtained by micro scissors, were treated with dispase 2 mg/ml and then transferred to the culture plates. This method is based on Gledhill et al[9] method with some modifications. The primary DP cells were cultured in DMEM/F12 supplemented with 20% FBS, 1% pen/strep at 37°C 5% CO_2. When reaching approximately 90% confluent, the DP cells were detached by Trypsin/EDTA 0.25%. The secondary cells were cultured in DMEM supplemented with 10% FBS, 10 ng/mL FGF-2 and 1% pen/strep (CCM medium). The CCM medium was changed every two days.

Characteristics of murine dermal papilla cells

Immunocytochemistry (ICC) staining with anti-vimentin and anti-α-SMA antibodies: The DP cells at passage 2 were fixed with cold methanol and incubated with 0.1% Triton X-100 to increase cellular permeability. Then, the cells were incubated with an anti-vimentin antibody (ab8979, Abcam) or anti-α anti-SMA antibody (ab7817, Abcam). The samples were then incubated with the secondary antibody (ab150113, Abcam) and incubated with DAPI for 30 minutes before being observed under a fluorescence microscope.

Mouse DP cells spheroid formation by Hanging Drops methodology: The DP cells in passage 3 were obtained by Trypsin/EDTA 0.25% and resuspended in the CCM medium. The cell-containing medium droplets (10^3 cells/40 µl CCM) were created on the inverted dish lids of a 90-mm Petri dish. Then, the lids were carefully turned back into Petri dishes and incubated at 37°C, 5% CO_2 for 3 days to create spheroids. This method was based on Topouzi et al[35] with some modifications. For haematoxylin and eosin (HE) staining, the spheroids were fixed twice in 10% neutral buffered formalin (NBF 10%) for one hour, then embedded in bovine fibrin, sectioned and stained with HE.

Cytotoxicity of CPC: The DP cells were seeded on a 96-well plate with 10^4 cells per well density. After one day of culturing in the CCM medium, the cells were treated by CPC at 0.5 - 2 mg/ml respectively. The cells cultured in the CCM medium were considered the control group. After 48 hours, an MTT assay was performed to assess the cytotoxicity of CPC. The OD values were measured at a wavelength of 590 nm.

Cell proliferation assay: The DP cells, seeded on a 96-well plate with 10^3 cells/well density, were cultured for 10 days in CCM medium containing CPC at concentrations of 0, 5, 10, 20, or 30 µg/ml (the control, P5, P10, P20, or P30 group). Cell proliferation was determined by the MTT method on days 2, 4, 6, 8 and 10 of culture. The OD values were measured at a wavelength of 590 nm.

Testing the ability of CPC to protect DP cells from oxidative stress: The DP cells were seeded on the 24-well plate with a density of $3x10^4$ cells/well. The culture medium containing CPC 5 µg/ml (P5) or 10 µg/ml (P10) was changed next day. Next, the DP cells were treated with H_2O_2 150 µM for 90 mins (the PC/H_2O_2-treated DP cells). After 90 mins, DCFH-DA and Hoechst 3342/PI staining were used to evaluate the total ROS and living/death rate[11,15,34].

Detection of ROS by DCFH-DA staining: DCFH-DA dye (Sigma) was diluted in DMEM to achieve a final concentration of 10 µg/ml. The PC/H_2O_2-treated DP cells were rinsed with PBS 1X and incubated in the 10 µg/ml DCFH-DA dye for 24 hours. Once incubation was complete, the cells were placed in PBS 1X, immediately observed and imaged using a confocal microscope. DCHF-DA can permeate the cells and can be converted to fluorescent DCF. The fluorescent intensity (FI) is correlated with total ROS.

Death rate (%) by Hoechst 3342 and PI staining: The PC/H_2O_2-treated DP cells were seeded on a 96-well plate after being treated with H_2O_2 and stabilized for 24 hours. The cells were washed with PBS 1X and incubated for 1 hour in the working solution of Hoechst 3342 (5 µg/ml) and PI dyes (5 µg/ml) (Abcam). After incubation, cells were stored in PBS for observing and capturing images by confocal microscope immediately. Hoechst 3342 dye exhibits blue fluorescence in all cells' nuclei while PI staining exhibits red fluorescence only in the dead cells.

Testing the ability of CPC to induce proliferation of H_2O_2-induced DP cells: The PC/H_2O_2-treated DP cells were seeded on a 96-well plate and cultured in a CCM medium containing CPC. Groups were listed in table 2. Cell growth and expression of senescence-associated ß-galactosidase were performed to check the capacity of CPC.

Cell growth: MTT assay was performed to test cell growth on days 1 (D1) and 7 (D7). The OD_{590} on D1 and D7 were measured and the ratio OD_{590} D7/OD_{590} D1 was calculated.

Senescence-associated ß-galactosidase (SA-Gal) staining: SA-Gal is a traditional biomarker for cellular senescence[23]. SA-Gal was stained by Senescence Cells Histochemical Staining Kit (Sigma). The staining protocol is according to the manufacturer's instructions. After staining, the cells with a blue color in the cytoplasm are positive.

Results

CPC extraction and purification: As detailed in table 2, the experimental results showed the concentration and purity of C-phycocyanin after purification with activated carbon, ammonium sulfate and AEC. The purity of CPC increased stepwise, underscoring the effectiveness of the purification methods. After two cycles of AEC, the fractions whose PI is higher than 4.0, were collected and concentrated for subsequent experiments on DP cells. The final PI of the CPC solution was 4.11. Moreover, the UV-Vis absorption spectrum of CPC solution showed two major peaks of one λ_1 = 280 nm (the absorption peak of protein) and second λ_2 = 620 nm (the absorption peak of CPC) (Fig. 2). These results showed that analytical grade CPC was purified successfully from *Arthrospira platensis*.

Table 1
Experimental groups in inducing proliferation of The PC/H_2O-treated DP cells

Group	Pre-treatment	H_2O_2 treatment	After H_2O_2 treatment	Test
Control	CCM medium	None	CCM medium	Proliferation, SA-Gal, ROS
P0-0	CCM medium	150 µM for 90 mins	CCM medium	Proliferation, SA-Gal, ROS
P5-5	CCM containing CPC 5 µg/ml for 1 day	150 µM for 90 mins	CCM medium containing CPC 5 µg/ml for 7 days	Proliferation, SA-Gal, ROS
P10-10	CCM containing CPC 10 µg/ml for day	150 µM for 90 mins	CCM medium containing CPC 10 µg/ml for 7 days	Proliferation, SA-Gal, ROS

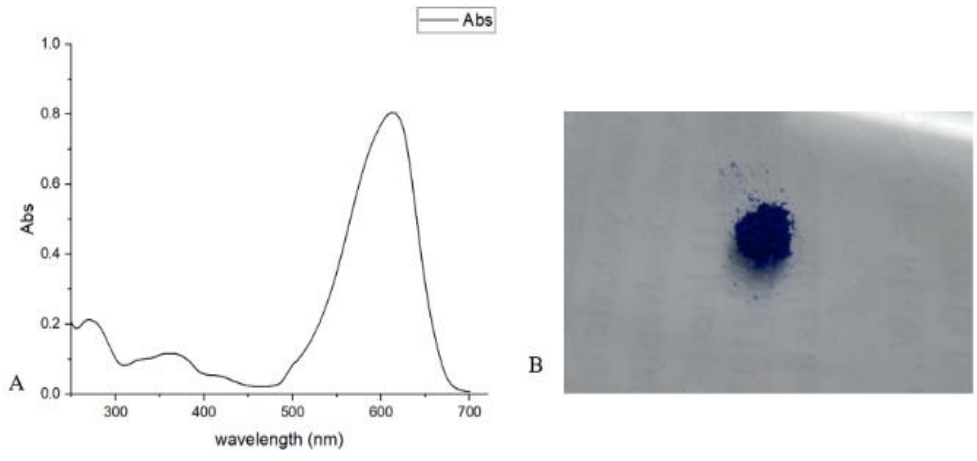

Figure 2: Phycocyanin purification. A: The absorption spectra of the purified phycobiliprotein(c-phycocyanin), B. Freeze-dried pure phycocyanin (>4.0) after anion exchange chromatography.

Isolation of Mouse Dermal Papilla cells: After five days of culture, over 90% of the dermal papillae were attached to the culture dish. The cells were spread out from the DP with an average area of 0.072 ± 0.013 cm² per DP. Fibroblast-like cells are predominant in culture. Their elongated, spindle-like morphology is a vital indicator of the mesenchymal lineage (Fig. 3). Moreover, in some cases, the attached cell moved and aggregated into clusters (Fig. 4A).

The characteristics of murine dermal papilla cells: Alpha-smooth muscle actin (α-SMA) is a marker commonly associated with smooth muscle cells and myofibroblasts and it serves as the marker for actin fibers of the DP cells in 2D culture[13,33]. The result showed that 98.85% ± 1.13% of the cultured cells are positive with the α-SMA antibody. Vimentin is a type III intermediate filament commonly found in mesenchymal cells. Dermal papilla cells belong to mesenchymal origin; the vimentin expression in dermal papilla cells is used as an indicator of their mesenchymal characteristics[24,30] .There are 97.63 ± 2.45% of the cultured cells expressed vimentin.

Mouse Dermal Papilla cells spheroid formation: When cultured in 2D, the important genes for triggering hair follicle development are weakened, so culturing in 3D (spheroid) restores the trichogenic properties of the cells[22,29]. After three days of culture in medium droplets, spheroids were formed successfully with a density of 10^3 cells/spheroid. The cells in individual drops are aggregated into small clusters, connecting to form one spheroid per drop with sizes from 110-130 µm in diameter. The edge surrounding the spheroid mass is visible (Fig. 3A). The H and E staining results showed the DP cells linked together by tight connections and the cells in the outermost layer made a cover. The cluster formation is due to the inherent property of dermal papilla cells to aggregate[3].

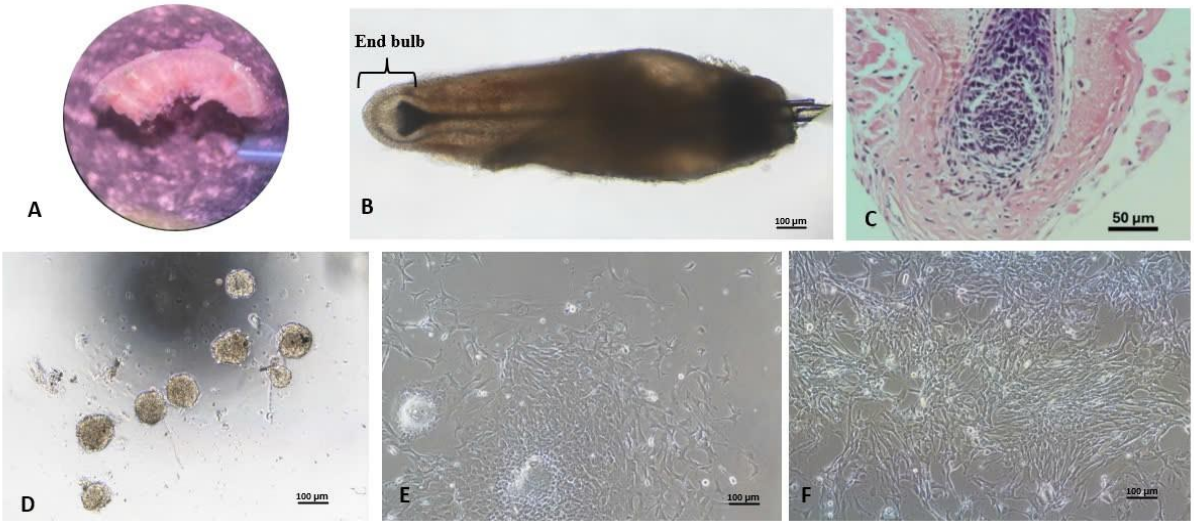

Figure 3: Isolation Dermal Papilla Cells from mouse vibrissa follicle. (A): A row of follicle was separated from whisker pad, (B): A mouse vibrissal hair follicle under microscope (scale 100 µm), (C): Histology of the end bulb part (scale 50 µm), (D): The mouse DPs were separated from the end bulb B (scale 100 µm), (E): DP cells were spread out off the tissue after 4 days of culture (scale 100 µm) and (F): DP cells are proliferating in passage 1 (scale 100 µm).

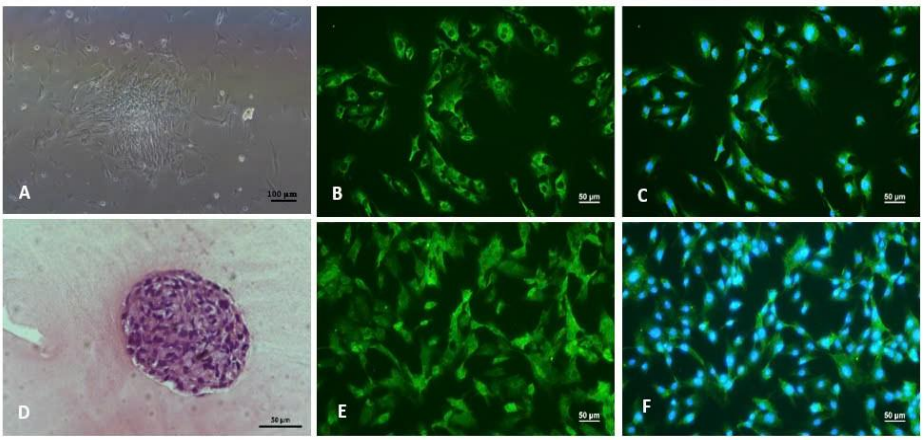

Figure 4: The main characteristics of cultured cell. (A): The cluster formation (scale 100 µm), (B): Expression of vimentin (scale 50 µm), (C): Merged image of vimentin and DAPI staining (scale 50 µm), (D): H and E staining of Dermal papilla cells' spheroid (scale 50 µm), (E): Expression of α-SMA, (F): Merged image of α-SMA and DAPI staining (scale 50 µm).

Table 2
Concentration and PI of CPC extracted from *Spirulina platensis*.

Methods	PI	Concentration (mg/mL)
Activated charcoal	0.91 ± 0.01^{a}	0.843 ± 0.005^{a}
$(NH_4)_2SO_4$	2.35 ± 0.02^{b}	0.575 ± 0.041^{b}
HiTrap Capto Q	4.11 ± 0.06^{c}	0.164 ± 0.005^{c}

($^{a, b, c}$: significant difference within the same column, $p < 0.05$)

Cytotoxicity of CPC: The results showed that the death rate increased when the concentration rose from 1 mg/ml to 2 mg/ml and the IC50 value for CPC was determined to be 1.445 mg/ml. According to the American National Cancer Institute (NCI), the extract is toxic for normal cells if its IC50 is lower than 10 µM after 2-3 days of exposure[5]. So, CPC is non-toxic for the DP cells.

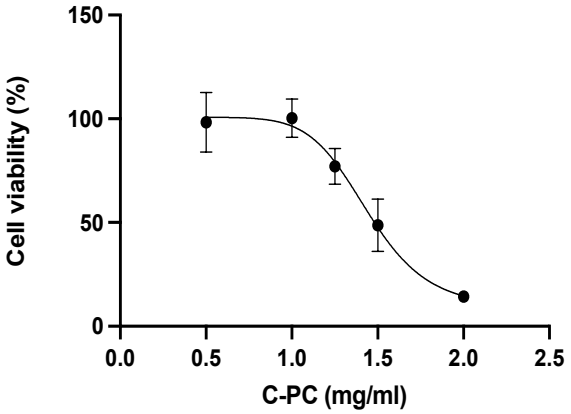

Figure 5: The percentage of viable DP cells in cytotoxicity assay

Proliferation assay: A CPC concentration range of 5 - 30 µg/ml was investigated. On day 8, the P5 and P10 groups exhibited significantly higher OD values (1.030 ± 0.027, 1.003 ± 0.065) compared to the remaining groups such as the control, P20 and P 30 group (0.695 ± 0.085, 0.690 ± 0.058, 0.695 ± 0.042). These results suggested that CPC at the 20 – 30 µg/ml concentration did not affect cell growth, while 5 and 10 µg/ml could enhance dermal papilla cell proliferation.

The ability of CPC to protect DP cells from oxidative stress: These experiments are designed to highlight the protective role of CPC at concentrations of 5 and 10 µg/ml against H_2O_2-induced damage (Fig. 7). The cells in the P0 groups (the cells were only exposed to H_2O_2) exhibited significantly higher cell death rates (18 ± 1.3%) than the control group (3.850 ± 0.35%). The cell death rate in the P0 group (18 ± 1.3%) was 1.7 times higher than in the P5 group (10.5 ± 0.5%) and over 15 times higher than in the P10 group (1.2 ± 0.6%). These results underscore the protective potential of CPC.

Total ROS by DCFH-DA staining: Fluorescence intensity (FI) per cell analysis revealed a significantly higher intensity in the PC0-0 group (1.011 ± 0.106) compared to the control group (0.423 ± 0.041) (Fig. 8). The PC0-0 group exhibited a significantly higher fluorescent intensity than the P5-5 group (0.428 ±0.002) and the P10-10 group (0.432 ± 0.002). In contrast, the fluorescence ratio of the PC10-10 (0.432 ± 0.002) and P5-5 (0.428 ±0.002) group did not differ significantly from the control group.

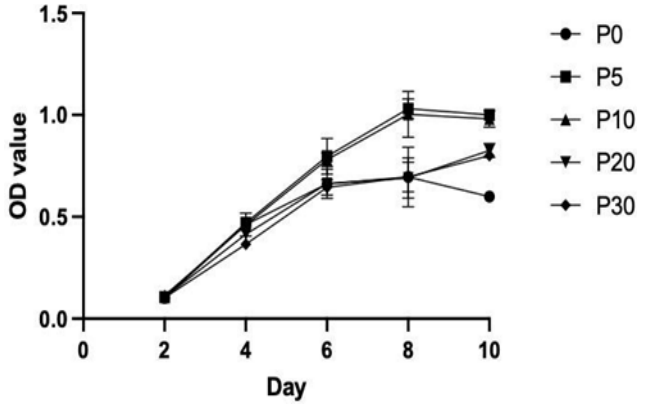

Figure 6: The growth curve of DP cells in CCM medium containing CPC

The ability of CPC to induce proliferation of H_2O_2-induced DP cells

Cell growth: The bar chart illustrated the ratio of OD value after H_2O_2 exposure (Fig. 10). After the exposing period, the cells were cultured continuously for the next seven days to evaluate the proliferation rate of each group by dividing the OD_{590} value of day 7 by those of day 1. The results indicated a significant increase in the ratio of the P5 and P10 groups, which was about twice as high as the data for the control group. In contrast, the ratio of the P0-0 group was maintained at about 1. Moreover, there are some alterations in cellular morphology in the P0-0 group. The cells are enlarged and flatter, with a fragmented cytoplasm and a larger nucleus. This condition is rarely observed in the P5-5 and P10-10 groups (Fig. 9). The results showed that CPC 5-10 µg/ml induced cell replication and suppressed the morphological changes.

SA-gal expression: The bar chart illustrated that the percentage of the SA-Gal-positive cells in P0-0 is highest in the experimental groups (73.32 ± 10.12%) (Fig. 11). The SA-Gal-positive percentages were ranked in descending order as follows: P5-5 (52.80 ± 6.41%), P10-10 (45.10 ± 7.86%) and control (6.46 ± 2.46%). The results showed that 5-10 µg/ml of CPC inhibited SA-Gal express ion, suggesting a potential role of CPC in regulating cell senescence.

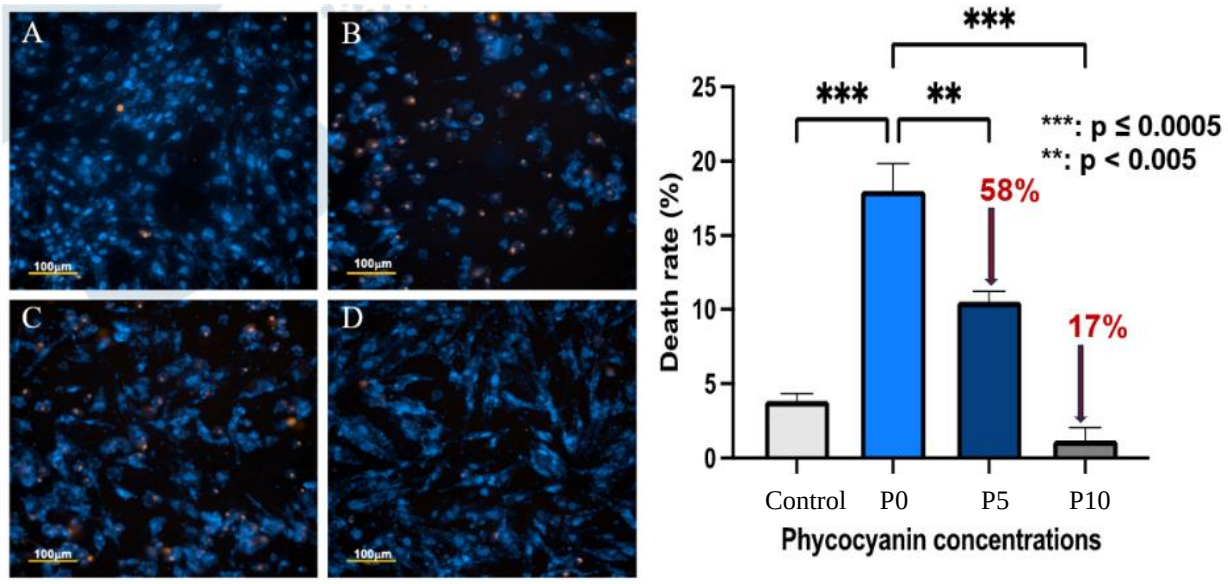

Figure 7: The results of cell death ratio of CPC/H_2O_2-treated DP cells. Left: The cells were stained by Hoechst 3342 and PI (scale 100 μm). A: Control, B: P0, C: P5, D: P10. Right: the bar chart of death ratio.

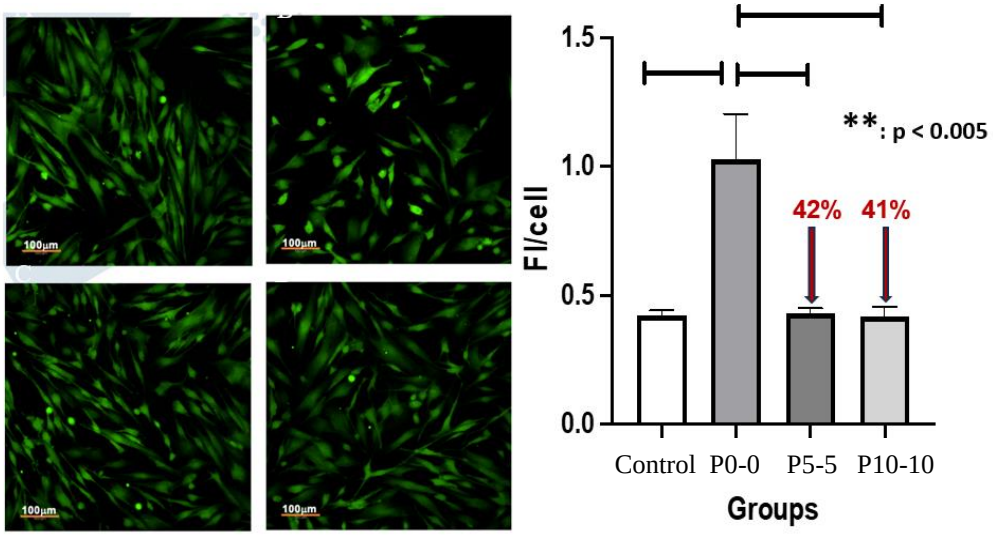

Figure 8: The result of total ROS of CPC/H_2O_2-treated DP cells. Left: DCHF-DA staining (scale 100 μm). A: Control, B: P0-0, C: P5-5, D: P10-10. RightL the bar chart.

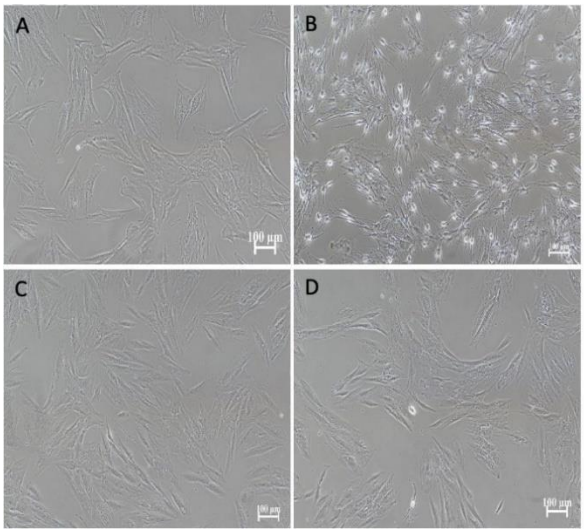

Figure 9: The cell morphological changes of DP cells exposed to H_2O_2. A: Control, B: P0-0, C: P5-5, D: P10-10.

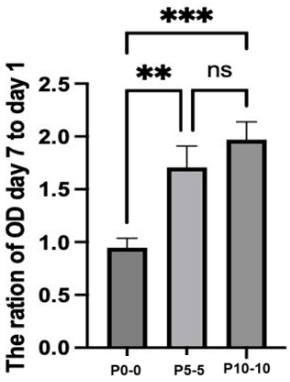

Figure 10: The ratio of OD_{590} D7/ OD_{590} D1 result of the PC cells.

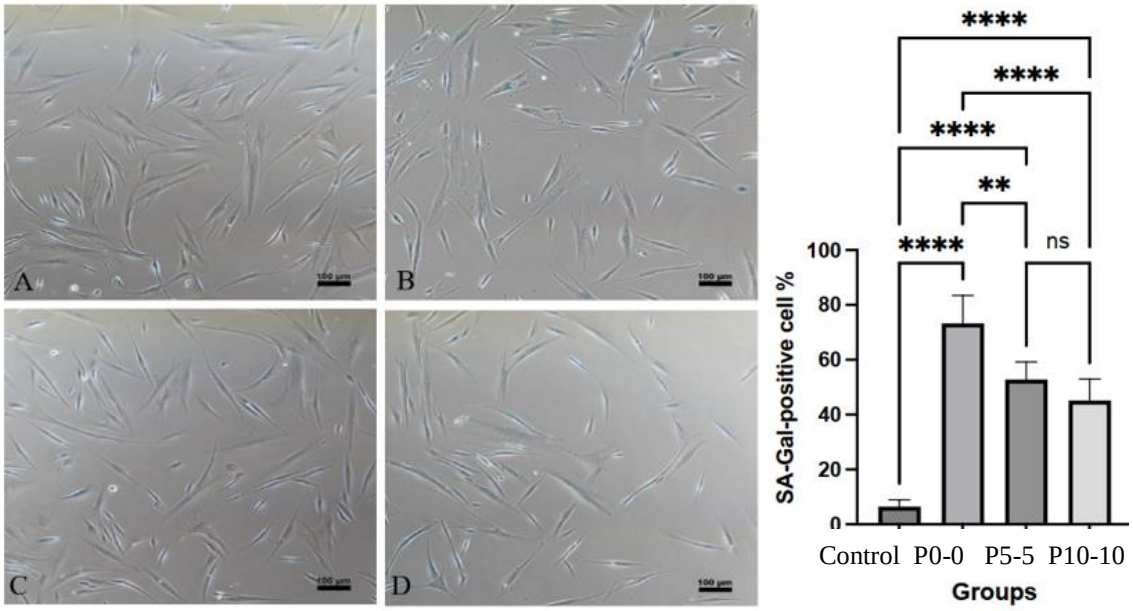

Figure 11: The results of SA-Gal expression in the experimental groups. Left: A. control, B. H_2O_2, C. P5-5, D. P10-10, Right: the result of SA-Gal-positive cell %.

Discussion

Mouse dermal papilla cells were isolated from vibrissa in anagen phase. In the CCM medium supplemented with 10 ng/ml FGF, murine DP cells have some characteristics similar to human DP cells[19]. The DP cell is sensitive to oxidative stress and rapidly loses its hair inductive characteristics. Hydrogen peroxide (H_2O_2) is a potential ROS. It can permeate the cell membrane and can destroy cellular molecules such as DNA, protein and lipid leading to cell senescence and death[7]. The hallmarks of cell senescence are cell growth arrest, expression of SA-Gal, enlargement of cell shape and cell nucleus[7,28]. The results showed that the treatment of 150 μM H_2O_2 for 90 min caused an increase in total ROS (DCFH-DA staining) and cell death (Hoechst/PI staining), cell senescence (cell growth arrest, morphological alternation and expression of SA-Gal).

Phycocyanin (CPC) is an antioxidant protein that can induce fibroblast and keratinocyte proliferation and wound healing[2]. Moreover, crude CPC is sold abundantly in the market in functional food and cosmetics. So, in this study, we checked the ability of CPC to activate DP cell proliferation, to protect DP cells from H_2O_2 and to trigger cell growth after damage. CPC was extracted from spirulina algae and was purified by many steps: filtered by active coal, precipitated by AS and purified by AEC. The purity increased stepwise. The actived coal is a porous structure absorbing cell fragments AS with optimal concentration (35% for the first time and 30% for the second time), can precipitate CPC from other proteins such as allophycocyanin (APC) and phycoerythrin (PE). Hitrap Q is a strong anion exchange with high resolution. In the pH = 6.5 solutions, CPC is an anionic substance that will attach to the column. NaCl 0.15-0.175 is the optimal concentration to elute and isolate CPC[1,32].

The results showed that CPC can stimulate DP cell growth, a key characteristic to maintain hair follicle development. Moreover, CPC can protect DP from oxidative stress. DP cells were pre-treated with CPC 24 hours prior to treatment with H_2O_2. After treatment, the total ROS and death rate increased in the H_2O_2 group but decreased in P5 and P10 (significant statistics). After H_2O_2 treatment, the DP cells were seeded into a 96-well plate and cultured in CPC 5 or 10

µg/ml for 7 days. The cells in the P5 and P10 group continuously replicated to day 7 (OD_{590} on day 7 is about 2-fold than those on day 1), cell shape was retained and SA-Gal expression is weaker than control. The CPC concentration of 10 µg/ml was better than 5 µg/ml in protecting and stimulating DP cell growth.

Conclusion

CPC at the concentration of 10 µg/ml enhanced the proliferation of dermal papilla cells. CPC 10 µg/ml protected cells against H_2O_2-induced damage and stimulated cell replication after H_2O_2 treatment. This study suggested that CPC has significant potential for encouraging hair follicle development.

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