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EVALUATION OF THE PROTECTION OF ASTAXANTHIN ON HUMAN HAIR FOLLICLE PAPILLA CELLS AGAINST THE AGING EFFECTS TRIGGERED BY HYDROGEN PEROXIDE

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ABSTRACT

Astaxanthin (ASX) is a powerful antioxidant with high efficacy in inhibiting reactive oxygen species (ROS), one of the most harmful factors leading to hair loss. Our research specifically evaluated the ability of ASX to protect human dermal papillae cells from hydrogen peroxide (H_2O_2). In this study, the human dermal papillae (hDP) cells were successfully isolated. The cells expressed DP cell markers in the 2D model, including alkaline phosphatase, alpha-smooth muscle actin (98.66 ± 0.02%), and vimentin (98.88 ± 0.02%), cluster aggregation and spheroid formation in the 3D model. The hDP cells (P3-P5) were pretreated with ASX and subsequently treated with H_2O_2 at a concentration of 550 µM for 4 hours. After 4 days of treatment, the percentage of cells expressing the marker b-galactosidase decreased from 87 ± 0.01% in the control group to 27.61 ± 0.01% in the ASX-treated group (1 µg/mL). Our results indicated that astaxanthin effectively protects against oxidative stress in dermal papillae cells, suggesting its potential application in hair loss treatment due to ROS.

Keywords: Anti-aging, Astaxanthin (ASX), Dermal papillae, Oxidative Stress.

INTRODUCTION

The hair follicle is a complex structure composed of a matrix, inner root sheath (IRS), outer root sheath (ORS), and associated structures such as dermal papilla (DP) and connective tissue sheath (Bakry et

al., 2014). The dermal papilla (DP) is a population of mesenchymal cells at the base of the hair follicle that regulates hair cycle phases (anagen, catagen, and telogen) and hair follicle growth by secreting nutrients and growth factors such as FGF-7 and IGF-1. So, cultured DP cells are often used as the cellular model to test the ability of reagents to stimulate hair growth or protect hair structure. Among some harmful reagents for hair, reactive oxygen species (ROS) are one of the main causes that impair DP function and lead to hair loss, including UV irradiation, hair dye ingredients, and pollutants, etc.(Yang & Cotsarelis, 2010). Exposure to high levels of reactive oxygen species (ROS) can give rise to DP cell senescence and death, and consequently, the hair follicle is miniaturized and lost. The condition takes place in alopecia areata (patchy hair loss) and androgenetic alopecia (male pattern baldness associated with androgen hormones).

Many antioxidants (vitamins A, C, and E, selenium, polyphenols, and niacinamide) are incorporated into hair-stimulating products to reduce hair loss. Astaxanthin (ASX) (3,3-dihydroxy-β, β-carotene-4,4-dione), a xanthophyll carotenoid, is one of the most potent natural antioxidants derived from various marine organisms, particularly the microalga *Haematococcus pluvialis* (Shah et al., 2016). ASX includes two β-ionone rings at its ends containing hydroxyl and keto moieties and an alternating double-bond polyene chain. The unique structure is responsible for its effective antioxidant properties and ability to interact with cell membranes. The polyene chain traps free radicals within the cell membrane, while the β-ionone rings can effectively scavenge free radicals inside and outside the cell membrane (Miki, 1991). In the pharmaceutical field, astaxanthin can be used to against oxidative stress, photo-aging, inflammation, cardiovascular diseases, and diabetes (Dong *et al*., 2014; Zou *et al*., 2013). ASX exhibits higher antioxidant activity than β-carotene and vitamin E, but the effect of ASX on hair protection has not been widely studied. This study examined the protective ability of astaxanthin extracted from *H. pluvialis* against the harmful effects of hydrogen peroxide-induced oxidative stress on human hair follicle dermal papilla cells. ASX was evaluated for the first time on an *in vitro* model of human dermal papilla cells to assess its potential for countering oxidative stress. This research is the cornerstone for developing hair-stimulating products containing ASX.

MATERIALS AND METHODS

Collection and cultivation of human hair follicle dermal papilla cells

Hair follicles were obtained from the scalps of male patients who underwent Follicle Unit Extraction (FUE) hair transplantation surgery. Collecting hair samples was implemented after obtaining written consent from the patients. The hair follicles were stored in DMEM/F12 at 4^{o}C and transported immediately to the laboratory. The cell isolation and cultivation were carried out based on Messenger AG's research (Messenger, 1984). Briefly, dermal papilla (DP) tissues were harvested from the base of the hair follicle by micro-dissection technique. Then, the DP tissue was digested by collagenase enzyme type IV for 30 minutes and cultured in DMEM/F12 medium (Biological Industries) supplemented with penicillin (100 U/mL), streptomycin (100 mg/mL), and 20% heat-inactivated fetal bovine serum (FBS –

Sigma-Aldrich) in a humidified environment with 5% CO_2 at 37°C. After reaching 80 - 90% confluency, cultured DP cells were sub-cultured into other culture flasks.

3D Suspension culture

3D culture was established by suspending cells in droplets to maintain the hair-inductive capacity of dermal papilla (DP) cells. DP cells were cultured in the medium drops placed on the underside of petri dish lids to form cellular spheroids (McElwee et al., 2003). DP cells were rinsed with PBS and treated with 0.25% trypsin-EDTA for 4 minutes, using DMEM/F12 supplemented with 10% FBS. After centrifugation, cells were seeded into medium drops on the inverted lid of a 90-cm petri dish at a density of $5x10^3$ cells/drop. Next, the lids were inverted to cover the bottom dish containing PBS and incubated at 37ºC, 5% CO_2 for two days.

Immunocytochemistry (ICC) staining with vimentin, αSMA, and alkaline phosphatase antibodies to assess DP cell characteristics

The alkaline phosphatase (ALP) activity has been utilized as a marker to detect the presence of DP and considered as an indicator of hair-inductive potential (Topouzi et al., 2017). The cells were washed with PBS and incubated with an alkaline phosphatase staining solution (85L1-1KT, Sigma-Aldrich) before being observed under a microscope.

In immunocytochemistry (ICC) staining, specific antibodies were employed to determine the characteristics of DP cells, namely Vimentin (ab8979, Abcam), a fibrous protein found abundantly in mesenchymal-origin cells, and α-Smooth muscle actin (αSMA, ab7817, Abcam), a marker for dermal papilla cells cultured in an *in vitro* environment.

Extraction of astaxanthin

Haematococcus pluvialis, containing ASX, was freeze-dried and stored at -20°C until use. After thawing, 0.5 g of algae was added to 15 mL of a solvent mixture (Dichloromethane: Methanol 1:3 (v/v)). The mixture was homogenized with a homogenizer at 10,000 rpm for 10 minutes, then centrifuged at 4000 rpm for 2 minutes to collect the supernatant. The pellet fraction was mixed with 15 mL of the same solvent mixture and homogenized for 10 minutes at 10,000 rpm. The extraction was repeated three more times to collect the entire extract. After centrifugating 60 mL of the extracted solution at 4000 rpm for 10 minutes, the mixture was filtered through a 0.22-micrometer nylon filter membrane to remove algal debris. The obtained ASX-containing liquid was evaporated completely before diluting in dimethyl sulfoxide (DMSO) with a concentration of 2000 µg/mL for subsequent use.

Evaluation of the effects of ASX toxicity on DP cells

DP cells were seeded into a 96-well plate and cultured in a new medium containing different concentrations of ASX (0, 0.1, 0.5, 1, 1.5, and 2 µg/mL). Cell toxicity of ASX was assessed by the formation of formazan crystals using the MTT assay (M6494, Thermo Fisher Scientific) to determine the number of viable cells through the optical density (OD) value at 570 nm. The experiments were performed in triplicate.

MTT assay for *In Vitro* proliferation assessment of DP cells

DP cells were seeded in a 96-well plate at a density of $2x10^3$ cells per well. The medium was removed after 24 hours and replaced with fresh medium containing various concentrations of ASX (0, 0.25, 0.5, and 1 µg/mL) (n = 3). The experiment was conducted for five days. At each time point (1-, 3-, 5-, and -8 days post-culturing), the cell proliferation was assessed by the formation of the MTT assay (M6494, Thermo Fisher Scientific). The assay qualitatively assessed viable cells through the optical density (OD) measurement at 570 nm.

Wound healing assay for *in vitro* evaluation of DP cell migration stimulated by ASX

The DP cell migration was assessed by the wound healing assay (Jonkman et al., 2014). The DP cells were seeded into a 24-well plate at a density of $5x10^4$ cells per well and cultured for 24 hours. After 24 hours, a tool used to create a wound in the cell monolayer is approximately 1 mm wide and a culture medium containing various concentrations of ASX (0, 0.25, 0.5, and 1µg/mL) was added subsequently. Cell migration into the wound was observed over 18 hours by a microscope.

Evaluation of ASX's protective effect on DP cells against H_2O_2-Induced oxidative Stress

The DP cells (P3 – P5) were seeded into the 24-well plate at a density of $5x10^4$ cells per well. After 24 hours, the wells were pre-treated with ASX (0, 0.25, 0.5, and 1µg/mL) for one day and subsequently treated with cell culture medium supplemented by H_2O_2 at the final concentration of 550 µM for 4 hours (Bae et al., 2014; Kim et al., 2014). The cell viability was determined by Trypan Blue staining. After five days of culture, a senescent marker, senescence-associated β-galactosidase, was assessed by a specific staining method.

Statistical analysis

The illustration and data in the study were designed and reported as mean ± standard deviation (SD) by the Excel program and the GraphPad Prism software using a 2-sample t-test. The p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Isolation of human dermal papilla cells (hDP)

As depicted in Figure 1A, the dermal papilla is on the lower region of the hair follicle. The dermal papilla tissues obtained through surgical dissection were cultured on a 4-well dish with sizes ranging from 100 – 200 µm (Figure 1B). After 3-5 days of cultivation (Figure 1C), dermal papilla cells began to expand from tissue and proliferate. After ten days, DP cells covered approximately 90% of cell culture plates, while the original dermal papilla tissues gradually diminished. Continuous cultivation increased cell proliferation, and the cell size became larger compared to cells from the newly formed tissue samples.

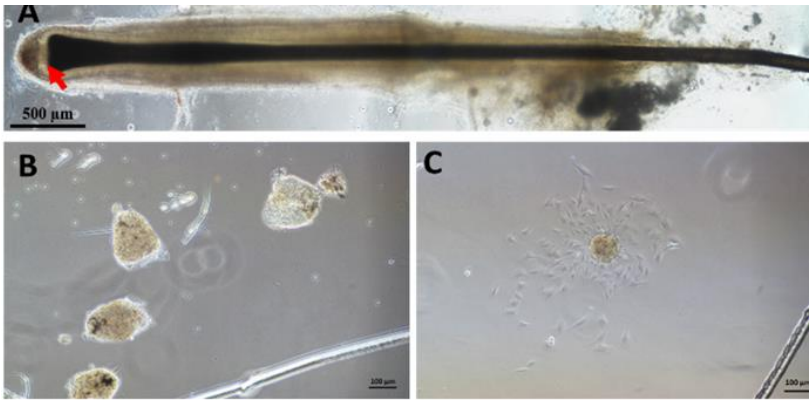

Figure 1. Human dermal papilla (DP) cells. (A) Human hair follicles harvested in vitro (red arrows indicate the hair bulb region). (B) collected DP tissues. (C) hDP migrated from the tissue after five days.

During continuous 2D cultivation, cells naturally formed clusters with an average size ranging from 100 – 300 µm (Figure 2A). However, a limitation of human dermal papilla cells in 2D *in vitro* culture is the loss of hair-inductive properties during the process (Rendl et al., 2008). When DP cells were grown in a 3D spheroid structure, they regained some signs of intact gene expression and inductive properties (Betriu et al., 2020). Cells were cultured in the hanging drops on the lid of a petri dish with a density of $5x10^3$ cells/ droplet. Cell clusters formed spheroids within 24 hours and remained viable for 48 hours of cultivation (Figure 2B). The results indicated that isolated cells retain the characteristics of DP cells with inductive properties and can form tissue-like structures at a low density of $5x10^3$ cells/droplet, which provides a foundation for hair follicle regeneration studies.

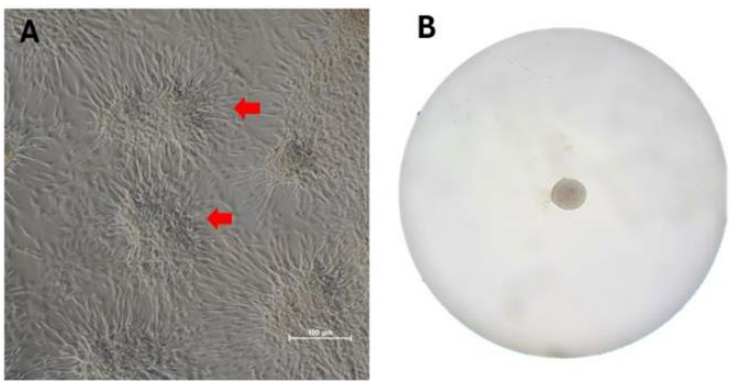

Figure 2. Cell aggregation in 2D and 3D culture. (A) Many cells aggregate to form the clusters in 2D culture. (B) The DP cells in 3D culture condition grow into 3D spheroidal pattern in 2 days (diameter is about 100–200 nm).

DP cells exhibit alkaline phosphatase expression

Alkaline Phosphatase (ALP) is considered an indicator of the inductive potential of DP cells (Iida et al., 2007; Topouzi et al., 2017). The DP cells exhibit strong ALP expression in the early anagen phase, which tends to decrease over time (Oh et al., 2016). In *in vitro* cultivation, ALP expression also decreased through passages, as several studies have reported a gradual loss of inductive potential in both DP and dermal sheath (DS) cells (Yuan & Chen, 1999). After staining, approximately 30.67 ± 0.13% of DP cells exhibited a blue color, which indicated the presence of ALP markers. Although not all cells expressed ALP due to the diminishing inductive potential, this method identified cells with inductive characteristics, confirming the isolated cells as DP cells (Figure 3).

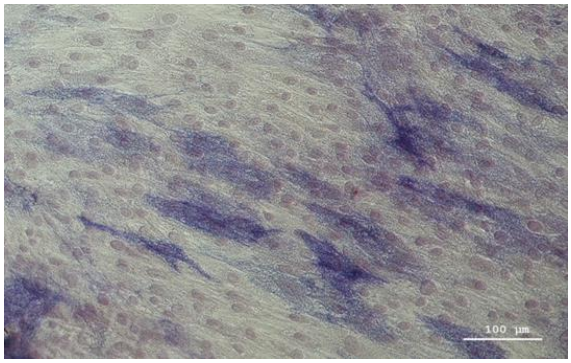

Figure 3. DP cells express ALP markers (stained purple) (20X). ALP staining positivity is observed scattered within cell areas interspersed with some sparsely negative cell areas.

Vimentin and α-Smooth muscle actin expression

The α-SMA expression is a marker of dermal papilla cells cultured *in vitro* but not *in vivo* (Jonkman et al., 2014). Actin fibers within 2D cultured DP cells were labeled with anti-αSMA antibodies and fluorescently stained with green fluorescence, with a proportion exceeding 98.66 ± 0.02% (Figure 4). The results align with previous studies that identified the isolated cells as having characteristics consistent with DP cells.

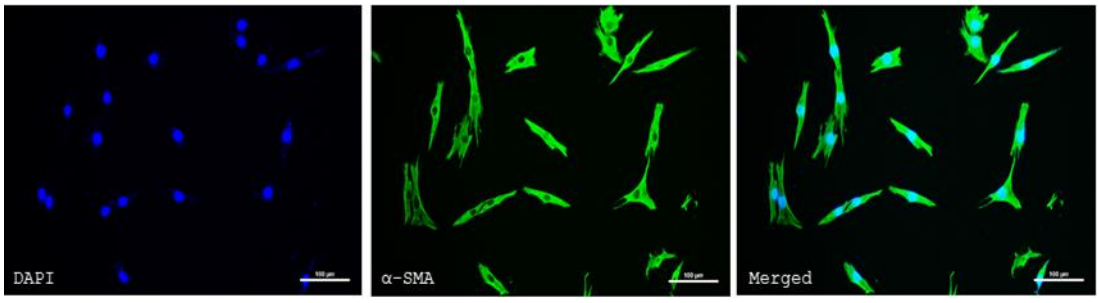

Figure 4. α-SMA marker expression of cultured hDP (20X). The DP cells were stained with DAPI (DAPI) and anti- α-SMA antiboy (α-SMA). Merged: combination of DAPI and α-SMA

Vimentin is a fibrous protein commonly found in mesenchymal cells. Following immunostaining with anti-vimentin antibodies, over 98.88 ± 0.02% of the isolated DP cells expressed the vimentin marker, as evidenced by the pairing with green fluorescently labeled antibodies observed under fluorescent microscopy (Figure 5).

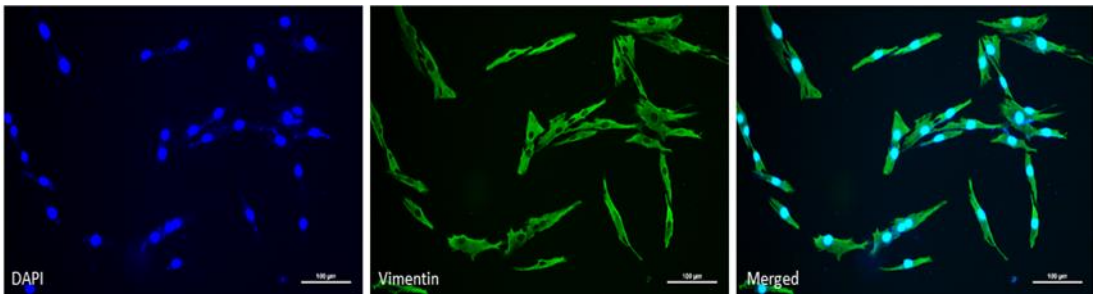

Figure 5. Vimentin marker expression of culuted hDP (20X). The DP cells were stained with DAPI (DAPI) and anti- vimentin antiboy (Vimentin). Merged: combination of DAPI and Vimentin

Based on staining results, it is evident that the isolated cells expressed the characteristic proteins α-SMA, vimentin, and ALP of DP cells. The observed variability in ALP enzyme expression is causative to the gradual loss of inductive potential during the cultivation process.

Astaxanthin (ASX) extraction from *H. pluvialis*

ASX presenting in the algae membrane contributes to the red-orange color of *H. pluvialis* algae (Figure 6A). ASX was released after extraction as the result of the algae transparency (Figure 6B). The average recovery rate of ASX was around 90%. The ASX extracted solution was dissolved in DMSO and diluted to assess its impact on cells.

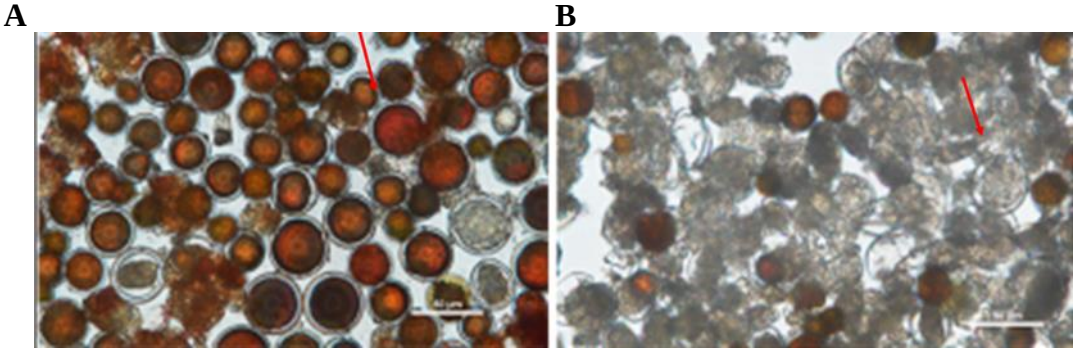

Figure 6. Biomass of *H. pluvialis* algae. (A): Biomass before extraction, (B): Algal residue after extraction, rate is approximately 90%.

The effects of ASX toxicity on DP cells

The toxicity assessment of ASX illustrated in Chart 1, showed that at a concentration of 1.5 - 2 µg/mL, the group exhibited the most significant cell impact, with the cell viability rate approximately 92.81 ± 0.04% and 84.91 ± 0.04%, and ASX at concentrations ranging

from 0.1 to 2 µg/mL ($\alpha > 0.05$) does not induce cellular toxicity. In this study, to ensure optimal and consistent effects, the ASX concentration for investigation was selected within the range from 0.25 to 1 µg/mL.

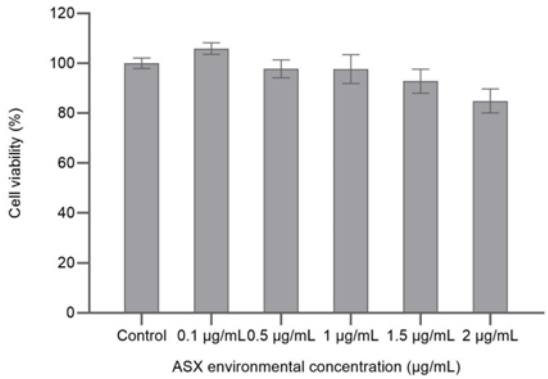

Chart 1. Toxicity results of ASX on DP cells (µg/mL). Concentrations ranging from 0.1 to 1 µg/mL show no difference compared to the control sample. Concentrations of 1.5 and 2 µg/mL exhibit lower cell viability rates of92.81% and 84.91%, respectively.

The effects of ASX on DP's proliferation

DP cells were cultured in medium containing different concentrations of ASX (0, 0.25, 0.5, and 1 µg/mL) for eight days. Overall, the hDF cells exhibited faster growth in the ASX-containing medium compared to those in the control medium. At ASX concentrations of 1 µg/mL, cells reached the peak of OD asorption on the 5th day of cultivation and were higher than other ASX concentrations. The cell density on day 5th was about three to four times as high as the control group. The results demonstrated that ASX concentrations of 1 µg/mL not only did not induce toxicity but also stimulated the proliferation of DP cells (Chart 2).

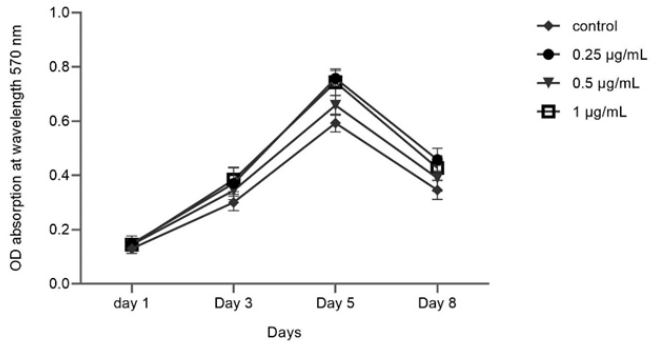

Chart 2. Results of DP proliferation ability in ASX-containing environment (µg/mL). The growth rate of DP cells peaks at day 5 across all concentrations. ASX 0.25 and 1 µg/mL exhibit the highest peaks, while the control group shows the lowest peak.

The effects of ASX on migration-stimulating ability of DP cells

After creating the scratch, DP cells migrated into the scratch and covered approximately

80% of the scratch area after 18 hours. Cells cultured in the medium containing various concentrations of ASX (0, 0.25, 0.5, and 1 µg/mL) showed equivalent migration with no significant difference, which indicated that ASX did not significantly impact the migration of cells (p = 0.009) (Figure 7, Chart 3).

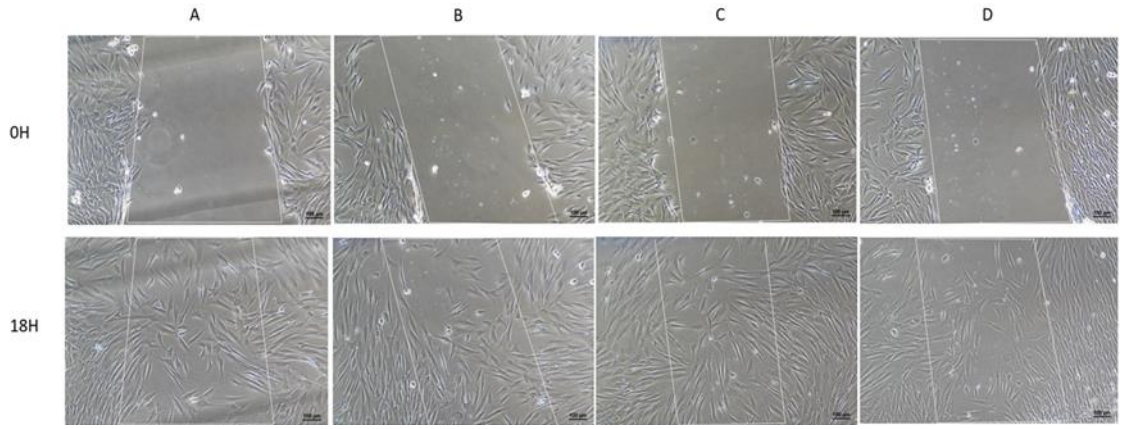

Figure 7. Migration of DP cells (A) control; (B) with ASX 0.25 µg/mL; (C) with ASX 0.5 µg/mL; (D) with ASX 1µg/mL. The migration was equivalent, ASX did not significantly impact the migration of cells. (p= 0.009<0.05)

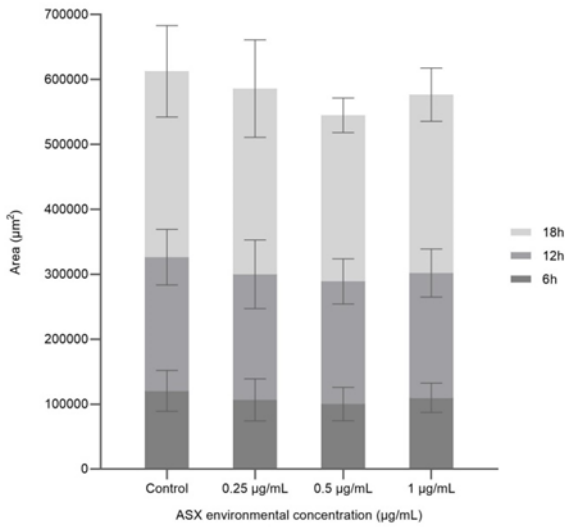

Chart 3. Results of DP cell migration area (µm^2) in ASX-containing environment (µg/mL). The migration area of cells at ASX concentrations of 0.25 and 1 µg/mL is the highest. However, there is no statistical difference compared to the remaining concentrations

Evaluation of ASX's protective ability on DP cells against oxidative stress - H_2O_2

The results presented in Table 1 and Figure 8 displayed that H_2O_2 deleteriously influenced DP cells, leading to cell death,

and detaching after 4 hours of exposure to H_2O_2 at a concentration of 550 µM. The cells became swelling and polygonal in shape, and cell nucleus areas increased after additional cultivation for five days. The results of β-galactosidase staining, a specific marker for long-term aging, showed that the hDF cells in all groups expressed galactosidase with different levels (Figure 8, Table 1), which were highest in the control group (87.12 ± 0.01%) and decreased in the ASX groups (from 31.79 ± 0.04 to 27.61 ± 0.01%). The higher cell death rate and galactosidase expression were observed in the control group without the antioxidant agent compared to cultured cells in an environment with ASX. The highest resistance of DP cells to free radicals was strengthened by ASX at the concentration of 1 µg/mL, with the lowest cell death rate and expression of aging markers (27.61 ± 0.01%).

Table 1: Results of the analysis of the antioxidant capacity of ASX

	Cell survival rate/total cell count (%)	**Percentage of cells positive for β-Gal (%)**
Control	86.59 ± 0.02	87.12 ± 0.01
ASX 0.25 µg/mL	89.27 ± 0.06	31.79 ± 0.04
ASX 0.5 µg/mL	82.70 ± 0.14	29.36 ± 0.03
ASX 1 µg/mL	87.77 ± 0.03	27.61 ± 0.01

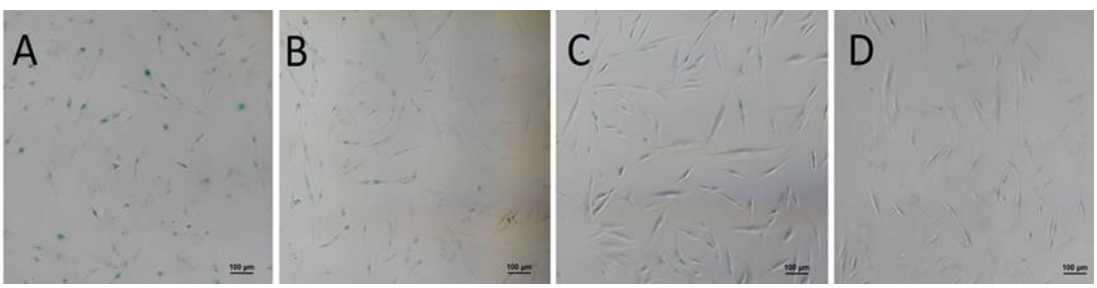

Figure 8: Expression of β-Galactosidase in DP after exposure to H_2O_2. A: the hDF cells cultured in the control group showed a relatively high positive rate (87.12 ± 0,01%). B: ASX 0.25 µg/mL significantly reduces the positive rate compared to the control group (31.79 ± 0,04%); C: ASX 0.5 µg/mL has a positive rate of 29.36 ± 0,03%; D: ASX 1µg/mL has the lowest positive rate at 27.61 ± 0,01%

CONCLUSION

This study represents the first trial of ASX on human hair follicle cells in Vietnam. The results demonstrated that ASX is a potent antioxidant agent that does not induce cytotoxicity but stimulates cell proliferation, with the highest protective activity in ASX concentrations extending from 0.25 to 1 µg/mL. Higher ASX concentrations extending from 1.5 - 2 µg/mL potentially influence cell migration and proliferation. One µg/mL of ASX is the optimal concentration to protect DP cells from the oxidative stress induced by H_2O_2. To further explore treatment options for hair loss, hair follicle cells have been successfully isolated and cultured using tissue culture techniques

with other characteristics of DP cells, namely expressing vimentin, α-SMA, ALP markers, and capable of self-aggregating naturally on 2D culture dishes or forming spheroids in a 3D state.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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