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The Effect of Tripeptide-Copper Complex on Human Hair Growth *In Vitro*

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The tripeptide-copper complex, described as a growth factor for various kinds of differentiated cells, stimulates the proliferation of dermal fibroblasts and elevates the production of vascular endothelial growth factor, but decreased the secretion of transforming growth factor-β1 by dermal fibroblasts. Dermal papilla cells (DPCs) are specialized fibroblasts, which are important in the morphogenesis and growth of hair follicles. In the present study, the effects of L-alanyl-L-histidyl-L-lysine-Cu^{2+} (AHK-Cu) on human hair growth *ex vivo* and cultured dermal papilla cells were evaluated. AHK-Cu (10^{-12}~10^{-9} M) stimulated the elongation of human hair follicles *ex vivo* and the proliferation of DPCs *in vitro*. Annexin V-fluorescein isothiocyanate/propidium iodide labeling and flow cytometric analysis showed that 10^{-9} M AHK-Cu reduced the number of apoptotic DPCs, but this decrease was not statistically significant. The ratio of Bcl-2/Bax was elevated, and the levels of the cleaved forms of caspase-3 and PARP were reduced by treatment with 10^{-9} M AHK-Cu. The present study proposed that AHK-Cu promotes the growth of human hair follicles, and this stimulatory effect may occur due to stimulation of the proliferation and the preclusion of the apoptosis of DPCs.

Key words: Hair follicle growth, Copper complex, Apoptosis, Dermal papilla cells

INTRODUCTION

Glycyl-L-histidyl-L-lysine, a tripeptide with affinity for copper (II) ions, was initially isolated from human plasma, and has been described as a growth factor for a variety of differentiated cells and a modulator of the extracellular matrix (Maquart *et al.*, 1993). Glycyl-L-histidyl-L-lysine-Cu^{2+} (GHK-Cu) stimulates the proliferation of dermal fibroblasts and elevates the production of vascular endothelial growth factor (VEGF) by dermal fibroblasts (Pollard *et al.*, 2005). However, GHK-Cu decreased the secretion of transforming growth factor-β1 (TGF-β1) from dermal fibroblasts (McCormack *et al.*, 2001). Interestingly, VEGF promoted hair growth and increased hair follicle and hair size by improving the vascularity around the hair follicle (Yano *et al.*, 2001), but androgen-inducible TGF-β1 derived from dermal papilla cells (DPCs) of androgenetic alopecia was involved in the suppression of epithelial cell growth (Inui *et al.*, 2003). From these reports, it was our supposition that the tripeptide-copper complex could have a stimulating effect on hair growth.

Dermal papilla (DP) consists of a discrete population of specialized fibroblasts, which are important in the morphogenesis of hair follicles in the embryo and in the control of the hair growth cycle in adults (Matsuzaki and Yoshizato, 1998; Jahoda *et al.*, 1993). Dermal papilla cells (DPCs) are thought to play these pivotal roles in hair formation, growth and cycling through the interaction with follicular epithelial cells (Krugluger, 2005). Recently, Han *et al.* studied the hair growth promoting effect of minoxidil using cultured DPCs *in vitro,* and reported that the stimulatory effect of minoxidil on hair growth may occur via its proliferative and anti-apoptotic influence on DPCs.

This study was performed to determine whether AHK-Cu affects hair growth, and elucidate the mode of action of AHK-Cu by investigating its effect on the proliferation and apoptosis of DPCs.

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MATERIALS AND METHODS

Drugs and reagents

AHK-Cu (11% stock solution in water) was obtained from Procyte Co. (Redmond, WA, U.S.A.), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) from Sigma (St Louis, MO, U.S.A.), and Annexin V-FITC from BD PharMingen (San Diego, CA, U.S.A.). Anti-poly ADP-ribose polymerase (PARP), anti-procaspase-3, and anti-cleaved caspase-3 were purchased from Cell Signal Technology Inc. (Beverly, MA, U.S.A.). Antibodies to Bcl-2 and Bax were from Dako (Glostrup, Denmark), and the β-actin antibody was from Santa Cruz Biotech Inc. (Santa Cruz, CA, U.S.A.).

Isolation of human hair follicles and dermal papillae

Follicles were obtained from the occipital scalp region of ten healthy volunteers (20-35 years of age) who had not received any medication for at least 1 month. Hair follicles were isolated under a stereo-dissecting microscopy, with the DP microdissected from individually isolated hair follicles, as previously described (Messenger, 1984). The study was approved by the Institutional Review Board of Seoul National University Hospital, and all subjects gave their written informed consent (IRB: H-0307-106-002).

Ex vivo human hair follicle organ culture

Isolated human scalp hair follicles were cultured *ex vivo,* as described previously (Philpott *et al*., 1996). Hair follicles were isolated from subcutaneous fat, and cut at about 2.5 mm from the base of the DP. Follicles were incubated at 37°C in 5% CO_2 and cultured for 12 days in 48 well-plates containing Williams' E Medium (Gibco BRL, Gaithersburg, MD, U.S.A.), supplemented with L-glutamine (2mM), insulin (10 mg/ml), hydrocortisone (40 ng/ml), and antibiotics (1%) (Thibaut *et al*., 2003). Hair follicles were re-fed three times a week. AHK-Cu was added to the culture media to final concentrations ranging from 10^{-13} M to 10^{-7} M. Hair follicle elongations were measured directly after 12 days of culture using an Olympus stereo microscope (Olympus America Inc., Center Valley, PA, U.S.A.) and real scale bar. A total of 240 hair follicles from 3 different volunteers were analyzed under each set of conditions (30 follicles/condition).

Primary culture of DPCs

Human DPCs were cultured, as described previously (Messenger, 1984), in a 35×10 mm culture dish containing 2 mL of Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Gaithersburg, MD, U.S.A.), supplemented with 20% fetal bovine serum (FBS) (Hyclone, Logan, UT, U.S.A.), 1×antibiotic antimycotic solution (1000 unit/mL of penicillin G sodium and 2.5 μg/mL of amphotericin B) and fungizone (2.5 μg/mL), at 37°C in a 5% CO_2 incubator. Fourth-passage DPCs were used.

Cell viabilities

Cell viabilities were determined using the MTT assay (Yu *et al*, 2007). DPCs (5.0×10^3 cells/well) were seeded into 96-well plates, and cultured for 24 h with AHK-Cu (10^{-13} ~10^{-7} M). 20 mL of MTT (5 mg/mL) was added to each well, and the cells incubated for 4 h at 37°C. The optical densities, not the actual cell numbers, were compared, with the results expressed as mean percentages of the control for six cultures.

Flow cytometric assay

Both Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) labeling for the detection of apoptotic cell death was performed according to the manufacturer's protocol (BD PharMingen, San Diego, CA, U.S.A.). Briefly, cultured DPCs were treated with AHK-Cu (10^{-9} M) for 72 h. 1×10^6 DPCs were washed twice with phosphate-buffered saline (PBS), and stained with 5 μL of Annexin V-FITC and 10 μL of PI (5 μg/mL) in 1×binding buffer (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM $CaCl_2$) for 15 min at room temperature in the dark. DPCs were analyzed by flow cytometry (Becton-Dickinson FACScan®, Mansfield, MA, U.S.A.). Annexin V, a protein that binds to phosphatidylserine residues, are exposed on the surface of apoptotic, but not normal cells. This test discriminates intact cells (Annexin V-/PI-), early apoptotic cells (Annexin V+/PI-) and late apoptotic necrotic cells (Annexin V+/PI+).

Western blotting

Western blot analysis was performed as follows; briefly, DPCs were cultured for 24 h in serum-free DMEM, and then treated for 24 or 72 h with 10^{-9} M AHK-Cu. Cells were then washed and scraped into 1×PBS, with proteins extracted using a buffer containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 100 μg/mL leupeptin, 20 μg/mL aprotinin and 100 mM NaCl. Soluble extracts were obtained by centrifuging at 13,000 rpm and 4°C for 15 min. Supernatants were collected and kept at -70°C until required. 50 μg of protein per lane was separated using 7.5 or 12% SDS-polyacrylamide gel electrophoresis and then blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were then washed twice with TBS containing 0.1% Tween 20 (TBST). After blocking, with TBS containing 5% nonfat milk, for 60 min, the membranes were incubated overnight at 4°C with the primary antibodies at appropriate dilutions (anti-Bcl-2 monoclonal antibody, 1:1000; anti-Bax monoclonal antibody, 1:1000; anti-actin monoclonal antibody, 1:2000; anti-procaspase-3 monoclonal antibody and anti-cleaved caspase-3 polyclonal antibody, 1:500; anti-PARP polyclonal

antibody, 1:1000) in TBST-5% bovine albumin, and then washed three times with TBST. The membranes were probed with anti-mouse IgG-Horseradish peroxidase (HRP) conjugates (1:2000), anti-rabbit IgG-HRP conjugates, or anti-goat IgG-HRP conjugates (1:2000) for 1 h at room temperature, and then washed three times with TBST. The antibody-antigen complexes were detected using an ECL plus system (Amersham Bioscience, Buckinghamshire, UK) and exposed to Kodak X-ray film, with the band intensities measured using the TINA software (Raytest Isotopenmeβgerate, Straubenhardt, Germany)

Statistical methods

Data are presented as the means ± S.E. Statistical comparisons were performed using Student *t*- and Wilcoxon-rank sum tests, with $p < 0.05$ considered to indicate statistical significance.

RESULTS

AHK-Cu stimulated the elongation of human hair follicles ex vivo

After 12 days of organ culture, the length of human hair follicles in the 10^{-12}~10^{-9} M AHK-Cu treated group were significantly increased compared with the vehicle-treated group (Fig. 1). 10^{-8} and 10^{-7} M AHK-Cu, however, significantly inhibited the hair follicle elongation by 14.8 ± 1.2 (2.3 ± 0.18 mm) and 81.5 ± 40.8% (0.5 ± 0.25 mm), respectively, as compared with the vehicle-treated control.

AHK-Cu induced the proliferation of cultured DPCs

According to the result of MTT assay, AHK-Cu significantly stimulated the proliferation of cultured DPCs at concentrations of 10^{-12}~10^{-9} M versus the vehicle-treated control (Fig. 2); however, 10^{-8} M AHK-Cu did not affect the proliferation of DPCs.

AHK-Cu decreased the number of apoptotic DPCs

Double staining with Annexin V-FITC and propidium iodide was performed to discriminate viable, apoptotic and late apoptotic/necrotic cells. The treatment with 10^{-9} M AHK-Cu reduced the number of apoptotic DPCs by 3.48 %, as shown by Annexin V+/PI- versus the vehicle-treated controls (Fig. 3); however, this decrease was not statistically significant.

AHK-Cu increased Bcl-2 expression and decreased Bax expression in DPCs

To evaluate any possible association in the changes on Bcl-2 family proteins, the effects of AHK-Cu on the expressions of Bcl-2 and Bax protein were investigated. When the cultured DPCs were treated with 10^{-9} M AHK-Cu for 24 h, the expression of Bcl-2 protein increased compared with the vehicle-treated control ($p < 0.05$), but the expression of Bax protein was decreased ($p < 0.05$) (Fig. 4). No remarkable differences were observed in the expressions of Bcl-2 and Bax between the AHK-Cu-treated group and vehicle-treated control when 10% fetal bovine serum was treated to both groups.

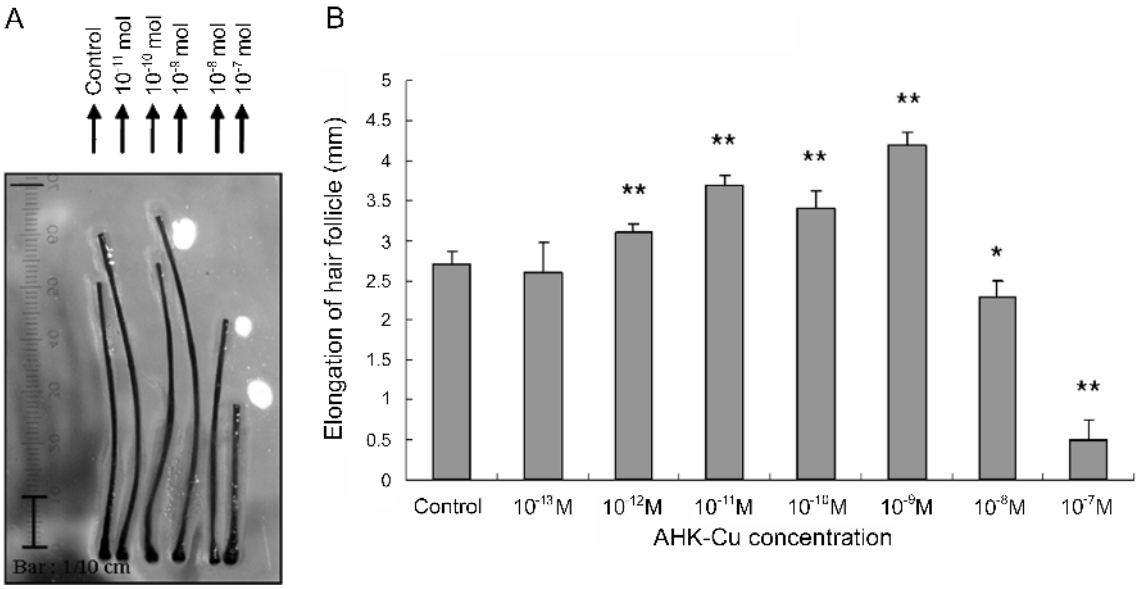

Fig. 1. Effect of AHK-Cu on a human hair follicle organ culture *in vitro*. Isolated human scalp hair follicles were cultured for 12 days in 48 well-plates containing Williams'E Medium (Gibco BRL, Gaithersburg, MD, U.S.A.), supplemented with L-glutamine (2 mM), insulin (10 mg/ml), hydrocortisone (40 ng/ml), and antibiotics (1%). AHK-Cu was added to the culture media to final concentrations ranging from 10^{-13} to 10^{-7} M. A total of 240 hair follicles from 3 different volunteers were analyzed under each set of conditions (30 follicles/condition). Values are the means±S.E. *p< 0.01, **p<0.001 compared with vehicle-treated controls.

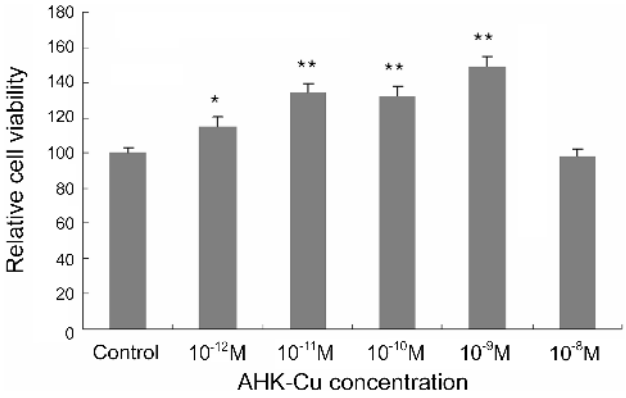

Fig. 2. Viabilities of human dermal papilla cells (DPCs) treated with AHK-Cu. DPCs (5.0×10^3 cells/well) were seeded into 96-well plates, and cultured for 24 h with AHK-Cu (10^{-12}~10^{-7} M). 20 μL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (5 mg/mL) was added to each well, with the cells then incubated for 4 h at 37°C. The optical densities were compared, with the results expressed as mean percentages of the controls for six cultures. Values are the means±S.E. from eight wells. *p<0.05, **p<0.001 compared with vehicle-treated controls.

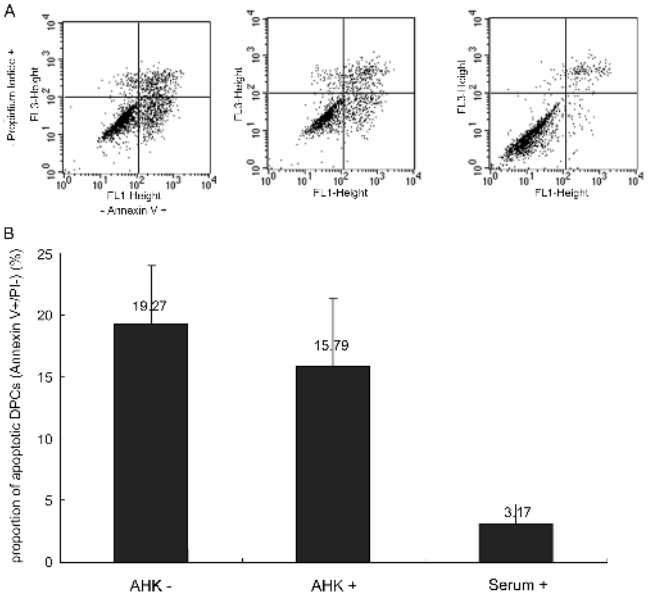

Fig. 3. Flow cytometric analysis of human dermal papilla cells (DPCs) treated with AHK-Cu. Cultured human DPCs were treated with AHK-Cu (10^{-9} M) for 72 h, and stained with Annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI). The serum (+) group is a positive control, where fetal bovine serum was added instead of AHK-Cu. This group represents the state of normal cell growth. The apoptotic cells are located in right lower quadrant (Annexin V+ / PI-) (A). Experiments were repeated in triplicate, with the values expressed as the means±S.E. (B).

Levels of cleaved caspase-3 and PARP cleavage fragments in DPCs were reduced by AHK-Cu treatment

Western blotting was performed to detect the pro-

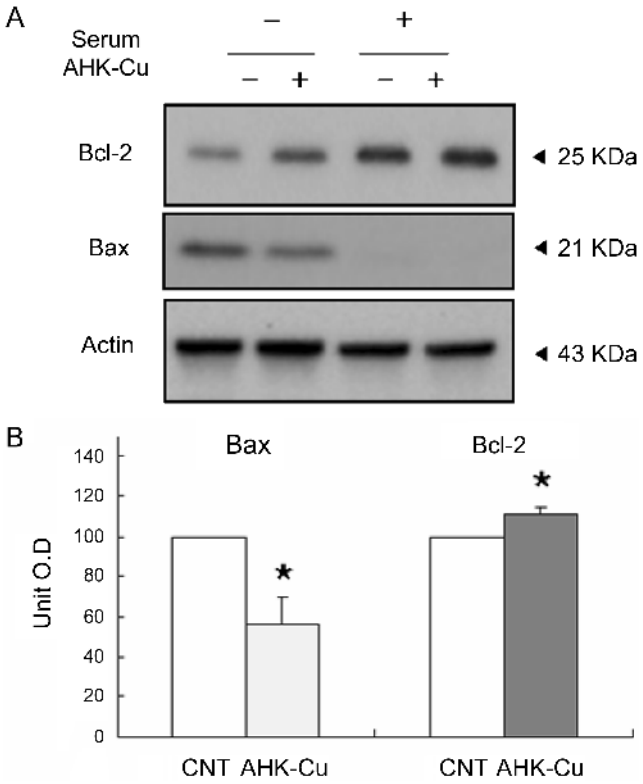

Fig. 4. Effect of AHK-Cu on the expressions of Bax and Bcl-2 in human dermal papilla cells (DPCs). DPCs were cultured in the presence or absence of 10^{-9} M AHK-Cu for 24 h. Cell lysates were subjected to western blot analysis using the indicated antibodies (A). Western blotting results of the DPCs cultured under serum-free conditions are expressed in histogram form as the percentages of the vehicle-treated controls. Values are the means±S.E. of three independent experiments (B). *p<0.05 compared with vehicle-treated controls.

caspase-3 and cleaved caspase-3, as well as the 115 and 85 kDa PARP cleavage fragments. Treatment of DPCs for 72 h, with 10^{-9} M AHK-Cu downregulated the levels of the cleaved caspase-3 and PARP cleavage fragments by 42.7% (p < 0.05) and 77.5% (p < 0.05), respectively, compared with the vehicle-treated control (Fig. 5). The expression of procaspase-3 showed no remarkable differences between the AHK-Cu-treated group and the control. When the cultured DPCs were treated with 10% fetal bovine serum, the AHK-Cu-treated group and vehicle-treated control showed similar expression levels of procaspase-3, cleaved caspase-3 and PARP cleavage fragments.

DISCUSSION

Hair follicle organ cultures have been used to evaluate the effects of various factors, such as insulin-like growth factor-1, epidermal growth factor, TGF-β and minoxidil, on hair growth *ex vivo* (Philpott *et al.*, 1996; Magerl *et al.*,

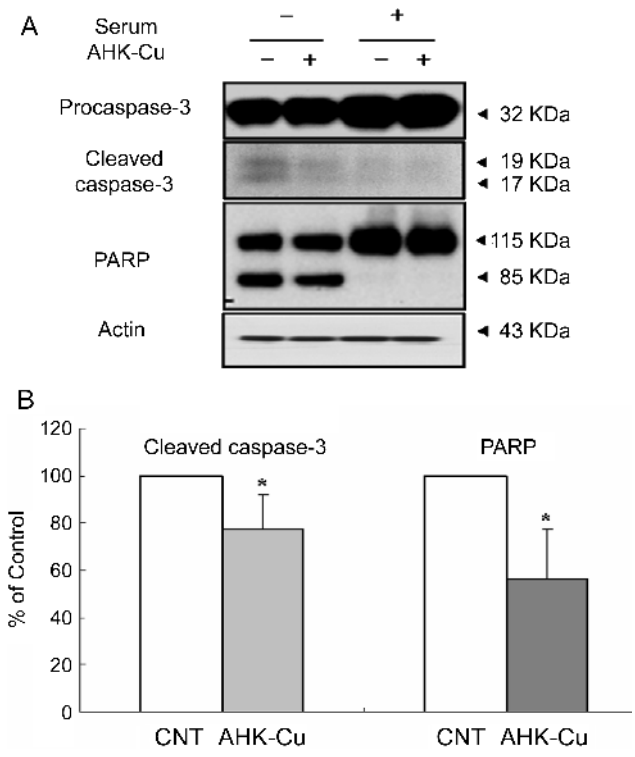

Fig. 5. Effect of AHK-Cu on the expressions of caspase-3 and PARP in human dermal papilla cells (DPCs). DPCs were cultured in the presence or absence of 10^{-9} M AHK-Cu for 72 h. Cell lysates were subjected to western blot analysis (A). Western blotting results of the DPCs cultured under serum-free conditions are expressed in histogram form as the percentages of the vehicle-treated controls. Values are the means±standard error of three independent experiments (B). *p<0.05 compared with vehicle-treated controls.

2004). In the present study, 10^{-12} ~ 10^{-9} M AHK-Cu was found to stimulate human hair growth *ex vivo*. It is generally believed that DPCs are primarily responsible for the proliferation and differentiation of hair matrix cells in the hair cycle. DPCs may secrete many kinds of growth hormones, as well as stimulate the proliferation and differentiation of the follicular epithelium (Rhee *et al.*, 2006). Hence, any cause that promotes the survival of DPCs may ultimately stimulate the proliferation and growth of hair matrix cells, as well as hair follicle elongation. In this respect, we supposed that AHK-Cu would have the proliferative or anti-apoptotic effect on DPCs. At concentrations between 10^{-12} and 10^{-9} M, AHK-Cu induced the proliferation of DPCs. In the flow cytometric analysis, the treatment of 10^{-9} M AHK-Cu did not significantly decrease the number of apoptotic DPCs. However, the reduction of apoptotic cells was not be negligible; therefore, Bcl-2, Bax, caspase-3 and PARP were further evaluated.

Bcl-2 family proteins are well-known regulators of apoptosis in both directions. Bcl-2 itself is an anti-apoptotic molecule; whereas, Bax is pro-apoptotic (Nunez and Clarke, 1994). The expression of Bcl-2 in human hair is dominant during telogen-anagen transition (Soma and Hibino, 2004). In the present study, AHK-Cu increased the expression of Bcl-2 in DPCs, decreased that of Bax, leading to a net sum in the anti-apoptosis arm. Caspase-3 is a critical component molecule in the apoptosis of many cell types (Sawaya *et al.*, 2002), with poly ADP-ribose polymerase (PARP) being one of the main cleavage targets of caspase-3 *in vivo* (Nicholson *et al.*, 1995). The apoptosis of DPCs induced by serum starvation was detected by monitoring the cleaved forms of caspase-3 (19, 17 kDa) and PARP (89 kDa). Our study showed that AHK-Cu treatment reduced the expressions of the cleaved forms of caspase-3 and PARP versus those in the vehicle-treated control.

In summary, the present study has provided strong *in vitro* evidence that AHK-Cu may stimulate hair growth by increasing the proliferation of DPCs, and by preventing their apoptosis. The effects of AHK-Cu on different cell types in hair follicles as well as the molecular basis for its promotion of hair growth both require further investigation.

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