

Efficacy of Caffeine in Promoting Hair Growth by Enhancing Intracellular Activity of Hair Follicles

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Abstract: Caffeine is widely used in cosmetics and hair care products. Although its efficacy in stimulating hair growth has been confirmed in recent studies, its mechanism of action remains unelucidated. The present study aimed to determine the effects of caffeine on hair growth, with a focus on intracellular hair follicle activity. Experiments included *in vitro* and *ex vivo* tests, and a clinical study. Caffeine enhanced the cellular activity and potassium channel opening. It also promoted human hair follicle elongation. Immunohistochemical staining showed that the Ki-67 signal was significantly higher in cells treated with caffeine. These efficacies of caffeine were comprehensively demonstrated in clinical results, wherein caffeine-containing shampoo improved hair density after 24 weeks of testing. Collectively, the results of this study demonstrated that caffeine promoted hair growth and inhibited the progression of hair loss by enhancing intracellular activity of hair follicles.

Keywords: Caffeine, Alopecia, Hair, Dermal papilla, Hair follicle

1. Introduction

Hair loss is a common problem that occurs in men and women of all ages. Individuals with hair loss may experience negative emotions such as loss of confidence and low self-esteem[1]. Nevertheless, to date, only two drugs (oral finasteride and topical minoxidil) have been approved by the United States food & drug administration (FDA) as a treatment for alopecia[2]. For decades, numerous studies have pursued the development of new drugs and the discovery of substances to prevent hair loss.

Caffeine is a well-known constituent of coffee[3] and is commonly consumed daily. It is a central nervous system (CNS) stimulant and has the effect of reducing fatigue and drowsiness[4-6]. The effects of caffeine on hair have also been studied. Caffeine was shown to promote hair growth against testosterone in *ex vivo* hair follicle cultures[7].

Additionally, caffeine enhanced insulin-like growth factor-1 (IGF-1) protein expression while downregulating transforming growth factor- β 2 (TGF- β 2) expression[8]. The mechanism by which caffeine promotes hair growth, however, remains to be determined.

On the basis of the findings obtained to date, we studied the effects of caffeine on hair growth with a focus on intracellular hair follicle activity. The present study examined hair follicle cell growth in the context of growth factors, energy generation, indirect vasodilation efficacy, histological changes, and clinical studies.

2. Experimental Materials and Methods

2.1. Human Hair Follicle and Cell Culture

The medical ethics committee of Dankook medical university (Cheonan, Korea) approved this study (institutional review board [IRB] number: DKUH 2013-08-012-001), and written informed consent was obtained from all subjects.

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Anagen human hair follicles were isolated from occipital scalp specimens obtained from the remainder of hair transplantation surgeries by using a reference method[9,10]. Briefly, hair follicles were cultured in William's E media (Sigma-Aldrich, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich), 10 $\mu\text{g}/\text{mL}$ insulin (Sigma-Aldrich), 10 ng/mL hydrocortisone (Sigma-Aldrich), 0.1% fungizone (Gibco, USA), and 1% antibiotic-antimycotic (Gibco).

Human dermal papilla (DP) cells were isolated from hair follicles, and NIH-3T3 cells were purchased from the Korean Cell Line Bank (KCLB, Korea). DP and NIH-3T3 cells were cultured in Dulbecco's Modified Eagle medium (DMEM; Hyclone Laboratories, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotic-antimycotic, and incubated at 37 °C in a 5% CO₂ atmosphere.

2.2. DP Cell Proliferation

DP cells were seeded in 96 well plates at the cell density of 2000 cells/well. After cell attachment, caffeine at a concentration of 10 to 50 parts per million (ppm) was added to FBS-free medium. As a positive control, 5% FBS medium was used. After 72 h, cell proliferation was measured using a commercially available kit (Cell Counting Kit-8 [CCK-8], Dojindo Molecular Technologies, Japan).

2.3. Growth Factor Analysis: KGF and VEGF

Keratinocyte growth factor (KGF), also known as fibroblast growth factor 7 (FGF7), promotes keratinocyte proliferation and differentiation[11], and mediates hair follicle growth, development, and differentiation[12]. Vascular endothelial growth factor (VEGF), which plays a role in promoting angiogenesis and vascular permeability, is highly expressed in DP cells[13] and influences hair growth [14,15]. To analyze these two growth factors, 2×10^5 DP cells were cultured in 6 well plates for 24 h, followed by exchange with FBS – free media containing caffeine at a concentration of 20 ppm. After additional 48 h incubation, the cells and supernatant were isolated for quantitative real-time polymerase chain reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. To quantify KGF gene expression level, mRNA was isolated

using a commercially available kit (RNeasy Mini Kit, Qiagen, United Kingdom) and cDNA was synthesized using PrimeScript™ 1st strand cDNA Synthesis Kit (Takara, Japan). Gene amplification and quantification were performed using a thermocycler (7500 Fast Real-Time PCR Instrument System, Applied Biosystems, USA) and TaqMan™ Universal Master Mix II (Applied Biosystems). VEGF produced by DP cells was quantified using a commercially available kit (Human VEGF Quantikine ELISA Kit, R&D Systems, USA). All experiments were performed according to the manufacturer's instructions.

2.4. ATP Assay

The ATP assay is based on a reaction with luciferase to measure the amount of ATP produced in cells [16] and was performed using the ATPlite 1step kit (Perkin Elmer, USA). DP cells were seeded into 96-well black plates at the cell density of 1×10^4 cells/well. Caffeine at a concentration of 10 – 100 ppm was added to the cells for 6 h, and 5% FBS was used as a positive control. After changing the media to 100 μL DMEM without phenol red (Welgene, Korea), the same volume of substrate solution contained in the kit was added. The reaction between ATP produced by the cells and D-luciferin/luciferase from the kit occurred during the 10 min incubation period. The ATP content in the cells was evaluated by measuring luminescence intensity.

2.5. Hair Follicle Elongation

Anagen hair follicles were cultured to determine their elongation. Cells treated with 10 ppm minoxidil (Sigma, USA) were used as the positive control, and caffeine was tested at the concentration of 20 ppm. Media containing each test material were changed every 2 - 3 days. The growth length of the hair follicle was measured by microscopic imaging (SZX16; Olympus, Japan) on day 2 and day 5. At least 10 samples were tested in each test group.

2.6. Potassium Channel Assay

The potassium channel assay is used to identify the effect on potassium channel opening according to the viability of NIH-3T3 cells co-treated with a potassium channel

blocker (tolbutamide [TBT]; Sigma-Aldrich). This experiment was performed according to a previously described method[17]. Briefly, NIH-3T3 cells were seeded in 96 well plates at the cell density of 8000 cells/well. One day after cell seeding, the cells were treated with 2.5 mM TBT for 10 min, followed by treatment with each concentration of caffeine in 2.5 mM TBT diluted media for 48 h. Minoxidil treatment served as the positive control. The efficacy of potassium channel opening was determined by the CCK-8 assay.

2.7. Ki-67 Immunohistochemistry

Ki-67 staining is a useful immunohistochemical tool for obtaining information about cell proliferation in hair follicles[18]. Ki-67 expression reduces as the hair follicle growth cycle passes from anagen to catagen[19]. To confirm the effect of caffeine on hair, human hair follicles were cultured in caffeine (50 ppm) diluted in culture medium for 3 days. The cryosectioned follicle samples were fixed in 10% paraformaldehyde for 10 min. Bovine serum albumin (4%) in TBST was used for blocking for 30 min, and the slides were then washed with TBST. The samples were incubated with Ki-67 monoclonal antibodies (Invitrogen, USA) at 1:50 dilution overnight at 4 °C. After washing, Alexa Fluor™ 488-conjugated anti-rat secondary antibodies (Invitrogen; 1 : 50 dilution) were added for 1 h. The samples were counterstained with DAPI (Fluoroshield with DAPI, Sigma-Aldrich), and images were captured using a microscope with a fluorescent light source (IX-73 with U-HGLGPS; Olympus, Japan) at 100× magnification.

2.8. Clinical Study

2.8.1. Participants

Forty Korean men and women between 18 and 54 years of age (mean ± SD age, 45.35 ± 7.06 years) were recruited as subjects for the clinical study. Subjects who were diagnosed to have hair loss were selected according to the guidelines of Korea food and drug administration (KFDA). Those who underwent procedures such as hair transplantation and those who took oral depilatory agents in the previous 6 months were excluded. This part of the study was performed according to the ethical principles of the

Declaration of Helsinki and was approved by the IRB (KC-IRB-014). All subjects provided written informed consent to participate.

2.8.2. Protocol

This part of the study was conducted in a double-blind manner. Each subject group used a shampoo containing a vehicle or 0.5% caffeine once per day for 24 weeks. Hair density was assessed at baseline and at 8, 16, and 24 weeks of treatment. To measure hair density, the area for evaluation was first designated and delineated into a pre-determined area of approximately 1 cm². The center of the measurement area was labeled with a dye to identify the area for hair counting. Hair density was determined by analyzing the total number of hairs in a 1 cm² area of the photographed image by using a phototrichogram instrument (Folliscope4.0; LeadM, Korea)[20]. To observe overall changes in hair density, global photographs of the vertex area were captured.

2.9. Statistical Analysis

SPSS version 23.0 (IBM corporation, USA) was used for statistical analysis. Data were compared using Student's t-test and expressed as mean ± standard deviation. Differences with $p < 0.05$ were considered as statistically significant. For the analysis of clinical study, Repeated - measure ANOVA and Friedman test were used to analyze the results of the present study.

3. Results and Discussion

3.1. Effect of Caffeine on DP Cell Proliferation

DP cell proliferation was measured by the CCK-8 assay. Caffeine increased DP cell population in a concentration-dependent manner. Compared to the control group, cell proliferation of the caffeine - treated (10 ppm) group increased by 10%, while that of DP cells treated with 20 and 50 ppm caffeine increased by 18% and 19%, respectively (Figure 1).

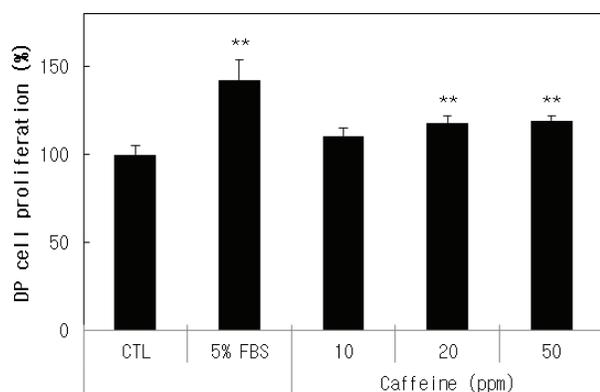
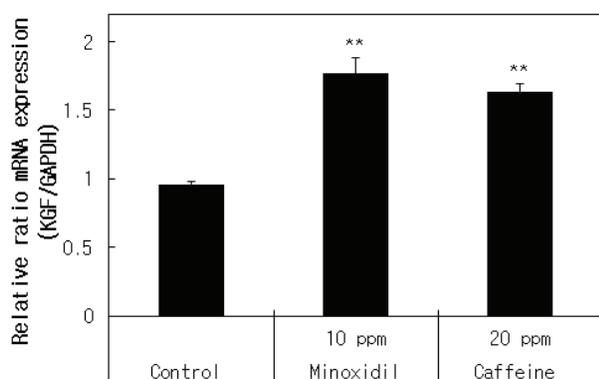
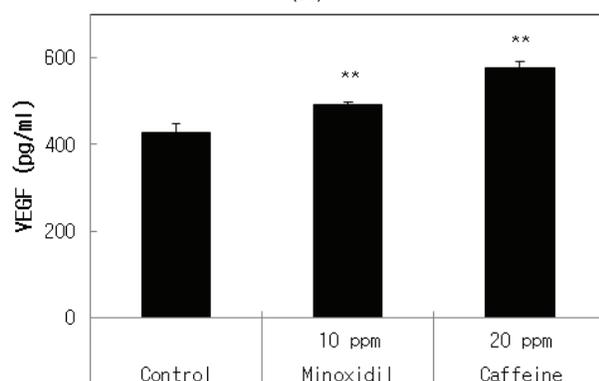


Figure 1. The effect of caffeine on DP cell proliferation. Caffeine enhanced the cell proliferation in a concentration-dependent manner. (** $p < 0.01$ vs. control)



(A)



(B)

Figure 2. Regulation of growth factor expression in DP cells. The expression of both KGF (A) and VEGF (B) was increased by caffeine. Minoxidil was used as a positive control. (** $p < 0.01$ vs. control)

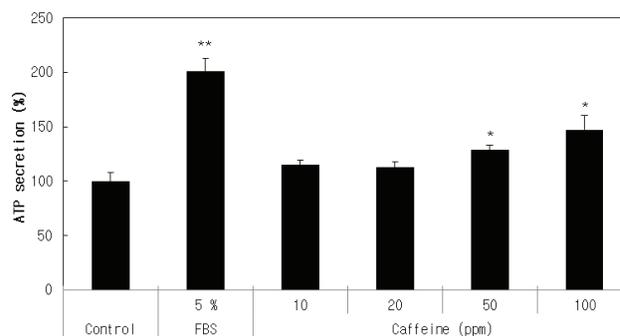


Figure 3. Increased ATP production in DP cells by caffeine. Caffeine increased ATP production at concentrations above 50 ppm. (* $p < 0.05$ vs. control, (** $p < 0.01$ vs. control)

3.2. Increase of Growth Factors (KGF and VEGF) in DP Cells

The mRNA level of KGF in DP cells was determined by qRT-PCR. KGF gene expression was enhanced by 1.76 fold in the positive control group (10 ppm minoxidil) and by 1.6-fold in the caffeine-treated group (Figure 2A). VEGF levels in caffeine-treated cells were 35% higher than those in the nontreated control group (Figure 2B). This increase in VEGF level was significantly higher than the 15% increase observed in the minoxidil - treated group. These results demonstrate that caffeine enhanced the action of important growth factors on hair follicles.

3.3. Induction of ATP Production by Caffeine

The ATP assay was performed to determine increased intracellular ATP production in DP cells. The results of the luminescence intensity assay were converted to the amount of ATP based on the serial dilution of an ATP standard. Caffeine increased ATP production in a concentration-dependent manner by 1.29 fold in cells treated with 50 ppm caffeine and by 1.47 fold in cells treated with 100 ppm caffeine (Figure 3). ATP is an energy source for living cells and drives many vital cellular processes[21]. Therefore, these results suggest that caffeine contributes to DP cell activity by promoting ATP production.

3.4. Function of Caffeine as a Potassium Channel Opener

The action of TBT, a potassium channel blocker, re-

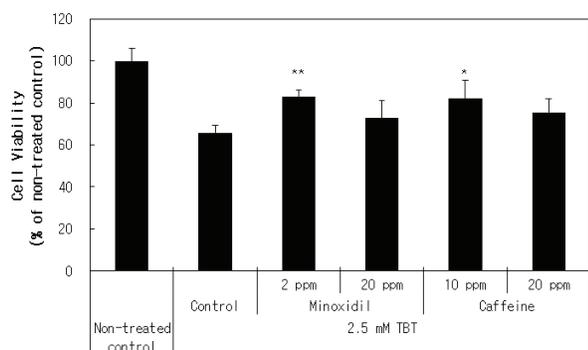


Figure 4. Caffeine as a potassium channel opener against TBT. Caffeine restored cell growth inhibition by TBT to levels similar to those restored by minoxidil. (* $p < 0.05$ vs. 2.5 mM TBT-treated control, ** $p < 0.01$ vs. 2.5 mM TBT-treated control)

duced cell viability to approximately 65%. In contrast, cell viability in the caffeine - treated group was approximately 80%, which is the same as that noted for the minoxidil - treated group (Figure 4). It appears that caffeine induced the opening of potassium channels against the action of TBT, which in turn improved cell survival. From these results, caffeine appears to have a vasodilation effect on hair follicles, with a mechanism of action similar to that of minoxidil[22,23]

3.5. Hair Follicle Elongation Promoted by Caffeine

During a 5 day culture with 20 ppm caffeine, hair follicles grew an average length of 1.16 mm, which was significantly greater than that of the control group, in which the hair follicles grew at an average of only 0.84 mm (Figure 5). Hair follicles in the positive control group grew

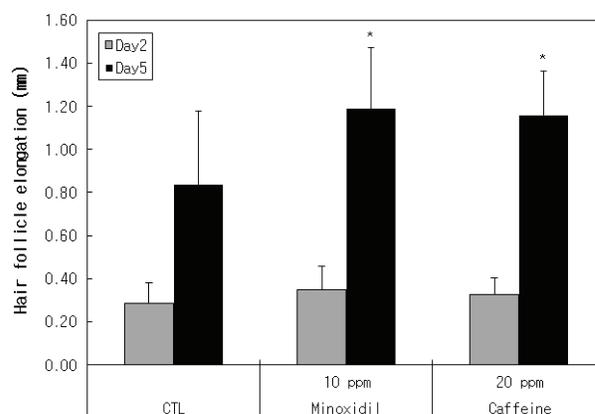


Figure 5. Hair follicle elongation by caffeine in *ex vivo* culture. Caffeine significantly promoted the growth of hair follicles. (* $p < 0.05$ vs. control)

an average of 1.19 mm, suggesting that caffeine has an efficacy comparable to that of minoxidil in promoting hair follicle growth.

3.6. Ki-67-Positive Cells in Hair Follicles Treated with Caffeine

After 3 days of *ex vivo* culture, it was difficult to detect the Ki-67 signal in follicles of the control group (Figure 6A). Otherwise, follicles treated with 50 ppm caffeine exhibited Ki-67-positive cells in the matrix (Figure 6C). The positive signal was particularly strong in the upper part of the matrix above the DP. During 3 days of culture, many nontreated hair follicles turned over to the catagen phase, thereby losing proliferative matrix cells in the process. In contrast, caffeine enhanced cell proliferation in hair follicles and maintained the anagen phase.

Table 1. Hair Density at Baseline and at 8, 16, and 24 Weeks

| Group | Density (n/cm ²) | | | |
|----------------------------|------------------------------|------------------|----------------|------------------|
| | Before | 8 w | 16 w | 24 w |
| Control | 118.15 ± 25.31 | 115.95 ± 26.05 | 115.45 ± 25.01 | 116.65 ± 25.16 |
| Change Rate (%) | | -2.20 ± 4.01 | -2.70 ± 5.34 | -1.50 ± 4.06 |
| Test | 110.90 ± 16.86 | 114.10 ± 19.09** | 114.55 ± 18.73 | 115.75 ± 19.07** |
| Change Rate (%) | | +3.20 ± 4.66 | +3.65 ± 5.68 | +4.85 ± 6.59 |
| <i>p</i> -value (vs Group) | - | 0 | 0.001 | 0.001 |

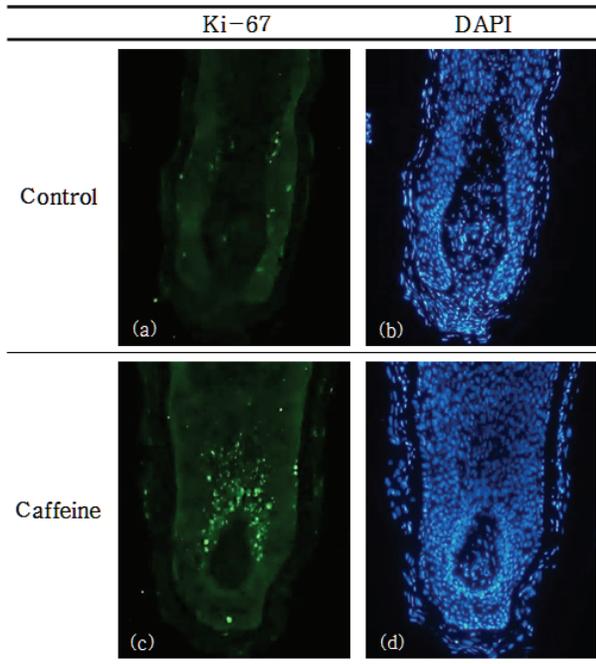
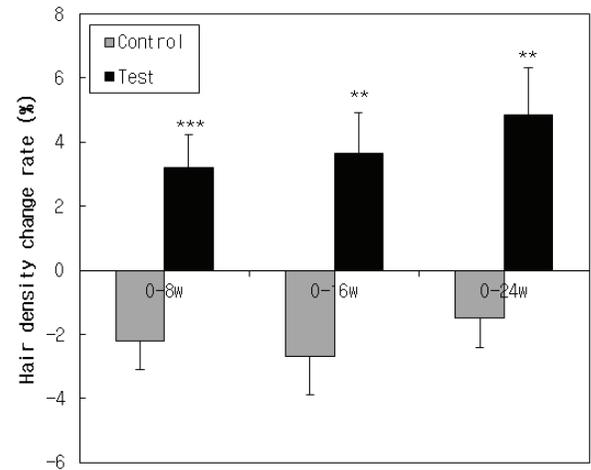


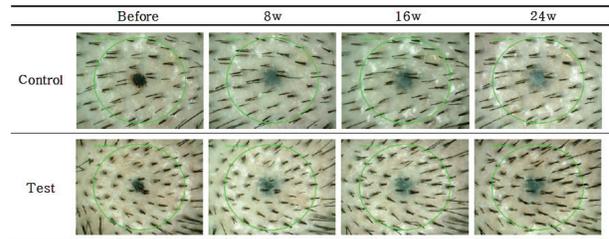
Figure 6. Ki-67 immunohistochemistry staining of hair follicles. Control group (A) showed few Ki-67-positive cells; however, caffeine-treated hair follicles (C) had stronger Ki-67 positive signal. DAPI staining (B, D) was used as a counterstaining method. (Ki-67: green, DAPI: blue)

3.7. Effect of Caffeine Shampoo on Hair Loss in the Clinical Study

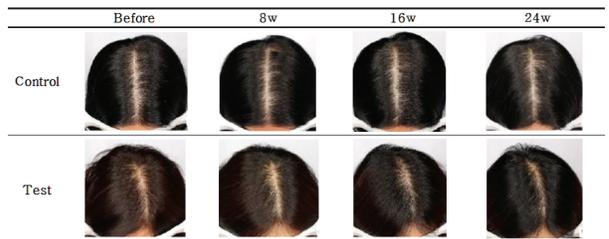
Phototrichogram results revealed that the control group (vehicle shampoo) did not exhibit any statistically significant difference in hair density during the 24 week trial. In the test group (0.5% caffeine shampoo), however, a significant improvement was noted after 8 and 24 weeks (Table 1). Comparison of hair density changes between the control and test groups revealed significant differences after 8, 16, and 24 weeks of product use (Figure 7A). During the 24 week test period, hair density increased by 4.85% in the test group but decreased by 1.5% in the control group. Efficacy results were also visually confirmed using clinical photographs from both phototrichogram and global vertex images (Figure 7B, C). From these results, caffeine appears to prevent hair loss progression and induce new hair growth in patients experiencing hair loss.



(A)



(B)



(C)

Figure 7. Clinical study of caffeine. The graph showed hair density change rate from baseline (A). The test group that used caffeine-containing shampoo showed increased change rate from baseline, while the control group had a negative change rate at every three measurement periods. The results of phototrichogram (B) and photographs of the vertex (C) showed a change in hair density during the 24-week clinical period. (** $p < 0.01$ vs. control, *** $p < 0.001$ vs. control)

4. Conclusion

Many potent ingredients are used in products formulated to prevent hair loss. Caffeine has been shown to stimulate hair growth and follicular penetration[24]. Despite its anti-hair loss effects, to date, the mechanism by which caffeine exerts its effects on hair follicle cells remains unclear. To elucidate this mechanism, we performed efficacy experiments involving cellular assays and tissue culture, and a clinical study.

Caffeine positively affected hair growth, which was reflected by an increase in DP cell proliferation and the expression of growth factors VEGF and KGF. This finding also demonstrated the potential to enhance energy sources through ATP production in DP cells. Results of the potassium channel opening assay indicated that caffeine can contribute to improved nutrient supply by facilitating vasodilation, which is similar to the action of minoxidil[25]. Caffeine also induced hair follicle elongation in *ex vivo* culture. An examination of the inner portion of hair follicles revealed that more Ki-67-positive cells were present in the caffeine-treated group. Unlike the control group, the caffeine – treated group appeared to have an extended anagen phase while maintaining cell proliferation. The effects of caffeine were also confirmed in clinical studies, which demonstrated its efficacy in increasing hair density and preventing the progression of hair loss.

In conclusion, the results of the present study suggest that caffeine maintains the health of hair follicles and induces growth by increasing energy levels inside hair follicle cells and thereby promoting cell growth by enhancing nutrient supply.

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