Original article

Cornu cervi pantotrichum Pharmacopuncture Solution Facilitate Hair Growth in C57BL/6 Mice

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Abstract

Objectives: *Cornu cervi pantotrichum* (CCP) has been widely used in Korean and China, as an anti-fatigue, anti-aging, and tonic agent to enhance the functions of the reproductive and the immune systems. Because CCP has various growth factors that play important roles in the development of hair follicles, we examined whether CCP pharmacopuncture solution (CCPPS) was capable of promoting hair growth in an animal model.

Methods: One day after hair depilation, CCPPS were topically applied to the dorsal skin of C57BL/6 mice once a day for 15 days. Hair growth activity was evaluated by using macro- and microscopic observations. Dorsal skin tissues were stained with hematoxylin and eosin. Expressions of bromodeoxyuridine (BrdU), proliferating cell nuclear antigen (PCNA), and fibroblast growth factor (FGF)-7 were examined by using immunohistochemical staining. A reverse transcription polymerase chain reaction (RT-PCR) analysis was also conducted to measure the messenger RNA (mRNA) expression of FGF-7.

Results: CCPPS induced more active hair growth than normal saline. Histologic analysis showed enlargement of the dermal papilla, elongation of the hair shaft, and expansion of hair thickness in CCPPS treated mice, indicating that CCPPS effectively induced the development of anagen. CCPPS treatment markedly increased the expressions of BrdU and PCNA in the hair follicles of C57BL/6 mice. In addition, CCPPS up regulated the expression of FGF-7, which plays an important role in the development of hair follicles.

Conclusion: These results reveal that CCPPS facilitates hair re-growth by proliferation of hair follicular cells and up-regulation of FGF-7 and suggest that CCPPS can potentially be applied as an alternative treatment for patients with alopecia.

1. Introduction

An increasing number of people suffer from hair loss due to their stressful lifestyles. Up to now, various causes of hair loss, such as genetic factors [1], androgen [2], aging, topical circulatory disorders, stress [3-5], diet, and autoimmune diseases [6, 7], have been described. However, owing to the complexity of the molecular signals that orchestrate hair growth, the underlying

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mechanisms of baldness are not yet fully understood [8].

Hair follicles in the scalp, continuously repeat a three phase cycle: the anagen (active growth) phase, the catagen (transitional) phase, and the telogen (resting) phase. The initiation of, the time spent in, and the completion of each phase are controlled by a number of growth factors released from the follicular cells, including dermal papilla and overlying matrix cells, in a hair. The length and the thickness of a hair shaft are mainly determined by the duration of anagen. Therefore, enhancing the induction of anagen or prolonging its duration should be a good strategy fo treating patients with hair loss.

Cornu cervi pantotrichum (CCP), a horn of *Cervus nippon temminck* or *C. elaphus L.*, has been widely used in Korea and China as an anti-fatigue, anti-aging and tonic agent to strengthen bones and muscles [9, 10]. CCP extracts were reported to have tonifying, anti-aging, wounds healing, and infection-suppressing effects [11]. In addition, CCP pharmacopuncture solution (CCPPS) processed by using hot water extraction has valid clinical effects on arthritis and osteoporosis [12]. CCP contains various nutrients such as proteins, amino acids, minerals, lipids, and polysaccharides, as well as growth factors that play important roles in hair follicle development [13, 14]. Therefore, CCP might have a positive influence on promoting hair growth. Thus, in this study, we examined the effects of CCPPS on the induction of anagen in the hair follicles of C57BL/6 mice.

2. Materials and Methods

Seven-week-old male C57BL/6 mice were purchased from Samtaco Bio Korea, Ltd. (Osan, Korea) and allowed to adapt to their new environment for 1 week. The mice were housed in certified, standard laboratory cages, and provided with food and water *ad libitum* prior to the experiment.

The animal protocol used in this study was reviewed and approved by the Pusan National University – Institutional Animal Care and Use Committee (PNU–IACUC) in accordance with established ethical procedures and scientific care (approval number PNU-2014-0581). CCPPS was purchased from the Korean Pharmacopuncture Institute (Seoul, Korea).

To observe the anagen phase promotion effect, we depilated a patch of dorsal hair in the telogen phase from 8-week-old C57BL/6 mice in order to synchronize anagen induction. The mice were divided into three groups: group 1 was treated with normal saline (negative control); group 2 was treated with 5% Minoxidil (positive control); group 3 was treated with CCPPS. Four mice were randomly allocated to each experimental group. All applications were implemented for 15 days. We dropped 300 uL of normal saline or 5% Minoxidil or CCPPS onto the depilated skin lesion topically once a day. Bromodeoxyuridine (BrdU, 50 µg /g body weight) was injected into the peritoneum twice a day for three days after hair depilation in order to observe the cellular proliferative activity.

The color of the hair, the state of hair re-growth and the thicknesses of the hair shafts at the depilated skin lesions were examined by using gross examination and dermoscopy (Sometech, Inc., Seoul, Korea) on the 14th and the 16th day after hair depilation.

At the 16th day after hair depilation, the mice were euthanized for histological examination of the hair follicles in the skin. The dorsal skins were removed from the areas of hair re-growth, were washed, and were then subjected to the processes of dehydration, clarification, and infiltration. Then, tissue was embedded in paraffin, which was then cut into 7-µm thick section. We performed hematoxylin and eosin (H&E) staining and observed the changes in the hair follicles in the dermis by using an optical microscope (Fig. 1).

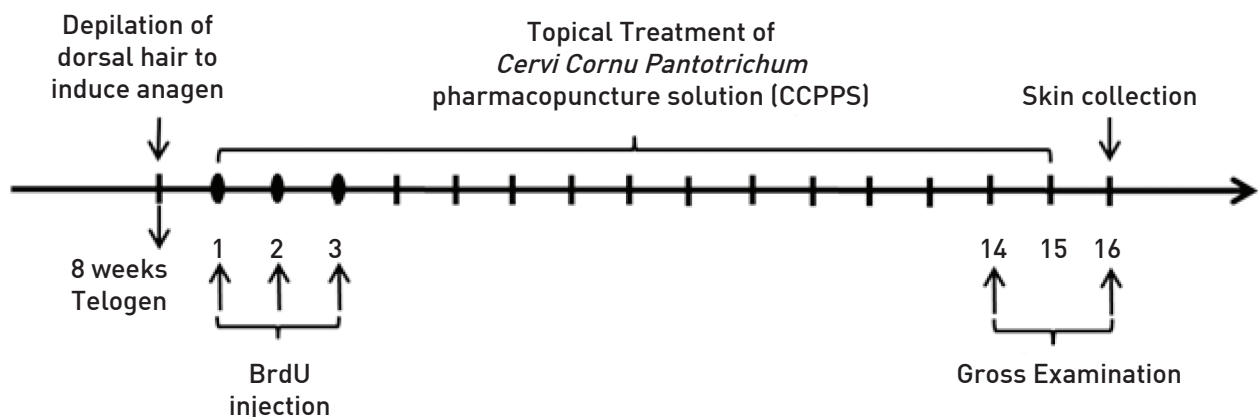Primary antibodies used in this study were mouse anti-

Figure 1 Experiment design. Dorsal hair in the telogen phase of 8-week-old C57BL/6 mice was depilated to synchronize anagen induction. The mice were divided into three groups with four mice allocated to each group: group 1 was treated with normal saline (negative control); group 2 was treated with 5% Minoxidil (positive control); group 3 was treated with CCPPS. One day after hair depilation, the mice were topically sprayed with normal saline, 5% Minoxidil or CCPPS. BrdU was administered twice a day on the 1st, 2nd and 3rd days after hair depilation. The dorsal skin was collected from the mice on the 16th day after hair depilation. Skin tissues were processed for H&E and immunohistochemical staining.

CCPPS, *Cervi cornu pantotrichum* pharmacopuncture solution; BrdU, bromodeoxyuridine; H&E, hematoxylin and eosin.

BrdU antibody (1:200; Santa Cruz, CA, USA) for the detection of BrdU; proliferating cell nuclear antigen antibody (1:1000; Santa Cruz, CA, USA) for the detection of proliferating cell nuclear antigen (PCNA); fibroblast growth factor (FGF)-7 antibody (1:200; Santa Cruz, CA, USA) for the detection of FGF-7.

The tissues were rinsed with phosphate-buffered saline (PBS) after application of 0.05% proteinase K for 20 minutes. Primary antibodies were dropped onto the tissue, which was then allowed to stand at room temperature for 2 hours. Bound antibodies were sequentially reacted with biotinylated goat anti-mouse immunoglobulin G (IgG) and avidin-biotinylated peroxidase complex (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 minutes in a moisture chamber. Immunoreactivity was visualized using diaminobenzidine.

Total ribonucleic acid (RNA) was isolated from the skin tissue by using TRIzol reagent (Invitrogen). cDNAs were synthesized by using AccuPower RT PreMix (Bioneer, Daejon, Korea) according to the manufacturer's instructions. Polymerase chain reactions (PCRs) on the specific deoxyribonucleic acid (DNA) sequences were performed with AccuPower PCR PreMix (Bioneer, Daejon, Korea). Specific primer sequences used in this study were as follows: FGF-7, forward 5′-AGATCATGCTTCCACCTCGT-3′ and reverse 5′-TGGGTCCCTTTCACTTTGCC-3′; GAPDH, forward 5′-GGAGCCAAAAGGGTCATCAT-3′ and reverse

5′-GTGATGGCATGGACTGTGGT-3′. Amplified products were analyzed in 1.0% agarose gel under ultraviolet light, and the images were captured using the GelDoc-It TS Imaging System (UVP, LLC, Upland, CA, USA).

Data were expressed as the means ± standard deviations (SDs). Statistical differences between means were determined by using the one-way analysis of variance (ANOVA) for repeated measures. *P* values less than 0.05 were considered significant.

3. Results

CCPPS was topically applied daily onto the depilated dorsal skin of C57BL/6 mice. 5% Minoxidil was applied daily to the depilated dorsal skin of mice in the positive control group. The dorsal skin lesions were observed on the 14th and the 16th days after hair depilation. CCPPS promoted hair re-growth as efficiently as 5% Minoxidil (Fig. 2, middle and right panels); however, a small amount of hair growth was observed in the mice treated with normal saline (negative control group) (Fig. 2, left panel). Also, the density of hair and the thickness of the hair shaft were increased by treatment with CCPPS (Fig. 3).

Development of hair follicles was observed by using microscopic examination. Fully developed anagen phases were observed in the hair follicles of the CCPPS treated
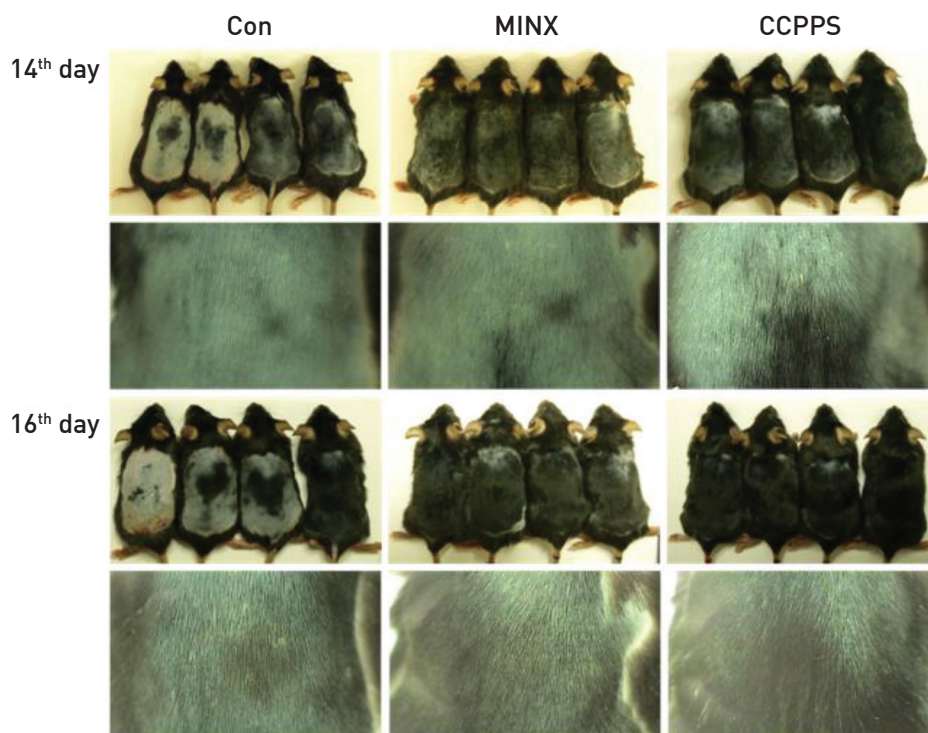

Figure 2 Gross observations of hair re-growth in C57BL/6 mice. On the 14th and the 16th days after depilation, skin color and hair growth on the depilated skin lesions were examined by gross observation. The mice had been treated with topical applications of normal saline or 5% Minoxidil or CCPPS once a day for 15 days. Depilated dorsal skin lesions were photographed on the 14th and the 16th days after hair depilation. Hair re-growth, as well as skin darkness, was increased in mice treated with CCPPS as compared to mice treated with normal saline (positive control group).

CON, normal saline; MINX, Minoxidil; CCPPS, *Cervi cornu pantotrichum* pharmacopuncture solution.
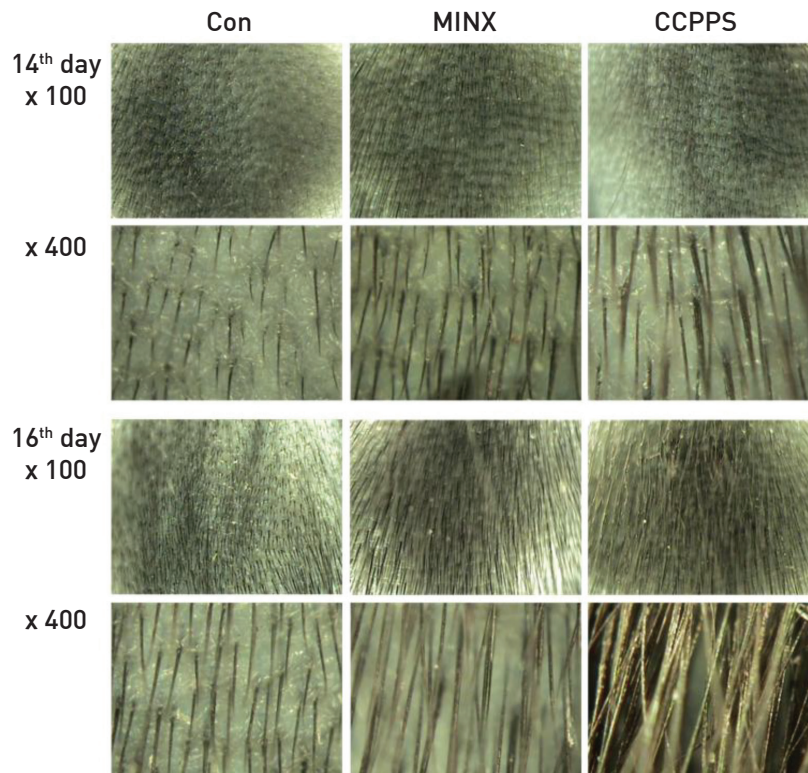
Figure 3 Dermoscopic observations of hair re-growth in C57BL/6 mice. The dorsal skins of the mice were photographed by using dermoscopy on the 14th and the 16th days after depilation. The thicknesses of the shafts of the re-grown hair were greater in the mice treated with CCPPS and the mice treated with Minoxidil (negative control) than they were in the mice treated with normal saline (positive control).

CON, normal saline; MINX, Minoxidil; CCPPS, *Cervi Cornu Pantotrichum* pharmacopuncture solution. Original magnifications were × 100 and × 400.
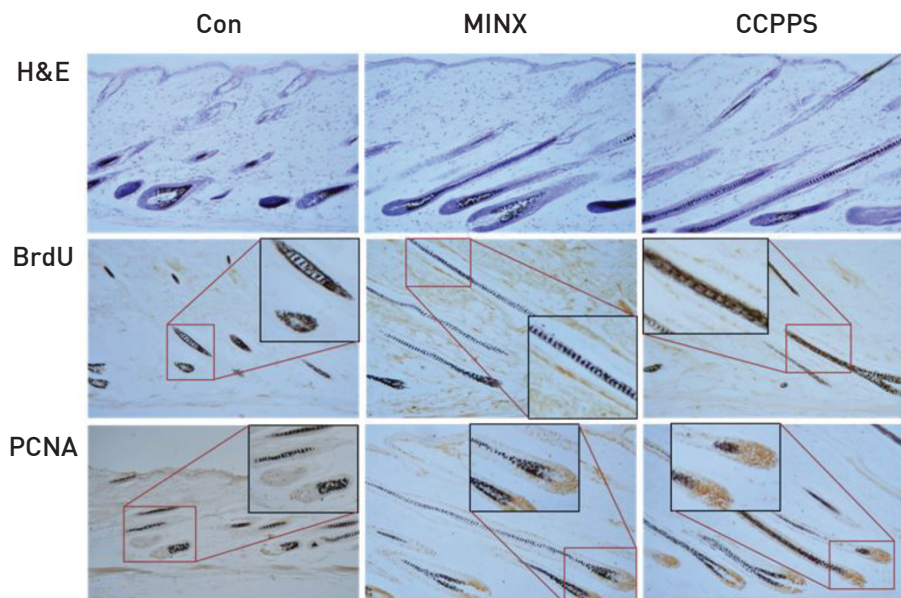

Figure 4 Microscopic observations of hair follicles in C57BL/6 mice. Histologic examination revealed hair-follicle development in C57BL/6 mice treated with normal saline, with MINX, and with CCPPS for 15 days. Dorsal skin sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed to examine the expressions of BrdU and PCNA. The original magnification was × 100; that of the large box was × 200.

CON, normal saline; MINX, Minoxidil; CCPPS, *Cervi cornu pantotrichum* pharmacopuncture solution; H&E, hematoxylin and eosin; BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen.
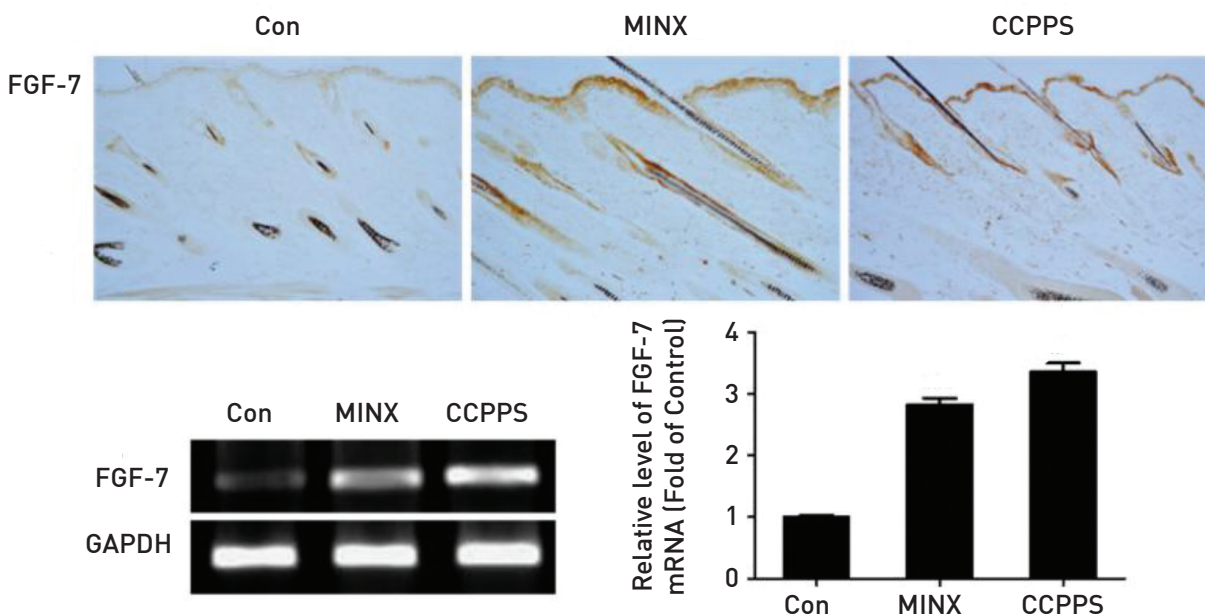
Figure 5 Effects of CCPPS on the expression of FGF-7. The dorsal skins of 16-week-old male C57BL/6 mice treated with normal saline, MINX, or CCPPS were collected. (A) An immunohistochemical analysis of the hair follicles against FGF-7 was performed. (B, left) The mRNA expression of FGF-7 was analyzed by using a RT-PCR analysis. (B, right) The relative levels of the expressions of FGF-7 mRNA are shown as means ± SDs for the saline (CON), Minoxidil (MINX), and CCPPS-treatde mice. $^*P < 0.0001$ compared to control.

CCPPS, *Cervi cornu pantotrichum* pharmacopuncture solution; FGF-7, fibroblast growth factor-7; mRNA, messenger RNA; RT-PCR, reverse transcription polymerase chain reaction; SDs, standard deviations.

mice. The hair follicles of the mce treated with normal saline remained in the anagen phase Ⅳ (Fig. 4, upper left panel) while those of Minoxidil or CCPPS treated mice had developed to the anagen phase Ⅵ (Fig. 4, upper middle and right panels). The expression of BrdU, a marker of cellular proliferation, was moderately increased at the outer root sheath in CCPPS treated mice (Fig. 4, middle and right panels). The expression of PCNA, another marker of cellular proliferation, was also increased by treatment with CCPPS. An especially strong expression was observed at the bulge lesion including dermal papilla (Fig. 4, lower right panel).

We also observed the expression of FGF-7, which plays critical roles in anagen induction in hair follicles. CCPPS increased the expression of FGF-7 at the epidermis, the inner and the outer root sheaths, and the dermal papilla in the hair follicles of the depilated skin lesions to an extent that was similar to the increase due to the use Minoxidil (Fig. 5A, middle and right panels). A reverse transcription polymerase chain reaction (RT-PCR) analysis revealed that the messenger RNA (mRNA) expression of FGF-7 at the same skin lesions was increased by the use of CCPPS (Fig. 5B).

4. Discussion

The incidence of alopecia, which is due to the multiple genetic factors, hormones, and stressful lifestyles, is increasing [2, 15-18]. Alopecia, which is a stressful condition, can negatively affect the quality of life, including personal relationships and social lifestyle [19].

Minoxidil and finasteride are commonly used to treat androgenic alopecia (AGA) [8, 20]. However, various side effects of these agents, such as itching, redness, exfoliation, abrupt hypotension and impotence, have been reported [21-25]. Therefore, the need for research to find safer and more effective drugs persists.

CCP has been used in East Asian countries, including China and Korea, for over 2000 years to promote virility, to replenish vital essence, and to prevent diseases [26]. CCP has been reported to contain many different polypeptides, carbohydrates, sterols, and inorganic substances [27-30]. Especially, the insulin-like growth factor-1 (IGF-1) contained in CCP [28], is known to promote hair growth by affecting follicular proliferation and tissue remodeling in the hair growth cycle [31].

In this study, we first examined whether CCPPS had hair growth-promoting effects. Gross observations showed a small amount of hair re-growth in positive control group treated with normal saline whereas robust hair re-growth was induced by topical treatment with CCPPS or Minoxidil (Fig. 2). The thicknesses of the hair shafts were examined by using dermoscopy on the 14th and the 16th days after hair-depilation. The density of hair and the diameters of the hair shafts were greatly improved in the mice treated with CCPPS compared to those treated with normal saline (positive control group) (Fig. 3). Histological observation showed that in the positive control group (normal saline), although enlarged hair bulbs were observed, the newly-formed hair shafts had not reached the middle of follicles, indicating that the hair follicles had not fully developed into the anagen phase (Fig. 4, upper left panel). However, in the CCPPS-treated group, the tips of the in-

ner root sheaths and the hair shafts reached the hair canal and the bulbs resided deep in the subcutis, indicating that hair follicles had reached anagen VI (Fig. 4, upper right panel). The same skin tissue was examined by using immunohistochemical staining against BrdU and PCNA in order to observe cellular proliferation of the hair follicles. BrdU combines with DNA instead of thymidine base during the S phase of the cell cycle [32]. Slightly positive reactions to BrdU were observed around the sebaceous gland in the positive control group (normal saline; Fig. 4, middle left panel) whereas moderate levels of immune responses against BrdU were observed at the outer root sheath in the CCPPS-treated group (Fig. 4, middle right panel). PCNA, another marker of cellular proliferation, can be stained in the G1, S, and G2 phases of the cell cycle [33]. The expression of PCNA was low in the positive control group (normal saline; Fig. 4, lower left panel) whereas strong immune signals against PCNA were observed at the dermal papilla in the CCPPS and the Minoxidil-treated (negative control) groups (Fig. 4, lower middle & right panels), indicating that CCPPS induced active cellular proliferation in the dermal papilla of hair follicles.

The induction and the maintenance of the anagen phase are determined by several growth factors released from dermal papilla cells. Among them, FGF-7 is known to regulate proliferation and differentiation of hair follicular stem cells, thereby playing a critical role in hair development [34]. Hence, by observing the expressions of FGF-7 in the same skin tissues as were used for the other observations, we were able to determine whether the cellular proliferative potential of CCPPS was related with FGF-7 or not. Protein expression of FGF-7 was also examined by using immunohistochemical analyses, and the levels of mRNA expression were measured by using a RT-PCR analysis. Expectedly, CCPPS increased the expression of FGF-7 at both the protein and the mRNA levels (Fig. 5).

5. Conclusion

The hair re-growth effect of CCPPS was found to be related to the proliferation of dermal papilla cells and overlying keratinocytes, such as matrix cells, and to the increased expression of FGF-7, the growth factor to initiate anagen. Thus, we conclude that the hair re-growth effect of CCPPS on C57BL/6 mice is excellent, and that CCPPS can be used for the treatment or prevention of alopecia.

Acknowledgment

Conflict of interest

The authors declare that there are no conflict of interest.

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