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ORIGINAL ARTICLE

Effect of *Chrysanthemum zawadskii* Extract on Dermal Papilla Cell Proliferation and Hair Growth

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Background: *Chrysanthemum zawadskii* (CZ) belongs to the genus *Chrysanthemum*, also known as 'Gu-Jeol-Cho' in Korea. CZ has been used as herbal remedy to manage cough, hypertensive disorders, pharyngitis, bronchitis, gastroenteritis, pneumonia, bladder diseases and common cold. However, its effect on hair growth has not been documented. **Objective:** The aim of present study was to elucidate the beneficial effects of CZ on hair growth. **Methods:** Proliferation of follicular dermal papilla (DP) cells from human scalp skin was evaluated by MTT assay. The expression of various molecules in DP cells was checked by western blot assay. Effect of CZ extract on the hair growth was evaluated by hair organ culture and C57BL/6 mice model. **Results:** Cultivation of DP cells with CZ extract increased cellular proliferation, increased expression of phosphorylated protein kinase B (p-Akt), p-ERK, B-cell lymphoma 2, and decreased expression of Bax. Treatment of human hair follicles with CZ extract significantly enhanced hair growth. Additionally, CZ markedly shortened telogen period, increased anagen transformation and stimulated hair growth in the animal study. **Conclusion:** These results suggest that CZ extract has an effect of promoting hair growth and may therefore be a useful a therapeutic remedy for preventing hair loss. **(Ann Dermatol 32(5) 395～401, 2020)**

-Keywords-
Chrysanthemum zawadskii, Dermal papilla cells, Hair follicle , Hair growth

INTRODUCTION

Hair follicle typically undergoes three successive cyclical phases of anagen, catagen, and telogen[1,2]. A lot of genes and signaling pathways are involved in the hair cycle regulation and morphogenesis of hair follicle. Hair cycle dysregulation has been known to be closely related to the pathogenesis of androgenetic alopecia (AGA)[3-5]. The main characteristic of AGA is a gradual shortening of anagen phase and a gradual miniaturized hair follicles[6,7]. As consequence, the ability of promoting or prolongation of anagen phase is a key factor in the treatment and prevention of AGA.

Various attempts have been concentrated on the detection of effective management for hair loss with herbal remedies. For example, *Polygonum multiflorum*[8], *Schisandra nigra*[9], *Asiasari radix*[10], and Ginseng[11] have long been used in the prevention of hair loss in traditional medicine. These herbal remedies might either have a supportive role by targeting non androgen-mediated alternative mechanisms of hair loss or exert synergistic role with the anti-androgenic medicines. *Chrysanthemum* is well known perennial flower belongs to the Asteraceae family that is native plant to northeastern Europe and Asian continent[12]. *Chrysanthemum zawadskii* (CZ) belongs to the genus *Chrysanthemum*, known as 'Gu-Jeol-Cho' in Korea. It has been used in oriental medicine to cure gastroenteritis, pneumonia, cough,

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bronchitis, hypertension, pharyngitis, bladder diseases, and common cold[12,13]. Also, CZ has been known to have various pharmacological effects such as cancer prevention, anti-inflammatory, anti-oxidative, and liver cell-protecting effects[14-18]. However, the beneficial effect of CZ on hair loss has not been documented.

The present study was performed to elucidate the beneficial effect of CZ on hair growth. Proliferation of dermal papilla (DP) cells obtained from human scalp tissue was evaluated by MTT assay. The expression of various molecules in DP cells was evaluated by western blot assay. Promoting effect of CZ extract on the hair growth was evaluated by hair organ culture and C57BL/6 mice model.

MATERIALS AND METHODS

Ethical considerations

This study was reviewed and approved by the Ethics Committee of the Institutional Review Board of Yonsei University Wonju College of Medicine (Approval number: YWMR-13-04-009). A written informed consent was collected from all subjects according to the Declaration of Helsinki. Animal study was conducted according to the institutional guidelines for animal care, at experimental facility for animal in Yonsei University Wonju College of Medicine.

Preparation of CZ extract

One hundred gram of whole dried plants of CZ (Bioland, Cheonan, Korea) were extracted with 1,000 ml of 95% ethanol for 120 minutes under reflux and then filtered with filtering paper (Whatman No. 5; Whatman International Ltd., Kent, England, UK). The liquid from the filtering process was concentrated in a vacuum evaporator. The resultant residue was weighed as 19.5 g, and then dissolved in 70% ethanol for treatment for *in vivo* study subsequently. CZ extracts were then filtered to sterilize for *in vitro* study using 0.45 μm-filter units (Millipore, Burlington, MA, USA).

Isolation of human hair follicles

Occipital scalp skin samples were obtained from healthy volunteers with no previous or current scalp diseases. Anagen hair follicles were isolated under a stereo microscope as mentioned previously[19,20] and carefully moved onto a 24-well plate containing 500 μl of William's E medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10 ng/ml of hydrocortisone (Sigma, St. Louis, MO, USA), 10 μg/ml of insulin (Sigma), 2 mM of L-glutamine (Gibco), 100 μg/ml of streptomycin and 100 IU/ml of penicillin (Gibco) at 37°C in 5% CO_2 incubator.

Dermal papilla cell culture

Isolation and cultivation of DPs from hair follicles were done as mentioned previously[21]. DPs were moved to a culture dish, and cultivated in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% of fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 μg/ml of streptomycin and 100 IU/ml of penicillin (Gibco) at 37°C in 5% CO_2 incubator. DP cells in second and third passage were used in this experiment.

Viability assay

Viabilities of DP cells were measured by MTT assay. DP cells (1×10^4) were inoculated into 96-well plates, and cultured with CZ extract (0~1,000 ppm) for 24 hours. MTT solution (5 mg/ml, 20 μl) was added per well and incubated at 37°C for 4 hours. The supernatant fluid was then aspirated, and the crystalized formazans were dissolved in 100 μl of dimethyl sulfoxide. Absorbance was spectrophotometrically checked at 570 nm using ELISA reader. Cell viability was calculated as the percentage of absorbance of CZ treated groups divided into the absorbance of control group (CZ untreated cells).

Western blot analysis

After treatment, whole cell protein was collected using Qproteome Mammalian Protein Prep Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol, and then protein content was measured using a Bradford protein assay. Equal amounts of cell proteins were separated by SDS-PAGE and immunoblotted with primary antibodies that against total protein kinase B (Akt), phosphorylated Akt (p-Akt), total extracellular signal-related kinase (ERK), p-ERK, B-cell lymphoma 2 (Bcl-2), Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (Sigma) and then were identified using horseradish peroxidase (HRP)-labeled secondary antibodies. Blots were visualized with enhanced chemiluminescence detection reagents (Santa Cruz Biotechnology).

Human hair follicle organ culture

CZ extract was supplemented to culture media at final concentrations of 0.1, 1, and 10 ppm at 2 day interval and the hair shaft prolongation in each follicle was calculated under a stereomicroscope (Olympus, Tokyo, Japan). In addition, follicular morphology was defined and then calculated the hair cycle score as mentioned previously: anagen VI follicles, 100; early catagen follicles, 200; mid catagen follicles, 300; late catagen follicles, 400[2].

Immunofluorescence staining

Ki-67 immunofluorescence staining was performed on 4 μm tissue slices of hair follicles as mentioned previously[2]. After de-paraffinized and rehydrated process, slices were fixed in 4% paraformaldehyde supplemented with 0.1% Triton X-100 for 10 minutes, equilibrated in phosphate buffer saline for 15 minutes and blocked in 4% normal donkey serum for 60 minutes. The slices were then incubated with anti-ki67 mouse monoclonal antibody (Abcam, Cambridge, MA, USA) overnight at 4℃, followed by incubation with Alexa Flour 488 conjugated donkey anti-mouse IgG (Abcam) for 60 minutes. And then, fluorescent images were analyzed using a confocal microscope (Leica Microsystems GmbH, Bannockburn, IL, USA).

Anagen induction assay *in vivo*

Six-week-old female C57BL/6 mice obtained from Orient Bio Inc. (Seongnam, Korea) were housed under a standard laboratory conditions. After a week of adjustment, anagen induction was performed on the dorsal skin of mice (seven-week-old, in telogen phase) by depilation, as reported previously[2,22]. The CZ extract (3%) was applied daily for 30 days. Minoxidil topical solution (3%, Hyundai Pharmaceuticals Co. Ltd., Cheonan, Korea) was employed as positive control. The dorsal skin of the mice was photographed once every 10 days for 30 days while observing changes in hair regrowth. Additionally, hematoxylin and eosin staining was performed for the histological examination. Dorsal skin of mice was fixed in 10% formaldehyde solution (pH 7) and embedded in paraffin blocks. Serial slices were longitudinally cut into 4 μm interval and coated on glass slides. Subsequently, slices were de-paraffinized, hydrated and stained with hematoxylin and eosin.

Statistical analysis

All statistical analyses were determined by the Student's t-test or multivariate ANOVA using the IBM SPSS statistical software (ver. 20, IBM Corp., Armonk, NY, USA). The results are marked as mean±standard deviation of at least triplicate experiments. A *p*-value of less than 0.05 was regarded to have a statistical significance.

RESULTS

Effect of CZ extract on the proliferation of follicular DP cells

Effect of CZ extract on the DP cells proliferation was measured by MTT assay. CZ extract markedly increased the DP cells proliferation compared to the vehicle-treated negative control, and the DP cells were proliferated in 1ppm and 10ppm CZ more efficiently (Fig. 1). The enhancement was greatest at 10ppm CZ (Fig. 1). Furthermore, at concentrations of 1,000 ppm, CZ showed cytotoxic effects (Fig. 1).

Expression of various molecules in CZ extract treated DP cells

Western blot analysis was performed to evaluate the expression of various factors that are closely associated to cell apoptosis and proliferation as reported previously[19,23]. CZ dose dependently enhanced phosphorylation process of Akt and ERK, increased Bcl-2 expression, and reduced Bax expression (Fig. 2). These results show that CZ prevents cellular death and influences intracellular signaling cascades by increasing Bcl-2/Bax ratio, promotes cellular survival by phosphorylation of Akt and ERK upregulation.

CZ prolongs anagen and promotes human hair growth

Human hair follicles were treated every other day with 0.1, 1, or 10 ppm CZ, and were cultivated for 8 days. One or 10 ppm CZ increased the length of hair follicle significantly (1,591.67±86.59 μm or 1,636.04±77.01 μm) ($p<0.05$) compared with control hair follicles (1,327.43±79.78 μm) (Fig. 3A). CZ treatment retarded development of catagen phase in quantitative morphometric evaluation of follicles (Fig. 3B). Hair cycle score was reduced significantly in the 1 or 10 ppm CZ treated hair follicles (185.32±10.68 or 175.68±12.74) ($p<0.05$) compared with control hair follicles (245.78±10.90) (Fig. 3C). Proliferation marker ki-67 positive cells were increseased significantly in 10 ppm CZ treated follicles (62.33±4.50) (*p*

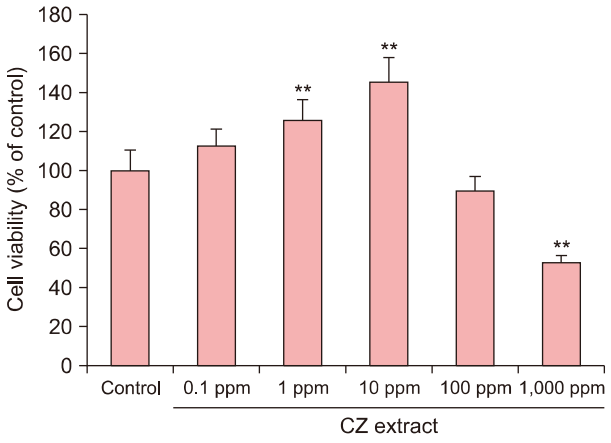

Fig. 1. Viability assay of follicular dermal papilla (DP) cells. Follicular DP cells were cultured with *Chrysanthemum zawadskii* (CZ) extract (0~1,000 ppm) for twenty-four hours. Values are presented as mean±standard deviation. $^{**}p<0.01$, compared to the vehicle treated control.

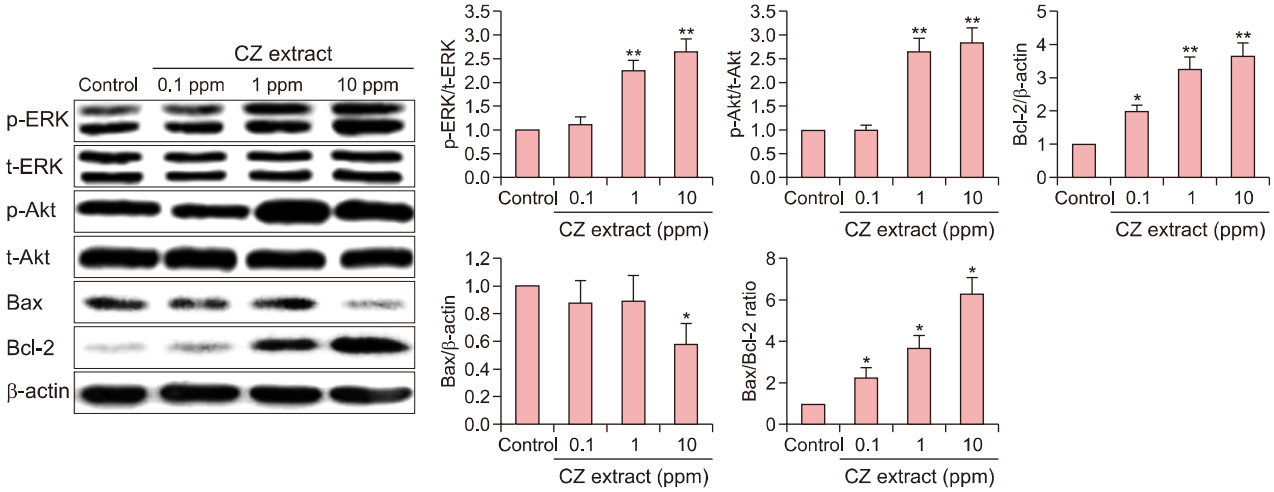

Fig. 2. Western blot analysis. Follicular dermal papilla (DP) cells were cultured with varying concentrations of CZ extract and analyzed by western blot. Values are presented as mean±standard deviation. CZ: *Chrysanthemum zawadskii*, ERK: extracellular signal-related kinase, Akt: protein kinase B, Bcl-2: B-cell lymphoma, Bax: Bcl-2-associated X protein. *$p<0.05$, **$p<0.01$, compared to the vehicle treated control.

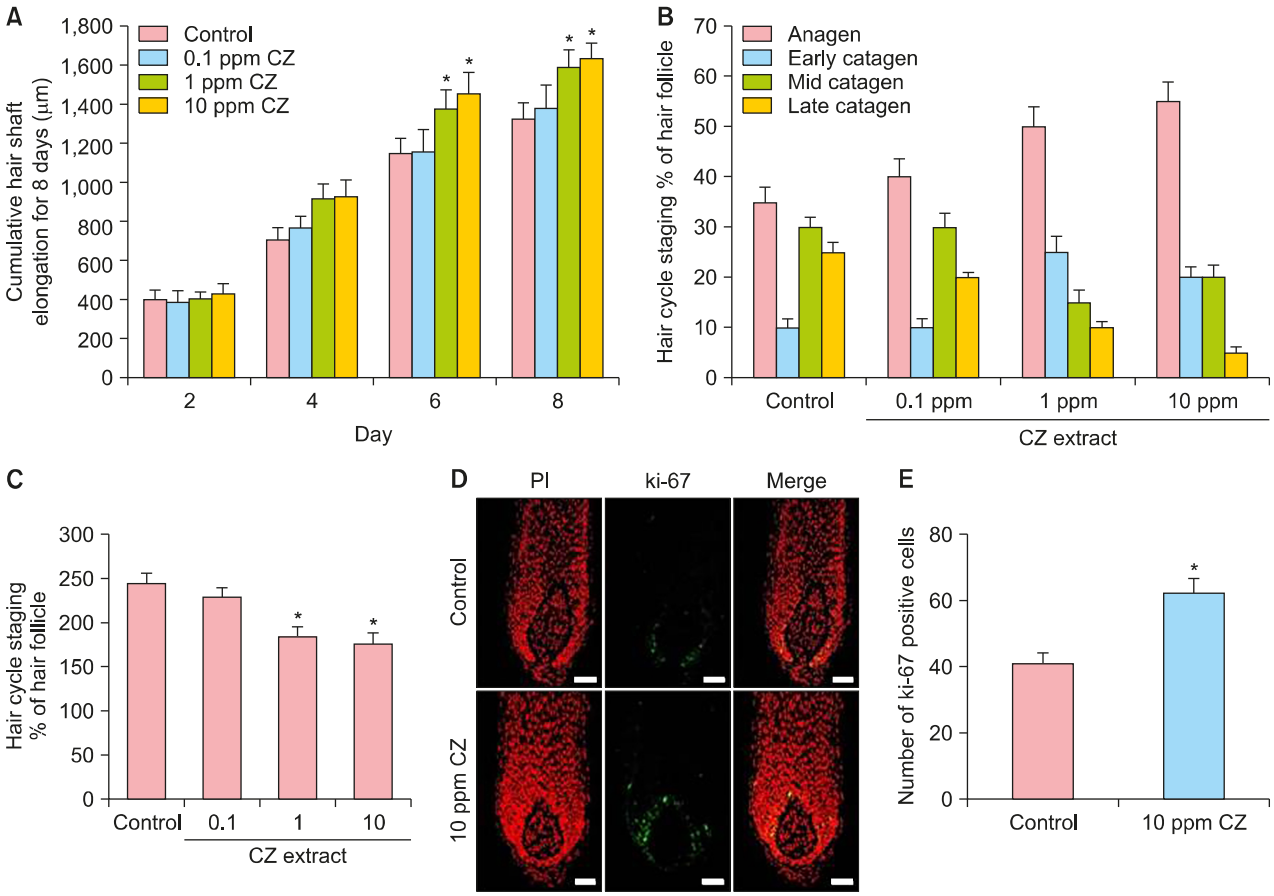

Fig. 3. Effects of *Chrysanthemum zawadskii* (CZ) extract on human hair growth and cycling. Hair follicle organ culture was performed over 8 days. (A) Hair follicle length was evaluated every other day until 8 days. (B) Evaluation of staging of hair cycle (i.e., anagen follicles, early catagen follicles, mid catagen follicles, late catagen follicles). (C) Evaluation of score of hair cycle. (D) Immunofluorescence staining of ki-67 (green fluorescence for ki-67-positive cells; red fluorescence for propidium iodide [PI]). Bar=0.1 mm. (E) Quantification of proliferating dermal papilla (DP) cells. Values are based on 20~30 hair follicles per group, and are presented as mean±standard deviation (*$p<0.05$).

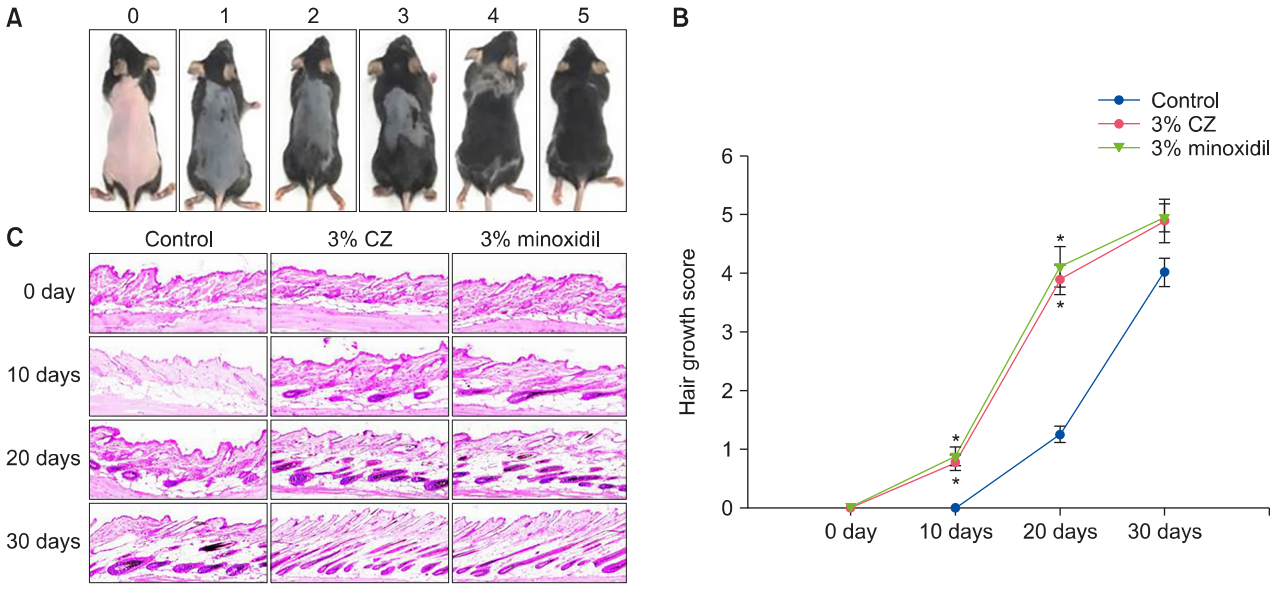

Fig. 4. Anagen induction assay in C57BL/6 mice. After depilation, anagen induction was performed on the dorsal skin of mice. (A) The score of mice hair regrowth was assessed as follow: 0, no hair regrowth; 1, 0%～20% regrowth; 2, 20%～40% regrowth; 3, 40%～60% regrowth; 4, 60%～80% regrowth; 5, 80%～100% regrowth. (B) Quantitative analysis of the score of mice hair regrowth (C) Hematoxylin and eosin stain. Bar=0.1 mm. Values are presented as mean±standard deviation. CZ: *Chrysanthemum zawadskii*. *$p<0.05$, compared to the control.

<0.05) compared with the control follicles (41.17±3.19) in quantitative histomorphometric study (Fig.3D, E).

Effect of CZ extract on hair growth in C57BL/6 mice

Promoting effects of CZ extract on hair growth was studied using 6 weeks old C57BL/6 mice, as mentioned previously[22]. The back skin of C57BL/6 mice is well known to show an age dependent cyclic hair growth. While depilated skin is pink in the telogen stage, it changes as initiation of anagen phase with a dark color, subsequently becomes gray. We studied whether induction of anagen phase was stimulated by CZ extract after depilation induced hair cycle synchronization. Treated groups with both 3% minoxidil and 3% CZ extract revealed remarkable induction of hair growth and their hair shaft were seen at 10 days. On the other hand, the color of control group was left in pink until 10 days as shown in Fig. 4. Histological examination revealed that CZ profoundly increased the length, size and number of hair follicles compared to the control mice. These data indicate that CZ extract treatment promotes hair growth and anagen induction in C57BL/6 mice.

DISCUSSION

DP cells are specialized fibroblasts forming a diamond or oval shaped mass that is totally encapsulated by the epithelial cells of the matrix. The size of the DP is associated with the overall size of hair follicle and hair shaft. In general, a larger DP produces larger hair follicles and thicker shafts[24,25]. In this experiment, CZ was shown to stimulate proliferation of follicular DP cells at concentration range from 0.1 to 10 ppm. These results show that promoting effects of CZ in hair growth may be mediated through mitogenic effects in the DP region.

A number of signaling pathways leading to cell survival in DP cells include Akt, ERK, and Bcl-2 family et al. The effects of the ERK signaling pathway in cellular growth and mitogenesis have been well documented[26]. Akt, a serine/threonine protein kinase, is known to be a major regulator of cell proliferation and is initiated by growth factors in a PI3K-dependent manner[27]. In our study, CZ significantly increased the phosphorylation process of Akt and ERK, suggesting that activation of Akt and ERK by CZ extract may increase the proliferation of DP cells. Also, further studies are needed to perform blocking experiment with specific inhibitor of the ERK and Akt/PI3K pathway. The Bcl-2 protein is composed of more than a dozen members that are basically either pro- or anti-apoptotic and seem to have a role of gatekeepers in the apoptotic process[28]. It is well documented that the Bcl-2 gene product, as an inhibitor of apoptosis, forms a heterodimer complex with pro-apoptotic substance Bax and neutralizes the pro-apoptotic process[28]. In our study, CZ was found to significantly

increase the expression of an anti-apoptotic molecule Bcl-2, but decrease the expression of of a pro-apoptotic molecule Bax in cultured DP cells. These results suggest that CZ increases survival of DP cells at least through the down-regulation of Bax, and up-regulation of Bcl-2 signaling.

The culture system of hair follicle has been known to be a useful model for evaluating various follicular function[29]. In our study, 1 or 10 ppm CZ increased length of hair follicle significantly compared to the control group ($p<0.05$). CZ treatment retarded development of catagen phase in quantitative morphometry of follicles. Hair cycle score was decreased significantly in 1 or 10 ppm CZ treated follicles. Matrix cell proliferation in hair bulb is an important aspect for hair growth in anagen phase[5]. Ten ppm CZ treated hair follicles revealed marked increase of ki-67 reactive cells, showing that CZ induces cellular proliferation of hair bulb matrix. We also studied promoting effect of CZ extract on hair growth using 6 weeks old C57BL/6 mice, which are useful model for evaluating various agents for hair growth due to their truncal pigmentation[22]. In our study, CZ extract were topically applied onto the back skin of mice for 30 days after depilation. Treatment of CZ remarkable induced hair growth and their hair shaft were seen at 10 days. On the contrary, no definite hair growth was found in the control group at same time. In addition, the histo-morphometric evaluation showed that CZ extract treatment accelerated the earlier transformation of telogen hair follicle to the anagen phase. These data suggest that CZ extract have a direct promoting effect on anagen induction in C57BL/6 mice.

In conclusion, the present study was performed to clarify the beneficial effects of CZ on hair growth by *in vitro* and *in vivo* assays. CZ extract treatment increased cellular proliferation, increased expression of p-Akt, p-ERK, Bcl-2, and decreased expression of Bax in DP cells. CZ extract significantly enhanced human hair growth and profoundly promoted anagen induction in C57BL/6 mice. All these results indicate that CZ extract has an effect of promoting hair growth. Accordingly, it may be used as an agent for hair loss prevention. As like many of the materials studied previously, further studies would be required for CZ to validate its actual effect on hair growth in the human body.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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None.

DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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