Hair Growth Promoting Effect and Action Mechanism of *Chrysanthemum Zawadskii* Extract

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Hair Growth Promoting Effect and Action Mechanism of *Chrysanthemum Zawadskii* Extract

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Hair Growth Promoting Effect and Action Mechanism of *Chrysanthemum Zawadskii* Extract

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Hair loss, medically referred to as alopecia, is a common condition that causes serious distress to those affected by this disease. The key feature of hair loss is a progressive shortening of anagen duration with a resultant progressive miniaturization of terminal hairs to vellus-like hairs. Therefore, promoting or prolonging hair follicle anagen duration is a key element in the development of remedies for the treatment and prevention of hair loss.

This study was conducted to evaluate the promoting effect and action mechanism of *Chrysanthemum zawadskii* (CZ) extract on hair growth. CZ extract was applied topically onto the back of depilated C57BL/6 mice every

day for 30 days. Hair shaft elongation was measured using an organ culture model of human scalp hair follicles. Proliferation of human dermal papilla (DP) cells was determined by MTT assay. mRNA expression level of growth factors related to hair growth was determined by reverse transcription polymerase chain reaction.

CZ extract was shown to induce an earlier conversion of telogen to anagen and promoted hair growth in the C57BL/6 mouse model. When human hair follicles were cultured in the presence of CZ extract for 8 days, CZ extract significantly promoted hair growth and prolonged anagen duration by reducing apoptosis and inducing proliferation of hair follicle cells in the bulb region. Treatment of human DP cells with CZ extract resulted in an increase of cell proliferation, increase of hair growth promoting factor (vascular endothelial growth factor, VEGF; insulin-like growth factor 1, IGF-1) expression and decrease of hair inhibitory factor (transforming growth factor β1, TGF-β1) expression. In particular, isolated jaceosidin and eupatilin, components from CZ extract, promoted the growth of human hair follicles, increased the proliferation of DP cells, induced VEGF, and reduced TGF-β1 expression.

These results suggest that CZ extract and its principal components, jaceosidin and eupatilin, have hair growth promoting potential through the regulation of growth factors in DP cells and promotion of DP cell proliferation.

Key Words: *Chrysanthemum zawadskii*, Hair follicle, Dermal papilla, Growth factor, Hair cycle.

I. INTRODUCTION

1.1. Structure of the hair follicle

A hair follicle is a complex mini-organs in the skin appendage that produces hair (i.e., a hair shaft). The mature hair follicle can be divided into three functional segments, the lower segment (bulb and supra-bulb), the middle segment (isthmus), and the upper segment[1]. The upper segment, called the infundibulum, extends from the entrance of the sebaceous gland duct to the follicular orifice. The isthmus is a shortened segment of the hair follicle, extending from the attachment of the erector pili muscle into the entrance of the sebaceous gland duct. The lower isthmus harbors epithelial and melanocytic hair follicle stem cells in the so-called bulge region[2]. The supra-bulbar area of the follicle is the region below the isthmus and above the bulb. The hair bulb, which is the actual hair shaft factory, is mainly composed of epithelial and mesenchymal compartments. The interaction between the dermal papilla (DP), a group of hair follicle specific mesenchymal cells, and matrix cells, a group of hair follicle specific epithelial cells, plays an important role in the morphogenesis and growth of the hair follicle[3-4].

The DP consists of an oval mass of fibroblasts that is completely encapsulated by the matrix epithelium. The size of the DP correlates with the size of the hair follicle and the size of the produced hair shaft, meaning a

larger DP correlates with larger hair follicles and produces thicker hair shafts[2, 4].

Matrix cells in the lower part of hair bulb have a high mitotic rate, while matrix cells in the upper part have a low mitotic rate and can differentiate (keratinize) into six different types of epithelial cells, including the Henle layer of the inner root sheath (IRS), the Huxley layer of the IRS, the cuticle of the IRS, the cuticle of the hair, the cortex, and the medulla, each of which keratinizes at a different level. The outermost layer of the IRS, the Henle layer, keratinizes first, thus establishing a firm coat around the soft central parts. The two apposed cuticles covering the inside of the IRS and the outside of the hair keratinize next, followed by the Huxley layer, and the hair cortex and medulla. The innermost cuticle layer of the IRS is interlocked with the cuticle of the hair shaft surface. This allows the IRS and hair shaft to move together during the growth period. At the level near or slightly below the sebaceous gland duct, the IRS breaks down and the hair shaft is separated from the IRS[4].

The hair shaft is generally constructed of three layers that are derived from the differentiated and keratinized matrix cells, the medulla (innermost part of hair shaft), the cortex (middle part of hair shaft), and the cuticle (outermost part of hair shaft). The major structural proteins of hair shaft are cysteine-rich keratins[5].

1.2. Hair growth cycle

During postnatal life, hair follicles cyclically undergoes three alternating phases of rapid growth and hair production (anagen), apoptosis-mediated regression (catagen), and relative quiescence (telogen)[1]. In the anagen phase, the hair matrix cells, which represent transient amplifying cells derived from epithelial hair follicle stem cells in the bulge, proliferate intensively and differentiate into distinct epithelial hair lineages[2-3, 6-7]. During catagen, the hair follicle rapidly regresses due to apoptosis of the matrix, IRS and outer root sheath(ORS) keratinocytes, while the bulge hair follicle stem cells escape apoptosis. Eventually, the lower hair follicle becomes reduced to an epithelial strand, bringing the DP into close proximity with the bulge[2]. During telogen, hair follicles enter a phase of relative quiescence, lasting for several days in the first telogen stage and typically up to at least 3 weeks in the second telogen stage[2].

1.3. Molecular regulation of hair growth

1.3.1. Action of androgens on hair growth

Androgens regulate human hair growth, but their effects vary depending on body site. After puberty, the conversion of short and thin hair (vellus hair) into long and thick hair (terminal hair) takes place. Androgens stimulate hair

growth in the beard, but suppress general hair growth in androgenetic alopecia (AGA); this reciprocal effect is known as the 'androgen paradox'[8-9].

The primary and most well-known androgen is testosterone. In general, testosterone can freely penetrate cellular membranes and subsequently enter into the cytoplasm, where it is converted to the more potent androgen dihydrotestosterone (DHT) by cytoplasmic 5α-reductase. DHT strongly binds to androgen receptors (AR) located in the cytoplasm of target cells and the AR-DHT complex is translocated to the nucleus after dimerization. AR co-activators are recruited to the AR-DHT complex, which can then bind to the androgen-response elements on the DNA[10]. AR is localized in DP, but not in epithelial cells, indicating that the DP is an actual target site for androgen in hair follicle[10-12]. Previous studies have demonstrated that AR expression is significantly higher in AGA DP cells than in non-balding DP cells[13-15], indicating that AR is one of the key molecules that regulate androgen sensitivity in the DP.

1.3.2. Action of growth factors and apoptosis related molecules in hair growth

Although the precise regulatory mechanism of the hair growth cycle is still unclear, interactions between the DP and overlying follicular matrix epithelial cells are critical for hair growth control[3]. The DP is a known target site for

androgen action. Androgen affects DP cells though the production of paracrine signals, such as the transforming growth factor-β1 (TGF-β1), insulin like growth factor-1 (IGF-1), and vascular endothelial growth factor (VEGF)[16-20].

IGF-1, via binding to IGF-1 receptors, regulates cell proliferation, differentiation and survival[21]. IGF-1 has been identified as one of the androgen-dependent paracrine growth factors in the DP cells[22]. IGF-1 has been found to stimulate human hair growth *in vitro* at physiologic concentrations and to prevent the premature entry of cultured hair follicles into catagen[23].

TGF-β1 is a secreted polypeptide that performs many cellular functions, including the control of cell growth, cell proliferation, cell differentiation and apoptosis[24]. TGF-β1 is known to inhibit human keratinocyte growth *in vitro*[25] and induce catagen progression in mouse hair growth *in vivo*[26]. In AGA progression, TGF-β1 as an androgen-inducible growth suppressor derived from balding DP cells suppresses hair growth through follicular keratinocyte growth inhibition[27].

VEGF is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions[28]. Transgenic over-expression of VEGF in hair follicle epithelial cells strongly induced perifollicular vascularization and led to accelerated hair regrowth after depilation. Moreover, VEGF as a major mediator of hair follicle growth and cycling that could promote hair growth[29].

Recently, several groups have investigated the role of apoptosis in alopecia [30-33]. Testosterone, the primary and most well-known androgen, induces apoptosis in balding DP cells in a dose-dependent and time-related manner. In addition, testosterone reduces expression of the B-cell lymphoma 2 (Bcl-2), an anti-apoptotic molecule, but induces the expression of Bcl-2-associated X (Bax), a pro-apoptotic species leading to an increase in the Bax/Bcl-2 ratio[33]. Finasteride, which is a type II 5α-reductase inhibitor, also influences the expression of caspase and X-linked inhibitor of apoptosis (XIAP) in hair follicle cells, thereby signaling anagen induction[30].

1.4. Treatment of hair loss

AGA is the most common hair loss disorder, affecting both men and women. The key feature of AGA is a progressive shortening of anagen duration and a resultant progressive miniaturization of terminal hairs to vellus-like hairs[34]. Therefore, the ability to prolong or promote anagen duration of the hair follicle is a key element in the development of remedies for the treatment and prevention of AGA.

Minoxidil and finasteride are two drugs that have been approved for AGA treatment by the Food and Drug Administration(FDA)[35]. Minoxidil, an oral hypotensive drug that causes hypertrichosis when given systemically, is available as a topical solution. Minoxidil promotes the survival of DP cells,

prolongs the anagen phase, and results hair shaft diameter enlargement. Clinically, apparent success is best in early cases (<10 years) of limited extent (<10 cm diameter bald area on the vertex) where pretreatment hair density is above 20 hairs/cm^2 [36]. Finasteride is given orally at a dose of 1.0 mg daily, and is effective in preventing further hair loss and in increasing hair count to the point of cosmetically appreciable results in men aged 18 to 41 with mild to moderate hair loss at the vertex, the anterior midscalp, and the frontal region[36]. However, the fact that 20-30% of AGA patients receiving these drugs are non-responders encourages a search for alternative substances for treatment.

1.5. *Chrysanthemum zawadskii*

Many attempts have focused on the discovery of effective hair loss treatments from traditional herbal medicines. For instance, *Polygonum multiflorum*[37], *Schisandra nigra*[38], *Asiasari radix*[39], and *Ginseng*[40] have been traditionally used for treating hair loss in oriental medicine. These substances may either exert a complementary effect by targeting alternative non-androgen-related mechanisms of AGA or act synergistically with the established anti-androgenic drugs.

Chrysanthemum is a perennial flowering plant in the *Asteraceae* family that is native to Asia and northeastern Europe[41]. *Chrysanthemum zawadskii* (CZ) is one of the species of the genus *Chrysanthemum*, known as Gu-Jul-Cho in

Korea and is used in traditional medicine to treat pneumonia, bronchitis, cough, common cold, pharyngitis, bladder-related disorders, gastroenteric disorders, and hypertension[42]. CZ has a variety of pharmacological properties, including cancer protective, anti-oxidative, anti-inflammatory and liver-protective effects[43-47]. However, the effect of CZ on hair growth has not yet been reported.

II. PURPOSE

Hair loss, medically referred to as alopecia, is a common condition that causes serious distress to those affected by this disease. The key feature of hair loss is a progressive shortening of anagen duration with a resultant progressive miniaturization of terminal hairs to vellus-like hairs. Therefore, promoting or prolonging hair follicle anagen duration is a key element in the development of remedies for the treatment and prevention of hair loss.

This study was conducted to evaluate the promoting effect and action mechanism of CZ extract on hair growth. In addition, this study was conducted to find active component through isolation of CZ extract and to evaluate the promoting effect and its action mechanism of active components of CZ extract.

III. MATERIALS & METHODS

3.1. Preparation of CZ extract

CZ(Bioland, Chunan, Korea) was extracted with 80% ethanol (ETOH) at room temperature for 2 h under reflux and filtered through filter paper (Whatman Grade No.5). The extract manipulation was repeated and the combined filtrates were evaporated under reduced pressure to obtain CZ extract.

3.2. Isolation of active components from CZ extract

CZ extract was evaporated in vacuum, suspended in water, and was stepwise fractionated with ethyl acetate and butanol. The active compounds isolated from the ethyl acetate fraction were isolated by High Performance Liquid Chromatography (Alliance 2695, Waters, Zellik, Belgium) and identified as eupatilin and jaceosidin (Figure 1) through comparison of their spectroscopic data with those previously reported in the literature[48].

3.3. Animal study

Female C57BL/6 mice (6 weeks of age) were purchased from Orient Bio (Seongnam, Korea) and provided with a standard laboratory diet and water *ad libitum*. Protocols approved by the Institutional Animal Care and Use

Committee (IACUC) of the Aekyung Corporation Central Research Laboratories were used to care for all animals. Anagen was induced on the back skin of C57BL/6 mice that were in the telogen phase of the cycle by depilation, as described previously[7]. Briefly, 6-week-old female C57BL/6 mice were allowed to adapt to their new environment for one week. Anagen was then induced in the back skin of the 7-week-old female C57BL/6 mice by depilation, which led to synchronized development of anagen hair follicles[7]. On the following day, 0.2 ml of 3% CZ extract in 50% ethanol was topically applied every day for 30 days. Minoxidil (5%, MINOXYL™, Hyundai Pharm.Co.Ltd., Chunan, Korea) was used as a positive control. The back skin of the mice was then observed and photographed at 0, 10, 20 and 30 days after depilation.

3.4. Isolation and culture of human hair follicles

Human occipital scalp skin specimens were obtained from hair transplantation surgery after informed consent. A total of 10 donor samples were obtained from five different individuals. The Institutional Ethics Committee of the Yonsei University Wonju College of Medicine, Wonju, Korea, approved all described studies. The study was conducted according to the Declaration of Helsinki Principles. Human hair follicles in anagen stage VI were isolated as previously described[49]. Briefly, after separation of the

epidermis and dermis from the dermo-subcutaneous interface, anagen hair follicles were isolated from the subcutaneous fat under a light microscope with watchmaker's forceps and maintained in Williams E medium (Invitrogen, Gaithersburg, MD, USA) supplemented with 10 μg/ml insulin (Sigma, St. Louis, MO, USA), 10 ng/ml hydrocortisone (Sigma, St. Louis, MO, USA), 2 mM L-glutamine (Invtrogen, Gaithersburg, MD, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Gaithersburg, MD, USA). Hair follicles were maintained free-floating at 37°C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. Control groups were treated with vehicle (DMSO diluted 1:1000 in Williams E medium). Test groups were treated with CZ extract (1.0 ppm, 10 ppm) or Jaceosidin (1 μM , 10 μM) or Eupatilin (0.1 μM , 1 μM).

3.5. Measurement of hair follicle length and morphology

Hair follicle length was defined as the entire length from the base of the hair bulb to the tip of the hair shaft using measuring scales attached to the objective lens of the microscope. Measurements were taken at 2 day intervals until the 8^{th} day of cultivation and were then statistically analyzed. At the same time, hair follicle morphology (anagen, early catagen, mid catagen, and late catagen) was observed and the hair cycle score was measured according to the

following system: anagen VI, 100; early catagen, 200; mid & late catagen, 300 (Figure 10).

3.6. Isolation and culture of DP cells

DP cells were isolated from hair follicles according to the methods previously described[50]. Subsequently, DP cells were transferred onto a plastic dish, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD, USA) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. Sec and third passage DP cells were used in this study.

3.7. Isolation and culture of follicular keratinocytes (FKCs)

For the culture of FKCs, anagen hair follicles were cut off from the hair bulb region, and then the dermal sheaths were removed from the upper part of hair follicles. Hair shafts, including part of ORS were treated with 0.05% trypsin-EDTA (Invitrogen, Gaithersburg, MD, USA). The dissociated cells were rinsed in DMEM (Gibco BRL) supplemented with 10% FBS (Hyclone) and centrifuged for 5 min at 1500 rpm. Cells were then resuspended in EpiLife medium (Cascade Biologics, Portland, OR, USA) with EpiLife Defined Growth Supplement (Cascade Biologics, Portland, OR, USA), penicillin (100 IU/ml), streptomycin (100 μg/ml), seeded onto culture dishes, and cultured in

an atmosphere of 5 % CO_2 and 95 % air in a humidified incubator. Sec passage FKCs were used in this study.

3.8. Cell viability assay

Cell proliferation was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays[51]. Briefly, 1×10^4 cells were seeded in each well containing 100 μl growth medium in a 96-well plate. Cells were allowed to adhere for 24 hours, and then treated with serial dose of CZ extract (from 0.1 ppm to 1000 ppm), jaceosidin (from 0.1 μM to 1000 μM) or eupatilin (from 0.01 μM to 1000 μM) for 1 to 2 days. After treatment, the medium in each well was removed and replaced with PBS solution containing 5 mg/ml MTT, and then the plate was incubated at 37°C for 4 h. All remaining supernatant was then removed and 100 μl DMSO was added to each well and mixed thoroughly to dissolve the crystallized formazan. After 10 min of incubation to ensure all formazan crystals were dissolved, the optical density at 570 nm was determined using an ELISA reader. The mean absorbance of the treated group were expressed as the cell viability percentage of the control group absorbance.

3.9. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from isolated DP cells using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol Reagent; Gibco, Rockville, MD, USA). The concentration of RNA was determined by UV absorbance at 260 nm. Aliquots (1.0 μg) of RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (MML-V RTase, Promega). RNA samples were incubated at 70℃ for 10 min with molecular biology grade water. After chilling on ice, primer extension and reverse transcription were conducted by the addition of 1X RT-buffer, 5.0 mM Mgcl2, 1.0 mM deoxynucleotide triphosphates (dNTPs), 2.5 μM Oligo d(T)16 (Roche, Mannheim,Germany) and MML-V RTase (2.5 U/μl) in 20 μl reaction volume. Samples were then incubated at 42℃ for 45 min before storage at -20℃. One microliter of cDNA was then subjected to PCR cycles as follows: 95ºC denaturation for 5 min, followed by 35 cycles of 95ºC for 1 min, 60 ºC for 1 min, and 72 ºC for 1 min. An additional extension for 10 min at 72 ºC was carried out after the last cycle. The primers used for amplifying the respective fragments are listed in Table 1. The PCR product was visualized on a 2% agarose gel.

3.10. Hematoxylin and eosin (H&E) staining

Mouse dorsal skin was collected from each group on days 0, 10, 20 and 30 days of treatment. The dorsal skin was fixed in 10% buffered formaldehyde,

pH 7, and embedded in paraffin. Serial sections of 4 μm were cut and mounted on slides. Sections were deparaffinized with xylene, hydrated in a descending series of graded ethanol, and stained with hematoxylin for 2 min, followed by washing for 2 min and eosin staining for 5 seconds.

3.11. Immunofluorescence staining

To evaluate apoptotic cells in hair follicles, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) was performed using the ApopTag Plus peroxidase in situ apoptosis detection kit (Chemicon, Billerica, MA, USA) according to the manufacturer's instructions. In brief, paraffin sections were digested with 20 μg/mL of proteinase K for 15 min at room temperature and reacted with terminal deoxynucleotidyl transferase enzyme for 60 min at 37°C. TUNEL-positive cells were visualized by an antidigoxigenin fluorescein antibody. Sections were then counterstained with propidium iodide and visualized using a Leica TCS-SPE confocal microscope (Leica Microsystems, Bannockburn, IL, USA). The number of cells positive for TUNEL immunoreactivity was counted per hair bulb.

3.12. Statistical analysis

Data handling and drawing were processed using the SPSS (15.0) for Windows statistical package (SPSS Inc, Chicago, IL, USA). The results are

expressed as the mean ± standard deviation(SD). The Student's t-test was used to analyze data between the two groups and a p value <0.05 was considered statistically significant.

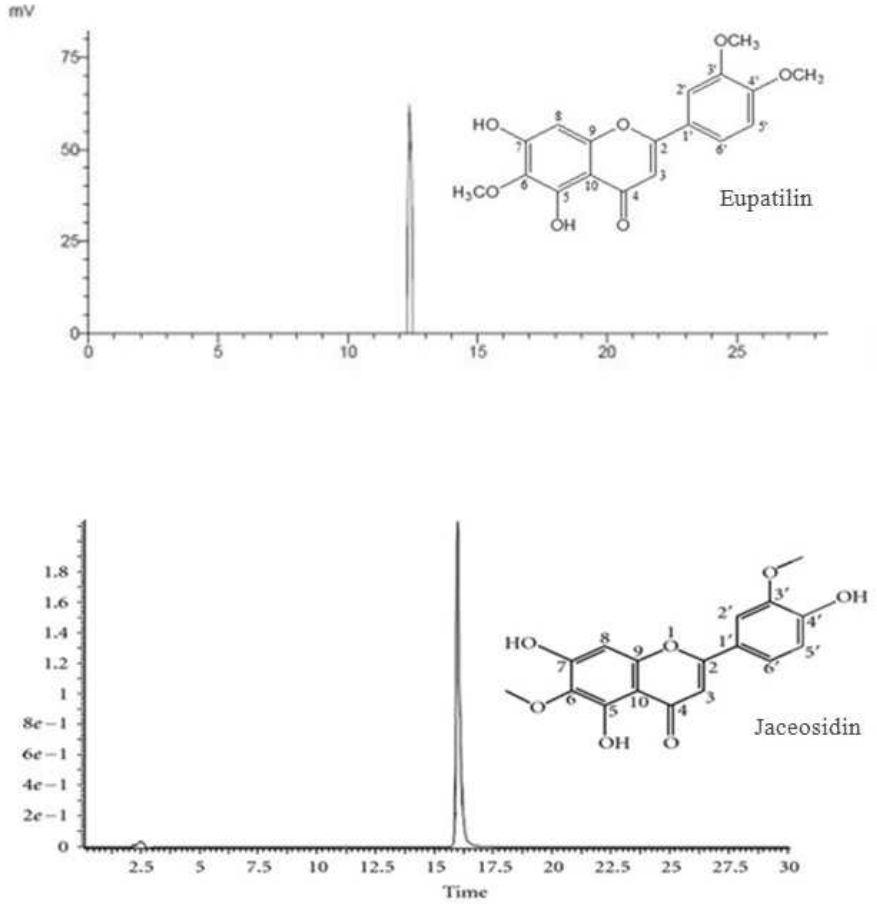

Figure 1. High performance liquid chromatography (HPLC) chromatogram and eupatilin and jaceosidin structures isolated from CZ extract.

Table 1. Primer Sequences for RT-PCR

Primers	Forward	Reverse
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC
IGF-1	ATGCACACCATGTCCTCCTC	GGTCTTCCTACATCCTGTAG
VEGF	CCCTGATGAGATCGAGTACATCTT	ACCGCCTCGGCTTGTCAC
TGF-β1	ACCGCCGCACAACTCCGGTGAC	ATCTATGACAAGTTCAAGCAGAGTA
Bax	ACCAAGAAGCTGAGCGAGTGTC	TGTCCAGCCCATGATGGTTC
Bcl-2	CGACGACTTCTCCCGCCGCTACCGC	CCGCATGCTGGGGCCGTACAGTTCC

GAPDH: glyceralbehydes-3-phosphate dehydrogenase, IGF-1: insulin like growth factor -1, VEGF: vascular endothelial growth factor, TGF-β1: transforming growth factor β1, Bcl-2: B-cell lymphoma, Bax: Bcl-2-associated X.

IV. RESULTS

4.1. The effect of CZ extract on hair growth

4.1.1. The effect of CZ extract on anagen induction in C57BL/6 mice

The dorsal hair of C57BL/6 mouse is known to have a time dependent hair growth cycle. After synchronization of the hair cycle with depilation, we investigated whether anagen induction was promoted by CZ extract. As shown in Figure 2, minoxidil(5%) and CZ extract treated groups showed post-hair growth induction and their hair shaft were visible at 20 days, while the control group remained pink until 20 days. Histological studies showed that CZ extract markedly increased the depth and size of the hair follicles as compared with the negative control group (Figure 3). Overall, these results indicate that CZ extract induced early telogen-to-anagen conversion of the hair follicle in C57BL/6 mice.

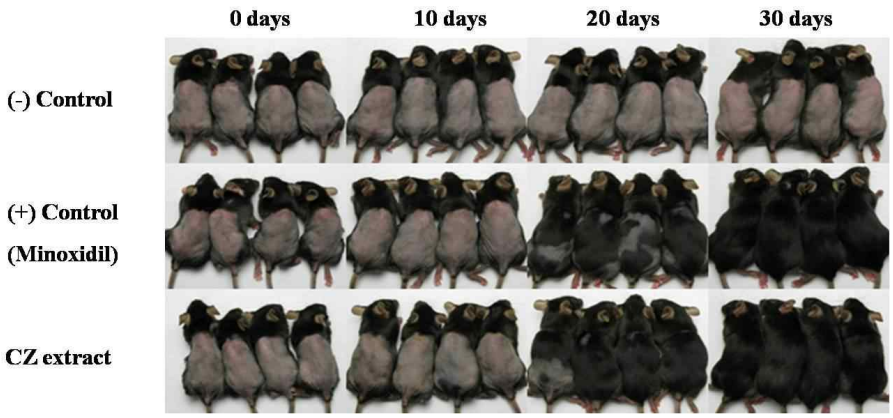

Figure 2. The effect of CZ extract on anagen induction in C57BL/6 mice. After depilation, 0.2 ml of 3% CZ extract in 50% ethanol was topically applied on dorsal skin every day for 30 days. Minoxidil (5%) was used as a positive control and 50% ethanol was used as the vehicle control. The dorsal skin of the mice was photographed at 0, 10, 20 and 30 days after depilation.

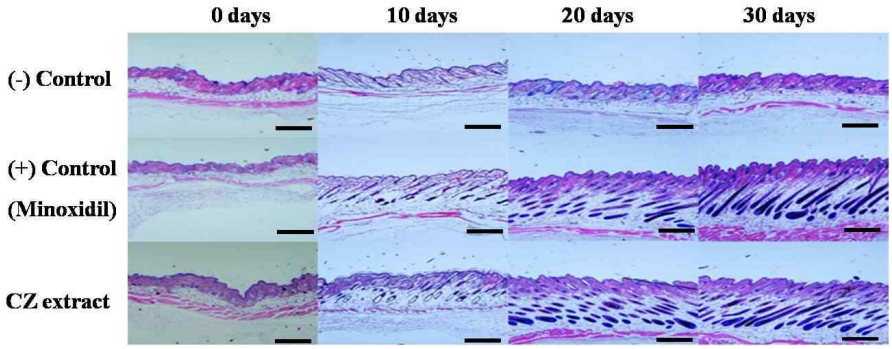

Figure 3. Histological analysis. The dorsal skin of the mice was stained with routine hematoxylin and eosin at 0, 10, 20 and 30 days after depilation. (Bar=0.1 mm.)

4.1.2. The effect of CZ extract on hair follicle derived cell proliferation

MTT assays were performed to evaluate the effect of CZ extract on hair follicle derived cell proliferation. Human hair follicle DP cells and FKCs were treated with serial doses of CZ extract and the mitogenic effect on DP cells and FKCs were examined. CZ extract promoted the proliferation of DP cells at concentrations of 1.0 ppm and 10 ppm ($p<0.001$) compared with the vehicle (0.1%DMSO) treated control (Figure 4). However, CZ extract did not effect FKC proliferation (Figure 5). Furthermore, CZ extract showed cytotoxic effects for both DP cells and FKCs at concentrations of 1000 ppm ($p<0.001$) (Figures 4, 5).

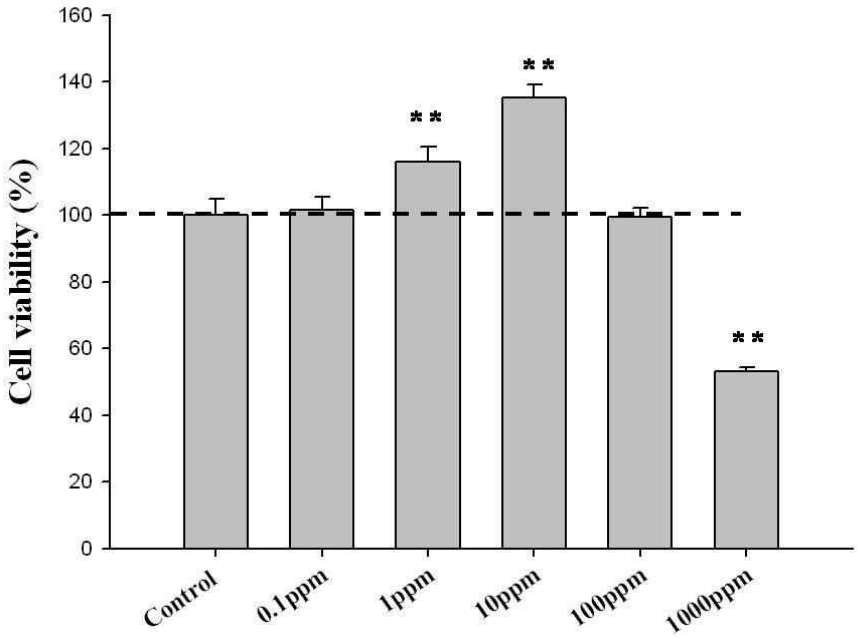

Figure 4. Viability of DP cells after treatment with CZ extract. DP cells were treated with serial doses of CZ extract (from 0.1 to 1000 ppm) for 24 hours. Cell viability (%): (mean absorbency in test wells)/(mean absorbency in control wells)×100. Data are presented as the mean ± SD. ** $p<0.001$, compared with the vehicle (0.1% DMSO) treated control.

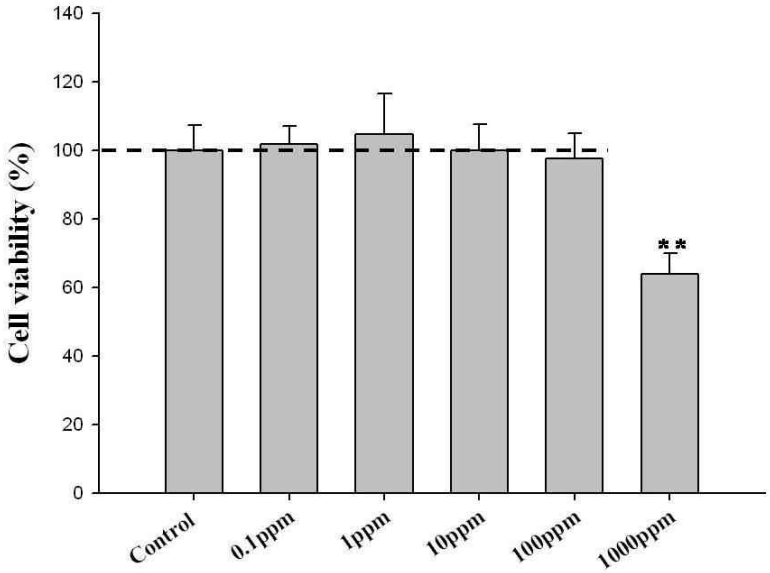

Figure 5. Viability of FKCs after treatment with CZ extract. FKCs were treated with serial doses of CZ extract (from 0.1 to 1000 ppm) for 24 hours. Cell viability (%): (mean absorbency in test wells)/(mean absorbency in control wells)×100. Data are presented as the mean ± SD. ** $p<0.001$, compared with the vehicle (0.1% DMSO) treated control.

4.1.3. The effects of CZ extract on growth and anagen duration of organ cultured human hair follicles

We investigated whether CZ extract directly exerts growth-modulating effects on human hair follicles. Human hair follicles treated with 1 ppm or 10 ppm of CZ extract every other day showed a significant increase in hair follicle length compared with the vehicle control (0.1% DMSO) (Figure 6). Determination of the hair cycle score also showed a high value for the control group and a lower value for the CZ extract treated group (Figure 7), demonstrating that catagen progression in CZ extract treated hair follicles is significantly delayed.

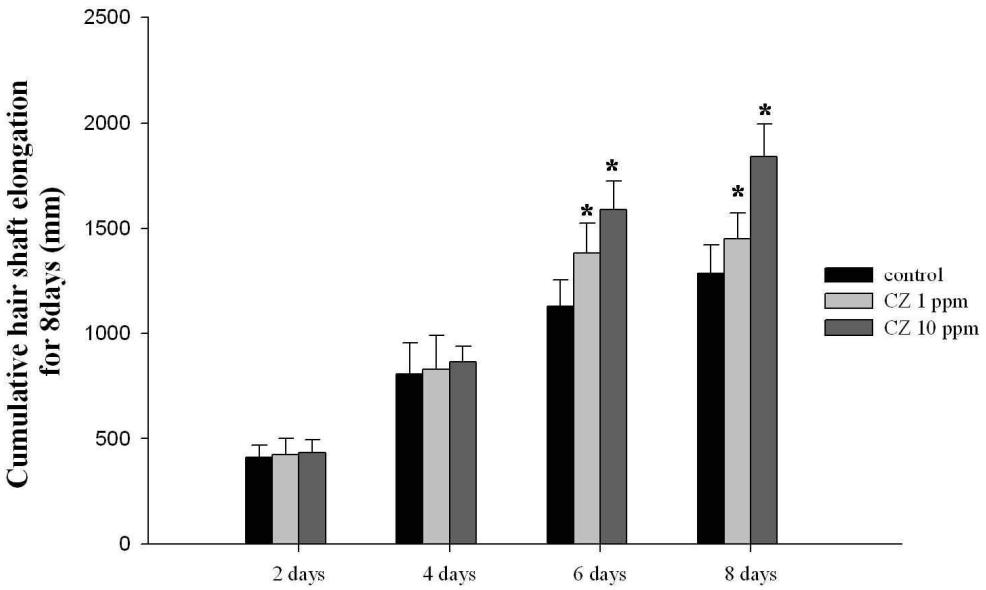

Figure 6. Quantitative analysis of hair elongation after treatment with CZ extract. Hair follicle length was measured in 2 day interval. The data was based on 15 hair follicles per group, and are presented as the mean ± SD. * $p<0.01$, compared with the vehicle (0.1% DMSO) treated control.

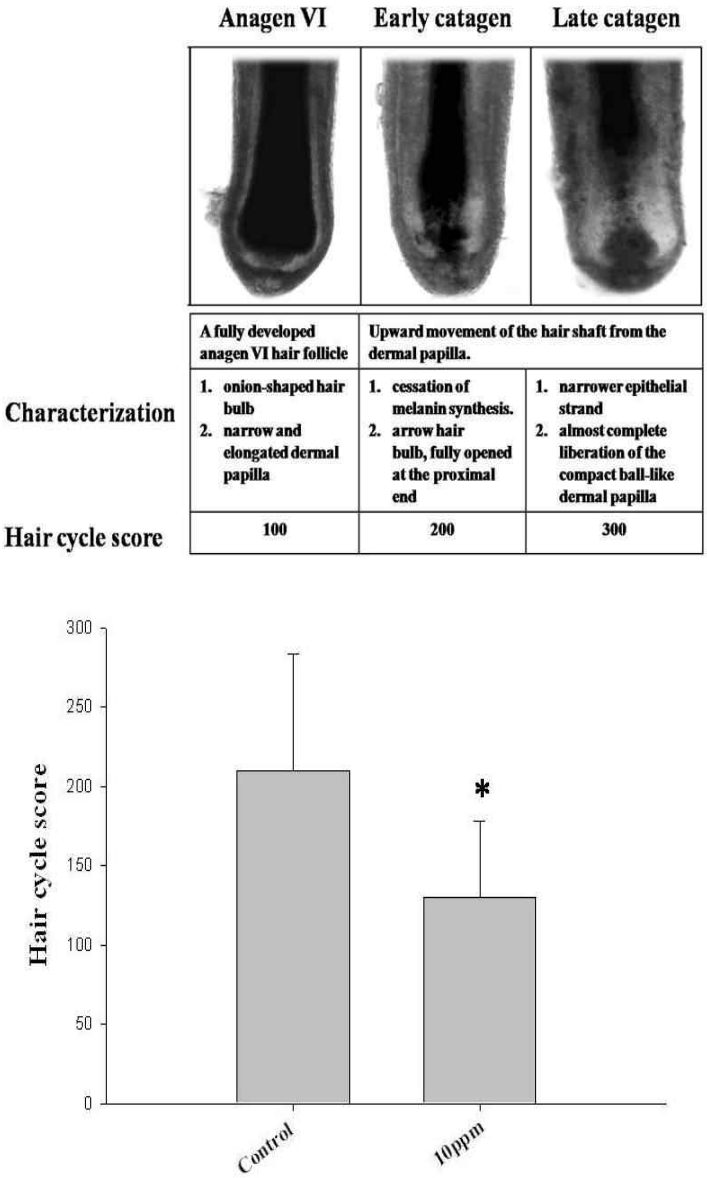

Figure 7. Hair cycle score after treatment with CZ extract. All hair follicles of each group were staged and scored as follows: anagen VI, 100; early catagen, 200; mid-catagen & late catagen, 300. Data are presented as the mean ± SD. * $p<0.05$, compared with the vehicle (0.1% DMSO) control.

4.1.4. The effect of CZ extract on apoptosis of the hair follicle

Since CZ extract was found to promote human hair growth and extend anagen duration of hair follicles, the effect of CZ on human hair follicle apoptosis was also evaluated. The results showed that significantly reduced cell death is observed in the ORS and matrix epithelial cells surrounding the DPs in response to 10 ppm CZ extract compared with the vehicle control (0.1% DMSO) (Figure 8).

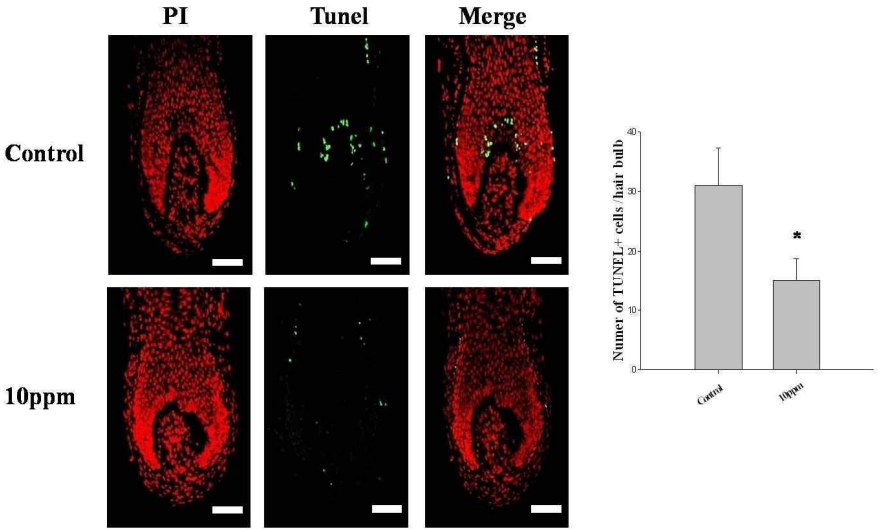

Figure 8. Analysis of TUNEL positive cells in human hair follicles after treatment with CZ extract. Human hair follicles were treated in the absence or presence of 10 ppm CZ extract for 4 days and stained with ApopTag Plus peroxidase in situ apoptosis detection kits. TUNEL positive apoptotic cells (green) in the hair were counted and data shown are the mean ± SD from five hair follicles (*$p<0.05$). Bar=0.1 mm.

4.1.5. The effects of CZ extract on the expression of various factors related to hair growth

Various factors that correlate with the hair growth cycle have been reported [18-19, 23, 26, 32]. To determine whether CZ extract affects the expression of various factors related to hair growth, semi-quantitative RT-PCR analysis was performed. The results showed that CZ extract induces the mRNA expression of VEGF and IGF1 (hair growth promoting factor), moreover, reduces the mRNA expression of TGF-β1 (hair growth inhibitory factor).

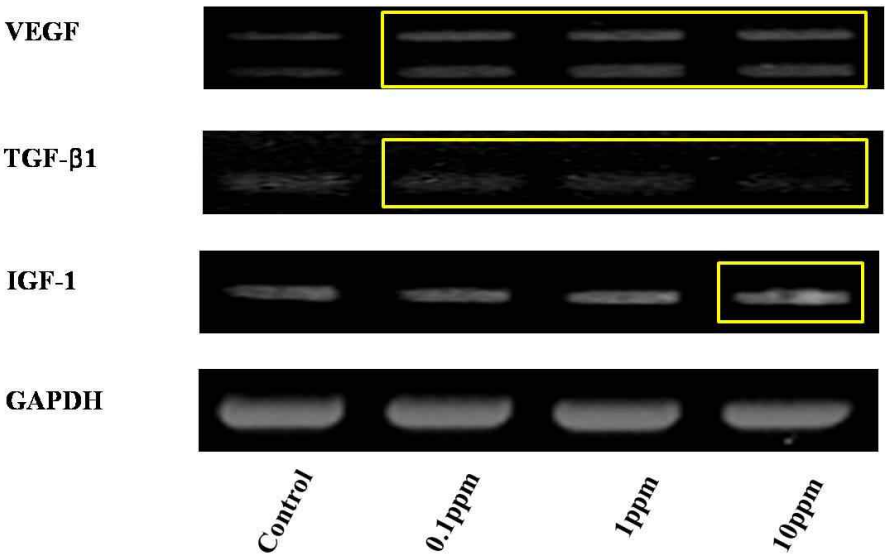

Figure 9. Semi-quantitative RT-PCR analysis of factors related to hair growth after treatment with CZ extract. DP cells were treated with varying concentrations of CZ extract for 18 hours and analyzed by RT-PCR.

4.2. The effect of jaceosidin on hair growth

4.2.1. The effect of jaceosidin on DP cell proliferation

MTT assays were performed to evaluate the effect of jaceosidin on DP cell proliferation, Human hair follicle DP cells were treated with serial doses of jaceosidin and the mitogenic effects on the DP cells were examined. jaceosidin promoted the proliferation of DP cells at concentrations from 0.1 μM to 100 μM ($p<0.05$) compared with the vehicle (0.1% DMSO) treated control. In addition, jaceosidin showed cytotoxic effects at concentrations of 1000 μM (Figure 10).

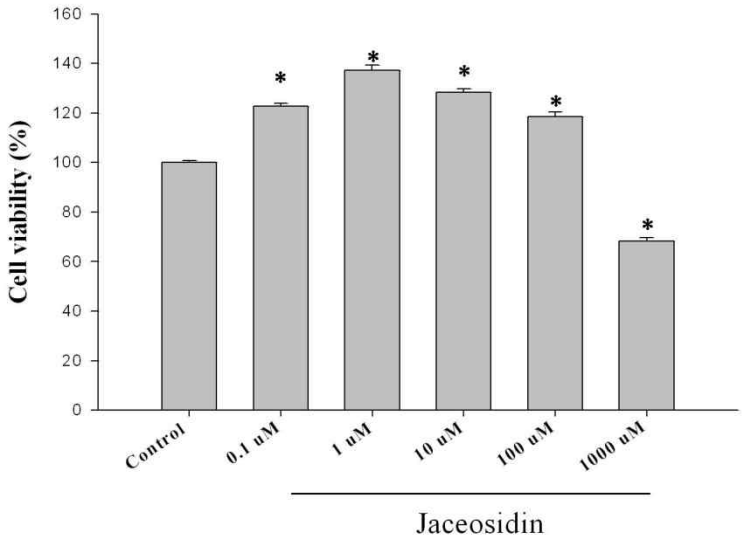

Figure 10. Viability of DP cells after treatment with jaceosidin. DP cells were treated with serial doses of jaceosidin for 24 hours. Cell viability (%): (mean absorbency in test wells)/(mean absorbency in control wells)×100. Data are presented as the mean ± SD. * $p<0.05$, compared with the vehicle (0.1% DMSO) treated control.

4.2.2. The effects of jaceosidin on the growth and anagen duration of organ cultured human hair follicles

Hair follicles were incubated with or without jaceosidin for 8 days. The length of the hair follicles was measured by stereomicroscope, and the growth rate was compared to control hair follicles. Up to days 2 and 4, both tested concentrations (1.0 and 10 μM) jaceosidin had a statistically significant effect on hair follicle elongation. However, after 4 days, there was no significant difference in hair follicle length between the treated and control groups. Data are presented as the mean ± SD. *$p<0.05$ versus control cells (Figure 11).

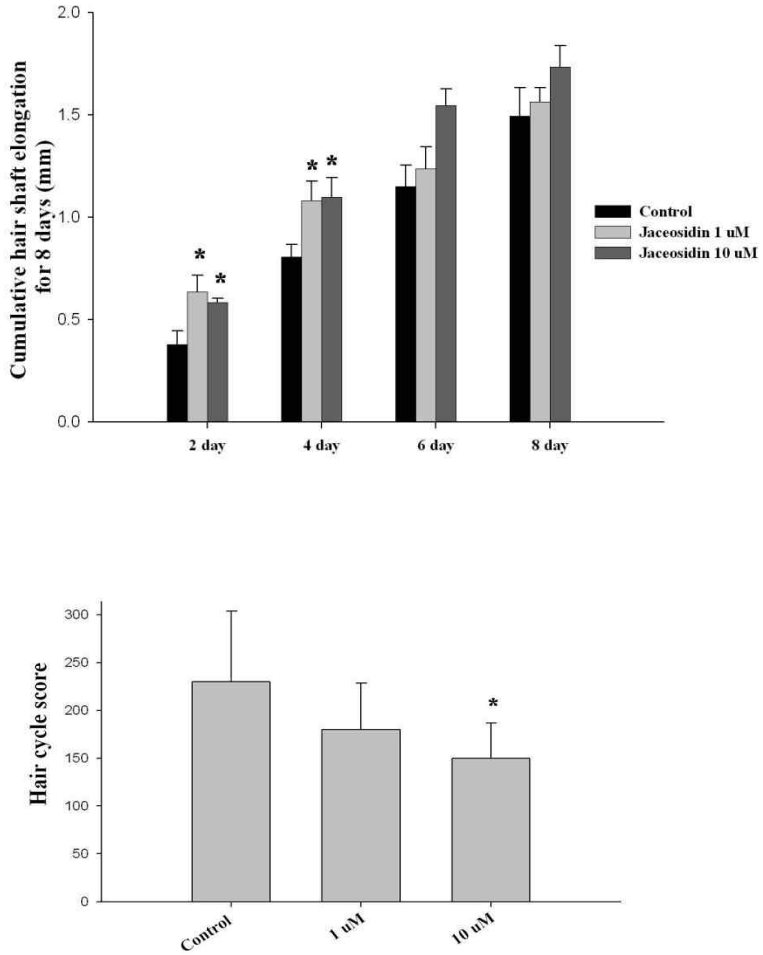

Figure 11. Quantitative analysis of hair shaft elongation and hair cycle score after treatment with jaceosidin. The hair follicle length was measured at 2 day intervals. Data is based on 15 hair follicles per group, and presented as the mean ± SD. * $p<0.05$, compared with the vehicle (0.1% DMSO) treated control. All hair follicles of each group were staged and scored as follows: anagen VI, 100; early catagen, 200; mid-catagen & late catagen, 300. Data are presented as the mean ± SD. *$p<0.05$ versus control cells incubated with control. * $p<0.05$, compared with the vehicle (0.1% DMSO) treated control.

4.2.3. The effect of jaceosidin on hair follicle apoptosis

To investigate the influence of jaceosidin on apoptosis of hair follicles, TUNEL positive cells in the hair bulb was counted. The green spot indicates apoptotic cell in ORS. The 1.0 and 10 μM jaceosdin treatment groups show significantly lower expression levels compared with the control group indicating decreased apoptosis by jaceosidin (Figure 12).

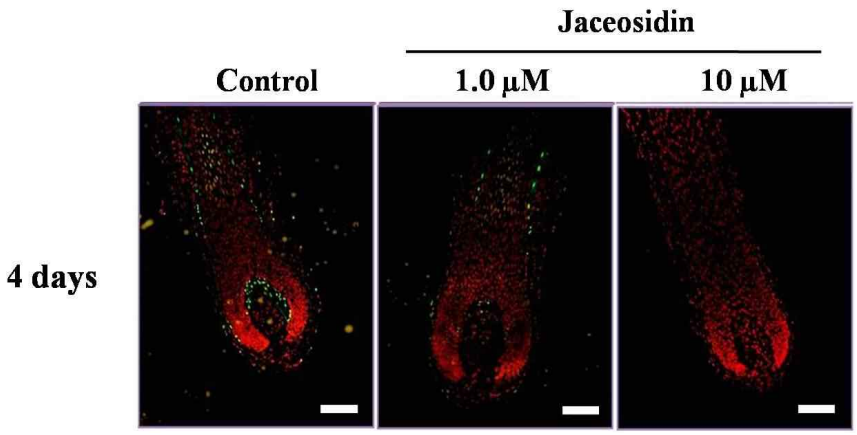

Figure 12. Analysis of TUNEL positive cells in human hair follicles after treatment with jaceosidin. Human hair follicles were treated in the presence or absence jaceosidin extract for 4 days and stained using ApopTag Plus peroxidase in situ apoptosis detection kits. Bar=0.1 mm.

4.2.4. The effects of jaceosidin on the expression of various factors related to the hair growth

RT-PCR analysis was performed to quantify the effect of jaceosidin on the expression of several growth factors implicated in hair growth regulation. As shown in Figure 13, while jaceosidin decreases TGF-β mRNA, it had no significant effect on VEGF and IGF-1. In this way, jaceosidin is believed to promote hair growth by inhibiting TGF-β1 and not by inducing hair growth promoting factors, such as VEGF and IGF-1.

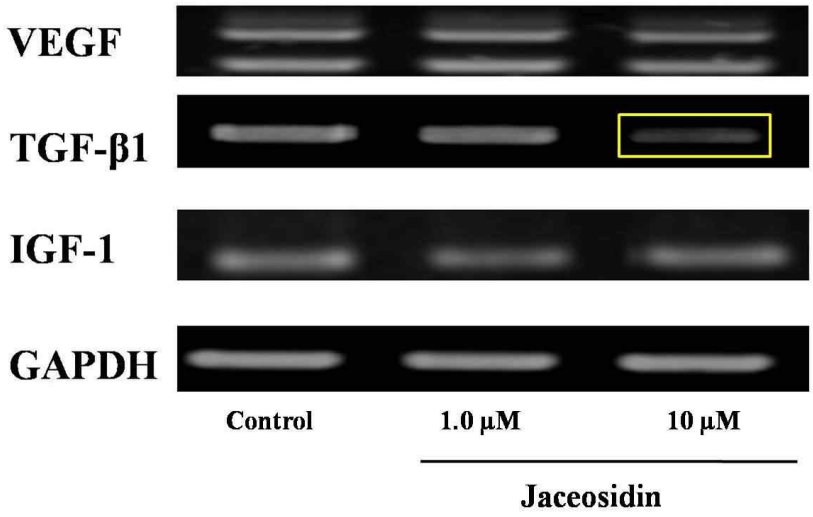

Figure 13. Semi-quantitative RT-PCR analysis of factors related to hair growth after treatment with jaceosidin. DP Cells were treated with varying concentrations of jaceosidin for 18 hours and analyzed by RT-PCR.

4.3. The effect of eupatilin on hair growth

4.3.1. The effect of eupatilin on DP cell proliferation

To evaluate the effect of eupatilin on DP cell proliferation, MTT assays were performed. Human hair follicle DP cells were treated with serial doses of eupatilin and the mitogenic effects were examined. Eupatilin promoted the proliferation of DP cells at concentrations ranging from 0.1 to 100 μM ($p<0.05$) compared with the vehicle (0.1% DMSO) treated control (Figure 14). In addition, eupatilin showed cytotoxic effects at concentrations of 1000 μM ($p<0.05$) (Figure 14).

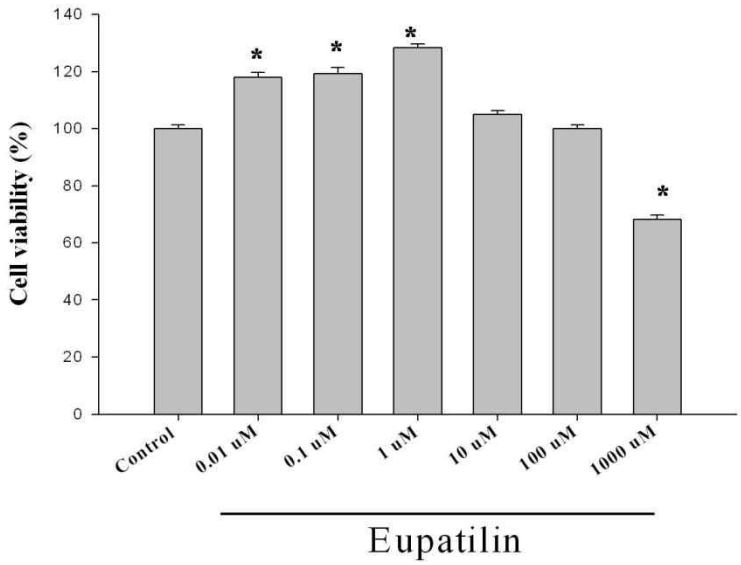

Figure 14. Viability of DP cells after treatment with eupatilin. DP cells were treated with serial doses of eupatilin for 24 hours. Cell viability (%): (mean absorbency in test wells)/(mean absorbency in control wells)×100. Data are presented as the mean ± SD. * $p<0.05$, compared with the vehicle (0.1% DMSO) treated control.

4.3.2. The effects of eupatilin on growth and anagen duration of organ cultured human hair follicles

Hair follicle organ cultures were used to determine the hair shaft elongation effect of eupatilin. Hair follicles were incubated with or without eupatilin for 8 days. The length of the hair follicles was measured by stereomicroscope, and the growth rate was compared to control hair follicles. Hair shaft elongation was promoted by treatment with 1 μM eupatilin compared to untreated hair follicles (Figure 15). Determination of the hair cycle score also showed a high value for the control group and a lower value for the eupatilin treated group (Figure 15), demonstrating that catagen progression in eupatilin treated hair follicles is significantly delayed.

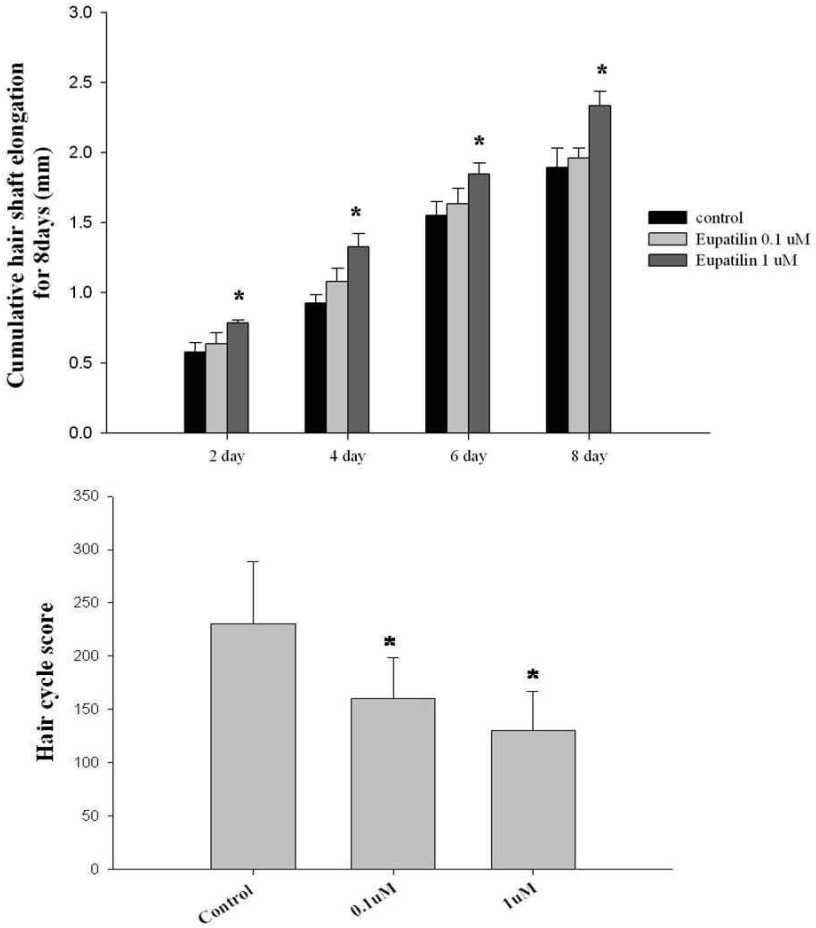

Figure 15. Quantitative analysis of hair shaft elongation and hair cycle score after treatment with eupatilin. Hair shaft elongation was measured at 2 day intervals. Data are based on 15 hair follicles per group, and presented as the mean ± SD. *$p<0.01$, compared with the vehicle (0.1%DMSO) treated control. All hair follicles of each group were staged and scored as follows: anagen VI, 100; early catagen, 200; mid-catagen & late catagen, 300. Data presented as mean ± SD. * $p<0.05$, compared with the vehicle (0.1% DMSO) treated control.

4.3.3. The effect of eupatilin on apoptosis of hair follicle

To investigate the influence of eupatilin on hair follicle apoptosis, TUNEL positive cells in the hair bulb were investigated. The results showed that significantly reduced cell death is observed in the ORS in response to 0.1 and 1.0 μM eupatilin compared with the vehicle control (0.1% DMSO) (Figure 16).

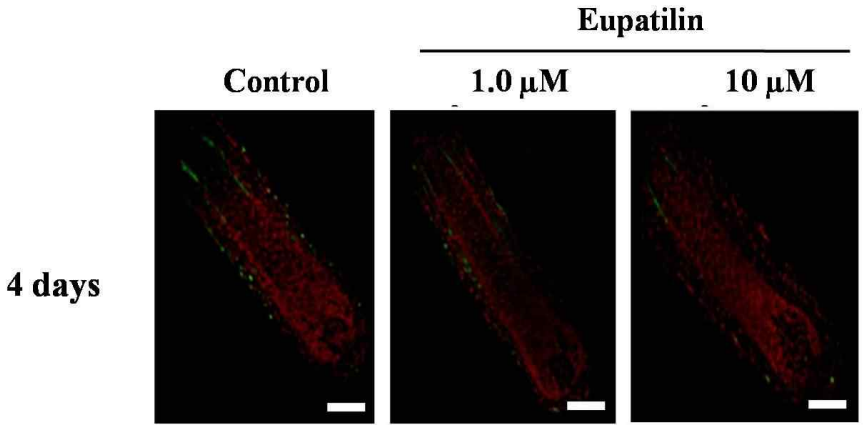

Figure 16. Analysis of TUNEL positive cells in human hair follicles after treatment with eupatilin. Hair follicles were treated in the presence or absence of eupatilin extract for 4 days and stained with ApopTag Plus peroxidase in situ apoptosis detection kits. Bar=0.1 mm.

4.3.4. The effects of eupatilin on the expression of various factors related to hair growth

RT-PCR analysis was performed to quantify the effect of eupatilin on the expression of several growth factors implicated in hair growth regulation. As shown in Figure 17, eupatilin induces the mRNA expression of VEGF (hair growth promoting factor) and Bcl-2 (anti-apoptotic factor), moreover, reduces the mRNA expression of Bax (pro-apoptotic factor).

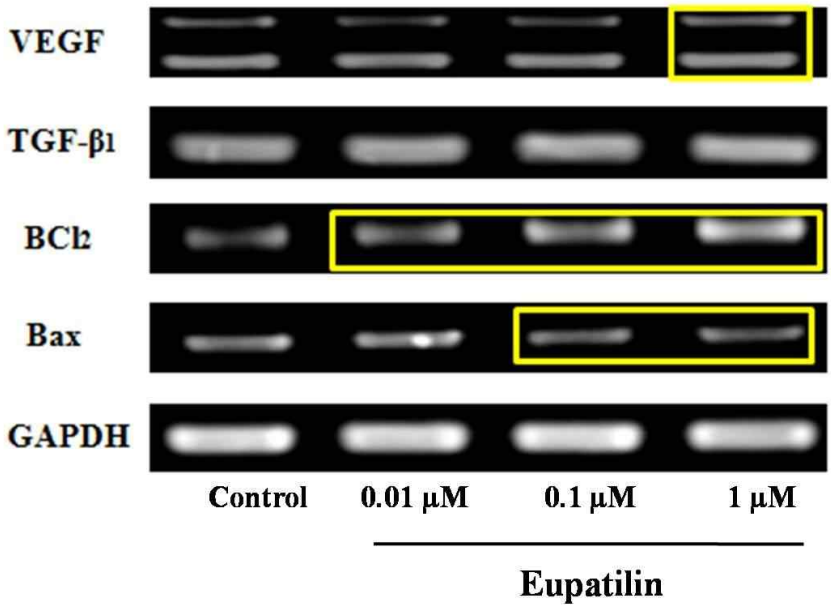

Figure 17. Semi-quantitative RT-PCR analysis of the factors related to hair growth after treatment with eupatilin. DP Cells were treated with varying concentrations of eupatilin for 18 hours and analyzed by RT-PCR.

V. Discussion

Hair loss, medically referred to as alopecia, is a common condition that causes serious distress to the persons afflicted with the disease. The key feature of hair loss is a progressive shortening of anagen duration and resultant progressive miniaturization of terminal hairs to vellus-like hairs. Therefore, promoting or prolonging anagen duration of the hair follicle is key to development of remedies for treatment and prevention of hair loss. Increased attention has been paid to herbal medicines that might exert hair promoting activity with minimal or no side effects. Several traditional herbal medicines have been widely used for the prevention of hair loss in Asia.

In the current study, the hair growth promoting effects of CZ extract *in vivo* and *in vitro* were investigated. We demonstrated that CZ and its main components, jaceosidin and eupatilin, have the potential to promote hair growth via upregulation of the hair growth promoting factors (VEGF and IGF-1) and downregulation of the hair growth inhibitory factor TGF-β1, as well as the proliferation of DP cells. First, we investigated the hair growth-promoting activity of CZ extract using 6-week-old C57BL/6 mice in the stable telogen phase. C57BL/6 mice are useful for screening hair growth promoting agents, because their truncal pigmentation is dependent on the follicular melanocytes, which produce pigment only during the anagen phase[7]. After depilation of the dorsal hair, C57BL/6 mice were topically applied with CZ extract for 30 days.

At 20 days, CZ extract (3%) significantly induced hair growth in telogenic C57BL/6 mice, whereas no visible hair growth was observed in the control group (Figure 2). Moreover, the histomorphometric analysis indicatesd that the topical application of CZ extract (3%) caused an earlier induction of the anagen phase, compared to the control (Figure 3).

The hair follicle culture system proved to be a powerful model for determining the direct effects of enzyme inhibitors, substrate deletions, and product additions on follicular function[49]. The results of this study showed that the CZ extract (10 ppm) increased the length of human hair follicles compared with the vehicle control (Figure 6). Determination of the hair cycle score also showed a high value for the control group and a lower value for the CZ extract (10 ppm) treatment group (Figure 7), demonstrating that catagen progression in CZ extract-treated hair follicles is significantly delayed.

DP cells consist of an oval mass of fibroblasts that is entirely encapsulated by the matrix epithelium. The size of the DP correlates with the size of hair follicle and the size of the produced hair shaft; e.g., a larger DP correlates with larger hair follicles and produces thicker hair shafts[2, 4]. To investigate the effect of CZ extract on the cell growth in the hair follicles, we examined the proliferation of DP cells and FKCs. CZ extract promoted the proliferation of DP cells compared with the vehicle treated control. However, CZ extract did not promote the FKC proliferation (Figure 4, 5). Taken together, these results

indicate that the hair growth promoting effect of CZ extract may be mediated through mitogenic effects that occur in the DP region.

Apoptosis, which is distinguished from necrosis, is involved not only in normal embryonic development but also in homeostatic mechanisms in various tissues including normal skin[52]. The regression phase of the hair cycle (catagen) is an apoptosis-driven process accompanied by terminal differentiation, proteolysis, and matrix remodeling[53]. Precise analysis of apoptotic cell death during the transition phase has revealed that sequential cell death occurs from the lower bulb to the upper ORS until the formation of club hair occurs[26]. The results of this study showed that CZ extract significantly reduced TUNEL positive apoptotic cells in hair bulb ORS cells and matrix cells. Taken together, these results indicate that the hair growth promoting effect of CZ extract may be mediated through anti-apoptotic effects that occur in the hair bulb region.

Various cytokine and growth factors play important roles in hair growth control. IGF-1 has been identified as one of the androgen-dependent paracrine growth factors in DP cells[22]. IGF-1 also stimulates human hair growth in vitro at physiologic concentrations to prevent the premature entry of cultured hair follicles into the catagen phase[23]. VEGF is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive function[28]. Transgenic over expression of VEGF in hair follicle epithelial cells

strongly induced perifollicular vascularization and led to accelerated hair regrowth after depilation. Moreover, VEGF as a major mediator of hair follicle growth and cycling that could promote hair growth[29]. TGF-β1 is a polypeptide member of the transforming growth factor β super-family. It is a secreted protein that performs many cellular functions, including control of cell growth, cell proliferation, cell differentiation and apoptosis. TGF-β1 is known to inhibit human keratinocyte growth in vitro[25] and induce catagen progression in mouse hair growth in vivo[26]. In AGA progression, TGF-β1 as an androgen-inducible growth suppressor derived from balding DP cells suppresses hair growth though follicular keratinocyte growth inhibition[27]. The results of this study showed that the CZ extract increases VEGF and IGF-1 expression and reduces TGF-β1 expression in DP cells. Taken together, these results indicate that the hair growth promoting effect of CZ extract may be mediated through upregulation of hair growth promoting factors (VEGF, IGF-1) and downregulation of hair growth inhibitory factor (TGF-β1).

We next examined which compounds of the CZ extract are responsible for its hair growth promoting activity of CZ extract. Silica gel column chromatography indicated that jaceosidin and eupatilin were found in CZ extract. Jaceosidin, the major pharmacologically active flavonoid in CZ extract, has a variety of biological activities in diverse experimental settings, including anti-oxidative, anti-inflammatory, immunosuppressive, and

proapoptotic activities[54-55]. Eupatilin, a natural flavonoid, is also major constituent of CZ extract. Eupatilin is reported to exert strong anti-inflammatory, and anti-oxidative activity as well as cytoprotective effects against experimentally induced gastrointestinal, hepatic, and pancreatic damage in vivo and in vitro[54]. To the best of our knowledge, this is the first study to demonstrate that jaceosidin and eupatilin promote hair growth. We documented for the first time that jaceosidin promotes the growth of human hair follicles, increases the proliferation of DP cells, reduces TGF-β1 expression, promotes hair follicle elongation in the early phase (until day 4), prolongs the anagen phase of the hair cycle in hair follicle organ culture, and reduces TUNEL positive apoptotic cells in hair bulb ORS cells. We also demonstrated that eupatilin promotes the growth of human hair follicles, increases the proliferation of DP cells, increases the expression of hair growth promoting factor - VEGF and anti-apoptotic factor – Bcl-2. Moreover, eupatilin reduces the expression of Bax, prolonges the anagen phase of the hair cycle in hair follicle organ culture, and reduces TUNEL positive apoptotic cells in hair bulb outer root sheath cells.

VI. Conclusion

This study was conducted to evaluate the promoting effect and action mechanism of *Chrysanthemum zawadskii* (CZ) extract on hair growth. CZ extract induced the earlier conversion of telogen to anagen phases and promoted hair growth in the C57BL/6 mouse model. When human hair follicles were cultured in the presence of CZ extract for 8 days, CZ extract significantly promoted hair growth and prolonged anagen duration by reducing apoptosis and inducing proliferation of hair follicle cells in the bulb region. Treatment of human DP cells with CZ extract resulted in an increase in cell proliferation, an increase of hair growth promoting factor (VEGF, IGF-1) expression, and a decrease of hair inhibitory factor (TGF-β1) expression. In particular, the isolated jaceosidin and eupatilin, components from CZ extract, promoted the growth of human hair follicles, increased the proliferation of DP cells, and induced VEGF and reduced TGF-β1 expression. These results suggest that CZ extract and its principal components, jaceosidin and eupatilin, have hair growth promoting potential through the regulation of DP cell growth factors and promote DP cell proliferation.

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Abstract in Korean(국문요약)

구절초추출물의 모발성장촉진효능 및 작용기전

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현재 국내 탈모 인구는 계속 증가하고 있으며 모발관리 대상자 60%가 20~30 대로 탈모환자의 연령대는 점점 낮아지고 있으며 여성 탈모 인구 또한 증가하고 있다. 지금까지 미국 Food and Drug Administration(FDA) 공인을 받은 탈모치료약물로는 고혈압치료제에서 발견된 물질로 만들어진 미녹시딜과 탈모의 원인이 되는 호르몬(dihydrotestosterone, DHT)의 생성을 억제하는 프로페시아 등이 있다. 그러나 20%~30%의 환자들은 이 두 종류의 약물들에 반응이 없는 것으로 나타난다. 또한 아주 극히 드물지만 일부 환자에서 피부자극 등을 비롯한 여러 부작용을 보여왔다. 최근 인체에 상대적으로

부작용이 적은 천연물로부터 새로운 발모성분 또는 소재를 발굴하려는 많은 연구가 활발히 진행되고 있다.

본 연구에서는 C57BL/6 마우스모델을 이용한 *in-vivo* 실험과 인체모낭기관배양모델을 이용한 *in-vitro* 실험을 통하여 구절초 추출물의 모발성장과 모발의 주기적 변화에 미치는 영향을 전반적으로 평가하고 또한 그 작용기전을 알아보고자 하였다.

6 주령 female C57BL/6 mice 의 등 (back skin)에서 털을 제거한 후 구절초 추출물의 발모효력을 형태학적 및 조직학적으로 평가하였다. 인체 생장기 모낭을 분리 및 배양하여 구절초 추출물이 모낭의 길이성장, 주기적변화, 세포고사에 미치는 영향을 조사하였다. 모유두세포를 분리 및 배양하여 구절초 추출물의 모발성장 관련 인자의 발현에 미치는 영향을 조사하였다.

그 결과, 구절초 추출물은 C57BL/6 마우스 모델에서 퇴행기에서 성장기로의 이행을 촉진시켰다. 구절초 추출물은 인체모낭기관배양 실험에서 통계적으로 유의하게 모발성장을 촉진하였고, 세포고사를 감소시키고 증식을 촉진시켜 성장기 기간을 신장시켰다. 구절초 추출물로 모유두세포를 처리한 결과 세포의 증식이 증가하였고 모발성장촉진인자인 VEGF 와 IGF-1 의 발현을 촉진시켰으며, 모발성장저해인자인 TGF-β1 의 발현을 감소시켰다. HPLC 를

활용하여 구절초 추출물의 주요성분인 Jaceosidin 과 Eupatilin 을 분리하였으며, Jaceosidin 과 Eupatilin 은 모유두세포의 증식을 촉진시키고 VEGF 의 발현을 증가시키고 TGF-β1 의 발현을 감소시켰다.

결론적으로 구절초 추출물과 그의 주요 성분인 Jeceosidin 과 Eupatilin 은 모유두세포의 증식을 촉진시키고 모유두세포내에서 모발성장인자의 발현을 조절하여 모발성장을 촉진시킴을 알 수 있었다.

핵심되는 말 : 구절초, 모낭, 모유두세포, 성장인자, 모발주기