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Therapeutic Potentiality of *Celtis choseniana* Nakai on Androgenic Alopecia through Repression of Androgen Action and Modulation of Wnt/β-catenin Signaling

Hui-Ju Lee[1], Geum-Lan Hong[2], Kyung-Hyun Kim[1], Yae-Ji Kim[1], Tae-Won Kim[1], and Ju-Young Jung[1,*]

[1]*Department of Veterinary Medicine & Institute of Veterinary Science, Chungnam National University, Daejeon 34134, Republic of Korea*
[2]*Department of Anatomy, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea*

Abstract – In this study, we investigated the efficacy of *Celtis choseniana* Nakai (*C. choseniana*) as complementary herbal medicine to ameliorate androgenic alopecia (AGA). The effects of *C. choseniana* on AGA were evaluated using testosterone propionate-induced AGA mouse model and dihydrotestosterone-treated human hair follicle dermal papilla cells. *In vivo*, *C. choseniana* treatment deactivated androgen signaling by reducing the concentration of serum dihydrotestosterone level and expressions of 5α-reductase 2 and androgen receptor. Next, *C. choseniana* treatment increased the hair regrowth rate. Histological studies demonstrated that *C. choseniana* induced the anagen phase in testosterone propionate-induced AGA mouse model. Cellular proliferation was promoted by *C. choseniana* treatment via increasing the expression of proliferation factors, such as proliferating cell nuclear antigen and cyclin D1. Furthermore, *C. choseniana* treatment increased the expression of proteins related to the Wnt/β-catenin signaling pathway. In addition, dickkopf-1, a Wnt inhibitor, was downregulated with *C. choseniana* treatment. Likewise, *C. choseniana* treatment promoted cellular proliferation *in vitro*. This study demonstrated the inhibitory effect of *C. choseniana* on androgen-induced AGA. Moreover, *C. choseniana* induced activation of Wnt/β-catenin signaling, resulting in prolonged anagen and cellular proliferation. Therefore, we suggest that *C. choseniana* can be used as a therapeutic agent to alleviate AGA.
Keywords – Androgenic alopecia, Androgen, Cellular proliferation, *Celtis choseniana* Nakai, Wnt

Introduction

Celtis, known as “hackberry” or “nettle tree”, is a genus of about 80 species broadly distributed in warm temperature regions, such as East Asia, eastern North America, and the Mediterranean.[1] It has been used as a folk remedy for therapeutic effects, such as appetite enhancement and alleviation of stomach disorders, neuralgia, urticaria, and lung abscess.[2] *Celtis choseniana* Nakai (family Cannabaceae, *C. choseniana*) is a species of *Celtis* endemic to Korea. *C. choseniana* regulates macrophage-mediated inflammatory responses and contains a variety of anti-inflammatory flavonoids, including quercetin, luteolin, and kaempferol.[3] Nevertheless, except for noted pharmacological effect of *C. choseniana*, not sufficient investigation has been conducted on therapeutic efficacy of *C. choseniana*. In a previous study, we confirmed the protective effects of *C. choseniana* on androgen induced benign prostatic hyperplasia through inhibition of 5AR2 and alleviation of Akt/nuclear factor kappa B (NF-κB)/AR pathway. Regarding, we hypothesized *C. choseniana* also suppresses androgenic alopecia (AGA), modulating androgen-induced signaling pathway.

Hair is very important for scalp protection and determining an individual’s impression. It undergoes regeneration through a regular and continuous hair cycle. The hair cycle consists of three main phases: anagen (growth phase), catagen (regression phase), and telogen (resting phase).[4] The endocrine system, genetic factors, and many environmental factors, such as age, sex, and the degree of nutrition, are related to maintaining the normal hair cycle. Problems with these factors cause alterations in the hair cycle, ultimately resulting in hair loss.[5] AGA, also known as male pattern baldness, is the most common type of hair loss. Although the definite mechanism of AGA has not yet been investigated, the effects of androgen on hair

*Author for correspondence
Ju-Young Jung, DVM, PhD, College of Veterinary Medicine, Chungnam National University, 220 Gung-dong, Yusung-gu, Daejeon 34134, Republic of Korea.
Tel: +82-42-821-8899, 7902; E-mail: jyjung@cnu.ac.kr

follicles are considered the main cause of AGA. Testosterone, a major circulating androgen, is converted to a more potent androgen, dihydrotestosterone (DHT), by 5α-reductase type 2 (5AR2).[6] DHT binds to the androgen receptor (AR) with high affinity, leading to hair follicle miniaturization, hair growth inhibition, and hair cycle alternation.[7] Finasteride, an inhibitor of 5AR2, is widely utilized as one of the drugs for the treatment of AGA. Several studies have demonstrated that finasteride has therapeutic effects, such as promoting hair growth and slowing hair loss.[8] However, there are some limitations to prescribing these drugs to patients with AGA because they can cause predictable side effects such as erectile dysfunction, loss of libido, and testicular pain, making the prolonged use of medication.[9] Therefore, the use of natural substance-based medication is suggested as an alternative and reasonable treatment for AGA.

In this study, we examined the effects of *C. choseniana* extract as a reasonable treatment for AGA using *in vivo* and *in vitro* models of androgen-induced AGA. We hypothesized that *C. choseniana* ameliorates AGA by inhibiting androgen signaling and promoting hair regrowth.

Experimental

Plant material and preparation of extract – The plant name, *Celtis Choseniana* Nakai, was confirmed at Plants of the World Online (http://www.plantsoftheworldonline.org/). The plant extract (001-011) used in this study was obtained from the Korea Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The plant was collected from Ulleung-gun, Gyeongsangbuk-do, Korea in 2005. A voucher specimen (KRIB 0004939) was kept in the herbarium of the Korea Research Institute of Bioscience and Biotechnology. The dried and powdered plant (96 g) was added to 1 L of 99.9% methyl alcohol and extracted through 10 cycles per day for 3 d (40 kHz, 1500 W, 15 min). Ultrasonication was performed for 120 min at room temperature using an ultrasonic extractor (SDN-900H, SD-ULTRASONIC CO., LTD).[1] *C. choseniana* extract (10.3 g) was obtained after filtration, concentration, and drying under reduced pressure.

HPLC analysis – The components of *C. choseniana* were analyzed using high-performance liquid chromatography (HPLC) according to the aforementioned conditions.[3] The conditions of the HPLC analysis are listed in Table 1.

Androgenic alopecia animal model – The animal experiments were approved by the Institutional Animal Ethics Committee 202006A-CNU-102). All research was conducted according to the principles of the Care and Use of Laboratory Animals of the National Institutes of Health. Five-week-old, male C57BL/6 mice were obtained from Orient Bio (Seongnam, Korea). Mice were acclimated under stabilized conditions (25 ± 0.2 ºC, 55 ± 5% humidity, and 12 h light/dark cycle) at the specific-pathogen-free animal facility for a week. After one week, all mice were depilated with a clipper to reduce individual differences and synchronize the hair cycle for telogen. The mice had one week after depilation to adjust to the environment that was set up for the experiment. The examination was performed according to the specified schedule (Fig. S1A).

Table 1. The conditions for luteolin analysis by high-performance liquid chromatography (HPLC)

Instrument	
Column	Eclips, C18 150 × 4.6 nm
Detector	UV detector
Solvent A	0.2% phosphoric acid in DW
Solvent B	MeOH
Standard	Dilution with MeOH
Injection volume	10 μl
Flow rate	1.0 ml/min
Gradient elution system	Isocratic
Time	15 min

One week after depilation, all mice except those from the control group received subcutaneous (S.C.) injections of 5 mg/kg testosterone propionate (TP; Tokyo Chemical Industry, Tokyo, Japan) dissolved in carboxymethyl cellulose sodium salt (SAMCHUN Chemical, Pyongtack, Korea) once a day to induce AGA. The mice of each group were orally administered each treatment as follows for 8 weeks: control (PBS, P.O.), AGA (TP S.C. + PBS P.O.), Fina (TP S.C. + 0.1 mg/kg finasteride, P.O.), C50 (TP S.C. + 50 mg/kg of *C. choseniana*, P.O.), and C100 (TP S.C. + 100 mg/kg of *C. choseniana*, P.O.). The dorsal skin of mice was observed once every two weeks. To investigate the hair regrowth that can be visually confirmed, we conducted hair regrowth scoring with obtained images of mice dorsal skin, utilizing the hair regrowth scale index (Fig. S1B). Each score on the scale corresponded to the rate of hair regrowth in the depilated area. After 8 weeks, none of the mice treated for two weeks. Ten weeks after administration, the mice were sacrificed, and their dorsal skin tissue was collected.

Serum DHT level measurement – Serum was collected and its DHT levels were measured using enzyme-linked immunosorbent assay (ELISA). All analyses were performed following the protocol of the DHT ELISA kit (My-

BioSource, San Diego, CA, USA).

Histological analysis – The skin tissue was collected, immediately fixed in a 10% buffered formalin phosphate solution (Sigma-Aldrich, MO, USA), embedded in paraffin, and cut into 5-μm-thick sections. After deparaffinization and dehydration, the skin sections were stained with hematoxylin and eosin (Dako, Denmark). All stained slides were analyzed under a light microscope (Nikon ECLIPSE Ni-U, Tokyo, Japan). Images were captured from 10 randomly selected areas at each magnification. The number of hair follicles and the length of dermal thickness, hair shaft, and inner root sheath (IRS) were measured using Image J software (Image J v46a, USA).

Cell culture and treatment – Hair follicle dermal papilla cells (HFDPCs) were purchased from PromoCell (Heidelberg, Germany). The cells were grown in Follicle Dermal Papilla Cell Basal Medium (Promocell, Heidelberg, Germany) supplemented with 1% penicillin-streptomycin (Gibco, NY, USA) and Supplement pack (Promocell, Heidelberg, Germany). These cells were incubated at 37 °C in 5% CO_2 humidified incubator. Cultured HFDPCs (1×10^5 cells/well) were seeded in 6-well collagen type I-coated plates (SPL, Pocheon, Korea) for 24 h. After 24 h, cells were treated without (control)/with 10 nM dihydrotestosterone (DHT; Sigma-Aldrich, MO, USA) except those of the control, finasteride (10 nM, Sigma, USA) and *C. choseniana* (50 and 100 μg/ml) which were additionally treated for 48 h.

Cell viability assay – The HFDPCs (1×10^4 cells/well) were seeded in 96-well collagen type I-coated plates (SPL, Pocheon, Korea) and incubated for 24 h. After 24 h, the cells were treated with various concentrations of *C. choseniana* (0, 10, 100, and 200 μg/ml) for 72 h. Absorbance was measured at 450 nm using a microplate reader (LTEK, Gyeonggi-do, Korea). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for the cell viability evaluation using the EZ-Cytox Cell Viability assay kit (Daeil, Korea). The evaluation was performed according to the manufacturer's instructions.

Western blotting – The protein concentration in the collected skin tissue samples and cells was determined using a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, USA). Protein samples (10 μg) were separated using 8-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Merck, New Jersey, USA) using a transfer system. The membrane was blocked with 5% skim milk and primary antibodies were added overnight. The used primary antibodies are revealed in Fig. S2. After incubation with the primary antibodies above, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse (AbFrontier, Seoul, Korea) and anti-rabbit (AbFrontier, Seoul, Korea) IgG antibodies for 2 h to detect each protein. The reacted protein bands were visualized using an enhanced chemiluminescence detection (ECL) kit (Biomax, Seoul, Korea) and quantified using the CS analyzer 4 program (Atto, Tokyo, Japan).

Statistical analysis – All experiment was conducted in double-blind. The results were selected randomly and reported as the mean ± standard deviation. All statistical analyses were performed with one-way analysis of variance by Tukey's method using Sigmaplot 12.0 (SYSTAT, CA, USA). Statistical significance was set at $p < 0.05$.

Results and Discussion

In this study, we investigated the effects of *C. choseniana* as AGA alleviating agents to inhibit androgen signaling and promote cellular proliferation using androgen-induced AGA animal and cellular models.

Initially, the chromatographic peak of luteolin in the *C. choseniana* extract sample was recognized based on the liquid chromatography uv spectrometry spectra and retention time, compared with our previous study.[1] The peak identities were further characterized by comparing the spike of the sample with the reference compound. The retention time of the chromatographic peak of luteolin in the sample was 5.9 min. The chemical structures of the reference (luteolin) and representative HPLC chromatogram standards are shown in Fig. S3. The luteolin content of the *C. choseniana* extract was 0.14%.

The therapeutic efficacy of *C. choseniana* was examined using TP-induced AGA mouse model. A significant ($p < 0.01$) increase in serum DHT level was observed in the AGA group relative to the control group. However, *C. choseniana* treatment resulted in a significant decrease ($p < 0.01$) in serum DHT level (Fig. 1A). As shown in Fig. 1B and C, the expression level of 5AR2 was significantly higher in the AGA group than in the control group, and *C. choseniana* treatment significantly ($p < 0.01$) downregulated 5AR2. The expression level of AR was significantly ($p < 0.01$) downregulated in both the C50 and C100 groups relative to that in the AGA group (Fig. 1B and C). In AGA, DHT, the most potent androgen, is strongly activated and highly expressed in the balding areas of the patient.[10] Drake et al. reported that decreased level of DHT in scalp skin delayed the progression of AGA.[11] DHT binds to AR and modulates a series of downstream signaling pathways, such as apoptosis and

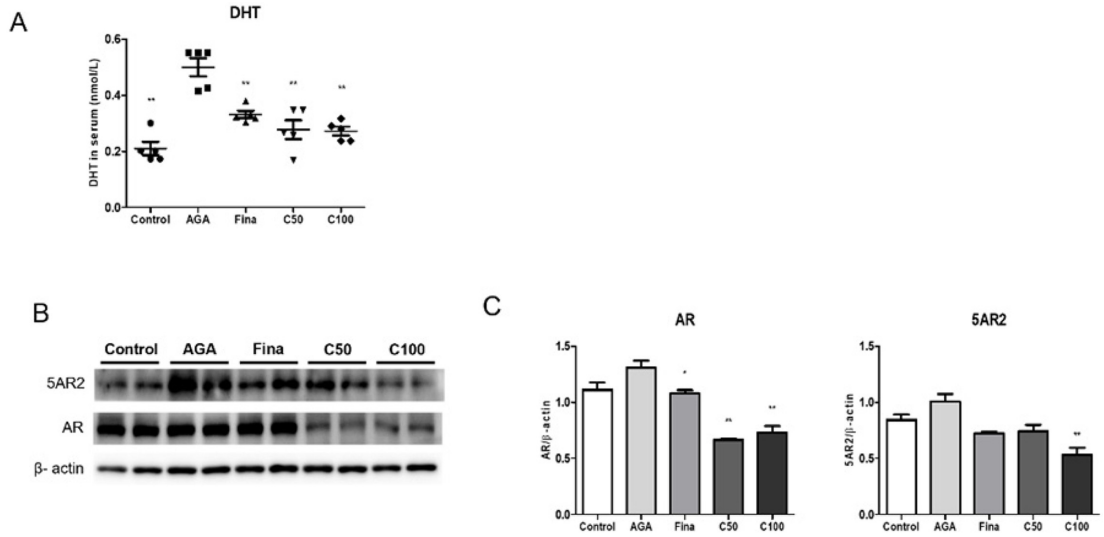

Fig. 1. Effects of *C. choseniana* on androgen action in TP-induced AGA mouse model.
(A) Concentration of DHT level in mice serum. (B) Western blot analysis of 5AR2 and AR expression levels *in vivo*. The expression intensities were normalized to those of β-actin. Values are expressed as the mean ± SEM. Significant differences were determined compared with the control group (#$p < 0.05$; ##$p < 0.01$) and with the AGA group (*$p < 0.05$; **$p < 0.01$). Abbreviation: Control (1X PBS P.O.), AGA (5 mg/ml TP, S.C. + 1X PBS P.O.), Fina (5 mg/ml TP, S.C. + finasteride 0.1 mg/ml P.O.), C50 (5 mg/ml TP, S.C. + *C. choseniana* 50 mg/kg P.O.), C100 (5 mg/ml TP, S.C. + *C. choseniana* 100 mg/kg P.O.)

inhibition of cellular proliferation in hair follicles.[12-13] These findings indicate that DHT plays an essential role in the progression of AGA. In our study, *C. choseniana* suppressed TP-induced upregulation of DHT, 5AR2, and AR in AGA. Considering this, we suggest that *C. choseniana* potentially prevented the progression of AGA by inhibition of androgen signaling.

Next, we monitored the proportion of hair regrowth and histological characteristics of AGA in TP-treated AGA mouse model. As shown in Fig. 2A and B, the hair regrowth score of the AGA group was lower than that of the control group every week. However, the hair regrowth score of the *C. choseniana*-treated group was higher than that of the AGA group every week. Although, the hair regrowth score of the C50 group remained the highest until the 8th week, the hair regrowth score of the C100 group was higher than that of the C50 group in the last 10th week. Histological images and analysis of the mouse skin tissue are depicted in Fig. 2C and D. Dermal thickness was significantly lower in the AGA group than in the control group ($p < 0.01$). However, the dermal thickness was greater in the *C. choseniana*-treated groups than in the AGA group. The number of hair follicles in the AGA group was significantly lower in the control group, whereas treatment with *C. choseniana* significantly ($p < 0.01$) increased the number of hair follicles in the AGA group. The analysis also showed the ratio of hair follicles to inner root sheath diameter (HF/IRS), a morphological parameter of the hair cycle. The HF/IRS ratio was lower in the AGA group than in the control group. However, *C. choseniana* significantly increased the HF/IRS ratio, in contrast to the case for the samples from the AGA group. According to the several studies, accelerated skin thickness and increased number of hair follicles are known to be associated with the anagen phase.[14-15] By comparing the characteristics of each group set for the same phase (telogen) at the beginning of experiment, it was shown that the *C. choseniana* induced anagen phase, whereas the AGA group was still passed the prolonged telogen phase. These results suggest that inhibition of androgen signaling through *C. choseniana* treatment regulated the hair cycle and induced the accelerated transition to the anagen.

Hair growth in hair follicle cells is induced by cellular proliferation. Especially, in the anagen phase, cellular proliferation is upregulated, and hair growth is activated.[16] In our study, cellular proliferation in the TP-induced AGA mouse model was monitored by investigating the expression of cyclin D1 and PCNA. The expression levels of cyclin D1 and PCNA were lower in the AGA group than in the control group. However, the expression of cyclin D1 and PCNA was significantly ($p < 0.01$) upregulated in the C50 and C100 groups, relative to that in the AGA group (Fig. 3A and B). Several studies showed that the expression levels of cellular proliferation factors, such as PCNA and cyclin D1, are increased during hair follicle

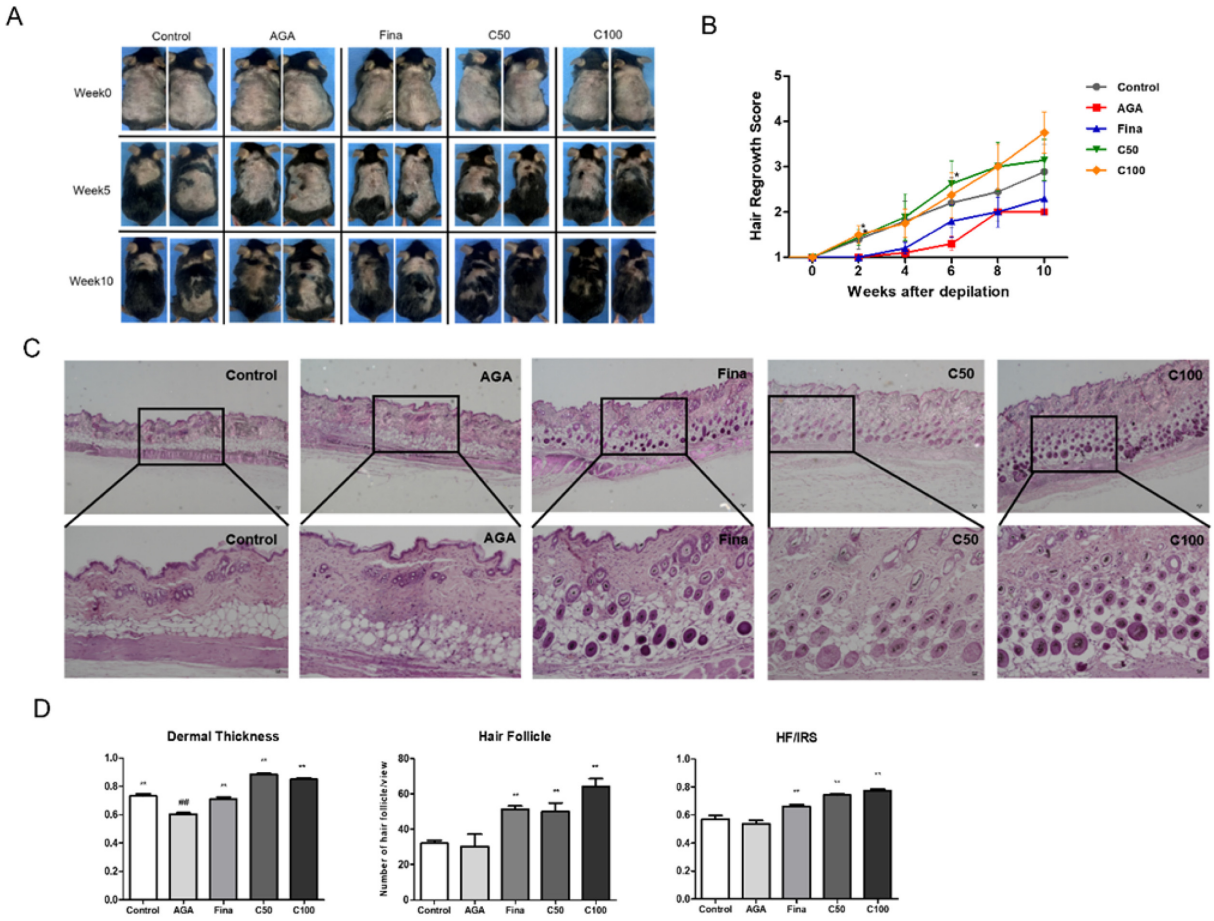

Fig. 2. Effects of *C. choseniana* on the regulation of the hair cycle in TP-induced AGA mouse model.
(A) Photographs of dorsal skins of all mice groups. Photographs were taken after week 0, 5, and 10 after depilation (B) Quantification of hair regrowth score in all mouse groups. (C) Histological images of dorsal skin of all mouse groups on week 10. All sectioned slides were stained with hematoxylin and eosin for same time (magnifications: 40X and 100X). (D) The number of hair follicles per view, quantification of dermal thickness (the ratio of dermis and epidermis) per view, and the ratio of IRS and hair shaft diameters. 10 images (magnifications: 100X) were randomly selected for observation in each group. Values are expressed as the mean ± SEM. Significant differences were determined compared with the control group (#$p < 0.05$; ##$p < 0.01$) and with the AGA group (*$p < 0.05$; **$p < 0.01$). Abbreviation: Control (1X PBS P.O.), AGA (5 mg/ml TP, S.C. + 1X PBS P.O.), Fina (5 mg/ml TP, S.C. + finasteride 0.1 mg/ml P.O.), C50 (5 mg/ml TP, S.C. + *C. choseniana* 50 mg/kg P.O.), C100 (5 mg/ml TP, S.C. + *C. choseniana* 100 mg/kg P.O.)

growth,[15,17] These studies suggest that cellular proliferation is considerably activated in the anagen phase, and the upregulation of cellular proliferation is significant in ameliorating AGA. Concomitant to these reports, our study showed that *C. choseniana* treatment promoted the cellular proliferation via upregulation of proliferation related-factors in TP-induced AGA mouse model.

We investigated a signaling pathway, related to the cellular proliferation in TP-induced AGA mouse model. Our study showed that *C. choseniana* treatment markedly enhanced the expression of Wnt3a and β-catenin in AGA group ($p < 0.01$). Furthermore, *C. choseniana* induced the phosphorylation of Akt and GSK-3β compared to those of AGA group ($p < 0.01$) (Fig. 3C and D). The Wnt/β-catenin signaling pathway plays a pivotal role in the maintenance and regrowth of hair follicles.[18] In particular, Wnt3a, secreted from the epithelium of hair follicles, maintains the anagen phase in dermal papilla cells.[19] It is associated with the expression of β-catenin, a regulator of pro-proliferation factor-encoding gene transcription. GSK-3β, the main regulator of the Wnt/β-catenin signaling pathway, acts as a key activator of hair follicle morphogenesis and hair growth.[20] DHT-AR inhibits the phosphorylation of GSK-3β, leading to the degradation of β-catenin.[21] Moreover, increased phosphorylation of GSK-3β is followed by increased phosphorylation of Akt.[22] We additionally investigated the expression of DKK-1 to confirm the regulation of the Wnt/β-catenin signaling pathway in hair

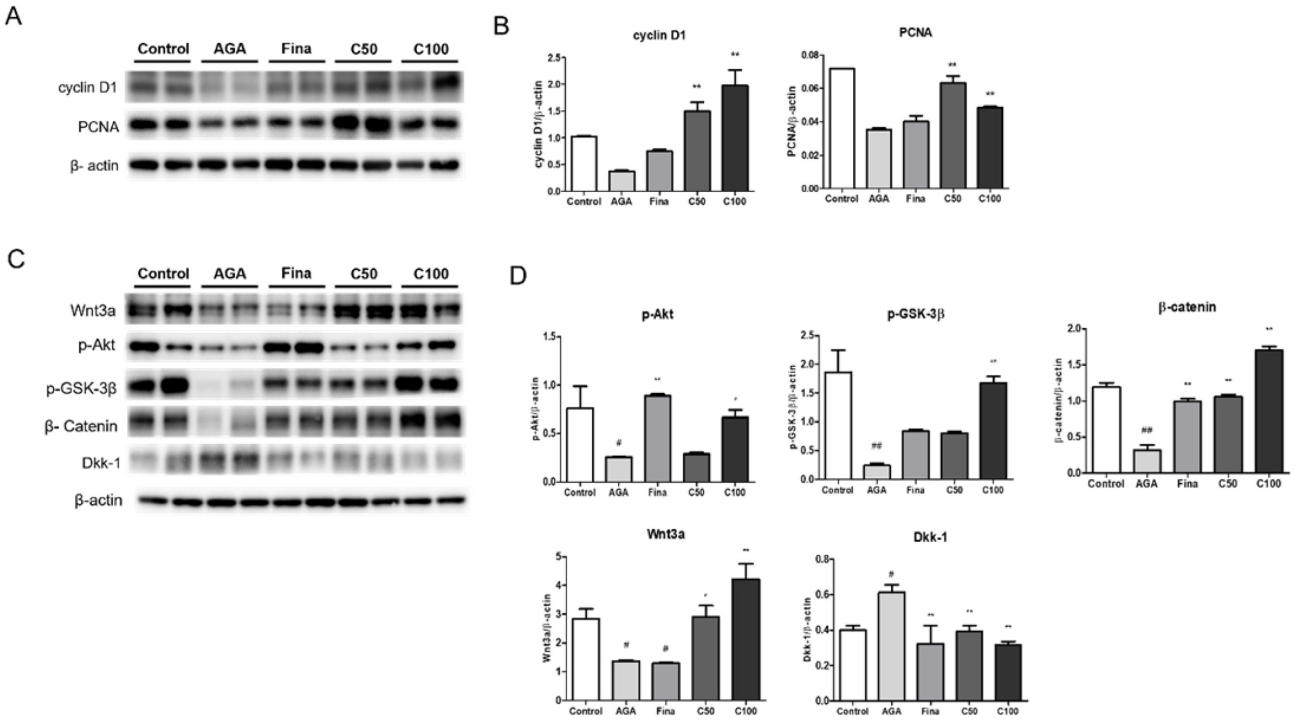

Fig. 3. Effects of *C. choseniana* on cell proliferation in TP-induced AGA mouse model.
(A-B) Western blot analysis of Cyclin D1 and PCNA expression levels *in vivo*. The expression intensities were normalized to those of β-actin (C-D) Western blot analysis of, p-Akt, p-GSK-3β, β-catenin, Wnt3a, and DKK-1 expression levels *in vivo*. The expression intensities were normalized to those of β-actin. Values are expressed as the mean ± SEM. Significant differences were determined compared with the control group (#$p < 0.05$; ##$p < 0.01$) and with the AGA group (*$p < 0.05$; **$p < 0.01$). Abbreviation: Control (1X PBS P.O.), AGA (5 mg/ml TP, S.C. + 1X PBS P.O.), Fina (5 mg/ml TP, S.C. + finasteride 0.1 mg/ml P.O.), C50 (5 mg/ml TP, S.C. + *C. choseniana* 50 mg/kg P.O.), C100 (5 mg/ml TP, S.C. + *C. choseniana* 100 mg/kg P.O.)

follicles. The expression level of DKK-1 was increased in the AGA group, whereas it was reversed by *C. choseniana* treatment in the AGA group (Fig. 3C and D). In contrast, it is reported DKK-1 acts as a natural Wnt inhibitor and disrupts the activation of the Wnt/β-catenin signaling pathway in AGA.[23] In addition, we suggest that *C. choseniana* also suppressed expression of DKK-1, contributing activation of Wnt/β-catenin signaling pathway. These results suggest that *C. choseniana* treatment promoted cell proliferation via modulation of the Wnt/β-catenin signaling pathway in TP-induced AGA mouse model.

Subsequently, we also investigated the effect of *C. choseniana* on AGA in vitro using DHT-treated HFDPC cells. Dermal papilla cells located in the hair follicles are well known to be related to regulate hair growth.[24] Especially, dermal papilla cells mediate the transition of telogen to anagen by various signaling pathway including Wnt/β-catenin signaling pathway.[15] In our study, *C. choseniana* treatment (10, 100, and 200 μg/ml) increased the cellular proliferation in MTT assay (Fig. 4A). To further investigate the cell proliferation of HFDPCs, the expression of cyclin D1 and PCNA was determined. Both cyclin D1 and PCNA expression levels in the DHT treatment group were similar to those in control HFDPCs. However, the expression levels of both cyclin D1 and PCNA were significantly ($p < 0.01$) higher in the *C. choseniana* treatment group than that in the DHT treatment group (Fig. 4B and C). The investigations of related signaling pathways involved in HFDPCs proliferation are shown in Fig. 4D and E. DHT treatment reduced the phosphorylation of both Akt and GSK-3β, whereas treatment with *C. choseniana* significantly ($p < 0.01$) increased the phosphorylation of both Akt and GSK-3β by over two-fold. β-catenin expression in the DHT treatment group was significantly lower ($p < 0.05$) than in control HFDPCs. However, the β-catenin expression level was higher in the *C. choseniana* treatment group than in the DHT treatment group. These *in vitro* results showed that *C. choseniana* treatment also promoted cellular proliferation in DHT treated HFDPCs.

In conclusion, this study demonstrates that *C. choseniana* alleviates androgen induced AGA by suppressing androgen signaling. Furthermore, *C. choseniana* promoted hair regrowth and cellular proliferation via the Wnt/β-catenin

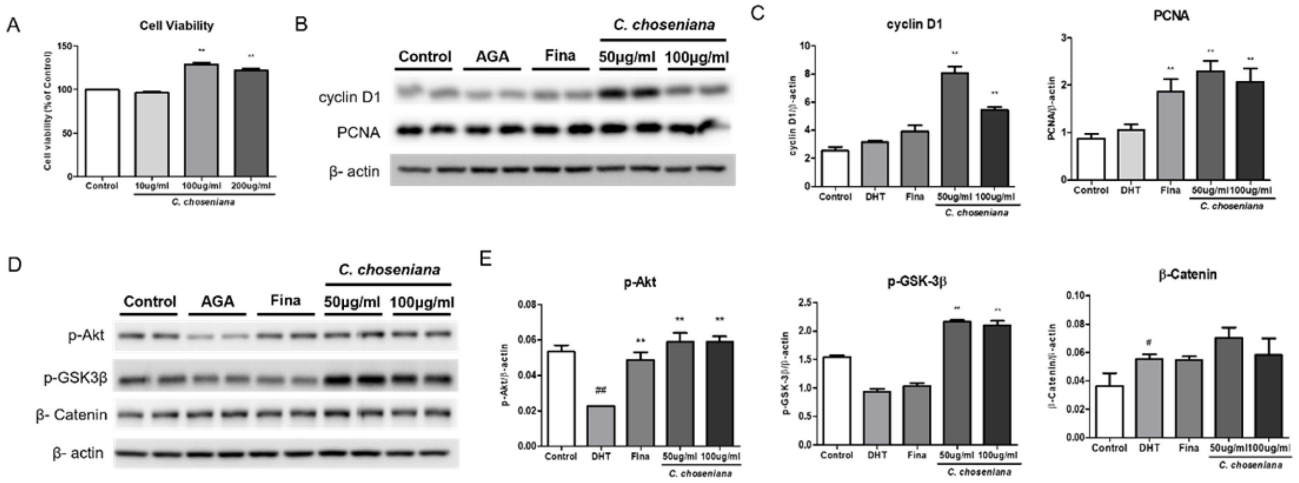

Fig. 4. Effects of *C. choseniana* on DHT-treated HFDPCs.
(A) Cell viability. (B-C) Western blot analysis of cyclin D1 and PCNA expression levels. The expression intensities were normalized to those of β-actin. (D-E) Western blot analysis of p-Akt, p-GSK-3β, and β–catenin expression levels. The expression intensities were normalized to those of β-actin. Values are expressed as the mean ± SEM. Significant differences were determined compared with control group (#$p < 0.05$; ##$p < 0.01$) and DHT group (*$p < 0.05$; **$p < 0.01$).

signaling pathway. Collectively, these results suggested that *C. choseniana* has potential to alleviate AGA.

Declaration of competing interest

The authors declare that they have no competing interests in this manuscript.

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Therapeutic Potentiality of *Celtis choseniana* Nakai on Androgenic Alopecia through Repression of Androgen Action and Modulation of Wnt/β-catenin Signaling

Hui-Ju Lee[1], Geum-Lan Hong[2], Kyung-Hyun Kim[1], Yae-Ji Kim[1], Tae-Won Kim[1], and Ju-Young Jung[1,*]

[1]*Department of Veterinary Medicine & Institute of Veterinary Science, Chungnam National University, Daejeon 34134, Republic of Korea*

[2]*Department of Anatomy, College of Medicine, Konyang University, Daejeon 35365, Republic of Korea*

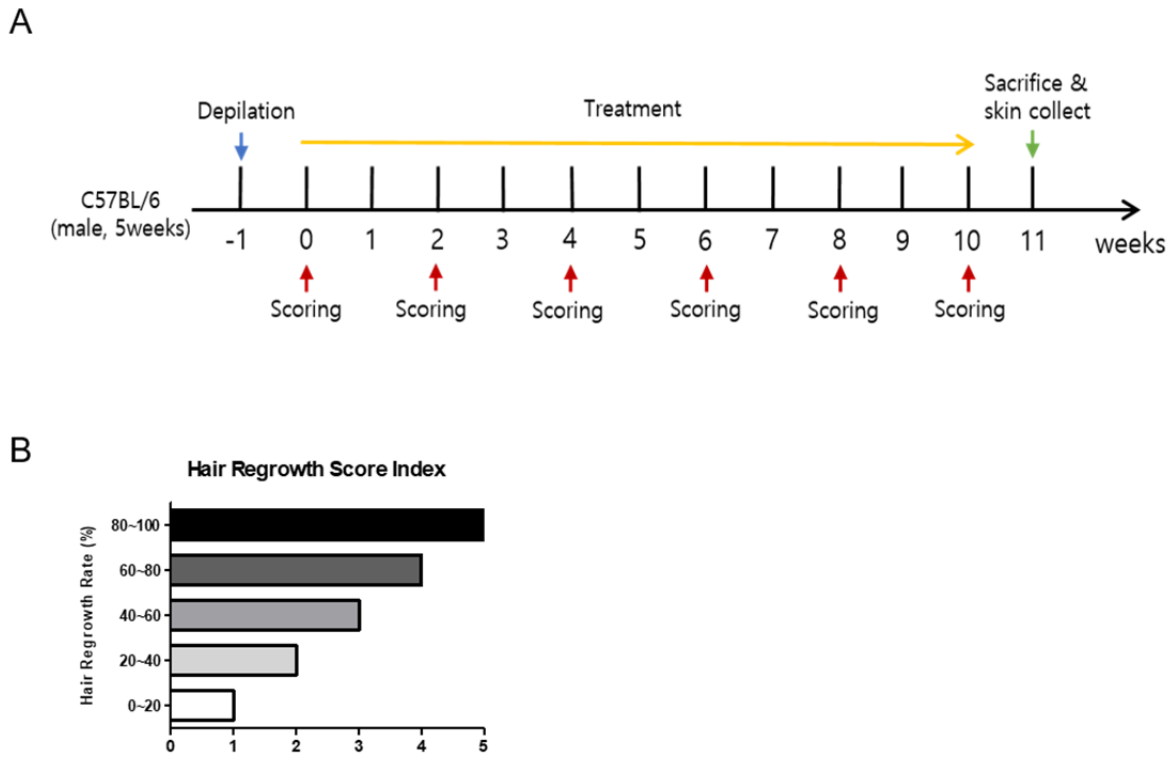
*Corresponding author: Ju-Young Jung, DVM, PhD

College of Veterinary Medicine, Chungnam National University, 220 Gung-dong, Yusung-gu, Daejeon 34134, Republic of Korea.

Tel: +82-42-821-8899,7902; Fax:+82-42-821-7926

E-mail : jyjung@cnu.ac.kr (J.Y. Jung)

Supplementary Fig. 1. (A) Diagram of mice treatment schedule. (B) Index of mouse hair regrowth score.



Supplementary Fig. 2. The list of primary antibodies used in this study

	Name	Dilution ratio	Company	State	Country
1	anti-beta-actin	1:1000	Abcam	Cambridge	United Kingdom
2	anti-5 alpha reductase2 (5AR2)	1:1000	Biorbyt	Cambridge	United Kingdom
3	Anti-androgen receptor (AR)	1:1000	Proteintech	Rosement	USA
4	anti-proliferating cell nuclear antigen (PCNA)	1:1000	Abcam	Cambridge	United Kingdom
5	anti-CyclinD1	1:1000	Abcam	Cambridge	United Kingdom
6	anti-phospho-Akt	1:1000	Cell Signaling	Danvers	USA
7	anti-phospho-glycogen synthase kinase 3 beta (p-GSK-3β)	1:1000	Cell Signaling	Danvers	USA
8	anti-beta-catenin	1:1000	Cell Signaling	Danvers	USA
9	anti-Wnt3a	1:1000	Abcam	Cambridge	United Kingdom

Supplementary Fig. 3. Chemical analysis of *C. choseniana*

(A) Chemical structure of compound, luteolin (B-C) HPLC chromatograms of *C. choseniana*