In vivo and *In vitro* hair growth promotion effects of extract from *Glycine soja Siebold et Zucc*

Jae Chan Yang¹ · Bo Ae Kim¹

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Abstract Hair is a dermal adjunctive organ that protects the body from external physical and chemical stimuli; hair undergoes anagen, catagen, and telogen phases, with hair-loss occurring during the telogen phase. Alopecia is a condition wherein a person undergoes hair-loss far exceeding the normal amount, owing to diverse external factors. Wild beans are rich in isoflavone and amino acids known to prevent hair-loss; compared to cultivated beans, many wild bean species have higher protein content. This study aimed to develop a hair growth promoting solution, with superior hair growth promoting effects and fewer side effects, using naturally obtained *Glycine soja Siebold et Zucc* (GSSZ) extracts. Seven-week-old C57BL/6N male mice were classified into different experimental groups. Hair growth was observed in GSSZ-treated mice, and compared against that seen in 3 % minoxidil (MXD, positive control)-treated mice. Visual observations revealed a greater reduction in hair-loss in MXD and GSSZ application groups, compared to that in TXN group (hair loss induction using 1 % testosterone). Evaluation using an image analysis software revealed that compared to the positive control, TXN + GSSZ group showed the highest hair growth. TXN + MXD and control groups exhibited similar follicular cell growth, while the hair growth promotion patterns were similar in the negative control (normal), TXN + GSSZ, and TXN groups, as observed via histological analysis. GSSZ did not induce cytotoxicity (even at 2 mg/mL) in keratinocytes and dermal papilla cells; alternately, dermal papilla cell proliferation was activated in a (GSSZ) concentration-dependent

manner. Therefore, the GSSZ extract promoted hair growth and increased hair growth-related cell activity, and could therefore be utilized in alopecia treatment.

Keywords Alopecia · Dermal papilla cells · *Glycine soja Siebold et Zucc* · Hair growth · Keratinocyte

Introduction

Human hair follows a repetitive cycle of maintaining the anagen phase for 3–8 years, undergoing hair loss during the catagen and telogen phases, and new hair growth at the site of hair-loss (Allegra et al. 1970; Arase and Sandamoto 1999). On an average, normal people experience a loss of 50–100 hairs on a daily basis; therefore, maintaining the balance between hair formation and loss allows for the retention of basic appearance. Alopecia is a generic term that describes hair loss from the scalp or body skin, owing to an increase in the extent of hair loss over that of hair formation; this mostly occurs on the scalp (Stenn et al. 1996; Arck et al. 2003). Recent social trends of gradual advancement and industrialization have led to the development of sociocultural factors that exert direct and indirect influences on alopecia, including excessive stress caused by increased social activities and lifestyle changes; consequently, this has resulted in a rapid increase in the number of people suffering from alopecia. Moreover, the population group suffering from alopecia has expanded from middle-aged males to young males and females; therefore, a number of studies have been performed to analyze the various causes of hair loss and identify potential solutions (Cash 1992). Causes of alopecia include genetic factors, secretion of androgens, excessive stress, blood circulation disorders, nutrient deficiencies, diet, smoking, drinking, and endocrine disorders; the major factors of alopecia are largely divided into internal factors and external environment factors (Stenn and Paus 2001).

Bo Ae Kim (✉)
E-mail: kba@mokwon.ac.kr

¹Division of Biomedical & Cosmetics, College of Sciences & Technology, Mokwon University, Daejeon 302-729, Republic of Korea

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Male pattern alopecia or male dominated-hair loss that displays a certain pattern in bangs is a type of progressive hair loss that occurs after the initiation of testicular secretion and is known to be caused by genetic factors, stress, scalp bloodstream disorders, and hypersecretion of male hormones (Messenger and Olsen 1994). Dihydrotestosterone (DHT), a byproduct of the male hormone testosterone, is known to be a cause of male pattern alopecia; a large quantity of DHT is produced in the head by 5- α -reductase. The presence of DHT in the follicles (of humans who are genetically predisposed to alopecia) gradually leads to follicle shrinkage and shortened hair length, i.e., follicle shrinkage and degeneration (Taksahima et al. 1970; Taksahima and Montagna 1971).

The number of alopecia patients has been observed to increase every year; however, the currently available medications and treatment (implants) methods for alopecia patients have not been entirely successful. Alopecia is known to be accompanied with pain and considerable adverse effects; therefore, there is an urgent need for the development of novel materials for overall clinical utilization.

Over the past few years, a number of universities and company research centers have been actively involved in conducting cytological, biochemical, and molecular biological investigations into hair growth and hair loss (Stenn 1991), and have attempted the development of therapeutic methods for the treatment of hair loss and promotion of hair growth (Buhl et al. 1990).

Minoxidil and finasteride, compounds that have been officially approved by the US Food and Drug Administration (FDA), and Propecia, are currently being used to promote hair growth. Minoxidil was originally prepared and sold for use as an antihypertensive (Buhl et al. 1990). However, following reports of side effects, such as hirsutism, it was further developed as a solution for hair growth (Burton and Marshall 1979). Minoxidil is known to induce hair growth by increasing the nutrient supply via vasodilation and opening the potassium channel (Meisheri et al. 1988; Buhl et al. 1990). Finasteride (Merck Sharp & Dohme Corp., Sumneytown Pike West Point, Pennsylvania, USA) and Propecia (MSD S.A., Opfikon, Switzerland) assist in promoting hair growth by inhibiting 5- α -reductase activity, which in turn affects the male hormone metabolism (Kaufman 1996).

Intensified research on anti-hair loss agents and hair growth promoting solutions over the past few years has resulted in the development of diverse products; however, the safety and efficacy of these anti-hair loss agents remain to be validated and developed. The major aim of this study was to investigate the hair loss prevention and hair growth promotion effects of extract from black beans, which has been classically (since ancient times) considered to prevent hair loss. Beans are a major protein source, containing 40 % qualified proteins, and an effective nutrient source. Beans contain cysteine, an essential component for hair growth, which has been shown to effectively prevent hair loss in humans

(Jones and Rivett 1997; Kovalenko et al. 2006). Among these, black beans contain abundant quantities of lecithin, phytoestrogen, unsaturated fatty acids, and anthocyanin, in addition to proteins, and exhibit a range of medicinal activities, including antioxidant, anti-cancer, anti-obesity, estrogenic, and immunomodulatory activities (Maeda et al. 1992; Liao et al. 2001; Zhao and Lou 2006; Jang et al. 2008). In addition, black beans are known to improve blood circulation by inhibiting the development of thrombi; previous reports have suggested that the vitamin E, unsaturated fatty acid, and anthocyanin content of black beans expand the blood vessels in order to facilitate blood circulation in the peripheral blood vessels (Shimizu et al. 2001; Kim et al. 2011).

A number of previous studies have utilized ethanol as the major solvent to evaluate the various effects of black beans. Black beans are known to promote hair growth and dermal papilla cell growth and exert a hemotherapeutic effect (Jeon et al. 2011); in addition, the use of black beans improves blood circulation by inhibiting the formation of thrombi, and it has excellent therapeutic effects against a number of diseases (Kim et al. 2011). Black beans provide nutrition to the roots of the hair by improving blood circulation in the scalp, effectively preventing hair loss; therefore, these are widely prescribed as food to prevent hair loss in folk medicine. Some studies have reported the effects of cultivated soybean and black bean extracts on hair cycle and growth (Kim et al. 2007); however, so far, the effect of wild beans (a variety of black beans) on hair growth has not been investigated. Wild beans are vines belonging to the Leguminosae family, which grow in annual cycles in the wild. Oriental medicine has identified the fruits of wild beans as Seomoktae (small black beans), and the stems and seeds as Yadaedudeung and Yaryodu respectively. Wild beans are used to prevent diseases affecting adults, such as breast cancer, prostate cancer, and heart disease, and contain antioxidants that are known to be effective against hyperlipidemia, atherosclerosis, and lung cancer. Several studies have attempted to devise methods for the effective utilization of wild beans; several species of wild beans have higher protein content than do cultivated beans. Wild beans have an average crude protein content of 45.4 % (37.4–50.2 %), which is approximately 5 % higher than that seen in general cultivated species (Kim and Park 2005). Based on the findings of the aforementioned studies, this study investigated the cytotoxic and cell proliferative effects of wild beans on keratinocytes and dermal papilla cells, which are generally used to stimulate hair growth in *in vitro* experiments. In addition, the effect of wild beans on C57BL/6 mice with testosterone induced-hair loss was analyzed *in vivo*. The skin was treated with wild bean extract in ethanol; the hair loss and hair growth characteristics of the experimental group was compared against that of the positive control treated with 3 % minoxidil, in order to investigate the possible hair growth-promoter characteristics of extracts from wild bean.

Materials and Methods

GSSZ extract preparation

The wild beans (*Glycine soja Siebold et Zucc*; GSSZ) used in this study were collected from Daedeok Mountain, Jeonbuk, Korea. Wild beans (500 g) were mixed with 20 % (v/v) ethanol in a shaker incubator (Jisico Co., Ltd., Bucheon, Korea) for 24 h at room temperature, with constant stirring, in order to obtain the extract. The extract was subjected to vacuum filtration and concentrated using a rotary evaporator (EYELA, Tokyo, Japan). An average yield of 18 % was obtained, and the extract was stored at -80°C until further analysis. The GSSZ extract was dissolved in dimethyl sulfoxide (DMSO) for vehicle; Sigma-Aldrich, St. Louis, MO, USA) at a final DMSO concentration not exceeding 0.2 %.

Lactate dehydrogenase (LDH) cytotoxicity measurement

The cytotoxic effect of the GSSZ extract was identified using a lactate dehydrogenase (LDH) release assay (CytoTox 96 Kit; Promega, Madison, WI, USA). The human keratinocyte HaCaT strain was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, New York, USA) supplemented with 100 U/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin (Gibco Inc., Life Technologies, Carlsbad, CA, USA), and 10 % heat-inactivated fetal bovine serum (FBS; Gibco Inc.) and incubated at 37°C in a 5 % CO_2 constant-temperature chamber; the cells were sub-cultured every 3 days. 1×10^5 cells/mL/well were seeded in 96-well plate, treated with 1, 5, 10, and 20 $\mu\text{g/mL}$ GSSZ extract, or 0.3 mM H_2O_2 (control), and incubated for 24 h. The culture solution and LDH reagent (50 μL each) were added to the cells, and allowed to react at room temperature for 30 min. Subsequently, the stop solution (50 μL) was added to arrest the reaction; the absorbance was then measured at 490 nm by using a micro-plate reader (Emax microplate reader; Molecular Devices, Sunnyvale, CA, USA).

Effect of GSSZ extract on dermal papilla cell proliferation

Dermal papilla cells (DPCs) were cultured in DMEM supplemented with penicillin-streptomycin and 10 % FBS (as described in the previous subsection), incubated at 37°C in a 5 % CO_2 constant-temperature chamber, and sub-cultured every 3 days. DPCs (1×10^4 cells/mL) were treated with GSSZ extracts at concentrations of 1, 5, 10, and 20 $\mu\text{g/mL}$ in a 96-well plate and incubated for 24 h. The positive control minoxidil (Sigma-Aldrich, St. Louis, MO, USA) was treated at a concentration of 10 μM . Cell proliferation was measured using the 4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) assay, per the manufacturer protocols.

Experimental animals Seven-week-old C57BL/6 male mice with an approximate weight of 25 g were obtained from Orient Bio (ORIENT Co., Ltd., Seongnam, Korea). These were subjected to dietary adaptation, under a controlled environment (average temperature, $25 \pm 1^{\circ}\text{C}$; relative humidity, 45 ± 5 %; and 12-h light/

dark cycle) in the Clean Animal Lack (Jeongdo Industry Co., Ltd., Seoul, Korea) for 1 week. The mice were allowed *ad libitum* access to commercially available experimental animal feed (Purina Inc., Seongnam, Korea) and purified tap water in a container, during the adaptation period.

Animal experimentation (hair removal and application methods)

The animals subjected to 1 week of dietary adaptation were divided into the experimental groups ($n = 6$ per group). Hair on the dorsal skin of the experimental animals was shaved off a day before treatment with the GSSZ extract; an electronic clipper was used to shave the small animals. Testosterone (1 %, 80 μL) was applied to the dorsal skin once a day for 7 days to induce hair loss; the GSSZ extract (80 μL) was applied to the skin using a dropping pipette and rubbed 3–4 times to accelerate the absorption; this was repeated every morning for 5 weeks. Purified water was used as the negative control (control, CON). Mice belonging to the first and second positive control (TXN and TXN-MXD) groups were treated with 1 % testosterone (to induce hair loss) and 3 % minoxidil (after inducing hair loss with testosterone), respectively. In the TXN + GSSZ experimental group, the mice were treated with 20 $\mu\text{g/mL}$ GSSZ for 5 weeks after induction of hair loss by testosterone; after hair loss induction, the 80 μL GSSZ extract was applied to the mouse dorsal skin surface once a day for 5 weeks.

Analysis of body hair and measurement of hair length using the image analysis software

Visual confirmation of hair growth was obtained by taking photographs of the experimental mice by using a D70 model camera (Nikon, Tokyo, Japan). The photos were analyzed using an image analysis software (Each system ships with 1 full version of Quantity One and unlimited copies of Quantity One Basic Mode; Bio-Rad, Hercules, CA, USA).

Histological analysis

The follicle changes in the skin were histologically analyzed; the mice were subjected to cervical dislocation, and the mouse dorsal skin showing hair growth was excised. The excised skin tissue was fixed with 10 % formalin buffer solution, and embedded in paraffin to create 4- μm -thick paraffin tissue sections by using a Microtome (HM-315 model, Microm Co., Walldorf, Germany). The sections were then stained with hematoxylin and eosin (H&E) stain. The histological changes in follicle tissues were observed using an optical microscope (Olympus, Tokyo, Japan); in addition, the rate and characteristics of follicle formation during the anagen phase were evaluated.

Statistical analysis

All results were expressed as means $\pm$ standard deviation or standard error of mean; the data was statistically analyzed using Student's *t*-test. *p*-values less than 0.05 were considered to be statistically significant.

Fig. 1 Cytotoxicity (LDH release) of GSSZ extracts in HaCaT cells. HaCaT cells were treated with various concentrations of GSSZ (0.1, 0.5, 1.0, 2.0 mg/mL) for 24 h. The release of LDH from HaCaT cells was analyzed by measuring absorbance at 490 nm as described in Materials and Methods. Bars represent an average of three independent measures. Data are presented as the mean $\pm$ SE (n=6). * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle treated control

Results

Cytotoxicity of keratinocytes

The cytotoxicity of keratinocytes subjected to GSSZ extracts was investigated. The keratinocytes were treated with GSSZ at concentrations of 0.1, 0.5, 1.0, and 2.0 μ g/mL; simultaneously, the positive control (0.5 mM) (H₂O₂) was used to induce cell damage. The H₂O₂-treated HaCaT cells showed high cytotoxicity (>80 %), while the cells exposed to GSSZ did not exhibit cytotoxicity (even at 2 mg/mL GSSZ concentration) (Fig. 1).

Rate of proliferation of dermal papilla cells

The addition of GSSZ extracts at 0.1, 0.5, 1.0, and 2.0 mg/mL, resulted in increased proliferation in the dermal papilla cells, by 102.10 $\pm$ 0.87, 106.17 $\pm$ 9.12, 108.82 $\pm$ 10.38, and 109.25 $\pm$ 16.57 %, respectively, compared to the negative control; this suggested a dose-dependent increase. The positive control minoxidil resulted in the highest increase in dermal papilla cell proliferation, at 119.28 $\pm$ 6.3 % (Fig. 2). These results confirmed the hair growth-inductive effect of GSSZ extracts, through the proliferation of dermal papilla cells.

In vivo measurement of hair growth in the anagen phase

The hair on the dorsal skin of C57Bl/6N mice were removed and subjected to 1 % TXN for 1 week to inhibit hair growth. Subsequently, MXD and GSSZ were applied for 7 weeks; on the day of experiment completion, the hair was collected and the hair growth in the anagen phase was examined up to 0.1 mm by using vernier calipers (Mitutoyo Corp., Tokyo, Japan). The measurement of the hair length after hair growth inhibition revealed that the CON and

Fig. 2 The effect of GSSZ extract on the inhibition of the proliferation of dermal papilla cells (DPCs). Immortalized vibrissa DPCs (1.0×10^4 cells/mL) were plated in 96 well plates. DPCs were treated with various concentration of GSSZ extract, as indicated. Cell proliferation was measured using a MTT assay for 4 days. Minoxidil (10 μ M) was used as a positive control. Data are presented as the mean $\pm$ the SD of three independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. control

Fig. 3 Comparative the length of the hair on induced alopecia male C57BL/6 mice. Hair growing on the dorsal skin of mice was collected on the day of experiment completion; hair growth during the anagen phase was examined using vernier calipers

TXN groups (normal and positive control with 1 % testosterone treatment, respectively) manifested the longest and shortest hair lengths at 0.55 $\pm$ 0.09 and 0.43 $\pm$ 0.05 mm, respectively. The TXN + MXD and TXN + GSSZ groups exhibited lengths of 0.51 $\pm$ 0.10 and 0.53 $\pm$ 0.03 mm, indicating greater hair growth compared to the positive control (TXN); however, it was lesser (shorter) than that observed in the normal group. The TXN + GSSZ group (testosterone + GSSZ) showed hair growth similar to that observed in the TXN + MXD group (Fig. 3).

Fig. 4 *In vivo* hair growth promotion the GSSZ extracts on male C57BL/6 mice of alopecia (Image analysis on dorsal skin). Hair length measurement using Scion Image 1.63 software. Image analysis software was used to quantify the percentage of hair length relative to the total area of the dorsal skin of C57BL/6

Analysis of hair growth using an image analysis software

Hair growth was visually confirmed by taking photos of the dorsal skin in the experimental mice every 2 weeks (once) after initiation of the hair growth experiment. The obtained images were analyzed using the Scion image analysis 1.63 software (Scion Image, Scion, Frederick, MD, USA). The TXN group showed the lowest value at 116.26 ± 4.39 , while the TXN + GSSZ and TXN + MXD groups showed values of 150.21 ± 5.61 and 114.61 ± 6.85 , respectively (Fig. 4).

Histological analysis

Compared to the CON group, the TXN group (treated with 1 % testosterone to induce hair loss) showed fixed follicular cells and circular hair roots. The TXN + MXD group showed longer hair roots in the follicles, suggesting active follicular cell growth. The TXN + GSSZ group, treated with the GSSZ extract, showed increased follicular cell growth, although this growth was slower than that observed in the positive control (TXN + MXD group) (Fig. 5).

Discussion

Recently, the number of males and females psychologically suffering from alopecia has increased drastically. Therefore, it is very important to develop medicinal substances that are effective for hair loss prevention and hair growth. The use of alternative medicine developed from oriental medicines or medicinal herbs has attained worldwide attention over the past few years. The morphological, histological, and biochemical aspects of hair loss in males were elucidated by the study of macaques. Representative male hair loss patterns include a prolonged telogen phase and a shortened anagen phase. The histological characteristic of hair loss, observed by H&E skin staining, was the insufficient increase in length of anagen follicles. The thickness of growing hair is determined by follicle size. Therefore, the length of the follicle, which grows between the initial anagen and middle anagen phase, is very important for the normal hair cycle (Takahima 1974). The C57BL/6 mice used in this study are known to enter the telogen phase of hair cycle within 6 weeks after birth. Mice at the telogen phase show a pink skin surface after shaving; the surface of the

Fig. 5 Histological observation of hair follicles (HF) in a male C57BL/6 mouse model of alopecia after topical application of samples for 4 weeks. A: Negative Control (CON), B: treated 1 % testosterone (TXN), C: treated 3 % minoxidil after treated 1 % testosterone (TXN + MXD), D: treated 2 % Glycine soja Siebold et Zucc extract after treated 1 % testosterone (TXN + GSSZ)

skin turned black with experiment progression, which indicates the resurgence of the anagen phase from the telogen phase (Tobin et al. 1998).

Minoxidil, a hair growth promotion agent that is currently seeing widespread usage, is used for percutaneous applications; finasteride and dutasteride, on the other hand, are administered orally. These agents are approved by the FDA and used extensively in clinical settings (Cash, 1992). However, clinical cases have indicated that the use of minoxidil may result in an increase in weight, edema, angina, dermatitis, and itching, while finasteride administration is known to impair male sexual function (McClellan and Markhan 1999; Hagemann et al. 2005). In addition, the treatment of HaCaT cells with 0.01 μ M minoxidil showed approximately 30 % higher cytotoxicity, compared to the normal group (Yoo et al. 2007). Therefore, it is important to develop hair growth materials derived from natural substances, which might avoid the drawbacks of conventional medicine, and might be safe for long-term utilization. Studies investigating the hair growth induced by reduction of oxidative stress in the scalp or by the application of natural plant-origin fermented substances, and hair growth promotion effected by plant extracts have been previously performed (Hee et al. 2012).

The results of previous studies have indicated that external stress induces cell oxidation; in addition, hair formation from the keratinocytes of dead skin cell layers is believed to be disrupted, resulting in weakening of the hair, leading to the catagen phase. The decreased follicle activity results in the progression of hair to the dystrophic anagen phase. In addition, stressed mice were reported to induce inflammation around the follicles via the Substance P (SP; a neuropeptide) route and through follicle development during the immature catagen phase, leading to inhibition of hair growth (Arck et al. 2003). Using an approach similar to the one described in the aforementioned study, another study reported that stress suppressed the keratinocyte proliferation in the telogen phase and accelerated apoptosis (Arck et al. 2001). This study investigated the cytotoxicity and cell proliferation rate using keratinocytes and dermal papilla cells. The oxidation of keratinocytes by hydrogen peroxide resulted in high cytotoxicity; on the other hand, the use of GSSZ extracts did not result in cytotoxicity even at high concentrations (20 μ g/mL). In addition, the GSSZ extract was observed to activate cell proliferation, thereby assisting in similar dermal papilla cell growth as that effected by minoxidil, a commonly used hair growth promoting solution. Dermal papilla cells, which have originated from mesoblasts and are located in the base of the follicles, are known to play a critical role in hair formation and growth by interacting with the matrix cells, including epithelial cells (Chi et al. 2013). In this study, *in vivo* experiments were performed in the C57BL/6 animal model; the size and morphology of follicles before and after experimentation was observed by histological analyses. The histological examination by H&E staining revealed distinct differences in the follicle length and size between the TXN + GSSZ (treated with the GSSZ extract)

and TXN (treated with 1 % testosterone) groups. The TXN group showed small and circular hair roots in the follicles, while the GSSZ group revealed follicles that were exposed to the skin because of the increased follicle length, suggesting the association of such a phenomenon with the hair growth effects of the GSSZ extract. A previous study used minoxidil, the positive control used in this study, to induce sufficient growth in follicles (length), and stimulate re-growth of the hair via follicle stimulation in C57BL/6 mice. Minoxidil sulfate is converted to active metabolites in the follicles, thereby improving blood circulation in the scalp (Bihl et al. 1990). The GSSZ extract also facilitated hair re-growth in C57BL/6 mice, similar to minoxidil. Although the mechanism underlying the hair growth promoting effects of the GSSZ extract remain to be elucidated, the results of this study confirmed that the GSSZ-induced accelerated follicle growth facilitated a resurgence of the anagen phase in the follicles. In other words, the time required for sufficient hair re-growth is shortened in the GSSZ extract-treatment group. Based on these results, GSSZ extract was believed to stimulate follicle growth to promote hair growth and increase the activity of hair growth-related cells in humans; therefore, the GSSZ extract may prove to be useful for the treatment of alopecia.

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