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Original Article

Phamacopuncture and Dermal Application of Sebalgukhwa-san: Effects on Hair Growth in a Mouse Model of Alopecia

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ABSTRACT

Background: This study was conducted to evaluate the effects of pharmacopuncture and dermal application of Sebalgukhwa-san extracts on hair growth in an alopecia mouse model.

Methods: Twenty-one C57BL/6 mice were divided into 3 groups; control group-normal saline injection or vehicle solution application, positive control group-minoxidil (MNXD), experimental group-pharmacopuncture and applied Sebalgukhwa-san (SGS) extract. The effects of the treatment on hair growth, were determined through photographs, and phototrichogram analysis by folliscope. Hair follicle morphometry by hematoxylin-eosin staining was performed, and hair growth-related protein expression of vascular endothelial growth factor, insulin like growth factor-1, and transforming growth factor-beta 1 were monitored by Western blotting. Serum levels of aspartate aminotransferase and alanine aminotransferase were measured for liver function test.

Results: Body weight increased consistently in all groups. Hair growth was improved in the MNXD and SGS groups compared with the control. Hair density and thickness improved statistically significantly in the MNXD and SGS groups compared with the control $p < 0.05$. The number of hair follicles improved in the MNXD and SGS groups compared with the control but the size did not. The expression of vascular endothelial growth factor and insulin like growth factor-1 increased, and there was a decrease in the expression of transforming growth factor-beta 1 in the MNXD and SGS groups compared with the control, however, there was no significant difference. Sebalgukhwa-san treatment had no toxicity in liver function tests.

Conclusion: Pharmacopuncture and dermal application of Sebalgukhwa-san extract may be therapeutically beneficial for the treatment of alopecia.

Introduction

Hair not only functions as protection but is also associated with beauty [1,2]. Hair production requires oxygenation and nutrients via the capillaries, with hair growth resulting from keratinized cells through cell division. Hair is distributed mainly in the scalp of a person's skin where it protects the brain and scalp from external factors such as temperature, ultraviolet rays, and impact. In addition, food intake, harmful heavy metals in the air and sebum secretion all affect the hair. The look of a person's hair can be associated with their attractiveness, or as an expression of their personal appearance.

Alopecia occurs in areas of the skin where hair should be present (generally over the scalp). The main causes of alopecia [3] are hormone imbalance, irregular lifestyle, excessive scalp stimulation (such as perming and dyeing), stress and diet (including weight loss, and iron deficiency). In Korean medicine, alopecia belongs to the category of oil, and together with wind, the hair falls out. Kidney deficiency, and Qi blood deficiency are known to represent internal causes, and heat and external wind are known to represent external causes [4]. In recent years, a mismatch of physical conditions causing abnormalities of the hormone and immune system, have been observed to result in the malnutrition of hair, and circulatory disorders [5]. Traditional medicine treatments for alopecia include herbal medicine [6-8], bee venom pharmacopuncture [9], pharmacopuncture [10,11] and the

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application of Saengbal-eum [12]. In recent years, plum-blossom needling [13], microneedle therapy system [14,15], and thread-embedding therapy [16] have also been used.

Sebalgukhwa-san is a prescription derived from Eyakwonbang [17], composed of *Chrysanthemi Indici Flos, Cnidii Rhizoma, Morus alba Linn, Viticis Fructus, Angelicae Dahuricae Radix, Asia Radix, Ecliptae Herba* and *Thujae Orientalis Folium*. It has the visual effect of darkening, and making the hair shine by treating dryness [18]. A study on the effects of Sebalgukhwa-san extract, and the composition of medicinal herbs for alopecia has been carried out using Gukhwasan extract shampoo [19], but no other studies have been reported to date. Therefore, this study aims to examine hair growth and the effects of using pharmacopuncture and skin treatment with Sebalgukhwa-san extracts using an alopecia mouse model (C57BL/6 mice).

Materials and Methods

Materials

Animals

In this study, 6-week old, female C57BL/6 mice, weighing approximately 15 g to 18 g were used. The mice were housed at 23°C ± 3°C, 50% ± 10% humidity, were exposed to 12 hours of light and dark per day, given sterile distilled water, and food. Experiments on the mice began 1 week after acclimatization. All procedures in this study were conducted in accordance with the regulations and policies of the Animal Experiment Ethics Committee of Daegu Haany University (Approval no: DHU2016-060).

Herbal medicine

Sebalgukhwa-san (Daewonherb, Daegu, Korea) herbal medicine was used in accordance with the dose described in Eyakwonbang [17] and is listed in Table 1.

Herbal medicine preparation and administration

The total amount of herbal medicine used was 270 g (Table 1), which was extracted with 2,700 mL of solvent mixture (ethanol and water at a ratio of 1:1), at 100°C, for 10 hours. The extract was filtered and concentrated by centrifugation at 7,000 rpm for 20 minutes. Secondary filtration of the extract was followed by lyophilization where 56.7 g of powdered sample was obtained (yield: 21.0%). The sample was stored at -70°C until use.

Table 1. Composition of Sebalgukhwa-san.

Herb scientific name	Origin	Dose (g)
Chrysanthemi Indici Flos	China	60
Cnidii Rhizoma	Korea	30
Morus alba Linn	China	30
Viticis Fructus	China	30
Angelicae Dahuricae Radix	Korea	30
Asia Radix	China	30
Ecliptae Herba	Korea	30
Thujae Orientalis Folium	Korea	30
Total		270

Pharmacopuncture preparation

Normal saline, and Sebalgukhwa-san (ethanol extracted, lyophilized, then dissolved in normal saline to make a 15% solution) pH 6.8 (adjusted with NaOH), were prepared.

Dermal application preparation

The dermal application was prepared using a vehicle solution mixed at a 3:1:1 ratio (normal saline, polyethylene glycol, 99.9% ethanol). Minoxidil (Moxidil Solution 3%, Hanmi Pharm Co Ltd, Korea) and Sebalgukhwa-san extract (Sebalgukhwa-san powder) were both dissolved in vehicle solution to make a 15% solution.

Methods

Alopecia induction

After diethyl ether was inhaled, and the mouse was anesthetized, the hair was removed using animal clippers to prevent damage to the skin. Then hair remover cream was applied to the primary hair removal site, and left for 5 minutes, hair that remained on the skin was removed using a hair removing spatula. Any remaining hair remover cream was washed away with warm water. The mice were left for 24 hours prior to the experiment.

Classification of the study groups

To evaluate the effects of pharmacopuncture and dermal application of Sebalgukhwa-san on hair growth, the mice were divided into 3 groups of 7 mice; 1) minoxidil-treated positive control group (MNXD, Minoxidil group), 2) Sebalgukhwa-san pharmacopuncture experimental group, and 3) Sebalgukhwa-san dermal application experimental group (SGS, Sebalgukhwa-san group). Each group was classified according to a randomized block design.

Treatment for each group

The experiment was carried out for 14 days, and all treatments were performed at 10.00 AM each day. Each control group received subcutaneous injections over the depilated area 7 times (2nd, 4th, 6th, 8th, 10th, 12th and 14th day), in 6 places including 3 right spinal regions, and 3 left spinal regions with 0.1 mL of normal saline. The vehicle solution (0.1 mL) was applied 7 times (1st, 3rd, 5th, 7th, 9th, 11th, and 13th day) percutaneously. In the MNXD positive control group, minoxidil (0.1 mL) was applied daily for 14 days, percutaneously to the depilated area. The SGS experiment group was injected subcutaneously over the depilated area 7 times (2nd, 4th, 6th, 8th, 10th, 12th, and 14th day), in 6 places including 3 right spinal regions and 3 left spinal regions using Sebalgukhwa-san pharmacopuncture (0.1 mL). Sebalgukhwa-san dermal application (0.1 mL) was applied 7 times (1st, 3rd, 5th, 7th, 9th, 11th, and 13th day) percutaneously to the depilated area.

Blood sampling and tissue section

On the 15th day, 1 day after the end of the experiment, the mice were fasted for 12 hours then sacrificed. After anesthesia (diethyl ether inhalation), blood was collected from the inferior vena cava of the abdomen, heparinized, and centrifuged at 1,000 rpm for 15 minutes at 4°C. The plasma was collected and stored at minus 70°C until analysis.

The skin tissue of the sacrificed mice was removed with scissors, washed several times with normal saline, the surface moisture removed and the skin cut into 3 sections. One section was quenched in liquid nitrogen and stored at minus 70°C until analysis using Western blotting, and 2 sections were fixed in 4% formaldehyde solution for 24 hours for use in folliscope analysis and hematoxylin-eosin staining.

Weight change

To examine whether subcutaneous injection or percutaneous application of Sebalgukhwa-san extract affected the body weight of the mice, they were weighed at 9.00 AM on the 1st, 8th and 15th day of the experiment.

Hair growth observation

To observe changes in the hair, a digital photo was taken on the 1st, 5th, 9th, 12th, and 15th day at 9.30 AM. Hair regrowth in the test area was visually scored; 0% to 19% (1 point), 20% to 39% (2 points), 40% to 59% (3 points), 60% to 79% (4 points), and 80% to 100% (5 points). The mean value for each mouse was calculated. Evaluations were made by averaging the judgment and scores of skilled researchers ($n = 10$), who had been blinded.

Folliscope image analysis

The skin tissue sections were flattened on Whatman filter paper and assessed using a folliscope. The same 2 regions of the image were designated, and hair density and thickness per unit area (cm^2) were calculated as an average value.

Hematoxylin-eosin staining

The flattened skin tissue was embedded in paraffin, and cut into 3 sections with a microtome. The slides were heated for 1 hour, deparaffinized with xylene, and dehydrated in serial dilutions (70%, 80%, 90%, and 100%) of alcohol; the slides were stained with hematoxylin and eosin and then sealed with a cover slip. Hematoxylin-eosin-stained tissue slides were photographed at a magnification of 10×0.25 using a microscope.

Western blotting

Lysis buffer was added to the defrosted skin tissue, and the supernatant was collected after centrifugation. Protein quantification was performed using the Bradford method [20] and electrophoresis was performed on 12% SDS-polyacrylamide gel. Proteins separated by size were transferred to a polyvinylidene fluoride microporous membrane using the electrical method. The membrane was immersed for 1 hour in 5% skimmed milk solution made up in phosphate buffered saline tween (PBST). Primary antibodies, insulin like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and transforming growth factor-beta 1 (TGF-β1) were diluted 1,000 times in 5% skimmed milk solution in PBST and left to react at 4°C for 12 hours. The polyvinylidene fluoride membrane was washed in 1 x PBST for 10 minutes, 3 times. The secondary antibody was diluted 1,000 times in 5% skimmed milk solution and left to react at room temperature for 1 hour. After 1 hour, the cells were washed with 1 x Tris buffered saline tween for 10 minutes, 3 times, and the fluorescence was developed with a small amount of enhanced chemiluminescence substrate. The degree of expression of each factor was analyzed using an image analyzer.

Liver function test

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are biomarkers of hepatocyte damage, were analyzed by spectrophotometer using the Reitman colorimetric method [21]. Substrate solution containing either AST (1 mL) or ALT (1 mL) was incubated at 37°C for 5 minutes, then 0.2 mL of plasma was added. AST plasma was incubated at 37°C for 60 minutes and ALT plasma was incubated at 37°C for 30 minutes. Then, 2,4-dinitrophenylhydrazine (color reaction solution) was added to the samples at room temperature and left to incubate for 20 minutes. The reaction was stopped using 0.4 N NaOH (10 mL) which was added to the sample and left to incubate for 10 minutes,

Table 2. Changes of the Body Weight in Alopecia Model of C57BL/6 Mouse.

Group	Body Weight (g)		
	Baseline	8th d	15th d
Control	17.13 ± 0.30	19.19 ± 0.35	21.00 ± 0.33
MNXD	17.14 ± 0.24	20.00 ± 0.35	21.57 ± 0.34
SGS	16.94 ± 0.27	19.06 ± 0.26	20.89 ± 0.27

Data are presented as mean ± SE of 7 mice per group.
Control, Mice were injected with normal saline and applied vehicle every other day; MNXD, Mice were applied with minoxidil every day; SGS, Mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.

before the absorbance was measured. To measure the reagent, a standard curve of serum hepatocyte enzyme was used as a calibration curve.

Statistical analysis

The results were analyzed using SPSS statistics 11.5 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was performed, and Duncan's post hoc test was used to confirm the significance of the mean value among the groups where $p < 0.05$.

Results

Weight change

Body weight measurement during the experimental period was shown to have continuously increased across all groups, and there was no significant difference between the groups (Table 2).

Hair growth observation

When the mice were depilated at 7 weeks of age, their hair was in the telogen stage, and their skin was pale pink; as the experiment proceeded, skin color on the dorsal area progressively turned black. Hair growth began in all groups on Day 5, and by Day 9 partial hair growth was observed with no difference between the groups. On Day 12, overall hair growth was observed in the dorsal area in all groups, but uniform growth was only observed in the MNXD and SGS groups. At Day 15 there was no difference between hair growth in the MNXD and SGS groups (Fig. 1).

Scoring hair growth by observation showed there was no significant difference between the groups at Day 9. On the 12th day of treatment, MNXD and SGS groups scored 2.86 and 2.43 respectively, which were statistically significantly different from the control group score of 1.71 ($p < 0.05$). In addition, both the MNXD and SGS groups scored 4.36 on Day 15 compared with the control group score of 3.64, showing a statistically significant difference in hair growth ($p < 0.05$, Table 3).

Folliscope image analysis

The MNXD and SGS groups showed an increased hair density and thickness compared with the control group (Fig. 2).

Hair density

Hair density was 28.67 ± 7.34 strands/cm^2 in the control group,

Table 3. Changes of Hair Growth Score in Alopecia Model of C57BL/6 Mouse.

Day	Hair Growth Score				
	1	5	9	12	15
Control	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.71 ± 0.13[a]	3.64 ± 0.27[a]
MNXD	1.00 ± 0.00	1.00 ± 0.00	1.07 ± 0.07	2.86 ± 0.18[b]	4.36 ± 0.23[b]
SGS	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	2.43 ± 0.20[b]	4.36 ± 0.17[b]

Data are presented as mean ± SE of 7 mice per group.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.
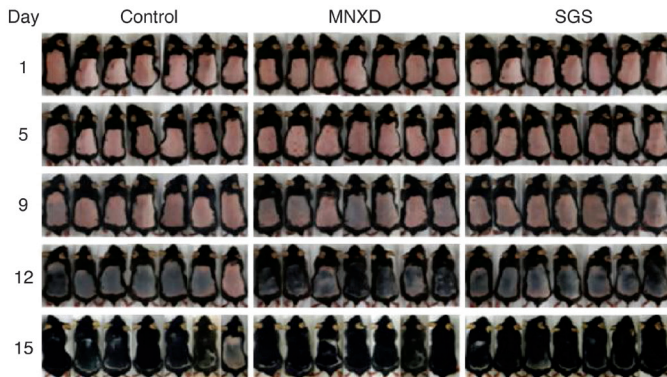[a,b]Means not sharing a common symbol are significantly different among groups at $p < 0.05$.

Fig. 1. Changes of hair growth in alopecia model of C57BL/6 mouse.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.
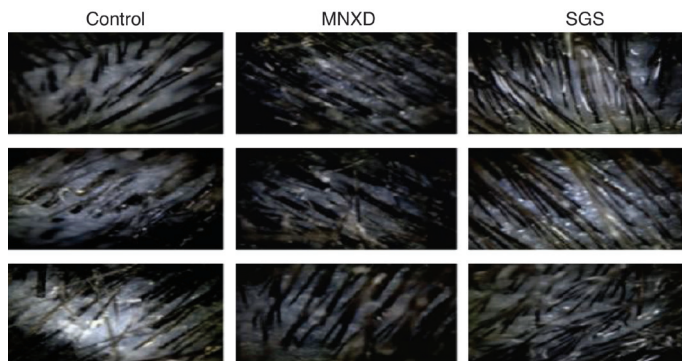

Fig. 2. Effects on hair density and thickness in alopecia model of C57BL/6 mouse.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.
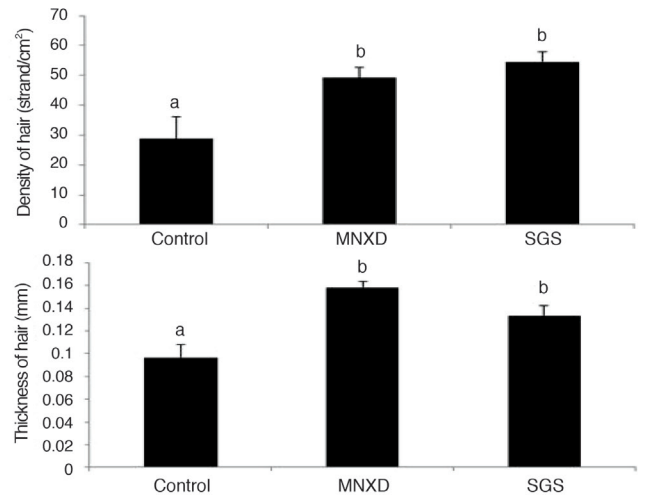

Fig. 3. Effects on hair density and thickness in alopecia model of C57BL/6 mouse.
Data are presented as mean ± SE of 7 mice per group.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.
[a,b]Means not sharing a common letter are significantly different among groups at $p < 0.05$.

49.17 ± 3.52 strands/cm^2 in the MNXD group, and 54.50 ± 3.45 strands/cm^2 in SGS group. There was a statistically significant difference between the MNXD and SGS groups, compared with the control group ($p < 0.05$, Fig. 3).

Hair thickness

Hair thickness was 0.10 ± 0.01 mm in the control group, 0.16 ± 0.01 mm in the MNXD group and 0.13 ± 0.01 mm in the SGS group. There was a statistically significant difference between the MNXD and SGS groups, compared with the control group ($p < 0.05$, Fig. 3).

Hematoxylin-eosin staining

In order to examine the effects pharmacopuncture and dermal application of Sebalgukhwa-san on the number and size of the hair follicles, hematoxylin-eosin staining of skin tissues was used. It revealed that the numbers of the MNXD and SGS groups hair follicles had increased, compared with the control group, but the size of hair follicles was similar (Fig. 4).

Western blotting

VEGF

The level of protein expression of VEGF was 0.928 ± 0.646 in the control group, 1.519 ± 1.107 in the MNXD group and 1.206 ± 0.697 in the SGS group. The higher level of VEGF in the MNXD and
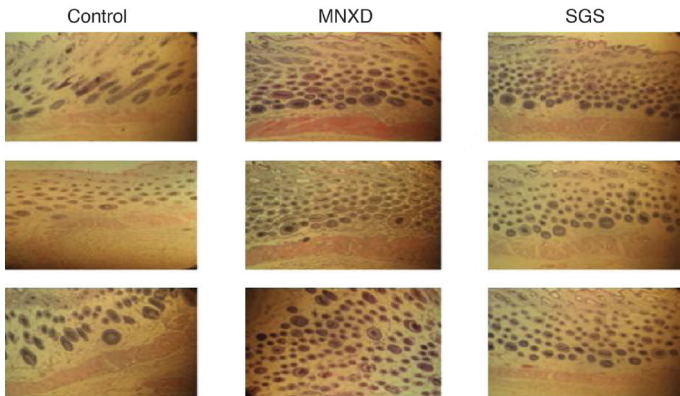
Fig. 4. Effects on the number and size of hair follicle in alopecia model of C57BL/6 mouse.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.

Fig. 6. Changes of AST and ALT in the alopecia model of C57BL/6 mouse.
Data are presented as mean ± SE of 7 mice per group.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.
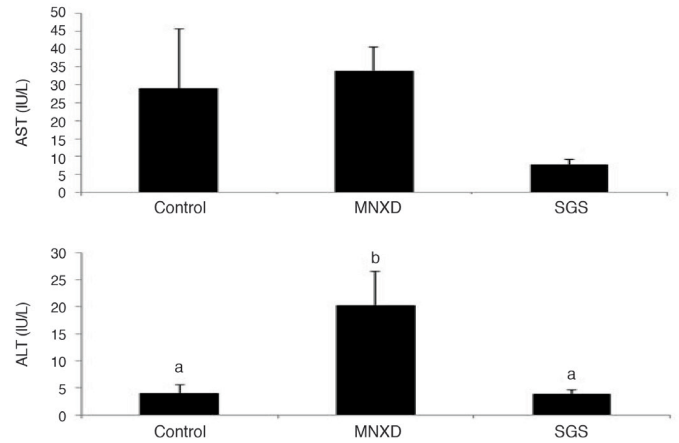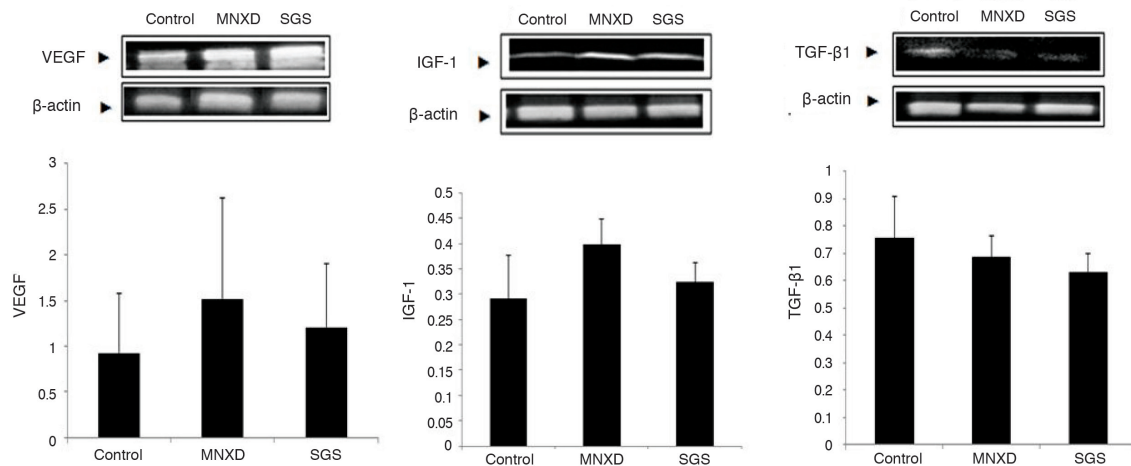[a,b]Means not sharing a common letter are significantly different among groups at $p < 0.05$.

Fig. 5. Effects on VEGF, IGF-1 and TGF-β1 protein expression in the alopecia model of C57BL/6 mouse.
Data are presented as mean ± SE of 7 mice per group.
Control, mice were injected with normal saline and applied vehicle every other day; MNXD, mice were applied with Minoxidil every day; SGS, mice were injected with Sebalgukhwa-san pharmacopuncture and applied extracts of Sebalgukhwa-san every other day.

SGS groups, compared with the control group were not statistically different (Fig. 5).

IGF-1

The level of protein expression of IGF-1 was 0.291 ± 0.085 in the control group, 0.398 ± 0.050 in the MNXD group and 0.324 ± 0.385 in the SGS group. The higher level of IGF-1 in the MNXD and SGS groups, compared with the control group were not statistically different (Fig. 5).

TGF-β1

The level of protein expression of TGF-β1 was 0.757 ± 0.153 in the control group, and 0.687 ± 0.777 in the MNXD group and 0.631

± 0.066 in the SGS group. The lower level of TGF-β1 in the MNXD and SGS groups were not statistically different (Fig. 5).

Liver function test

AST

The concentration of AST was 29.01 ± 16.69 IU/L in the control group, 34.01 ± 6.64 IU/L in the MNXD group and 7.76 ± 1.43 IU/L in the SGS group. In comparison with the control group and the SGS group, the MNXD group had a higher level of AST. The SGS group had a lower level of AST, compared with the control group. However, there were no significant differences between groups (Fig. 6).

ALT

The concentration of ALT was 4.06 ± 1.61 IU/L in the control group, 20.22 ± 6.20 IU/L in the MNXD group and 3.85 ± 0.82 IU/L in the SGS group. The amount of ALT was significantly different in the MNXD group, compared with the control group and the SGS group ($p < 0.05$, Fig. 6).

Discussion

Alopecia is a condition in which the blood circulation is reduced due to various causes. The cells that produce hair have an insufficient supply of nutrients and oxygen, and the hair falls out gradually [2]. Alopecia mainly affects men but, in recent years there has been an increase in incidence of alopecia in women [22]. Where the alopecia sufferer is young, they typically seek treatment. Alopecia not only causes the loss of physiological function of the hair, but also severely affects mental health, and causes problems such as depression, anxiety, and social isolation [23]. The esthetics in modern society, where beauty attracts attention, only heightens the psychological stress experienced by alopecia sufferers [24].

The production of a hair follicle is the start of the growth of hair, and there are 4 stages of the hair cycle: anagen, catagen, telogen, and exogen [25]. Of the total hair, 85% is in the anagen stage which receives nutrients and oxygen from the capillaries, and 1% is in the catagen stage which stops growth, shrinks the hair bulb, and slows metabolism. In the telogen stage, the hair follicles shrink and move upwards. As new hair grows, the period that the old hair exits is called the exogen stage. Approximately 10% to 15% of all hair is removed, and new hair grows [26].

Alopecia progresses when there is a lack of nutrient supply to the cells that make the hair, and the anagen stage becomes shortened. The transition time from the shortened catagen stage, to the telogen stage quickens. As the transition to the telogen stage accelerates, the average life span of the hair becomes shorter. When the proportion of hair in the telogen stage increases from 10% to 15%, to 20% or more, the lifespan of the strand of hair is shortened, and the number of lost strands of hair rises [2,27].

Minoxidil and finasteride are pharmaceutical drugs approved by the US Food and Drug Administration to promote hair growth. Minoxidil is a vasodilator developed in the United States primarily for hypertension, but when hair growth was observed as a side effect, it was approved as a hair growth treatment. Likewise, finasteride was developed as a 5α-reductase inhibitor for the treatment of prostatitis, but it also led to hair growth, and so it was approved for use as a hair growth treatment [4,28]. Minoxidil and finasteride are approved drugs but long-term use leads to side effects [29]. The side effects of minoxidil include; hypertrichosis (other than the scalp), erythema and pruritus, peeling of the epidermis, psoriasis, seborrheic dermatitis, and contact dermatitis. The side effects of finasteride are erectile dysfunction, gynecomastia, and birth defects. In addition, people with liver abnormalities should use with caution because the hair growth agent is metabolized in the liver [30].

In this study, Sebalgukhwa-san which is composed of *Chrysanthemi Indici Flos*, *Viticis Fructus*, *Cnidii Rhizoma*, *Angelicae Dahuricae Radix*, and *Asia Radix* work together promoting blood flow to eliminate blood stasis and remove wind, to detoxify [31]. *Thujae Orientalis Folium*, *Morus alba Linn*, and *Ecliptae Herba* work together to strengthen the Eum principle and blood phase allowing hair to grow [18]. Therefore, it is considered that Sebalgukhwa-san is effective for external wind and blood deficiency, which are among the main causes of alopecia. In particular, Kim et al [32] found that the most frequently used medicinal herbs in alopecia research were *Polygoni Multiflori Radix*, *Cnidii Rhizoma*, and

Thujae Orientalis Folium. Among them, *Cnidii Rhizoma* has vasodilating, antibacterial and sedative effects [33], and *Thujae Orientalis Folium* prevents alopecia, and promotes hair growth by nourishing the hair follicle to aid hair growth [34].

Bee venom acupuncture treatment was reported by Kim et al [9] to benefit hair growth in C57BL/6 mice. Lee et al [10] reported that hair growth was facilitated by pharmacopuncture in C57BL/6 mice treated with *Cornu Cervi Pantotrichum* pharmacopuncture solution. Yun et al [11] also reported hair growth following bee venom *Carthami Flos* herbal acupuncture. The alopecia mouse model is developed in C57BL/6 mice who have melanocytes only in hair follicles where black hairs are produced. The pigment is formed only during the anagen stage; thus, it is possible to confirm the growth cycle of the hair (a process widely used in the clinical research of hair) [35,36].

As a cause of alopecia, the male hormone testosterone (which affects hair growth and degradation) when converted by a reducing agent to atrophy, also causes atrophy of the hair follicle. Protein synthesis of the hair follicle is delayed as we become older, and the percentage of the hair in the telogen stage increases allowing alopecia to progress rapidly [37]. In this study, female mice were used to exclude the effects of male hormones.

Spontaneous or artificial stimulation causes hair growth from the telogen stage to the anagen stage. Artificial hair growth can be caused by trauma or injury, hair removal, excessive shaving, or exposure to chemicals [38]. Therefore, at the beginning of the experiment, the animals were observed after being depilated using animal clippers then hair remover on the dorsal area.

In this study, pharmacopuncture and dermal application of Sebalgukhwa-san was used to induce hair growth through subcutaneous stimulation. Treatment with Sebalgukhwa-san did not affect weight. It facilitated uniform growth of hair by Day 12 compared with the control group, and observation scores for hair growth were statistically significantly different to the control group on the 12th and 15th day ($p < 0.05$, Table 3). As a result of gross observation and scoring, pharmacopuncture and dermal application of Sebalgukhwa-san showed hair growth similar to that of minoxidil.

The folliscope was used to measure hair density and thickness, and is a high-magnification microscope that is often used for clinical evaluation of alopecia treatments and hair growth. With a folliscope, it is possible to assess the condition of the scalp by photographing and analyzing the hair density, and thickness, the growth rate, and the ratio of hair in the anagen stage, and the telogen stage to measure the patient's alopecia condition pre and post-treatment [39] with the advantage of observing using a non-invasive method [40].

In order to examine the effects of Sebalgukhwa-san on hair density and thickness, skin was taken with a folliscope. In both the MNXD and SGS groups, the hair density and thickness were significantly different from those of the control group ($p < 0.05$, Fig. 3). It was confirmed that pharmacopuncture and dermal application of Sebalgukhwa-san had similar effects to minoxidil.

To examine the effects of Sebalgukhwa-san on hair follicle number and size, skin tissue was stained with hematoxylin-eosin on the 15th day. The numbers of the MNXD and SGS groups hair follicles were increased, compared with the control group, but the size of hair follicles was similar (Fig. 4). Pharmacopuncture and dermal application of Sebalgukhwa-san had similar effects to minoxidil.

To determine the expression level of proteins affecting hair growth, VEGF, IGF-1 and TGF-β1 expression was measured by Western blotting. Cell growth and differentiation are caused by a variety of factors, including VEGF, IGF-1, and TGF-β1,

which are secreted by hair follicle cells [41]. There is no vascular distribution in the epidermis around the hair follicles and hair roots, but the growing hair follicles rapidly divide and increase oxygen and nutrient supply, leading to angiogenesis in the dermis. At this time, VEGF expression is increased to promote hair follicle growth, thereby increasing the size of the hair follicle, and the thickness of the hair [42,43]. IGF-1 is produced in mesenchymal cells, and is known to be an important growth factor with its receptors on epithelial cells. It plays a role in cell growth and migration by binding to cell surface receptors. In particular, IGF-1 secreted from the dermal papilla cells of the hair follicle, promotes epithelial cell proliferation, and significantly increases the length of follicular tissue [44,45]. In contrast to VEGF and IGF-1, TGF-β1 reduces hair growth and suppresses proliferation of epithelial cells, resulting in faster regression than normal [46,47]. In this study, VEGF and IGF-1 levels increased in the MNXD and SGS groups compared with the control group, but there was no significant difference between groups (Fig. 5). TGF-β1 levels tended to decrease in the MNXD and SGS groups compared with the control group, but there was no significant difference between the groups (Fig. 5). Although there was no significant difference, pharmacopuncture and dermal application of Sebalgukhwa-san appeared to be similar to minoxidil, which is thought to affect hair regrowth by controlling the growth regulator of hair.

The levels of AST showed no statistically significant differences between all groups (Fig. 6) however, the levels of ALT in the MNXD group were statistically significantly different from the control group and SGS ($p < 0.05$, Fig. 6). In this study, it was also confirmed that ALT levels did not increase in the SGS group (compared to the control and the MNXD group), despite dermal application, indicating that treatment with Sebalgukhwa-san was safer than the conventional hair growth agent, minoxidil.

Taken together, these results suggest that pharmacopuncture and dermal application of Sebalgukhwa-san promote the growth of hair follicles, increase hair density and thickness, and increase the number of hair follicles. This effect was confirmed both by visual inspection, folliscope and staining results. In addition, it was found that the alopecia treatments effects were enhanced by controlling the growth regulator of hair. Treatment with Sebalgukhwa-san was superior to the control group, and it was similar to the minoxidil treatment group which is an approved hair growth promoter. ALT, a hepatic enzyme, was significantly higher in the positive control group than in the control group and the experimental group. This implies that treatment with pharmacopuncture and dermal application of Sebalgukhwa-san is safer for metabolism than the conventional hair growth drug. This observation is expected to be very useful for clinical use in the future.

Sebalgukhwa-san is effective in promoting blood flow to eliminate blood stasis, remove wind, detoxify, and strengthen Eum the principle and blood phase, so that there is a hair growth effect [18]. In this study, treatment with Sebalgukhwa-san was judged objectively to be as effective as minoxidil at hair regrowth. The pharmacopuncture treatment is usually combined with pharmacological action and the action of acupuncture, which is an invasive treatment [48]. The combination of pharmacological and subcutaneous stimulant action of Sebalgukhwa-san pharmacopuncture is believed to have produced this distinctly effective outcome on this alopecia murine model.

This study has shown a safe treatment for alopecia, in mice, however before clinical application several follow-up studies are needed to determine safety, efficacy and stability of product.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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