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Original Article

Immunohistochemical Study on the Hair Growth Promoting Effect of *Yonnyuniksoogobon-dan*

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Objectives : This study evaluates the hair growth promoting effect of *Yonnyuniksoogobon-dan* on shaved C57BL6 mice.
Methods : *Yonnyuniksoogobon-dan* was administered orally (Group I) and both orally and by skin application (Group II) once a day for 12 days. The experimental groups were compared to Control, which was orally administered physiological saline solution. Hair regrowth was photographically and histologically determined during the experimental period. The levels of hair growth cycle related factors (EGF, TGF-β1) and vascular factors (VEGF, iNOS) were also determined by immunohistochemistry. On gross observation of hair growth, both Group I and Group II shaved C57BL6 mice showed accelerated hair regrowth.
Results : The hair regrowth index of Group I increased significantly from 7 days ($P<0.05$) to 12 days ($P<0.01$) after shaving and that of Group II was significantly higher at 12 days ($P<0.05$). On histological observation, both Group I and Group II demonstrated histological improvement and increases in the number and diameter of the hair follicles. EGF expression on the root sheath of hair follicles was up-regulated in both Group I and II. TGF-β1 expression on the root sheath of hair follicles was unchanged in both Group I and II. VEGF expression in the tissues surrounding hair follicles was up-regulated in both groups. iNOS expression in the tissues surrounding hair follicles was down-regulated in both groups.
Conclusions : These results suggest that *Yonnyuniksoogobon-dan* promotes hair growth and this effect is related to up-regulation of EGF and VEFG expression and down-regulation of TGF-β1 and iNOS expression on hair roots.

Key Words : *Yonnyuniksoogobon-dan* hair growth; C57BL6 mice; EGF; TGF-β1; VEGF; iNOS

Introduction

Hair has the primary defensive functions of protecting and buffering against external physical shocks, sunlight and cold along with the functions of absorbing heavy metals to excrete outside the body[1]. There are various causes of hair loss, including genetic causes, endocrinologic causes (especially excessive male hormone), psychological stresses, hemodynamic disorders, nutritional unbalance, environmental contamination (atmospheric contamination, excessive use of chemical agents for scalp and so on), several diseases including autoimmune diseases and certain drugs. Some of these causes have become

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more problematic with industrialization. Hair loss is considered to be an independent disease that can be accompanied with physical, psychological and social problems[2-4].

For the current study, we designed a preparation with the aim of both protecting against hair loss and promoting hair growth. This preparation comprised 11 components: *Polygoni Multiflori Radix, Lycii Radicis Cortex, Poria, Rehmanniae Radix, Rehmanniae Radix Preparat, Asparagi Radix, Liriopis Tuber, Lycii Fructus* and *Acori Graminei Rhizoma*, that have been described in Yonryunggobon-dan[5, 6] and *Yonnyuniksoobulro-dan*[6] and the agents of Angelicae Gigantis Radix[6] and pine needle[6], whose effects on hair loss protection have been reported. The preparation was named *Yonnyuniksoogobon-dan* and its stimulative effect on hair growth was examined.

The effects of *Yonnyuniksoogobon-dan* on hair growth were observed experimentally by histologically assessing the hair growth status of depilated C57BL/6 mice and by immunohistologically examining the expression levels of proteins involved in hair growth of these mice. These proteins were epidermal growth factor (EGF) and transforming growth factor-β1 (TGF-β1), which are involved in the growth of hair follicles and hair, and vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS), which are involved in the supply of blood to the surrounding tissue of hair follicles. The significant results are reported here.

Materials and Methods

1. Experimental animals

36 female C57BL/6 mice aged 5 weeks were purchased from Samtako Co. (Korea) and were used after one week of acclimation in a breeding room. Temperature and humidity were maintained at 21-23℃ and 40-60% respectively, and the light cycle was automatically maintained (12 hour light/darkness). Autoclaved water and food (Samyang Co., Korea) were provided freely. The study was approved by the Institutional Animal Care and Use Committee of Kyung Hee University and all procedures were conducted in accordance with the U.S. National Institutes of Health guidelines.

2. Preparation of medication

The contents of the *Yonnyuniksoogobon-dan* preparation are described in Table 1. Approximately

Table 1. Herbal Constituents of *Yonnyuniksoogobon-dan*

Herbal medicine	Scientific name	Medical Use	Dosage (g)
Polygoni Mutiflori Radix	Pleuropterus multiflorus	lumpy root	16.0
Lycii Radicis Cortex	Lycium chinense MILL L.	dry bark vel·a·men·tous	10.0
Polia	Poria cocas Wolf.	sclerotium	10.0
Rehmanniae Radix	Rehmannia glutinosa (GAERTN.)	unripe root	6.0
Rehmanniae Radix Preparat	Rehmannia glutinosa Liboschitz var. purpurea Makino	ripe root	6.0
Asparagi Radix	Asparagus cochinchinensis (Lour.) Merr	lumpy root	6.0
Liriopis Tuber	Liriope platyphylla Wang et Tang	lumpy root	6.0
Lycii Fructus	Liriope platyphylla Wang et Tang	fruit	4.0
Acori Graminei Rhizoma	Acorus gramineus SOLAND.	lumpy root	4.0
Angelicae Acutiloba Radix	Angelica gigas Nakai	root	4.0
Pini Folium	Pinus densiflora Siebold. et Zucccarini	leaf	4.0
Total			76.0

760g, the equivalent of 10 packs of medication, was boiled for 2 hours with 4,000 ml of water in a decocting machine that was loaded with a condenser. The boiled solution was filtered before vacuum concentration with a rotary evaporator and freeze drying to acquire 94.8g of powered extract. The amount of medication administered per dose was 10 times the calculated body weight ratio amount of 15.8 mg/10g body weight. This amount was diluted in 0.2 ml drinking water and administered orally once a day for 12 days.

3. Depilation and Classification of experiment groups

To observe hair growth, the black hair on the back of 6 week old C57BL/6 mice was cut using an animal hair clipper, any remaining hair was completely removed using NICLEAN (Ildong Pharmaceutical, Korea), and the skin was cleaned. The experimental groups were Control - orally administered physiological saline solution, Group I - orally administered *Yonnyuniksoogobon-dan*, and Group II - orally administered *Yonnyuniksoogobon-dan* plus skin application of *Yonnyuniksoogobon-dan*. 12 mice were assigned to each group, and the oral administration of the medication or saline and skin application were performed at 10:00 every morning for 12 days starting on the day after the depilation.

4. Observation of hair growth

Hair growth status was examined by lightly anesthetizing the mice with 2% isoflurane on days 1, 5, 7, 9 and 12. The image of the back of each mouse was photographed with a digital camera (EOS-10D, Canon, Japan). The tail of each mouse was marked with different colored oil based pens to distinguish between them.

5. Measurement of hair regrowth index (HRG index)

For the quantitative comparison of hair growth status, the back of each mouse was photographed with a digital camera and the image was inputted to a computer for measurement using the “ImageJ” software of NIH (ver. 1.36). The HRG index was calculated using the melanin forming phenomenon of theC57BL/6 mice that correlates to the hair growth stage whereby the skin color changes from

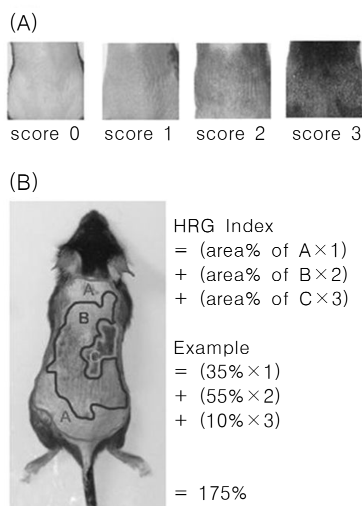

Fig. 1. Representative sections of scores (A) and example of hair regrowth index (HRG index) calculated with area percentages and scores (B).

pink to gray, dark gray, and then black[7]. Pink skin color of the depilated mouse was ranked as 4th grade and assigned a score of 0; gray skin color signifying the start of hair growth in hair follicles was ranked as 3rd grade with a score of 1; dark gray skin color when hair can first be seen outside of the skin with the naked eye was ranked as 2nd grade and assigned 2 points; and when the hair color became completely black as seen with the naked eye the ranking was 1st grade with a score of 3 points (Fig. 1-A). The ratio between the area with hair growth and total depilated area of the back was measured for each grade and expressed as a percentage, and this value was multiplied by the score assigned to that grade to give the HRG index. In this way, the HRG index had a minimum value of 0% and a maximum value of 300% (Fig. 1-B).

6. Treatment of skin tissue

On the 12th day of the experiment, the animals were sacrificed by anaesthetizing with an excessive amount of sodium pentobarbital, and some of back skin tissue was collected to freeze dry with dry ice-isopentane. 20 m sections were prepared to be used for immunohistochemical staining, and remaining tissue was fixed using 10% neutral formalin solution and dehydrated after washing to be formatted with paraffin. The paraffin-formatted skin tissue was sliced to a thickness of 8 m for hematoxyline and eosin (H&E) staining.

7. Immunohistochemical staining

After washing the freeze-dried skin tissue sections 3 times for 5 minutes with 0.05 M PBS, the sections were reacted with 1% H_2O_2 for 10-15 minutes and then washed 3 times before blocking with 10% normal horse serum (Vectastain) and bovine serum albumin (Sigma) in PBS for 1 hour. After washing 3 times with PBS the sections were treated with the appropriate primary antibody. The primary antibodies used were anti-EGF (1:200, sc-1342, Santa Cruz, USA), anti-TGF-β1 (1:100, sc-146, Santa Cruz, USA), anti-iNOS (1:200, 610329, R&D Bioscience, USA), and anti-VEGF (1:100, sc-152, Santa Cruz, USA), diluted with PBS and Triton X-100 mixed solution and incubated for 12 hours at 4℃. After the reaction, each tissue section was washed with PBS and reacted for 1 hour with avidin-biotin immunoperoxidase according to the manufacturer’s instructions (ABC Vectastain Kit). EGF and iNOS expression in the tissue sections was visualized by chromogenic reaction with diaminobenzidine-tetrachloride (Sigma, USA) for 5-10 minutes. TGF-β1 and VEGF expressions was visualized by reacting with diaminobenzidine-tetrachloride mixed with $NiCl_2$ (Sigma, USA) for 5-10 minutes. The reacted tissue sections were attached to poly-L-lysine coated slides and dried for 2-3 hours, then dehydrated and sealed to prepare the tissue samples. The immunohistochemical staining was observed by optical microscopy to evaluate the whole tissue. Unclear or weak staining was graded as ±, a weak positive reaction was graded as +, a medium positive reaction was graded as ++, and strong expression was graded as +++.

8. Statistical analysis

The experimental results were analyzed using SPSS® for Windows (version 10.0, SPSS, Inc., Chicago, U.S.A.) to perform one way ANOVA tests to compare the index value variation by the concentration of medication, and a P value <0.05 was considered to be significant. Scheffe’s test was performed as the post hoc test.

Results

1. Visible changes in hair growth status

In mice in the Control group, the depilated back skin was pink without any growth of hair until the 5th day after the depilation (Fig. 2, Control, 1st day), and new hair started to show on the 7th day when the skin color was partially gray, though parts of the skin were still pink (Fig. 2, Control, 7th day). On the 9th day, the growth of many new hairs from hair follicles was observed on the depilated back

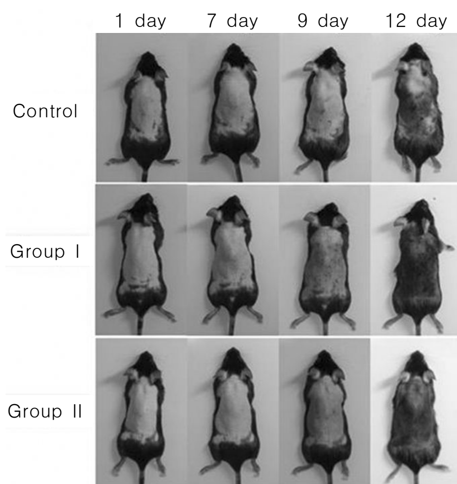

Fig. 2. Representative sections of shaved C57BL/6 mice for 12 days after shaving. *Yonnyuniksoogobon-dan* administration (Group I) and *Yonnyuniksoogobon-dan* administration plus skin application (Group II) accelerated hair regrowth of the shaved C57BL/6 mice.

skin overall accompanied with a change of skin color to dark gray. Growth of hair outside of the skin could be seen in parts while some of the skin was still pink (Fig. 2, Control, 9th day). On the 12th day, the full growth of black colored hair could be seen on part of the skin (Fig. 2, Control, 12th day). Group I (oral administration of *Yonnyuniksoogobon-dan*) showed similar hair growth as the Control up to and including the 9th day, though the area of skin changed to gray was larger than that in the Control group (Fig. 2, Sample I, 7th day, 9th day). On the 12th day, the area of skin with growth of black hair outside of the skin was larger than that of the Control group (Fig. 2, Sample 1, 12th day). Group II (combined oral administration and skin application of *Yonnyuniksoogobon-dan*) showed a similar hair growth promotion pattern to that of Group I, with no significant difference between the two treatment groups (Fig. 2, Sample II).

2. Change of HRG index

The HRG index of the Control group was 0% on the 1st day, 61.1 ± 3.3% on the 7th day, 122.5 ± 13.0% on the 9th day, and 219.2 ± 15.0% on the 12th day. In comparison, Group I (orally administered *Yonnyuniksoogobon-dan*) had HRG indices

Table 2. Effect of *Yonnyuniksoogobon-dan* on Hair Regrowth Index of Shaved C57BL/6 Mice (%)

Group	Days after shaving			
	1(n=12)	7(n=12)	9(n=12)	112(n=12)
Control	0	61.33 ± 3.3	122.5 ± 13.0	129.2 ± 15.0
Group I	0	75.4 ± 5.5*	180.0 ± 10.6**	268.3 ± 10.2*
Group II	0	69.6 ± 6.6	158.3 ± 13.7	259.2 ± 8.3*

Data presented as mean ± standard error.
Control: treated with saline after shaving
Group I: treated with *Yonnyuniksoogobon-dan* orally after shaving
Group II: treated with *Yonnyuniksoogobon-dan* orally plus skin application after shaving
Statistical significance was verified with repeated ANOVA among groups (*: P<0.05; **: P<0.01).

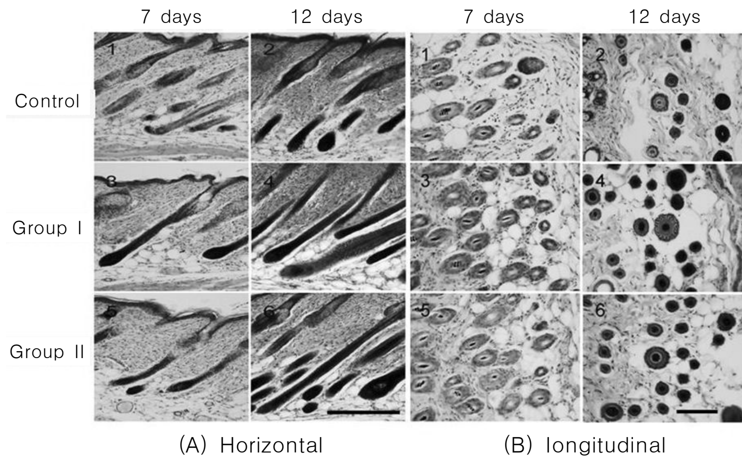

Fig. 3. Representative skin sections of shaved C57BL/6 mice.
(A) Longitudinal section of hair follicles. Group I (oral *Yonnyuniksoogobon-dan* administration) and Group II (oral *Yonnyuniksoogobon-dan* administration plus skin application) demonstrated histological improvement of the hair follicles. (B) Horizontal section of hair follicles. Group I and Group II demonstrated increases in number and diameter of the hair follicles. Sections are stained with H&E. Scale bar in section 6 is 200 μm.

of 0% on the 1st day, 75.4 ± 5.5% on the 7th day, 180.0 ± 10.6% on the 9th day, and 268.3 ± 10.2% on the 12th day. Group I had significantly higher HRG indices than Control from the 7th day to 12th day ($P<0.05$, $P<0.01$). Group II (combined oral administration and skin application of *Yonnyuniksoogobon-dan*) had HRG indices of 0% on the 1st day, 69.6 ± 6.6% on the 7th day, 158.3 ± 18.7% on the 9th day, and 259.2 ±8.3% on the 12th day. The HRG index was only significantly higher compared to the Control ($P<0.05$) on the 12th day (Table 2, Fig. 2).

3. Histological examination of hair growth

H&E stained skin tissues from the 7th and 12th day after depilation were used to observe the status of hair follicle and hair growth by optical microscopy. On the 7th day, the Control group had partial weak development of hair roots with areas without enough inner root sheath development (Fig. 4-1). In comparison, Group I and Group II had well-developed hair follicles and inner root sheath overall (Fig. 3-A, 3-B) and an increased growth of hairs compared to the Control group (Fig. 3-B-1, 3, 5). On the 12th day after depilation, while many fully grown hair follicles were observed in all three groups, the

Table 3. Effect of *Yonnyuniksoogobon-dan* on EGF, TGF-β1, VEGF, and iNOS Expression of Shaved C57BL/6 Mice

Groups	Immuno-reactivity			
	EGF	TGF-β1	VEGF	iNOS
Control	+	+	+(~5)	+++
Group I	++/+++	+/±	++/(10~)	+
Group II	++/+++	+/±	++/(10~)	+

Control: treated with saline after shaving
Group I: treated with *Yonnyuniksoogobon-dan* orally after shaving
Group II: treated with *Yonnyuniksoogobon-dan* orally plus skin application after shaving
Immuno-densities are demonstrated as ±, weak; +, mild; ++, moderate; and +++, strong.

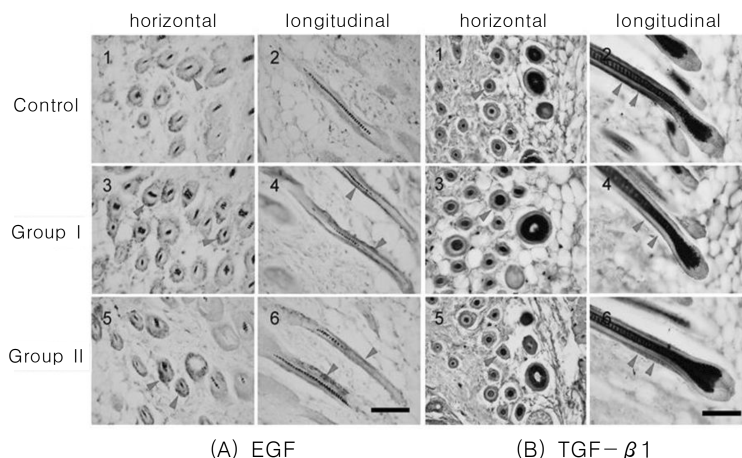

Fig. 4. Representative sections of EGF and TGF-β1 immuno-reacted skin tissues of shaved C57BL/6 mice at 12 days.
(A) EGF, Group I (oral *Yonnyuniksoogobon-dan*) and Group II (oral *Yonnyuniksoogobon-dan* plus skin application) demonstrated up-regulation of EGF expression on the root sheath of hair follicles. Red arrowheads indicate EGF expression.
(B) TGF-β1, TGF-β1 expression on the root sheath of hair follicles was not different between groups. Red arrowheads indicate TGF-β1 expression. Scale bar in section 6 is 100 μm.

cross sectional area of hairs from Group I and Group II was thicker. There were a greater number of developed hair follicles with more inner root sheaths and outer root sheaths in the two treatment groups than in the Control group (Fig. 3-A-2, 4, 6; Fig. 3-B-2, 4, 6).

4. EGF expression

On the 12th day after depilation, the EGF response of skin tissue was observed by immunohistochemical staining. The Control group showed a weak (± or +) positive response at the grown hairs of certain hair thickness and at the outer root sheath of thin vellus hairs, but a negative response was observed at the inner root sheath (Fig. 4-A-1, 2, Table 3). Group I showed a medium level (++) of positive response at the outer root sheath of fully grown hair of a certain thickness, and a very strong (+++) EGF positive response was observed at the outer root sheath of newly growing fine hairs (Fig. 4-A-3, 4). Group II had a similar pattern of EGF expression as Group I (Fig. 4-A-5, 6, Table 3).

5. TGF-β1 expression

On the 12th day, the TGF-β1 response of skin tissue was observed by immunohistochemical staining. The Control group showed a weak (+) positive response at the inner root sheath of fully grown hair of a certain thickness, and the few hairroots had a very strong (+++) positive response. The inner out outer root sheaths at the hair roots of thin vellus hairs had weak (± or +) positive responses (Fig. 4-B-1, 2, Table 3). Group I and Group II mice had similar expressions of TGF-β1 to the Control, though some cross sectional hair roots had decreased expression of TGF-β1 (Fig. 4-B-3, 4, 5, 6, Table 3).

6. VEGF expression

The immunohistochemical staining for VEGF of the skin on the 12th day was observed. The Control group had approximately 3-7 VEGF expressing cells in peripheral tissues of hair follicles and hair roots examined at 400 X magnification (Fig. 5-A-1, 2, Table 3). In contrast, Group I had 10-15 VEGF

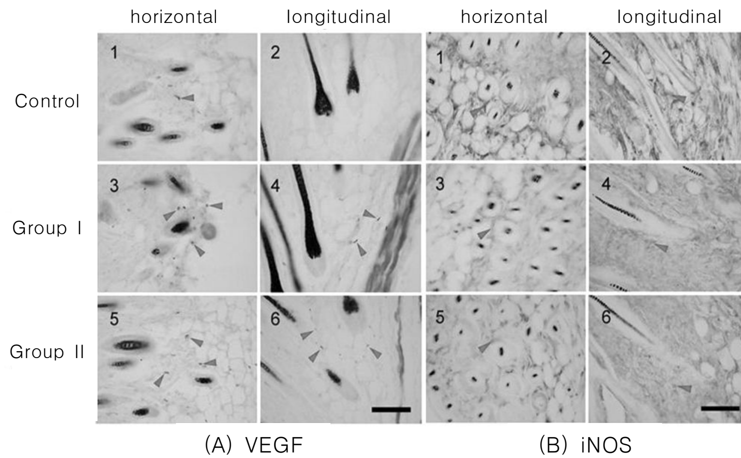

Fig. 5. Representative sections of VEGF and iNOS immuno-reacted skin tissues of shaved C57BL/6 mice at 12 days.
(A) VEGF, and iNOS Group I (oral *Yonnyuniksoogobon-dan* administration) and Group II (oral *Yonnyuniksoogobon-dan* administration plus skin application) demonstrated up-regulation of VEGF expression in the surrounding tissues of hair follicles. Red arrowheads indicate VEGF immuno-positive cells.
(B) iNOS, Group I and Group II demonstrated down-regulation of iNOS expression in the surrounding tissues of hair follicles. Red arrowheads indicate iNOS expressions. Scale bar in section 6 is 100 µm.

expressing cells in peripheral tissues of hair follicles and hair roots, so had increased expression of VEGF compared to the Control (Fig. 5-A-3, 4, Table 3). Group II had more than 10 VEGF expressing cells, so also demonstrated an increase in VEGF expression compared to the Control group (Fig. 5-A-5, 6, Table 3).

7. iNOS expression

Immunohistochemical staining for iNOS in the skin on the 12th day demonstrated strong (+++) iNOS expression in the peripheral tissues of hair follicles and hair roots of the control group (Fig. 5-B-1, 2, Table 3). In contrast, Group I had medium level (+ or ++) iNOS expression at the peripheral tissues of hair follicles and hair roots so had decreased expression compared to Control (Fig. 5-B-3, 4, Table 3). Group II had a similar iNOS expression pattern to Group I (Fig. 5-B-5, 6, Table 3).

Discussion

Human hair grows in cycles, with periods of growth and shedding over several months or even 2-4 years in some cases. Generally, this hair growth cycle can be classified into the anagen period with the most active growth, the catagen period when the degeneration of hair starts and the telogen period when the growth of hair is stopped or inactivated[8]. Hair loss is defined as the incidence of excessive hair shedding compared to normal due to hair growth abnormalities. Genetic causes, excessive secretion of male hormone, psychological stresses, hemodynamic disorders, nutritional disorders, aging, environmental contamination and dyeing have been shown to be causes of hair loss1[2]. Hair loss is also classified as an autoimmune disease[3]. Since hair loss is due to complex causes, it is very difficult to suggest broadly applicable effective treatment approaches.

Hair growth-promoting agents or hair tonics that have been developed so far usually aim to either promote hair growth or prevent hair lossby correcting

the indirect causes of hemodynamic disorders, nutritional disorders, seborrheic dermatitis and stress that induce hair loss. Consequently the effects of such treatments have been incomplete. Minoxidil is well known as a hair growth promoter. It was originally developed to treat high blood pressure, but hypertrichosis was reported as a side effect, leading it to be developed as a hair growth promoting agent. The mechanism of its hair growth effect is considered to be an increase of nutrition supply through vasodilation and K^+ channel opening[9]. Another hair growth promoter is finasteride, which inhibits the activation of 5α-reductase, so affecting the metabolism of male hormone[10]. Its disadvantage is that when its use is stopped, hair loss returns[11]. Recently, studies have progressed on promoting the activation of dermal papilla cells, blood circulatory improvement around dermal papilla cells, inhibition of sebum, inhibition of male hormone transformation and inhibition of the activation of TGF-β, which promotes the transition of the hair growth cycle from the anagen phase to the catagen or telogen phases[12].

From the perspective of oriental medicine, hair is controlled by the kidney, and vigorous blood status is responsible for a rich hair condition while deteriorated blood status is responsible for poor hair condition. Thus, normal hair growth is closely related to normal kidney and blood functioning[6]. On the other hand, blood heats, deficiency of both *ki* (vital energy) and blood, insufficiency of kidneys, insufficiency of lungs, and stresses have all been suggested as causes of hair loss[13].

When the studies of oriental herbal medicines for hair loss prevention or hair growth promotion were reviewed, the methanol extract of *Mori Cortex, Sophorae Fructus, Polygoni Multiflori Radix* and *Chebulae Fructus* have been reported to have effects on the promotion of hair growth, and the *Mori Cortex, Chaenomelis Fructus, Aucklandiae Radix, Angelicae Gigantis Radix, Polygoni Multiflori Radix* and *Angelicae Dahuricae Radix* have been reported to have effects on inhibiting 5α-reductase type II activation[14,15]. The *Sophorae Radix* extract's inhibiting effect on the activation of both 5α-reductase type I and type II has been reported to promote hair growth[16].

In a combined preparation, the *Shineungyangjindan*[17], *Dangguibohyultang-gamibang*[18], and Sangbaleum[19] has been shown to significantly prevent hair loss and promote hair growth, and hair sprays[18], hair essences[20] and soaps[21] containing oriental herbal medicine were reported to improve hair condition by increasing the hair density per unit area or by reducing the hair shedding ratio. In addition, Khil *et al.*[22] described an antioxidant effect that reduced the content of thiobarbituric acid reactive substance of serum and liver in aging-induced white rats. Heo *et al.*[23] reported the effects of *Yonnryunggobon-dan* on the sexual ability of aged rats, and found that it increased egg counts and the normal egg to fertilization ratio, and also effected fertility reduction by decreasing NOS gene expression at sexual glands. Park *et al.*[24] reported that it delayed cellular aging by increasing the cell doubling frequency and by reducing cell doubling time in dermatic fibroblastic cells, cardiac vascular endothelial cells and glomerulus mesangial cells. Many studies have reported that the oriental herbal medicines in *Yonnyuniksoogobon-dan* prevent hair loss and promote hair growth[25-29].

Oral treatment with *Yonnyuniksoogobon-dan* resulted in a similar pattern of hair growth to that of the Control group until the 7th and 9th day, though the amount of gray skin was greater than in the Control. The enlargement of the hair growth area outside of the skin was observable at the 12th day. Treatment with *Yonnyuniksoogobon-dan* both orally and by skin application revealed a similar growth pattern to that of mice treated with oral *Yonnyuniksoogobon-dan* alone. The histological observation of hair follicles and hair growth status of H&E-stained skin tissue on the 7th and 12th days revealed thick cross-sectional areas of hair in Groups I and II, with hair follicles that had more inner and outer root sheath development and thicker hairs compared to the Control group, indicating an improvement in hair growth status.

Measurement of the HRG index from hair growth status and growth area revealed that the group that was orally administered *Yonnyuniksoogobon-dan* showed significantly higher HRG indices from the 7th day to the 12th day of the experiment. Mice that were treated both orally and topically with *Yonnyuniksoogobon-dan* showed significant HRG index increases only on the 12th day of the experiment. The relative inferiority of combined oral and topical treatment might be attributable to supplementary problems, such as skin deposition and retention of precipitant by the direct skin spreading of the extracts of the oriental herbal medicine. These problems could be tested by additional experiments using technologically developed skin spreading agents.

The hair follicle is an organ that contains very complex components. It is comprised of mesenchymal cells, called dermal papilla, and epithelial cells, and has 3 growth periods: anagen period of growth, the degenerating stage of the catagen period; and the inactivated stage of the telogen period[8]. This growth cycle has been reported to be controlled by various factors, such as fibroblast growth factors, Sonic hedgehog, TGF-β, insulin-like growth factors, EGF, hepatocyte growth factors, platelet-derived growth factors, interferon and interleukins, thyroid hormone and Vitamin D, glial cell line-derived neurotrophic factor, and neurotrophins[8,9,12]. Among these factors, EGF has been reported to be expressed at the outer root sheath of hair follicles to stimulate the cell propagation and the formation of follicles[30,31], while TGF-β1 has been known to be expressed at the both of inner root sheath and outer root sheath of fully grown hair follicles to inhibit the induction of the anagen period and promote the induction of the catagen period[32,33].

Therefore, in this study the expression of EGF and TGF-β1 was observed by immunohistochemical staining. On the 12th day, the Control group had a weak (± or +) EGF positive response or a (-) negative response. Group I had medium a (++) positive response to EGF and part of skin showed very strong (+++) EGF positive response. Group II showed a similar EGF expression pattern to that observed for Group I. TGF-β1 expression in the Control group was observed with a weak (+) positive response, though the hair roots at parts of the skin had a very strong (+++) positive response. Neither oral-only treatment or oral plus topical treatment with *Yonnyuniksoogobon-dan* significantly effected TGF-β1 expression compared to Control, though parts of cross sectional hair roots had a reduction in TGF-β1 expression compared to Control. This result indicated that the hair growth effect of *Yonnyuniksoogobon-dan* was due to the promotion of EGF expression and the inhibition of the expression of TGF-β1 at hair follicles.

On the other hand, since the hair follicle it self and the epidermal tissue surrounding the hair roots do not have blood vessel distribution, the rapid cell division that occurs during the growth cycle of hair follicles increases oxygen and nutrition demand, which can lead to angiogenesis from the deep dermis. VEGF expression plays a major role at this process. Increased VEGF expression has been shown to promote the growth of hair follicles and increase the size of hair follicles and the thickness of hair[34,35]. The formation of nitric oxide in the skin tissue by nitric oxide synthase (NOS) is known to be involved in melanogenesis, wound healing and vasodilation[36,37]. In particular, since endothelial NOS (eNOS) is expressed in follicular papilla cells, it has been shown to have effects on the growth of hair follicles and on the hair growth cycle, and the expression of inducible NOS (iNOS) has been reported to be increased at the peripheral tissues of hair follicles by inflammatory stimulation and ultraviolet light[38,39].

For these reasons, the current study observed the expressionof VEGF and iNOS at the peripheral tissues of hair follicles through immunohistochemical staining. On the 12th day, 3-7 VEGF expressing cells were observed at the peripheral tissues of hair follicles and hair roots in the Control group, whereas Group I had 10-15 VEGF expressing cells at the peripheral tissues of hair follicles and hair roots

and Group II had more than 10 VEGF expressing cells, indicating that *Yonnyuniksoogobon-dan* treatment increased the number ofVEGF expressing cells. Control mice had very strong (+++) iNOS expression at the peripheral tissues of hair follicles and hair roots, while Group I and Group II mice had weak or medium (+ or ++) levels of iNOS expression at the peripheral tissues of hair follicles and hair roots. These findings indicate that *Yonnyuniksoogobon-dan* treatment decreased iNOS expression. These results suggest that *Yonnyuniksoogobon-dan* promotes hair growth by improving the hemodynamic environment at the peripheral tissues of hair follicles and by reducing inflammatory responses.

Combining all the findings of this study, oral *Yonnyuniksoogobon-dan* promotes hair growth by the stimulating EGF expression at hair follicles and by inhibiting the expression of TGF-β1. Topical application of *Yonnyuniksoogobon-dan* was not effective, and the evaluation of *Yonnyuniksoogobon-dan* effects when topically administered to the skin will require the development of a prototype that will solve the supplementary problems of skin spreading.

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References

1. Bressler RS and Bressler CH. Functional anatomy of the skin. Clin. Podiatr. Med. Surg. 1989;6(2): 229-46.
2. Hadshiew IM, Foitzik K, Arck PC and Paus R. Burden of hair loss: stress and the underestimated psychosocial impact of telogen effluvium and androgenetic alopecia. J Invest. Dermatol. 2004; 123(3):455-7.
3. Jaworsky C. and Gilliam AC. Immunopathology of the human hair follicle. Dermatol. Clin. 1999; 17(3):561-8.
4. Spencer LV and Callen JP. Hair loss in systemic disease. Dermatol. Clin. 1987;5(3):565-70.
5. Kong YH. Jeungbo manbyounghoechun. Seoul: Iljungsa. 1994:193-4.
6. Heo J. Donguibogam. Seoul: Namsandang. 1987: 78,85,307-9,449,738.
7. Peters EM, Maurer M, Botchkarev VA, Jensen K, Welker P, Scott GA and Paus R. Kit is expressed by epithelial cells in vivo. J. Invest. Dermatol. 2003;121(5):976-84.
8. Stenn KS and Paus R. Controls of hair follicle cycling. Physiol. Rev. 2001;81(1):449-94.
9. Price VH. Treatment of hair loss. N. Engl. J. Med. 1999;341(13):964- 73.
10. McClellan KJ and Markham A. Finasteride: a review of its use in male pattern hair loss. Drugs. 1999;57(1):111-26.
11. Trüeb RM. The value of hair cosmetics and pharmaceuticals. Dermatology. 2001;202(4):275 -82.
12. Cotsarelis G and Millar SE. Towards a molecular understanding of hair loss and its treatment. Trends. Mol. Med. 2001;7(7):293-301.
13. Chae BY. Oriental MedicalSurgery. Seoul: Komoonsa. 1971:285-6.
14. Choi W, Choi JH and Kim JH. Studies on the effects of medicinal plant extracts on the hair growth stimulation. J. Kor. Orient. Medi. Opthalmol. & Otolaryngol. & Dermatol. 2002;15(2): 80-103.
15. Lee HS, Yun SJ, Moon YK and Moon JY. Hair growth effects of Mori Cortex Radicis mixture on the hair of rat. Korean J. Seric. Sci. 2000; 42(2):83-5.
16. Roh HC andRoh SS. Studies on the effect of *Sophora flavescens* extract on the hair growth stimulation and acne inhibition. J. Kor. Orient.

Medi. Opthalmol. & Otolaryngol. & Dermatol. 2002;15(1):96-126.

17. Kim SY, Kim JH and Chae BY. An experimental study on the effect of *Sineunyangjin-dan* applied for the treatment of alopecia. J. Kor. Orient. Medi. Opthalmol. & Otolaryngol. & Dermatol. 1991;4(1):43-58.
18. Kim PS, Kim HT, Roh SS and Hwang CY. Effect of danguibohyultanggami-bang on the alopecia and hair growth stimulation. J. Kor. Orient. Medi. Opthalmol. & Otolaryngol. & Dermatol. 2004;17 (3):38-60.
19. Lee SH, Jung SH, Song MY and Shin HD. Hair growth promoting effect of saengbaleum application on hair-removed C57BL/6 mouse. J. Orient. Rehab. Medicine. 2007;17(2):101-21.
20. Park WS,Sung DS, Kim DK, Cho WH, Lee HK, Lee CH, Park SK and Sim YC. The effect of hair essence (HHRHG0202-80) containing five Herbal extracts on hair growth and the prevention of alopecia in vitro & vivo. J. Korean Oriental Med. 2004;25(1):152-60.
21. Kim NK,Du IS, Mun YJ and Woo WH. Clinical study on the effect of sangmogen on the alopecia. J. Orient. Physiol. 2005;19(1):270-8.
22. Khil HS, Lee SS, Lee SJ and Kim KH. Effect of *younnyeniksoobulrodan* on antioxidant capacity in D-galactose-induced aging rats. Kor. J. Orient. Prev. Med. 2002;6(2):112-27.
23. Heo SJ, Jo JH, Jang JB and Lee GS. Effects of *Yeonlyeonggobondan* on the reproductive competence of aged mice. J. orient. Obstet. & Gynecol. 2004;17(1):72-87.
24. Park YJ,Ahn YM, Ahn SY and Doo HK. Effects of *yeonryunggobondan* and *palmijihwangtangon* the population doubling number and the population time in rat fibroblasts, heart-endothelial cells, mesangial Cells. J. Korean Oriental Med. 2004;25(1):49-59.
25. Lee YG andKim JK. The Study of the oriental medicine extract on the hair growth effect : I. The effect of the mixture extract of *Polygoni multiflori* Radix, *Angelicae gigantis Radix* and *Lycii Fructus* on the hair growth. Kor. J. Herbol. 2004;19(2):83-90.
26. Lee YS, Kim HS, Son YW, Yoo KS, Lee JH, Lee KC, Choi KC, Shin HC and Park ST. Effect of Radix *Polygoni Multiflori* on cultured vascular endothelial cells damaged by xanthine oxidase and hypoxanthine. Korean J. Oriental Medical Physiology & Pathology. 2002;16(4):720-3.
27. Kim SB and Kim KJ. The effects of rehmannia glutinosa on the protein expression related to the angiogenesis, cell survival and Inflammation. J. Kor. Orient. Medi. Opthalmol. & Otolaryngol. & Dermatol. 2006;19(3):22-33.
28. Oh YS, Oh MS and Roh SS. The experimental study on the effect of herbal extracts on hair growth and acnes. J. Kor. Orient. Medi. Opthalmol. & Otolaryngol & Dermatol. 2006;19(3): 34-54.
29. Jiang C, Lee HJ, Li GX, Guo J, Malewicz B, Zhao Y, Lee EO, Lee HJ, Lee JH, Kim MS, Kim SH and Lu J. Potent antiandrogen and androgen receptor activities of an Angelica gigas-containing herbal formulation: identification of decursin as a novel and active compound with implications for prevention and treatment of prostate cancer. Cancer Res. 2006;66(1):453-63.
30. Hansen LA, Alexander N, Hogan ME, Sundberg JP, Dlugosz A, Threadgill DW, Magnuson T and Yuspa SH. Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. Am. J. Pathol. 1997;150:195975.
31. Moore GPM, Panaretto BA and Robertson D. Effects of epidermal growth factor on hair growth in the mouse. J. Clin. Endocrinol. 1981;88:2939.
32. Mori O, Hachisuka H and Ssasai Y. Effects of transforming growth factor β1 in the hair cycle.

J. Dermatol.1996; 23:89-94.

33. Wollina U, Lange D, Funa K and Paus R. Expression of transforming growth factor β isoforms and their receptors during hair growth phases in mice. Histol. Histopathol. 1996;11: 431-36.
34. Lachgar S, Moukadiri H, Jonca F, Charveron M, Bouhaddioui N, Gall Y, Bonafe JL and Plouët J. Vascular endothelial growth factor is an autocrine growth factor for hair dermal papilla cells. J. Invest. Dermatol. 1996;106(1):17-23.
35. Yano K, Brown LF and Detmar M. Control of hair growth and follicle size by VEGF-mediated angiogenesis. J. Clin. Invest. 2001;107(4):409-17.
36. Roméro-Graillet C, Aberdam E, Clément M, Ortonne JP and Ballotti R. Nitric oxide produced by ultraviolet-irradiated keratinocytes stimulates melanogenesis. J. Clin. Invest. 1997;99:635-42.
37. Cobbold CA. The role of nitric oxide in the formation of keloid and hypertrophic lesions. Med. Hypotheses. 2001;57:497-502.
38. Wolf R, Schönfelder G, Paul M and Blume-Peytavi U. Nitric oxide in the human hair follicle: constitutive and dihydrotestosterone-induced nitric oxide synthase expression and NO production in dermal papilla cells. J. Mol. Med. 2003;81(2): 110-7.
39. Sowden HM, Naseem KM and Tobin DJ. Differential expression of nitric oxide synthases in human scalp epidermal and hair follicle pigmentary units: implications for regulation of melanogenesis. Br. J. Dermatol. 2005;153(2):301-9.