1. Introduction

Hair gives ease to the external impact and protects the scalp from ultraviolet rays, and serves to discharge the waste material in addition to playing an important cosmetic\textsuperscript{1,2}. In other words, if you change the appearance of the hair, you also alter the image of a man, and change the way modern society values that man.

Alopecia (hair loss) is a detrimental disease not only because of society's interest on the external appearance, but also because it can also be accompanied with physical and mental disorders, such as depression and sociophobia,
so the interest and importance for hair growth, hair promotion, and hair loss prevention are increasing more and more.

The cause of hair loss has shown us that the circulation and nutritional disorder of the blood surrounding the hair papilla and hair follicles is the main factor in hair loss. Hair grows when it is supplied with water and nutrients through the capillary around the hair mother cell, so a lack of nutrition through the capillaries results in hair being unable to grow and falling out. Therefore, the development of blood capillaries around the hair is very important to the growth of the hair. In addition, recent increase in physical and mental stress also affects the growth of the hair. Accordingly, the population of males experiencing hair loss as well as female hair loss is growing and hence the hair loss market is also growing rapidly and recent statistics showed a growth of 20-30% annually.

However, treatment for hair loss, despite a developing medical technology, is very limited. The main treatment is hair transplant surgery, which promotes taking healthy hair and transplanting it to balding areas in the hopes that the transplanted hair will maintain their healthy growth in the future. However, this treatment is difficult and can have many side effects. As a result, attention has been focused on natural products that have fewer side effects, and for which therapeutic effects have been proven. Danggui, sukjihwang, hasuo, cheukbaekyeop, and Gugija-like natural herbal medicines have already been reported to be effective in hair growth.

SM-215 used pagoji, baekbugeun, sanggiseang, macmumdong, hyunsam, jimo, baekjain, Omija, and Bockboonja for this experiment. No studies have reported in detail on the effect of hair growth and hair loss prevention except the use of Omija and Bockboonja.

In this study, we aim to investigate the effects of SM-215 on hair growth by hair follicle stimulation.

2. Materials and Methods

2.1 Materials

2.1.1 Animals

Male ICR mice (8 weeks of age) were purchased from Orient Bio (Seongnam, Korea) and provided with a standard laboratory diet and water. After 2 weeks, we screened the 40g mice and divided them into a Control Group (CG), a Non-Treatment Group (NTG), and a SM-215 Application Group (SMG). Thirty mice were assigned to 3 groups with 10 mice in each.

2.1.2 Shaving

The back skin hair of NTG and SMG was shaved with an electric shaver (Joas, Korea) and hair removal cream (Sensitive, Korea).

2.1.3 Preparation and Application of SM-215

The composition herbs of SM-215: 750g of the herb (Table 1) was extracted with 3L of 100°C water at room temperature for 3 hours under pressure and filtered through filter paper (Whatman Grade No. 5). The extracted water was evaporated using a Rotary evaporator (NE-1001, EYELA, Japan) under reduced pressure to obtain the SM-215 extract. The final SM-215 yield was 23.75%. We diluted it in saline before the experiment and SM-215 extracts prepared in 15% concentration was applied 100 μl/day in SMG and saline was applied 100 μl/day in NTG for 3 weeks.

Table 1. The amount and composition of SM-215

| Name of Herbs | Pharmacognostic Name | Weight(g) |
|---------------|----------------------|-----------|
| Pagoji        | Psoralea corylifolia L | 100       |
| Baekbugeun    | Stemona japonica (Bl.) Miq. | 100       |
| Sanggiseang   | Loranthus parasitic(L) Merr. | 100       |
| Macmoondong   | Liriope platyphylla Wang et Tang | 75        |
| Hyunsam       | Scrophularia buergeriana Miquel | 75        |
| Jimo          | Anemarrhena asphodeloides Bunge | 75        |
| Baekjain      | Thuja orientalis Linne | 75        |
| Omija         | Schizandra chinensis Baillon | 75        |
| Bockboonja    | Rubus coreanus Miquel | 75        |
| **Total**     |                      | **750**   |

2.2 Methods

2.2.1 Tissue Samples Production

All mice were anesthetized with a sodium pentobarbital solution-3 weeks after the hair removal then the dorsal skin was excised and fixed in 10% NBF for 24 hours at room temperature. Fixed tissues were embedded in paraffin, and serial sections of 5 μM were produced by a conventional method.

2.2.2 Observation of External Morphologic Changes

Hair growth measurements of mice were taken using ProgRes C14 plus digital camera system.
2.2.3 Observation of Sebaceous Gland’s Changes

Masson trichrome staining was performed to observe the changes in the sebaceous gland. First a mordanting treatment was performed in 50-60°C Bouin solution for 1 hour. Then picric acid of Tissue samples were removed in 70% ethanol. This was followed by nucleus staining in Weigert iron hematoxylin for 15 minutes each. Staining of sebaceous gland (red) and collagen (blue) was performed in aniline blue for 5 minutes. Finally, stained tissue samples were observed with an optical microscope (BX50, Olympus, Japan).

Immunohistochemical staining of the sebaceous gland was used to investigate the Peroxisome Proliferator Activated Receptor (PPAR)-γ expression. The process of proteolysis was carried out for 5 minutes in proteinase K (20 μg/ Ml) and the skin sections were treated for 2 hours in a blocking serum, which is 10% normal goat serum. Reaction with the primary antibody was carried out for 72 hours in 4°C humidified chamber. Then, the secondary antibody was linked for 24 hours at room temperature, followed by treatment in an avidin biotin complex kit (Vector Lab, USA) for 1 hour at room temperature. After coloring in a 0.05M tris-HCl buffer (pH 7.4) containing 0.05% 3,3’-diaminobenzidine and 0.01% HCl, skin sections were stained with hematoxylin for counterstaining. A positive response of the sebaceous gland in PPAR-γ was measured to image analysis of divert image.

2.2.4 Observation of Capillary’s Distribution Changes

2.2.4.1 Image Analysis of Capillary’s Distribution

After cutting the dorsal skin, exposed capillaries were taken to x4 magnification. In the first, using a sharpened low – filter of the image function in Image pro Plus (Media Cybernetic, USA), we made the vessel clear. Selecting the invert function on binary morphology, we converted capillaries in intensity 180-200. After that we observed an emerged image.

2.2.4.2 Capillary’s Distribution Changes Around Hair Follicle

To observe the capillary’s distribution changes around a hair follicle, phloxine-tartrazine staining was performed. After nuclear staining for 5 minutes in a Mayer’s hematoxylin, skin sections were treated for 30 minutes in a phloxine solution. Then, the solution was observed after fractionation in tartrazine. We placed it in tartrazine until the capillary was red and all other tissue was yellow. After that we observed a distinguished image.

2.2.4.3 Vaso Dilatation Changes

In order to examine the eNOS distribution changes involved in the vasodilatation, immunohistochemical staining was performed using a primary antibody and a secondary antibody. Each group was compared with the image analysis.

2.2.5 Observation of Hair Follicle Activation Changes

2.2.5.1 Observation of Growth Stimulation Hormone Changes

In order to examine the Insulin-like Growth Factor-1 (IGF-1) around hair follicle, which was the growth stimulating hormone, immunohistochemical staining was performed using a primary antibody and a secondary antibody. Each group was compared with the image analysis.

2.2.5.2 Observation of Neurotransmitter Changes

In order to examine serotonin and Neuropeptide Y (NPY) around hair follicle, which were neurotransmitters, immunohistochemical staining was performed using primary antibodies and secondary antibodies. Each group was compared with the image analysis.

2.2.6 Image Analysis and Statistical Analysis

For evaluating the results of immunohistochemistry, image analysis was performed using Image pro Plus (Media Cybernetic, USA). The significance of the results was validated using the student T test using Sigma Plot 2000 (Sigma).

3. Result

3.1 External Morphologic Changes

A large number of hairs in SMG was observed as compared to NTG. The difference of two group was noticeable near the hip’s hair versus near the neck. Also the hairs in SMG was thicker and more glossy than NTG (Figure 1).
SM-215 was topically applied to the back of ICR mice for 3 weeks.

Figure 1. External morphologic changes. (A-E) Non-Treatment Group (NTG). (F-J) SM-215 application Group (SMG).

3.2 Sebaceous Gland’s Changes
A greater number of hair follicles were observed in the subcutaneous layer of SMG as compared to NTG. Sebum secretions around the hair follicles in SMG were observed more clearly than in NTG. And sebaceous gland distribution in SMG was more observed compared to NTG. (Figure 2 A and B).

In PPAR-γ immunohistochemistry, PPAR-γ positive response was observed in the sebocyte of the sebaceous gland. A positive response in SMG was noted, as it had increased 303% compared to the NTG in image analysis. (Figure 2 C and D, Table 2).

Figure 2. Sebaceous gland’s changes. (A) Skin structure in NTG (Masson trichrome method, x40). (B) Skin structure in SMG (Masson trichrome method, x40). (C) PPAR-γ positive reaction (arrow) in NTG (PPAR-γ immunohistochemistry, x1000). (D) Increase of PPAR-γ positive reaction (arrow) in SMG (PPAR-γ immunohistochemistry, x1000). (E and F) Diverted photographies of C and D.

3.3 Changes of Capillary’s Distribution
A large number of capillaries, especially in the hip region compared to the neck region in SMG, were observed compared to the NTG in external capillaries observation. (Figure 3 A and B)

Capillaries around the hair follicle, were more observed compared to the NTG in Phloxine-tartrazine staining. (Figure 3 C and D).

In eNOS immunohistochemistry, the eNOS positive response was observed around hair follicle in dermis and the positive response of SMG was increased 79712% compared to the NTG in image analysis. (Figure 3 E and F, Table 2).

Figure 3. Changes of capillary’s distribution. (A) External vessel distribution in NTG (x4). (B) External vessel distribution in SMG (x4). (C) Capillary around hair follicle (arrow) in subcutaneous layer of NTG (Phloxine-tartrazine method, x400). (D) Increased capillary around hair follicle (arrow) in subcutaneous layer of SMG (Phloxine-tartrazine method, x400). (E) eNOS positive reaction (arrow) in NTG, (eNOS immunohistochemistry, x400). (F) Increase of eNOS positive reaction (arrow) in SMG (eNOS immunohistochemistry, x400).

3.4 Changes of Hair Follicle Activation
3.4.1 Change of Growth Stimulation Hormone
In IGF-1 immunohistochemistry, the IGF-1 positive response was observed around hair follicle in subcutaneous
layer. And the positive response in SMG was increased 308% compared to the NTG in image analysis. (Figure 4 A and B, Table 2).

### 3.4.2 Change of Neurotransmitter

In serotonin immunohistochemistry, the serotonin positive response was observed in root sheet of hair follicle in subcutaneous layer. The positive response of SMG was increased 450% compared to the NTG in image analysis. (Figure 4 C and D, Table 2).

**Figure 4.** Changes of Hair follicle activation. (A) IGF-1 positive reaction (arrow) in NTG (IGF-1 immunohistochemistry, x400). (B) Increase of IGF-1 positive reaction (arrow) in SMG (IGF-1 immunohistochemistry, x400). (C) Serotonin positive reaction (arrow) in NTG (Serotonin immunohistochemistry, x400). (D) Increase of serotonin positive reaction (arrow) in SMG (Serotonin immunohistochemistry, x400). (E) NPY positive reaction (arrow) in NTG (NPY immunohistochemistry, x400). (F) Increase of NPY positive reaction (arrow) in SMG (NPY immunohistochemistry, x400).

In NPY immunohistochemistry, the NPY positive response was observed around hair follicle in subcutaneous layer and the positive response in SMG was increased 1770% compared to the NTG in image analysis. (Figure 4 E and F, Table 2).

**Table 2.** The image analysis of hair development

| Objective | Group  | CG       | NTG       | SMG       |
|-----------|--------|----------|-----------|-----------|
| PPAR-γ    |        | 5874±275 | 6158±243  | 24830±1180* |
| eNOS      |        | 53±4     | 55±5      | 43897±2389* |
| IGF-1     |        | 611±33   | 656±32    | 2677±173*   |
| Serotonin |        | 8145±623 | 8824±142  | 48562±2211* |
| NPY       |        | 3207±470 | 3453±212  | 64571±3187* |

### 4. Discussion

Hair is a solid cylindrical fiber made of tightly packed keratinized epithelial cells. It is one of the defining characteristics of mammals. Hair is present everywhere in human skin except for the palms of the hands, soles of the feet, fingers and toes of the distal border, the boundary of the mucous membranes, and the glans of the penis. Although not directly related to the life, hair has a number of roles depending on the site of the body. In addition, because hair has a cosmetic function as a means of expressing individuality, it plays an important role in human social life, so no hair on the head gives it great emotional stress.

Hair is formed from hair follicles. The lower part of the hair follicle consists of hair papilla, hair matrix, hair shaft, inner root sheath, and outer root sheath. Hair papilla is located in papilla-like projections of the hair follicle’s lower dermis, the source of hair growth, and consists of a number of blood tissues and cells. The hair matrix is a collection of epithelial cells called “hair mother cells”, often interspersed with the pigment-producing cells called melanocytes. The matrix wraps completely around the papilla and provides access for the capillary. The hair matrix epithelium is supplied with nutrients from the capillaries. Therefore, improvement of blood circulation in the scalp can have a very close relationship with healthy hair, and a blood circulation disorder caused by compression of the capillaries is one of the causes of hair loss.

Modern medical science has developed and applied various, but the treatment and improvement for chronic hair loss still has a limitation, as there are also numerous side effects of sustained drug use. In recent years there...
has been progress in research using a variety of natural substances and materials for hair loss and active hair growth, especially in cases of herbal extracts proven as a herbal medicine. In this study, we aim to investigate the effects of SM-215 on hair growth by hair follicle stimulation.

First, the results of external morphologic changes showed a large number of hairs became thicker and turned glossy in SMG compared to NTG, so we can confirm the promoting effect of SM-215 on the hair growth.

Observation of the changes in the sebaceous gland show promotion in the subcutaneous layer and hair follicle, the sebaceous glands around hair follicle, the sebum secretion, and a positive response of PPAR-γ was noted with an increase in SMG compared to NTG. Most of the sebaceous gland is connected with the hair follicle and helps promote hair growth by secreting sebum19. PPAR-γ plays a pivotal role in the lipid homeostasis and differentiation and maturation of seocytes in the sebaceous gland; and is the nuclear hormone receptor involved in maintenance of epithelial stem cells in the animal’s hair follicle19,20. When observing a lot of PPAR-γ, the secretion of sebum may be considered to be actively carried; a positive response of PPAR-γ in SMG in this experiment means SM-215 helps to promote hair growth by keeping epithelial stem cells in the hair follicle via promotion of expression of PPAR-γ and by applying moisture and luster to the hair via stimulation of sebaceous gland.

In SMG, changes of the capillary’s distribution, the number of capillaries around a hair follicle, the distribution of thick blood vessels, and the positive response of eNOS to dilate the blood vessels increased.

Endothelial NOS (eNOS, NOS III) is mostly expressed in endothelial cells. It keeps blood vessels dilated, controls blood pressure, and has numerous other vaso-protective and anti-atherosclerotic effects produced NO21. An increase of eNOS positive response means the expansion of blood vessels, and the results of this study indicate that SM-215 assists in the maintenance and growth of healthy hair with smooth nutrient supply through the promotion of blood flow and angiogenesis.

The factors that affect hair growth are the Insulin-like Growth Factor-1 (IGF-1), the Fibroblast Growth Factor (FGF)-1, 2 and 5, the Keratinocyte Growth Factor (KGF), the Hepatocyte Growth Factor (HGF), the Vascular Endothelial Growth Factor (VEGF), and the Transforming Growth Factor (TGF)-α and β1; all of them were reported to be expressed in the hair follicle22. Among them, IGF-1 that is secreted from the hair papilla cells is one of the important growth factors to control the hair growth by significantly increasing the length of the hair follicle tissue as well as promoting the proliferation of epithelial cells in culture systems23. Recently it has also been known to play an important role in the differentiation and migration of cells in the development stage of hair follicle cells. To complete its’ biological role, IGF-1 combines with a specific receptor, Type I IGF Receptor (IGF-1R), on the cell surface, and promotes the differentiation of cells, resulting in IGF-1 the differentiation of the hair follicle through this cell signaling pathway24.

In this study, it was confirmed that the positive response among the SMG IGF-1 increased 308% in the cells from the hair follicle at subcutaneous layer compared to NTG; this means that the role of SM-215 is to facilitate the expression of IGF-1 involved in hair growth by inducing the differentiation of the hair follicle.

Stress is one of many risk factors that cause hair loss25. The relevance of psychological and emotional stress as a contributor to hair loss is proven by various studies. Acute or chronic stress may cause the resting stage hair loss or act on the pathogenesis of a number of different hair loss diseases (male pattern baldness, alopecia areata) that aggravate hair loss.

In mice, stress induces suppressed hair growth, causes the induction of a catagen, generates hair damage, and the resting hair loss caused under various clinical conditions when the sudden stress is given is similar to humans26. Serotonin (5-Hydroxytryptamine, 5-HT) is a monoamine neurotransmitter. It is popularly thought to be a contributor to feelings of well-being and happiness. Biochemically derived from tryptophan, serotonin is primarily found in the Gastrointestinal tract (GI tract), blood platelets, and the Central Nervous System (CNS) of animals, including humans27. In addition, human skin produces serotonin, and converting this to melatonin is known to affect the growth of the hair28.

Peripheral nerve fibers that are distributed on the skin may have an important function in controlling the unique afferent sensory transfer function, and also have an efferent function to control various types of inflammatory and proliferative skin reactions29. Where the nerve fibers from skin structures that are distributed with the highest density are just hair follicles, a relative number of studies have reported on the follicular distribution around the nerve30–33. The hair follicles originate in ectoderm,
mesoderm, and generate hair through the interaction of the organic-derived neuro-ectodermal cells. Recent clinical and experimental observation in the generation of hair follicles suggests that the peripheral nervous system plays an important role in the generation of hair follicles, growth and hair cycle. These efferent functions of the hair follicle in the peripheral nervous system is considered to be mediated by a number of unique receptor materials, and these materials are released from the distal end of the sensory nerve fibers. The typical materials for such an action are the neuropeptide Substance P (SP) and Calcitonin Gene-Related Peptide (CGRP). Neuropeptide functions as a material for a wide range of actions generated in the nervous system by binding to receptors on the target cells. There are a lot of neuropeptides in human skin, such as neurokinin A, somatostatin, Vasoactive Intestinal Peptide (VIP), Neuropeptide Y (NPY), γ-melanocyte stimulating hormone, in addition to substance P and CGRP.

NPY is represented in the form of a variety of responses within the central nervous system, including feeding behavior, gonadotropin-releasing hormone release and stress responses. According to the latest research, anti-cancer agents used in cancer treatment cause a variety of side effects, to normal cells as well as to cancer cells; one of these side effects affecting normal cells can be seen in the hair loss during cancer treatment. But by using NYP materials’ various types of immune cells, especially bone marrow hematopoietic cells which do not die, it is possible to minimize side effects, and thus treatment is possible without hair loss.

In this study, the evaluation of using a sensitive neurotransmitter serotonin and NPY in the stress response for stimulating hair follicle activity. Serotonin immunohistochemical results from this study show that serotonin positive reaction was observed in the subcutaneous layer of the hair follicle roots connective tissue, and image analysis results indicate the SM-215 group tested positive 450% more when compared to the depilation. A NPY positive reaction was observed in the hair follicle in the dermis around and the image analysis results indicate SM-215 group tested positive 1770% more compared to the depilation group.

These results indicate that the SM-215 group had more coarse and shiny hair because the administration of SM-215 disturbed the hair growth suppression due to stress and; the increase in this neurotransmitter is thought to have been generated to promote hair growth.

Through the above these results, SM-215 was shown to facilitate the supply of nutrients through the vascular increase and the hair follicle surrounding the capillaries, to stimulate the sebaceous gland thereby increasing the secretion of sebum and inhibiting hair growth by suppressed the generation of stress and neurotransmitters. It is thought to increase by that promote hair growth. And this study is considered to be significant in that herb medicine can be used as an important functional component of the hair growth stimulant or hair restorer.

5. Conclusion

After treating SM-215 to rodents for three weeks in order to investigate the effects of SM-215 on hair growth stimulation of the hair follicle in mice, then we observed the condition, angiogenesis, cell activity changes of hair follicle and obtained the following results.

- A large number of hairs were observed to become thicker and turn glossy in SMG compared to NTG.
- It has been shown to promote subcutaneous layer and hair follicle, sebaceous glands around hair follicle, sebum secretion and positive response of PPAR-γ were increased in SMG compared to NTG.
- The number of capillaries around a hair follicle, the distribution of thick blood eNOS, IGF-1, serotonin and NPY positive response showed an increase in SMG compared to NTG

Through the above these results, this study is considered to be significant in demonstrating that herb medicine has the possibility to be used as an important functional component of the hair growth stimulant or hair restorer.

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