Action Mechanism of Natural Plant Extracts for Hair Loss Prevention and Hair Growth Promotion in C57BL/6 Mice

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ABSTRACT

The Natural Plant Extracts (NPE), produced by mixing seven natural plant extracts including sweet flag, contains asacurin 100 as its main ingredient and shows improvement in skin, but no hair growth effect has been reported. This study was conducted to evaluate the effects of NPE on mouse hair loss prevention and hair growth promotion and to determine the mechanism of action of NPE. Thirty male C57BL/6 mice were randomized into three treatment groups of 10 mice each: Control group (saline), NPE and minoxidil 5% application. For the NPE application group, the first hair growth was observed on day 9 and the density, length and thickness of their hair were significantly higher than those of the control group. Compared to the control group, the anagen hair growth in the C57BL/6 mice of the NPE application group was significantly stimulated. Compared to the control group, the expression of VEGF and KGF mRNA was up-regulated in the NPE and minoxidil 5% application groups, but the expression of TGF-β1 mRNA was down-regulated on the contrary. This result suggests that NPE promotes hair growth while inducing anagen hair growth in animals through genetic regulation while, simultaneously preventing hair loss.

Key words: Natural plant extract, hair growth, anagen, gene expression

INTRODUCTION

Hair is a component of the skin that grows from the follicles of the dermis and protects the body. A human has about 100,000 follicles, which are small tissues that grow hair. The hairs growing from the follicles go through four hair cycles of anagen, catagen, telogen and exogen, repeating growth and alopecia (Fuchs, 2007; Kim et al., 2014). During the anagen phase of the skin, the follicles in the dermal papilla layer of the derma grow, which during the catagen phase, the thickness of the dermis decreases and the follicles get closer to the epidermis. After the dermal papilla cells drop out, a new hair growth cycle begins (Cotsarelis and Millar, 2001; Botchkarev and Kishimoto, 2003). Alopecia is a phenomenon in which the hair does grow normally, falls out easily and become thin. As a result, the anagen phase becomes shorter and the telogen phase becomes longer. Alopecia has been known as an aging phenomenon, but it is associated with many factors, such as genetics, stress, diet, nutritional imbalances and eating habits (Kerscher et al., 2007; Aljuffali et al., 2014). Vascular Endothelial Growth Factor (VEGF) and Keratinocyte Growth Factor (KGF) stimulate hair growth and transforming growth factor-β1 (TGF-β1) is a gene that suppresses hair growth (Inui et al., 2003; Weger and Schlake, 2005). One method to prevent alopecia is to improve blood flow around the follicles and supply nutrients to the hair root, while another method is to activate the follicles by improving the immune function using the many lymphocytes gathered around the follicles that have lost hair (Muller-Rover et al., 2001; Van Mater et al., 2003). About 2% of global population is estimated to be suffering from alopecia. Finasteride and Minoxidil (Rogaine) received approval by the FDA as alopecia remedies, but adverse reactions, such as itching and dermatitis have been reported (Messenger and Rundegren, 2004; Rogers and Avram, 2008). The mutation of testosterone acceptor has been found to be responsible for about 80% of male alopecia cases, but stress is also an important factor that influences gray hair and alopecia (Garza et al., 2012).
Although, female alopecia has a lower prevalence than male alopecia, over 30 million women in the U.S. are suffering from it. The age group of alopecia sufferers is getting lower. Some people in their 20 and 30s and even some teenagers are suffering alopecia (Garza et al., 2011).

Natural products are being applied to cosmetics and in hair care products and different plant extracts have been researched in relation to hair growth (Rathi et al., 2008). Sweet flag (Acorus calamus Linn.), morus bark (Mori cortex radicis), licorice root (Glycyrrhiza uralensis), pine needles (Pinus densiflora), sophora root (Sophora angustifolia), cnidium (Ligusticum chuanxiong Hort) and Korean angelica (Angelica gigas) have been reported to prevent alopecia and gray hair, to treat inflammation, improve blood circulation and improve the scalp (Kim, 2014). In South Korea, it has been traditionally known that washing one’s hair in sweet flag water makes the hair glossy and prevents the hair from falling out easily. Sweet flag is also known as Bacha and the main ingredient or the extract is a sweet flag extract is asarone, an essential oil, which has been researched in association with alopecia treatment and antibacterial functions (Radusiene et al., 2007). The main ingredients of morus bark are α-Amyrin, kaempferol, morin, quercetin and umbelliferone, which have been known to have hair growth effects for alopecia (Lee et al., 2000). The main ingredients of the flavonoids of licorice root are liquiritigenin, licquiritin, licurasiside and glycyrrhizin, which anti-inflammatory effects (Han et al., 2014). The main ingredients of pine needle extracts are cumaric acid, terpene, polyphenolic tannin and proanthocyanidie, which prevent alopecia, improve skin tissue and lightening the skin, treat hypertension and diabetes and have anticancer and antibacterial effects (Choi, 2007). Sophora root contains various types of alkaloids and flavonoids and their main ingredients have been found to be d-matrine and d-oxymatrine, which have been reported to be associated with hair growth, skin anti-aging, skin care and anti-inflammatory effects (Hwang et al., 2005; Han et al., 2014). Cnidium contains volatile essential oils and the alkaloids dl-tetrahydropalmatine, protopine, ferulic acid, ligustilide and butylidenepthalide, which have been associated with skin lightening, antibacterial and anti-oxidant effects (Heo and Ha, 2011; Ran et al., 2011). The main ingredients of Korean angelica are coumarin derivatives, such as decursin, decursinol and nodakenin and essential oils such as α-pinene, limonene, β-eudesmol and elemol, which have been found to have skin health (Lee et al., 2012) and anti-inflammatory effects produced by mixing the seven natural plant extracts listed above, NPE has been proven from previous studies to contain asacurin 100 and have preventive effects against atopic dermatitis through anti-inflammatory reaction (Park et al., 2012). In this study, the hair growth effect and mechanism of action of NPE produced by mixing seven natural plant extracts, including sweet flag, in C57BL/6 mice were investigated.

MATERIALS AND METHODS

Preparation of NPE: Sweet flag, morus bark, licorice root, pine needles, sophora root, cnidium and Korean angelica were mixed at the ratios suggested by NJY Biotechnology Co., Ltd (Seoul, South Korea). The mixture was then extracted by hot water using a heating mantle (1:10 w/v). Furthermore, the aforementioned seven natural plants were mixed by hot water extraction and then extracted in pure ethanol (1:10 w/v) for three hours three times in a reflux condensing system. Each tract was filtered through Whatman No. 2 filter paper and vacuum concentrated with a Rotary vacuum evaporator (EYELA, Rikakiki Co., Tokyo, Japan). After concentration, NPE that promoted hair growth was obtained (hot water extraction yield 7.5%, ethanol extraction yield 5.1%). Each NPE product was analyzed for ingredients (Pang et al., 2008) with the GC/MSD (Gas chromatograph/Mass selective detector, Agilent 7890A, 5975C, Agilent Technologies, USA). The main ingredients were found to consist of 80% asarone, 18% cumaric acid and 2% of others. This matter was named asacurin 100. The NPE as an experimental material was produced by concentrating the seven natural plant extracts, which were obtained from hot water extraction and mixing it with an ointment base supplied from NJY Biotechnology.

Experimental animals and experiment design: Animal experiments were conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the European Community guidelines (EEC Directive of 1986; 86/609/EEC) (Park and Park, 2015) and were approved by the Animal Ethics Committee of Kangwon National University, Republic of Korea (IACUC, KW-141 027-1). Thirty male C57BL/6 mice were purchased from Daehan Biolink Co., Ltd. (Eumseong Chungbuk, South Korea). After adapting them to the breeding environment for one week, the hair on their dorsal skin was completely removed before NPE was applied to the skin. The animals were randomized to three treatment groups for three repetitions with 10 animals per cage. The treatment plots were classified into saline solution (control group), NPE and minoxidil 5% application groups. The experimental diet was prepared in accordance with the nutrient demands of mouse proposed by AIN-93 in the USA and pelleted. The crude protein and total energy contents were adjusted equally for each meal. The experimental diet (w/w, g 100 g⁻¹) consisted of casein-vitamin free 20, corn starch 39.75, maltodextrin 13.20, sucrose 10.00, soybean meal 7.00, cellulose 5.00, AIN 93G mineral mix. 3.50, AIN 93G vitamin mix. 1.00, L-cystine 0.30, choline bitartrate 0.25 and t-butylhydroquinone 0.0014 (Park and Park, 2015). The breeding room was maintained at 22±2 with a relative humidity of 55±5% and a 12 h lighting cycle. The animals were allowed to freely ingest water and the experimental diet.
Application of NPE: Seven-week old mice that reached the telogen phase of hair growth in which the dorsal skin turns pink were anesthetized with an intraperitoneal injection of a 10:1 diluted mixture of Zoletil (Virbac, Paris, France) and Rompun (Bayel Korea Co., Seoul, Korea) mixed at 8:2 (10 µL 10 g of weight⁻¹). The skin hairs were removed with an epilator at first and then with Niclean Cream (Ildong Pharmaceutical Co., Ltd., Seoul, Korea). Immediately after the hair removal, the treatments including NPE, were applied to the entire hair removal site on the dorsal skin of each animal twice a day until the end of hair growth. Among the three treatment groups, the first hair growth was found on day 9 after the start of application in the NPE application group. Thus, all the items related to hair growth were measured at this time.

Hair growth area and appearance: On day 9, 12, 15 and 18 after NPE application, the animals were anesthetized by aforementioned method (Park and Park, 2015). Then the degree of hair growth was visually evaluated and scored. The hair growth area was measured as the percentage of the hair growing area in the hair removal site area. The appearance was recorded with a digital camera.

Measurement of density, length and thickness of hair: The density, thickness and length of hair was measured with a 300X microscope using the hair analysis system (PSI 2003, Hair and Scalp Microscope System, SIF-1, Korea) on day 9, 12, 15 and 18 after NPE application. For the hair density, the number of hairs per 0.6 mm² was measured and the length and thickness were measured with the program embedded in the system.

Physiological findings of skin tissues: The skin tissues were collected on day 9 after NPE application and fixed with 10% neutral formalin. The site to be observed was trimmed and paraffin processing for animal tissues (sampling, fixation, washing, dehydration, clearing, infiltration, embedding) was carried out. For the embedding paraffin block, 3-5 µm microtome cuttings were made with a microtome (HM-315 model, Microm Co., Germany) and stained with Hematoxylin and Eosin (H and E). The stained tissues were observed under the light microscope (CX41, Olympus, Tokyo, Japan) and the number of follicles were counted in each growth step and represented as percentage.

Gene expressions: The genetic expression was measured in accordance with the protocol provided by Eco Real-Time PCR (Illumina Inc., USA). On day 9 after NPE application, the dorsal skin tissues of the mice were collected and rapidly frozen with liquid nitrogen before being store at -80. Total RNA was extracted from a 30 mg sample with the lysis buffer of Xprep Tissue RNA Mini Kit (Phile Korea Technology, PKT). The RNA concentration was measured at 260 nm of absorbance with NanoDrop ND-1000 Spectrophotometer and 300-500 ng µL⁻¹ of total RNA was obtained. From 1 µg of the extracted RNA, the 1st strand cDNA was synthesized using a cDNA synthesis kit (PKT). The cDNA was amplified for 5 min at 70, 30 min at 42 and 5 min at 85 and used as the template for Eco RT-PCR. For RT-PCR, the QuantiMix SYBR Kit (PKT) was used. After cDNA was diluted (5:1) with distilled water, the primers were mixed. The proper primers of the target gene were designed for the KGF, TGF-β1 and VEGF of the mice as follows: KGF forward 5’-GGCAAGTAAAA GGGACCCA-3’, reverse 5’-AAGGCACCGGATTTCC C-3’; TGF-β1 forward 5’-CATCAACGGGTTTCACTACCG-3’, reverse 5’-AGTTGGGATAGTACCTT-3’; VEGF forward 5’-CAAGGCCACGACATAGGAGA-3’, reverse 5’-GCAACGCGAGTCTGTGTTT-3’; GAPDH forward 5’-GGACCCTAAAAGGCTGTCAT-3’, reverse 5’-GTCGTGG GCATGGACTGTTG-3’. The relative expression levels of the target gene’s mRNA was normalized with the GAPDH internal standard.

Statistical analysis: The analysis data were statistically processed with the SAS application. The average and standard error of each treatment plot were obtained and variance analysis was performed. The significance (p<0.05) was tested at 95% level by the Duncan's multiple range test (SAS., 2004).

RESULTS AND DISCUSSION

The visual observation results of the hair growth are shown in Fig. 1-3. The first hair growth started on day 9 after NPE application and the hair growth ratio was 17.06%. The minoxidil 5% application group showed the hair growth ratio of 19.15% on day 9, but the control group to which saline solution was applied maintained their hair-removed pink skin and showed no hair growth. The NPE and minoxidil 5% application groups showed the hair growth ratios of 50.67 and 60.92% on day 12, respectively, 90.25 and 92.47% on day 15, respectively and 100% for both on day 18. The control group started hair growth on day 13 after saline solution application and showed the hair growth ratios of 25.17, 40.25 and 60.28%...
Fig. 2: Effect of natural mixed plant extracts (NPE) on hair regrowth promoting in C57BL/6 mice
on day 13, 15 and 18. Their hair growth was not completed on day 18, when the hair growth of the NPE and minoxidil groups was completed. As the hair growth started in every treatment group, the skin changed from pink to gray and stayed black as hair grew. The skin changes from gray or black as the hair enters the anagen phase (Kawano et al., 2009). The minoxidil 5% application group showed an adverse reaction: The skin shrunk from day 10 after application and inflammation was found up to the neck. This result suggests that NPE and minoxidil have similar hair growth effects, but minoxidil has adverse reactions. This result coincides with the reports that edema, dermatitis and itching appeared as hypersensitivity reactions to minoxidil and hair loss and horny substances appeared over time phase (Kawano et al., 2009).

Black solid hairs started to grow from day 9 after application of NPE and minoxidil, but no hair grew in the control group (Table 1, Fig. 4). On day 9, when hair growth started, the density, length and thickness of hair were the greatest in the NPE group, followed by minoxidil 5% and saline solution groups. The hair density of the NPE group was 599.8, 166.7 and 136.6% greater compared to the control group on day 9, 12 and 15, respectively. The hair density of the minoxidil 5% group showed no change on day 9 and was 366.1 and 132.9% greater compared to the control group on day 12 and 15, respectively (p=0.05). The hair length of the NPE group was 122.2, 171.4 and 116.9% greater compared to the control group on day 9, 12 and 15, respectively. The hair length of the minoxidil 5% group was 116.7, 152.4 and 121.9% greater compared to the control group on day

<table>
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<th>Parameters</th>
<th>Control</th>
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<th>Minoxidil 5%</th>
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<td>Density</td>
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<tr>
<td>9 days</td>
<td>0.100±0.02</td>
<td>0.600±0.05</td>
<td>0.400±0.02</td>
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<td>12 days</td>
<td>0.600±0.55</td>
<td>16.000±0.80</td>
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<td>16.400±0.58</td>
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<td>Length</td>
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<tr>
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<td>0.020±0.004</td>
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<td>Thickness</td>
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<tr>
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<td>15 days</td>
<td>0.010±0.0002</td>
<td>0.020±0.001</td>
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Mean values±standard deviation (n = 10), *p<0.05

Fig. 3: Appearance of dorsal skin by minoxidil 5% in male C57BL/6 mice

Fig. 4: Changes of hair regrowth promoting in male C57BL/6 mice applied with natural mixed plant extracts (NPE)
The hair thickness of the NPE group was 181.8, 200.2 and 180.7% greater compared to the control group on day 9, 12 and 15, respectively. The hair thickness of the minoxidil 5% group was 101.1, 120 and 121.9% greater compared to the control group on day 9, 12 and 15, respectively (p<0.05). When the grown hairs were removed by pulling with tweezers on day 12, those of the control group and minoxidil 5% group were removed easily, whereas almost none of the NPE group were removed which suggests that NPE keeps the hair roots strong. In the anagen phase, the dermis and subcutaneous layers of the skin are developed and follicles grow in the dermal papilla layer (Mou et al., 2006). In the catagen phase, the dermis thickness decreases and the follicles get closer to the epidermis. Furthermore, dermal papilla cells in the follicles regenerate the follicles and promote the formation of follicles (Randall et al., 2001; Jahoda et al., 2003). During the anagen phase of the hair growth, hair loss occurs because the hair does not grow sufficiently long. As the thickness of growing hair is determined by the follicle length, the follicle length of the growing hair from the early to middle stages of growth is very important in the normal hair lifecycle (Cotsarelis and Millar, 2001).

The follicle growth steps and the pathological findings of the skin tissues after NPE application are shown in Fig. 5 and 6. The hair growth stages are divided into anagen, catagen and telogen phases. The growth stages of every follicle observed on the dorsal skin were separately indicated. On day 9 when the first hair growth was observed in the NPE group, the growth stage of the hair on the dorsal skin of the mice in the anagen and catagen phases was the highest in the NPE group, followed by the minoxidil 5% group and the control group. In the telogen phase, the hair growth stage was the highest in the control group, followed by the minoxidil 5% group and the NPE group. Compared to the control group, the hair growths of the NPE and minoxidil 5% groups were significantly 163.2 and 139.7% higher, respectively, in the anagen phase, but they were 85.50 and 53.20% lower, respectively, in the telogen phase (p<0.05). The hair shaft looks like a ladder in the middle of a follicle. The NPE group had more follicles that reached the anagen phase, less follicles in their catagen phase and their follicles are longer and larger compared to the minoxidil 5% group and the control group. The follicles that grow in the dermal papilla layer of the dermis get closer to the epidermis and the dermal papilla cells in the follicles undergo a new hair growth cycle. The hair growth control of the dermal papilla cells has been known to

![Fig. 5: Changes of the cycles of hair follicle growth in male C57BL/6 mice after 9 days applied with natural mixed plant extracts (NPE). Bars represent standard deviation of means (n = 10)].

**Fig. 5:** Changes of the cycles of hair follicle growth in male C57BL/6 mice after 9 days applied with natural mixed plant extracts (NPE). Bars represent standard deviation of means (n = 10) a,b,c;p<0.05

**Fig. 6(a-c):** Skin photomicrographs on the hair follicles growth in male C57BL/6 mice after 9 days applied with natural mixed plant extracts (NPE) (a) Control (b) NPE and (c) Minoxidil (5%)
be associated with the size of hair papilla. With regard to the follicle cycle, it has been found that as the dermal papilla cells that were shrunk in the catagen phase become larger, the epithelial cells increase and form large hair bulbs, which cause hair growth (Cotsarelis and Millar, 2001; Botchkarev and Kishimoto, 2003).

The expressions of VEGF, KGF and TGF-β1 mRNA after NPE application are shown in Fig. 7. Compared to the control group, the VEGF and KGF mRNA expressions were greater in the NPE group than in the minoxidil 5% group, but the expressions of TGF-β1 mRNA were significantly lower on the contrary (p<0.05). Compared to the control group, the expressions of VEGF mRNA of the NPE and minoxidil 5% groups were 426.5 and 278.2% higher, respectively, but their expressions of TGF-β1 mRNA were 17.35 and 14.19% lower, respectively. These results show that in the NPE group the expressions of VEGF and KGF mRNA, which stimulate hair growth, were significantly up regulated and the expression of TGF-β1 mRNA that suppresses hair growth was down regulated. Therefore, it follows that NPE stimulates the hair growth factors and helps angiogenesis, thereby creating a beneficial environment for hair growth. The increased expressions of VEGF and KGF genes suggest that NPE has hair loss suppression effects by maintaining the anagen phase of the hair. The VEGF has been found to promote angiogenesis in the telogen phase and induce a new anagen phase of hair and KGF has been found to stimulate hair growth as a member of the family of fibroblast growth factors. The KGF protein has been found to have effects on the division and growth of fibroblasts and endothelial cells as well as horny cells. Thus, the significant increase in the expression of KGF must have given strong signal stimulation to the hair in the anagen phase (Inui et al., 2003; Weger and Schlake, 2005). The TGF-β1 is active in the late anagen phase and early catagen phase of the hair, suppresses the proliferation of epithelial cells and induces cell death, thereby suppressing hair growth (Cotsarelis and Millar, 2001; Alonso et al., 2005).

In summary, the findings from this study suggest that NPE, which was produced by mixing various natural plant extracts including sweet flag (Acorus calamus var. angustatus), keeps the hair roots strong, stimulates the growth of follicles and promotes hair growth through the regulation of genes.

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