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Effects of *Eclipta prostrata* Extract and Copper Peptide on Hair Growth Promotion in an Experimental Animal Model

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ABSTRACT

The present study was carried out to investigate promotional effect of *Eclipta prostrata* extracts on hair growth in an animal model of mice. There were four experimental groups including distilled water as negative control (NC), 3% minoxidil (MXD) as positive control (PC), Tricomin as copper peptide (PC) and *Eclipta prostrata* extract (EE) groups. The EE was extracted with ethanol and diluted with distilled water. The 6 week-old C57BL/6 male mice were shaved with an electric clipper and the test materials were topically treated with 0.2 mL per mouse daily for 3 weeks. The hair re-growth was photographically evaluated weekly during the experimental periods. The number of mast cells was counted on the dorsal skin section of mice. The enzymes, alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT), were determined using an biochemical autoanalyzer. There were no clinical signs in all experimental groups. As the results of photometric analysis, PC, CP and EE groups accelerated hair regrowth significantly faster than that of NC group after treatments for 2-3 weeks ($p < 0.05$). PC, CP and EE groups showed a significant decrease in mast cell population compared to NC group. Activities of ALP and γ-GT were significantly increased in PC and EE groups compared to NC group ($p < 0.05$). Taken together, these results suggest that the *Eclipta prostrata* extract may have hair-growth promoting activity equal to that of MXD or copper peptide.

Key words : *Eclipta prostrata,* Hair growth, Mast cell, Alkaline phosphatase, γ-glutamyl transpeptidase

Introduction

Hair loss is not life threatening, but their profound impact on social interactions and on patients' psychological well-being is undeniable. In recent years, the population which suffering from hair loss is increasing and the suffering age is coming to be low gradually because of mental stress, nutritional imbalances and environmental pollution. Since people become more self-conscious on what others think, young men and working women are concerned of their thinning hair and hair loss problems as much as middle-age and elderly men. Therefore, the demands of therapeutics which prevent from hair loss and promote hair growth are growing. Androgenic alopecia is a partial or complete loss of hair that occurs in a progressive pattern in genetically predisposed individuals. A variety of genetic and environmental factors likely play a role in androgenic alopecia, and most of contributing factors remain unknown.

The hair follicle undergoes a life-long cyclic transformation from a resting (telogen) phase to a growth (anagen) phase with rapid proliferation of follicular keratinocyes and elongation and thickening of the hair shaft, followed by a regression (catagen) phase leading to involution of the hair follicle [1,2]. These cyclic changes are profoundly influenced by numerous growth factors, cytokines, hormones, neuropeptides, and pharmaceutical products [3,4] and the relative duration of these phases

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varies with the hormone factors [5] and other physiologic and pathologic factors as well as body site, age, and nutritional status [6,7].

In the hair cycle, hair growth and hair loss are caused by a variety of factors. Mast cells are distributed around hair follicles, and the murine hair cycle is largely associated with the degranulation of mast cells [8]. The degranulated perifollicular mast cells significantly increase during late anagen VI just before the onset of catagen. The catagen development is retarded by the inhibition of mast cell degranulation [8]. In addition, when hair is induced into the anagen phase, there is an increase in the activity of the enzymes, γ-glutamyl transpeptidase (γ-GT) and alkaline phosphatase (ALP), which are indicators of hair growth [9].

Presently, the US-FDA-approved minoxidil (MXD) is a vasodilatory medication used primarily as antihypertensive drug. MXD is a potassium channel agonist that bears the chemical structure of nitric oxide (NO), a blood vessel dilator, and may be a nitric oxide agonist. This appears to explain its vasodilatory effect [10] but may also be linked to Minoxidil's ability to stimulate hair growth and treat hair loss [10]. But, the side effects, including seborrhoeic dermatitis, erythema, allergy dermatitis, angina and haematological problems were reported [11].

The aqueous extract of *Eclipta prostrata* has been traditionally used in hair growth and blackening hair. In addition, its extract has some biological activities including anti-inflammatory, antioxidant, antitumor and lipid-lowering activities [12-15].

Copper peptide is a naturally occurring copper complex of a glycyl-L-histidyl-L-lysine peptide. It is described as a growth factor for various kinds of differentiated cells and it stimulates the proliferation of dermal fibroblasts and elevates the production of vascular endothelial growth factor [16-18]. The copper peptide has been reported to promote hair growth *in vivo* and *in vitro* [16-18].

The C57BL/6 mice are thought to be particularly useful as an animal model for studying the biology of hair growth [19] and phenomenon of anagen-coupled melanogenesis. This model offers the opportunity to study large numbers of biologically homogeneous follicle populations, because hair growth in mice can be synchronized; in contrast to humans. Hair follicles enter or leave anagen waves covering large skin area. Anagen phase in C57BL/6 mice (grey to black skin) can easily be distinguished from telogen phase (pink skin) by the skin color [1].

In comparison with Tricomin containing copper peptide, the extract of *Eclipta prostrata* that have been used traditionally in oriental medicine was used to evaluate if it has hair growth promoting effect in C57BL/6 mice.

Materials and Methods

1. Materials

Eclipta prostrata extract was supplied by Damocosmetics Inc. (Seoul, Korea). The 1.0 kg of dried *Eclipta prostrata* plant was extracted with 10 L of 100% ethanol and then the ethanol extract was diluted with distilled water (DW) with a ratio of 1 : 1. The other chemicals were mainly purchased from Sigma Co. (St. Louis, MO, USA).

2. Animals

C57BL/6 mice (6 weeks old) were purchased from Joungang Lab Animal Co. (Seoul, Korea). The mice were acclimated for 1 week before the experiment. Five mice per group were housed in each plastic cage, at temperature of 22±2°C in lighting for 12 h of a light/dark cycle, with humidity of 50±10%. They were freely given with pelleted rodent diet and tap water. The animal experiment was conducted in compliance with "Guide for care and use of laboratory animals" of Chungbuk National University. The body weight was measured weekly for 3 weeks.

3. Experimental design and treatment

Anagen phase from the telogen phase of the hair cycle in the back skin of mice was induced by shaving. The dorsal areas of the mice were clipped with an electric clipper, then remaining hair was removed by a shaver with shaving foam as not to damage the skin. In the next morning, mice without visible scratches were selected, randomized and assigned to four experimental groups including distilled water (DW) as negative control (NC), 3% minoxidil (MXD) as positive control (PC), Tricomin as copper peptide (CP) and *Eclipta prostrata* extract (EE) as experimental treatment. Each material (0.2 mL) was sprayed on the back of mice and rubbed with a brush so that fluid could spread evenly and absorb easily. The topical application was performed daily for 3 weeks at the same time.

4. Quantification of hair re-growth activities

After mice were anesthetized lightly with diethyl ether, their photographs were taken at week 0, 1, 2, and 3. Analysis of hair

re-grown area and shaved area was performed by a digital image analysis system.

5. Histological examination

At week 3, all mice were sacrificed by diethyl ether and their dorsal skin was removed and trimmed according to the lines marking the areas. Dorsal skin was cut into two parts of which one was used for histological examination and another for enzyme assays. The dorsal skin was fixed in 10% neutral buffered formalin (pH 7.4).

6. Mast cell staining and counts

Histochemical staining with toluidine blue was performed to identify mast cells in tissues. Five μm paraffin sections were stained for 3 min with 0.1% w/v toluidine blue (pH 2.3) at room temperature. Mast cells were clearly identified by their granules which exhibit metachromatic violet/red purple staining. The number of mast cells (MC) was counted in the dermal and subcutaneous layer by toluidine blue staining (× 200).

7. Alkaline phosphatase and γ-glutamyl transpeptidase activity assay

The dorsal skin samples from C57BL/6 mice were homogenized in 0.1 M phosphate buffered saline (PBS, pH 7.4) to obtain a 20% (w/v) homogenate. The homogenate was centrifuged at 12,000 rpm, 4°C, for 20 min. The supernatant was collected and stored in a freezer at −70°C until required. Alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) activities were analyzed using an auto biochemistry analyzer.

8. Statistical analysis

Data were expressed as mean±standard deviation (SD). A one-way ANOVA analysis was conducted by using SPSS 17.0 for Windows (SPSS Inc., USA) statistics program, and a significant difference between the groups was carried out by using Duncan's multiple range test or student's t test at $p < 0.05$.

Results

1. Changes of the body weight

The animal body weights were increased gradually with increasing experimental period but there were no significant differences in the body weights among the experimental groups within the same period (Fig. 1).

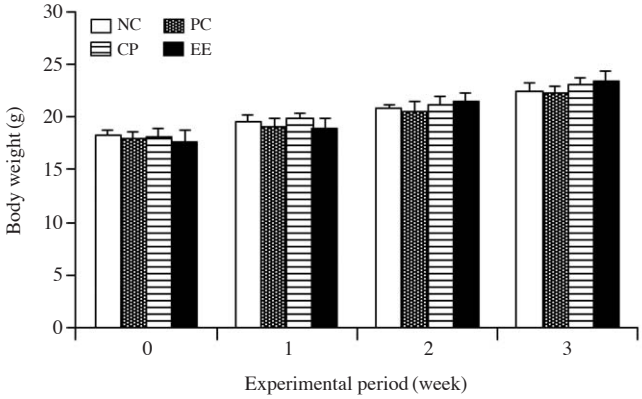

Fig. 1. Changes in body weight of C57BL/6 mice topically treated with test materials for 3 weeks. No significant differences were found among experimental groups. Negative control (NC), positive control (PC), copper peptide (CP) and *Eclipta prostrata* extract (EE). Data represent mean±SD (n=5).

2. Planimetric changes of hair re-growth

Test materials were applied on the back of mice daily for 3 weeks. During the experimental period, any visible abnormal change was not observed in the skin of all the groups. At week one the skin color was darkened slightly observed in PC, CP, and EE groups. At week two, all experimental groups showed hair re-growth. PC, CP and EE groups showed an evident promotion of the hair growth compared with NC group (Fig. 2). At week 3, the hair growth in PC and EE groups was almost completely accomplished compared with NC group (Fig. 2).

The ratios of hair re-grown area to shaved area were summarized in Table 1. At week 1, although there was change in skin color, hair regrowth in PC, CP, or EE group was not significantly different from NC group. However, at week 2 and 3, hair growth area of the mice in PC, CP and EE groups was significantly increased compared to that of NC group ($p < 0.05$).

3. Mast cell population

Mast cell is an important modulator of hair follicle cycling, especially during anagen development. PC, CP and EE groups significantly decreased mast cell population compared with NC group. EE group showed most decreased population of mast cells among experimental groups (Fig. 3).

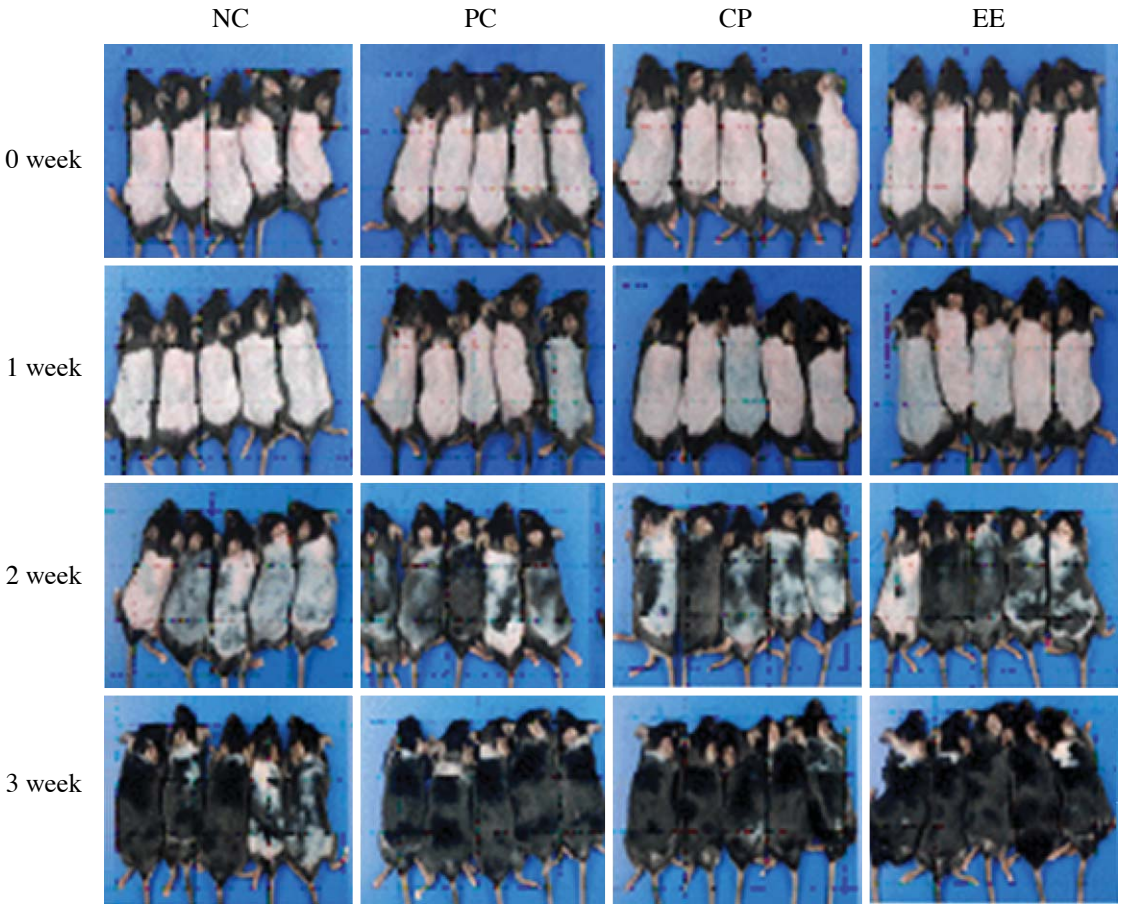

Fig. 2. Photographs of hair re-growth changes for 3 weeks in C57BL/6 mice. Normal control (NC), positive control (PC), copper peptide (CP) and *Eclipta prostrata* extract (EE). PC, CP and EE groups showed a remarkably fast hair growth compared with NC group.

Table 1. Quantitative measurement of hair re-growth in mice

Group	Hair re-growth area/shaved area (%)		
	1 week	2 week	3 week
NC	20.5 ± 2.4^{a}	56.9 ± 6.9^{a}	82.4 ± 6.1^{a}
PC	24.2 ± 1.7^{a}	88.9 ± 5.4^{b}	98.6 ± 3.2^{b}
CP	24.8 ± 2.0^{a}	82.7 ± 5.9^{b}	98.4 ± 2.7^{b}
EE	24.4 ± 1.9^{a}	86.2 ± 6.7^{b}	97.2 ± 3.6^{b}

The animals were treated topically with test materials daily for 3 weeks. Negative control (NC), positive control (PC), copper peptide (CP) and *Eclipta prostrata* extract (EE). Data represent mean ± SD (n=5). [ab]The means with different letters within the same column were significant different each other at ($p < 0.05$).

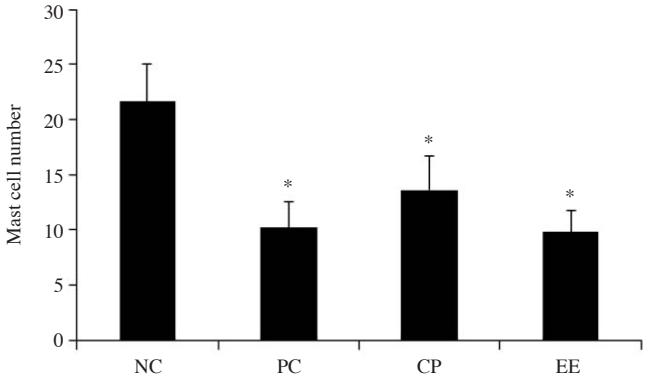

Fig. 3. Population of mast cells in the dorsal skin section of the mice. The animals were treated topically with test materials daily for 3 weeks. Negative control (NC), positive control (PC), copper peptide (CP) and *Eclipta prostrata* extract (EE). Data represent mean ± SD (n=5). *Significantly different from NC group at $p < 0.05$.

4. ALP and γ-GT activities

ALP activity, a biochemical indicator of angiogenesis in hair cycle, was determined after application of test materials for 3 weeks (Table 2). PC and EE groups showed significant high levels of ALP activity compared with NC or CP group ($p < 0.05$). The ALP activity of CP groups was also significantly different from NC group ($p < 0.05$) (Table 2).

γ-GT activities of PC and EE groups were also significantly higher than NC or CP group (Table 2). However, there was no

Table 2. Activities of alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) in the dorsal skin of mice

Group	Enzyme activities (IU/mg protein)	
	ALP	γ-GT
NC	225.6±45.2[a]	30.4±4.5[a]
PC	453.6±34.4[b]	52.3±5.7[b]
CP	353.3±21.8[c]	30.5±5.2[a]
EE	481.0±23.7[b]	48.8±6.4[b]

The animals were treated topically with test materials daily for 3 weeks. Negative control (NC), positive control (PC), copper peptide (CP) and *Eclipta prostrata* extract (EE). Data represent mean±SD (n=5). [abc]The means with different letter within the same column were significant different each other at ($p<0.05$).

significant difference between PC and EE groups or NC and CP groups (Table 2).

Discussion

In recent years, many people suffer from hair loss or hair thinning. Therefore, it is important to develop novel therapeutical agents that prevent hair loss and promote hair growth. Although it has not yet been incorporated into mainstream of medical care, due to limited scientific evidence and incomplete knowledge of the mechanisms involved, alternative medicine has become an increasingly attractive approach worldwide.

The aqueous extract of *Eclipta prostrata* has been reported to have anti-inflammatory, antixoidant and antitumor activities [12-15]. The traditional *Eclipta prostrata* extract are also used in the East Asia to promote hair growth and thickening. Copper peptide (GHK-Cu) and its analogues were found to stimulate hair growth. In some circumstances, the efficiency of synthetic analog of GHK-Cu was similar to that of 5% minoxidil [17]. Stimulating effect on hair growth by copper peptides might be due to improves microcirculation, reduces inflammation, and blocks dihydrotestosterone (DHT) and TGF-beta formation [16-18]. Unlike Propecia, it doesn't inhibit DHT to stop the cause of hair loss, and unlike Minoxidil, it doesn't use Potassium Channel openers to stimulate hair growth [16-18].

In present study, *Eclipta prostrata* plant was extracted with absolute alcohol and it was diluted with distilled water at a rato of 1 : 1. We demonstrated that the *Eclipta prostrata* extract had hair-growth promoting activities in our *in vivo* animal model. In macroscopic observation on hair re-growth at week 2 and 3, PC, CP, and EE groups increased significantly the hair regrowth compared with NC group ($p<0.05$). At the third week, the hair regrowth in PC, CP and EE groups was completely accomplished compared with NC group ($p<0.05$).

It is now widely accepted that hair follicle transformation during cycling is caused by alterations in the local signaling milieu. This local signal includes numerous growth factors, cytokines, hormones, and neuropeptides [20-22]. One of the hair cycle modulator is mast cells that are preferentially located around hair follicles [19,23]. Moreover, mast cells are the key mesenchymal compartment of the hair follicle that apparently secrete the main signals dictating hair follicle cycling [2,19,23]. The number of histochemically detectable mast cells reportedly increase substantially in the first week of anagen and drop in the following days until telogen [22,23]. Paus et al. have demonstrated that mast cells act via their secretory products as stimulators of anagen development in mice [23]. In this study, PC, CP and EE groups significantly decreased mast cell population at week 3 compared with NC group. It suggests that MXD, copper peptide or *Eclipta prostrata* extract delayed the catagen phase induction from anagen phase during experiment period.

ALP activities increase hair re-growth [24]. Although the precise mechanism is still unknown, it is thought that ALP activity was increased with the process of angiogenesis [24]. It suggests that the increase in ALP activity might be occurred as a result of thickening of the skin in the anagen phase [24]. In this study, PC and EE groups showed an increased ALP activity compared with NC group. This result may be associated with hair growth stimulation by increasing blood supply.

γ-GT is membrane-bounded enzyme functioning absorption or secretion of amino acid or peptide through cell membranes and glutathione metabolism [25]. It is known to promote cysteine moiety release from glutathione tripeptide and synthetize keratin by obtaining cysteine from hair follicles. It was reported to be present only in outer root sheath of hair follicles and be expressed a lot in cells whose proliferation and division are active and decrease when entering pause stage and increase at growth stage [26]. In this study, At week 3 of the experiment, activity of γ-GT appeared significantly higher in PC and EE groups than in NC groups. These results supported the strong hair growth effects by treatments of MXD and *Eclipta prostrata* extract.

In conclusion, as compared with MXD and copper peptide, *Eclipta prostrata* extract exhibited a strongly promoting effect on hair growth as based on its abilities to stimulate ALP and γ-GT activities and to decrease mast cell number. It is possible that the hair follicles were stimulated to enter into anagen by the *Eclipta prostrata* extract resulting in the shortening of time

required for hair re-growth. However, more research can be necessary to clarify the mechanisms of the extract compounds. Then it may lead to discover a new candidate for treatment of human alopecia.

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