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ORIGINAL ARTICLE

Efficacy of a Complex of 5-Aminolevulinic Acid and Glycyl-Histidyl-Lysine Peptide on Hair Growth

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Background: Pattern hair loss is a very common problem. Although effective therapeutics for the treatment of pattern hair loss have been used, novel therapeutic modalities are still required to enhance hair growth. **Objective:** We investigated the efficacy and safety of a complex (ALAVAX) of 5-aminolevulinic acid (5-ALA) and glycyl-histidyl-lysine (GHK) peptide for the treatment of pattern hair loss. **Methods:** Forty-five patients with male pattern hair loss were treated with ALAVAX 100 mg/ml (group A), ALAVAX 50 mg/ml (group B) or placebo (group C) once a day for 6 months. Total hair count, hair length, hair thickness, patient's assessment and adverse events were evaluated at month 1, 3, and 6. **Results:** An increase in hair count for 6 months was 52.6 ($p<0.05$) in group A, 71.5 ($p<0.05$) in group B, and 9.6 in group C. The ratio of changes in hair count between group B (2.38) and group C (1.21) at 6 months showed a statistically significant difference ($p<0.05$). The proportion above good satisfaction was higher in group A (26.7%) than in the other groups (group B: 14.3%, group C: 7.1%). There was no statistically significant difference in hair length and hair thickness among 3 groups at 6 months. There was no adverse event in 3 groups. **Conclusion:** Our study showed that a complex of 5-ALA and GHK peptide may be considered as one of the complementary agents for the treatment of male pattern hair loss. **(Ann Dermatol 28(4) 438~443, 2016)**

-Keywords-
5-aminolevulinic acid, Glycyl-histidyl-lysine peptide, Pattern hair loss

INTRODUCTION

Pattern hair loss of male and female is a very common problem that has been gradually increasing in incidence[1]. U.S. Food and Drug Administration-approved medications, newly developed medications and medical devices have been used for the treatment of pattern hair loss[2]. Minoxidil and finasteride have been approved as main therapeutics for the treatment of pattern hair loss. Dutasteride has been introduced as a new therapeutic agent to the patients with pattern hair loss[3]. Novel devices like the laser hair comb recently are used for the treatment of pattern hair loss[4]. However, other novel therapeutic modalities are still required to enhance treatment efficacy.
As a growth factor, the tripeptide-copper complex has been known to have effects on hair growth. It stimulates the proliferation of dermal fibroblasts and increases the production of vascular endothelial growth factor. L-alanyl-L-histidyl-L-lysine-Cu2+, one of the tripeptide-copper complex, promotes the growth of human hair follicles, as is caused by stimulation of the proliferation and the preclusion of the apoptosis of dermal papilla cells[5]. Another tripeptide-copper complex, glycyl-L-histidyl-L-lysine-Cu2+, is able to activate a plethora of wound remodeling-related processes, such as chemoattraction of wound repair cells, anti-inflammatory actions, increase in protein synthesis, and cellular proliferation[6]. In addition, it increases hair fol-

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licle size leading to improvement of hair loss[7]. Furthermore, it promotes the survival of basal stem cells in the skin. It has been said that copper-free glycyl-L-histidyl-L-lysine peptide can be used to obtain the effects of glycyl-L-histidyl-L-lysine-Cu2+[8].

In this study, a complex (ALAVAX) of 5-aminolevulinic acid (5-ALA) and glycyl-histidyl-lysine (GHK) peptide were used to investigate the efficacy and safety on hair growth in patients with male pattern hair loss.

MATERIALS AND METHODS

Preparation of ALAVAX

The peptide of the GHK was produced according to a chemical synthesis method known in the relevant art, especially, solid-phase synthesis techniques. ALAVAX was synthesized with 9-fluorenylmethoxycarbonyl (as an amino acid protector against aminolysis) by solid-phase peptide synthesis, linked with amino acid residues using N-hydroxybenzotriazol-N,N-di-cyclohexylcarbodiimide.

The peptide moiety, GHK was prepared with standard solid-phase peptide synthesis method. In detail, Fmoc-lysin–loaded resin was added into a vessel and swelled for 20 min in N-methylpyrrolidinone (NMP). Then, piperidine (20%) with NMP (80%) solution was mixed for 15 min. The mixture was washed three times with methylene chloride (MC) and NMP solvent, respectively. To activate next amino acid H, Fmoc-histidin amino acid (10 molar equivalent to Fmoc-lysin–loaded resin) was mixed with N-hydroxybenzotriazole (HOBt) (10 molar equivalent to a Fmoc-lysin–loaded resin), N,N-diisoproylcarbodiimide (DIC) (10 molar equivalent to Fmoc-lysin–loaded resin) into NMP. The dissolved Fmoc-histidin -OH, HOBt, DIC (in NMP) was mixed with the Fmoc-removed Fmoc-lysin–loaded resin. To complete synthesis GHK, same cycle with lysin was repeated to give tripeptide, GHK. The tripeptide, GHK on the resin was mixed with the dissolved aminolevulinic acid (10 molar equivalent to a Fmoc-lysin–loaded resin), HOBt (10 molar equivalent to a Fmoc-lysin–loaded resin), DIC (10 molar equivalent to Fmoc-lysin–loaded resin) in NMP. After the upling with aminolevulinic acid was finished, the mixture was washed with DCM (three times), NMP (three times), and dichloromethane (DCM) (three times) respectively. The reaction mixture was vacuumed and was washed three times with MC (three times), NMP (three times), and MC (three times) respectively. The synthesized ALAVAX was cleaved from resin using phenol, distillated water, ethandithiol, trifluoroacetic acid, and thioanisole. Purification of pure ALAVAX was conducted by reversed-phase high-performance liquid chromatography (RP-HPLC) with Bondapack C18 column (Waters system). The usal purity was more than 95%.

Matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS) assay (linear mode, α-cyano-4-hydroxy-cinnamic acid matrix) was performed to ensure the synthetic quality of ALAVAX (molecular weight and chemical structure). A MALDI-TOF MS instrument was used, along with an Axima curved field reflectron (Shimadzu/Kratos, Manchester, UK) instrument, in which a gauge pressure was set to 8.0×10^{-4} pascals, and the samples along with a matrix were put into a 96 square-well sample plate with linear modes to be analyzed. The matrix used together with the analysis has used cinnamic acid (a-cyano-4-hydroxycinnamic acid; CAS Number, 28166-41-8).

Concentration of ALAVAX

ALAVAX were dissolved in pure water to a concentration of 100 mg/ml or 50 mg/ml to be used.

Study design for clinical evaluation of ALAVAX

This was a randomized, double blind, 6-month prospective study conducted at department of dermatology, Kyungpook National University Hospital. Eligible men were aged 20 to 60 years with male pattern hair loss classified as type II～V according to the Norwood-Hamilton classification. Exclusion criteria included endocrine disorders, immune system disorders, systemic infectious disorders, recent treatment for hair loss within three months, surgical treatment for hair loss and scalp disorders. A screening period (up to 1 month) was followed by 6-month period of treatment. We measured total hair count, hair length and hair thickness at the same frontal scalp site using a plastic headhand connected with a tapeline at the center of band. In addition, we shaved the frontal scalp site 1 cm in diameter to double-check the location and to make it easy to measure hair length. Patients were randomized to ALAVAX 100 mg/ml (group A, n=15), ALAVAX 50 mg/ml (group B, n=14) or placebo (group C, n=14). All patients sprayed the assigned spray on the scalp once a day before sleep at home. Investigators and patients were blinded to treatment allocation until study completion. The study protocol was approved by the Institutional Review Board of Kyungpook National University Hospital (IRB no. KNUH 2013-08-023). Written informed consent forms were given by patients before this study.

Assessment

1) Hair count

Changes in hair count within a 1-cm-diameter circle at the frontal scalp of each group at month 1, 3 and 6 comparing with baseline were evaluated using a phototrcihogram technique (Folliscope; LeadM Corp., Seoul, Korea). In addition, the ratio of changes in hair count was evaluated at month 1, 3 and 6 comparing with baseline.

2) Hair length and thickness

Changes in hair length and hair thickness at the frontal scalp of each group from baseline to 6 months were measured.

3) Patient self-assessment

Subjects' perceived change in hair growth and satisfaction at each group were assessed using the following 5-point scale at 6 months: (4) excellent, 75%～100% improvement; (3) good, 50%～74% improvement; (2) fair, 25%～49% improvement; (1) poor, 0%～24% improvement; and (0) bad, 25%～1% aggravation.

4) Adverse events

Adverse events were monitored during this study.

Statistical analyses

Analysis of variance (ANOVA) (the difference among 3 groups at 6 months), and repeated-measure ANOVA (the difference between baseline and each visit at each group) were used for the statistical analysis with PASW Statistics for Windows ver. 18.0 (SPSS Inc., Chicago, IL, USA). A p-value of <0.05 was considered statistically significant.

RESULTS

Confirmation of ALAVAX structure by MALDI-TOF MS

ALAVAX was synthesized to combine glysyl-histidyl-lysine as a peptide to support physiological activity and 5-ALA as a phytochemical agent. A MALDI-TOF MS assay (linear mode, α-cyano-4-hydroxy-cinnamic acid matrix) was conducted to confirm the molecular weight and chemical structure of ALAVAX (Fig. 1). The Chemical formula of ALAVAX is $C_{19}H_{31}N_7O_6$ and its molecular weight is 453.23.

Subject demographics at baseline

Baseline characteristics of subjects were similar across treatment groups (Table 1). All subjects completed this study. All 45 patients who completed the study evaluation were male. Their average age was 42.2 years (range, 25～60 years). According to a degree of hair loss, 45 patients were divided into II (n=13), III (n=18), IIIa (n=4), IV (n=6) and V (n=4). In the group A, 15 patients were divided into II (n=5), III (n=4), IIIa (n=2), IV (n=1), and V

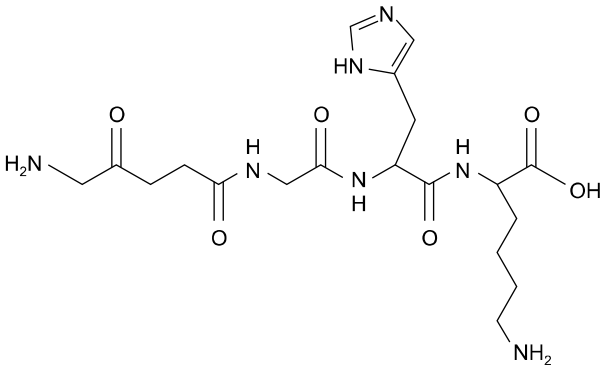

Fig. 1. The structure of ALAVAX composed of 5-aminolevulinic acid and glycyl-histidyl-lysine peptide.

Table 1. Subjects' baseline demographic characteristics

Variable	ALAVAX 100 mg/ml (group A)	ALAVAX 50 mg/ml (group B)	Placebo (group C)
No. patients	15	15	15
Age (yr)	43.6±9.5	39.3±10.0	43.7±11.3
Stage of male pattern hair loss			
II	5 (33.3)	3 (20.0)	5 (33.3)
III	4 (26.7)	9 (60.0)	5 (33.3)
IIIa	2 (13.3)	1 (6.7)	1 (6.7)
IV	1 (6.7)	2 (13.3)	3 (20.0)
V	3 (20.0)	0	1 (6.7)
Hair count (number/1-cm-diameter area)	108.83±31.49	89.54±39.42	120.33±30.53

Values are presented as number only, mean±standard deviation, or number (%).

(n=3). In the group B, 15 patients were divided into II (n=3), III (n=9), IIIa (n=1), and IV (n=2). In the group C, 15 patients were divided into II (n=5), III (n=5), IIIa (n=1), IV (n=3), and V (n=1). Baseline hair count in the circle 1 cm in diameter was 108.83 in the group A, 89.54 in the group B, and 120.33 in the group C. There was no significant difference in the degree of hair loss and hair count at baseline among 3 groups.

Hair count (1 cm in diameter)

Hair count was measured every visit. There was an increase to some degree in hair count at every visit time at each group. The increase in hair count for 1 month was 10.5±27.9 in group A, 11.3±19.2 in group B, and −3.6±17.1 in group C. The increase in hair count for 3 months was 54.5±37.2 in group A, 37.9±52.1 in group B, and 24.9±40.3 in group C. The increase in hair count for 6 months was 52.6±45.7 in group A ($p<0.05$), 71.5±44.9 in group B ($p<0.05$), and 9.6±45.1 in group C (Fig. 2A). The ratio of changes in hair count between group B (n=2.38) and group C (n=1.21) only at 6 months showed a statistically significant difference ($p<0.05$; Fig. 2B).

Hair length and hair thickness

Change in hair length for 1 month was 0.96±0.17 cm in the group A, 0.88±0.2 cm in the group B, and 0.88±0.32 cm in the group C. Change in hair length for 3 month was 3.26±0.7 cm in the group A, 2.88±0.2 cm in the group B, and 2.57±0.57 cm in the group C. Change in hair length for 6 months was 6.56±1.63 cm in the group A, 6.47±2.06 cm in the group B, and 5.06±2.31 cm in the group C (Fig. 3). There was no statistically significant difference among 3 groups at each visit. Hair thickness for 1 month was changed from 0.029 cm to 0.0025 cm (−0.004±0.008 cm) in the group A, from 0.040 cm to 0.033 cm (−0.007±0.016 cm) in the group B, and 0.029 cm to 0.026 cm (−0.003±0.007 cm) in the group C. Hair thickness for 3 months was changed from 0.029 cm to 0.023 cm (−0.006±0.008 cm) in the group A, from 0.040 cm to 0.027 cm (−0.013±0.018 cm) in the group B, and 0.029 cm to 0.021 cm (−0.008±0.010 cm) in the group C. Hair thickness for 6 months was changed from 0.029 cm to 0.029 cm (0±0.008 cm) in the group A, from 0.040 cm to 0.030 cm (−0.01±0.011 cm) in the group B, and 0.029 cm to 0.023 cm (−0.006±0.010 cm) in the group C (Fig. 4). There was no statistically significant difference between baseline and each visit at each group. In addition, there was no statistically significant difference among 3 groups at each visit.

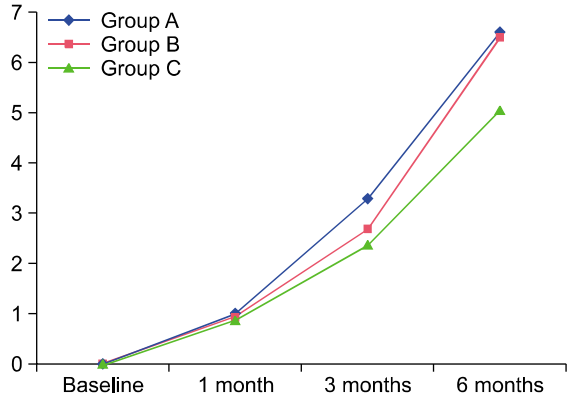

Fig. 3. Hair length. There was no statistically significant difference among 3 groups at each visit.

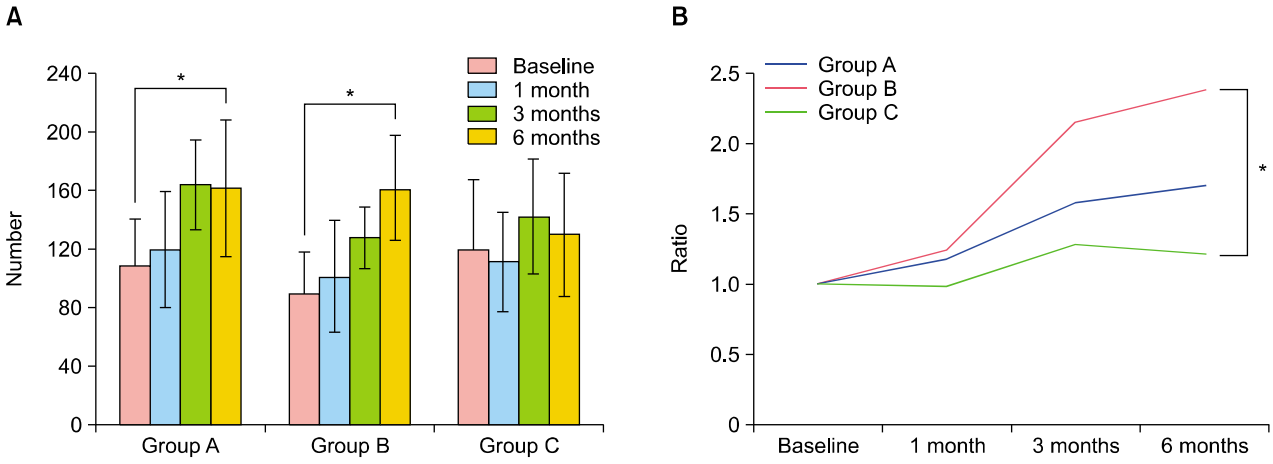

Fig. 2. Hair count. (A) An increase in hair count for 6 months was 52.6 in group A (*$p<0.05$), 71.5 in group B (*$p<0.05$), and 9.6 in group C. (B) The ratio of changes in hair count between group B (n=2.38) and group C (n=1.21) only at 6 months showed a statistically significant difference (*$p<0.05$).

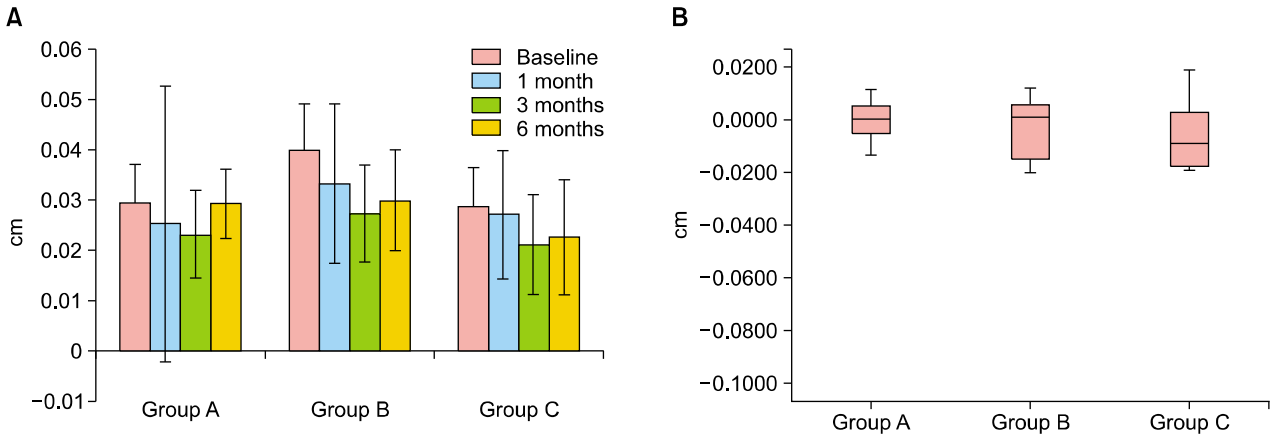

Fig. 4. Hair thickness. (A) There was no statistically significant difference in hair thickness between baseline and each visit at each group. (B) There was no statistically significant difference in the change of hair thickness among 3 groups at 6 months.

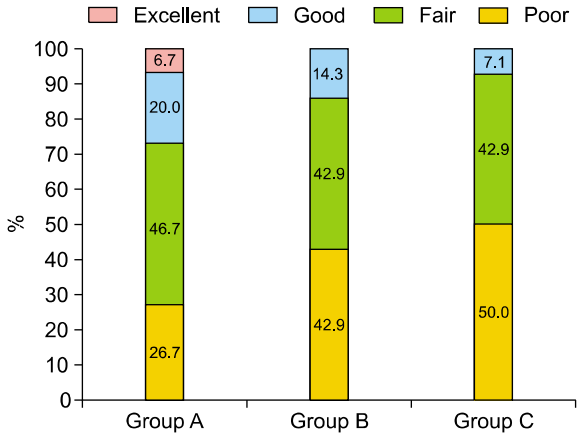

Fig. 5. Patient's satisfaction. The proportion of good and excellent satisfaction was higher in the group A than in the other groups.

Patient's satisfaction

Patient's satisfaction assessment at 6 months was as follows: poor (26.7%), fair (46.7%), good (20.0%), and excellent (6.7%) in the group A, poor (42.9%), fair (42.9%), and good (14.3%) in the group B, and poor (50.0%), fair (42.9%), and good (7.1%) in the group C. The proportion above good satisfaction was higher in group A (26.7%) than in the other groups (group B: 14.3%, group C: 7.1%) (Fig. 5).

Adverse events

There was no adverse event in 3 groups.

DISCUSSION

5-ALA is a photosensitizer precursor that is transformed by cells into protoporphyrin IXa, which can be activated by light. Therefore, 5-ALA has been used for photodynamic therapy for a variety of dermatologic disorders like tumorous skin conditions and inflammatory diseases[9-11]. Photodynamic therapy is involving the administration of a photosensitizer or a precursor followed by its activation with light to generate a therapeutic effect. It was reported that a complex of 5-ALA and iron ion can also stimulate murine hair growth *in vivo* independent of epithelial and mesenchymal cells. So, this complex may have the potential to become a beneficial new treatment for alopecia[12]. On the contrary, there has been a report by Bissonnette et al.[13] that photodynamic therapy was ineffective in the treatment of alopecia areata.

The tripeptide-copper complex has been known to have effects on hair growth. L-alanyl-L-histidyl-L-lysine-Cu2+ and glycyl-L-histidyl-L-lysine-Cu2+ are tripeptide-copper complexes to promotes the growth of human hair follicles[5,8]. In addition, it was revealed that copper-free glycyl-L-histidyl-L-lysine peptide can be used to obtain the effects of glycyl-L-histidyl-L-lysine-Cu2+[8]. On the basis of these reports about photodynamic therapy using 5-ALA and tripeptide-copper complex, we made a complex of 5-ALA and GHK called ALAVAX. In addition, 5-ALA has drawbacks, such as low skin penetration and skin toxicity by ultraviolet, but peptides are photoprotective and skin-regenerative. So ALAVAX was developed to improve the drawbacks of 5-ALA and to add the usefulness of the peptides into the treatment of pattern hair loss. ALAVAX was proven through MALDI-TOF MS. In this study efficacy and safety of ALAVAX on the male pattern hair loss were evaluated through clinical study.

In this clinical study, it was suggested that ALAVAX is a complementary agent for the treatment of pattern hair loss

through the investigation using hair count, hair length, hair thickness and patient's satisfactory assessment. An increase in hair count for 6 months showed statistically significant difference in group A and group B. In addition, the ratio of changes in hair count between group B and group C at 6 months showed a statistically significant difference. Furthermore, there was no adverse events occurred after the treatment of patients with ALAVAX.

The tripeptide-copper complex has been known to have effects on hair growth through various mechanisms including dermal fibroblast stimulation and increased expression of vascular endothelial growth factor. It is also known to decrease the secretion of transforming growth factor-β1 by dermal fibroblasts. In addition, it reduces the number of apoptotic dermal papilla cells, showing the elevated ratio of Bcl-2/Bax and the reduced levels of the cleaved forms of caspase-3[5]. Surely, further studies are needed to evaluate the mechanism of ALAVAX for hair growth.

In conclusion, a complex of 5-ALA and GHK may be considered as one of the safe and complementary agents for the treatment of male pattern hair loss.

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