A Clinical Evaluation of an Anti-Aging Cosmetics Containing Functional Peptides and an Epidermal Penetrating Peptide

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Abstract: ***Despite considerable interest and research on the efficacy of peptides, clinical and academic studies are yet to demonstrate that their components reduce wrinkles. The purpose of the present study was to investigate the effects of anti-aging peptides; copper glycine-histidine-lysine (Cu-GHK), glycine-histidine-lysine (GHK), palmitoyl oligopeptide-1 (Pal-GHK), palmitoyl pentapeptide-4 (Pal-KTTKS), palmitoyl tetrapeptide-3 (Pal-GQPR), acetyl hexapeptide and a skin penetrating peptide (arginine oligomer peptide, R6) on human skin, by analyzing the effects of a cosmetic formulation containing this substance in terms of skin wrinkle (Antera 3D), skin brightness (Chromameter CR400) and skin melanin measurement (Mexameter MX18). A clinical efficacy test was conducted on 23 adult women aged 30 to 60 years and 3 subjects dropped out of the study so a total of 20 subjects were tested. Wrinkles of the eyes and melanin pigments of the skin significantly decreased, and skin brightness significantly increased after 4 weeks of using the test product ($p<0.05$). No side effects, such as erythema, edema, scaling, itching, tingling, burning sensation, rashes, and thorns, were observed. Therefore, the test products (Biotoc Regen Cosmetics) containing wrinkle improving peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, acetyl hexapeptide) and a skin penetrating peptide (arginine oligomer, R6) are considered to have beneficial effects on improvement of skin wrinkle, skin brightness, skin melanin of 4 weeks use.***

Index Terms: Anti-wrinkle Cosmetics, Epidermal Penetrating Peptide, Clinical Evaluation, Functional Peptides.

I. INTRODUCTION

Skin aging is divided into intrinsic aging over time and photogenic aging, which occurs when degenerative changes occur in the exposure area of the sun's combined with natural aging. In the intrinsic aging process, collagen and dermis in the upper layer of the dermis are regressed, resulting in reduced elasticity, wrinkles, and wrinkles are worsened due to reduction of the subcutaneous fat layer. The characteristics of photoaged skin are dry skin, rough, coarse and deep wrinkles, elastic fibrosis accumulates, and skin becomes thick and loosens like leather [1].

Many functional ingredients have been developed in the cosmetics field to improve and prevent such wrinkles. Among the antioxidants that have been developed so far, representative examples of highly-recognized and highly effective consumers are vitamin A, which is effective in collagen synthesis and inhibition of degradation. In addition, vitamin C, which is essential for collagen synthesis and is effective for pigmentation, and AHA, which improves the skin by promoting cell activity and exfoliation, are also widely used. However, it has been pointed out that most of the skin improvement efficacy described above have problems with safety and stability to be taken as cosmetic raw materials [2]. In contrast, anti-wrinkle cosmetics containing peptide components, which have recently been improved in safety and stability, have been evaluated as effectively improving symptoms of skin caused by aging [3-4]. The commonly used peptides include copper glycine-histidine-lysine (Cu -GHK), glycine-histidine-lysine (GHK), palmitoyl oligopeptide-1 (Pal-GHK), palmitoyl pentapeptide-4 (Pal-KTTKS), palmitoyl tetrapeptide-3 (Pal-GQPR), acetyl hexapeptide, etc. [5-6]. However, the outermost layer of the epidermis, the stratum corneum, hinders the skin penetration of compounds, especially hydrophilic peptides [7].

Recently, drug delivery using cell-penetrating peptides (CPPs) has been researched for the intracellular delivery of many therapeutic molecules such as siRNA [8-9], protein [10] and peptides [11]. However, use of CPPs as the transdermal delivery system is still in its infancy. A few studies have reported that CPPs such as trans-activator of transcription (TAT) [12], polyarginine [13], meganin [14], penetratin [15] can enhance the transdermal delivery of various therapeutic molecules like siRNA, cyclosporine A, insulin, etc.

Short oligomers of arginine efficiently cross biological membranes and are more efficient than TAT [16-17] and the third helix of Drosophila antennapedia [18,20,21,22,23,24,25,26,27]. Short arginine oligomers facilitated transport across the cutaneous barrier when applied topically to either mouse or human skin [13]. The results of these studies raise hope for short arginine oligomers to be used as a transdermal delivery system.

It was recently reported by the present author that transdermal absorption was enhanced when functional peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS,

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Pal-GQPR, acetyl hexapeptide) are contained with a short arginine oligomer, R6 [19]. There are few clinical studies of skin aging that contain both functional peptides and skin penetrating peptides. In this study, we investigated clinical evaluation of skin wrinkle, brightness and melanin index when functional peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, acetyl hexapeptide) and skin penetrating peptides (R6) are contained at the same time and examined feasibility of anti-aging functional cosmetics.

II. MATERIAL AND METHODS

A. Test Formulations

The cosmetics used in this test are "Biotoc Regen" manufactured by Dermafirm Co., Ltd., which consists of 5 products (ampoule, serum, cream, peel, and apple-zone patch). The main ingredients contained 500 ppm (0.05%) of each of 6 wrinkle-improving peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, and acetyl Hexapeptide), and 100 ppm (0.01%) of epidermal penetrating peptide (R6), respectively. The other ingredients contained 99.69% of emulsifier, oil, humectants, fragrance and deionized water. 6 wrinkle-improving peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, and acetyl Hexapeptide) and epidermal penetrating peptide (R6 are manufactured from Dermafirm Co., Ltd., in Korea and have a purity of at least 99.0%, respectively.

B. Study protocol

In this study, KC Skin Research Center conducted the body efficacy evaluation according to the tenets of the Declaration of Helsinki and complied with the Guideline of Bioethics and Safety Act by the Ministry of Health and Welfare. The study was approved by the Institutional Review Board of KC Skin Research Center Co., Ltd., in Korea (KC-IRB-016).

23 healthy female subjects who live in Seoul and the metropolitan area over 30 years of age and who did not have acute or chronic physical illnesses including skin diseases participated in this study after having given their written informed consent. 3 of them dropped out and finally 20 females were selected as subjects.

Women who were pregnant or breastfeeding or who did not consent to the protocols prescribed by the protocol were excluded. Also, women who did not agree with the methods of contraception, cosmetics, and medicines, or women with severe or allergic reactions to daily light exposure or skin products containing steroids were excluded. Women who had used similar cosmetics or medicines in the study area within 3 months prior to the start of the study were also excluded from the study. A woman who could not carry out a human body test, who could not participate in the test due to a skin disease during the human body application test, who had a serious adverse reaction after using the product, or those who violated the prescribed methods of application or schedules were dropped from the study. In addition, individuals were dropped from the study following withdrawal of consent, failure of follow-up, and inadequate compliance.

Those who satisfied the criteria for selection as subjects and those who did not satisfy criteria were identified using a homogeneity test to determine individuals who were relatively similar based on their basic living environments, skin conditions, skin care, and cosmetics use.

In vivo test was carried out during the period of four weeks with test subjects. A total of twenty female subjects were selected after homogeneity test. The individual instructions over how to use each product are as follows. First, subjects were instructed to use "Biotoc Regen" ampoule, serum and cream individually for four weeks, by applying a proper amount of the product onto their faces and patting the contents to be absorbed, twice a day (morning and night). Second, the subjects were instructed to use "Biotoc Regen" peel individually for four weeks, once a week (evening). Third, subjects were instructed to apply "Biotoc Regen" apple-zone patch onto the area around the eyes and onto the forehead for four weeks, three times a week (evening).

The evaluation of adverse events was carried out by the investigator during every visit in the course of the skin condition measurement and analysis activities. Erythema, edema, scaling, itching, stinging, burning sensation, tightness, prickling, and other abnormalities were evaluated. Severities were classified as weak, moderate, or severe. The test was recorded in the case record by checking whether the test was stopped or omitted. If a subject was no longer able to participate in the examinations even though it was not a visiting day, they would have to fill out the "Attendance Abandonment Agreement" and enclose their signature.

C. Facial skin measurement and analysis

To minimize errors in the measurement of the skin conditions of the subjects, the subjects first rinsed their skin with the same cleanser and then stabilized their skin for 30 minutes in a constant temperature and humidity chamber (temperature: 22 ± 1°C, humidity: 4 5% ± 5%). Skin wrinkles, skin brightness, and changes in skin melanin were then measured. Measuring instruments used were the most commonly used instruments for skin wrinkles (Antera 3D, Miravex Ltd, lreland), skin brightness (Chromameter CR400, KONICA MINOLTA Inc, Japan), and melanin (Mexameter MX18, Courage Inc, Germany).

The wrinkles of the skin of the subjects were analyzed as follows. The subjects were asked to use each test product for 4 weeks. Measurements were obtained before the test (0 weeks), 2 weeks after use, and after the completion of use (4 weeks after use). The extent of changes in skin wrinkles was determined. Using Antera 3D, an area of the eye was photographed before using the test product. 2 and 4 weeks after using the product, the subject's eye area was photographed using Antera 3D, and the effect on wrinkles effect was examined. A questionnaire, which is a subjective method of evaluation, was also administered.

The subjects were asked to analyze changes in facial brightness. The subjects were instructed to use each test product for 4 weeks and the degree of facial brightness was checked using a Chromameter CR400. The selected facial area was photographed using a Chromameter CR400T before using the test product, 2 weeks after using a product, and 4 weeks after using a product. The evaluation of the effect of the product on facial brightness was also carried out by administering a questionnaire, which is a subjective method of evaluation.

Melanin changes in hyperpigmented lesions of the subjects were analyzed. The subjects could use each test product for 4 weeks. Melanin pigmentation was checked using a Mexameter before use (0 weeks), 2 weeks after use, and 4 weeks after use. Using a Mexameter, the area of the face with hyperpigmentation was photographed before using the test product, 2 weeks after using the product, and 4 weeks after using the product, and the wrinkle improvement effect was evaluated by conducting a questionnaire evaluation, which is a subjective method of evaluating the subject.

D. Statistical analysis

Statistical analysis of the data in the present study was conducted in IBM SPSS Statistics 23.0 for Windows (IBM-Armonk, NY, USA). A paired t-test was used to analyze the significant changes in the results for various skin characteristics. Differences were accepted as statistically significant at $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Selection of the subject

The mean age of the subjects who participated in the present study was 47.565 ± 3.75 years old. The general characteristics and living environment indices for the homogeneity tests were gender, age, residence, sleeping time, and life stress. Basic skin condition indices included wrinkles, skin brightness, and enhancement of melanin. For the 20 selected subjects, the faces, eye wrinkles, and hyperpigmented areas were assigned to the tested sites based on product type. homogeneity tests

B. Eye winkle change of the subject

The changes in the eye wrinkles were measured 3 times, including before application (0 weeks), after 2 weeks, and after 4 weeks. Measurement of facial wrinkles after use of the test products showed a decrease to 15.035 ± 5.498 after 2 weeks and 14.830 ± 5.576 after 4 weeks, from 15.750 ± 5.218 (Table 1, $p<0.05$).

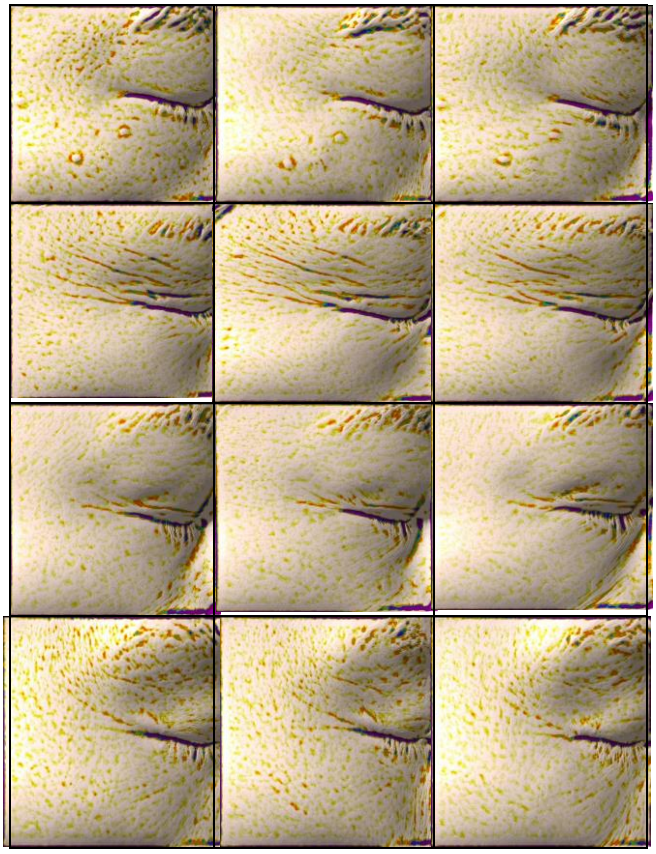
To analyze the rates of improvements, when the degrees of change on the 2nd and 4th week were 100% based on the degree of facial flushing before the use of the products, the degree of wrinkles decreased by 5.245% after 2 weeks, 6.937% after 4 weeks (Table 1). In other words, the analysis of wrinkles after using the test product revealed a statistically significant decrease 2 weeks after using the product and 4 weeks after using the product ($p<0.05$). Therefore, it was concluded that using the test product facilitated the improvement of wrinkles. Figure 1 shows clinical pictures of wrinkle improvement of major subjects using Antera 3D.

Table 1. Results of skin wrinkle measurement

Time	Average ± STD (Overall size)	Improvement rate[a] (%)	Probability[b] (*p* value)
product use	15.750 ± 5.218	-	-
After 2 weeks	15.035 ± 5.498	-5.245	0.016*
After 4 weeks	14.830 ±5.576	-6.937	0.001*

• Improvement rate[a] (%) = [(After product use –Before product use) / Before product use] x 100

• Probability[b] (*p value*) *: $p<0.05$ by Paired samples T-test



(a) Before product use (b) After 2 weeks (c) After 4 weeks

Figure 1. Photographs of major subjects using Antera 3D.

C. Analysis of changes in brightness of complexion

The changes in the skin brightness were measured 3 times, including before application (0 weeks), after changes in the brightness of complexion were measured three times, before using the products (week 0), two weeks after using them (week 2), and four weeks after using them (week 4). Brightness on face were measured after using the products. The results showed that the brightness increased from 64.369 ± 2.626 to 64.687 ± 2.578 after 2 weeks and to 64.698 ± 2.693 after 4 weeks (Table 2, $p<0.05$).

The degrees of the improvement in the second and fourth weeks were calculated as percentage to analyze improvement rate for each week, by setting the brightness of complexion after using test products as 100%. Brightness increased by 0.504% after two weeks, while brightness increased by 0.510% after four weeks (Table 2). Skin brightness were measured after using the test products.

According to the results, wrinkles around the eyes showed a statistically significant decrease 4 weeks after using the product, compared to the measurement before using the product (p<0.05). From these results, it was found that using the test products has a positive effect on the brightness of complexion.

Table 2. Results of skin brightness measurement

Time	Average ± STD (**L** value)	Improvement rate[a] (%)	Probability[b] (*p* value)
product use	64.369 ± 2.626	-	-
After 2 weeks	64.687 ± 2.578	0.504	0.089
After 4 weeks	64.698 ± 2.693	0.510	0.009*

• Improvement rate[a] (%) = [(After product use –Before product use) / Before product use] x 100

• Probability[b] (*p value*) *: p<0.05 by Paired samples T-test

D. Analysis of changes in facial pigmentation

Changes in facial pigmentation were assessed 3 times, including before using the test products (0 weeks), after 2 weeks (2), and after 4 weeks (4) of using the test products. Using the test products resulted in a decrease of the index from 180.967 ± 44.313 before use, to 174.667 ± 41.426 after 2 weeks of use and to 173.200 ± 41.212 after four weeks.

Analysis of the rate of improvement in facial pigmentation revealed that the percentage of change between the 2nd and 4th weeks was 100% and the extent of wrinkles in the eyes decreased 3.304% after 2 weeks and 4.180% after 4 weeks (Table 3). Therefore, analysis of facial pigmentation after using the test product revealed a statistically significant decrease after 2 weeks and after 4 weeks of using the products (p<0.05). There, it was concluded that using the test products improved facial pigmentation.

Table 3. Results of skin melanin measurement

Time	Average ± STD (Melanin Index)	Improvement rate[a] (%)	Probability[b] (*p* value)
product use	180.967 ± 44.313	-	-
After 2 weeks	174.667 ± 41.426	-3.304	0.000*
After 4 weeks	173.200 ± 41.212	-4.180	0.000*

• Improvement rate[a] (%) = [(After product use –Before product use) / Before product use] x 100

• Probability[b] (*p value*) *: p<0.05 by Paired samples T-test

E. Evaluation of skin adverse reactions

In the test subjects, the presence of adverse skin reactions such as erythema, edema, scaling, itching, stinging, burning, tightness, ting (rickets, swelling, scurvy, itching, aching, burning, stiffness, tingling) among others was investigated every time subject presented themselves for analysis. No specific skin adverse events were observed in all subjects participating that participated in the present study (Table 4).

Table 4. Assessing skin adverse events

Time	Erythema	Edema	Scaling	Itching
After 2 weeks	-	-	-	-
After 4 weeks	-	-	-	-

Time	Stinging	Burning	Tightness	Prickling
After 2 weeks	-	-	-	-
After 4 weeks	-	-	-	-

Step=1: Weak, 2: Medium, 3: Severe

IV. CONCLUSIONS

This study focused on verifying whether the use of a cosmetics containing 6 functional peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, and acetyl Hexapeptide) and epidermal penetrating peptide (R6) has a positive effect on facial skin. In vivo test was carried out during the period of four weeks with test subjects. A total of twenty female subjects were selected after homogeneity test. The individual instructions over how to use each product are as follows. First, subjects were instructed to use Biotoc Regen ampoule, serum and cream individually for four weeks, by applying a proper amount of the product onto their faces and patting the contents to be absorbed, twice a day (morning and night). Second, the subjects were instructed to use Biotoc Regen peel individually for four weeks, once a week (evening). Third, subjects were instructed to apply Biotoc Regen apple-zone patch onto the area around the eyes and onto the forehead for four weeks, three times a week (evening). Subjects were asked to wash with their facial skin the same cleanser and after 30 minutes of stabilization in an indoor environment maintained at constant temperature and humidity, 3 kinds of skin testers (Antera 3D, Miravex Ltd, lreland, Chromameter CR400, KONICA MINOLTA Inc, Japan, Mexameter MX18, Courage Inc, Germany) were used to evaluate different facial characteristics.

When the test products were applied, changes of the eye wrinkles, facial brightness and the changes in pigmentation were analyzed by the parking lot and it was observed that wrinkles of the eyes decreased by 5.24% after 2 weeks and to 6.93% after 4 weeks. The test product had a very significant effect on the improvement of eye wrinkles. In the case of facial brightness, it increased to 0.50% in the two weeks after using the products use and 0.51% 4 weeks after using them. The test product had a very significant positive effect on facial brightness. Finally, in the case of facial pigmentation, it decreased to 3.304% at the 2 weeks use and to 4.18% at the 4 weeks after using them.

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The test product also had a very significant positive effect on facial pigmentation.

Therefore, the test products containing wrinkle improving peptides (Cu-GHK, GHK, Pal-GHK, Pal-KTTKS, Pal-GQPR, acetyl hexapeptide) and a skin penetrating peptide (R6) are considered to have beneficial effects on improvement of skin wrinkle, skin brightness, skin melanin of 4 weeks use.

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