A Clinical Study on Anti-wrinkle Efficacy of a Cosmetics Containing Oligoarginine Conjugation of Palmitoyl-GHK peptide for Skin Penetrating

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Abstract: In this study, Arg 4 (R4) was conjugated with Pal-GHK to get palmitoyl heptapeptide, Pal-GHK-R4 and wrinkle improvement clinical trials of cosmetics containing Pal-GHK-R4 were conducted. As a result of total 21 subjects used the test products 4 weeks use on the face area; 1) measurement results of crow's feet after 2 and 4 weeks of test products use, analysis value of crow's feet statistically significantly decreased after 2 and 4 weeks of test products use compared to before use of products ($p<0.05$); 2) measurement results of skin moisturization after 2 and 4 weeks test products use, measured value of skin moisture content statistically significantly increased after 2 and 4 weeks use compared to before use of products ($p<0.05$); 3) measurement results of TEWL after 2 and 4 weeks use, measured value of TEWL statistically significantly decreased after 2 and 4 weeks use compared to before use of product($p<0.05$); 4) there was no skin adverse event reported after using the products during the study period. Therefore, the test products containing both wrinkle improving and skin penetrating peptides, Pal-GHK-R4 are considered to have beneficial effects on improvement of skin wrinkle, moisturization, transepidermal water loss(TEWL) after 2 and 4 weeks use of test product.

Keywords: Cell penetrating peptides, transdermal delivery, conjugated liposomes, Skin permeation

1. Introduction

The aging population structure is not limited to developed countries, but is a phenomenon that is being carried out worldwide including developing countries. The increase of the elderly causes various social problems, but also leads to the growth of the senior industry [1]. In the cosmetics market, senior generation women have emerged as a high consumer and a high purchasing power, and they are a big part of important consumers [2]. As the number of senior generation women who want young and resilient skin increases, the demand for functional cosmetics that improve wrinkles with excellent efficacy has increased, and the cosmetics industry is actively pursuing research on this [3].

Recently, there has been a growing interest in using peptides, an amino acid polymer, as a functional material for wrinkle improvement in cosmetics. Peptides have excellent physiological activity, are safe because they are in vivo, are stable in light and air, and are smaller than proteins, so they can be synthesized, so they are highly likely to be used as cosmetic materials [4-5]. GHK (glycyl-histidyl-lysine), which was first isolated from human serum and known as a hepatocellular growth factor, is a typical wrinkle-improving functional peptide. GHK exists in the blood about 200 mg/mL at the age of 20, but it decreases as aging progresses and is about 80 mg/mL at the age of 60. This increases the synthesis of extracellular matrix (ECM) such as collagen, elastin, and glycosaminoglycans (GAGs), which can cause skin regeneration and wound healing. GHK is commonly present in the form of GHK-Cu with high affinity to copper ions, and Pal-GHK, which is bound with palmitate, a fatty acid, is also present to enhance the permeability and stability of GHK. These GHK, GHK-Cu, and Pal-GHK are popular as functional cosmetic materials for wrinkle improvement. However, the problem is that the peptide has difficulty in penetrating the stratum corneum due to its hydrophilic properties, making it difficult to actually exhibit efficacy in the skin [6-11-19].

On the other hand, there have been active studies to deliver hydrophilic macromolecules without destroying the cell membrane and without showing cytotoxicity for effective intracellular drug delivery. In this regard, many studies on cell penetrating peptides (CPPs) have been conducted. Cell penetrating peptides are generally composed of 10-30 short amino acids, and most contain a large number of basic amino acids, lysine (K) and arginine (R). TAT peptides derived from HIV-1 are one of the most studied cell penetrating peptides. TAT peptides are composed of 86 amino acids, among which the sequence of RKKKRRQRRR is known to be the minimum site for cell penetrating peptides. The experiments that substituted or deleted amino acid sequences of TAT peptides revealed that lysine (K) and arginine (R) play a key role in the intracellular permeability of TAT peptides as cell penetrating peptides [12-16]. Furthermore, since both the cell membrane and intercellular lipids have structural similarity in the structure of lipid bilayer, it is considered that the cell penetrating peptides can be applied to the intercellular lipids'permeation of functional materials [17-18].

In this study, arginine 4 (R4) was conjugated with Pal-GHK to get palmitoyl heptapeptide, Pal-GHK-R4 and clinical study on improvement of crow's feet, moisturization, transepidermal water loss (TEWL) cosmetics containing Pal-GHK-R4 were conducted.

2. Materials and Methods

2.1 Study Protocols and Measurements

The cosmetics used in this test are "Biotoc Regen" manufactured by Dermafirm Co., Ltd., which consists of 4 products (ampoule, serum, cream and peel). The main ingredients contained 1,000 ppm (0.1%) of Pal-GHK-R4. The other ingredients contained 99.9% of emulsifier, oil, humectants, fragrance and deionized water. Pal-GHK-R4 is manufactured from Dermafirm Co., Ltd., in Korea and have a purity of at least 99.0%, respectively.

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). 21 healthy female subjects who aged 30 ~ 60 years and who have voluntarily signed the Consent form after understanding study object and contents, and who can be observed and traced during the study period. 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). The evaluation of adverse events was carried out by the investigator during every visit during 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. 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: $22 \pm 1°C$, humidity: $45\% \pm 5\%$). Crow's feet, skin moisturization and TEWL were measured. Measuring instruments used were the most used instruments for skin wrinkles (Antera 3D, Miravex Ltd, lreland), moisturization (Corneometer, Courage Inc, Germany), and TEWL (Vapometer, Courage Inc, Germany).

Crow's feet were measured by using Antera 3D to photograph crow's feet before using the test product, 2 weeks after using the product, and 4 weeks after using the product. A specific area of the photograph was specified and analyzed and used as an evaluation material for crow's feet. The analyzed wrinkle variable is the overall size, and since the analysis value and the improvement degree of wrinkle are inversely proportional, it means that the lower the analysis value, the better the wrinkle.

Skin moisturization was measured by using Corneometer before using the test product, 2 weeks after using the product, and 4 weeks after using the product. The skin moisture content of the facial cheeks was measured. It was measured 3 times and averaged to evaluate skin moisture content. The unit of skin moisture content is Arbitrary Unit (A.U.), and the measured value is proportional to the skin moisture content, it means that the higher the measured value, the better the skin moisturization.

Transepidermal water loss (TEWL) was measured in the facial cheeks by using Vapometer before using the test product, 2 weeks after using the product, and 4 weeks after using the product. The value measured once is used as evaluation data, and the unit is g/m2h. Since the measured value and the improvement degree of TEWL are inversely proportional, it means that the lower the measured value, the better the TEWL.

2.2 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 $p < 0.05$.

3. Results and Discussions

3.1 Crow's Feet Changes of the Subject

The changes in the crow feet 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 15.700 ± 4.201 after 2 weeks and 15.417 ± 3.998 after 4 weeks, from 16.995 ± 4.625 (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 7.199% after 2 weeks, 8.528% 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 Crow's Feet Changes

Time	Average ± STD (Overall size)	Improvement rate[a] (%)	Probability [b] (*p* value)
Before product use	16.995 ± 4.625	-	-
After 2 weeks	15.700 ± 4.201	-7.199	0.000*
After 4 weeks	15.4710 ±3.998	-8.258	0.000*

• Improvement rate[a] (%) = [(After product use –Before product use) / Before product use] x 100
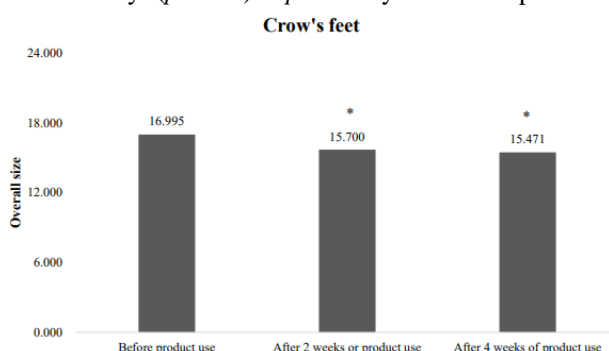• Probability[b] (*p value*) *: *p*<0.05 by Paired samples T-test

Figure1. Results of Crow's Feet Changes Using Antera 3D

3.2 Measurement Results of Skin Moisturization

The changes in the skin moisturization were measured 3 times, including before application (0 weeks), after changes in the moisturization 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). Skin moisturization on face were measured after using the products. The results showed that the moisturization increased from 60.383 ± 8.466 to 65.632 ± 8.409 after 2 weeks and to 65.162 ± 8.113 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 skin moisturization after using test products as 100%. Moisturization increased by 9.013% after two weeks, while moisturization increased by 8.287% after four weeks (Table 2). According to the results, measurement results of skin moisturization after test products use, measured value of skin moisture content statistically significantly increased 2 weeks after and 4 weeks after use of test product compared to before use of product(p<0.05).

Table 2. Results of Skin Moisturization Measurements

Time	Average ± STD (A.U.)	Improvement rate[a] (%)	Probability [b] (*p* value)
Before product use	60.383 ± 8.466	-	-
After 2 weeks	65.632 ± 8.409	9.013	0.000*
After 4 weeks	65.162 ± 8.113	8.287	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
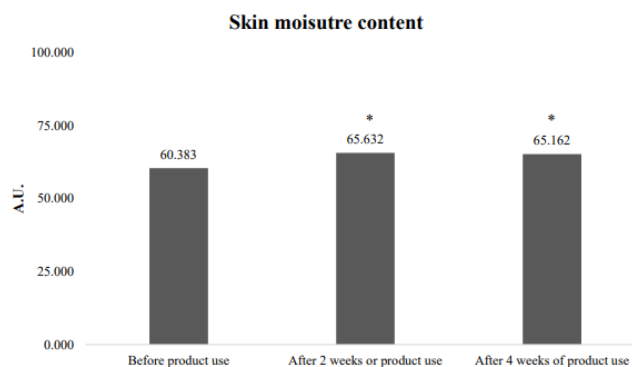
Skin moisutre content

Figure 2. Results of Skin Moisturization Using Corneometer

3.3 Measurement Results of TEWL

Changes in TEWL 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 14.048 ± 2.539 before use, to 13.005 ± 2.472 after 2 weeks of use and to 13.090 ± 2.510 after four weeks.

Analysis of the rate of improvement in TEWL revealed that the percentage of change between the 2nd and 4th weeks was 100% and the extent of wrinkles in the eyes decreased 7.306% after 2 weeks and 6.559% after 4 weeks (Table 3). Therefore, measurement results of TEWL after test products use, measured value of TEWL statistically significantly decreased 2 weeks after and 4 weeks after use of test product compared to before use of product($p<0.05$).

Table 3. Results ofTEWL

Time	Average ± STD (g/m²h)	Improvement rate[a] (%)	Probability[b] (p value)
Before product use	14.048 ± 2.539	-	-
After 2 weeks	13.005 ± 2.472	-7.306	0.001*
After 4 weeks	13.090 ± 2.510	-6.559	0.005*

• 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

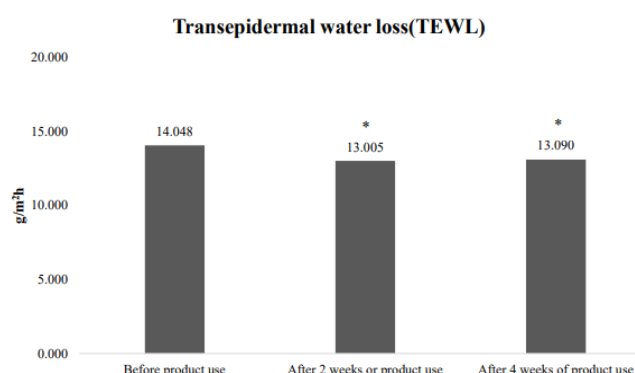**Transepidermal water loss(TEWL)**

Figure 3. Results of the TEWL Using Vapometer

3.4 Evaluation of Skin Adverse Reaction

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).

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Table 4. Assessing Skin Adverse Events

Time	Erythem	Edema	Scaling	Itching
After 2weeks	-	-	-	-
After 4 weeks	-	-	-	-
Time	Stingin	Burnin	Tightne	Prickin
After 2weeks	-	-	-	-
After 4 weeks	-	-	-	-

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

I. Conclusions

This study focused on verifying whether the use of a cosmetics containing 1,000ppm Pal-GHK-R4 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 21 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. Second, the subjects were instructed to use Biotoc Regen peel individually for four weeks, once a week. 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 for crow's feet, Corneometer for skin moisturization and Vapometer for the TEWL were used to evaluate different facial characteristics.

When the test products were applied, changes of the crow's feet, skin moisturization and TEWL were analyzed by the parking lot and it was observed that crow's feet decreased by 7.199% after 2 weeks and to 8.258% after 4 weeks. The test product had a very significant effect on the improvement of crow's feet. In the case of skin moisturization, it increased to 9.013% in the two weeks after using the products use and 8.287% 4 weeks after using them. The test product had a very significant positive effect on skin moisturization. Finally, in the case of TEWL, it decreased to 7.306% at the 2 weeks use and to 6.559% at the 4 weeks after using them. The test product also had a very significant positive effect on TEWL. Therefore, the test products containing wrinkle improving peptides, Pal-GHK-R4 are considered to have beneficial effects on improvement of crow's feet, skin moisturization and TEWL of 4 weeks use.

4. Acknowledgment

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