The anti-aging effect of COLinus, human collagen-derived peptide, *in vitro* and *ex vivo* study

So Hun Lee, Byung Kuk Kim, Ha Yeon Kim, Jae Seok Lee, Kang Hyuk Lee, Eun Young Jung and **Song Seok Shin***

Life Science R&D Center, HYUNDAI BIOLAND Co., Ltd., Chungcheongbuk-do, South Korea

***Song Seok Shin, 22, Osongsaengmyeong 2-ro, Osong-eup, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, South Korea, +82-43-249-6801, ssshin@hyundaibioland.co.kr**

Background: As life expectancy increases, reducing visible signs of skin aging caused by various factors such as intrinsic and extrinsic factors has become a major issue. There are several phenomena in skin aging, but the reduction of collagen synthesis and regeneration in human skin is a major feature of skin aging.

Methods: Cell viability was measured using MTT assay for cell cytotoxicity and proliferation. Wound healing was confirmed through Scratch Assay. qRT-PCR and immunoblotting were performed to investigate the collagen and Elastin mRNA and collagen protein expression, respectively. Skin permeability was measured using the Franz diffusion cell of the transdermal absorption system, and the effect of improving eye wrinkles was evaluated using ANTERA 3D® CS in *clinical trials*.

Results: MTT assay revealed that COLinus was no significant cytotoxicity in NHDFs. COLinus improved cell proliferation and wound healing. COLinus increased mRNA expressions and protein expressions of COL-I, COL-III and Elastin. As a result of performing a skin permeation test in *ex vivo* human skin, it was confirmed that the COLinus penetrated the human skin. COLinus improved the wrinkles around the eyes in the *clinical trials*.

Conclusion: The findings of this study confirmed that COLinus was well absorbed into the human skin and improved cell proliferation, wound healing, collagen synthesis and elastin synthesis in NHDFs. COLinus helped alleviate wrinkles around the eyes by improving the levels of various wrinkle-related factors in *clinical trials*. In Conclusion, COLinus, human

collagen-derived peptide, can be used as a future anti-aging cosmetic ingredient for improving skin conditions.

Keywords: COLinus, Human Collagen-derived Peptide, Anti-Aging, Collagen and Elastin synthesis, Cell regeneration.

Introduction.

As life expectancy increases, reducing visible signs of skin aging caused by various factors such as intrinsic (genetic and hormonal factors) and extrinsic factors (UV irradiation, continuous environmental stimuli, food and lifestyle) has become a major issue [1]. Due to many of these factors, wrinkles and reduced elasticity are typical phenomena of skin aging. One of the main mechanisms of skin aging is thought to be a reduction in the amount of extracellular matrix (ECM), particularly collagen in the dermis [2]. In aged skin, the production of collagen decreases and its degradation increases, which leads to an overall reduction in collagen amount [3]. A major component of the extracellular matrix is collagen; in skin, type I and type III collagen comprise 85 - 90% and 8 -11 % of the total collagen synthesized, respectively [4]. Type I collagen is an heterotrimer, triple helix protein, composed of two α1 chains and one α2 chain respectively coded by COL1A1 and COL1A2 genes which is involved in skin elasticity, flexibility, and tension [5]. Type III collagen is more prevalent in young skin than aged skin and is particularly involved in wound healing [6].

In previous studies, many products containing collagen isolated from land animals or marine animals have been used for a wide range of purposes in the cosmetics and food industries [7]. Collagen has been isolated from land animals including bovine and porcine and many marine organisms such as fish, sponges, jellyfish, squid. However, associated with these sources, there is the risk of the transference of zoonotic diseases such as BSE (bovine spongiform encephalopathy) and FMD (Foot and Mouth Disease) or even religious constraints [8]. Besides, because these sources can cause immune or irritation reactions such as allergies, safety is not guaranteed for human use. In addition, collagen used in foods and cosmetics is controversial because it is not efficiently absorbed in the body due to the large molecular weight of natural collagen or partially hydrolyzed oral collagen [9]. Currently, since

researches on the skin efficacy of human collagen-derived peptide have not been investigated, we started the following study for human collagen-derived peptide, which has relatively few side effects and is easily absorbed into the skin.

In this study, we developed hCOL1A2 (COLinus), small human-derived collagen peptide, with higher absorption and safer compared to natural collagen and studied for anti-aging efficacy in human skin.

Materials and Methods.

Preparing of hCOL1A2, human collagen-derived peptide (COLinus)

We generated a His-tagged human COL1A2 (hCOL1A2, COLinus) and cloned it into the pET-28a (His-tag) vector. Recombinant proteins were overexpressed in the E. *coli* strain and recombinant hCOL1A2 were purified from the supernatants using Ni-NTA resin (GE Healthcare). Purified recombinant hCOL1A2 was eluted and visualized as a single band around 20 kDa. Purified recombinant hCOL1A2 was eluted and visualized as a single band around 20 kDa. Isolated hCOL1A2 was identified using an anti-His antibody.

Cell culture

Normal human dermal fibroblasts (NHDFs) were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Welgene, Korea) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotics (Gibco) at 37°C in a humidified incubator with 5% CO_2. The cells were then sub-cultured with 0.25% trypsin-0.53 mM EDTA, which was replaced with fresh medium every two or three days.

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. NHDFs were seeded in 24-well plate and incubated for 24 h. Then the cells were exposed to various concentrations of samples for 24 h. After that, culture medium was replaced with fresh medium containing MTT. Following 4 h incubation, MTT formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Duksan, Korea) and the absorbance was measured at 570 nm. Cell viabilities were calculated as a percentage of control absorbance.

Cell proliferation assay

NHDFs were seeded in 12-well plate and incubated for 24 h. Then the cells were exposed to various concentrations of samples for 72 h. After that, culture medium was replaced with fresh medium containing MTT. Following 4 h incubation, MTT formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Duksan, Korea) and the absorbance was measured at 570 nm. Cell viabilities were calculated as a percentage of control absorbance.

Wound healing assay

NHDFs were seeded in 24-well plate and incubated for 24 h. After scratching the cells with scraper (ScarTM Scrather, SPL), photography was performed with a microscope (Day 0). After that, culture medium was replaced with fresh medium and treated samples for 24 h. Then, photography was performed with a microscope (Day 1). Compare the photos of Day 0 and Day 1 to determine the extent to which the cells stretch out.

RNA isolation and Quantitative Real-Time PCR(qRT-PCR)

NHDFs were seeded in culture-plates and cultured in medium with supplement for 24 h. After 1 day, the cells were washed with PBS and then treated samples were culture in medium without supplements for 24 h. Total RNA was isolated from the cells with QiaZol (Invitrogen, USA) according to the manufacturer's instructions. 1 μg of RNA was reserved transcribed into cDNA using a qPCRBIO cDNA synthesis kit (PCRBIO, PCR Biosystems, USA). Using qPCRBIO SyGreen Blue Mix Lo-ROX (PCR Biosystems, USA) following the manufacturer's protocol, qRT-PCR reactions were performed in triplicates.

Immunoblotting analysis

NHDFs were incubated with lysis buffer(iNtRON, Korea). Equal amount of protein (10 μg) were dissolved in NuPAGE LDS Sample buffer (Invitrogen). Lysates were boiled at 95℃ for 5 min and loaded and run on 7% NuPAGE Tris-Acetate gels (Invitrogen). The proteins were transferred onto polyvinylidene fluoride membranes (Invitrogen) and blocked in 5% Skim milk. Membranes were probed with anti-Collagen Type I, anti- Collagen Type III and anti-beta actin antibody for overnight at 4℃. The secondary antibodies used were anti-mouse horseradish peroxidase-conjugated IgG antibody and anti-rabbit horseradish peroxidase-

conjugated IgG antibody for 1 h at room temperature. Protein bands were detected suing the Chemi Doc (ATTO). Densitometric analysis of protein bands was performed using Image software (CS analyzer, ATTO).

in vitro Ocular Irritation (OECD TG 491)

SIRC cells were incubated for four days after seeding into 96-well plates. Each sample should tested in triplicate in each repetition by exposing the cells to the appropriate test or control chemical for five minutes at room temperature. After exposure, cells are washed twice with PBS and MTT solution is added. After a two-hour reaction time in an incubator, the MTT solution is decanted, MTT formazan is extracted by adding 0.04N hydrochloric acid-isopropanol for 60minuates in the dark at room temperature, and the absorbance of the MTT formazan solution is measured at 570nm with a plate reader.

ex vivo skin permeability and human skin tissue absorption image evaluation

ex-vivo skin permeability and human skin tissue absorption image evaluation tests were conducted by requesting KSRC Korea Skin Clinical Research Center (South Korea).

To evaluate the skin permeability of the test substance, human skin tissue to which the test substance was not applied (control group) and human skin tissue to which the test substance was applied (test group) were used. The test substance was used for testing after fluorescent labeling. Saline and fluorescence-labeled test substance were instilled in the human skin tissue of the control group and test group in the donor chamber of the static diffusion device, reacted for 24 h, and the sample was collected in the receptor chamber. The amount of the test substance that was collected and penetrated the human skin tissue was analyzed with Multimode Microplate Reader (Fluorescence mode). In addition, in order to confirm the test substance absorbed into the human skin tissue, a tissue slide was prepared by fixing the human skin tissue at 24 h after the reaction, and it was observed using a fluorescence microscope.

Clinical trials

Clinical trials for wrinkle improvement were implemented in CRA Korea (South Korea). The *Clinical trials* were conducted to evaluate the eye wrinkle improvement effect of using

the test product for 4 weeks. A total of 10 subjects who satisfied the inclusion criteria and did not satisfy exclusion criteria were recruited. All measurements were conducted after the subjects were stabilized for at least 30 minutes in constant temperature and humidity (22±2℃, 50±10% relative humidity (Rh)) without air movement and direct exposure to sunlight. The selected subjects visited a research institution to be photographed and to undergo instrument measurements. Eye wrinkles were photographed using ANTERA 3D® CS (Miravex Limited, Ireland). Wrinkles around the subjects' eyes were photographed before product use (0 week) and 2 weeks and 4 weeks after product use, and wrinkle-related factors of pre- and post-test were compared.

Results.

1. *in vitro* assay

1.1. Cytotoxicity of COLinus in NHDFs

To evaluate the cytotoxicity of COLinus, samples were treated at various concentrations in NHDFs, and MTT assay was performed. COLinus showed no cytotoxicity at 1 and 30 μg/ml in NHDFs. (Fig. 1)

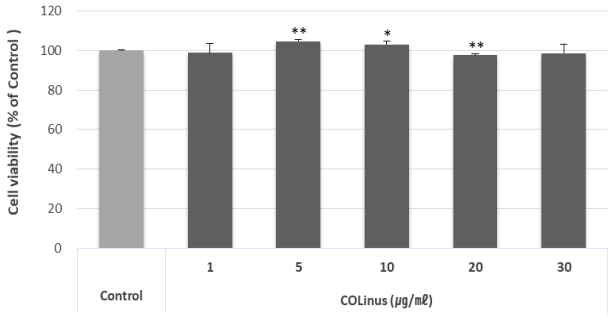

Fig. 1 Cytotoxicity of COLinus. Cell viability was measured by MTT assay with the concentrations of COLinus for 24h in NHDFs. The data are presented as the mean ± Standard deviation from three independent experiments performed in triplicate (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 vs. the respective control group.

1.2. Effects of COLinus on the proliferation and wound healing in NHDFs

We investigated the effects of COLinus on the proliferation of NHDFs by MTT assay. As shown in Fig. 2, COLinus enhanced NHDFs growth by approximately 29% at a concentration of 10 μg/ml ($p<0.001$), compared to that of the control.

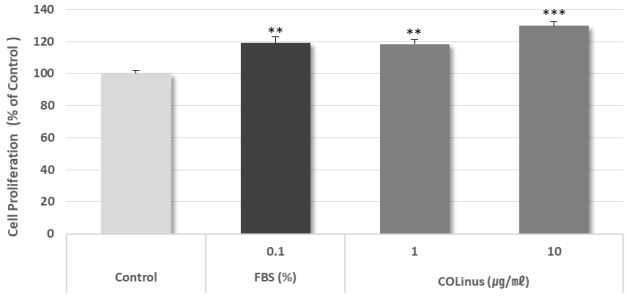

Fig. 2 Proliferation of COLinus. Cell proliferation was measured by MTT assay with COLinus for 72h in NFDFs. The data are presented as the mean ± Standard deviation from three independent experiments performed in triplicate (n = 3). **p < 0.01, ***p < 0.001 vs. the respective control group.

In addition, we then investigated the effects of COLinus on the wound healing and observed that COLinus enhanced the migration of NHDFs (Fig. 3). When a confluent monolayer of NHDFs was scratched, the migration of cells into the wounded area was increased in the presence of COLinus compared to the migration of control.

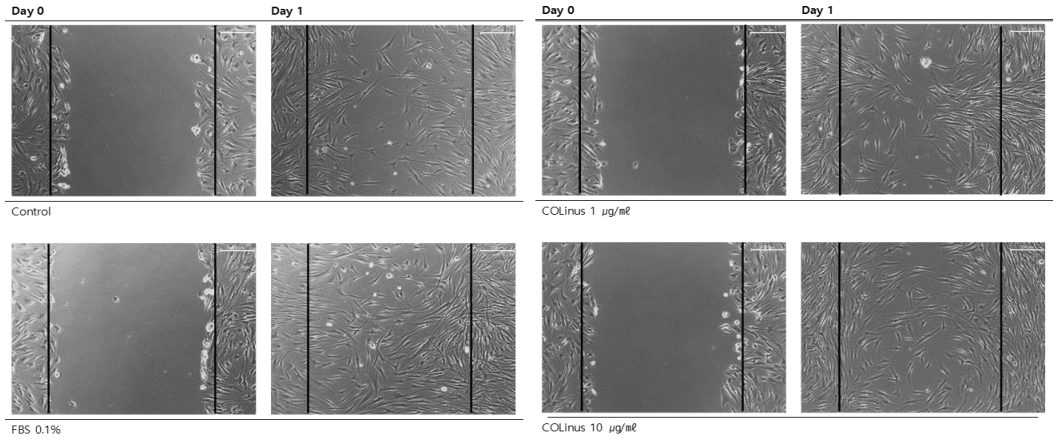

Fig. 3 Wound healing of COLinus. Wound healing was performed by scratch assay with COLinus for 24h in NHDFs.

1.3. Effect of COLinus on the production of COL-I, COL-III and Elastin expressions in NHDFs

To investigate skin elasticity efficacy of COLinus, we measured COL-I, COL-III and Elastin mRNA levels by qRT-PCR and Col-I and Col-III protein levels by immunoblotting. As

shown in Fig. 4, after treatment with COLinus, COL-I, COL-III and Elastin mRNA levels were increased by approximately 46.8%, 33.6%, and 151.7%, respectively ($p < 0.001$), compared to that of the control.

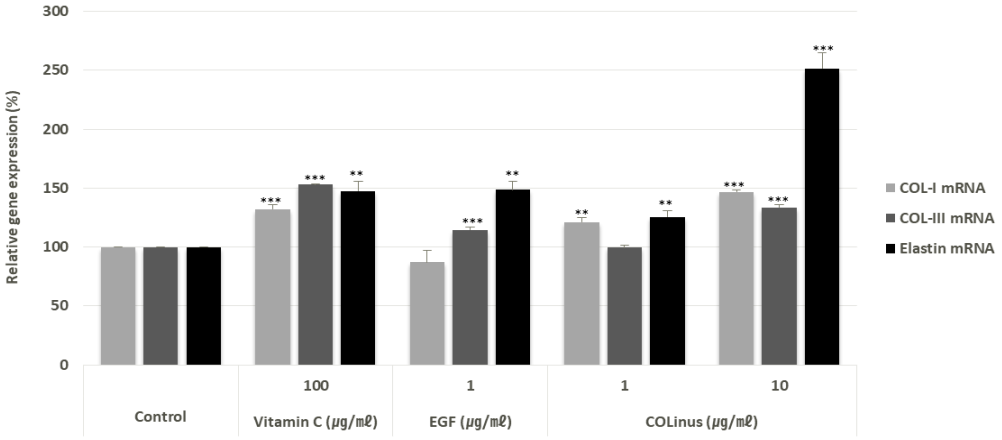

Fig. 4 The mRNA expressions of skin elasticity of COLinus. NHDFs were treated with COLinus for 24h. The mRNA expression of COL-I, COL-III and Elastin were determined by real-time PCR. β-actin was used as a house keeping gene. The data are presented as the mean ± Standard deviation from three independent experiments performed in triplicate (n = 3). **p < 0.01, ***p < 0.001 vs. the respective control group.

Protein levels, after treatment with COLinus, COL-I and COL-III protein levels were increased by approximately 43.7%, 30.3%, and 25.1%, respectively ($p < 0.001$), compared to that of the control (Fig. 5). These results suggested that COLinus has an effect on improvement of skin elasticity.

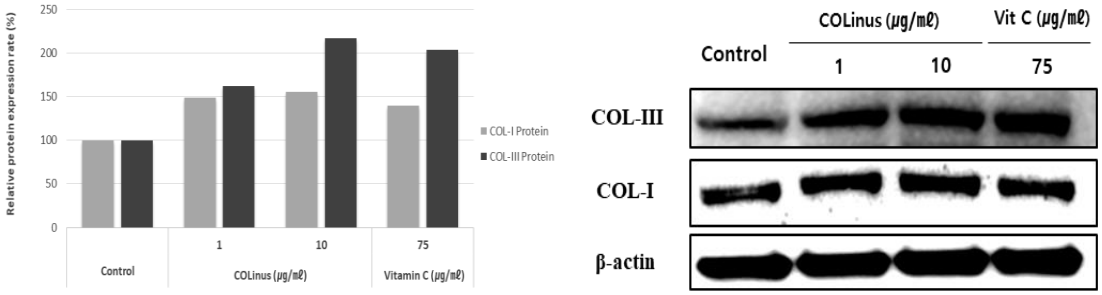

Fig. 5 The protein expressions of skin elasticity of COLinus. NHDFs were treated with COLinus for 24h. The protein expressions of COL-I and COL-III were evaluated by immunoblotting. β -actin was used as a house keeping gene.

1.4. *in vitro* ocular irritation test (STE assay)

To evaluated *in vitro* ocular irritation test for COLinus, we conducted STE assay with OECD guidelines (OECD TG 491) whether it will induce eye irritation. According to the test result of cell viability (Table 1), it was concluded that COLinus is non-irritant material.

Sample		Avg.	Stdev.	UN GHS Classification
Solvent control (0.9% NaCl)		100.00	3.88	
0.01% SLS		29.64	2.15	
COLinus	5% (w/w)	98.45	1.54	No Category
	0.05% (w/w)	106.18	1.76	

Cell viability		UN GHS Classification
At 5%	At 0.05%	
> 70 %	> 70 %	No Category
≤ 70 %	> 70 %	No prediction can be made
≤ 70 %	≤ 70 %	Category 1

Table. 1 *in vitro* ocular irritation test of COLinus.

2. *ex-vivo* assay

2.1. *ex-vivo* skin permeability and human skin tissue absorption image evaluation

As a result of comparing the cumulative permeation amount of the test substance, 38.25×10^4 fluorescence intensity was confirmed at 24 h after application of the test substance for the control group, and fluorescence intensity of $1{,}407.41 \times 10^4$ was confirmed for the COLinus group at 24 h after application of the test substance. Although there was no significant difference in comparison between groups, the cumulative transmittance of the test substance at 24 h was increased 36.80 times in the COLinus group compared to the control group (Fig. 6(A)). In addition, as a result of observing a cross section of human skin tissue using a fluorescence microscope, the FITC labeled test substance was observed in the epidermal and dermal layers (Fig. 6(B)).

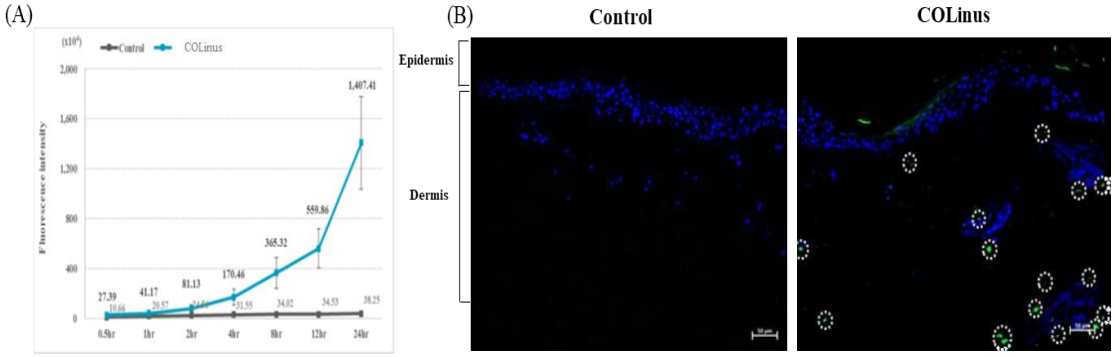

Fig. 6 *ex-vivo* skin permeability and human skin tissue absorption image of COLinus. Fluorescence intensity analysis result graph of the permeated filtrate by time point (A) and representative fluorescence microscopy image of human skin cross section (x200, Scale bar= 50 μm) (B).

Clinical trials

The results of measuring the wrinkles around the eyes using ANTERA 3D® CS (Miravex Limited, Ireland) for 10 subjects to evaluate the effect of improving skin wrinkles according to the use of the test product for 4 weeks are as follows. Average width had significantly decreased by 13.68% at 4 weeks ($p<0.01$) after product use compared to before use (0 week). Average depth had significantly decreased by 24.24% at 4 weeks ($p<0.001$) after product use compared to before use (0 week). Maximum depth had significantly decreased by 33.40% at 4 weeks ($p<0.001$) after product use compared to before use (0 week). Indentation index had significantly decreased by 26.70% at 4 weeks ($p<0.001$) after product use compared to before use (0 week). Therefore, it was confirmed that wrinkles were improved by reducing the measured values of the four factors.

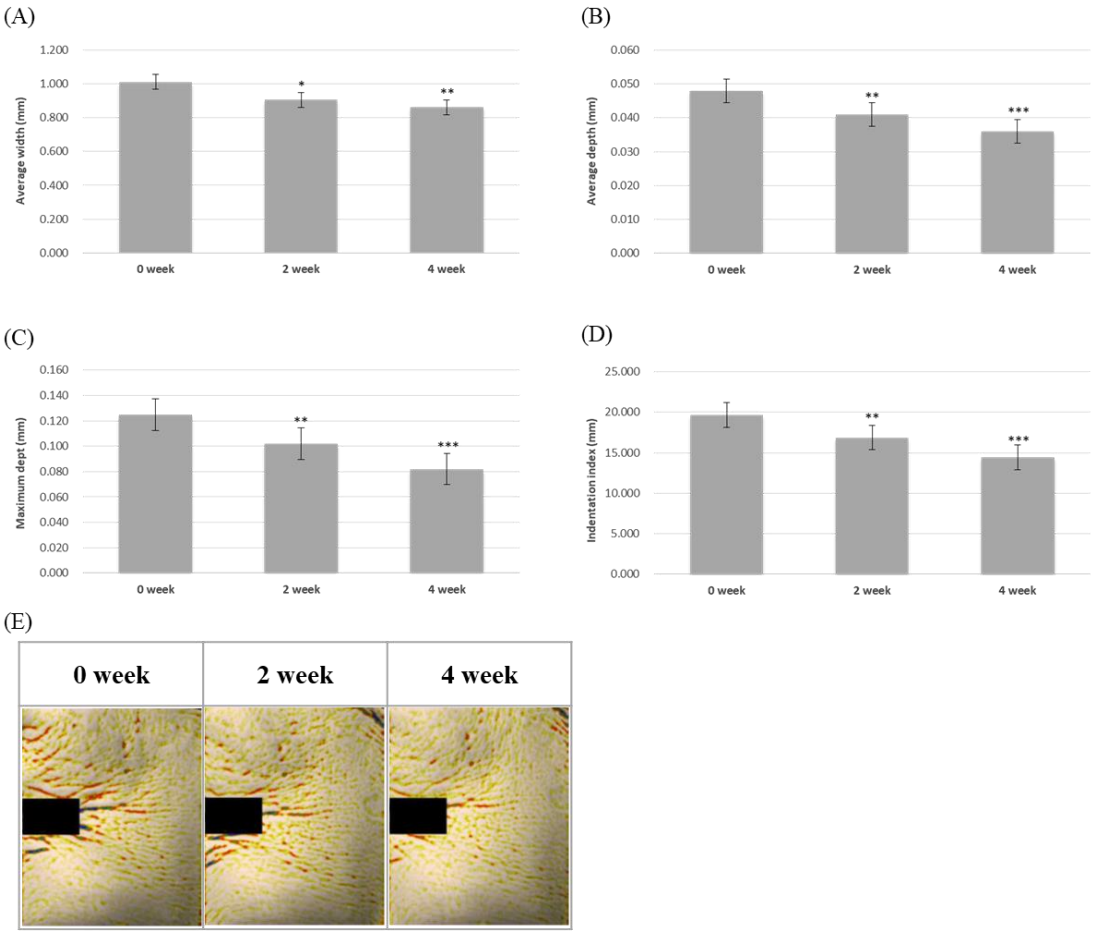

Fig. 7 Graph and image of eye wrinkles measurement results of COLinus. Average width; The average width of the selected wrinkles (A), Average depth; The average depth of the selected wrinkles (B), Maximum depth; The maximum depth of the selected wrinkles (C), Indentation index; The average depth of all indentations under the surface (D) and image of eye wrinkles (E). *p<0.05, **p<0.01, ***p<0.001

Discussion and Conclusion.

Skin aging, one of the most concerned problems in people, can be noticed as wrinkles, sagging, uneven skin tone, and dull or dry skin [10]. The causes of skin aging can be categorized into intrinsic and extrinsic factors. These factors cause the aging skin by which interferes collagen synthesis, degrades collagen and elastin [11]. Collagen synthesis in skin plays a major role in skin rejuvenation. The reduction of types I and III collagen synthesis is a critical feature of aged skin leading to skin thinning and the increased fragility of skin [12]. Hence, collagen is a very important factor and a key component in skin aging since the inhibition of collagen synthesis or a loss in the function of collagen results in chronologically aged skin.

For this reason, we have been interested in collagen, which is considered an important factor in skin aging, and we have developed COLinus, small human-derived collagen peptide, that is safe and permeable into the skin rather than the currently widely used animal collagen.

The findings of this study confirmed that COLinus increased growth and enhanced cell migration in NHDFs. Moreover, COLinus significantly induced COL-I, COL-III, and Elastin synthesis in NHDFs. Based on these results, COLinus is thought to be effective in preventing skin sagging and creating solid skin by not only increasing skin elasticity but also increasing tissue regeneration. In addition, we conducted an eye irritation test in accordance with OECD guidelines to evaluate the presence or absence of stimulation of COLinus and we confirmed that COLinus was a non-stimulating material. Furthermore, it was confirmed that the COLinus had excellent permeability and penetrated into dermis of skin, and that it helped alleviate wrinkles around the eyes by improving the levels of various wrinkle-related factors through *clinical trials*.

In conclusion, COLinus, human collagen-derived peptides, can be used as a future anti-aging cosmetic ingredient for improving skin conditions.

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