How to deliver to the dermis: First-in-class dermal delivery technology in human skin.

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Abstract

Background: This study reports a novel peptide which has capability to deliver to dermis without covalent bond with various cargos. Furthermore, we aimed to demonstrate the mode of action of skin permeation in the stratum corneum and the epidermis which are skin barriers.

Methods: A novel peptide (INCI: Oligopeptide-191, 12 mer peptide) is prepared using solid-phase-peptide-synthesis. FITC-conjugated collagens of 3 k to 300 kDa and hyaluronic acid of 800 kDa and fluorescein liposome of 200 nm are prepared as a cargo. Section images by confocal are observed 3 hours after cargo treated. To investigate the mechanism of skin permeation the SC and epidermis were peeled off the skin and frozen section slides are prepared.

Results: It has been investigated that novel peptide delivers all different size of cargo to the dermis, from 3 kDa collagen, the smallest size used in the experiment, to 800 kDa hyaluronic acid. A novel peptide delivered not only biopolymers closed to hydrophilic but also liposome closed to hydrophobic. As a result of analyzing the fluorescence of the SC and epidermis treated with hyaluronic acid, it was investigated that it penetrates through intercellular lipid in the SC and it penetrates through intracellular in epidermis.

Conclusion: This study will contribute to the development of innovative cosmetics that deliver various substances to the dermis. It would be a kind of topical filler that deliver the biopolymers to the dermis with topical treatment and fat reduction technology also the technology that target cells in the dermis such as senolytic.

Keywords: Transdermal delivery system, TDDS, Skin penetration peptide, Peptide

Introduction.

The skin, which occupies the largest part of the human body, is composed of the epidermis, dermis, and subcutaneous tissue, and performs various functions from the external environment [1,2]. In particular, it is known that the molecular weight that can be delivered through the skin is about 500 Da or less due to the inherent characteristics of the stratum corneum constituting the skin barrier [3]. However, it is known that low molecular weight substances have low skin penetration efficiency, and high molecular weight substances such as growth factors have lower penetration efficiency. Therefore, there is a great need for a safe method for the effective delivery of active substances in the cosmetic and pharmaceutical industries [4,5].

Physical delivery systems such as microneedles can cause irreversible damage to the skin, and existing materials for skin penetration often use fat-soluble substances or polymers, which can cause various side effects and unknown toxicity in the human body. It is necessary to safely deliver active substances to the dermis using peptides, which are fragments of proteins existing in the human body [6,7].

Many of active compounds for topical therapeutics including cosmetics should deliver to dermis. This is because many skin-related problems, including cosmetic point of view, occur in the dermis [8]. The dermis under the epidermis is a connective tissue composed of collagen, elastic fibers and a mixture of extracellular matrix proteins. Unlike the epidermis, the dermal layer is supplied with blood and lymphatic vessels, nerve endings, fibroblasts and various types of immune cells, so it should be the target of the transdermal delivery system (TDDS). Various TDDS represented by active and passive systems have been intensely researched and the possibility has been reported [9]. However, reach or penetrate the dermis is still challenging. More precisely, few studies have been reported that can deliver cargo to dermis and applied without limitation to the chemical properties or tens of thousands Da or larger or even particles using human model [10,11]. Therefore, to our knowledge, this is the first study to report that various substances range from small/large

size and hydrophobic/ hydrophilic properties and nanoparticle can be delivered to the dermis using a human skin model.

Under this background, the a novel peptide for transdermal delivery system (TDDS) technology developed in this study is a cutting-edge biotechnology that greatly improves the absorption rate of raw materials and increases the bioavailability. As it is a new biomaterial platform technology, we intend to apply it to the development of cosmetics and cosmeceuticals for skin penetration.

Materials and Methods.

1. Synthesis and purification of Oligopeptide-191

Peptides were synthesized using 2-chlorotrityl chloride resin based solid-phase chemistry. Nα-Fmoc deprotection was achieved with 20% piperidine /N,N-Dimethylmethanamide (DMF) (15min). All standard Fmoc amino acid derivatives and resins were purchased from Novabiochem® (Darmstadt, Germany). At the completion of synthesis, resins were washed with diethyl ether and dried. Peptides were cleaved from the resin, and side chains were deprotected using modified reagent (trifluoroacetic acid [TFA], 95% [v/v]; triisopropylsilane [TIS], 2.5% [v/v]; water, 2.5% [v/v]; for 3h). Post-cleaving solutions were collected and peptides precipitated by the addition of ice-cold diethyl ether. The precipitated peptides were separated by centrifugation, washed using cold diethyl ether, spun down again, and dried in vacuo. Peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) to >95% homogeneity. The purity of peptides was evaluated by analytical RP-HPLC using a Shimadzu LC Solution System (Shimadzu Corp., Kyoto, Japan) connected to a photodiode array detector (monitoring at 220 nm), using a 4.6 450mm C18 column (Shimadzu Corp., Kyoto, Japan) (pore diameter: 100 Å; particle size: 5 μm) in a linear gradient from 5% to 90% (v/v) solvent B (0.1% trifluoroacetic acid [TFA] in acetonitrile) in solvent A (0.1% TFA in water) over 30 minutes (flow rate: 1ml/min). The molecular weight of each peptide was confirmed using an TSQ Quantis Plus Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, USA).

2. Cell culture

Human keratinocyte cells (HaCaT) were obtained from Thermo Fisher Scientific (Waltham, MA, USA) and cultured in Dulbecco's Modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Carlsbad, MA, USA) and 100 μg/mL penicillin-streptomycin (Gibco) at 37℃ in a humidified atmosphere containing 5% CO_2.

3. MTT assay

HaCaT cells (5×10^5 /mL) were cultured in the presence of Oligopeptide-191 (25-200 μM) for 24 hr and stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma, USA) solution (0.5 mg/mL) for 4 hr at 37℃. Formazan crystals were dissolved in DMSO (dimethyl sulfoxide) for 30 min, and the absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

4. Ames test

The strains used in Ames test were frame shift mutants TA98, TA1537 among Salmonella typhimurium mutant strains, and base-pair mutants TA100, TA1535 and Escherichia coli mutants WP2 (uvrA), WP2 (Moltox, NC , USA). Nutrient Broth, Top Agar/L-Histidine & D-Biotin, Minimal Glucose Agar, 2-Aminoanthracene, Sodium Azide, Methyl Methane-sulfonate, and ICR191 ACRIDINE are all included in Moltox's Test Kit. S. Typhimurium The tendency according to the presence or absence of S9 mixture (hepatic metabolic activity) was examined using TA98 and TA100, TA 1537, and TA 1535. Add 100 μL of diluted sample and 100 μL of each strain to 2 ml of top agar containing 0.05M histidine/biotin and mix. (Add 500 μL of S9 mix for metabolic activity method.) The number of colonies was counted after incubation at 37℃ for 48 hours in the minimal glucose agar plate. E.coli Wp2 is add 100 μL of diluted sample and 100 μL of each strain to 2 ml of top agar containing 0.05M Tryptophan and mix. (Add 500 μL of S9 mix for metabolic activity method.) Likewise the number of colonies was counted after incubation at 37℃ for 48 hours in the minimal glucose agar plate. If the number of return mutations formed on the plate of each strain more than doubled compared to the negative control group, it was determined that it was mutagenic.

5. Franz diffusion cell assay

Human cadaver skin was obtained from the UMC science (Goyang-si, Seoul). Full-thickness (Epidermis + Dermis) frozen skin was from female donor age from 66 years and

was stored at -80℃ and thawed to room temperature before use. The skin used in this experiment was 3cm × 3cm, and skin permeability was measured using a Franz Diffusion cell instrument. The skin tissue was placed upward on the receptor compartment of the skin penetration absorbing device, the donor compartment was fixed on the keratin layer, and a magnetic stirring bar was put inside the receptor compartment, and 16 ml of PBS, which is a receptor buffer, was filled to fit the skin and receptor compartment. The indoor temperature of the experiment was maintained at 37℃, and the skin penetration absorption device was put in a beaker containing 32℃ ± 1℃ water to maintain the internal temperature and stabilize it for 30 minutes, and 1 ml of the prepared sample solution was dropped onto the skin. In order for the skin-transmitted sample to be uniformly mixed in the receptor buffer, the magnetic stirring bar was maintained at 600 rpm, and after 4 to 6 hrs, 0.2 ml of the receptor buffer was collected through the sampling port, and the same amount of buffer was supplemented. The Receiver buffer sample was quantitatively analyzed using HPLC (Agilent, USA).

6. Confocal microscope

Collagen of various molecular sizes (3 Kda, 30 Kda and 300 Kda) was conjugated with FITC using the FITC conjugation kit (Lightning-Link® (ab188285), Abcam, Cambridge, MA, USA). Fluorescein liposome was prepared using a commercially available liposome kit (L4395; Sigma-Aldrich, St. Louis, MO, USA) and according to the manufacturer's protocols. Skin samples were fixed with 4% paraformaldehyde for 24 hr at 4℃ and cryoprotected with 30% sucrose for 24 hr at 4℃. Skin sections were snap frozen and embedded in optimal cutting temperature media and sectioned on a cryostat (Leica CM 3050 S, Leica Microsystems, Wetzlar, Germany) at 20 μm. The skin slices were placed on cover slips using mounting medium with DAPI. All tissue samples were observed using confocal scanning laser microscopy (LSM800 confocal microscope system, ZEISS, Oberkochen, Germany).

7. Statistical analysis

All data are expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed using GraphPad Prism version 7 (GraphPad Software Inc., San Diego, CA, USA) to assess between-group differences. Multiple group comparisons were

performed using one-way ANOVA, followed by post-hoc Tukey tests. Differences at $p < 0.05$ were considered statistically significant.

Results.

We first performed an MTT assay to determine the cytotoxicity effects of Oligopeptide-191 in human keratinocyte (HaCaT) cell line. The result of this assay demonstrated that Oligopeptide-191 is not cytotoxic effect at up to 200 μM (Figure 1).

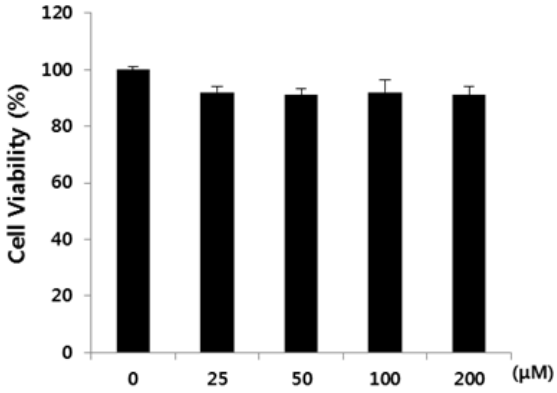

Figure 1. Effect of Oligopeptide-191 on cell viability in HaCaT cells. HaCaT cells were treated with various concentrations of Oligopeptide-191 for 24 hr, and then cell viability was measured by MTT assay. The data represent the mean ± SD of triplicate determinations.

To test the safety of Oligopeptide-191, a mutagenicity test was performed. As a result, in the untreated condition of the S9 mixture treated with Oligopeptide-191 at a concentration of 0.125 ~ 1 μM/plate, a dose-dependent increase in the number of revertant colonies exhibiting mutagenicity was not observed for the five strains, and 0.125 ~ 1 μM/plate A dose-dependent increase in the number of revertant colonies exhibiting mutagenicity was not observed for the five strains under the treatment conditions of the S9 mixture treated with the concentration. Therefore, it was confirmed that the Oligopeptide-191 is a safe raw material that does not induce reversal mutation of bacterial strains (Figure 2).

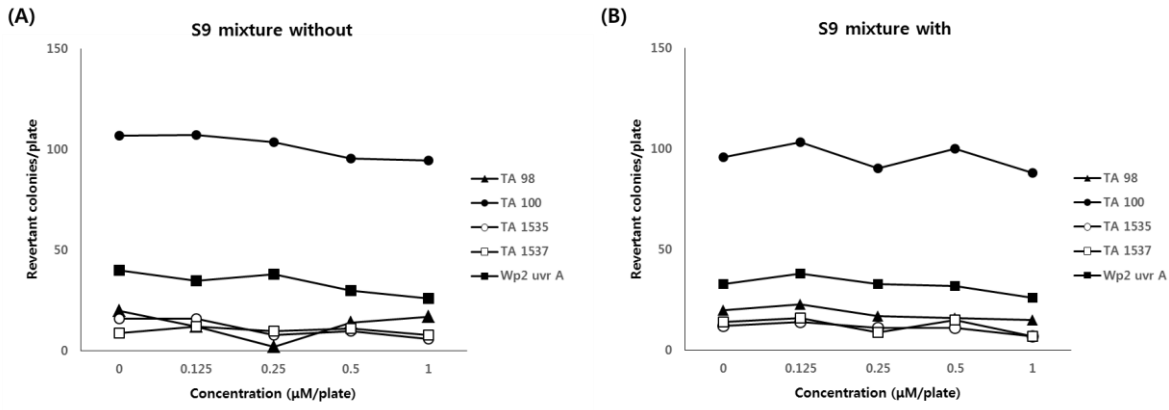

Figure 2. Effect of Oligopeptide-191 on mutagenicity. The data represent the mean ± SD of triplicate determinations.

We performed a Franz diffusion cell assay to determine how much Oligopeptide-191 increased the skin permeability of caffeine compared to TAT peptide, a positive control [12]. As a result of HPLC analysis, it was confirmed that caffeine permeated more than twice in the group treated with oligopeptide-191 and caffeine compared to TAT peptide, a positive control (Figure 3).

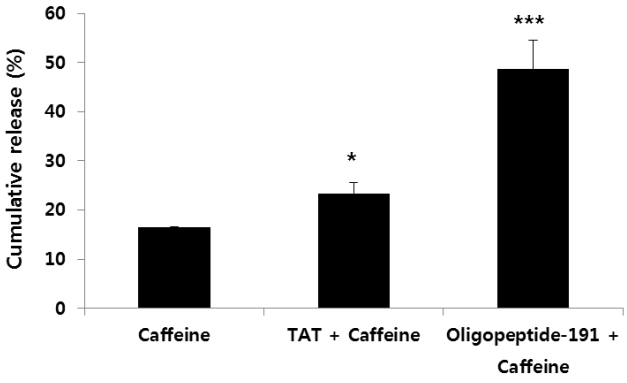

Figure 3. Enhance effect of Oligopeptide-191 on skin permeability in Human cadaver skin. Human cadaver skins were treated with caffeine mixed TAT peptide or Oligopeptide-191 for 3 hr. The skin permeability was measured using a Franz Diffusion cell instrument. The Receiver buffer sample (Caffeine) was quantitatively analyzed using HPLC. The data represent the mean ± SD of triplicate determinations. (one-way ANOVA; *$p < 0.05$, ***$p < 0.001$ vs. Caffeine only).

Collagen of various sizes (3kda ~ 300kda) was conjugated using the FITC® Conjugation Kit (Fast) - Lightning-Link® according to the manufacturer's instructions, and then

assembled with Oligopeptide-191 and treated on human cadaver skin for 3 hours. As a result of confocal analysis of frozen section slides, it was confirmed that collagen of various sizes migrated to the dermis in the group treated with Oligopeptide-191 compared to collagen alone (Figure 4).

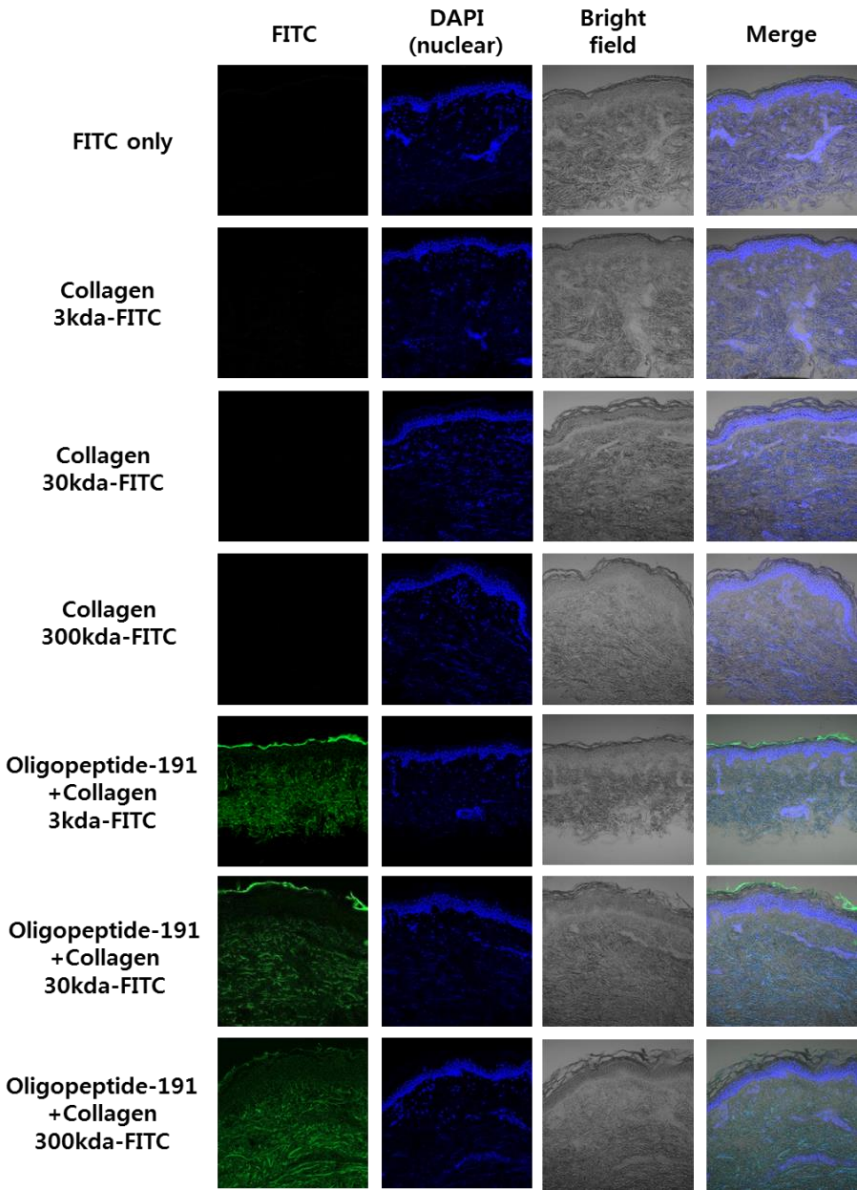

Figure 4. Enhance effect of Oligopeptide-191 on various size collagens skin penetration in human cadaver skins. Human cadaver skins were treated with FITC conjugated various size collagens or Oligopeptide-191 for 3 hr. The skin slices were placed on cover slips using mounting medium Hoechst (blue). All tissue samples were observed using confocal scanning laser microscopy. The data presented are representative of three independent experiments.

After assembling Oligopeptide-191 and 200 nm liposomalized Fluorescein and treating it on human cadaver skin for 3 hours, confocal analysis showed that liposomes migrated to the entire skin layer. Fluorescein liposomes were prepared using Sigma-Aldrich's Liposome kit for liposomes, and it was confirmed that the liposome-type material was also able to move within the skin. In the group treated with only liposomes, it was specifically observed to exist only in the stratum corneum, which is consistent with the results of the previous report [13] (Figure 5).

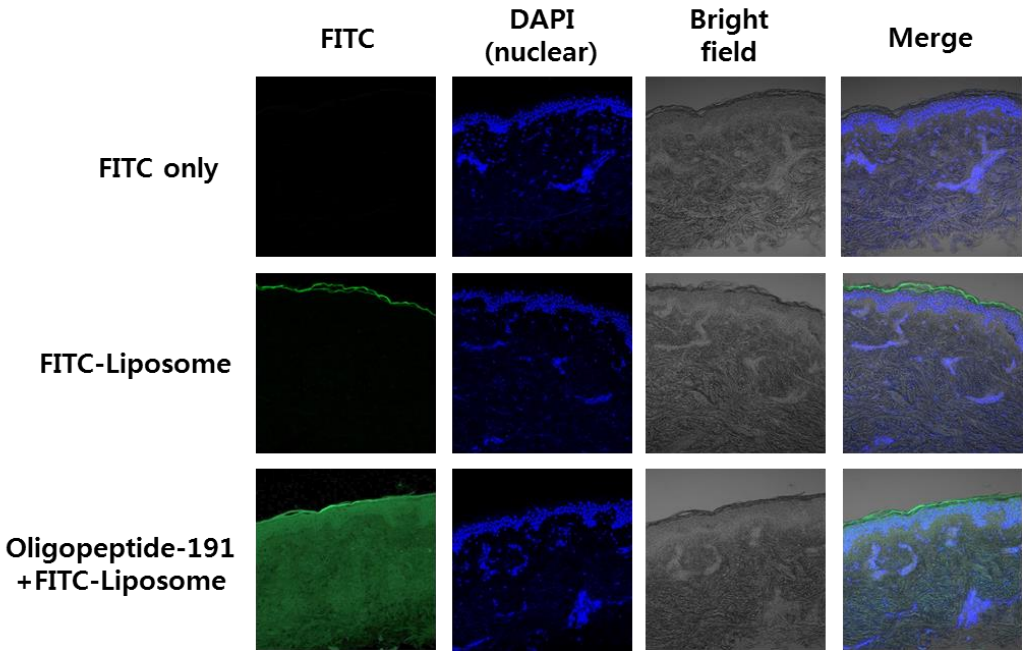

Figure 5. Enhance effect of Oligopeptide-191 on fluorescein liposome skin penetration in human cadaver skins. Human cadaver skins were treated with fluorescein liposome or Oligopeptide-191 for 3 hr. The skin slices were placed on cover slips using mounting medium with Hoechst (blue). All tissue samples were observed using confocal scanning laser microscopy. The data presented are representative of three independent experiments.

After assembling Oligopeptide-191 and FITC-hyaluronic acid (molecular weight 800 kDa HA) and treating it on human cadaver skin for 3 hr, a frozen section slide was prepared and analyzed by confocal analysis. It was confirmed that it moved to the dermis (Figure 6).

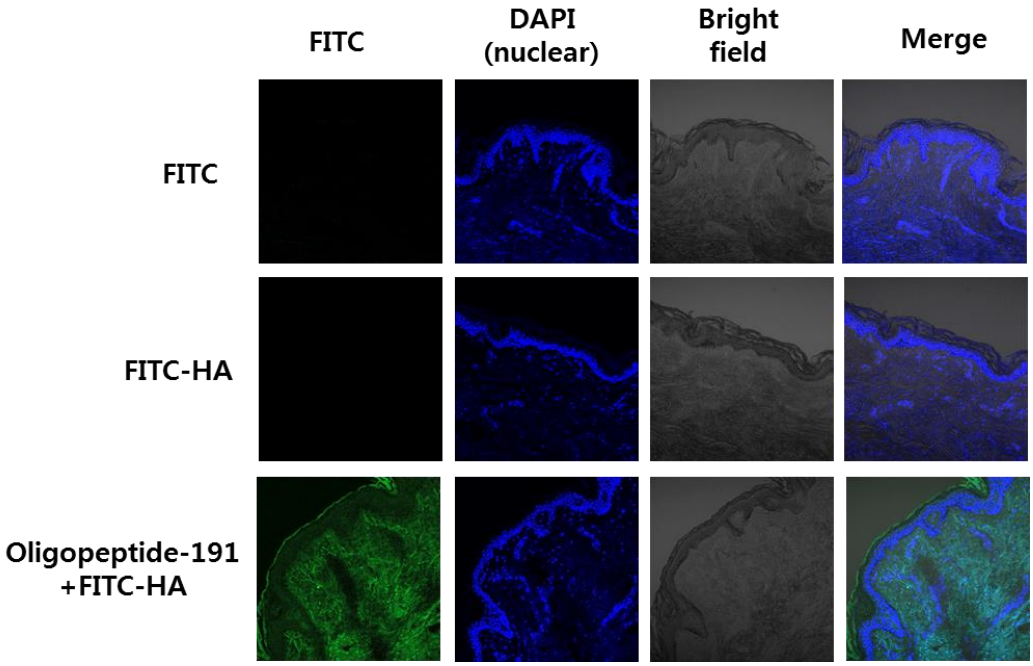

Figure 6. Enhance effect of Oligopeptide-191 on skin penetration in human cadaver skins. Human cadaver skins were treated with FITC conjugated hyaluronic acid or Oligopeptide-191 for 3 hr. (A) The skin slices were placed on cover slips using mounting medium Hoechst (blue). All tissue samples were observed using confocal scanning laser microscopy. The data presented are representative of three independent experiments.

After assembling Oligopeptide-191 and FITC-hyaluronic acid and treating it on cadaver skin for 3 hours, only the stratum corneum was made into a slide and analyzed by confocal analysis. As a result, it was found that migrates to lipids in the stratum corneum and then in the epidermal layer, cell-to-cell transmission (Figure 7).

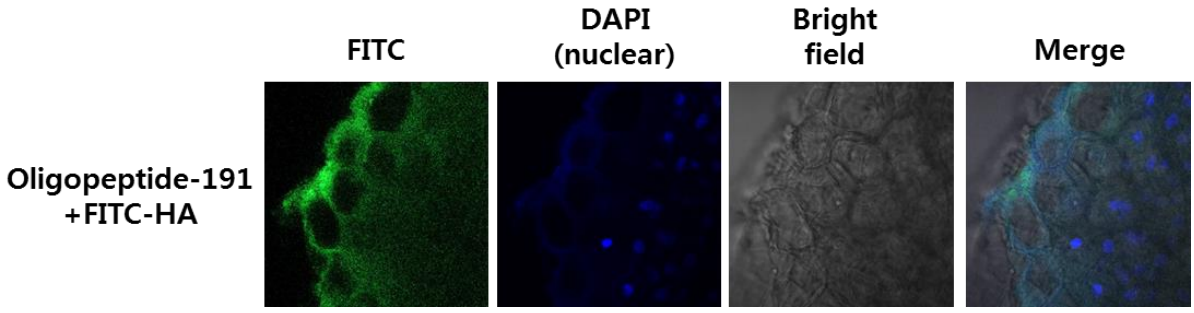

Figure 7. Skin surface fluorescence image of human cadaver skin. Human cadaver skins were treated with FITC conjugated hyaluronic acid for 3 hr. Skin surface frozen section were mounted using mounting medium with Hoechst (blue). Skins were than visualized using confocal laser scanning microscopy (10 X). The data presented are representative of three independent experiments.

Discussion.

Although the demand for anti-aging due to the increase in human lifespan continues to increase, the discovery of safe and effective biomaterials that can lead the market change is insufficient. Accordingly, if the development of TDDS cosmetic ingredients that can effectively penetrate the skin membrane is successfully achieved, it can lead the paradigm of cosmetics. Therefore, we studied to prove the effect of a novel peptide on TDDS [14,15]. First of all, in the study on the skin penetration study, all of cargo with peptide treated group, florescence was observed in dermis. In contrast in the cargo without peptide, was not observed in dermis. In the study on delivery capability by cargo siz, It has been investigated that novel peptide delivers all different size of cargo to the dermis, from 3 kDa collagen, the smallest size used in the experiment, to 800 kDa hyaluronic acid. In addition, the 200 nm liposome, is known to be the size that can capture dozens of 150 kDa of immunoglobulin, were delivered to the dermis. In the study on delivery capability by cargo properties at hydrophilic/hydrophobic surface, novel peptide delivered not only biopolymers closed to hydrophilic but also liposome closed to hydrophobic. In the study on pathway of skin permeation, as a result of analyzing the fluorescence of the SC and epidermis treated with hyaluronic acid, it was investigated that it penetrates through intercellular lipid in the SC and it penetrates through intracellular in epidermis. This result means that as the skin penetration pathway, cargo moves to the lipid of the stratum corneum and then the cell-to-cell transmission takes place in the epidermal layer [16,17]. In conclusion, when the Oligopeptide-191 and materials of various sizes and properties were assembled and then treated, it was confirmed that the materials moved to the dermis of the human cadaver skin.

The TDDS to be developed in this study is a cutting-edge biotechnology that greatly improves the absorption rate of raw materials and increases the bioavailability. We intend to apply this to the development of TDDS cosmetics by using existing functional materials.

Conclusion.

This study aims to report a novel peptide for TDDS and to demonstrate it has a capability to deliver to the dermis in various cargos, and to the human skin model. We used substances

of various sizes ranging from 3 k to 800 kDa and 200 nm of liposome as a cargo, which were also aimed at studying different chemical properties of cargo. (hydrophilic/hydrophobic). In order to investigate the skin penetration pathway, the SC and epidermis were peeled off from skin to analyze by confocal, it was confirmed that cargo penetrates through intercellular lipid in the SC and through intracellular (cell-to-cell) permeation in epidermis. It shows that the novel peptide delivers passed through skin barrier of two different characteristics through self-assembling. This study will contribute to the development of innovative cosmetics that deliver various substances to the dermis. It would be a kind of topical filler that deliver the biopolymers to the dermis with topical treatment and fat reduction technology also the technology that target cells in the dermis such as senolytic [18~20].

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Conflict of Interest Statement. NONE.

References.

1. Ji-Yeon Chung, Hyo-Sun Han, (2014) The Recent Trend of Percutaneous Absorption Used in Cosmetics. Kor. J. Aesthet. Cosmetol 12(5):597-605.
2. Jin Sun Kang, Ha Na La, et al (2019) Development of Dermal Transduction Epidermal Growth Factor (EGF) Using A Skin Penetrating Functional Peptide. J. Soc. Cosmet. Sci. Korea 45(2):175–184.
3. M. R. Prausnitz and R. Langer, (2008) Transdermal drug delivery. Nat. Biotech., 26(11):1261-1268.
4. Z. Nemes, P. M. Steinert, (1999) Bricks and mortar of the epidermal barrier. Experimental and Molecular medicine 31(1):5-19.
5. R. Vanbever, V. Preat, (1999) In vivo efficacy and safety of skin electroporation. Adv. Drug Deliv. Rev. 35(1):77-88.

6. Dubey V, Mishra D, Jain NK, (2007) Melatonin loaded ethanolic liposomes : physicochemical characterization and enhanced transdermal delivery. Eur. J. Pharm. Biopharm. 67:398-405
7. T. Hikima, S. Ohsumi, et al (2009) Mechanisms of Synergistic Skin Penetration by sonophoresis and iontophoresis. Biol. Pharm. Bull. 32(5):905-909.
8. Honeywell-Nguyen PL, Bouwstra JA, (2005) Vesicles as a tool for transdermal and dermal delivery. Drug Discovery Today. Technologies 2:67-74.
9. Elsayed MA, Abdallah Y, et al (2007) Deformable liposomes and ethosomes: Mechanism of enhanced skin delivery. Int. J. Pharm. 332:60-66.
10. M. Essendoubi,C. Gobinet, et al (2015) Human skin penetration of hyaluronic acid of different molecular weights as probed by Raman spectroscopy. Skin Research and Technology 22(1):55-62.
11. Huey-Jine Chai, Jing-Hua Li, et al (2010) Effects of Sizes and Conformations of Fish-Scale Collagen Peptides on Facial Skin Qualities and Transdermal Penetration Efficiency. BioMed Research International 2010:757301.
12. A. Eguchi, T Akuta, et al (2001) Protein transduction domain of HIV-1 Tat protein promotes efficient delivery of DNA into mammalian cells, J. Biol. Chem., 276(28), 26204-26210.
13. Jes Dreier, Jens A. Sørensen, Jonathan R. Brewer, (2016) Superresolution and Fluorescence Dynamics Evidence Reveal That Intact Liposomes Do Not Cross the Human Skin Barrier. PLOS ONE 11(1):e0146514.
14. Tejashree Waghulea, Gautam Singhvi, et al (2019) Microneedles: A smart approach and increasing potential for transdermal drug delivery system. Biomedicine & Pharmacotherapy 109:1249-1258.
15. Vineet Mathur, Yamini Satrawala, Mithun Singh Rajput, (2010) Physical and chemical penetration enhancers in transdermal drug delivery system. Asian Journal of Pharmaceutics https://doi.org/10.22377/ajp.v4i3.143.
16. Sorkin A, Puthenveedu MA, (2013). Clathrin-Mediated Endocytosis. Springer New York. pp. 1–31. doi:10.1007/978-1-4614-6528-7_1. ISBN 978-1-4614-6527-0.
17. Kaksonen M, Roux A, (2018). Mechanisms of clathrin-mediated endocytosis. Nature Reviews. Molecular Cell Biology 19(5):313–326.

18. Howe CL (2005). Modeling the signaling endosome hypothesis: why a drive to the nucleus is better than a (random) walk. Theoretical Biology & Medical Modelling 2 (1):43.
19. Aung Than,Ke Liang, et al (2017) Transdermal Delivery of Anti-Obesity Compounds to Subcutaneous Adipose Tissue with Polymeric Microneedle Patches. Small Methods 1(11): 1700269.
20. J L Kirkland 1 , T Tchkonia, (2020) Senolytic drugs: from discovery to translation. Journal of internal medicine 288(5):518-536.