Biology and Medicine
ISSN: 0974-8369

Takenokuchi et al., Biol Med (Aligarh) 2018, 10:5
DOI: 10.4172/0974-8369.1000448

Research Article Open Access

Evaluation of Absorbability of Macromolecular Substances in the Oral Mucosa and Skin using a Three-Dimensional Tissue Culture Model

Mariko Takenokuchi[1], Keiichi Kadoyama[1], Daisuke Yoshida[2], Shigeki Takaki[3], Ryoma Yamamoto[4], Katsuyasu Saigo[1] and Taizo Taniguchi[5*]

[1]*Faculty of Pharmaceutical Sciences, Himeji Dokkyo University, Himeji, Hyogo, Japan*

[2]*NIKKOL GROUP Nikoderm Research Inc, Nikko Chemicals Co. Ltd., 1-4-8, Nihonbashi-Bakurocho, Chuoku, Tokyo 103-0002, Japan*

[3]*Biostir Inc, Annex Shin-Osaka 4A, 2-9-1 Miyahara, Yodogawa-ku,Osaka 532-0003, Japan*

[4]*Amaze plus Co. Ltd., 451-0046 Nishi-ku Nagoya-shi Nishi-ku 5-21 Fujiko, Japan*

[5]*Pharmacrea Kobe Co. Ltd., Kobe, Hyogo 650-0047, Japan*

[*]**Corresponding author:** Taizo Taniguchi, Representative Director and President, Pharmacrea Kobe Co. Ltd., Kobe, Hyogo 650-0047, Japan, Tel: +81 90 8386 0288; E-mail: pharma-taniguchi@hotmail.com

Received date: July 11, 2018; **Accepted date:** August 17, 2018; **Published date:** August 24, 2018

Abstract

Introduction: Collagen exists in various connective tissues and helps to provide them mechanical strength and elasticity. The main component of the tendon is properly arranged collagen fibers withstanding very strong forces. The compactly packed collagen fine fibers inside the bone and cartilage increase their elasticity. Collagen also helps skin elasticity and strength. Hyaluronic acid widely exists in extracellular matrix, such as joints and skin. It plays an important role in maintaining cartilage function by making ultra-macromolecular complexes with aggrecan or protein. These collagen and hyaluronic acid decrease with age. Therefore oral supplementation or cosmetics containing them are developed day by day and catch interests of people trackleing anti-aging. The objective of this study was to evaluate and compare the absorbability of macromolecular substances in the oral mucosa and in the skin.

Methods: As animal experiments cannot be performed for the evaluation of cosmetics, we used a three-dimensional oral mucosa culture model and a three-dimensional epidermis culture model. The absorbability of macromolecular substances with molecular weights from 4,000 to 2,000,000 Da was compared between the oral mucosa model and the skin model.

Results: In the skin model, the amount permeated were quite low on all molecular weight. In the oral mucosa model, it absorbed much more compared with that of skin model, though the amount decreased according to the molecular weight.

Conclusion: This result was considered due to the absence of the stratum corneum in the oral mucosa. High absorbability through oral mucosa shows the possibility of a new absorption pathway of macromolecular substances.

Keywords: Absorbability of macromolecular substances; Oral mucosa; Collagen; Hyaluronic acid

List of Abbreviations:

HOE: Human Oral Epithelium; RHE: Reconstructed Human Epidermis; IgG: Immunoglobulin G

Introduction

Cosmetics or functional foods intended for anti-aging containing macromolecular substances such as collagen and hyaluronic acid are developed day bay day. Many people are spending huge money, believing that taken collagen or hyaluronic acid would play a role as collagen or hyaluronic acid in the body. We started our study to correct this superstition. In the skin, collagen exists mainly in the dermis and plays an important role of skin elasticity and strength [1,2]. Hyaluronic acid is a polysaccharide involved in moisture retention in the skin [1,2]. Although these macromolecular substances are expected to be absorbed into the skin from cosmetics, they are in fact poorly absorbed. As augmented in the 500-Da rule [3], the stratum corneum in the skin, just a few micrometers thick effectively forms a barrier and inhibited penetration of high molecules [4]. As foods, they are absorbed from the gastrointestinal tract. However, collagen and hyaluronic acid are broken down by digestive enzymes and finally absorbed in the body as amino acids or monosaccharides, respectively [5], and thus do not exert effects as macromolecules in the body. Accordingly, we propose an administration method through the oral mucosa [6]. Little enzymes that degrade collagen or hyaluronic acid exist in the oral cavity and the mucosa has higher absorption efficiency than the skin. We evaluated the oral mucosa as a novel absorption route for the delivery of macromolecular substances. For the evaluation, studies in the past mainly used skin or mucosa isolated from animals, but alternative methods have recently been recommended from the viewpoint of animal welfare. Particularly for cosmetics, use of alternative methods has been accelerated by regulations in the European Union, and guidelines for safety evaluation using a three-dimensional tissue model have been established [7]. This three-dimensional tissue model is prepared by culturing isolated human cells. Advantages using alternative methods

include reduction in variability due to individual differences in animals, in addition to ethical considerations.

Thus, we used two three-dimensional tissue models, HOE, an oral mucosa model and RHE, a skin model in this study.

Materials and Methods

Materials

Fluorescein isothiocyanate-dextrans (FD) with molecular weight 4,000Da (FD-4), 40,000Da (FD-40), 250,000Da (FD-250), 500,000Da (FD-500) and 2,000,000Da (FD-2000) were purchased from Sigma-Aldrich. Type-I collagen was purchased from Rockland Immunochemicals Inc. Fluoresceinamine-labeled sodium hyaluronate was purchased from PG Research.

An oral mucosa model, SkinEthic^{TM}-HOE, and an epidermis model, SkinEthic^{TM}-RHE, were purchased from EPISKIN.

Experiments

Absorption experiment using FDs: The outside of acclimated SkinEthic^{TM}-HOE (HOE) and SkinEthic^{TM}-RHE (RHE) models was treated with 150 µL each of 0.01% FD-4, FD-40, FD-250, FD-500 and FD-2000 solutions, and the models were incubated at 37°C for 6 hours. Phosphate-buffered saline (PBS)(-) was used as a receiver solution. At 0.5, 1, 2, 4 and 6 hours after the application, sampling from the receiver solution was performed and the amount of FD was determined. After completion of the study, frozen sections were prepared and observed with a fluorescence microscope.

Absorption experiment using collagen: The outside of acclimated HOE and RHE models was treated with 100 µL of 0.5 mg/mL type-I collagen (average molecular weight approximately 130,000), and the models were incubated at 37°C for 3 and 6 hours, respectively. PBS(-) was used as receiver solution. The amount of collagen in tissue and in receiver solution were determined by the way discussed in amount of collagen in tissue and amount of collagen permeation through tissue sections respectively.

Absorption experiment using hyaluronic acid: The outside of the acclimated HOE and RHE models was treated with 100 µL of 0.5% fluoresceinamine-labeled sodium hyaluronate (average molecular weight 600,000 to 1,120,000), and the models were incubated at 37°C for 3 and 6 hours, respectively. PBS(-) was used as receiver solution. The amount of hyaluronic acid in tissue and in receiver solution were determined by the way discussed in amount of hyaluronic acid in tissue and amount of hyaluronic acid permeation through tissue sections respectively.

Observation of tissue sections (FDs and hyaluronic acid): The rinsed tissue models were embedded in a compound for the preparation of frozen sections, placed in a plastic mold and frozen. The compound removed from the plastic mold was fixed on a sample stage in a device for the preparation of frozen sections (cryostat). A section of each applied sample was prepared so that the thickness was 4 µm, and then the section was mounted on a glass slide and observed with a fluorescence microscope (FDs, Ex/Em=490/514 nm; hyaluronic acid, Ex/Em=494/520 nm).

Observation of tissue sections (collagen): The tissue models were frozen in M.E CRYO COMPOUND, and were subjected to immunostaining of sections. Frozen tissue sections with a thickness of 4 µm were prepared and mounted on a glass slide. The tissue section on the glass slide was fixed with 4% paraformaldehyde, followed by blocking with 1.0% bovine serum albumin. Then, the tissue section was treated with a 100-fold diluted anti-human type-I collagen rabbit antibody at 4°C overnight (primary antibody reaction), and treated with 500-fold diluted anti-rabbit IgG goat Alexa Fluor488 at 37°C for 1 hour (secondary antibody reaction). The immunostained tissue section was observed with a fluorescence microscope for fluorescence derived from type-I collagen applied to the outside (100 × magnification; BP 485, FT 510).

Determination

Amount of FD in tissue: The application surface of the HOE and RHE test samples was rinsed after completion of the study with 1.5 mL (0.5 mL × 3 times) of purified water by pipetting. The rinsed tissue was transferred into an Eppendorf tube, and 1 mL of purified water and stainless beads were added, followed by crushing with TissueLyser (Qiagen). Then, the fluorescence intensity (Ex/Em=490/514 nm) of the supernatant obtained by centrifugation was measured with a microplate reader.

Amount of FD permeation in tissue: The fluorescence intensity (Ex/Em=490/514 nm) of the collected PBS(-) was measured with a microplate reader.

Amount of collagen in tissue: The application surface of the HOE and RHE test samples was rinsed after completion of the study with 1.5 mL (0.5 mL × 3 times) of purified water by pipetting. The rinsed tissue was transferred into an Eppendorf tube, and 1 mL of purified water and stainless beads were added, followed by crushing with TissueLyser. Then, the amount of type-I collagen in the supernatant obtained by centrifugation was measured by enzyme-linked immunosorbent assay (ELISA).

Amount of collagen permeation through tissue: The amount of collagen in receiver solution was measured by ELISA.

Amount of hyaluronic acid in tissue: The application surface of the HOE and RHE test samples was rinsed after completion of the study with 1.5 mL (0.5 mL × 3 times) of purified water by pipetting. The rinsed tissue was transferred into an Eppendorf tube, and 1 mL of purified water and stainless beads were added, followed by crushing with TissueLyser. Then, the fluorescence intensity (Ex/Em=494/520 nm) of the supernatant obtained by centrifugation was measured with a microplate reader.

Amount of hyaluronic acid permeation through tissue: The fluorescence intensity (Ex/Em=490/514 nm) of receiver solution was measured with a microplate reader.

Results

Comparison of absorbability using different molecular weights: Figure 1 shows the amount of FD tissue permeation, and Figure 2 shows the amount in tissue. For all FDs, HOE showed higher tissue permeation and a higher amount in tissue compared with those in RHE. In both HOE and RHE, the tissue permeation decreased depending on molecular weight. The amount in tissue decreased depending on molecular weight in RHE, while the amount in tissue increased with an increase in the molecular weight in HOE (Figure 3).

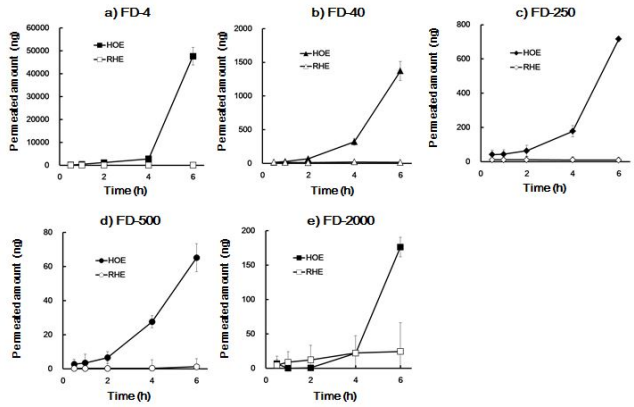

Figure 1: Dextran permeating through tissue.

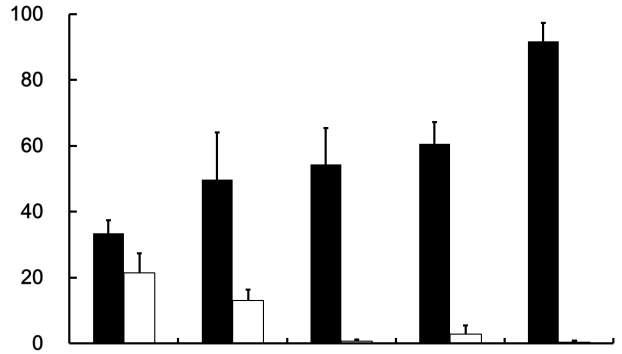

Figure 2: Dextran remaining in tissue.

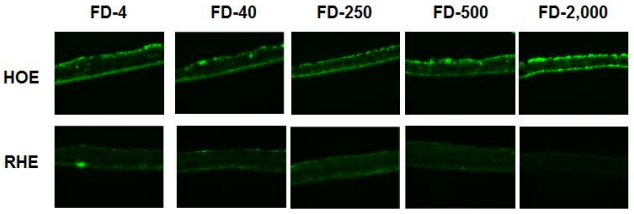

Figure 3: Localization of dextran in tissue.

Absorbability of collagen and hyaluronic acid: Figure 4 shows the tissue permeation of collagen and hyaluronic acid, and Figure 5 shows the amount remaining in tissue. The tissue permeation was higher in HOE for both collagen and hyaluronic acid. In tissue, however, the amount of collagen was higher in RHE, and the amount of hyaluronic acid was higher in HOE (Figure 6). This unexpected observation will be discussed later.

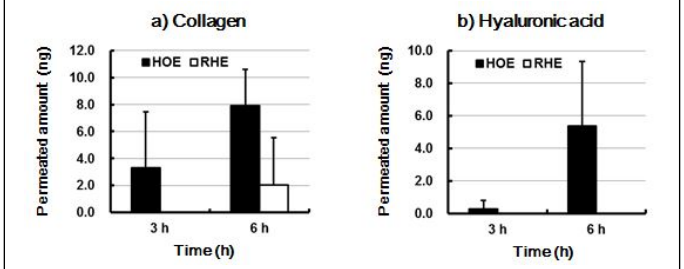

Figure 4: Collagen and hyaluronic acid permeating through tissue.

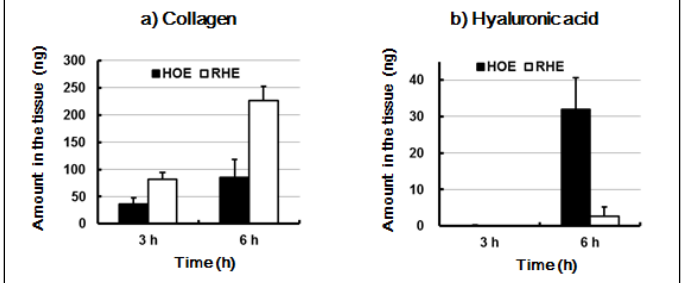

Figure 5: Collagen and hyaluronic acid remaining in tissue.

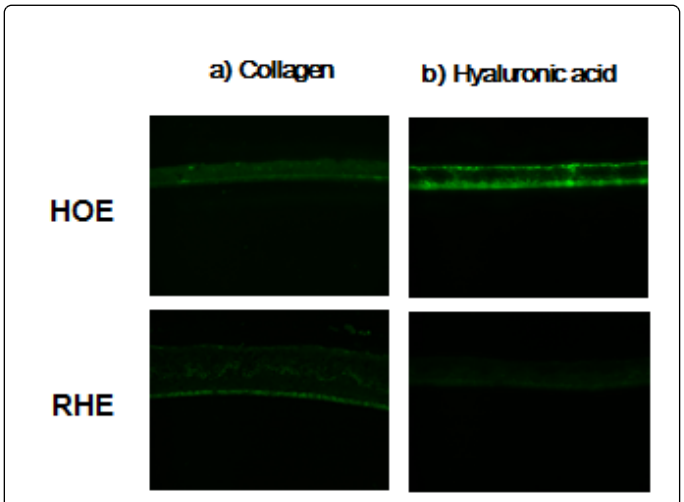

Figure 6: Localization of collagen and hyaluronic acid in tissue.

Discussion and Conclusion

Collagen and hyaluronic acid play a key role as components of many tissues such as skin, cartilage and bone. However, these decrease with aging [2]. Thus, maintenance of the amount of collagen and hyaluronic acid in the body may aid in the prevention of aging. Therefore, in order to prevent various phenomena caused by a decrease in collagen and hyaluronic acid, namely an increase in wrinkles in the skin and decrease in the cartilage in joints, many cosmetics and health foods containing collagen and hyaluronic acid have been developed and are regularly taken by many people [8-10]. Cosmetics are expected to be

absorbed from the skin and foods are expected to be absorbed from the gastrointestinal tract. Therefore, in the case of cosmetics, whether or not macromolecules such as collagen and hyaluronic acid are actually absorbed into the skin must be determined.

Among the epithelial tissues, the skin has a stratum corneum and its original function is to act as a barrier to protect areas inside the body from the external world [11]. Meanwhile, mucosal tissues have no or little stratum corneum and high absorbability, and their sole function is the nutrient uptake from the surrounding environment [12]. In fact, as shown in Figure 1, macromolecular substances show almost no permeability in the skin model, and as shown in Figure 2, only a few low-molecular-weight substances are observed in tissue. On the other hand, in the oral mucosa model, although tissue permeation decreases according to molecular weight, the absorbability is significantly higher than that in skin. The amount in tissue increased in proportion to molecular weight in the oral mucosa model. This may be because higher-molecular-weight substances move slower and increasingly remain in the tissue (Figures 1 and 2). After completion of the experiments, tissue sections were prepared to confirm permeability under fluorescence, and the oral mucosa model showed higher permeability for all molecular weights (Figure 3).

Similar studies were also conducted for collagen and hyaluronic acid. For hyaluronic acid, both tissue permeation and the amount in tissue were higher in the oral mucosa model, consistent with the evaluation results of the effects of molecular weight (Figures 4 and 5). Meanwhile, as for collagen, the permeation amount was higher in the oral mucosa model, but the amount in tissue was higher in the skin model, contrary to expectations (Figures 4 and 5). Therefore, we further examined the localization of collagen in the tissue. Collagen was found to remain in the middle layer of the skin (Fig. 6). This means that collagen applied to the skin did not reach the dermal layer, which is the original site where collagen plays roles. In other words, applied collagen is present in the stratum corneum and is expected to be effective for maintaining the water-retention capacity of the skin, but is not expected to contribute to the elasticity of the dermis. In the oral mucosa, some collagen reached the dermis, though whether they play roles there as collagen is not clear from the result.

We should keep in mind that this is only speculation based on the results of alternative experiments, where *in vivo* investigations cannot be performed from the viewpoint of animal welfare.

In conclusion macromolecular substances such as collagen and hyaluronic acid are not absorbed from the skin and the oral mucosa would be superior to the skin as a pathway to incorporate macromolecular substances.

References

1. Fore J (2006) A review of skin and the effects of aging on skin structure and function. Ostomy Wound Manage 52: 24-35.
2. Farage MA, Miller KW, Elsner P, Maibach HI (2008) Intrinsic and extrinsic factors in skin ageing: a review. Int J Cosmet Sci 30: 87-95.
3. Bos JD, Meinardi MMHM (2000) The 500 Dalton rule for the skin penetration and of chemical compounds and drugs. Exp Dermatol 9: 165-169.
4. Kolarsick PAJ, Kolarsick MA, Goodwin C (2011) Anatomy and Physiology of the Skin. J Dermatol Nurses' Assoc 3: 203-213.
5. Goodman BE (2010) Insights into digestion and absorption of major nutrients inhumans. Adv Physiol Educ 34: 44-53.
6. Montenegro-Nicolini M, Morales JO (2017) Overview and Future Potential of Buccal Mucoadhesive Films as Drug Delivery Systems for Biologics. AAPS PharmSciTech 18: 3-14.
7. EU Cosmetics Directive 76/768/EEC.
8. Asserin J, Lati E, Shioya T, Prawitt J (2015) The effect of oral collagen peptide supplementation on skin moisture and the dermal collagen network: evidence from an ex vivo model and randomized, placebo-controlled clinical trials. J Cosmet Dermatol 14: 291-301.
9. Addor FAS, Cotta Vieira J, Abreu Melo CS (2018) Improvement of dermal parameters in aged skin after oral use of a nutrient supplement. Clin Cosmet Investig Dermatol 11: 195-201.
10. Kalman DS, Heimer M, Valdeon A, Schwartz H, Sheldon E (2008) Effect of a natural extract of chicken combs with a high content of hyaluronic acid (Hyal-Joint®) on pain relief and quality of life in subjects with knee osteoarthritis: a pilot randomized double-blind placebo-controlled trial. Nutr J 7: 3.
11. Hänel KH, Cornelissen C, Lüscher B, Baron JM (2013) Cytokines and the Skin Barrier. Int J Mol Sci 14: 6720-6745.
12. Van Spaendonk H, Ceuleers H, Witters L, Patteet E, Joossens J, et al. (2017) Regulation of intestinal permeability: The role of proteases. World J Gastroenterol 23: 2106-2123.