Note

Non-invasive Intradermal Delivery of Hyaluronic Acid *via* Iontophoresis

Shinya Inoue,[a] Yasufumi Oshima,[a] and Kentaro Kogure*[,b]

[a] *Graduate School of Pharmaceutical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan: and*
[b] *Graduate School of Biomedical Sciences, Tokushima University, 1 Shomachi, Tokushima 770–8505, Japan.*
Received June 15, 2023; accepted September 7, 2023

Hyaluronic acid (HA) is a hydrophilic supra-macromolecule, with a molecular weight (MW) 1000000<. HA is recognized as a biomaterial for skin moisturization. HA solution is typically injected into the skin using a needle. However, needle injection is invasive and does not result in homogeneous distribution of HA over a large area of skin. Therefore, non-invasive and effective technologies for homogenous intradermal delivery of HA are needed. Recently, we demonstrated the use of iontophoresis (ItP) for non-invasive intradermal delivery of various macromolecules, such as small interfering RNA (siRNA) (MW: 12000) and antibodies (MW: 150000). Based on our previous studies, we hypothesized that HA can also be delivered non-invasively into the skin by ItP. In this study, we applied ItP to fluorescence-labeled HA (MW: 600000–1120000 and 1200000–1600000) on rat dorsal skin. Following treatment, fluorescence was observed to be widely distributed in the skin, demonstrating successful intradermal delivery of HA *via* ItP. In addition, the relative moisture content and elasticity of skin treated with ItP/HA was temporarily higher than that of control skin. This is the first report demonstrating successful non-invasive intradermal delivery of HA and improvement of skin conditions by high-molecular weight HA delivered by ItP. In conclusion, ItP would be a useful technology for non-invasive intradermal delivery of high-molecular weight HA for treatment of skin diseases and cosmetology applications.

Key words iontophoresis, hyaluronic acid, non-invasive intradermal delivery

INTRODUCTION

Hyaluronic acid (HA) is a long polysaccharide composed of repeating units of *N*-acetyl-D-glucosamine and glucuronic acid. HA is a hydrophilic supra-macromolecule, with a molecular weight (MW) ranging from 5000 to more than 1000000. HA is a component of the extracellular matrix (ECM) in connective, neural and epithelial tissues, and is recognized as a biocompatible and biodegradable material.[1,2] HA is a hygroscopic molecule, capable of binding water at 1000 times its volume. Due to its hydrophilic nature, HA can be used as a moisturizing component in skincare formulations. It has been reported that low-molecular weight HA (MW: around 5000–50000) is able to penetrate into the stratum corneum and epidermis layers of the skin. The mechanism of penetration of low-molecular weight HA is suggested to be *via* receptor-mediated transdermal delivery, hydration of the stratum corneum and hydrophobic interactions with the stratum corneum.[3] However, penetration of high-molecular weight HA (MW: 600000<) through the skin is difficult.[3] Distribution of high-molecular weight HA inside the skin may allow for improvements in skin moisture and elasticity. HA is typically delivered by injection of HA solution into the skin using a needle. However, the needle injection method is invasive and can be painful. Non-invasive and effective technologies would be the ideal administration method for delivering HA into the skin.

Iontophoresis (ItP) is a non-invasive intradermal delivery technology that has been recognized as a useful technology in the field of drug delivery systems. ItP relies on the use of weak electric current to facilitate penetration of charged molecules across the skin by electric repulsion and electroosmosis.[4] Molecular characteristics that are generally compatible with ItP include hydrophobic, ionic and small molecular weight molecules; however, we recently succeeded in the intradermal delivery of hydrophilic macromolecules, such as small interfering RNA (siRNA) (MW: 12000)[5] and antibodies (MW: 150000).[6] The mechanism of intradermal penetration of hydrophilic macromolecules by ItP was found to occur by weak electric current-induced cleavage of intercellular junctions, such as gap-junction and tight-junction, *via* activation of intracellular signal transduction pathways.[7,8] Thus, we expected that HA should be able to be delivered non-invasively into the skin by ItP because of the negative charge on the carboxyl group,[1] even though its molecular weight is much higher than that of siRNA and antibodies.

In the present study, we examined the non-invasive intradermal delivery of high-molecular weight HA (MW: 600000<) *via* ItP on rat dorsal skin. In addition, we evaluated the moisture and elasticity of the skin following ItP of high-molecular weight HA. This is the first report of non-invasive and effective intradermal delivery of high-molecular weight HA.

MATERIALS AND METHODS

Materials and Animals Fluorescein amine-labeled sodium hyaluronates of varying molecular weights (MW: 600000–1120000 and 1200000–1600000) were purchased from PG research Inc. (Tokyo, Japan). Non-labeled sodium hyaluronates (MW: 1000000<) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All reagents used in this study were special grade. Seven-week-old male HWY/Slc hairless rats and Wistar rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). All animal protocols were evaluated and approved by the Animal and Ethics Review Committee of

* To whom correspondence should be addressed. e-mail: kogure@tokushima-u.ac.jp

Tokushima University.

ItP of HA on Rat Dorsal Skin ItP of HA was performed according to our previous report.[6] Rats were anesthetized by isoflurane inhalation, and chloral hydrate dissolved in phosphate-buffered saline (PBS) (50 mg/mL) was administered (400 mg/kg rat) by intraperitoneal injection. HWY/Slc hairless rats were used for ItP of fluorescently-labeled HA. Wistar rats with shaved dorsal skin hair were used for ItP of non-labeled HA to evaluate moisture content and elasticity of the skin. At first, we tried using hairless rats, but we could not measure moisture and elasticity. Because we thought that the reason for this was that skin was hardened by exposure to the outside, we used Wistar rats. For ItP of HA, nonwoven fabric (4 cm^2) containing 0.35 mg (in 350 μL PBS) of fluorescently-labeled HA solution was placed as a cathode on the dorsal skin of rats, and nonwoven fabric moistened with 350 μL of PBS was placed on the skin as an anode. Each nonwoven fabric containing fluorescently-labeled HA or PBS was attached to Ag–AgCl electrodes (3M Health Care, Minneapolis, MN, U.S.A.) with surface areas of 4 cm^2 connected to a power supply (TCCR-3005, TTI Ellebeau Inc., Tokyo, Japan). ItP was performed with a constant current of 0.4 mA/cm^2 for 1 h. After ItP treatment, we removed electrodes while lightly wiping off the formulation with non-woven fabric attached to the electrodes. Moisture and elasticity of the skin after ItP of HA were evaluated by a commercially-available skin checker device designed for human use (PM-907, Peipai, Shenzhen, China).

Observation of Skin Sections Following ItP of HA The skin under the cathode was collected 3 h after ItP application with fluorescently-labeled HA, and then embedded in OCT compound (Sakura Finetek Japan Co.,Ltd., Tokyo, Japan), followed by freezing with a dry ice–ethanol bath. Then, frozen tissue sections (10 μm) were prepared with a cryostat. After mounting the sections with Dako Fluorescence Mounting Medium (Dako North America, Inc., Carpinteria, CA, U.S.A.), fluorescence in the skin section was observed using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

Statistical Analysis Statistical differences were evaluated by one-way ANOVA with Tukey's *post hoc* test. Data are presented as mean ± standard deviation (S.D.). Differences for which $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

In this study, we used fluorescently-labeled HA to determine the intradermal distribution of exogenous high-molecular weight HA following application with ItP. We first sought to detect HA in the skin by an immunohistochemical method using anti-HA antibody; however, it was difficult to distinguish between HA that had penetrated from outside the skin from that originally present in the skin using this method. We therefore used fluorescently-labeled HA as an easy marker in the skin, and observed skin sections following ItP application by confocal laser scanning microscopy.

We applied ItP to fluorescently-labeled HA, with molecular weights ranging from 600000 to 1120000, on the dorsal skin of hairless rats. As shown in Fig. 1c, green fluorescence associated with labeled HA was observed to be widely distributed in the skin section following ItP. In contrast, no significant

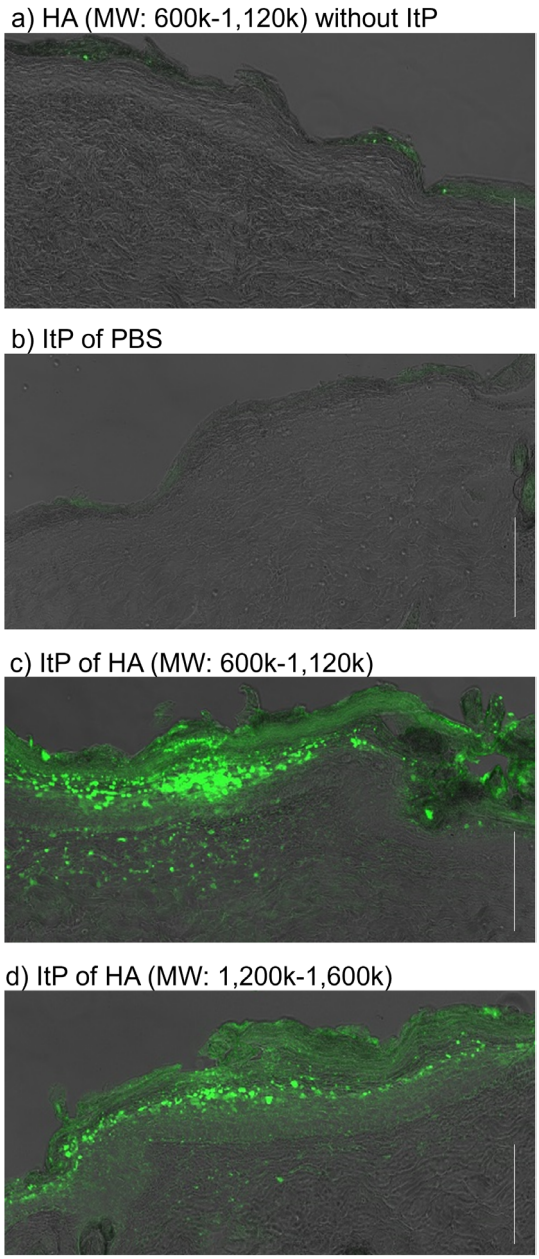

Fig. 1. Intradermal Distribution of Fluorescein Amine-Labeled HA after ItP

Skin sections a) applied 600000–1120000 Fluorescein amine-labeled HA without ItP and b) iontophoretic treatment alone. Skin sections after ItP treatment with c) 600000–1120000 Fluorescein amine-labeled HA and d) 1200000–1600000 fluorescein amine-labeled HA. Frozen skin sections following ItP treatment with HA were observed by confocal laser scanning microscopy. Green fluorescence indicates fluorescein amine-labeled HA. The white scale bar denotes 100 μm.

fluorescence signal was observed in the skin treatment for both topical skin application of fluorescently-labeled HA and iontophoretic treatment alone (Figs. 1a, b). HA fluorescence intensity was strongest in the epidermis layer (100 μm from the surface). Further, green fluorescence was even observed in deep regions of the skin (at depths of approximately 200 μm or more) (Fig. 1c). Taken together, these results demonstrate that even high-molecular weight HA can be delivered into the skin by ItP, and widely distributed, penetrating into the epidermis and dermis layers.

We also investigated intradermal delivery of even higher-molecular weight HA (MW: 1200000–1600000) by ItP. As

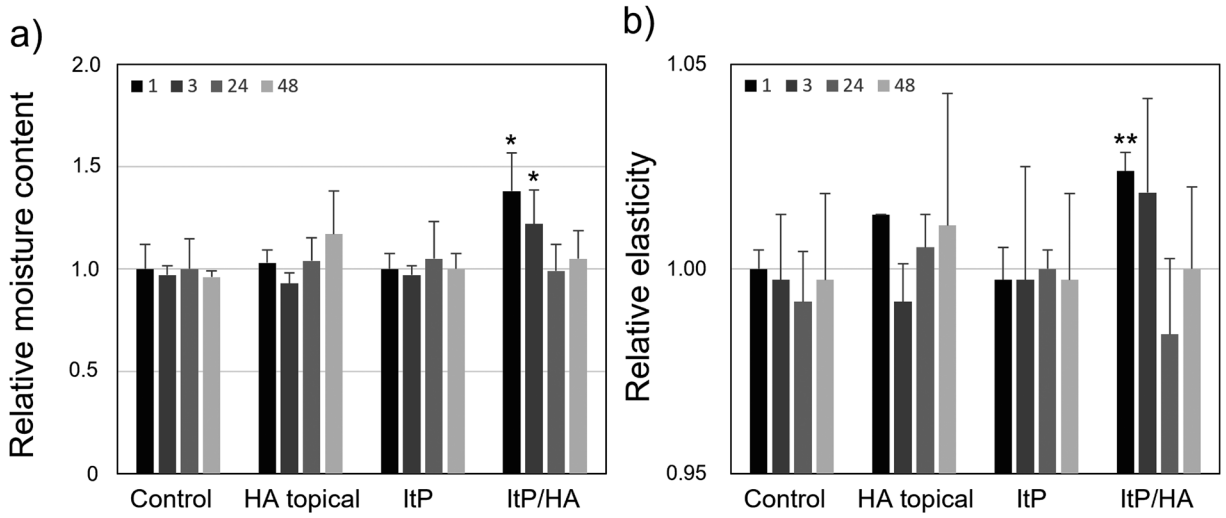

Fig. 2. Effect of ItP Treatment with HA on Moisture Content and Elasticity of the Skin

Relative values for a) moisture content and b) elasticity of rat skin, as measured at 1, 3, 24, and 48 h after ItP application with non-labeled HA (MW: 1000000<) by a skin checker device designed for human use. Control, HA topical, ItP and ItP/HA indicate non-treated skin, skin treated with topical HA, skin treated with ItP without HA, and skin treated with ItP and HA, respectively. The data represent mean ± S.D. calculated from at least three different experiments. *$p < 0.05$ *vs.* Control and ItP and **$p < 0.01$ *vs.* Control, HA topical and ItP.

shown in Fig. 1d, green fluorescence associated with labeled HA was observed in the skin, although the intensity of the fluorescence was weaker than that seen for HA with molecular weights of 600000–1120000 (Fig. 1c). Fluorescence was also seen in the deep region (dermis) of the skin, indicating that ItP can non-invasively deliver even higher-molecular weight HA (MW: 1200000–1600000) into the skin. The mechanism associated with effective permeation of high-molecular weight HA into deep regions of the skin is suggested to be due to cleavage of the intercellular junction of skin tissue *via* activation of intracellular signal transduction pathways induced by the weak electric current of ItP, as suggested in our previous report.[7)]

We also evaluated the effect of ItP treatment with non-labeled HA (MW: 1000000<, 1 mg/mL) on the relative moisture content and elasticity of the skin at different time points after ItP. ItP treatment alone did not affect skin moisture (Fig. 2a). The relative moisture content at 1 and 3 h after ItP treatment with HA was higher than that of control (non-treated) skin, skin treated with topical HA and ItP without HA. In the topical HA, significant fluorescence signal was hardly observed in the skin surface (Fig. 1a). Therefore, "HA topical" did not affect moisture content. Furthermore, the relative elasticity of skin at 1h after ItP treatment with HA was significantly higher than that of control skin, skin treated with topical HA and ItP alone (Fig. 2b). These results indicate that HA delivered intradermally by ItP can improve temporarily the moisture content and elasticity of the skin. Based on our previous report,[9)] some of the HA delivered intradermally may be taken up by endocytosis, but it seems that most of HA is distributed extracellularly because of its large molecular weight. We think this extracellularly distributed HA affects the water content and elasticity.

CONCLUSION

In summary, the present study we demonstrated that intradermal delivery of hyaluronic acid, a hydrophilic supra-macromolecule, is possible using ItP. The delivered HA observed in deep skin at depths of approximately 200 μm. Transdermal delivery of HA temporarily enhanced skin functionality of moisture content and elasticity. Taken together, these results suggest that ItP is a useful technology for non-invasive and effective intradermal delivery of high-molecular weight HA to improve skin conditions for treatment of skin diseases and for cosmetology applications.

Acknowledgments This study was supported by a research program for the development of intelligent Tokushima artificial exosome (iTEX) from Tokushima University, Japan.

Conflict of Interest The authors declare no conflict of interest.

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