Permeation of hyaluronan tetrasaccharides through hairless mouse skin: an *in vitro* and *in vivo* study

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Abstract Hyaluronan (HA) is a well-known active ingredient for cosmetic and drug applications. However, based on its varying molecular size, HA may have limited skin permeation. Therefore, the aim of the present study was to investigate the *in vitro* skin permeability of HA tetrasaccharide (HA4). In addition, the effects of HA4 on *in vivo* skin barrier function were examined. The cumulative amounts of HA4 through stratum corneum (SC)-stripped skin and full-thickness skin after 8 h were 2109.6 μg/cm^2 and 0.8 μg/cm^2, respectively. Furthermore, the cumulative amounts of HA4 permeated after 8 h were 784.4 ng/cm^2 for a HA4 solution with a pH 4 and 70.0 ng/cm^2 with a pH 7 on full-thickness skin. Next, the *in vivo* effects of HA4 on the water content of the SC and transepidermal water loss (TEWL) were investigated. The dorsal skins of hairless mice were irradiated to a UVA dose of 22.3 J/cm^2/d, 5 times a week. In the control group, the water content of the SC was decreased and TEWL and epidermal thickness were increased with UVA irradiation than the normal group. However, the water content of the SC was increased in the HA4 group than that of the control group in the non-UVA irradiation groups. In addition, the water content of the SC was increased and TEWL and epidermal thickness were decreased in the HA4 group than those of the control and HA groups. These results suggest that treatment with HA4 improved skin functional recovery after UVA irradiation by skin penetration of HA4.

Keywords: Hyaluronan oligosaccharide · Skin permeation · Barrier function · UVA · Transepidermal water loss · Water content of stratum corneum

Introduction

The stratum corneum (SC), the uppermost layer of the skin, functions as a primary barrier against the penetration of compounds. When formulations are applied on the skin surface, they are generally permeated through the skin barrier by passive diffusion. Generally, skin permeability of lipophilic compounds is higher than that of hydrophilic compounds. Molecular weight is also an index which affects the skin permeation profiles of compounds. Only low molecules, usually less than 500 Da, can penetrate the skin membrane [8]. However, tacrolimus (804.02 Da), used in the treatment of atopic eczema, is reported to have a low skin permeation [1].

Hyaluronan (HA), which is composed of repeated β-1,4-glucuronic acid-β-1,3-*N*-acetylglucosamine disaccharide units, is a non-sulfated glycosaminoglycan with a molecular weight of over 1,000 kDa. HA is abundant in the extracellular matrix of the skin. HA exists freely in extracellular matrix spaces, but is also involved in many biological processes such as tissue homeostasis, cell proliferation, cell migration, cell differentiation, angiogenesis, tumor biology, and repair processes by the interface of each protein [23]. The amount of HA in the skin is equivalent to 50% or more of the total amount of HA in the body.

HA plays different biological roles, depending on its molecular weight. High molecular weight HA elicits anti-inflammatory and anti-angiogenic responses [3, 20]. In contrast, low molecular weight HA has been implicated in several biological processes including angiogenesis, cell proliferation, maturation, migration, activation of protein tyrosine kinase cascades, and inflammatory gene expression [5, 6, 9, 17, 18, 21, 22]. HA oligosaccharides, which are smaller than low molecular weight HA, up-regulate heat shock protein 72 expression [27].

The amount of ultraviolet (UV) A radiation reaching the earth's surface is approximately 20 times greater than that of UVB radiation, and solar UVA radiation contributes to photoaging and photocarcinogenesis. UVB irradiation to the skin induces a variety of responses including erythema, hyperproliferation of keratinocytes, and skin barrier function alterations. UVB irradiation also induces disruptions in epidermal permeability barrier function. Diminished permeability barrier function has been reported in response to UVB; combined UVA and UVB; or UVC. UV irradiation of either human or rat skin results in increased percutaneous absorption of xenobiotics [7].

Due to its strong water binding potential, HA is a well-known active ingredient for cosmetic and

drug applications. Cosmetics combined with HA impede transepidermal water loss from skin by forming a coated skin surface. Additionally, injectable HA fillers are well known for improving skin contour defects related to aging (wrinkles and lines), depressed acne scars, and other traumatic or congenital conditions. However, based on its varying molecular size, skin penetration of HA may be limited.

Therefore, we focused our attention on a tetrasaccharide (HA4) containing two units, with a single unit being -β-1,4-glucuronic acid- β-1,3-*N*-acetylglucosamine, and the *in vitro* skin permeability of HA4 across hairless mice skins was assessed. In addition, the *in vivo* effect of HA4 on skin function was investigated.

Materials and methods

Materials

HA4 (99.14%) (776.3 Da) were provided by Glycoscience Laboratories Inc. (Tokyo, Japan). High-molecular weight hyaluronan (HA) (>1200 kDa) was used. All other chemicals and solvents used were analytical grade.

Animals

Seven-week-old male hairless mice (HR-1) were purchased from Hoshino Experiment Animal Center (Ibaraki, Japan). All experiment animals had free access to food and water, and were housed in rooms where the lighting was automatically regulated on a 12 hour light and dark cycle. All animal experiments and maintenance were performed under conditions approved by the animal research committee of Josai University.

HA4 sample preparation

A test solution was prepared with 1 mL of distilled water containing HA4 at three different concentrations: 0.02, 0.1, and 0.5%. Additionally, pH 4 and pH 7 adjustment of 0.5% HA4 solution was performed using formic acid and 1.5 M ammonium acetate.

Skin membrane preparations

Permeation studies were conducted with excised intact hairless mouse skin (8-10 weeks old) as a diffusion membrane. To obtain SC-stripped skin, adhesive tape was applied to hairless mouse skin with uniform pressure. The procedure was repeated about 20 times, until the SC was entirely removed from the skin.

In vitro permeation experiments

The diffusion cell used in this study was a modified Franz type diffusion cell. The receiver compartment had a volume of 5 mL, and the effective surface area was 1.77 cm^2. Donner solution was applied with 1 mL of distilled water containing HA4. The receiver solution was distilled water (5 mL) and was stirred by a magnetic bar. The receiver solution was maintained at a temperature of 32ºC using a thermally controlled circulating water bath. At appropriate intervals, 300 μL aliquots of the receiver medium were withdrawn and immediately replaced by an equal volume of fresh distilled water. Samples were frozen at -18ºC prior to assay of HA4 using LC/MS.

Determination of HA4

Determination of HA4 was performed using a LC/MS system. Mass spectra were obtained using an LCQ DECA XP plus (Thermo Fisher Scientific Inc., MA, USA). The electrospray interface was set in negative ionization mode with 10 mM ammonium acetate containing methanol (8:2). LC/MS separation was done using a Paradigm MS4 (Microm Bioresources Inc., CA, USA), and performed on a TSK-gel ODS-80Ts column (2.0×150 mm) from TOSOH (Tokyo, Japan). The flow rate was remained at 100 μL/min throughout the assay.

Animal treatment and UVA irradiation

Hairless mice were topically treated with either 0.1% HA or HA4 in 70% ethanol or 70% ethanol alone after UVA irradiation. Hairless mice were divided into the following 4 groups (n=3 in each group): group 1, no treatment and no UVA irradiation (Normal/UVA(-)); group 2, vehicle application after irradiated with UVA (Control/UVA(+)); group 3, application of HA after irradiated with UVA (HA/UVA(+)); group 4, application of HA4 after irradiated with UVA (HA4/UVA(+)). The dorsal skin of adult hairless mice aged 8-10 weeks was irradiated using a high performance UV transilluminator UVP® (Funakoshi, Tokyo, Japan). The distance between the UV lamps and the dorsal skin of mice was approximately 5 cm. Hairless mice were exposed to a UVA dose of 22.3 J/cm^2/day five times a week for three weeks. Hairless mice were sacrificed by cervical dislocation after the last measurement of transepidermal water loss (TEWL), water content of the SC, and skin viscoelasticity. Full thickness dorsal skins were removed and stored at -80ºC for further analysis.

Evaluation of skin properties

All measurements were performed in triplicate for each skin, and the mean values were obtained.

Measurement of the water content of the SC: To measure the water content of the SC, a Cutometer® MPA 580 (Courage+Khazaka, Cologne, Germany) was used. The procedure was performed five times a week for three weeks after the course of UVA irradiation. Measurement of TEWL: To measure cutaneous water evaporation, a VAPO SCAN AS-VT100RS (Asahibiomed, Yokohama, Japan) was used. The procedure was performed five times a week for three weeks after the course of UVA irradiation.

Histological procedures

Cryosections were prepared from tissue samples in optimal cutting temperature (OCT) embedding compound. Hairless mice skin sections (6 μm) were stained with haematoxylin and eosin (HE), and analyzed for structural differences using light microscopy. Epidermal thickness was defined as the distance (in μm) from the top of the stratum granulosum to the bottom of the stratum basale. Thickness of the SC was measured from the top of the SC to the bottom. The thickness of the epidermis and SC was measured at the horizontal midpoint of each visual field. Approximately 50 individual measurements were made along the wound margin for each histological section, and the mean thickness was evaluated.

Data analysis

Analysis was performed using Statistical Analysis SAS statistical software ver. 9.2 (SAS Institute, Cary, NC). Indicated *P*-values were derived from a Tukey's *post-hoc* multiple comparison test.

Results

Passive skin permeation of HA4 across stripped skin and full-thickness skin

The effect of the SC on skin permeation of 0.5% HA4 solution was investigated in *in vitro* skin permeation experiments. Figure 1 shows the permeation profile of the cumulative amount of HA4 that permeated through SC-stripped skin and full-thickness skin, and Table 1 summarizes the calculated permeation parameters. The cumulative amounts of HA4 through SC-stripped skin and full-thickness skin after 8 h were 2110 ± 176 μg/cm^2 and 0.8 ± 0.3 μg/cm^2, respectively (Fig. 1). The flux and permeability coefficient (*P*) was increased in SC-stripped skin than that of full-thickness skin (Table 1).

Figure 1

Table 1

Comparison of the effects of HA4 concentrations on the skin permeation

Three different HA4 solutions (HA4 concentration; 0.02, 0.1, 0.5%) were applied to SC-stripped skin and full-thickness skin. Figure 2a and b show permeation profiles of the cumulative amounts of various concentrations of HA4 solutions that permeated through SC-stripped skin and full-thickness skin, and Table 2 summarizes the calculated permeation parameters. As shown in Fig. 2a, the cumulative amounts of HA4 permeated after 8 h were 58.3 ± 10.7 μg/cm^2 after application of a 0.02% HA4 solution, 392 ± 44 μg/cm^2 for a 0.1% HA4 solution, and 2365 ± 47 μg/cm^2 for a 0.5% HA4 solution on SC-stripped skin. Figure 2b shows that the cumulative amounts of HA4 permeated after 8 h were 12 ± 7 ng/cm^2 after application of a 0.02% HA4 solution, 22 ± 12 ng/cm^2 for a 0.1% HA4 solution, and 815 ± 319 ng/cm^2 for a 0.5% HA4 solution on full-thickness skin. Flux and *P* showed similar increases (Table 2).

Figure 2

Table 2

Effect of solvent pH on the *in vitro* skin permeation of HA4

The effect of pH on skin permeation of 0.5% HA4 solution was investigated in *in vitro* skin permeation experiments. Different HA4 solutions (pH 4 and pH 7) were applied to full-thickness skin. Figure 4 shows the permeation profile of the cumulative amount of HA4 solutions with a pH 4 and pH 7 that permeated through full-thickness skin, and Table 3 summarizes the calculated permeation parameters. Fig. 3 shows that the cumulative amounts of HA4 permeated after 8 h were 784 ± 371 ng/cm^2 for a HA4 solution with a pH 4 and 70 ± 13 ng/cm^2 with pH 7 on full-thickness skin. Flux and *P* were increased in the pH 4 HA4 solution than that of the pH 7 HA4 solution (Table 3).

Figure 3

Table 3

Effect of HA or HA4 on the measurement of water content of the SC and TEWL

The effect of HA or HA4 on skin in hairless mice was assessed by water content of the SC and TEWL. Regarding changes in the water content of the SC, water content in the UVA irradiation groups was decreased than that of the non-UVA irradiation group with UVA irradiation. However, the water content of the SC was significantly increased in the HA4/UVA(+) group than that of the Control/UVA(+) group and HA/UVA(+) group. In addition, the water content of the SC was significantly increased in the HA4/UVA(-) group than that of the Control/UVA(-) group (Fig. 4). TEWL was increased in the UVA irradiation groups than that of the non-UVA irradiation group with UVA irradiation, but TEWL were significantly decreased in the HA4/UVA(+) group than that of the Control/UVA(+) group and HA/UVA(+) group (Fig. 5).

Figure 4 and 5

Effect of HA or HA4 on skin thickness

The effect of HA or HA4 on skin thickness in hairless mice was assessed by measurement of the thickness of the epidermis and SC. Figure 6a and b show the measurement of epidermal thickness, and Fig. 6c-i shows histological sections with HE staining. Epidermal thickness was significantly decreased in the HA4/UVA(-) group than that of the Control/UVA(-) group, and this level was similar to that in the Normal group (Figure 6a). Furthermore, epidermal thickness was significantly incleased in the Control/UVA(+) group and HA/UVA(+) group than that of the Normal group. In addition, epidermal thickness was significantly decreased in the HA4/UVA(+) group than that of the Control/UVA(+) group (Fig. 6b).

Figure 6

Discussion

The barrier function of the skin is principally attributed to the SC. HA can't penetrate into the skin membrane because of high water solubility and a high average molecular weight of 100 kDa. The *in vitro* skin permeability of HA4 was initially investigated. As a result, HA4 has been suggested to cross skin by passive diffusion.

Various investigations have already been conducted to improve skin permeation of drugs into or through the SC using techniques such as application of chemical enhancers [13, 19, 28], sonophoresis [12], iontophoresis [25], and electrophoration [16]. The small peptide like arginine-vasopressin (1084.23 Da) had negligible passive permeation through rat skin, while the permeation rate with application of iontophoresis was much increased than the passive permeation rate [14]. In addition, high-molecular compounds like fluorescein isothiocyanate (FITC) -dextrans (FD-10) (9.6 kDa) permeated through SC-stripped skin [26]. In our study, HA4 resulted in a concentration dependent increase in skin permeability through SC-stripped skin and full-thickness skin (Fig. 2). The cumulative amount of HA4 permeated after 8 h was 815 ± 319 ng/cm^2 after application of 0.5% HA4 solution on full-thickness skin. At this time, *P* was 26 × 10^{-6} ± 0.5 × 10^{-6} cm/s, and the lag time was 4.6 ± 0.04 h. The cumulative amount of HA4 permeated after 8 h was 2365 ± 47 μg/cm^2 after application of 0.5% HA4 solution on SC-stripped skin. At this time, *P* was 26 × 10^{-6} ± 0.6 × 10^{-6} cm/s, and the lag time was 2.9 ± 0.1 h. Skin permeability was about 2600-fold decreased in full-thickness skin than that of SC-stripped skin (Fig. 1). However, this study has demonstrated that HA4 permeated through skin by passive diffusion. The skin permeation of HA4 was presumed to be rate-limiting in the SC because the cumulative amount of HA4 was significantly increased. Nevertheless, the lag time through SC-stripped skin was not drastically increased. Consequently, HA4 was presumed to have a slow partition and diffusion.

In general, drug percutaneous permeation conformed to the pH-partition theory, and only unionized forms of drugs are able to pass through skin. The flux of salicylic acid was dependent upon the vehicle pH, and the amount of salicylic acid permeation was related to the degree of ionization of the solute [11]. The p*K*a of HA is 3 [10, 29]. The unionized form of HA was increased in HA4 solution with a pH 4 than that of HA4 solution with a pH 7. HA4 percutaneous permeation also conformed to the pH-partition theory, and the cumulative amount of HA4 was increased with a pH 4 than that with a pH 7 (Fig. 3).

Next, the effects of HA and HA4 treatment on *in vivo* skin properties were verified using the dorsal skins on hairless mice. It has recently been shown that irradiation of UVA on hairless mice increased the water content of the SC [24]. In addition, it has been reported that chronic UVB irradiation induces an increase in TEWL in the dorsal skin of hairless mice [2, 5]. TEWL is the outward diffusion of water through the skin [15], and measurements are carried out to gauge skin water barrier function. In our study, the water content of the SC decreased and TEWL increased with UVA irradiation (Fig. 4b and 5b). In the group treated with HA4, the water content of the SC and TEWL was notably improved over that of the Control/UVA(+) and HA/UVA(+) groups. The sample treatment and UVA irradiation caused no significant change in body weight in any of the groups (data not shown). The water content of the SC increased with HA4 treatment in the without UVA irradiation group (Fig. 4a).

Chronic UVA irradiation significantly increased epidermal thickness [2]. In this study, skin thickness was increased by chronic UVA irradiation (Fig. 6a). The epidermal thickness of Control/UVA(+) and Control/UVA(-) groups was about 7.3- and 1.5-fold increased than that of the Normal group. In contrast, the epidermal thickness level was similar to the HA4 treatment group than the Normal group (Fig. 6a).

These results show that HA4 permeated through skin by passive diffusion, but presumed to have a slow partition and diffusion. However, it was revealed that the HA4 of a small molecular weight penetrated skin with the water-soluble compound. In addition, these results show that treatment of HA4 improved skin functional recovery after UVA irradiation. Therefore, these data suggest that HA4 is involved in the healing process in the skin after UVA irradiation. In has been reported that high molecular weight hyaluronan-mediated CD44 activation regulates skin differentiation [4]. If the skin differentiation happens by HA4, it may be involved in the skin functional recovery after UVA irradiation. Accordingly, it is necessary to clarify the mechanism of skin functional recovery.

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Table 1 *In vitro* skin permeation of HA4 across SC-stripped skin and full-thickness skin

	Cumulative amount of HA4 (μg/cm^2)	Flux (μg/cm^2/h)	× 10^{-7} P (cm/s)	ER
SC-stripped skin	2109.6±175.9	418.0±30.6	232.2±17.0	2587.5
Full-thickness skin	0.8±0.3	0.2±0.09	0.1±0.05	-

ER = Enhancement ratio = cumulative amount of SC-stripped skin / cumulative amount of full-thickness skin

Table 2 *In vitro* skin permeation of HA4 at different concentrations across SC-stripped skin and full-thickness skin

	HA4 concentration (%)	Cumulative amount of HA4 (μg/cm^2)	Flux (μg/cm^2/h)	× 10^{-6} P (cm/s)	ER
SC-stripped skin	0.50	2364.8±46.5	461.6±10.0	25.6±0.5	40.6
	0.10	392.4±44.3	76.8±6.9	21.3±1.9	6.7
	0.02	58.3±10.7	12.0±2.2	16.7±3.1	-
	HA4 concentration (%)	Cumulative amount of HA4 (μg/cm^2)	Flux (μg/cm^2/h)	× 10^{-7} P (cm/s)	ER
Full-thickness skin	0.50	0.8±0.3	0.2±0.1	0.1±0.05	67.9
	0.10	0.2±0.1	0.003±0.001	0.009±0.003	1.9
	0.02	0.1±0.07	0.003±0.002	0.03±0.02	-

ER = Enhancement ratio = cumulative amount of HA4 / cumulative amount of 0.02% HA4

Table 3 *In vitro* skin permeation of HA4 at different pH across full-thickness skin

pH	Fraction unionized	Cumulative amount of HA4 (μg/cm^2)	Flux (μg/cm^2/h)	× 10^{-7} P (cm/s)	ER
4	0.09	0.8±0.4	0.2±0.1	0.1±0.06	11.3
7	0.0001	0.07±0.01	0.03±0.004	0.01±0.007	-

Fraction un-ionized = 1 / (1 + antilog (pH – pKa)).

ER = Enhancement ratio = cumulative amount of pH 4 HA4 / cumulative amount of pH 7 HA4

Fig. 1 Passive permeation of HA4. Cumulative amount of HA4 permeated through SC-stripped skin. Each point represents mean ± S.D. of 3 to 5 determinations. Symbols: SC-stripped skin (●) and full-thickness skin (◆).

Fig. 2 Effect of HA4 concentrations on *in vitro* skin permeation of HA4 across SC-stripped skin and full-thickness skin. Cumulative amount of HA4 permeated through SC-stripped skin (a) and full-thickness skin (b). Each point represents mean ± S.D. of 3 to 5 determinations. Symbols: 0.02 (▲), 0.1 (◆) and 0.5% (●) HA4 solutions.

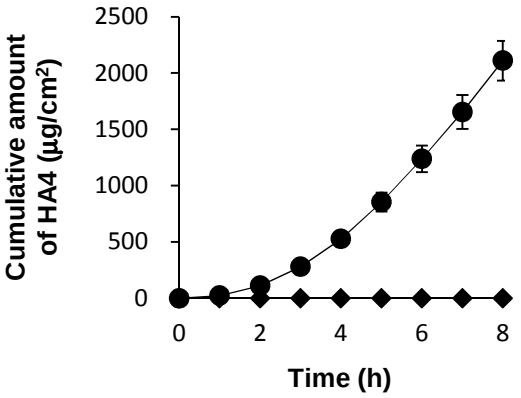
Fig. 3 Effect of pH on *in vitro* skin permeation of HA4. Cumulative amount of HA4 permeated through full-thickness skin. Each point represents mean ± S.D. of 3 to 5 determinations. Symbols: pH 4 (●) and pH 7 (◆) HA4 solutions.

Fig. 4 Changes in water content in the SC after HA4 treatment. Water content in the SC was measured for 0-21 days in non-UVA-irradiated mice (a) and UVA-irradiated mice (b). Values were the mean ± S.D. (n = 3). $*p < 0.05$, $**p < 0.01$ (versus control), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (versus HA), Tukey's *post-hoc* multiple comparison test. Symbols: normal (△), control (◇), HA (●) and HA4 (■) groups.

Fig. 5 Changes in TEWL after HA4 treatment. TEWL was measured for 0-21 days in non-UVA-irradiated mice (a) and UVA-irradiated mice (b). Values were the mean ± S.D. (n = 3). $*p < 0.05$, $**p < 0.01$ (versus control), $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (versus HA), Tukey's *post-hoc* multiple comparison test. Symbols: normal (△), control (◇), HA (●) and HA4 (■) groups.

Fig. 6 Changes in epidermal thickness and skin morphology after HA4 treatment. Epidermal thickness and skin morphology were treated for 21 days in non-UVA-irradiated mice (a, d-f) and UVA-irradiated mice (b, g-i). Skin samples were fixed and stained with HE. HE-stained sections were photographed, and epidermal thickness was assessed (a, b). Non-UVA-irradiated skin specimens of normal (c), control (d), HA (e), and HA4 (f) groups after each sample treatment. UVA-irradiated skin specimens of control (g), HA (h), and HA4 (i) groups after each sample treatment and irradiation. Values were the mean ± S.D. (n = 3). $*p < 0.05$, $**p < 0.01$, Tukey's *post-hoc* multiple comparison test.

Fig. 1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2000
1500
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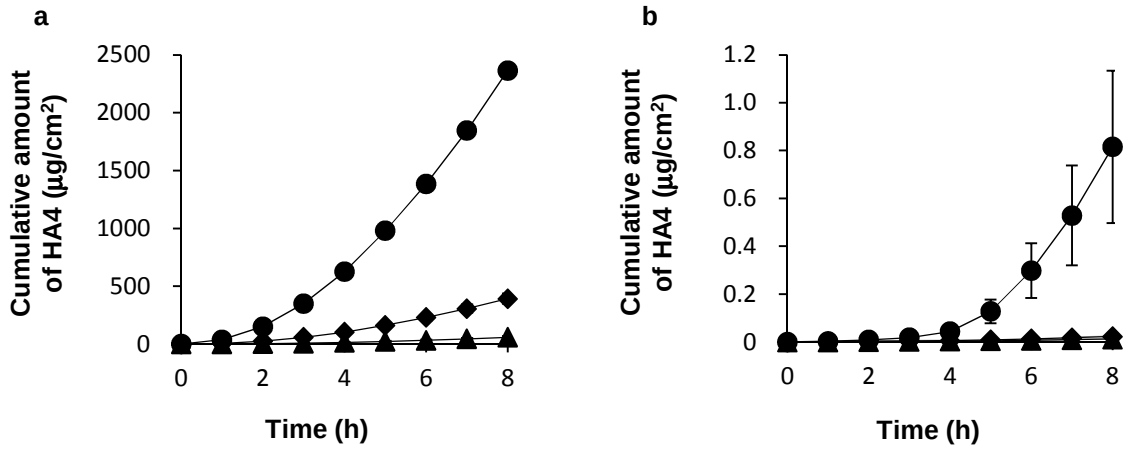
500
0
0
2
4
6
8
Time (h)

Fig. 2


a
Cumulative amount of HA4 (μg/cm²)
2500
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6
8
Time (h)
b
Cumulative amount of HA4 (μg/cm²)
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Time (h)

Fig. 3

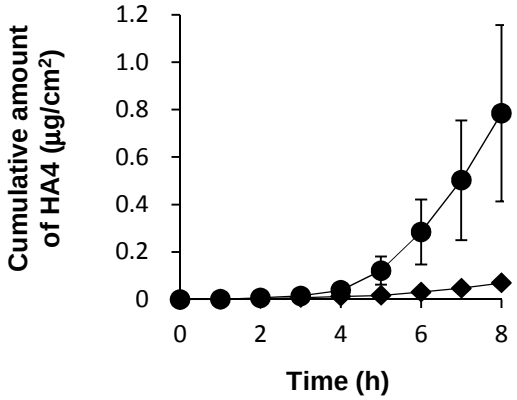


Fig. 4

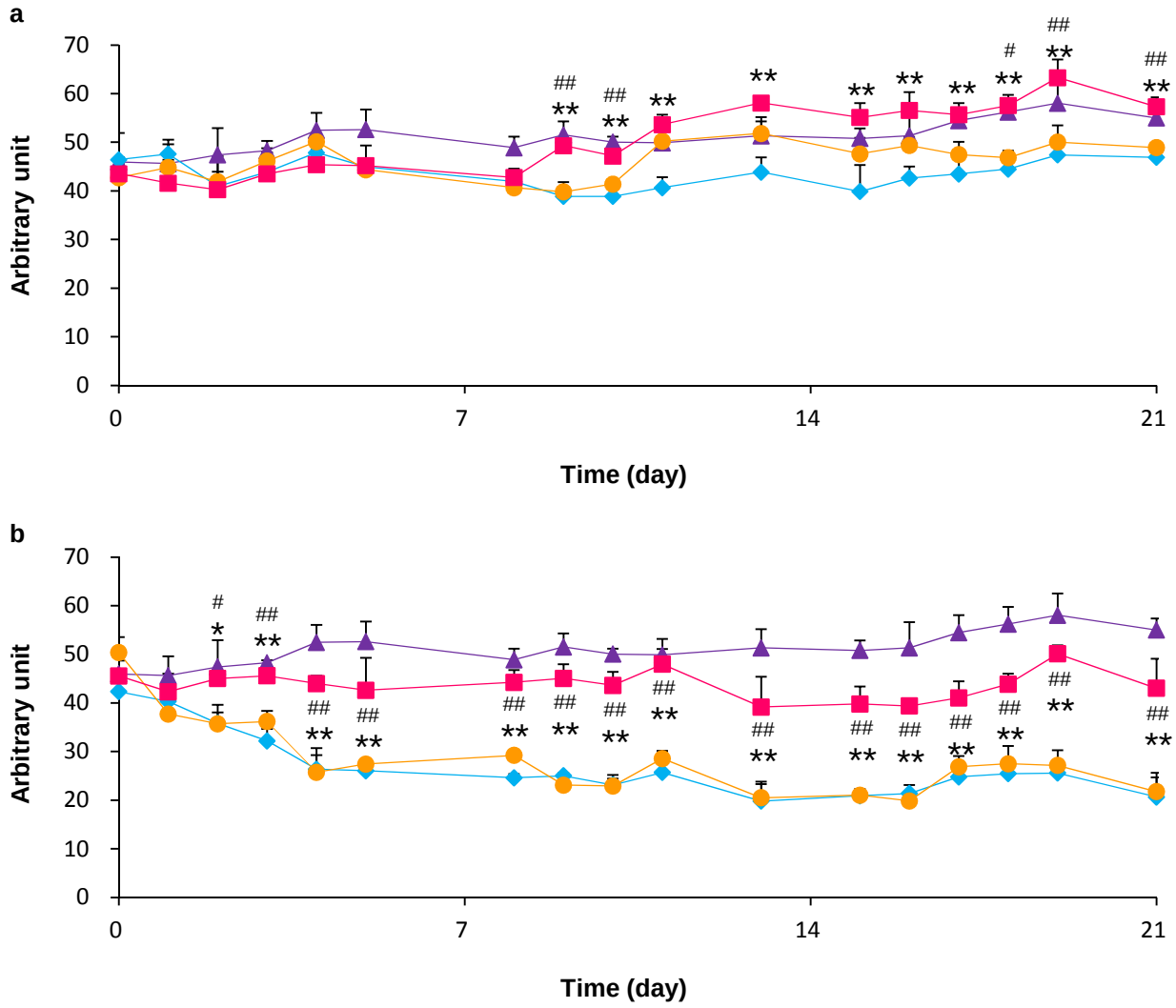

a
Arbitrary unit
70
60
50
40
30
20
10
0
0
7
14
21
Time (day)
b
Arbitrary unit
70
60
50
40
30
20
10
0
0
7
14
21
Time (day)

Fig. 5

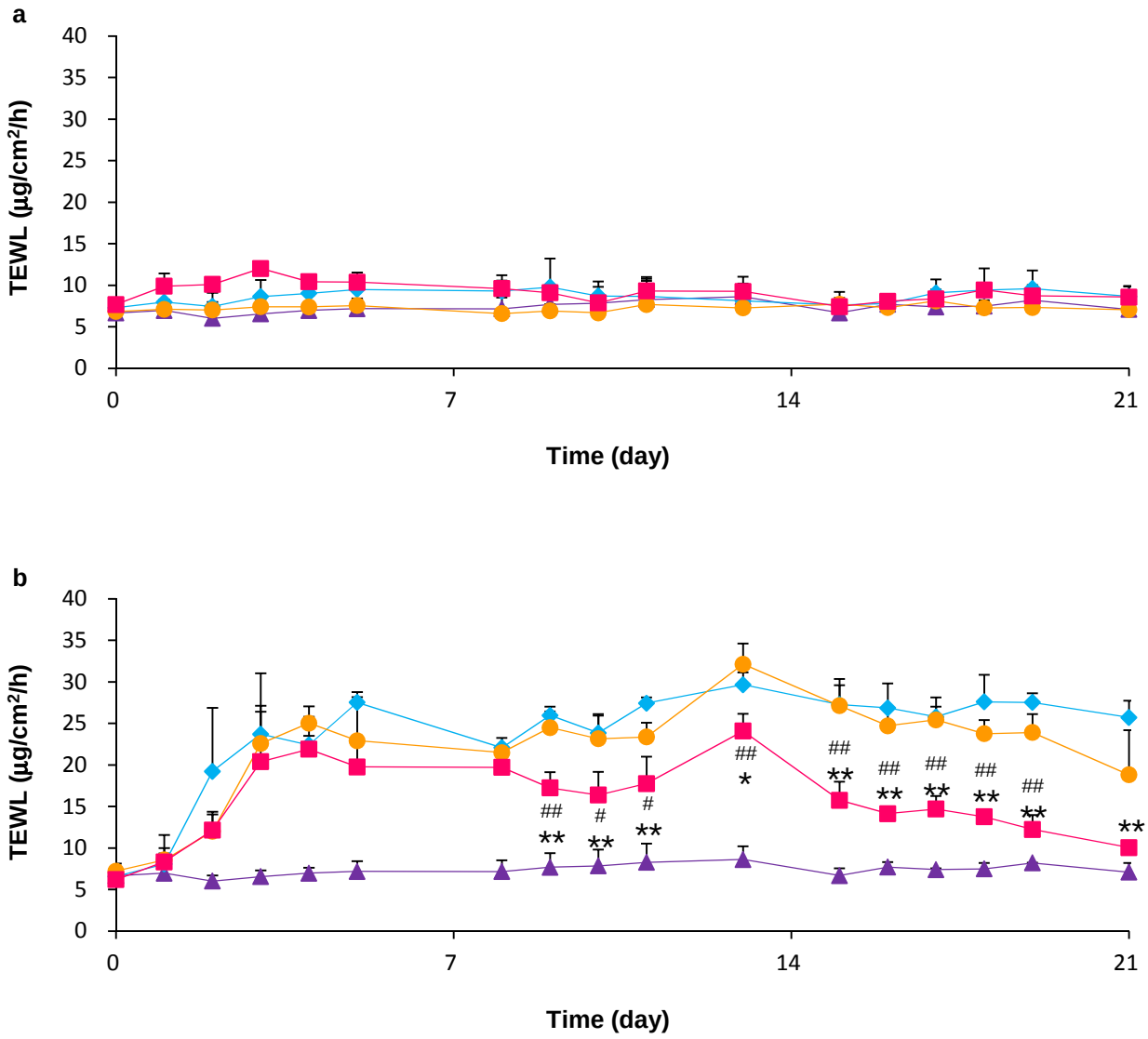
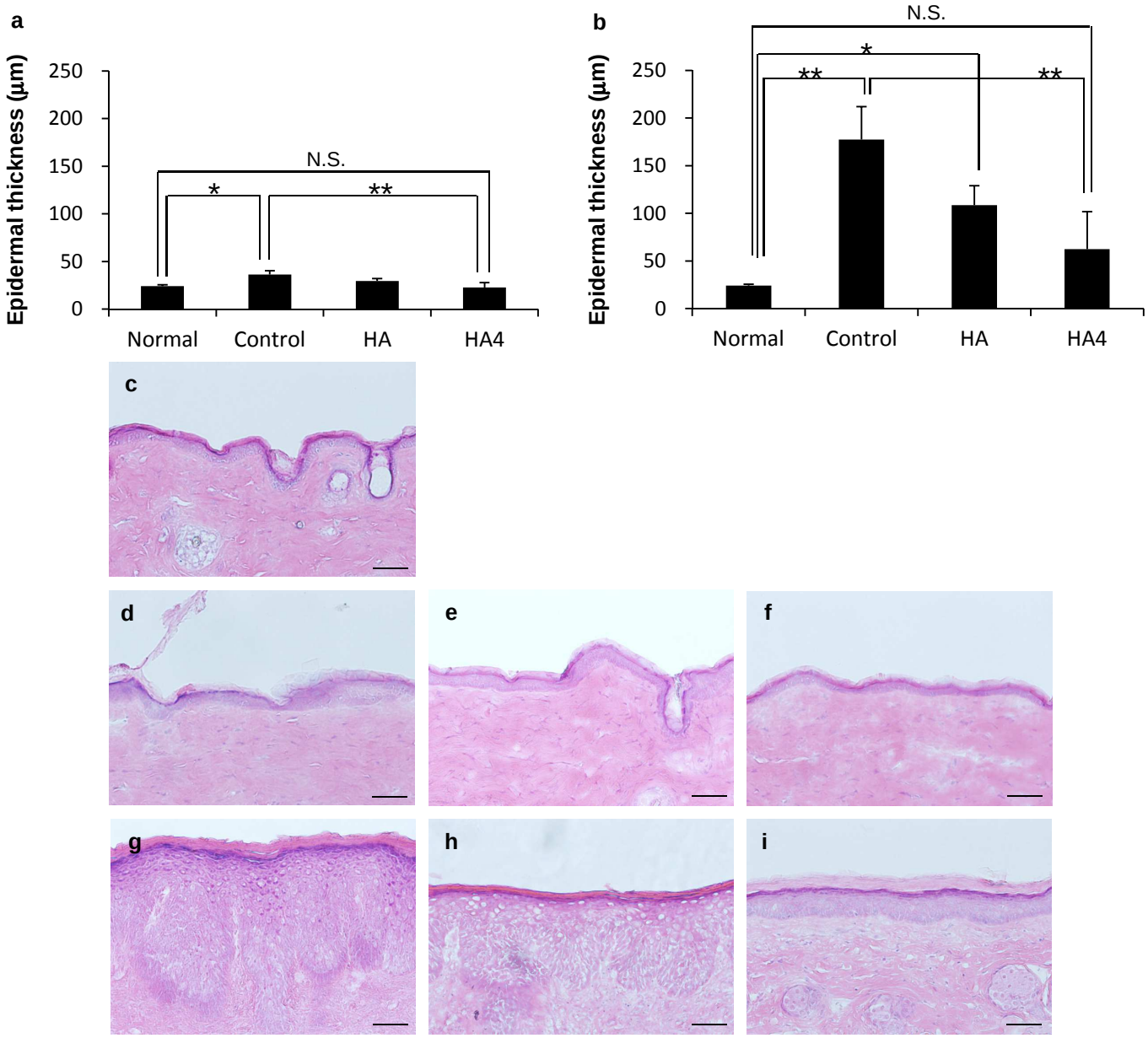

a
TEWL (μg/cm²/h)
40
35
30
25
20
15
10
5
0
0
7
14
21
Time (day)
b
TEWL (μg/cm²/h)
40
35
30
25
20
15
10
5
0
0
7
14
21
Time (day)
##
**
#
*

Fig. 6


a
Epidermal thickness (μm)
250
200
150
100
50
0
N.S.
*
**
Normal
Control
HA
HA4
b
Epidermal thickness (μm)
N.S.
*
**
**
Normal
Control
HA
HA4
c
d
e
f
g
h
i