



## Aging enhances maceration-induced ultrastructural alteration of the epidermis and impairment of skin barrier function

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### ABSTRACT

**Background:** Skin maceration is recognized as a risk factor for the development of certain skin lesions. In health care settings, incontinence-associated skin maceration is highly prevalent in the elderly. However, the effect of senescence on maceration has not been fully elucidated.

**Objective:** To reveal the enhancement of the maceration-induced ultrastructural alteration and barrier function of the epidermis by aging.

**Methods:** Skin maceration was reproduced by exposure to agarose gel in human and rat. The ultrastructural alterations in human and rat tissue were observed by transmission electron microscopy. The skin barrier function was evaluated by noninvasive methods in human, and by the transdermal penetration of small- and large-fluorescent molecules in rat. In order to reveal the effect of aging on the skin maceration, we compared these parameters between young and aged rats.

**Results:** In macerated skin, we observed expansion of the interstices of the stratum corneum, spinosum, and basale of the epidermis; disruption of the intercellular lipid structure in the stratum corneum; a decreased number of cell processes in the stratum spinosum and basale. The transdermal penetration test in the rat using two types of fluorescein indicated that maceration disrupted skin barrier function. Furthermore, senescence-enhanced ultrastructural and functional alterations were revealed in the rodent studies.

**Conclusion:** This study demonstrates that aging enhances skin maceration. Considering that maceration is a risk factor for the skin damage, the development of technology to promote skin barrier recovery after maceration in the elderly is warranted.

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## 1. Introduction

Skin maceration, induced by long-term exposure to excessive moisture, is recognized as a risk factor for the development of skin lesions including pressure ulcers and moisture-associated skin damage [1–4]. In health care settings, most skin maceration is related to incontinence. It has been reported that urinary and/or fecal incontinence is prevalent in more than half of nursing home residents [5] and in 28% of elderly people living at home

[6]. The prevalence of incontinence is higher in the elderly [6,7], who also comprise a population with skin vulnerabilities due to senescence [8].

Shigeta et al. [9] reported that 36% of patients with urinary and/or fecal incontinence in long-term medical facilities in Japan had skin lesions. This result supports the opinion that maceration is a risk factor for the development of skin lesions. Loss of the buffering function and disruption of the skin barrier are generally recognized as the pathways of maceration-induced skin lesions. However, direct evidence of these two pathways is quite limited. To reveal the vulnerability of macerated skin, histological analysis is important. Several studies have revealed structural changes in the stratum corneum. However, these results cannot explain the decreased skin tolerance to mechanical stimulation because skin strength is supported by intercellular junctions in the stratum

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spinosum and basale of the epidermis and extracellular matrix of the dermis.

The skin barrier function is often measured by transepidermal water loss (TEWL) [9–12], which is a parameter of water penetration from the inside to the outside of the body. However, the transdermal penetration of chemical irritants and macromolecules from the outside to the inside should be considered direct evidence for the development of maceration-induced skin lesions. We observed ultrastructural changes in all skin layers, which provided detailed evidence related to skin strength, and revealed transdermal penetration by applying fluorescent molecules in our evaluation of skin barrier function in the experimentally reproduced macerated skin.

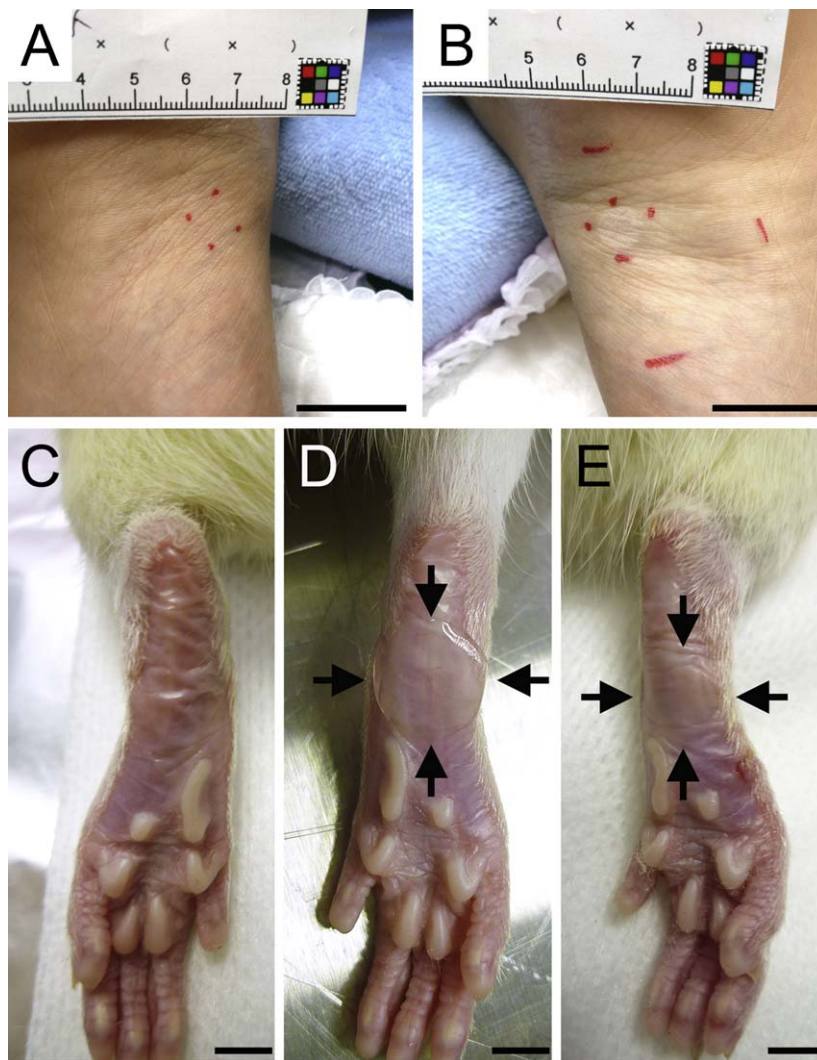
Several researchers have reported morphological and functional skin alterations due to senescence [8,13,14]. However, few studies have examined the effect of senescence on maceration-induced changes. Because the elderly are a high-risk population for the development of maceration-induced skin lesions and pressure ulcers [15,16], the need for structural and functional studies on the effect of skin maceration is a pressing theme.

In this report, we demonstrated ultrastructural changes in the stratum spinosum and basale of the epidermis, induced by exposure to excessive water in humans and rats, *in vivo*. The transdermal penetration of small- and large-molecular-weight fluoresceins were revealed in rat macerated skin. Furthermore, ultrastructural changes and transdermal penetration of fluorescein were compared between young and aged rats to reveal the enhancement of skin maceration by aging.

## 2. Materials and methods

### 2.1. Subjects

Three males (23, 24, and 37 years old) and three females (28, 31, and 39 years old) with no dermatopathies volunteered for the study. They were given detailed information on the purpose, experimental design, methodology, and possible risks, and their written informed consents were obtained. This study was conducted according to the principles expressed in the Helsinki Declaration and was approved by the Ethical Committee of the Graduate School of Medicine of The University of Tokyo.



**Fig. 1.** Agarose droplet attachment-induced skin maceration on the foot sole of the human and rat. Skin maceration was reproduced on the foot sole of the human ( $n = 6$ ) and 9-week- and 6-month-old rats ( $n = 15$  and 9, respectively) by attaching 1% agarose gel droplets for 6 h. (A) and (B) show a case of 28 females in which the right macerated foot sole skin is clearly whitened (B). Noninvasive measurements and split-thickness skin tissue harvests were carried out within the area enclosed by red markers. (C–E) Rat foot soles were macerated by the same method used in the human experiment. The area enclosed by arrows was exposed to agarose gel (D). After a 6-h maceration period, the sole skin appeared whiter and more swollen (E) compared with the contralateral corresponding area (C). Bar = 2 cm in (A) and (B), and 5 mm in (C)–(E).

Nine-week- and 6-month-old male Sprague–Dawley rats were purchased from Japan SLC (Shizuoka, Japan). Animals were maintained in a temperature-controlled facility (24 °C) with a 12-h light–dark cycle and were fed a regular diet. The animal experiments were carried out in accordance with the Guidelines for Animal Experimentation published by the Japanese Association for Laboratory Animal Science (1987), and were approved by the Animal Research Committee of The University of Tokyo.

## 2.2. Reagents

Agarose S was purchased from Nippon Gene (Tokyo, Japan); 5-carboxyfluorescein diacetate (CFDA) was purchased from AAT Bioquest (Sunnyvale, CA); dextran conjugated with FITC (Dex) was purchased from Invitrogen (Carlsbad, CA); Xylocaine (10 mg/mL lidocaine chloride solution) was purchased from AstraZeneca (London, UK); Bosmin (1 mg/mL adrenaline solution) was purchased from Daiichi-Sankyo (Tokyo, Japan) and diluted five times with normal saline (0.9% NaCl) for hemostasis; Somnopentyl (64.8 mg/mL pentobarbital sodium solution) was purchased from Kyoritsu Seiyaku (Tokyo, Japan).

## 2.3. Skin maceration

Previous study indicated that substance can penetrate into the skin through transdermal and transfollicular route [17]. In this study, foot sole in which hair follicle is absent was selected for skin maceration.

Approximately 50- $\mu$ L droplets of 1% agarose gel dissolved in normal saline were used for maceration. In some rat studies,

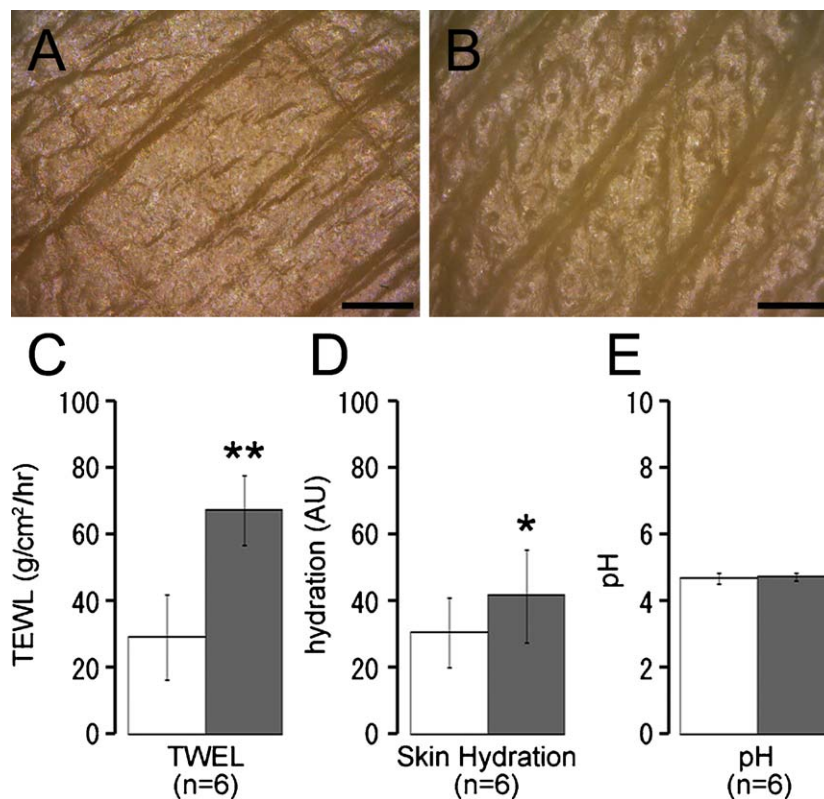
agarose gel droplets were immersed in 10  $\mu$ M CFDA or 100  $\mu$ g/mL Dex for 1 h or more.

To induce skin maceration, an agarose gel droplet was attached to one foot sole (Fig. 1) using a polyurethane film dressing (Tegaderm transparent dressing, 3M, St. Paul, MN) for 30 min, 2 h, or 6 h. The maceration side was randomly selected and the opposite side was used as a control. In the histological analysis of both human and rat skin as well as in the CFDA penetration test, the control consisted of intact skin tissue without any treatment. To reveal the penetration of Dex in macerated skin, agarose gel containing Dex was attached to both the control and macerated soles in the initial 30-min period, then agarose gel without Dex was attached to only the macerated sole for an additional 1.5 or 5.5 h (total maceration periods were 2 and 6 h, respectively).

## 2.4. Noninvasive evaluation of skin maceration in the human

Following the skin maceration period in the human, TEWL, skin hydration, and skin pH were measured on the surface of the macerated area and corresponding control area using the VapoMeter (Delfin Technologies, Kupio, Finland), Derma Unit with Corneometer, and Skin-pH-Meter (Courage + Khazaka electronic GmbH, Köln, Germany), respectively. Measurements were repeated until the values became steady, and the means of the last three measurements were used for analysis. The difference in values between macerated and control soles was analyzed by the paired *t*-test, and values of *p* < 0.05 were considered statistically significant. Values are presented as means  $\pm$  standard deviations.

The appearance of the skin surface was recorded by a digital camera (Lumix DMC-FX35, Panasonic) and skin microscope (i-



**Fig. 2.** Noninvasive evaluation of skin maceration in humans. (A and B) Following a 6-h maceration, the appearance of the skin surface was observed using a skin microscope (*n* = 6). In the macerated skin (B), the sulcus cutis width was increased and the sweat pores were remarkably more noticeable compared with the control skin (A), probably due to swelling. Bar = 1 mm. (C–E) TEWL (C), skin hydration (D), and skin pH (E) measurements of the macerated foot sole (gray bar) and contralateral skin (white bar) were performed. The significant elevation in TEWL and skin hydration in the macerated skin compared with those in the control skin indicated skin overhydration, whereas there was no difference in skin pH. Values were presented as means  $\pm$  SD. Differences between the two groups were analyzed using paired *t*-tests. \**p* < 0.05, \*\**p* < 0.01.

Scope USB2.0, Moritex, Tokyo Japan). The white balance of the digital images was adjusted using Adobe Photoshop.

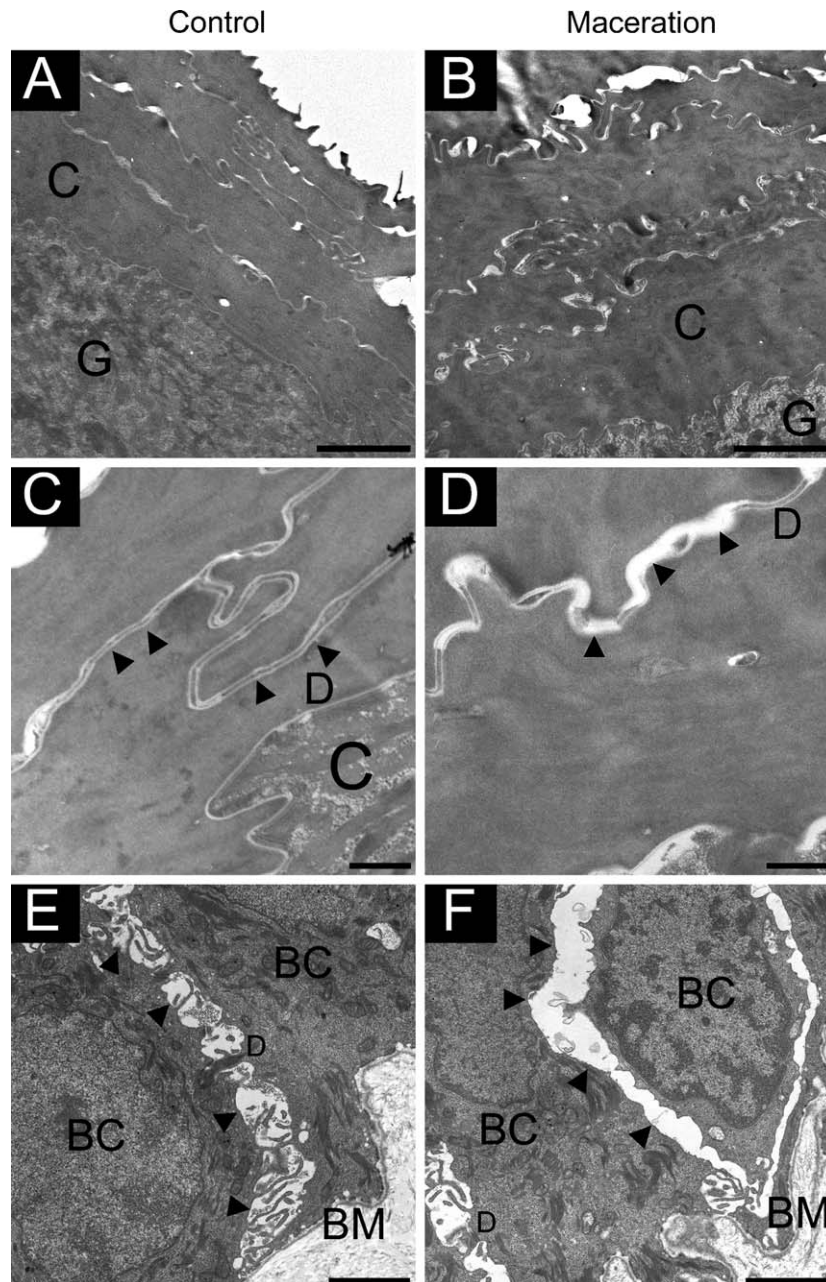
### 2.5. Tissue sampling

In the human, split-thickness skin tissues (approximately 5 mm × 5 mm) were harvested from the macerated sole and the contralateral corresponding area under local anesthesia with lidocaine following hemostasis using styptic cotton containing adrenaline. In the rat experiment, full-thickness macerated and control skin tissues were harvested under general anesthesia induced by pentobarbital sodium. After tissue collection, the wound sites were treated with calcium alginate and/or hydrocol-

loid dressing. The collected tissue was immediately divided into two pieces and fixed with one of two fixatives: 4% paraformaldehyde in phosphate buffer (PB) (pH 7.4) for fluorescent microscopy, or 2% glutaraldehyde and 2% paraformaldehyde in PB for electron microscopy.

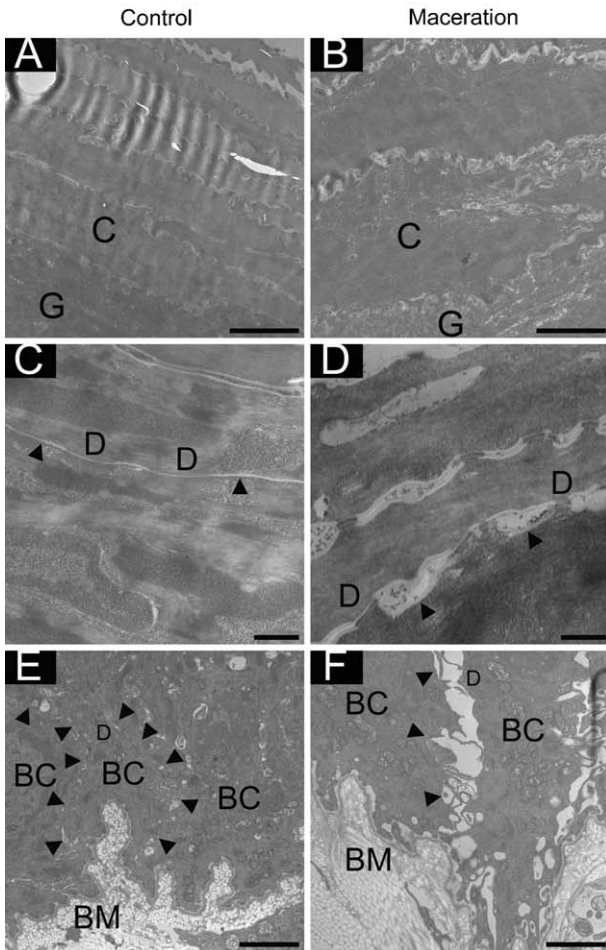
### 2.6. Transmission electron microscopy

The fixed samples were rinsed with 3% sucrose in PB and postfixed with 1% OsO<sub>4</sub> in PB at 4 °C for 1 h. Following dehydration in a graded series of ethanol concentrations, tissues were embedded in epoxy resin. The ultra-thin sections (approximately 70-nm thickness) were stained with 2% uranyl acetate in 70%

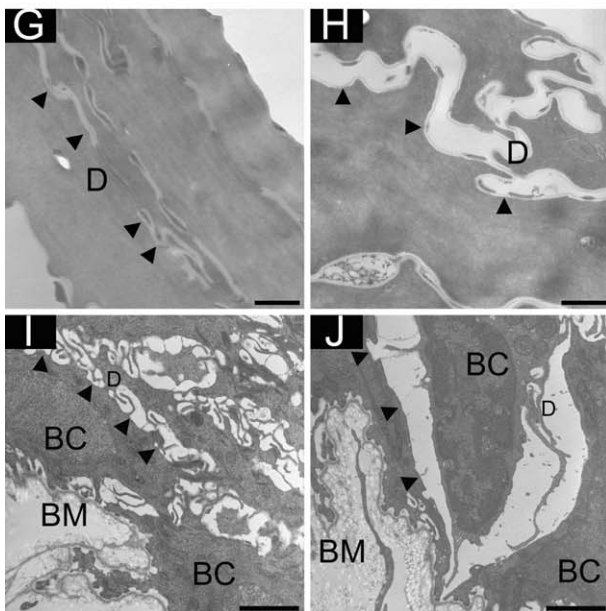


**Fig. 3.** Transmission electron microscopy of the epidermis after exposing human skin to maceration for 6 h. After exposure to agarose gel for 6 h, the ultrastructure of the human epidermis was observed by transmission electron microscopy. (A), (C), and (E) show the morphology of control skin ( $n = 6$ ), while (B), (D), and (F) show the morphology of macerated skin ( $n = 6$ ). (A and B) Observation of the stratum corneum in low magnification (1500 $\times$ ) revealed increased thickness. Bar = 3  $\mu$ m. (C and D) Observation with high magnification (20,000 $\times$ ) of the stratum corneum revealed expansion of the interstice (arrowheads) and disruption of the intercellular lipid layer. Bar = 500 nm. (E and F) In the stratum basale (6000 $\times$ ), the interstitial space (arrowheads) was expanded and the number of cell processes was decreased. Bar = 1.7  $\mu$ m. BC: basal cell; BM: basement membrane; C: corneum; D: desmosome; G: granular cell.

## 9-week-old



## 6-month-old



**Fig. 4.** Ultrastructural changes in the epidermis after 6-h maceration in young and aged rat skin. Skin maceration was reproduced on the sides of the foot soles of 9-week-old (A–F) and 6-month-old (G–J) rats, and the ultrastructural characteristics were compared. The increase in corneum thickness (A and B); the expansion of the interstitial space (arrowheads) and disruption of interstitial lipid lamellae in the stratum corneum (C and D and G and H); and the expansion of the intercellular

ethanol for 5 min, then with Reynolds' lead staining solution for 5 min. The ultrastructure of the epidermis was observed with the Hitachi H-7500 transmission electron microscope (TEM). The white balance and brightness of the images were adjusted using Adobe Photoshop.

### 2.7. Fluorescent microscopy

Skin tissues exposed to CFDA or Dex were fixed and embedded in OCT compound. Approximately 5- $\mu\text{m}$ -thick frozen sections were counterstained with DAPI, then observed with an inverted fluorescence microscope (Leica DMI 4000B). The obtained digital image was not processed.

## 3. Results

### 3.1. Reproduction of skin maceration in humans and rats

We attempted to reproduce skin maceration by exposure to a hydrogel of the polysaccharide agarose, which forms a solid containing excess liquid. A droplet of 1% agarose gel was attached for 6 h on the foot soles of the rat and human. The attached areas developed a whitened skin color and decreased wrinkles (Fig. 1).

Noninvasive measures commonly used to evaluate skin maceration, including skin microscopy and measurements of TEWL, skin hydration, and skin pH [9–12], were applied to human skin. These measurements were not applied in the rat experiments because the measuring probes of these instruments were too large for the rat foot sole. Observation of the skin surface revealed an increased sulcus cutis width and very noticeable sweat pores in the agarose-attached skin, probably due to increased swelling, as compared with control skin (Fig. 2A and B). Agarose attachment induced significantly higher levels of TEWL ( $67.37 \pm 10.552 \text{ g}/(\text{cm}^2 \text{ h})$ ,  $p = 0.003$ ) and skin hydration ( $41.48 \pm 13.905 \text{ AU}$ ,  $p = 0.012$ ) than in control skin ( $29.16 \pm 12.951 \text{ g}/(\text{cm}^2 \text{ h})$  and  $30.38 \pm 10.405 \text{ AU}$ , respectively), whereas there was no difference in skin pH ( $4.68 \pm 0.168$  in control skin and  $4.73 \pm 0.125$  in macerated skin,  $p = 0.296$ ; Fig. 2C–E). These results suggest that skin swelling and overhydration, i.e., skin maceration, can be reproduced by agarose gel attachment for 6 h to human skin.

### 3.2. Ultrastructure of macerated and control skin

We performed an ultrastructural analysis of the epidermis by TEM in human intact and macerated skin (Fig. 3). In the stratum corneum of macerated skin, an increased thickness of the corneum (Fig. 3A and B), expansion of the interstices, and disruption of the intercellular lipid lamella (Fig. 3C and D) were observed. Expansion of the interstices was also observed in the stratum basale and spinosum of the epidermis (Fig. 3E and F), as well as in the stratum corneum. The number of cell processes of basal and prickle cells was decreased in macerated skin compared with that in intact tissue (Fig. 3E and F).

The ultrastructural changes in the epidermis of both young and aged rat intact and macerated skin were similar to the observations in the human experiment (Fig. 4). These observations demonstrated that skin maceration was reproduced in the rat model as well as in human skin. The differences in ultrastructure between young

space and decrease in cell processes in the stratum basale (E and F and I and J) were observed in both young and aged rats. These ultrastructural changes were enhanced in aged rats compared with young rats. Magnification: 1500 $\times$  in A and B, 20,000 $\times$  in C and D and G and H, and 6000 $\times$  in E and F and I and J. Bar = 3  $\mu\text{m}$  in A and B, 500 nm in C and D and G and H, and 1.7  $\mu\text{m}$  in E and F and I and J. bc: basal cell; bm: basement membrane; c: corneum; d: desmosome; gc: granular cell.

and aged animals were noticeable. In young rat intact skin, corneocytes from the stratum basale to the corneum were tightly connected (Fig. 4A, C, and E). On the other hand, a wider interstice between corneocytes was observed in aged rats (Fig. 4G and I). Furthermore, ultrastructural changes, including the expansion of interstices, disruption of the intercellular lipid layer, and a decrease in cell processes by skin maceration, were remarkable in aged rats (Fig. 4H and J) compared with young animals (Fig. 4B, D, and F). These observations indicate that aging enhances ultrastructural changes due to skin maceration.

### 3.3. Transdermal penetration of 5-carboxyfluorescein diacetate

To evaluate the barrier function in macerated skin, two types of fluoresceins, CFDA (MW 460 Da) and Dex (MW approximately 3 kDa), were applied to agarose gels. Their transdermal penetrations were then observed.

A randomly selected side of the foot sole was exposed to agarose gel containing CFDA for 30 min or 6 h in 9-week-old rats. The contralateral intact skin without agarose gel attachment was used as a control. Fig. 5 shows the frozen sections of these tissue samples. In the control tissue, slight autonomous fluorescence was observed in the hair follicles only (Fig. 5A–C). After 30 min of exposure, CFDA remained on the skin surface and in the lumen of the hair follicles (Fig. 5D–F). After 6 h of exposure, however, fluorescence was detected through the epidermis and dermis (Fig. 5G–I), indicating that long-term exposure to excessive solution induced penetration and diffusion of solution and small

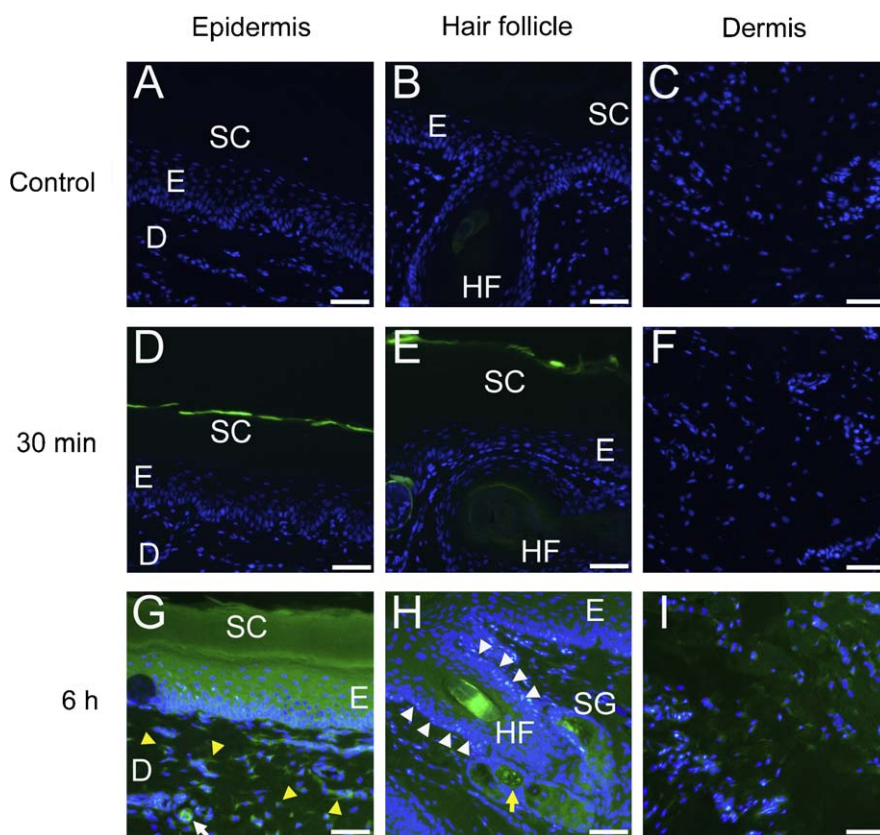
molecules. Remarkable fluorescent signals were detected within hair follicles, sebaceous glands, and cells within blood vessels (Fig. 5G and H).

### 3.4. Transdermal penetration of dextran conjugated with fluorescein

Agarose gel droplets containing Dex were attached to both the left and right foot soles of 9-week-old rats in the initial 30-min period because it was confirmed that the fluorescein did not penetrate the stratum corneum during this short time period in the CFDA penetration assay described above. In subsequent 1.5- or 5.5-h periods (total maceration period of 2 or 6 h, respectively), one randomly selected side was exposed to agarose containing only normal saline; the contralateral side without additional gel attachment was used as a control. This experimental design was conducted to compare maceration-enhanced penetration with voluntary diffusion of Dex. In the control tissue, Dex was localized to the surface of the epidermis and within hair follicles (Fig. 6A–E). On the other hand, diffusion of Dex in all layers of the epidermis was observed in 2-h macerated skin, and penetration of the dermis and concentrated signals in hair follicles and sebaceous glands were observed in 6-h macerated skin (Fig. 6F–J).

### 3.5. Effect of aging on transdermal penetration

Similar to young rats, 6-month-old rats were also subjected to the transdermal penetration test using CFDA and Dex. In aged skin, the distribution of fluorescein was similar to that in young skin in



**Fig. 5.** Penetration of CFDA into the skin of young rats during maceration. One side of the foot sole was exposed to agarose gel containing a small amount of molecular fluorescent dye, CFDA, for 30 min (D–F) and 6 h (G–I) in 9-week-old rats ( $n = 3$  each). The contralateral intact sole tissue without exposure to agarose gel was used as a negative control (A–C). The frozen sections counterstained by DAPI were observed using inverted fluorescence microscopy (magnification  $20\times$ ). The observation of control tissue revealed autonomous fluorescence, which was not observed in skin tissue except within hair follicles. In skin tissue with a 30-min exposure, CFDA green fluorescence was observed only on the surface of the stratum corneum and in the lumens of hair follicles. Six hours later, a strong CFDA signal was observed in all layers of the epidermis; within blood vessels (white arrow), hair follicles (enclosed by white arrowheads), and sebaceous glands (yellow arrow); on the fibroblasts in the dermis (yellow arrowheads), indicating penetration of CFDA solution. Bar = 50  $\mu\text{m}$ . SC: stratum corneum; E: epidermis; D: dermis; SG: sebaceous gland; HF: hair follicle.

both the CFDA and Dex experiments (Fig. 7). However, the intensity of fluorescence in aged skin was stronger than that in young skin. Specifically, the fluorescein signal in the deep layer of the dermis was significantly increased in aged skin compared with young skin (Fig. 7F and L). This result suggests that more fluorescein molecules and a greater volume of solution penetrated

the skin barrier, and that aging enhanced the maceration-induced disruption of the skin barrier function.

#### 4. Discussion

##### 4.1. Ultrastructural alteration by skin maceration

Previous studies have revealed ultrastructural changes induced by skin maceration [18–22]. They focused on the stratum corneum, particularly the intercellular lipid structure, which largely contributes to regulation of skin permeability of water-soluble factors [23]. However, to better understand skin maceration, it is also important to analyze the skin layers consisting of living corneocytes because proliferation and differentiation of corneocytes are essential to maintain barrier function [24] and because the intercellular junctions of living corneocytes support skin tolerance [25,26]. Our observations of the human and rat stratum corneum agreed with the results of previous studies, although the detailed structure of the lipid layer was not observed using our method. Interestingly, we observed a decreased number of cell processes of basal and prickle cells in macerated skin compared with that of intact tissue, in addition to an expansion of interstices in the stratum basale and spinosum.

Considering the above findings, an attractive hypothesis for the mechanism of maceration-induced skin lesion development (pressure ulcer- and moisture-associated skin damage) is as follows. Moisture-induced swelling of cells causes increased tension of the plasma membrane with a resultant reconstruction of the actin filament network. This, in turn, causes a decrease in cell processes, weakened cell-to-cell junctions, and vulnerability of macerated skin. Further study on ultrastructural changes due to skin maceration and vulnerability is warranted.

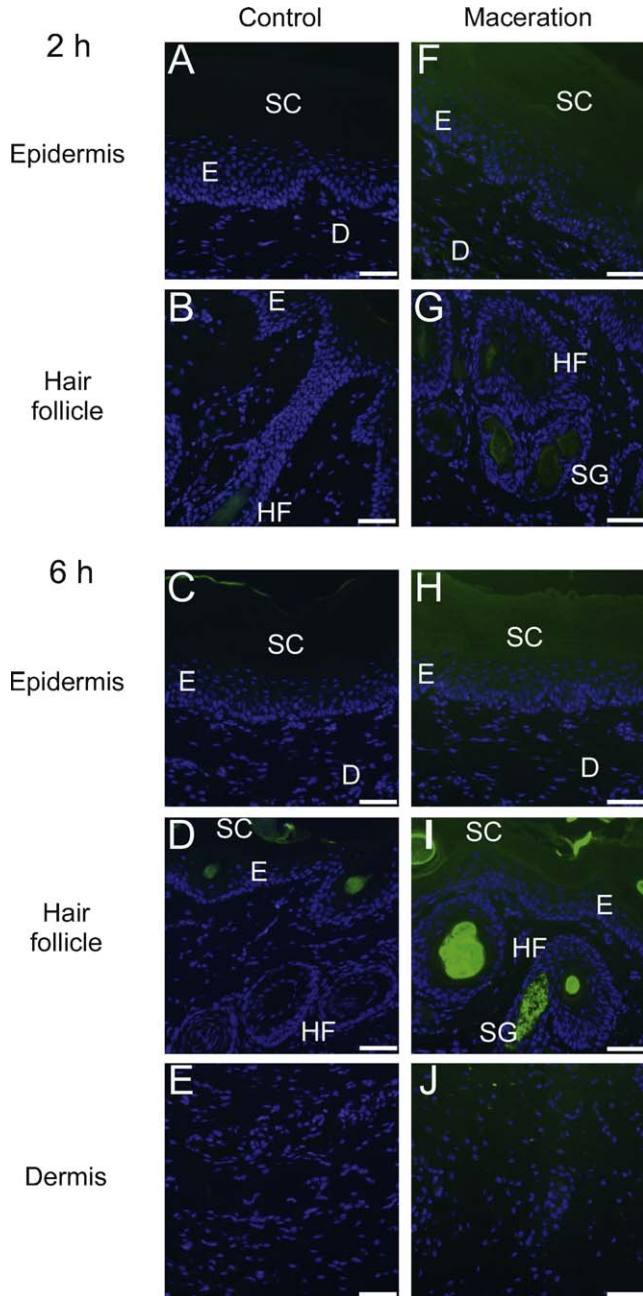
##### 4.2. Ultrastructure of aged skin and maceration-induced alteration

Numerous previous studies have revealed several structural, biochemical, and functional skin changes secondary to aging [8,13,14,27]. Ghadially et al. [28] reported a decreased number of intercellular lamellae, extensive intercellular clefts, and a decreased number of secreted lamellar bodies in the stratum corneum of the aged human and mouse. Our results revealed that aging expands the intercellular space throughout the epidermis. Several researchers have reported a reduction in proteoglycans [29,30], natural moisturizing factors, and intercellular lipid contents [28,31–33] in senescent skin. These alterations decreased the water-holding function of the epidermis. It is speculated that the lower water-holding capacity resulted in enhancement of skin maceration, and such ultrastructural changes are closely related to the decrease in the barrier function in aged skin.

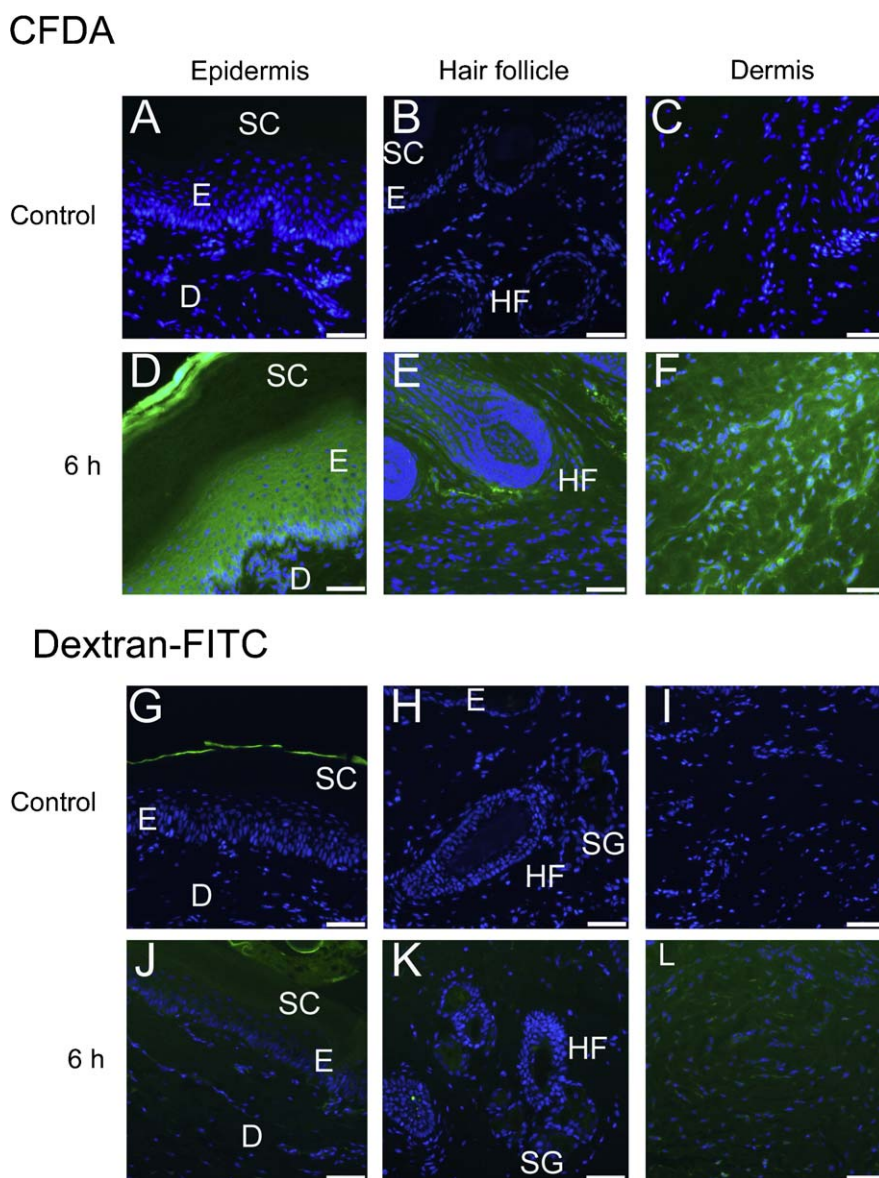
In this experiment, we observed no morphological changes in the dermis of macerated skin (data not shown). This lack of a change may indicate a potential limitation of our study. Although we used normal saline, skin maceration is induced by exposure to urine, diarrhea, and wound fluid in the clinical setting. These exposures could affect the conditions of the solution, including osmotic pressure and pH. Stool and wound fluid contain several proteinases and lipases. In addition, environmental conditions such as temperature and humidity are also related to skin maceration [34]. In future studies, the effects of the solution and environment on skin structure should be analyzed.

##### 4.3. Skin barrier function in young rat skin

The measurement of TEWL is widely used to evaluate skin barrier function, even in macerated skin [9,12]. However, we



**Fig. 6.** Penetration of dextran-FITC into the macerated skin of young rats. Initially, agarose gel droplets containing 3 kDa dextran conjugated with FITC (Dex) were attached to both the right and left foot soles for 30 min. Agarose gel without Dex was continuously attached to one side, while the contralateral side had no attachment of agarose gel until the endpoint of the experimental period (2 or 6 h). Following a 2-h treatment ( $n = 3$ ), Dex was observed in all layers of the epidermis, within hair follicles and sebaceous glands, and in the dermis just below the epidermis in macerated skin (B and D); however, Dex was observed only on the surface of the epidermis and within hair follicles in control skin (A and C). The total 6-h treatment ( $n = 3$ ) enhanced the penetration of Dex in the sebaceous glands and deeper layers of the dermis in macerated skin (F, H, and J), while Dex remained on the surface of the epidermis (E, G, and I). Magnification: 20 $\times$ . Bar = 50  $\mu$ m. SC: stratum corneum; E: epidermis; D: dermis; SG: sebaceous gland; HF: hair follicle.



**Fig. 7.** Penetration of CFDA and dextran-FITC into the macerated skin tissue of aged rats. One side of the foot soles of 6-month-old rats were exposed to agarose gel containing CFDA for 6 h (D–F), and the contralateral control soles had no exposure (A–C). Fluorescein-conjugated 3-kDa dextran (Dex) was applied to both control and macerated skin in the initial 30 min. Agarose gel was then attached to the macerated side for 5.5 h (Control: G–I; Macerated: J–L). Each experiment was replicated three times. In both experiments, green fluorescence was observed in all layers of macerated skin. The signal in the deep layer of the dermis of the aged rats was stronger than that of the young rats. Magnification: 20 $\times$ . Bar = 50  $\mu$ m. SC: stratum corneum; E: epidermis; D: dermis; SG: sebaceous gland; HF: hair follicle.

experienced difficulty accurately measuring TEWL in hydrated skin. Measurements were repeated several times immediately after the removal of agarose gel. The values rapidly decreased over the first three or four measurements, even when the skin surface was completely wiped; this was likely due to moisture held in the stratum corneum. Furthermore, although TEWL is a parameter of the transdermal evaporation of internal moisture, invasion of foreign bodies through the skin barrier should be evaluated to better understand maceration-associated skin lesions.

In this report, we evaluated the barrier function by applying two types of fluorescein to both control and macerated young rat skin. First, we used fluorescein with a small molecular weight of 460 Da (CFDA). According to the “500 Dalton rule” [35], it was expected that CFDA would easily pass through the stratum corneum. Although CFDA did not penetrate the skin barrier within a 30-min exposure, the fluorescein penetrated and diffused through both the epidermis and dermis by 6 h. In this experiment, the remarkable fluorescent signal

within hair follicles and sebaceous glands suggested the significance of the pilosebaceous route and transdermal drug delivery during the progression of skin maceration [17]. Additionally, the strong staining of cells within blood vessels probably reflected the removal of excessive moisture by blood flow.

Next, we used fluorescein with a larger molecular weight of 3 kDa (Dex), which was applied to both control and macerated young rat skin. We observed diffusion within the epidermis only by 2 h, while a 6-h exposure was required for Dex penetration into the dermis. Tan et al. [19] also analyzed the transdermal penetration of the macromolecule fluorescein-conjugated bovine serum albumin (FITC-BSA, MW 67 kDa) in macerated skin. They harvested full-thickness skin tissue from newborn piglets and exposed it to fully wetted gauze for 4–10 h (highly hydrated) or environmental conditions for 2–3 h (natively hydrated). FITC-BSA, which loaded on the surface of skin tissue placed in a petri dish for 1 h, penetrated into the deep layer of the dermis. In their report,



fluorescein penetration was extremely rapid, even though FITC-BSA is twice as large as the Dex we used. It is speculated that the rapid penetration in their experiment was caused by the long period of tissue treatment after harvest; living corneocytes may have been damaged during this period. Therefore, comparison between Tan's and our results suggest that the living cell layers, such as the stratum basale and spinosum of the epidermis, also contribute to the obstruction of macromolecules in the skin barrier function.

#### 4.4. Enhancement of transdermal penetration of fluorescein by aging

According to Jung et al. [36], the water and glycosaminoglycan content of the skin of Sprague–Dawley rats rapidly decreased until 3 months of age, then remained constant from 3 to 21 months of age. Similarly, Monterio-Riviere et al. [37] reported that the epidermal thickness in Fischer-344 rats decreased from 2 to 3 months of age, then remained constant until 24 months of age. These results indicate that age-related morphological and functional changes are drastic until 3 months of age in rats. Therefore, we used 6-month-old rats without clinical dermatopathies as aged skin models. Our study revealed fluorescence with a similar distribution as, but greater intensity in, senescent rat skin. As noted in Section 3, this suggests that more fluorescein molecules and a greater volume of solution penetrated the skin barrier; aging enhanced the maceration-induced disruption of the skin barrier function. It follows that the development of technology that promotes skin barrier recovery after maceration in the elderly is warranted to help prevent the development of pressure ulcer- and moisture-associated skin damage.

Ghadially et al. [28] studied the skin barrier function by measuring TEWL in young and senescent mice. While the TEWL value was normal even in the aged mice, delayed recoveries of the skin barrier disrupted by acetone treatment and tape stripping were reported. They also observed lipid lamellar bodies in the stratum granulosum by electron microscopy. Disruption of the intercellular lipid structure promotes the formation and secretion of lamellar bodies filled with lipid material [38]. Although numerous lamellar bodies were formed 3 h after barrier disruption, they were not replete with lipid material. The reduced activities of acid sphingomyelinase and ceramide synthase [32] probably resulted in the formation of lamellar bodies with poor lipid quality, as well as a delayed recovery of skin barrier function.

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