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Skin aging modulates percutaneous drug absorption: the impact of ultraviolet irradiation and ovariectomy

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Abstract Ultraviolet (UV) exposure and menopause are known as the inducers of damage to the skin structure. The combination of these two factors accelerates the skin aging process. In this study, we aimed to evaluate the influence of UV and ovariectomy (OVX) on the permeation of drugs through the skin. The role of tight junctions (TJs) and adherens junctions (AJs) in the cutaneous absorption of extremely lipophilic permeants and macromolecules was explored. The OVX nude mouse underwent bilateral ovary removal. Both UVA and UVB were employed to irradiate the skin. The physiological and biochemical changes of the skin structure were examined with focus on transepidermal water loss (TEWL), skin color, immunohistochemistry, and mRNA levels of proteins. UVB and OVX increased TEWL, resulting in stratum corneum (SC) integrity disruption and dehydration. A hyperproliferative epidermis was produced by UVB. UVA caused a pale skin color tone due to keratinocyte apoptosis in the epidermis. E-cadherin and β-catenin showed a significant loss by both UVA and UVB. OVX downregulated the expression of filaggrin and involucrin. A further reduction was observed when UV and OVX were combined. The in vitro cutaneous absorption demonstrated that UV

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increased the skin permeation of tretinoin by about twofold. However, skin accumulation and flux of estradiol were not modified by photoaging. OVX basically revealed a negligible effect on altering the permeation of small permeants. OVX increased tretinoin uptake by the appendages from 1.36 to 3.52 μg/cm^2. A synergistic effect on tretinoin follicular uptake enhancement was observed for combined UV and OVX. However, the intervention of OVX to photoaged skin resulted in less macromolecule (dextran, molecular weight=4 kDa) accumulation in the skin reservoir because of retarded partitioning into dry skin. The in vivo percutaneous absorption of lipophilic dye examined by confocal microscopy had indicated that the SC was still important to controlling topical delivery, although the role of epidermal junctions could not be simply ignored.

Keywords Percutaneous absorption · Ovariectomy · Photoaging · Ultraviolet · Epidermal junction

Introduction

The skin ages intrinsically as a result of the passage of time together with hormone deficiency, such as that occurring with menopause (Calleja-Agius and Brincat 2012). Sex hormones have important functions in regulating skin development and physiology. For example, a lack of estrogen results in abnormality of the epidermis, dermis, vasculature, and appendages (Parchami and Fatahian Dehkordi 2010). Cutaneous aging can be accelerated by ultraviolet (UV) radiation from sun exposure. Photoaging is becoming significant due to today's lifestyle and severe climate change. Optimal care is essential to prevent aging or to treat aged skin. Some topical drug therapies, including sunscreens, antibiotics, retinoids, and hormone supplements, are useful for skin aging management (Farage et al. 2009). It is anticipated that drug absorption via intrinsically or extrinsically aged skin is quite different from that via intact skin. Assessment of the efficacy and possible over-absorption of topically applied drugs for aged skin is important to avoiding adverse risk. This issue is especially critical for the elderly since a hormone shortage increases skin sensitization to photoaging (Šitum et al. 2010). In this study, we aimed to examine percutaneous drug absorption as influenced by menopause and UV radiation.

Skin barrier function cannot be attributed to stratum corneum (SC) alone but also to the tight junctions (TJs) and adherens junctions (AJs) just below the SC (Yamamoto et al. 2008; Andrews et al. 2013). Junctions are intercellular junctions in the viable epidermis controlling paracellular permeability and epithelial adhesion (Niessen 2007). Since the resistance of TJs and AJs for drug transport cannot be ignored, the second aim of this work was to elucidate the role of junctions on drug permeation into/across aged skin.

Viable skin offers a pronounced barrier for extremely lipophilic molecules (Hung et al. 2012). We selected tretinoin (log P=6.30) as a model permeant since both the SC and the epidermis provide resistance against tretinoin diffusion (Chen et al. 2013). Tretinoin is a retinoid used for photoaging treatment. Estradiol (log P=4.01) was also chosen in this study. It induces epidermal growth factor upregulation and anti-inflammation in UVB-irradiated skin (Röck et al. 2012). Transdermal estradiol provides hormone replacement therapy (HRT) for menopausal women to improve postmenopausal symptoms such as dermal wrinkling, skin flushing, and cardiovascular risk (Egras and Umland 2010). A macromolecule of dextran with a molecular weight (MW) of 4 kDa was also employed to examine skin permeation since some macromolecular proteins and nucleotides have been proved to inhibit UV-induced skin damage (Antoniou et al. 2010). The findings of this report may have important implications for evaluating the feasible dose of topically applied drugs for the elderly, especially the menopausal population.

Materials and methods

Materials

Tretinoin, estradiol, fluorescein isothiocyanate (FITC)-conjugated dextran with a MW of 4 kDa, and rhodamine B were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used in this work were of analytical grade.

Animals

Female normal nude mice (ICR-Foxn1nu) and ovariectomy (OVX) mice at 8 weeks old were supplied by Biolasco (Taipei, Taiwan). The OVX procedure was

performed according to a previous study (Tsukahara et al. 2004). Briefly, the flanks were opened bilaterally. The ovaries were carefully removed after ligation of oviducts with catgut. The fascia and skin were then sutured. Animal experimental protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung University. All animals used in this work were treated under the institutional guidelines.

UV irradiation

UV treatment was started 1 week after operation. A Bio-Sun illuminator (Vilber Lourmat, Marne-la-Vallée, France) was used to produce UVA (365 nm) or UVB (312 nm). The distance between the nude mice and the lamp was 10 cm. The spectral irradiance was 10 J/cm^2 and 175 mJ/cm^2 for UVA and UVB, respectively. The dorsal region was irradiated with UVA every other day for 5 days. They were exposed to UVB once a day for 5 days.

Transepidermal water loss and colorimetry

Transepidermal water loss (TEWL) and skin color of the dorsal region were examined 1 h after the accomplishment of UV irradiation course. A Tewameter® (TM300, Courage and Khazaka, Köln, Germany) was employed for measuring TEWL (g/m^2/h). A spectrocolorimeter (CD100, Yokogawa, Tokyo, Japan) was used to quantify skin lightness (L*) and erythema (a*).

Histological analysis

The dorsal skin was excised from the mice after sacrifice. The skin species were immersed in a 10 % buffered formaldehyde using ethanol, embedded in paraffin wax, and sliced at a thickness of 3 μm. The samples were stained with hematoxylin and eosin (H&E) and imaged under light microscopy (IX81, Olympus, Tokyo, Japan).

Immunohistochemistry

For immunohistochemical observation, primary anti-E-cadherin and anti-β-catenin antibodies were incubated with the skin specimens at room temperature for 1 h. The skin sections stained with E-cadherin were visualized by fluorescence microscopy (IX81, Olympus). Immunoreactivity was detected with an Alexa Fluor 594 goat anti-rabbit immunoglobulin antibody. The biopsies stained with β-catenin antibody were monitored by light microscopy.

Enzyme-linked immunosorbent assay

Levels of E-cadherin in skin were measured by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Abchem, San Francisco, CA, USA) with a colorimetric method. β-Catenin was also detected by the ELISA kit (BioVision, Milpitas, CA, USA). This assay employed antibodies specific for mouse E-cadherin and β-catenin coated on a 96-well plate. The samples were pipetted into the wells, and E-cadherin/β-catenin present in the sample was bound to the wells by the immobilized antibody. The wells were washed, and biotinylated anti-mouse antibodies were added. After washing away unbound biotinylated antibody, streptavidin was pipetted to the wells. The wells were again washed; a stop solution was added to the wells, and color developed in proportion to the amount of E-cadherin/β-catenin bound. Following addition of the stop solution, the optical density of each well was determined immediately by ELISA plate reader at 450 nm.

Reverse transcription polymerase chain reaction

Total RNA was extracted from the skin with the employment of RB buffer containing β-mercaptoethanol (1 %). RNA was subsequently isolated using Total RNA Mini Kit (Geneaid, New Taipei City, Taiwan). Reverse transcription polymerase chain reaction (RT-PCR) was carried out with a Deoxy$^+$ HiSpec Reverse Transcriptase Kit (Yeastern, Taipei, Taiwan). The following primers were used to analyze target mRNA expression: filaggrin: 5′-AGTCCAGTGGGAGAGGACAC-3′ (forward) and 5′-TCCAAGGGGAACACATGGAGA-3′ (reverse); integrin β: 5′-GTGTTGGGAGGCACTGTGAA-3′ (forward) and 5′-TTGGTGAGATTGAAGTGGGAGC-3′ (reverse); involucrin: 5′-ACCTGAACCAGAACTG CACC-3′ (forward) and 5′-TCCGGTTCTCCAATTC GTGT-3′ (reverse). GADPH was an internal control.

In vitro percutaneous absorption

This experiment was conducted with Franz diffusion cell. The excised skin was mounted between the donor and receptor compartments with SC facing upward into

the donor. The receptor contained 30 % ethanol in pH 7.4 buffer with a volume of 5.5 ml for tretinoin and estradiol. Receptor medium was pH 7.4 buffer for dextran. The donor (0.5 ml) was loaded with tretinoin (0.1 %*w*/*v*), estradiol (0.1 %), or dextran (0.05 %) in 30 % propylene glycol/water, 30 % ethanol/pH 7.4 buffer, and water, respectively. The effective diffusion region for permeants was 0.785 cm^2. The temperature and stirring rate of the stirrer was kept at 37 °C and 600 rpm, respectively. At determined intervals, a 300-μl aliquot was withdrawn from the receptor. After a 24-h application, the skin was removed from Franz cell. The permeant amount within skin reservoir was extracted by methanol for tretinoin and estradiol or 0.1 N HCl for dextran as described previously (Hsieh et al. 2013). The samples of tretinoin and estradiol were analyzed by high performance liquid chromatography (HPLC) (Fang et al. 2001; Lin et al. 2013). The samples taken from dextran application were quantified by a fluorescence spectrophotometer (F2500, Hitachi, Tokyo, Japan).

Hair follicle uptake

Differential stripping and cyanoacrylate skin surface casting were utilized to detect the content of permeants in hair follicles (Teichmann et al. 2005). Subsequent to stripping the SC, a follicular cast was prepared. A drop of superglue was added on a glass slide, which was pressed onto the surface of SC-stripped skin. The cyanoacrylate polymerized, and the slide was expelled with one quick movement after 5 min. The superglue remaining on the slide was scraped off and positioned in a tube with 2 ml methanol. The tube was shaken for 3 h. The final product was vacuumed to evaporate methanol. Mobile phase or water was added to dissolve the residuals for HPLC or fluorescence spectrophotometry assay.

In vivo percutaneous absorption

A glass cylinder with a hollow area of 0.785 cm^2 was attached to mouse back by superglue. An aliquot of 0.2 ml of 30 % ethanol/pH 7.4 buffer containing rhodamine B (0.03 %) as the dye was pipetted into the cylinder. The application period was 2 h. The animal was then sacrificed, and the treated skin area was excised. A confocal laser scanning microscope (TCS SP2, Leica, Wetzlar, Germany) was used to observe a horizontal section of the skin. The thickness of the skin was scanned at 5-μm increments via *z*-axis. Images were taken by summing 15 fragments at different depths from the skin surface.

Statistical analysis

Statistical analysis of differences between the groups was performed using Kruskal–Wallis test. The post hoc test used for checking individual differences was Dunn's test. A 0.05 level of probability ($p<0.05$) was taken as the level of significance.

Results

Transepidermal water loss and colorimetry

In order to confirm the success of OVX, the biopsy was examined. The OVX mice lacked ovaries and had atrophied uteri (data not shown), whereas the normal mice showed typical and thickened uteri. We first determined how UV and OVX affected cutaneous physiology. As indicated in Table 1, the TEWL of UVA-treated skin did not show statistical significance ($p>0.05$) compared to that of nontreated skin. TEWL increased following UVB irradiation ($p<0.05$). OVX induced a significantly greater TEWL than the nontreated and UVA groups. UVB exposure of the OVX mouse did not further elevate the TEWL. UVA treatment resulted in a tendency toward an increase in skin lightness (L*, $p<0.05$) in both normal and OVX animals. This lightness increment reflected a reduction in skin redness (a*) in UVA-treated groups, although no significant difference ($p>0.05$) was detected. The combination of OVX and UVB also augmented the L* value from 54.2 to 57.4.

Histology and immunohistochemistry

Figure 1a shows close-up images of nude mice. A normal mouse treated with UVA revealed a paler white skin tone as compared to a mouse that did not receive treatment. Sagging in the back, especially near the neck, was observed. UVB treatment in non-OVX skin was documented by desquamation. Visual assessment after OVX showed a paler tone than untreated skin. As with a normal mouse receiving UVA and UVB, the skin surface of OVX/UVA and OVX/UVB mice demonstrated sagging and desquamation, respectively. As shown in the top images of Fig. 1b, H&E-stained histology

Table 1 In vivo physiologic parameters of nude mouse skin treated with ovariectomy, UVA, and UVB, determined by TEWL, lightness (L*), and erythema (a*)

Mouse	Radiation	TEWL ($g/m^2/h$)	L*	a*
Normal	None	9.38±2.78	54.22±0.64	2.76±0.89
	UVA	11.58±2.85	57.47±1.18*	1.22±1.09
	UVB	16.10±5.08*	53.79±1.69	3.25±0.62
Ovariectomy	None	22.30±8.19$^{\#}$	54.20±1.48	1.79±0.92
	UVA	17.73±2.78$^{\#}$	57.36±1.94*	1.23±0.68
	UVB	18.30±2.91	57.37±0.65*$^{\#}$	2.32±0.46

All data are presented as the mean of ten experiments ± S.D.

*$p<0.05$ as compared to non-radiation group; $^{\#}p<0.05$ as compared to non-ovariectomy group

exhibits an intact structure of untreated skin. The nuclei of keratinocytes in the upper epidermis were lost in normal/UVA and OVX/UVA animals, indicating the occurrence of cell apoptosis and epidermal degeneration by UVA irradiation. The groups receiving UVB (normal/UVB and OVX/UVB) produced epidermal hyperplasia, which had at least doubled the epidermal thickness. This hyperkeratosis was associated with a thickening of the stratum granulosum region (arrows in Fig. 1b).

E-cadherin contributes to epidermal TJs for providing diffusion resistance. As shown in the top images of Fig. 2a, E-cadherin is observed in the lower epidermis of the control skin (arrows). E-cadherin was diminished following UVA exposure (normal/UVA and OVX/UVA). Only some signals were seen near the epidermal–dermal junctions (arrows). UVB did not significantly downregulate E-cadherin in the normal/UVB group. However, this protein had nearly disappeared in the epidermis treated with the OVX and UVB combination. This indicates an increased sensitivity of OVX skin to UVB. The amount of E-cadherin was quantified by colorimetric assay as shown in the bottom images of Fig. 2a. The results of ELISA were similar to the immunohistochemistry. Treatment of UVA significantly reduced ($p<0.05$) E-cadherin level in normal mice. UVB irradiation also decreased E-cadherin level in OVX mice, although the statistical analysis did not reach a significant level. Expression of β-catenin, an AJ-related biomarker, is detected as a distinct line in the intercellular regions of intact skin (arrows in the top images of Fig. 2b). A selective loss of β-catenin occurred in the focal regions of UVA- and UVB-treated skin (normal/UVA and normal/UVB). β-Catenin with abundant amounts remained localized in the epidermis after treatment of OVX alone. OVX/UVA and OVX/UVB mice expressed fragmented β-catenin in a discontinuous pattern. It seemed that β-catenin expression was less for OVX skin than for normal skin in the presence of photoaging. As illustrated in the bottom images of Fig. 2b, the management of UVA or UVB decreases β-catenin amount in both normal and OVX skins. This decrease showed a significant difference ($p<0.05$) as compared to the groups without photoaging (normal and OVX), except the β-catenin decrease by normal/UVA intervention.

Reverse transcription polymerase chain reaction

To determine the effect of photoaging and OVX on gene expression involved in epidermal junctions, total RNA was isolated from skin for examining the mRNA levels of integrin β, filaggrin, and involucrin (Fig. 3). Either UV radiation or OVX caused a significant downregulation of integrin β. A total deficiency of filaggrin was detected in the UVA-treated animals. Although filaggrin was also significantly decreased by UVB, the inhibition level was minor compared to UVA. The result was in line with the immunohistochemistry of E-cadherin. There was a marked decrease of filaggrin in the skin treated with OVX. In the normal mouse, involucrin mRNA expression was found to be unaltered by UVA and UVB irradiation. Involucrin was partially lost after OVX treatment. A marked decrease of involucrin was demonstrated for OVX/UVA and OVX/UVB. This synergistic inhibition by combining UV and OVX was found to parallel the expression of β-catenin.

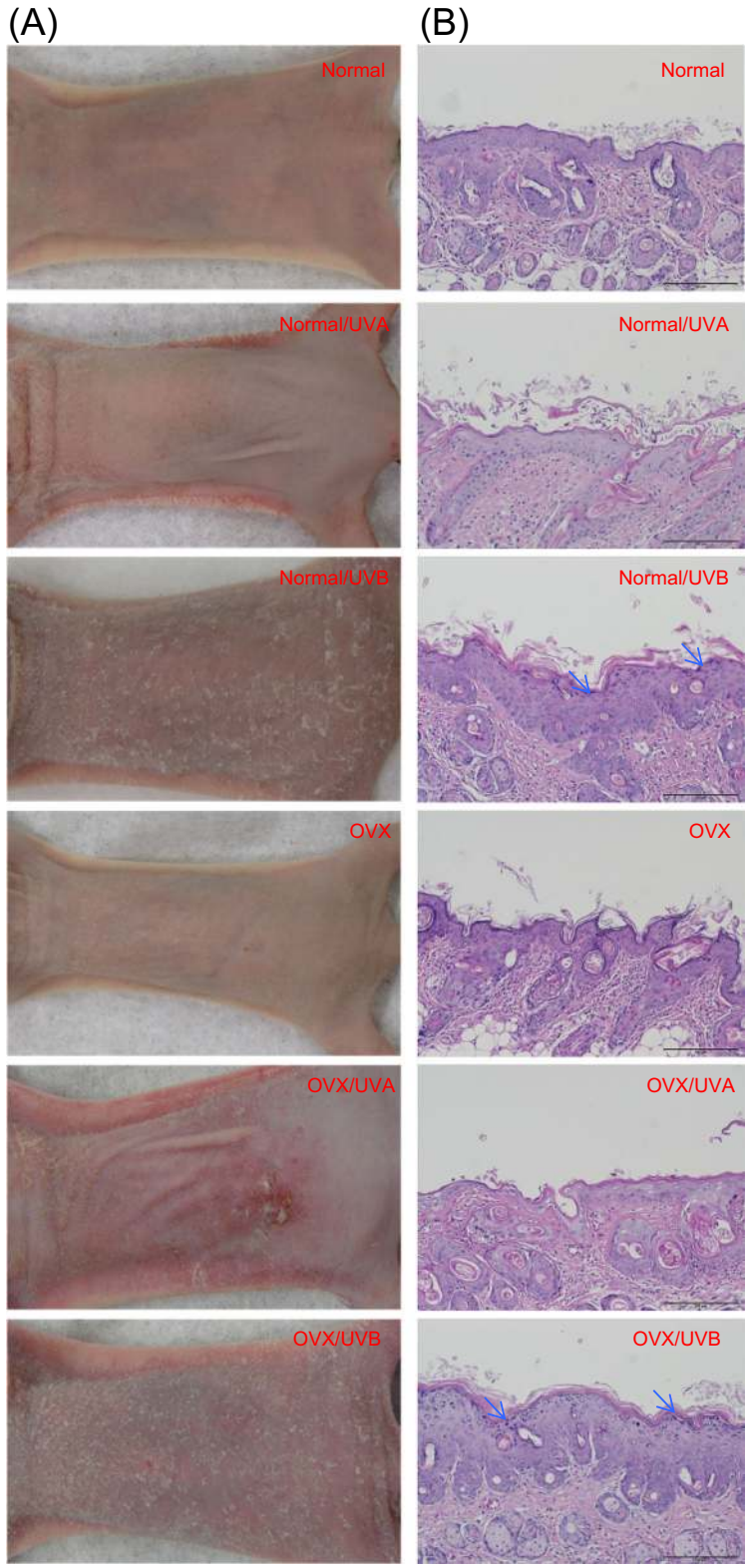

Fig. 1 Macroscopic observation (**a**) and hematoxylin and eosin (H&E)-stained histological sections (**b**) of nude mouse skin (8 weeks old) undergoing UVA or UVB in the absence or presence of ovariectomy (*OVX*) treatment. *Scale bar* 100 μm

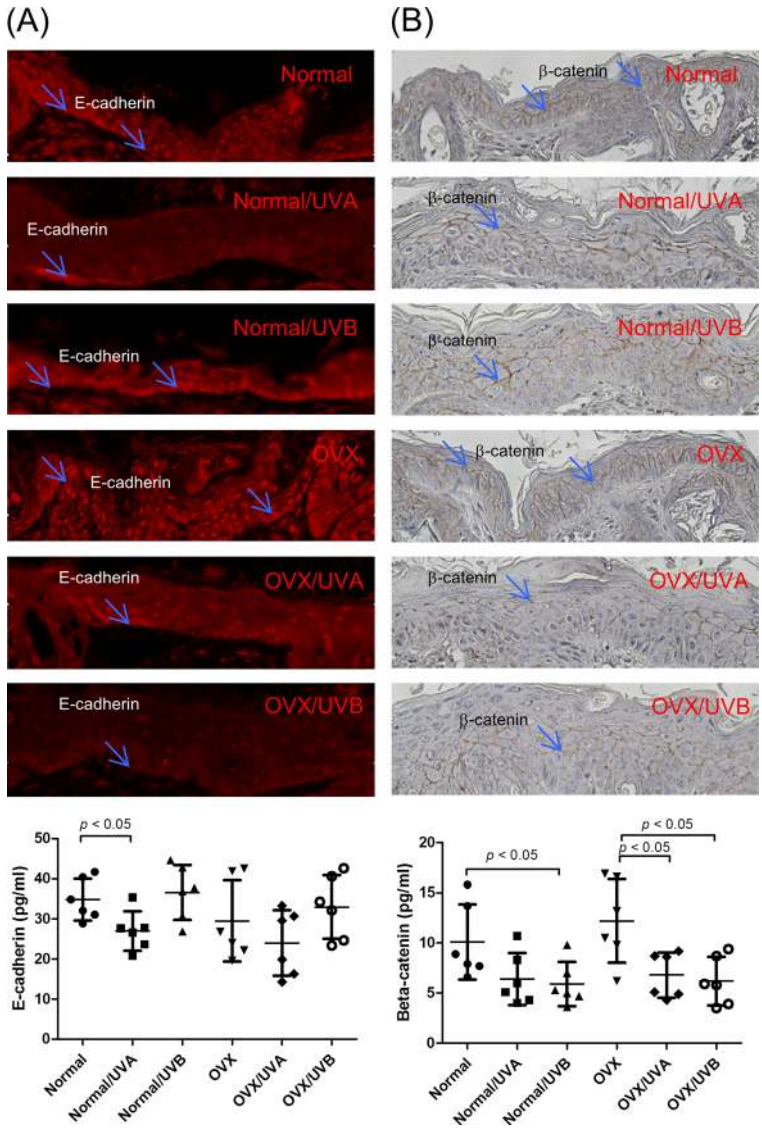

Fig. 2 Immunohistochemical histology and ELISA stained by E-cadherin (**a**) and β-catenin (**b**) of nude mouse skin (8 weeks old) undergoing UVA or UVB in the absence or presence of ovariectomy (*OVX*) treatment

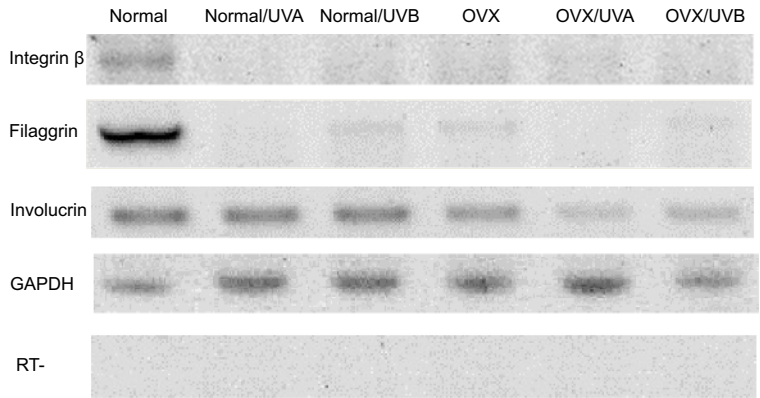

Fig. 3 The mRNA levels of integrin β, filaggrin, and involucrin extracted from nude mouse skin (8 weeks old) undergoing UVA or UVB in the absence or presence of ovariectomy (*OVX*) treatment

In vitro percutaneous absorption

Both the skin reservoir and flux of drugs into/across the skin were examined in the in vitro Franz cell. The accumulation in the skin indicates cutaneous uptake by topical delivery, whereas the flux predicts transdermal delivery to systemic circulation. Table 2 summarizes the permeation profiles of tretinoin, a drug with an extremely high lipophilicity. The skin deposition of tretinoin via normal skin without UV radiation was 0.37 μg/mg. The same value was obtained by the OVX skin, suggesting that OVX did not affect tretinoin absorption into the skin. UVA and UVB irradiation increased tretinoin deposition in both the normal and OVX groups more than in the respective controls. UVA and UVB revealed an increased skin reservoir of 1.5- and 2.0-fold over normal skin, respectively. With respect to OVX animals, tretinoin deposition of UVA- or UVB-treated skin increased by a factor of 1.5. The flux was calculated by the slope of linear penetration in a cumulative amount in receptor–time profiles. Similar to the skin reservoir, tretinoin flux was increased by photoaging. UVA and UVB showed a 1.8- and 2.8-fold higher tretinoin flux compared to that in normal skin. The flux of tretinoin was 2.2 times higher after OVX/UVA treatment compared to OVX alone. Although UVB also increased tretinoin flux via OVX skin, the difference did not achieve statistical significance ($p>0.05$). We evaluated permeant uptake by follicles to evaluate the effect of aging on appendageal pathways. As depicted in Table 2, UV did not alter the follicular uptake of tretinoin in normal skin ($p>0.05$). It was surprising that OVX caused a significant increase in follicular tretinoin ($p<0.05$) from 1.36 to 3.52 μg/cm^2. UVB, but not UVA, further promoted tretinoin uptake in follicles treated with OVX ($p<0.05$).

Estradiol is another lipophilic permeant with less lipophilicity (log $P=4.01$) than tretinoin (log $P=6.30$). Table 3 compares the skin permeation profiles of estradiol via normal and aged skin. Different from the case of tretinoin, estradiol skin deposition was not influenced by either photoaging or OVX ($p>0.05$). A minor influence of UV and OVX was also observed in estradiol flux, except that UVA increased the flux via OVX-treated skin from 2.29 to 3.59 μg/cm^2/h ($p<0.05$). Estradiol uptake in the follicles of the normal/UVA group displayed a 3.5-fold increase compared to that of intact skin. UVB (normal/UVB) exhibited a negligible effect on the follicular estradiol amount ($p>0.05$). UVA (OVX/UVA) significantly enhanced ($p<0.05$) the follicular amount of estradiol compared to the OVX control by 3.7-fold. The follicular uptake of OVX skin was slightly but significantly ($p<0.05$) increased by UVB from 1.08 to 1.59 μg/cm^2.

Table 4 demonstrates the skin permeation of dextran with an MW of 4 kDa. There was no dextran detected in the receptor during a 24-h application due to the large size of the macromolecules. Dextran deposition in normal skin was established as 1.49 ng/mg. When the normal skin was treated by UVA, dextran deposition increased to 5.75 ng/mg ($p<0.05$). A comparable enhancement was observed with the treatment of UVB. There was no significant difference ($p>0.05$) between normal and OVX skin without photoaging. Compared to the normal skin, UV application led to less enhancement on dextran accumulation in OVX skin. There was not even any dextran deposition in OVX skin treated by UVB. Dextran in hair follicles was not affected by either UV or OVX ($p>0.05$) except the group of OVX/UVB, which showed a negligible follicle accumulation due to the lack of dextran deposition within the skin reservoir.

Table 2 Skin accumulation (μg/mg), flux (μg/cm^2/h), and follicular amount (μg/cm^2) of tretinoin permeation via skin treated with ovariectomy, UVA, and UVB

Mouse	Radiation	Skin accumulation (μg/mg)	Flux (μg/cm^2/h)	Follicular amount (μg/cm^2)
Normal	None	0.37±0.05	0.26±0.13	1.36±0.23
	UVA	0.57±0.05*	0.48±0.08*	1.04±0.33
	UVB	0.71±0.39*	0.72±0.16*	1.38±0.46
Ovariectomy	None	0.37±0.15	0.25±0.10	3.52±0.91$^{\#}$
	UVA	0.54±0.05*	0.56±0.13*	4.07±0.59$^{\#}$
	UVB	0.56±0.05*	0.30±0.14$^{\#}$	5.02±0.29*$^{\#}$

All data are presented as the mean of four experiments ± S.D.

*$p<0.05$ as compared to non-radiation group; $^{\#}p<0.05$ as compared to non-ovariectomy group

Table 3 Skin accumulation (μg/mg), flux (μg/cm^2/h), and follicular amount (μg/cm^2) of estradiol permeation via skin treated with ovariectomy, UVA, and UVB

Mouse	Radiation	Skin deposition (μg/mg)	Flux (μg/cm^2/h)	Follicular amount (μg/cm^2)
Normal	None	0.32±0.07	2.41±0.31	0.87±0.19
	UVA	0.26±0.09	3.11±0.60	3.08±1.04*
	UVB	0.25±0.03	2.58±0.80	0.84±0.15
Ovariectomy	None	0.30±0.05	2.29±0.41	1.08±0.23
	UVA	0.24±0.03	3.59±0.33*	4.00±1.17*
	UVB	0.22±0.07	1.59±0.34	1.59±0.10*$^{\#}$

All data are presented as the mean of four experiments±S.D.

*$p<0.05$ as compared to non-radiation group; $^{\#}p<0.05$ as compared to non-ovariectomy group

In vivo percutaneous absorption

Levels of rhodamine B, a lipophilic dye, distributed in skin were determined following in vivo application to the dorsal area of nude mice. Figure 4 shows the CLSM profiles. We acquired the *x*–*y* planed sectional imaging from the skin surface with a ~5-μm increment. Figure 4a shows the summary of 15 fragments. The results suggested that UVA and UVB could enhance the fluorescence intensity of rhodamine B distributed in the skin. The most intense signal was seen in the hair shafts. More fluorescein permeants penetrated the skin barrier by OVX operation. Similar to the normal skin, the fluorescence in the OVX/UVA and OVX/UVB groups was greater than that in OVX alone. To demonstrate the dye accumulation in SC and epidermis, Fig. 4b represents the fluorescence at depths of ~5 and ~20 μm from the skin surface, which is the location of the nude mouse SC and epidermis. A faint fluorescence in the SC of normal skin was observed. Normal/UVA skin showed a comparable fluorescence in the SC as compared to intact skin. The fluorescence intensity in the SC of the OVX group did not surpass that of normal skin. This indicates that the greater dye distribution in whole OVX skin was derived from the skin layers below the epidermis. Photoaging increased rhodamine B accumulation in the SC and epidermis of OVX mice. The fluorescence in the epidermis was generally higher than that in the SC. The trend of fluorescence in the epidermis was the same as that in the SC, demonstrating that the SC may govern the process of rhodamine B transport.

Discussion

It is our aim to investigate the influence of barrier function on drug delivery via the skin undergoing UV exposure and OVX. The extremely lipophilic drugs and macromolecules were employed in this work due to the considerable resistance of viable skin for these permeants. Our results revealed SC integrity disruption and dry skin by UVB and OVX according to the

Table 4 Skin accumulation (μg/mg), flux (μg/cm^2/h), and follicular amount (μg/cm^2) of dextran permeation via skin treated with ovariectomy, UVA, and UVB

Mouse	Radiation	Skin accumulation (ng/mg)	Follicular amount (μg/cm^2)
Normal	None	1.49±0.75	0.82±0.38
	UVA	5.75±2.84*	0.68±0.22
	UVB	4.43±1.89*	1.05±0.34
Ovariectomy	None	1.93±0.42	0.98±0.01
	UVA	2.81±1.51$^{\#}$	0.87±0.12
	UVB	0*$^{\#}$	0*$^{\#}$

All data are presented as the mean of four experiments ± S.D.

*$p<0.05$ as compared to non-radiation group; $^{\#}p<0.05$ as compared to non-ovariectomy group

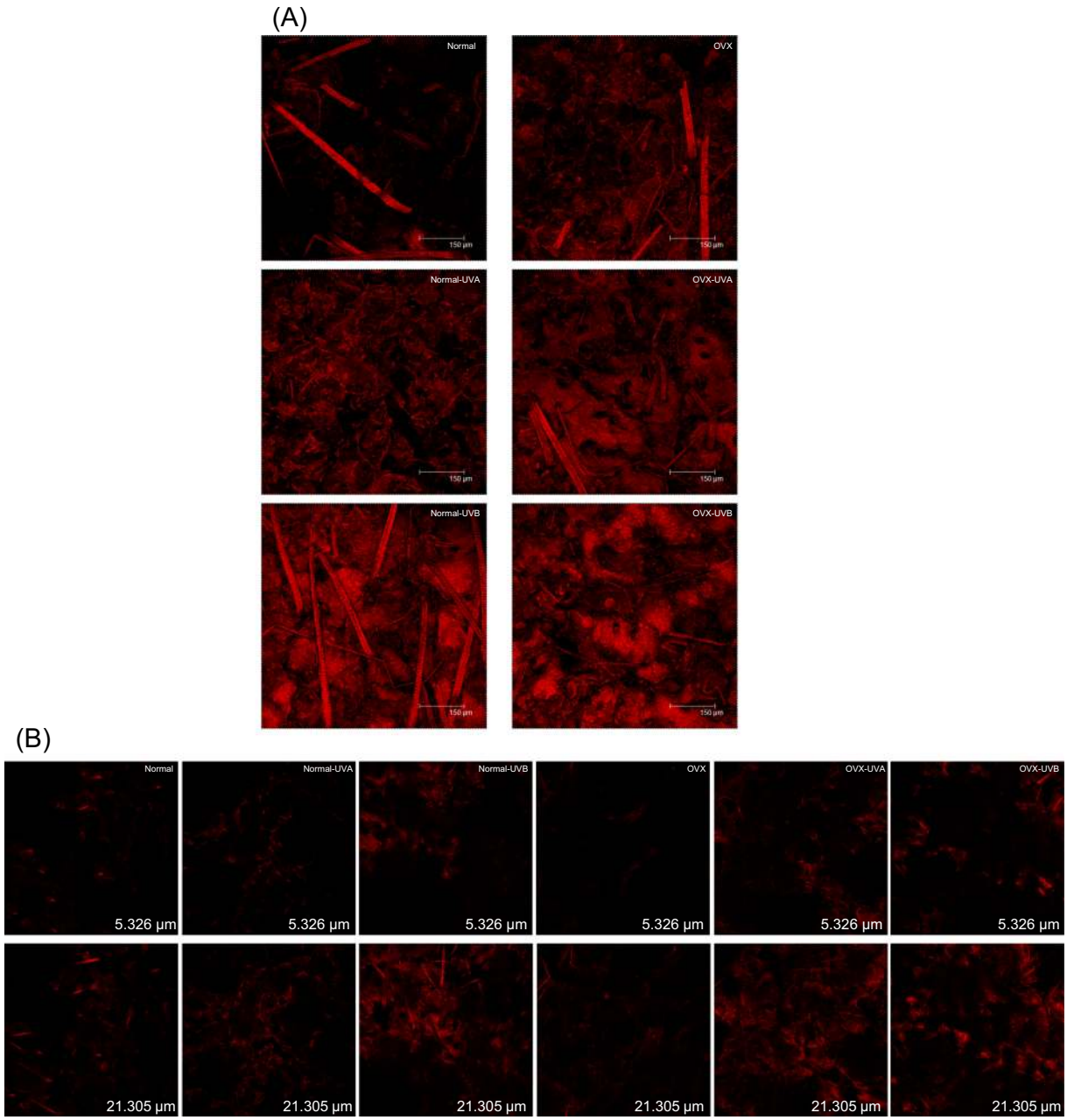

Fig. 4 Confocal micrographs of nude mouse skin (8 weeks old) undergoing UVA or UVB in the absence or presence of ovariectomy (OVX) treatment: **a** summary of 15 fragments at various skin depths, **b** fragment at the depth of ~5 μm (*upper panel*) and ~20 μm (*lower panel*) from skin surface. *Scale bar* 150 μm

increased TEWL. UVB exerted stronger damage to the SC as compared to UVA. Contrary to this result, UVA disorganized junctions with a greater level than UVB. Some proteins related to the barrier function of SC and epidermis were downregulated by OVX. OVX generally did not affect drug delivery into photoaged skin, except a synergistic effect of combined UV/OVX for tretinoin follicular uptake. OVX intervention even reduced macromolecule absorption via photoaged skin.

TEWL is an indicator of SC damage or loosening. UVB, but not UVA, enhanced the TEWL values in our study. In accordance with previous studies (Tsukahara

et al. 2008; Yuki et al. 2011), chronic UVB exposure increases TEWL. Tsukahara et al. (2005) also indicated that UVA increased TEWL only under conditions of very high energy and prolonged duration. UVA generally penetrates deeper into the skin than UVB, resulting in less influence on the superficial SC layer. Çömelekoğlu et al. (2012) also suggest that OVX compromised skin strength and toughness via oxidative stress in rats. OVX by itself generated skin aging; the barrier function of SC was thus compromised. The increased TEWL by OVX may decrease the water content of the skin, the major symptom of postmenopausal skin (Calleja-Agius et al. 2007). The blood flow of dermal microcirculation decreases significantly in both OVX mice and menopausal women (Tsukahara et al. 2008; Calleja-Agius and Brincat 2012). The macroscopic observation of OVX skin in this study also showed a paler skin color as compared to normal skin. The paler skin tone was also detected in UVA-treated skin. The keratinocyte apoptosis and skin necrosis may contribute to the decreased blood supply in UVA-exposed skin.

We had inspected some biomarkers related to TJs and AJs. In general, photoaging and OVX showed a capability to deteriorate junctions in the epidermis. Integrin β compromises the barrier function of the skin, especially in epidermal–dermal adhesion (Has et al. 2012). Both UV and OVX were harmful to integrin β with a significant reduction. This suggests that UV and OVX have the effect of disrupting the permeability barrier in viable skin. OVX could aggravate suppression of epidermal junctions by photoaging according to involucrin and β-catenin expression. Involucrin serves as a substrate that covalently binds to ceramides in the SC (Proksch et al. 2008). β-Catenin plays a role in the regulation of intercellular mobility. It is fundamental to maintaining AJs under externally applied stress (Ray et al. 2013).

Filaggrin is a structural protein, which is postulated to participate in the mechanical strength and integrity of epidermal barrier function (Kezic et al. 2009). OVX had a dramatic effect on filaggrin downregulation. Corneocytes in the SC are anchored by keratin–filaggrin attachments (Levin et al. 2013). Filaggrin is important in maintaining skin hydration. Filaggrin deficiency results in the loss of natural moisture factors (NMF) in SC (Kawasaki et al. 2012). Dry skin is always a significant indication of postmenopausal individuals. The filaggrin suppression by OVX contributed to SC integrity compromise and TEWL increase. Another observation was that UVA showed a greater reduction of filaggrin than UVB. The same trend was shown in the result of E-cadherin. Adherens junctions and desmosomes mediate cell–cell cohesion via E-cadherin (Tunggal et al. 2005). E-cadherin absence leads to permeable TJs and loss of epidermal resistance. Nakai et al. (2012) demonstrated that the E-cadherin decrease in atopic dermatitis is due to a filaggrin deficiency. Our results also verified the relationship between E-cadherin and filaggrin in photoaged skin.

Junctions are substantial for the selective passage of molecules via the skin (Kirschner et al. 2010). Our results suggested that UV generally increased skin permeation of drugs. This phenomenon was especially significant for tretinoin. UVA induced a barrier defect by junction loosening because of the loss of E-cadherin, β-catenin, integrin β, and filaggrin. The integrity of the barrier was weakened, allowing augmented skin transport of tretinoin. A similar result was detected by UVB, although the harmful effect on TJs was less than that of UVA. Inflammation and the resulting accumulation of reactive oxygen species (ROS) exert an essential role on skin aging (Pillai et al. 2005). ROS produce damage to lipids, proteins, and DNA within the skin. It is reported that ROS disrupt junctions and cell adhesion molecules such as cadherins and integrins (Mittal et al. 2014). The more severe disruption of epidermal junctions by UVA may be associated with keratinocyte apoptosis, which is a mechanism of reducing barrier function (Yamamoto et al. 2008). An increase of mast cells and neutrophils has been shown in the photoaged skin. Mast cells modulate skin aging process by damaging many surrounding cells, including keratinocytes and fibroblasts (Mansouri et al. 2014). Nevertheless, UVA and UVB basically showed a comparable effect on drug permeation. Based on TEWL data, this may be due to UVB damaging the SC integrity. This SC disruption was not observed in the case of UVA.

OVX exerted a minor effect to modulate skin accumulation and flux of small molecules (tretinoin and estradiol). However, appendageal transport showed an effortless way for tretinoin absorption via OVX skin. The volume of sebaceous glands is increased after OVX, although the sebaceous activity and function are decreased (Parchami and Fatahian Dehkordi 2010; Šitum et al. 2010). In vivo skin distribution of the dye monitored by CLSM showed a similar profile to tretinoin, with greater absorption in photoaged skin. The

tendency of rhodamine B accumulation in the SC region was in line with that in the epidermis. This suggests that the SC still cannot be ignored as a primary barrier governing permeant transport.

The role of epidermal junctions as a penetration barrier was less essential for estradiol than for tretinoin due to the minor enhancement of the skin reservoir and flux by the aging process. Although estradiol can be categorized as a lipophilic permeant, it is less lipophilic than tretinoin. Another possibility is the saturation of the skin reservoir by estradiol. Further skin disruption by UV or OVX could not increase estradiol absorption because of the limited capacity for drug loading. The results suggest that epidermal junctions may become an important permeation barrier for the permeants with a log *P* of>4. UVA exposure opened up follicular routes for estradiol delivery, indicating the ability of UVA for enhancing estradiol uptake into the follicles, although this effect was not shown for tretinoin. Corneocytes in the follicles are small and crumbly, producing a deficient barrier (Knorr et al. 2009). Follicular transport may accelerate estradiol diffusion into the deeper skin strata and the following receptor compartment. It was the consequence of the greater estradiol flux in normal/UVA and OVX/UVA groups.

The measurement of in vitro penetration over 24 h revealed no dextran transport across the skin. TJs manifest an important permeation obstruction for macromolecules (Kawasaki et al. 2012). Our results indicated that barrier disruption by UVA and UVB increased dextran deposition by four- and threefold, respectively. It is noticeable that OVX decreased dextran deposition into the skin treated by photoaging. The combination of UVB and OVX did not even show any skin accumulation after dextran application. SC dehydration is inferred to be a reasonable explanation. TEWL is utilized as a surrogate indicator of skin dryness (Proksch et al. 2006). Both UVB and OVX could induce the elevation of TEWL. The loss of water results in increased SC lipophilicity (Hung et al. 2012). Dextran can be classified as a hydrophilic permeant. Hydrophilic molecules preferentially deposit in aqueous skin tissues such as the epidermis (Gattu and Maibach 2010). The skin partitioning of hydrophilic macromolecules to SC could be reduced due to SC dryness. A previous study (Chiu et al. 2005) verifies that UVB produced skin dryness. Dry skin is also the most common dermatological condition of postmenopausal women (Roberts 2006). Thus, the dry SC retarded partitioning and subsequent penetration of dextran into the skin; even the SC integrity was disrupted. The dryness status of the SC by aging was further confirmed by PCR profiles. The loss of filaggrin leads to NMF deficiency and dehydration of the upper SC layers (Levin et al. 2013). Involucrin reduction in the SC decreases the water holding capability (Jensen et al. 2000).

Topical tretinoin is utilized to treat clinical signs associated with photoaged skin. Patients often use this drug for the long term (Cho et al. 2005). The safety of topical tretinoin should be of concern since prolonged use of tretinoin can cause epidermal atypia in photoaged skin and accelerate UV-induced tumor growth, erythema, and scaling (Miura et al. 2012). These adverse effects are concentration dependent (Antoniou et al. 2010). Our results demonstrated a significant increase of tretinoin skin accumulation caused by UV. Photoaging becomes a risk factor to induce skin toxicity of tretinoin. The possibility of a mutagenic effect due to systemic tretinoin should be of concern since photoaging was also shown to increase tretinoin flux. OVX was less important for tretinoin over-absorption. The increase in follicular uptake by OVX did not further increase skin accumulation and flux of this drug. A reduction of the tretinoin dose for photoaged skin should be considered to lessen the adverse effects. HRT generates the risks associated with thrombotic and cancerous complications in selected cases (Mueck 2012). Our results showed that UV and OVX basically did not alter estradiol absorption, although UVA enhanced follicular uptake. Postmenopausal women exposed to UV may not be at increased risk of topical estradiol administration.

Conclusions

Some indicators or biomarkers showed that the intervention of OVX increased the sensitivity of photoaging. These included the loss of β-catenin, filaggrin, and involucrin. Although OVX further reduced the cohesion of junctions, this effect did not largely alter skin accumulation and the flux of lipophilic permeants. Except that OVX increased the follicular uptake of tretinoin in UV-irradiated skin, OVX intervention did not synergistically increase skin absorption of small molecules. CLSM profiles suggest that SC still possesses a main role in controlling cutaneous permeation, although the role of epidermal junctions cannot be neglected. The

results of dextran permeation indicate that OVX does not necessarily enhance the transport of permeants. The combination of photoaging and OVX led to a significant reduction of dextran accumulation within the skin, possibly due to SC dehydration by both aging processes. It is concluded that either UV or OVX gave synergistic, additive, or inhibitory effects based on the different cases of the permeants employed. We reported for the first time, as far as we know, the influence of combined UV and OVX on skin absorption of drugs.

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