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Immunofluorescent Distribution of SIRT1, SIRT2, SIRT6 Protein in the Skin of SKH1 Mouse after Ultraviolet B Irradiation for 10 Weeks

Distribución Inmunofluorescente de la Proteína SIRT1, SIRT2, SIRT6 en la Piel del Ratón SKH1 Después de la Irradiación Ultravioleta B Durante 10 Semanas

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SUMMARY: The skin, located on the outermost part of the body, is always exposed to external stimuli such as sunlight. The exposure of skin to ultraviolet B (UVB) radiation from sunlight is known to be a major environmental factor in inducing photoaging. After exposure to UVB, an increase in reactive oxygen species can affect the expression and activity of many critical proteins depending on the duration and dose of the UVB radiation. Mammalian sirtuins (SIRTs), which are nicotinamide dinucleotide-dependent protein deacetylases, are well known for playing a role in cellular longevity. However, little is known about SIRT protein alterations in keratinocytes upon UVB irradiation according to SIRT subtypes. Therefore, in this study, the distribution of non-mitochondrial SIRT1, SIRT2, and SIRT6 proteins was investigated by immunofluorescence (IF) staining of the skin of SKH-1 mice (n=12) after UVB irradiation for 10 weeks. After UVB irradiation for 10 weeks, the IF of both SIRT1 and SIRT6 was significantly increased in the UVB-irradiated mice group (UG), but the difference in SIRT2 IF was not statistically significant between the control group (CG) and the UG. The translocation of both SIRT1 and SIRT6 IF from the nucleus to the cytoplasm of keratinocytes was observed in the upper epidermis of the UG, whereas SIRT2 IF was localized in the cytoplasm of keratinocytes in the epidermis in both the CG and the UG. The translocation of SIRT1 and SIRT6 IF from the nucleus to the cytoplasm of keratinocytes may account for the physiologically protective action of keratinocytes against UVB irradiation. However, the exact role of SIRT1 and SIRT6 translocation in keratinocytes, where SIRT1 and SIRT6 shuttle from the nucleus to the cytoplasm, is not well known. Therefore, further studies are needed to understand the molecular mechanisms of SIRT1 and SIRT6 translocation in keratinocytes upon UVB irradiation.

KEY WORDS: SIRT1; SIRT2; SIRT6; UVB; Keratinocyte.

INTRODUCTION

The skin plays a crucial role as a barrier in maintaining body homeostasis from external impacts and is a visual indicator of the aging of the body. The skin consists of continuous layers from the outer epidermis to the deep dermis. The outermost layer of the skin, the epidermis, which is mainly composed of keratinocytes, melanocytes, Langerhans cells, and Merkel cells, is responsible for many biological functions. Since the epidermis is the contact layer that divides the outermost part of the body from the external environment, biological and physical activities in the epidermis play crucial roles in response to external stimuli such as ultraviolet radiation (UVR) (D'Orazio *et al*., 2013). Solar UVR contains both ultraviolet A (UVA, 320–400 nm) and ultraviolet B (UVB, 290–320 nm) radiation (D'Orazio *et al*., 2013). Due to the different wavelengths of UVA and UVB, UVA radiation penetrates both the epidermis and the dermis, whereas UVB radiation penetrates the epidermis only, leading to numerous photo-responses in the skin (Wang *et*

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al., 2019). Accordingly, UVB irradiation of the skin induces epidermal photo-responses, such as erythema, epidermal thickening, and wrinkle formation (Yano *et al*., 2004). The initial photo-response showed a marked increase in inflammatory cell infiltration, edema, and skin thickness in SKH1 hairless mice (Konger *et al*., 2016). These epidermal responses to direct UVB radiation exposure were closely related to a significant increase in reactive oxygen species (ROS) (Avadhani *et al*., 2017). In addition, increases in ROS following exposure to UVB may affect the function of other critical macromolecules, such as proteins and lipids, depending upon both the duration and dosage of UVB radiation (Avadhani *et al*., 2017) and may trigger molecular signaling cascades that affect epidermal cells, of which the most abundant are keratinocytes.

Keratinocytes, the principal cells in the epidermis, are in contact with the external environment, contribute to many barrier functions, and respond to external stimuli by modifying biological activity in the epidermis (Kanfi *et al*., 2008). These cells are partially protected from UVB radiation damage because UVB radiation is absorbed in the basal layer of the epidermis (Barresi *et al*., 2011). Absorption is determined by the extinction coefficient and the spatial distribution of chromophores such as melanin pigments, aromatic amino acid residues of proteins, and DNA (Barresi *et al*., 2011). As melanin pigment absorbs UV light, photobiological studies have mainly been performed in albino hairless mice like SKH1 mice to reduce experimental variability (Konger *et al*., 2016). After acute exposure to UVB radiation, SKH1 hairless mice exhibited a characteristic reaction similar to the human sunburn response (Sahu *et al*., 2012).

The sirtuin gene family (Sirt) appears to regulate longevity in a wide variety of living systems from yeast to mammals and plays a role in the cellular stress response, inflammation, and repair (Poulose & Raju, 2015). Mammalian sirtuins (SIRTs) consist of seven nicotinamide dinucleotide (NAD^+)-dependent protein deacetylases, SIRT1 to SIRT7 (Poulose & Raju, 2015). The subcellular localization of SIRT1, 6, and 7 is mainly confined to the nucleus and SIRT2 is confined to the cytoplasm, whereas SIRT3, SIRT4, and SIRT5 are known as mitochondrial proteins (Poulose & Raju, 2015). SIRT1 plays a role in promoting keratinocyte differentiation through transcription factors such as peroxisome proliferator-activated receptors a and g (Blander *et al*., 2009). The overexpression of SIRT1 in skin fibroblasts was shown to protect against UVB-induced cellular senescence and oxidative stress by inhibiting p53 acetylation (Chung *et al*., 2015). In mouse fibroblasts, SIRT2 bound to forkhead box O3a (FOXO3a) and increased manganese superoxide dismutase expression, resulting in a decrease in H_2O_2-induced ROS and cell death (Wang *et al*., 2007). Interestingly, in UVB irradiation, an increase in SIRT6 expression in human keratinocytes was observed in vitro while UVB-induced apoptosis was increased by inhibiting its expression (Ming *et al*., 2014). UVB irradiation studies have analyzed SIRT1 effects on transcription factors, cellular signaling in the skin. (Chung *et al*., 2015). However, few studies have investigated and compared the morphological distribution of SIRT1, 2, and 6 proteins in skin keratinocytes.

Therefore, in the present study, the distribution of non-mitochondrial SIRT1, 2, and 6 protein immunofluorescence was investigated in the skin of SKH1 hairless mice after UVB irradiation.

MATERIAL AND METHOD

Animals. Male albino hairless SKH-1 mice were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). Twenty hairless mice (age 8 weeks) were randomly assigned to two groups after a two-week stabilization period: 1) control group (n=10, CG), 2) UVB-irradiated group (n=10, UG). The mice were kept under controlled conditions (ambient temperature of 22 ± 2 ºC, relative humidity of 55 ± 5 %, 12-h light/dark cycle from 8:00 am to 8:00 pm). Food (Samtako Bio Korea, Osan, South Korea) and water were supplied *ad libitum*. The animals were maintained in the Laboratory Animal Research Center of Dankook University (DKU). The National Institutes of Health (NIH) guidelines for animal research were followed for all animal procedures and the study was approved by the Dankook University Institutional Animal Care and Use Committee (DKU-19-041), which adheres to the guidelines issued by the Institution of Laboratory Animal Resources.

UVB irradiation. A fluorescent lamp emitting light at a wavelength of 312 nm (UV Crosslinker, Vilber Lourmat GmbH, Eberhardzell, Germany) was used as the UVB source. The mice were exposed to UVB radiation three times a week for 10 weeks. Briefly, the initial dose was set at 36 mJ/cm2, which was increased to 54, 72, 108, 144, 162, 180, and 198 mJ/cm2 at weekly intervals and finally, to 216 mJ/cm2 on the ninth and tenth weeks.

Skin processing for immunofluorescence staining. Mice were deeply anesthetized and perfused transcardially with cold phosphate-buffered saline (0.1 M PBS [pH 7.4]), followed by cold 4 % paraformaldehyde (PFA) in 0.1M PBS. The skin tissues were post-fixed in PFA for 24 h. The tissues were then washed three times in cold sodium phosphate buffer (0.1 M, pH 7.6) and cryoprotected with gradient sucrose solutions (10 %, 20 %, and 30 % for 2 – 3 h each), and then with 30 % sucrose overnight. The tissues were then

embedded in optimal cutting temperature (OCT) compound embedding medium (Sakura Finetech, Japan) and frozen rapidly in 2-methylbutane pre-cooled to its freezing point with liquid nitrogen. Tissue specimens were cut into 12 μm sections on a Leica cryostat, thaw-mounted on gelatin-coated microscopic slides, and stored at -20 °C until required for histological studies.

Immunofluorescence staining. The primary antibodies used were goat anti-rabbit SIRT1 (cat# 07-131, EMD Millipore, USA), SIRT2 (cat# 09-843, EMD Millipore), and SIRT6 (cat# ab62739, Abcam, USA). IF staining was performed using an Alexa Fluor 555-conjugated secondary antibody (cat# A31572, Thermo Fisher, USA). IF staining was performed as follows. Briefly, the sections were incubated sequentially in (1) 5 % normal donkey serum for 1 h at room temperature; (2) primary antibody diluted 1:200 overnight at 4 °C; (3) secondary antibody diluted 1:200 for 1 h at room temperature; and (4) 4¢,6-diamidino-2-phenylindole (DAPI) stain for nucleic acid staining. The stained sections were observed by fluorescence microscopy (Zeiss, Germany).

Masson's trichrome staining. Masson's trichrome staining was performed on the skin samples, as described in previous protocols (Bancroft & Stevens, 1996). Briefly, the samples were stained with Weigert's iron hematoxylin for 5 min, Biebrich scarlet-acid fuchsin for 5 min, phosphotungstic/phosphomolybdic acid for 5 min, Aniline Blue solution for 5 min, and then rinsed, dehydrated with gradient alcohol solutions from 50 % to 100 %, cleared in xylene and mounted with PolyMount Mounting media (Bancroft & Stevens, 1996).

Statistical analysis. The intensity of SIRT1, 2, and 6 IF was measured in the epidermis using ImageJ software (https://imagej.nih.gov/ij/). All densitometric data are expressed as mean density, i.e., the average of the gray values of all pixels within the measured area (maximum level was 256). The data were presented as means ± standard deviation (SD).

RESULTS

The mouse skin structures observed by Masson's Trichrome staining generally consisted of a thin epidermal layer composed of three more layers of keratinocytes and collagen-rich dermis (Fig. 1). Compared to the control mice group (CG), The thickening of the epidermis was conspicuous in the UVB-irradiated mice group (UG). The epidermal thickness of the UG measured by image analysis was about 3.46-fold more than that of the CG ($p < 0.001$, Table I). The number of keratinocytes was increased in the epidermis of the UG and the height of the keratinocytes seemed to be increasedin the UG as well (Fig. 1).

Table I. The effects of UVB irradiation on skin epidermal thickness.

	CG	UG
Epidermal thickness (μm)	23.23 ± 4.58	80.35 ± 19.09

Epidermal thickness of the control mice group (CG) and UVB irradiated mice group (UG) in the SKH1 mouse skin tissue, which was measured in photographed field using NIH ImageJ program (Abramoff *et al*., 2004). The data were presented as means±SD. The acquired data were analyzed by Student t-test (*$p<0.001$). CG, control mice group; UG, UVB-irradiated mice group.

SIRT1 IF in the CG was distributed in the epidermis, where it was mostly localized in the keratinocytes. In the dermis of the CG, SIRT1 IF was observed in follicular sheaths, sebaceous glands, and connective tissue like collagen fibers. Although SIRT1 IF was not observed as a layer-specific distribution in the epidermis of the CG, SIRT1 IF was localized to some nuclei of the keratinocytes in the epidermis (Fig. 2A). In the UG, SIRT1 IF was also observed in the epidermis as well as skin appendages in the dermis (Fig. 2D). Since the epidermal thickness in the UG was thicker than that of the CG, it was relatively easy to distinguish the epidermal layers in the UG. Furthermore, SIRT1 IF in the basal and spinous layers in the UG was prominently localized in the nuclei of the keratinocytes but SIRT1 IF in the granular layers was localized in the cytoplasm of the keratinocytes (Fig. 2D).

SIRT2 IF was observed throughout the epidermis without layer-specific localization, in which SIRT2 IF was mostly localized in the epidermal keratinocytes of both the CG and the UG. In both groups, SIRT2 IF was mainly found in the cytoplasm of keratinocytes (Fig. 3A). In the dermis, SIRT2 IF was also observed in follicular sheaths, sebaceous glands, and connective tissue in both groups.

SIRT6 IF was prominently observed in the keratinocytes of the epidermis, which were mostly localized in the nucleus in the CG (Fig. 4A). In the dermis of the CG, SIRT6 IF was also observed in follicular sheaths, sebaceous glands, and connective tissue. In the UG, SIRT6 IF was also observedin the epidermis, as well as skin appendages in the dermis (Fig. 4D). Since the epidermal layer in the UG was thicker than that of the CG, it was possible to differentiate each layer of the epidermis in the UG. Moreover, in the UG, SIRT6 IF was more prominently observed in the upper layers than in the lower layers of the epidermis, in which the SIRT6 IF was localized in the cytoplasm of the keratinocytes (Fig. 4D).

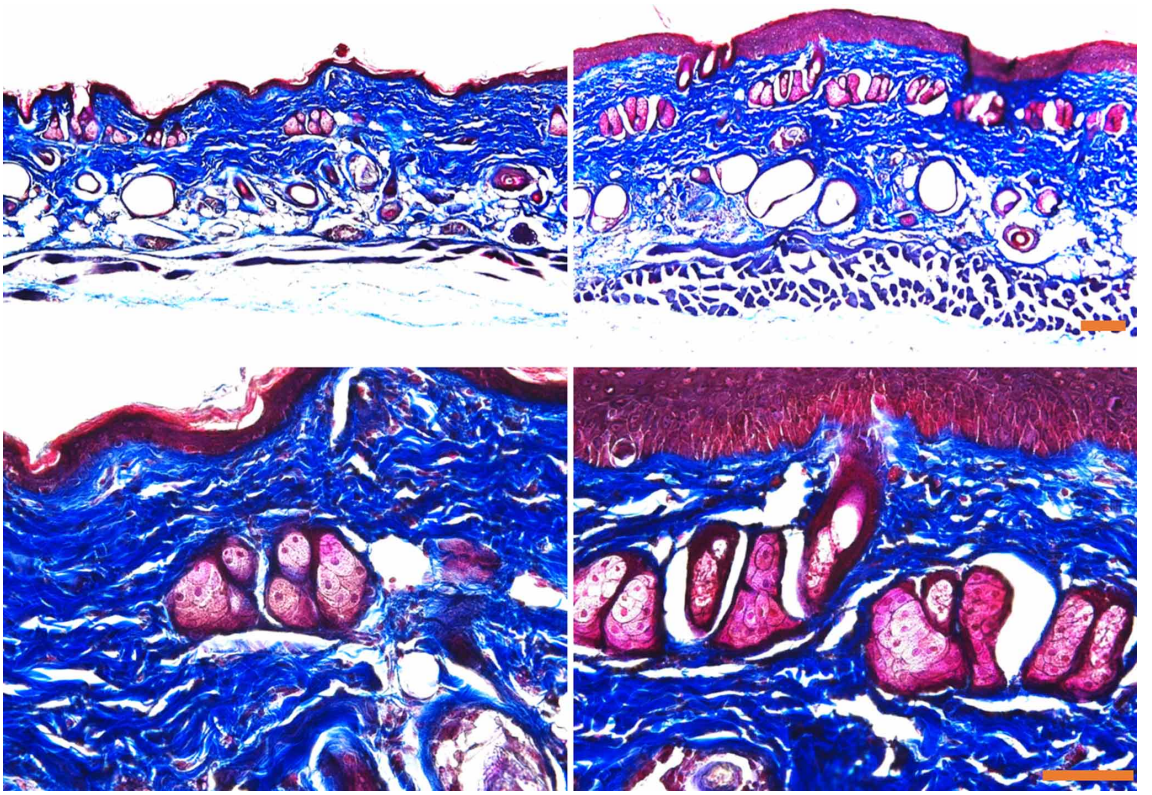

Fig. 1. Histopathological analyses of SKH1 mouse skin tissue stained with Masson's Trichrome methods. Owing to the epidermal hyperplasia, the thickness of the UVB irradiated mice group (B, D) was thicker than that of the control mice group (A, C) with the same magnification. B, D are magnified images of A, C, respectively. The staining of collagen appears blue in the dermis. Scale bar=50 μm.

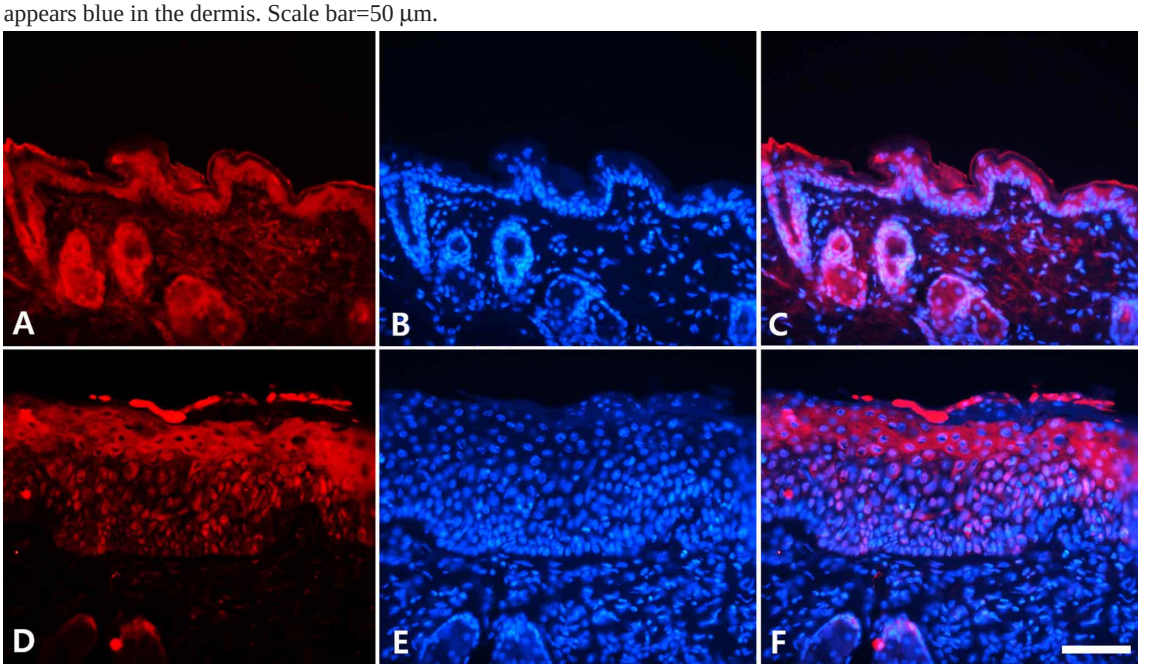

Fig. 2. The representative SIRT1 (red) immunofluorescence staining images of the control mice group (CG; A, B, C) and UVB irradiated mice group (UG; D, E, F) in the SKH1 mouse skin tissue. SIRT1(red) Immunofluorescences (IFs) were increased in the epidermis of UG(D) compared with that of CG (A). A more prominent SIRT1 IFs were also denoted in the suprabasal epidermis in UG compared to the basal epidermis. DAPI (blue) was used to label the nucleus (B, E). The areas of SIRT1 and DAPI IFs colocalization appear as purple in the merged image (C, F). Scale bar=50 μm.

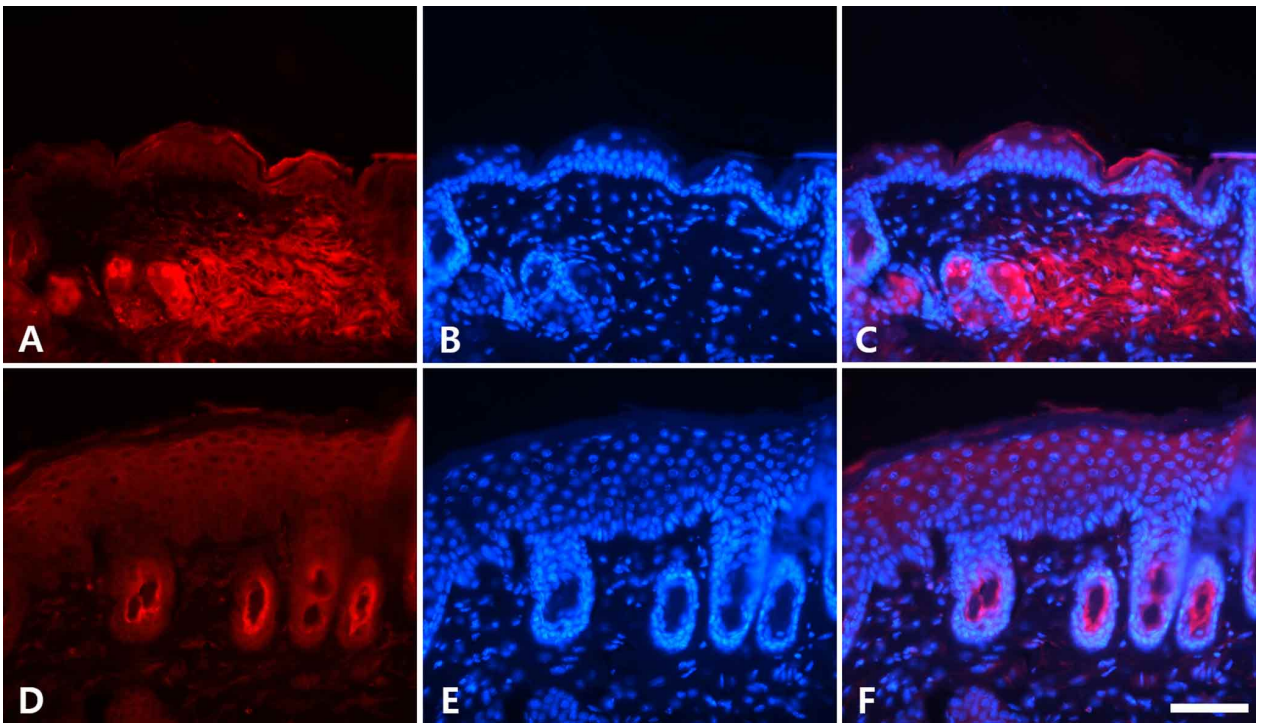

Fig. 3. The representative SIRT2 (red) immunofluorescence staining images of the control mice group (CG) and UVB irradiated mice group (UG) in the SKH1 mouse skin tissue. SIRT2 (red) Immunofluorescence (IFs) were not displayed significant differences between CG (A, B, C) and UG (D, E, F). DAPI (blue) was used to label the nucleus (B, E). The areas of SIRT2 and DAPI IFs colocalization appear as purple in the merged image (C, F). Scale bar=50 μm

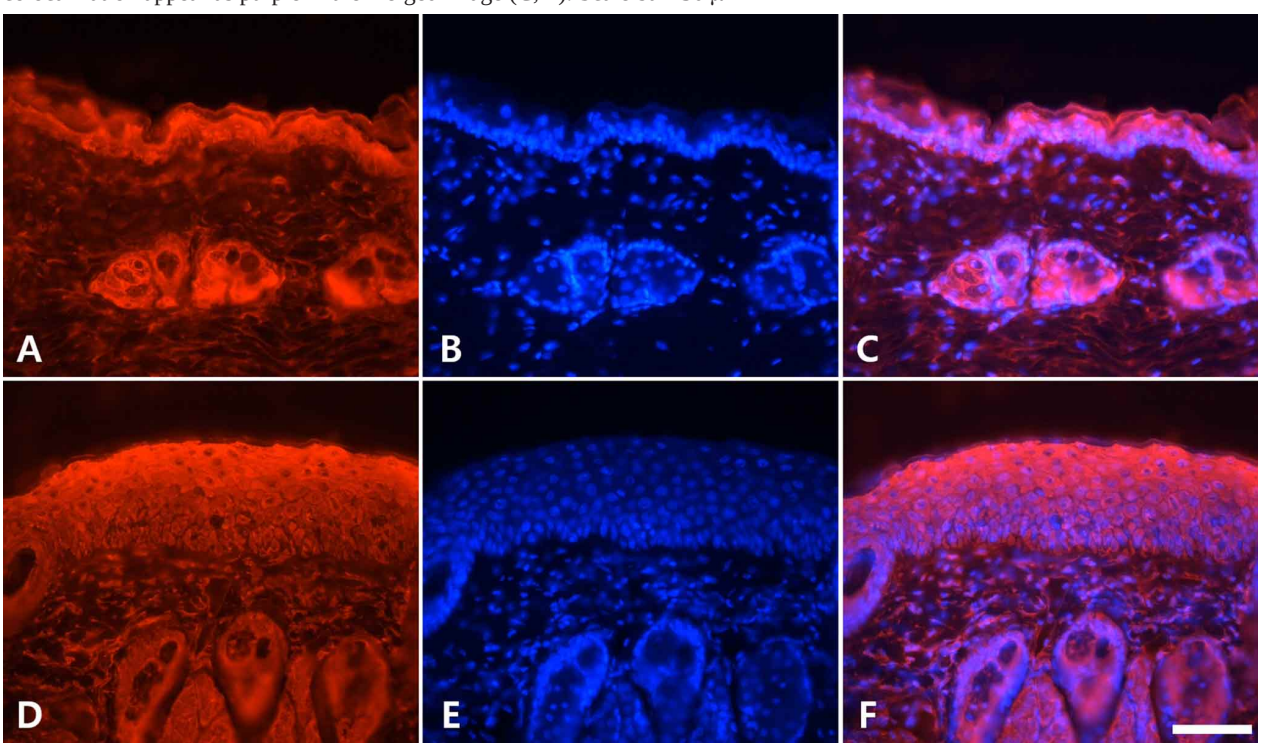

Fig. 4. The representative SIRT6 (red) immunofluorescence staining images of the control mice group (CG; A, B, C) and UVB irradiated mice group (UG; D, E, F) in the SKH1 mouse skin tissue. SIRT6 (red) Immunofluorescences (IFs) were increased in the epidermis of UG(D) compared with that of CG (A). DAPI (blue) was used to label the nucleus (B, E). The areas of SIRT6 and DAPI IFs colocalization appear as purple in the merged image (C, F). Scale bar=50 μm

SIRT1 and SIRT6 IF showed statistically significant increases in the UG ($P < 0.001$ and $P < 0.05$, respectively) (Fig. 5). However, no statistically significant difference in SIRT2 IF was observed between the CG and the UG (Fig. 5).

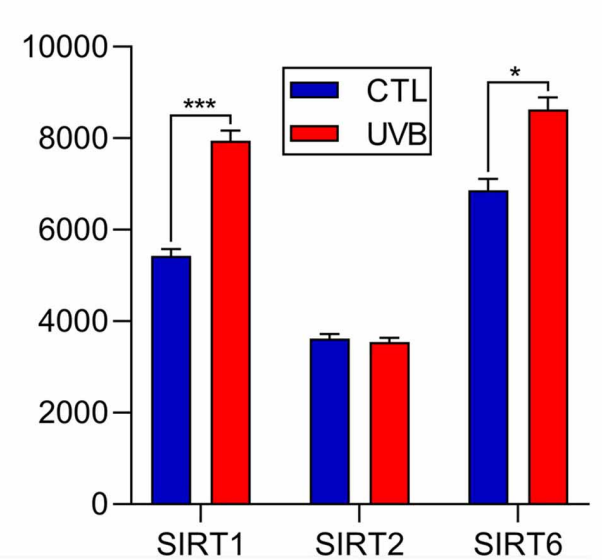

Fig. 5. Bar graph showing quantitative assessment of SIRT1, SIRT2, and SIRT6 immunofluorescences in the SKH1 mouse skin epidermis. The acquired data (means±SD) were analyzed by Student t-test by comparing with those of the control mice group (*** P<0.001, *P<0.05).

DISCUSSION

Skin photoaging is a gradual and complex biological process mainly induced by environmental UV exposure from sunlight, which is primarily linked to an imbalance between free radical production and resistance in the skin (Poljsˇak *et al*., 2012). The increased generation of ROS triggers oxidative stress, affecting cellular processes like DNA replication (Lee *et al*., 2022). Simultaneously, structural and physiological alterations accumulate in the skin layers (Addor, 2018), which can cause wrinkle formation, subcutaneous vascularization, and hyperplasia of the epidermis.

SIRT1 belongs to the NAD+-dependent deacetylase family and promotes keratinocyte differentiation, suggesting that SIRT1 is required for normal differentiation (Blander *et al*., 2009). The deacetylation activity of SIRT1 in various protective mechanisms was related to stress responses through interactions with substrates, including important transcription factors (Anastasiou & Krek, 2006).

In response to DNA damage and other stressors, SIRT1 was found to interact with p53 to suppress UVB-induced p53 acetylation (Chung *et al*., 2015). Keratinocyte-specific conditional SIRT1 knockout mice exhibited increased sensitivity to UV damage, concomitant with the accumulation of acetylated p53 (Ming *et al*., 2015), indicating that SIRT1 negatively regulated p53 activity to protect the epidermis from UV stress. In vitro, SIRT1 mRNA expression was significantly increased up to 6-fold in NHEK and 3-fold in HaCaT cells 8 h after UV irradiation compared to controls. However, the level of SIRT1 protein was decreased by approximately 30 % in NHEK and HaCaT cells at 6, 8, and 24 h after UV irradiation (Benavente *et al*., 2012). These results suggest that SIRT1 expression in keratinocytes from UV irradiation was time- and dose-dependent, although the correlation between SIRT mRNA expression and changes in protein levels was not properly addressed in the study.

In this study, in response to UVB irradiation for 10 weeks, SIRT1 IF was increased in the skin of SKH1 hairless mice in the UG. The plausible explanation for the increase in SIRT1 IF by UVB irradiation is that the oxidative stress produced during these processes led to compensatory or protective increases in SIRT1 expression to deal with the decline in the SIRT1 activity (Elibol & Kilic, 2018). In other words, the increase in SIRT1 protein might have a role in alleviating oxidative stress, which was not well correlated with previous in vitro experimental results. The reasons for this discrepancy are not clear. However, SIRT1 expression may be regulated differently according to the experimental conditions, such as in vitro and in vivo experiments, as well as time and doses of UVB irradiation. Interestingly, a previous study on the role of SIRT1 using keratinocyte-specific SIRT1 deletion SKH1 hairless mice suggested that the onset of skin cancer induced by UVB irradiation was dependent upon the gene dose (Ming *et al*., 2015), implying that species-specific differences between mice may affect the level of SIRT1 expression in the skin as well.

In skin fibroblasts, UVB induced the nuclear translocation and acetylation of FOXO3a while SIRT1 overexpression significantly reduced the UVB-induced acetylation of FOXO3a (Chung *et al*., 2015), suggesting that the effect of SIRT1 in response to UVB is associated with its interaction with FOXO3a. Under starvation conditions, SIRT1 upregulated SIRT6 in mice by forming a complex with FOXO3a and nuclear respiratory factor 1 (NRF1) at the NRF1-binding site of the SIRT6 promoter (Kim *et al*., 2010). Similar to FOXO3a, P53 also serves as a positive regulator of SIRT1 in another molecular cascade, and SIRT6 was upregulated by P53 under starvation

conditions (Kanfi *et al*., 2008). Our results similarly showed an increase in both SIRT1 and SIRT6 IF in the UG after UVB irradiation. Together with our results, it was possible to deduce the regulatory function that the SIRT6 expression level may be mediated by SIRT1 expression levels through an unknown mechanism.

The present study also showed the translocation of both SIRT1 and SIRT6 IF from the nucleus to the cytoplasm of keratinocytes in the upper epidermis of the UG (Figs. 2 and 4), although SIRT2 IF was observed only in the cytoplasm of keratinocytes in the epidermis of both groups. The shuttling of SIRT1 and SIRT6 from the nucleus to the cytoplasm may be considered a cell-protective response to physiological and/or pathological stimuli. However, the molecular mechanism of SIRT1 and SIRT6 movement from the nucleus to cytoplasm in the skin was not completely elucidated. Thus, it is necessary to investigate the role of SIRT1 and SIRT6 shuttling in the skin epidermis to fully understand photoaging due to UVB irradiation.

Taken together, the present study showed that the IF of both SIRT1 and SIRT6 was increased, but differences in SIRT2 IF between the two groups were not statistically significant in the skin of UVB-irradiated SKH1 hairless mice. The translocation of SIRT1 and SIRT6 IF from the nucleus to the cytoplasm in the upper epidermis may reflect a physiological, protective action of keratinocytes against UVB irradiation, although further investigation is needed to confirm this.

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RESUMEN: La piel, situada en la parte más externa del cuerpo, está siempre expuesta a estímulos externos como la luz solar. Se sabe que la exposición de la piel a la radiación ultravioleta B (UVB) de la luz solar es un factor ambiental importante en la inducción del fotoenvejecimiento. Después de la exposición a los rayos UVB, un aumento en las especies reactivas de oxígeno puede afectar la expresión y la actividad de muchas proteínas críticas según la duración y la dosis de la radiación UVB. Las sirtuinas de mamíferos (SIRT), que son proteínas desacetilasas dependientes de dinucleótidos de nicotinamida, son bien conocidas por desempeñar un papel en la longevidad celular. Sin embargo, se sabe poco sobre las alteraciones de la proteína SIRT en los queratinocitos tras la irradiación UVB según los subtipos de SIRT. Por lo tanto, en este estudio, se investigó la distribución de las proteínas SIRT1, SIRT2 y SIRT6 no mitocondriales mediante tinción de inmunofluorescencia (IF) de la piel de ratones SKH-1 (n = 12), después de la irradiación con UVB durante 10 semanas. Posterior a la irradiación, el IF de SIRT1 y SIRT6 aumentaron significativamente en el grupo de ratones irradiados con UVB (UG), pero la diferencia en SIRT2 IF no fue estadísticamente significativa entre el grupo control (CG) y el UG. La translocación de SIRT1 y SIRT6 IF desde el núcleo al citoplasma de los queratinocitos se observó en la epidermis superior de la UG, mientras que SIRT2 IF se localizó en el citoplasma de los queratinocitos en la epidermis, tanto en el GC, como en la UG. La translocación de SIRT1 y SIRT6 IF del núcleo al citoplasma de los queratinocitos puede explicar la acción protectora fisiológica de estos contra la radiación UVB. Sin embargo, el papel exacto de la translocación de SIRT1 y SIRT6 en los queratinocitos, donde SIRT1 y SIRT6 se trasladan desde el núcleo al citoplasma, no se conoce bien. Por lo tanto, se necesitan más estudios para comprender los mecanismos moleculares de la translocación SIRT1 y SIRT6 en los queratinocitos tras la irradiación UVB.

PALABRAS CLAVE: SIRT1; SIRT2; SIRT6; UVB; Queratinocito.

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