Research article

Dietary immature *Citrus unshiu* alleviates UVB- induced photoaging by suppressing degradation of basement membrane in hairless mice

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**ABSTRACT**

Ultraviolet (UV) irradiation induces physiological and morphological skin damage, resulting in skin dryness, wrinkle formation, and loss of elasticity. The basement membrane (BM) has been shown to play crucial roles in binding epidermis to dermis tightly, regulating cell differentiation and proliferation, and signaling protein production. Dietary flavonoids have been revealed to improve the damage caused by UV exposure. Immature *Citrus unshiu* is known to contain high concentrations of flavonoids such as hesperidin and narirutin. In this study, the effects of immature *Citrus unshiu* powder (ICP) on photoaged skin were demonstrated using UVB irradiated hairless mice. Oral administration of ICP improved loss of skin hydration and increase of transepidermal water loss. The histological analyses of hairless mice dorsal skin revealed that oral administration of ICP improved UVB-induced overgrowth of epidermal cell, suppressed epidermal cell mortality and BM destruction. Therefore, the administration of ICP could improve photoaging by protecting the tissues around BM.

1. Introduction

Skin aging is divided into two types: age-related intrinsic aging and ultraviolet (UV) irradiation-induced photoaging. Photoaging is characterized by several clinical features, including wrinkles, dryness, roughness, and pigmentary change [1]. The physiological and biochemical features of photoaged skin include skin dehydration and increase in epidermal thickness [2]. Basement membrane (BM) is known as an important multi-molecular structure which firmly attaches the epidermis and dermis, regulating epidermal differentiation, proliferation, as well as epidermal-dermal interaction. BM is damaged and multilayered by UV irradiation, promoting aging process in dermis and epidermis, hence improving BM repair is important to maintain skin health [3].

One of the major factors of photoaging is thought to be an increase in reactive oxygen species (ROS) production. ROS is generated in both epidermis and dermis by UV irradiation, followed by degradation of BM and extracellular matrix and apoptosis [4]. One way to reduce ROS and oxidative stress is dietary antioxidants, which can enhance endogenous antioxidant activity. Consequently, there is increasing interest in dietary antioxidants that can induce skin anti-aging effects [5].

*Citrus unshiu* (Satsuma mandarin) is one of the most popular fruits in Japan, China and Korea. *Citrus unshiu* contains various bioactive substances such as flavonoids, amino acids, and carotenoids, so that its fruits and peel have been widely used as a traditional medicine to treat common colds, bronchial discomfort, and indigestion for centuries [6]. Interestingly, the constituents and the biochemical composition of *Citrus unshiu* change during its maturation [7]. In immature *Citrus unshiu* (ICP), the flavonoids content is higher than mature one [8]. Among the flavonoids, hesperidin and narirutin are well known as dominant flavonoids in *Citrus unshiu*. These flavonoids have been shown to possess antioxidant and anti-inflammatory activities [9] and protective effect against apoptosis [10]. Chiang *et al.* showed that hydrolysates of *Citrus* plants stimulate melanogenesis protecting against UV-induced dermal damage [11]. And *Citrus* has reported to be used as a skin moisturizing and...
protective agent on UV-induced damage [11, 12]. Choi et al showed that anti-photoaging effects of immature *Unripe Citrus* extracts including inhibiting the expression of matrix metalloproteinases (MMPs) and enhancing the type I collagen [13]. However, the effects of immature *Citrus unshiu* on BM remains unknown. In this study, we examined the effect of oral administered immature *Citrus unshiu* on photoaging in the skin of hairless mice and confirmed that orally supplementation of ICP might be a useful strategy to protect from photoaging via repairing BM damage.

2. Materials and methods

2.1. Reagents

Histological and immunohistochemical analyses were conducted with Mayer’s hematoxylin (Muto pure chemicals, Tokyo, Japan), anti-laminin polyclonal antibody DyLight488 (Thermo Fischer Scientific, MA, USA), 10 % normal goat serum and MAX-PO (rabbit) (Nichirei bioscience, Tokyo, Japan), and ImmPACT DAB SK-4105 (Vector laboratories, CA, USA). All other reagents were obtained from Wako (Wako Pure Chemicals, Osaka, Japan).

2.2. Fruits and sample preparation

ICP was obtained from Mikkabi – cho, Hamamatsu – city, Shizuoka, Japan. ICP was stored at -30 °C before used for experiment, and was crushed and freeze-dried. Whole fruits including peel and pulp were used in the experiment. The content of hesperidin and narirutin in ICP for three consecutive years were analyzed (Figure 1). In 2019, fruits in June were not available due to bad harvest of ICP. There were no change in flavonoid content since September. They were determined with HPLC according to the method for *Citrus unshiu* peel analysis on The Japanese Pharmacopoeia 17th edition [14]. The HPLC analysis was performed on DP8020, AS 8021, UV8020, and CO8020 (Tosoh, Tokyo, Japan). The chromatographic separation was performed on an ODS – 80 TsQA column (4.6 × 150 mm) (Tosoh, Tokyo, Japan). ICP containing 22.9 μg hesperidin, 7.2 μg narirutin/100 g, which was processed from *Citrus unshiu* harvested in June 2017, was used for animal experiments.

2.3. Animals and administration of ICP

The experimental protocol was approved by the Ethics Committee of Tokyo University of Agriculture and Technology (Tokyo, Japan; approval No. 29–71). Six-week old male Hos:HR-1 hairless mice (Japan SLC,

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Hesperidin and narirutin contents in *Citrus unshiu* from the different developmental stages. The whole fruits were cut and homogenized. Following to lyophilized and grind into fine powder, hesperidin and narirutin was extracted with methanol for HPLC analysis. (A) hesperidin; (B) narirutin.
Shizuoka, Japan) were purchased from Sankylolabo service (Tokyo, Japan). Mice were housed in collective cages at 24 ± 2 °C and at 40 ± 10% humidity on a twelve hour light and dark cycle, with free access to water and Lab MR stock diet (Nihon nosan kogyo, Tokyo, Japan). Following one week of acclimation, mice were divided into three groups: non-irradiated control mice [UV(-)] control, n = 6], UV irradiated control mice [UV(+) control, n = 7], and UV irradiated and ICP administered mice [UV(+) ICP, n = 7]. The ICP was dissolved in 0.5% tragacanth gum (Wako Pure Chemicals, Osaka, Japan) and orally administered at 200 mg/kg body weight/day for seven weeks. Only 0.5 % tragacanth gum was orally administered to the control groups. In accordance with the UV irradiation method of Tanaka et al. [15], the mice were housed in cages and subjected to UVB irradiation. Mice were irradiated with UVB three times a week for seven weeks. UVB irradiation time was gradually extended. Mice were exposed to UVB irradiation for three times 60 s for the first week. Exposure time was then increased to 90, 120, 150 s for the second week, 120, 120, 150 s for the third week, 150, 180, 180 s for the fourth week, three times 210 s for the fifth week, 225 s each for the sixth and seventh weeks.

The total energy of UVB that each mouse received was 2.42 J/cm² over seven weeks. Skin moisture content and transepidermal water loss (TEWL) in the dorsal skin were measured with Corneometer CM 825 and Tewameter TM 300 (Courage + Khazaka Electronic, Koln, Germany).

2.4. Histological and immunohistochemical analyses

After seven weeks of UVB exposure, mice were sacrificed under anesthesia (SEVOFRANE; Maruishi Pharmaceutical, Osaka, Japan). Dorsal skin biopsy samples were obtained using a biopsy punch BP-80F (Kai medical, Tokyo, Japan) with a diameter of eight mm. Dorsal skin samples were fixed in tissue tek Ultra (Sakura-finetek Japan, Tokyo, Japan) and embedded in paraffin. Paraffin sections (4 μm) were deparaffinized and stained with hematoxylin and eosin (H&E). Immunostaining was conducted according to the method of Amano et al. [16]. Three sites were randomly selected in each section and photographed using microscope and a camera (C – 3040 ZOOM, OLYMPUS, Tokyo, Japan). The epidermal thickness of three groups were calculated for a total of 10 places in three visual fields and analyzed with software Image J. The means of these 30 measurements in each group were used to calculate the mean for each experimental group.

2.5. Statistical analysis

Data are presented as the means ± standard deviation (SD). After a one-way ANOVA was performed, statistical analysis was performed using Student’s t-tests followed by Statcel version 4.0. P - values <0.05 were considered significant.

3. Results

3.1. Skin moisture content and TEWL of dorsal skin from UVB irradiated hairless mice

As a result of ICP administration for seven weeks, restoration of skin moisture content and TEWL were observed (Figure 2). Comparing the skin moisture content and TEWL of hairless mice back skin on the UV(-) and UV(+) control, the influence of UVB irradiation was confirmed. A photoaging model was created without causing erythema formation on the back skin. At the start of the experiment, skin moisture content in UV(-) control, UV(+) control and UV(+) ICP were 70.2 ± 3.4 and 70.5 ± 3.1, and 70.1 ± 3.4, respectively. Skin moisture content decreased with UVB irradiation. seven weeks later, skin moisture content of UV(-) control, UV(+) control and UV(+) ICP were 71.7 ± 4.7, 54.1 ± 2.6 and 60.7 ± 5.9, respectively. Skin moisture content in UV(+) ICP was significantly higher than that in UV(+) control (p < 0.05).

In this study, ICP improved photoaging skin by protecting BM in UVB irradiated hairless mice. UVB irradiation triggers skin dysfunction through generating intracellular and extracellular ROS, which has negative effects.
BM is known as an important structure which strongly attaches the epidermis to the dermis. At the dermal-epidermal junction (DEJ) of sun-exposed skin, severe disruption and reduplication of BM were reported [23]. Laminin, a glycoprotein which provides links between keratinocyte and dermal extracellular matrix, is confirmed to promote the formation of BM at the DEJ in skin equivalent model [24]. Therefore, degradation of laminin may induce BM deficiency, and consequently result in cell proliferation abnormality and skin barrier dysfunction. Hence enhancing BM repair could be an effective way to suppress photoaging [3]. In this study, we could not determine the function of polyphenols in Immature Citrus unshiu on the improvement of basement membrane with UV irradiation damage. It is considered that this finding suggested that ICP maintained epidermal homeostasis, normalizing dermal-epidermal adhesion and signal transduction by protecting from BM destruction. BM is conceived to be disrupted by UV radiation through activating MMP-1, 2, 3, 9 [3]. In previous reports, immature C. unshiu extract suppressed MMP-1 production in human dermal fibroblasts, MMP-2 expression in UVB-irradiated hairless mice [12], and hesperidin inhibited UVB-induced MMP-9 activation in hairless mice [25]. From these reports, it is conceivable that ICP suppressed UVB-induced BM degradation via MMPs suppression. Moreover, we confirmed that methanol extract from ICP increased HA production in HaCaT keratinocytes (data not shown). Extracellular HA in epidermis plays a crucial role to hold water and maintain cell cycle and function normally [26]. A previous study showed that decreasing of the HA content was observed in UVB-damaged epidermis such as epidermal hyperplasia and increase of the number of the dead cells in stratum spinosum. Epidermal thickening is known as one of the characteristics of photoaged skin. Previous reports revealed that dietary supplementation with antioxidants such as green tea polyphenols had anti-photoaging activities including epidermal hyperplasia [22]. Although dietary antioxidants do not directly neutralize free radicals, they attribute to maintain the cell’s redox state, resulting in activating the endogenous antioxidant enzymes, which work as ROS scavengers [5]. We confirmed that ICP had high antioxidant activity with ORAC score 2260 μmol Trolox equivalent/L (data not shown). Our findings suggested that orally administered ICP suppressed UV-induced cell death in epidermis and prevent increase in the epidermal thickness via alleviating ROS generation.
E. Tamaru: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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