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# Topical Hesperidin Enhances Epidermal Function in an Aged Murine Model

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## To The Editor

As skin ages, the epidermis is thinner with reduced epidermal proliferation, abnormal differentiation, impaired lipid synthesis and elevated skin surface pH. These alterations have profound consequences for barrier function, skin cohesion, antimicrobial defense, inflammatory threshold, and cutaneous wound healing (Choi et al., 2007; Mauro et al., 1998; Ghadially et al., 1995; Rodriguez-Martin et al., 2011). These abnormalities have been linked, in part, to reduced epidermal IL-1α expression (Ye et al., 2002), reduced epidermal expression of CD44 and its ligand, hyaluronic acid (Bourguignon et al., 2013), and reduced epidermal lipid synthesis.

Among these many changes, much attention has been paid to the epidermal permeability barrier, because of its dominant role in regulating cutaneous homeostasis. Studies have demonstrated that epidermal permeability barrier regulates epidermal proliferation, differentiation, lipid production, as well as innate immunity. Therefore, strategies that enhance epidermal proliferation, differentiation and/or lipid production, while also reducing stratum corneum (SC) pH could prove useful for preventing and/or treating the functional abnormalities, including permeability barrier homeostasis in aged skin. Our previous studies demonstrated that topical applications of a readily available herbal ingredient, hesperidin, improve epidermal permeability barrier function in young mice by stimulating epidermal proliferation, differentiation, and lamellar body formation/secretion (Hou et al., 2012), all of which are likely independent of the anti-oxidant properties of hesperidin. Here, we show that topical applications of hesperidin improve a multiple key epidermal functions in aged mouse skin. Following 9 days of treatment, the gross appearance of mouse skin treated with vehicle



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and hesperidin appeared similar. Histological analysis showed that aged epidermis was thinner than young epidermis, while PCNA staining indicated that aged epidermis displayed less robust proliferative activity as compared to young epidermis, hesperidin treatment did not stimulate epidermal proliferation in aged skin, as indicated by PCNA positive cells per cm epidermal length (2.70 ± 0.10 vs. 2.45 ± 0.13 for vehicle-treated vs. hesperidin-treated skin, NS; 3.46 ± 0.17 for young skin; young vs. vehicle- or hesperidin-treated aged skin, $P<0.001$). These results indicate that topical hesperidin does not stimulate epidermal proliferation in aged mice.

After 9 days of topical hesperidin treatment, baseline SC hydration in hesperidin-treated mice also was no different from that in vehicle-treated mice (60.77 ± 1.32 for vehicle-treated vs. 58.80 ± 2.27 for hesperidin-treated). However, skin surface pH significantly declined in hesperidin- than in vehicle-treated skin (Fig. 1a). Although basal transepidermal water loss rates increased slightly in hesperidin-treated skin as compared to vehicle-treated skin (Fig. 1a), these levels still fell well within the normal range of young skin. Consistent with previous findings in young mice (Hou et al., 2012), topical hesperidin significantly accelerated barrier recovery at both 2 and 4 hours after acute barrier disruption of aged skin (Fig. 1b). These results demonstrate that topical hesperidin improves epidermal permeability barrier homeostasis, while also lowering skin surface pH in aged murine skin.

We next examined the basis for improved barrier function and acidification in aged epidermis. Our previous studies demonstrated that topical hesperidin stimulates epidermal differentiation, accounting in part for improved epidermal permeability barrier homeostasis in young mice. Hence, we next assessed whether topical hesperidin also stimulates epidermal differentiation in aged epidermis. As shown in Fig 1c, topical hesperidin significantly increased the mRNA levels of filaggrin and loricrin in aged mouse epidermis, consistent with the results of immunostaining (Suppl. Fig 1.). Consistently, hesperidin also increased the mRNA levels of filaggrin, involucrin and loricrin in adult keratinocyte cultures (Fig 1d). These results indicate that hesperidin stimulates epidermal differentiation, providing one potential mechanism whereby hesperidin improves barrier function in aged skin.

Epidermal lipid synthesis is required for formation and maintenance of the epidermal permeability barrier. Synthesis of three key barrier-related lipids, cholesterol ceramides, and fatty acids requires their respective rate-limiting enzymes, HMGCoA, SPT1, and FAS. Basal mRNA levels for all three key lipid synthetic enzymes were lower in aged as compared with young epidermis (demonstrated by the dotted line in Fig 1e), consistent with concept that the lower lipid synthesis rates in aged epidermis could reflect the reduced expression of their synthetic enzymes. Topical hesperidin treatment significantly increased the mRNA levels of HMGCoA, SPT1 and FAS in aged mouse epidermis, as assessed by Q-PCR.

Epidermal permeability barrier function depends on newly-synthesized epidermal lipids delivered to the SC through the secretion of lamellar bodies from the stratum granulosum. Therefore, we next assessed whether topical hesperidin stimulates lamellar body formation and/or secretion. Since ABCA12, a trans-membrane glycosylceramide transporter, is required for normal lamellar body assembly (Thomas et al., 2009), we next evaluated the

changes in epidermal mRNA levels of ABCA12 in hesperidin-treated aged epidermis. While untreated aged epidermis displayed lower levels of ABCA12 mRNA in comparison with young mice, topical hesperidin induced a marked increase in ABCA12 mRNA expression in aged mouse epidermis (Fig 1g) and adult keratinocyte cultures (Fig 1h). While the density of lamellar bodies did not increase in aged epidermis following hesperidin treatment (Fig 1f), quantitative analyses revealed that extent of lamellar body secretion was enhanced by topical hesperidin treatment (Fig 1f). In comparison with young epidermis, the increased number of lamellar bodies in aged epidermis is likely due to the retardation of secretion. Together, these results suggest that hesperidin induced an increase in ABCA12 mRNA expression that results in an apparent acceleration in the delivery of newly-synthesized lipids to the SC.

Both epidermal NHE1 and sPLA2 (in particular SPLAg2f) are key factors that selectively influence the SC pH (Ilic et al., 2014). Previous studies from our group have shown that aged skin exhibits higher pH (Choi et al., 2007), due at least in part to reduced NHE1 expression (Choi et al., 2007). To determine whether the hesperidin-induced acidification of SC pH results from upregulation of NHE1 and/or the parallel acidifying mechanism, sPLA2g2f, we next assessed the changes in epidermal mRNA levels of these two genes in aged epidermis after herperidin treatments by Q-PCR. Topical hesperidin provoked a dramatic elevation in mRNA levels for both NHE1 and sPLA2g2f in aged epidermis (Fig. 1i). These results suggest that hesperidin-induced acidification of aged epidermis results from stimulation of NHE1 and sPLA2g2f, accounting for the lower skin surface pH, and likely improved epidermal permeability barrier homeostasis in hesperidin-treated aged mouse skin.

Our prior studies demonstrated that epidermal permeability barrier and antimicrobial function are co-regulated and independent (Aberg et al., 2008). Aged humans are predisposed to develop both cutaneous and extracutaneous infections, and expression of the epidermal cathelicidin antimicrobial peptide, CAMP/LL37, is reduced in aged skin (Rodriguez-Martin et al., 2011). To determine whether hesperidin enhances epidermal antimicrobial defense, we next assessed changes in the mRNA levels of mBD3, a homologue of hBD2, following hesperidin treatment. Hesperidin treatment significantly increased epidermal mBD3 mRNA levels (Suppl. Fig 2a). To further validate these in vivo results, the effects of hesperidin on antimicrobial mRNA expression were evaluated in cultured keratinocytes from aged human skin. While no changes in constitutive hBD2 mRNA expression were observed (Suppl. Fig 2c), addition of hesperidin to aged human keratinocyte cultures markedly up-regulated not only hBD3 mRNA, but also CAMP/LL37 expression (Suppl. Figs 2b and d). These results demonstrate that hesperidin stimulates antimicrobial peptide mRNA expression in aged keratinocytes.

In the present study, we demonstrated that topical hesperidin improves a wide spectrum of functional abnormalities in aged epidermis, including abnormalities in epidermal permeability barrier function, epidermal differentiation, lipid production, and SC acidification. Although the molecular mechanisms of hesperidin induced functional changes in aged skin is not clear, but the antioxidant property of hesperidin could be involved. Aged skin displays lower antioxidant capacity and excessive accumulation of oxidative products,

and hesperidin shows high antioxidant capacity (Pękal et al., 2011). Hesperidin inhibits production of reactive oxygen species in rat kidney and human hepatocytes, reduces plasma malondialdehyde levels and increases superoxide desmutase activity in diabetic rats. Oral hesperidin administration increases blood glutathione peroxidase activity in Type 1 diabetic patients, whereas antioxidants stimulate keratinocyte differentiation. Our recent study demonstrated that topical hesperidin applications increased epidermal mRNA levels of antioxidant enzymes such as glutathione reductase and superoxide dismutase in murine skin (Man et al., 2014). Moreover, antioxidants such as vitamin E and C increase lipid production in keratinocyte cultures. Pertinent to antioxidant, Nrf2, a transcription factor, regulates epidermal differentiation and antioxidant defense (Schäfer et al., 2012). Nrf2 function is impaired in aged heart (Gounder et al., 2012), and expression levels were lower in aged epidermis (unpublished observation by Man MQ and Elias PM). Hesperidin up-regulates Nrf2 in the heart (Elavarasanet al., 2012) and aged epidermis (unpublished observation by Man MQ and Elias PM). Hence, hesperidin-induced improvement of epidermal permeability barrier function in aged skin may be mediated via Nrf2. Nevertheless, the present study indicates that hesperidin could be a valuable approach for anti- aging of skin.

## Materials and Methods

### Experimental protocols and functional studies

All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Since hesperidin is not soluble in 100% ethanol, 70% ethanol was used as vehicle. Since the turnover time for hairless is about 8–9.5 days in normal young mice (Potten et al., 1987), we chose to treat aged mice for 9 days. Both flanks of 12–15 month old mice were treated topically with 60 μl of 2% hesperidin or 70% ethanol twice daily for 9 days. Basal epidermal permeability barrier function was assessed by measuring transepidermal water loss (TEWL) using TM300 connected to MPA5 (C&K, Cologne, Germany) (Mao-Qiang et al., 2004; Man et al., 2012). For barrier recovery, TEWL was measured using an electrolytic water analyzer (Meeco, Warrington, PA) at 0, 2 and 4 hours after tape stripping (10-fold increase in TEWL), and percent barrier recovery was calculated as described earlier (Liu et al., 2010; Mao-Qiang et al., 2004; Man et al., 2012).

### Keratinocyte Culture

Second-passage keratinocytes isolated from adult human (donor aged 60–65 year old) were cultured in serum-free keratinocyte growth medium containing 0.07 mM calcium (Clonetics, San Diego, California). Cells at 60%–70% confluence were switched to a medium containing 1.2 mM calcium and treated with either 0.02% hesperidin or vehicle alone (0.02% ethanol). After 24 and 48 hrs of treatment, keratinocytes were collected for Q-PCR analysis (Hou et al., 2013).

### Immunohistochemistry

Immunohistochemical staining for assessing changes in epidermal differentiation was performed as described earlier (Hou et al., 2013; Mao-Qiang et al., 2004). Briefly, 5 $\mu$m paraffin sections were incubated with the primary antibodies (Covance, Emeryville, CA)

overnight at 4°C. After washes ×3, sections were incubated with the secondary antibody for 30 minutes. Staining was detected with ABC-peroxidase kit from Vector Lab (Burlingame, CA). Sections were examined with a Zeiss fluorescence microscope (Jena, Germany) and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany).

### Q-PCR for mRNA expression

Total RNA was isolated from cultured human keratinocytes using TRI Reagent (Sigma). First strand cDNA was synthesized from 1ug of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μl of 2× LightCycler 480 SYBR Green I Master in a final volume of 20 μl in 96-well plates using Mx3000P™ Real-time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative $C_T$ method with 36B4 or Cyclophilin A used for normalization. The primers for lipid synthetic enzymes such as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), serine–palmitoyl transferase 1 (SPT1), fatty acid synthase (FAS), lipid transporters (ATP-binding cassette A12 (ABCA12)), mouse beta defensin 3 (mBD3), sodium-hydrogen exchanger 1(NHE1), secretary phospholipase A2g2f (sPLA2g2f), filaggrin, involucrin, loricrin and Cyclophilin A are listed in supplemental table 1. Relative expression of the mRNAs compared to control mRNA was calculated. Data are expressed as percentage of control (as 100%) (Hou et al., 2013).

### Electron Microscopy

Skin biopsies from both vehicle and hesperidin-treated mice were taken for electron microscopy (Bourguignon et al., 2006; Hou et al., 2012 and 2013). Briefly, samples were minced to <0.5 $mm^3$, fixed in modified Karnovsky's fixative overnight, and post-fixed in either 0.2% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. After fixation, all samples were dehydrated in a graded ethanol series, and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further contrasting with lead citrate, in a Zeiss 10A electron microscope (Carl Zeiss, Thornwood, NJ), operated at 60 kV.

### Measurement of Lamellar Body Density and Secretion

LB numbers were determined in granular cells two to three layers below the stratum granulosum-stratum corneum (SG–SC) junction as previously described (Elias et al., 1983). The number of LBs was counted at 4800 magnification using a calibrated grid. Total 10 random pictures from each biopsy sample were assessed. For quantification of LB secretion, number of LB protrusion at the SG–SC junction were measured at a magnification of 5800 and correlated with the length of the bottom surface of the first SC layer on 10 random images at 5800 magnification.

### Statistics

Data are expressed as the mean ± SEM. GraphPad Prism 4 software (San Diego, CA, USA) was used for all statistical analyses. Unpaired two-tailed student's *t*-test with Welch's

correction was used to determine the statistical significances when two groups were compared. One-Way ANOVA with Tukey correction was used when three or more groups were compared.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
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| **SC** | stratum corneum |
| **NHE1** | sodium/hydrogen exchanger 1 |
| **sPLA2** | secretory phospholipase A2 |
| **ABCA12** | ATP-binding cassette transporter 12 |
| **PCNA** | Proliferating cell nuclear antigen |
| **HMGCoA** | 3-hydroxy-3-methyl-glutaryl-CoA reductase |
| **SPT** | serine palmitoyltransferase 1 |
| **FAS** | fatty acid synthase |
| **mBD-3** | mouse beta-defensin 3 |
| **hBD-2** | human beta-defensin 2 |
| **Nrf2** | nuclear factor (erythroid-derived 2)-like 2 |

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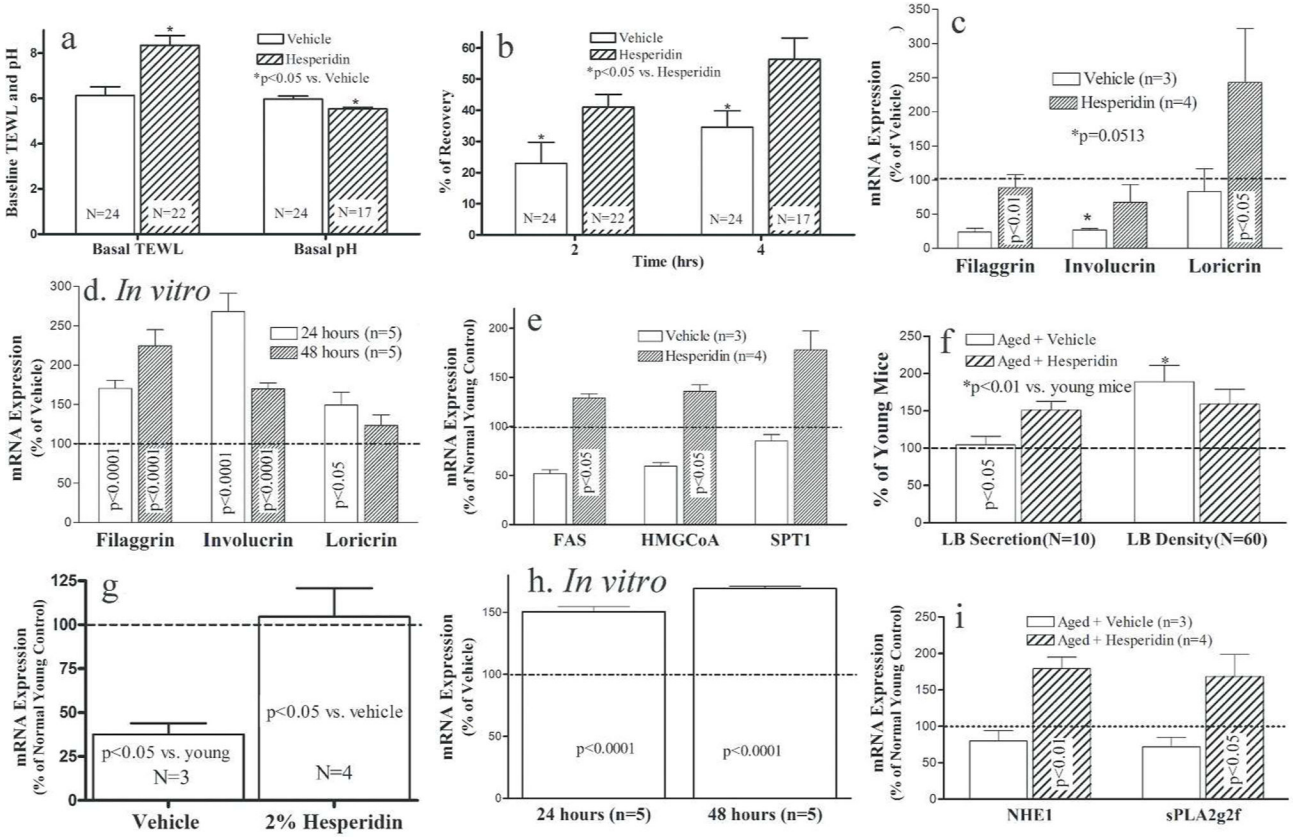


**Figure 1. Topical Hesperidin Improves Epidermal Permeability Barrier Homeostasis in Aged Murine Skin**

The values present the data *in vivo* in mice unless otherwise specified. Figure 1a displays basal TEWL and skin surface pH in mice; 1b, barrier recovery in mice; Fig. 1c shows the levels of epidermal mRNA in mice; 1d exhibits the levels of mRNA expression *in vitro* in human keratinocyte cultures, expressed as % of vehicle treated samples setting the levels of vehicle treated as 100% (dotted line); Fig. 1e shows epidermal mRNA levels in mice, expressed as % of normal young mice setting the levels of young mice as 100% (dotted line); Fig. 1f is the results of quantitative analysis of lamellar body density and secretion in mice; Fig. 1g and h present the expression levels of ABCA12 in mice and *in vitro* in human keratinocyte cultures, respectively; Fig. 1i shows the expression levels of epidermal NHE1 and sPLA2 in mice. Significances and numbers of samples are indicated in the figures.