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A pilot study of the photoprotective effect of almond phytochemicals in a 3D human skin equivalent

Julie A. Evans-Johnson [a,1], Jonathan A. Garlick [b], Elizabeth J. Johnson [a], Xiang-Dong Wang [a], C.-Y. Oliver Chen [a,*]

[a] Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, 711 Washington Street, Boston, MA 02111, USA
[b] Division of Cancer Biology and Tissue Engineering, Tufts University, 55 Kneeland Ave, South Cove Room 116, Boston, MA 02111, USA

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ABSTRACT

UV exposure causes oxidative stress, inflammation, erythema, and skin cancer. α-Tocopherol (AT) and polyphenols (AP) present in almonds may serve as photoprotectants. Our objectives were to assess the feasibility of using a 3D human skin equivalent (HSE) in photoprotectant research and to determine photoprotection of AT and AP against UVA radiation. AT or AP was applied to medium (25 and 5 μmol/L, respectively) or topically (1 mg/cm^2 and 14 μg/cm^2), followed by UVA. Photodamage assessed 96 h post UVA included HSE morphology, keratinocyte proliferation, apoptosis, and differentiation. UVA induced disorganization of basal layer, alteration of epidermal development, and fibroblast loss which were alleviated by all nutrient pretreatments. UVA significantly decreased keratinocyte proliferation compared to controls, and all pretreatments tended to negate the reduction though only the medium AT effect was statistically significant ($p \leqslant 0.05$). UVA led to a significant 16-fold increase in apoptosis of fibroblasts compared to the control which was alleviated by topical AP pretreatment and completely negated by topical AT ($p \leqslant 0.05$). In conclusion, we validated the feasibility of using HSE in evaluation of photoprotectants and found that AT and AP, applied to medium or topically, provided some degree of photoprotection against UVA.

1. Introduction

Skin is the largest organ in the human body and is exposed to an array of environmental insults. Among them, UV light is thought to be the most harmful because cumulative UV exposure can cause oxidative stress, inflammation, erythema, breakdown of the extracellular matrix, wrinkling and non-melanoma and melanoma skin cancer [1–4]. While a majority of photodamage research has focused on UVB irradiation, recent research has revealed that UVA damage can be significant, including deoxyribonucleic acid (DNA) mutations and altered DNA repair, immune function, cell integrity and cell cycle regulation [5,6], partially due to UVA's deeper penetration into the dermal layer than UVB [7]. There are many means to protect against UV damage, including avoidance of the sun and wearing protective clothing and topical sunscreen. Compared to topical sunscreens, which require reapplication and have localized effects, dietary sunscreen delivery could also play a significant role in continual protection of whole body and skin against UV irradiation.

α-Tocopherol (AT) is well-known for its role as a chain-breaking antioxidant during lipid peroxidation. Photoprotection of AT through dietary or skin application, as evaluated with erythema, wrinkling, sunburn cell formation, and skin tumor incidence, has been demonstrated in clinical and preclinical studies [8–11]. Its concentration in skin is approximately 7 and 13 ng/mg wet weight in dermal and epidermal compartments, respectively [12]. Its level can be increased with oral consumption (5-fold increase in skin surface lipid) or topical application (>20-fold increase) [13,14] and decreased by 50% in the stratum corneum with UV exposure [15]. Flavonoids are produced in plants as a phytoalexin, responding to environmental insults including excess light. They exert an array of bioactions, e.g., radical scavenging, UVA absorption, anti-inflammatory, anti-apoptotic, DNA protection, and modulating signaling pathways [16,17]. Topically applied or orally consumed flavonoids have shown protection from UV-induced erythema and oxidative stress [18–21].

Clinical human trials, animal, and monolayer cell culture studies are generally employed to examine the efficacy of photoprotectants from either topical or oral application. However, conducting human studies appears to be very challenging because of high cost and scar-inducing skin biopsies. While it is less challenging to conduct *in vitro* and animal experiments, it is well appreciated that ex-

* Corresponding author. Tel.: +1 617 556 3128.
E-mail address: oliver.chen@tufts.edu (C.-Y. Oliver Chen).
[1] Current address: University of Arkansas for Medical Sciences, 4301 Markham Street, Slot 820, Little Rock, AR 72205, USA.

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tra precaution is required to extrapolate preclinical animal data to human skin health. A novel, *in vitro* three dimensional (3D) human skin equivalent (HSE) has been developed and provides a highly-predictive system to assess histological, biochemical and molecular outcomes under conditions similar to those found *in vivo*. While HSE has previously been used to study the impact of UV-irradiation, studies designed to characterize the effect of photoprotectants using this model are scarce. A few ground-breaking nutrient photoprotection studies in similar HSE models, commercially available EpiDerm™ Full thickness, have shown promising results from other flavonoids [22-24]. Therefore, we undertook a pilot study to examine the feasibility of using our HSE in photoprotectant research and to determine the photoprotective effect of α-tocopherol (AT) and almond polyphenols (AP) on UVA induced damages. The outcome measures include morphology, basal cell proliferation, apoptosis, strata-specific differentiation, and growth factor production. Almond nutrients were investigated because almonds are among the richest dietary sources of AT and contain an array of antioxidant polyphenols. Predominant APs include the flavonoids isorhamnetin, catechin, kaempferol, epicatechin, and quercetin [25].

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium was purchased from Invitrogen (Carlsbad, CA), Bromodeoxyuridine (BrdU) antibodies and terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) kits were purchased from Roche (Indianapolis, IN). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Deep-well polycarbonate tissue culture inserts, Type I collagen, and low and normal calcium epidermal growth medium were obtained from Organogenesis (Canton, MA). Vectastain ABC kit was purchased from Vector Labs (Burlingame, CA), cytokeratin-10 from Abcam (Cambridge, MA) and DuoSet HGF, IL-1α and keratinocyte growth factor (KGF) ELISA kits from R&D Systems (Minneapolis, MN). Quercetin dihydrate, UDP-glucuronic acid, alamethicin, dimethyl sulfoxide (DMSO), BrdU, α-tocopherol, collagenase, protease, high performance liquid chromatography (HPLC) grade ethanol, methyl-tert-butyl ether, ammonium acetate (analytical grade), retinyl acetate, and all other chemical and reagents were obtained from Sigma–Aldrich (St. Louis, MO). HPLC water was obtained from Water Purification Systems (Millipore Corp., Billerica, MA). Liver microsomal protein was purchased from BD Biosciences (San Jose, CA) and optimal cutting temperature (OCT) compound from Sakura Finetek USA (Torrance, CA).

2.2. Fabrication of HSE

3D HSEs were constructed as previously described [26]. Briefly, human dermal fibroblasts (HDF) were derived from newborn foreskin and grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS. HDF were mixed with bovine Type I collagen and allowed to contract for 7 days in deep-well polycarbonate tissue culture inserts. After human keratinocytes were seeded onto the collagen gel, tissues were then maintained submerged in low calcium epidermal growth media for 2 d, in normal calcium media for an additional 2 d, and then brought to the air–liquid interface for 7 d. Subsequently, developed HSEs were used in experiments with 2–4 replications for each experimental condition.

2.3. Alpha-tocopherol treatment

HSEs were treated with pure AT in the medium at a final concentration of 0 or 25 μmol/L for 48 h. DMSO concentration was 0.1% of media. The dose was selected because it is in the range of physiologically achievable circulating concentrations in humans. For topical application, AT was dissolved in 45 μL acetone and applied to the HSEs at 0 or 1 mg/cm^2 for 2 h. The dose was selected based on our preliminary work which established its safety in HSEs at 0.5–5 mg/cm^2.

A portion of HSEs was separated into dermal and epidermal compartments with forceps, weighed, and stored at −80 °C until analysis. AT in epidermal and dermal layers was extracted as previously described with minor modifications [27]. Briefly, the tissue was first digested with collagenase at 37 °C for 1 h followed by homogenization on ice for 2 min (IKA Ultra-Turrax T8, Wilmington, NC) and digestion with protease at 37 °C for 30 min. After the addition of 1% sodium dodecyl sulfate in ethanol, AT was extracted with hexane, dried under nitrogen air, and reconstituted in ethanol for analysis using a reverse phase gradient HPLC method with a YMC C30 carotenoid column (S-3 3.0 × 150 mm). AT concentration was calculated using a standard curve constructed with authentic AT standard with adjustment of internal standard recovery (retinyl acetate).

2.4. Almond polyphenol treatment

For topical application of AP to HSEs, polyphenols were extracted from Nonpareil almond skins according to Chen et al. [28]. Total phenolics content of the resulting extract was quantified using the Folin–Ciocalteu's reaction and expressed as gallic acid equivalents (GAE) [28]. AP was resuspended in 45 μL of 40% acetone in phosphate buffered saline (PBS) for topical application for 2 h at the dose of 14 μg/cm^2 GAE. The dose and incubation time were selected based on our preliminary safety experiment (ranging 14–840 μg/cm^2 GAE).

For medium application, quercetin glucuronides (Q_{gluc}) were produced according to Bolling et al. [29] to mimic the flavonoid form found in circulation after almond consumption. Quercetin was selected as a representative almond flavonoid because it is found in almond skins, is readily available and inexpensive. Human liver microsomal protein (1 mg/ml, final concentration) were pre-incubated with alamethicin at 37 °C for 5 min in microcentrifuge tubes containing quercetin (600 μmol/L). Glucuronidation was initiated with the addition of a cofactor solution of UDP-glucuronic acid, magnesium chloride, and potassium phosphate buffer. After 3-h incubation at 37 °C, Q_{gluc} were extracted with methanol, dried under nitrogen gas, and stored at −80 °C until use within 2 wk. Q_{gluc} were quantified using an established HPLC method [29]. Dried residues were reconstituted in PBS for application to culture medium at a final concentration of 5 μmol/L total Q_{gluc} for 2 h. The dose and 2-h incubation period were selected based on physiologically achievable concentrations normally found in flavonol pharmacokinetic studies. For vehicle control, the dry residue from glucuronidation reaction without quercetin was used.

2.5. UV irradiation and tissue preservation

The HSE surface was gently washed with PBS twice, and then the HSEs were transferred to a sterile platform for UVA irradiation. A 200-W UV radiation source (Lightningcure™ 200, Hamamatsu, Japan) was used in combination with a dichroic mirror assembly reflecting most of the visible and infrared wavelengths, to reduce the heat load on the skin, and with UG11 and WG335 filters for wavelength delivery in the UVA range (315–400 nm). A liquid light guide was mounted to a stand for uniform and accurate UVA deliv-

ery 3 cm above the HSE surface. UVA dose was 0 or 35 J/cm^2 with an intensity of 75 mW/cm^2 as measured by a radiometer (Fluke 289 True RMS Multimeter, Everett, WA). The 35 J/cm^2 dose was selected based on our preliminary data demonstrating mild, nontoxic tissue damage where morphology and apoptosis were assessed after UVA exposure (0–100 J/cm^2, data not shown). After UVA exposure, HSEs were cultured with fresh medium for an additional 96 h and then harvested for tissue analysis. The corresponding media were also collected after overnight incubation. For tissue analyses, HSEs were normally divided into three equal portions. One third was fixed in formalin, embedded in paraffin, and serially sectioned at 6 μm thickness. Paraffin-embedded HSEs were stained with hematoxylin and eosin (H&E), images were captured using a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) equipped with a SPOT RT camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed using SPOT Advanced software. Tissue architecture was evaluated by the degree of organization of basal cell layer, presence of fibroblasts, and the maturational development of the multiple layers of epidermis (spinous, granular and stratum corneum). The second part was snap frozen in OCT compound over liquid nitrogen vapor after immersion in 2 mol/L sucrose solution at 4 °C for >24 h. The remaining third was preserved for TUNEL assay by immersion in 4% paraformaldehyde for 1 h and 2 mol/L sucrose solution at 4 °C for >24 h and snap frozen in OCT compound over liquid nitrogen vapors. All frozen tissues were serial sectioned at 6 μm thickness and mounted onto gelatin-chrome alum coated slides and used for immunohistochemical staining (IHS).

For photoprotection studies, HSE were incubated with the study nutrients or vehicle controls (at previously established concentrations and durations) prior to UVA exposure. Photodamage was assessed 96 h post UVA by morphology, keratinocyte proliferation, apoptosis, and differentiation.

2.6. Proliferation, differentiation, and apoptosis

Paraffin-embedded HSEs were used to determine proliferation using IHS to detect nuclear BrdU staining after it was added to tissue culture media (20 μg/mL) 5 h prior to HSE harvesting using a monoclonal BrdU antibody and Vectastain ABC kit (Vector Labs, Burlingame, CA). The paraffin-embedded HSEs were used to assess differentiation using a monoclonal antibody against cytokeratin-10 (CK10) and Vectastain ABC kit. 3,3′-diaminobenzidine tetrahydrochloride was used as chromogen in both BrdU and CK10 assays, producing a brown stain. The TUNEL assay was used to identify apoptotic cells in epithelium and dermis. Paraformaldehyde-fixed HSEs were treated with 0.1% triton in 0.1% sodium citrate, incubated with the TUNEL reaction mix for 1 h at 37 °C in the dark, and then counterstained with 40, 6-diamidino-2-phenylindole. The number of BrdU and TUNEL-positive cells were determined and expressed as a percentage of total cells. Differentiation of keratinocytes was evaluated by visual comparison of the strata-specific localization of CK10-positive cells.

2.7. Determination of growth hormones and IL-1α in medium

To assess secretion of growth hormones and cytokine into the medium, DuoSet HGF, KGF and IL-1α ELISA kits (R&D Systems, Minneapolis, MN) were employed. The assays were run in duplicate.

2.8. Statistical analysis

All data of quantitative analyses are expressed as mean ± SEM. All 5 controls, including 1 no treatment control and 4 vehicle and UVA sham controls, were performed in duplicate for a total of 10 HSE samples. After reviewing values of all 10 HSE controls in a systematic manner and noting no marked difference between 5 controls, they were grouped as one control for statistical analyses. There were a total of 4 treatments (2 nutrients× 2 application routes) in the study. The effect of each treatment on UVA-induced damages was assessed using one-way ANOVA, followed by Post Hoc analysis using Tukey's honestly significant difference test. All statistical analyses were performed using Prism 5 (GraphPad Software Inc., La Jolla, CA).

3. Results and discussion

Our pilot study successfully demonstrated the feasibility of using a 3D HSE, that closely mimics the function of human skin, to investigate absorption and photoprotection of almond phytonutrients applied in medium and topically.

3.1. AT absorption

Absorption and physiologically relevant localization of nutrients in skin are critical steps in their photoprotection. We found that AT applied to the medium or surface of the HSEs was absorbed into both the dermis and epidermis in time and dose-dependent manners. The kinetics of AT absorption via medium and topical applications were examined in the epidermis vs. dermis after the tissue compartments were physically separated with $n = 2$/time point. The medium treatment of 9 μmol/L led to progressive AT accumulation in both epidermis and dermis with the epidermal concentration being much greater than dermal (Fig. 1A). AT concentrations in both epidermis and dermis appeared to accumulate rapidly up to 6 h after medium application, but continued to increase at a slower rate up to 48 h. Similarly, the topical application led to increased AT accumulations in epidermis and dermis, and the concentrations were at least 100-fold larger than those observed from the medium application (Fig. 1B). At 2 h after the topical treatment, AT concentration in epidermis was markedly elevated and reached >10 μg/mg wet weight.

The dose response of AT accumulation at 48 h after the applications was apparent (Fig. 1C and D). The 25 μmol/L AT medium dose led to greater AT accumulations in both epidermis (40%) and dermis (127%) than the 15 μmol/L dose. The 1 mg/cm^2 topical AT dose resulted in an 884% greater accumulation in epidermis than the 0.5 mg/cm^2 dose. The dermis appeared to become saturated with the 0.5 mg/cm^2 dose as the AT accumulation was not augmented by the higher dose. Importantly, the 1 mg/cm^2 topical dose led to 1251- and 300-fold greater AT concentrations in epidermis and dermis, respectively, than those achieved with the 25 μmol/L medium dose.

Absorption of flavonoids to HSEs after AP applications was assessed, but their concentrations were below the detection limits of our HPLC method with electrochemical detection.

We noted that AT concentrations in HSEs treated with AT in medium were similar to those found in normal human skin (7 and 13 ng/mg wet wt in dermis and epidermis, respectively) [12]. Also consistent with what is found in normal human skin, AT preferentially localized and accumulated in the epidermis of HSEs, regardless of the means of application. Even though permeability of 3D skin tissues is higher than human skin [30] and sebaceous glands primarily accountable for AT transport to the skin's surface are not present in our HSEs [14], our results suggest comparability of HSE to human skin in nutrition absorption and inform the adequacy of HSE for future *in vitro* investigations of nutrient absorption and metabolism.

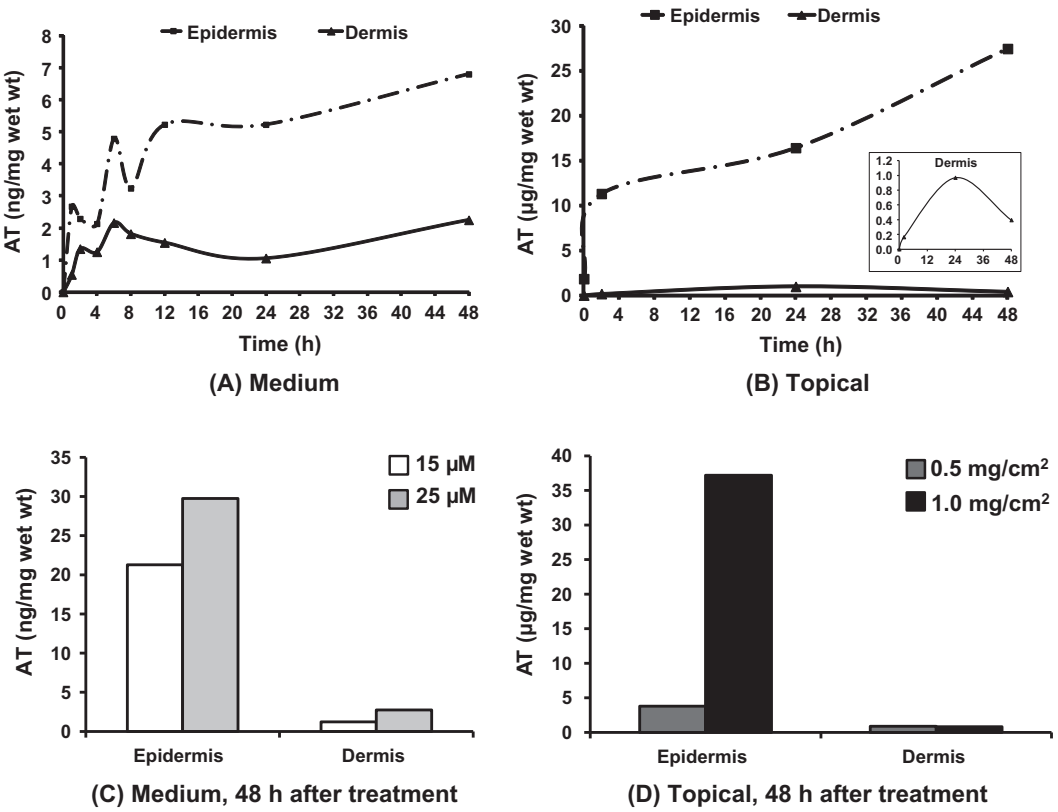

Fig. 1. Absorption of AT into epidermis and dermis of 3D HSEs. (A) AT kinetics after medium treatment with 9 µmol/L AT, $n = 2$; (B) AT kinetics after topical treatment with 1 mg/cm^2 AT, $n = 2$; (C) AT concentrations 48 h after medium treatment AT, n = 2; (D) AT concentrations 48 h after topical treatment AT, $n = 2$.

3.2. Photoprotection

The potential UVA photoprotection of AT, Q_{gluc} and AP was examined in HSEs through topical and medium applications with the intention to mimic topical sunscreen application and dietary consumption. Topical AP treatment consisted of a mixture of polyphenols extracted from almond skins (predominantly isorhamnetin, catechin, kaempferol, epicatechin, and quercetin [25]). To better mimic the *in vivo* condition, the polyphenol treatment added to the media was glucuronidated quercetin, Q_{gluc}, intended to mimic metabolites found in circulation after consumption of flavonol-rich almonds. Of the known almond polyphenols, quercetin is among the predominant 5. A physiologic dose of 35 J/cm^2 UVA irradiation (equivalent to <5 h mid-day summer sun exposure [31]) was administered in the study, based on our preliminary data demonstrating this dose induced nontoxic tissue damage.

3.2.1. Morphology

UVA irradiation of 35 J/cm^2 induced keratinocyte disorganization, flattening of the basal cell layer, altered epidermal development (spinous, granular and stratum corneum layers), and fibroblast disappearance (Fig. 2A and B). Representative examples of altered basal cell morphology are observed (inset of Fig. 2B and C2) and contrast the normal cuboidal to columnar architecture seen in the control (inset of Fig. 2A) and tissues that were UVA exposed and nutrient treated (inset of Fig. 2C3). Sham irradiated HSEs treated with vehicle controls (picture not shown) were comparable to the untreated controls. Morphology of HSEs receiving the treatments alone (Fig. 2C1, 2D1, 2E1, and 2F1) was comparable to the control (Fig. 2A) and demonstrated normal tissue architecture characterized by a fully-developed spinous cell layer, polarized basal cells, and dermal fibroblast presence. However, vehicles employed for both AT applications (Fig. 2C2 and 2D2) and topical AP (Fig. 2F2) were not protective of UVA-induced morphological changes while medium Q_{gluc} vehicle (Fig. 2E2) showed slightly less UVA-induced basal layer disorganization, delayed epidermal development, and fibroblast disappearance when compared to the UVA-treated HSEs (Fig. 2B). However, the HSEs treated with each of the 4 nutrient treatments prior to UVA exposure blocked UVA-induced morphological changes (Fig. 2C3, 2D3, 2E3, and 2F3) as tissues demonstrated normal tissue architecture very similar to control (Fig. 2A) and tissues treated with nutrients alone (Fig. 2C1, 2D1, 2E1 and 2F1). In addition, medium Q_{gluc} and UVA treated HSEs had more crowding in the basal layer and fewer pyknotic fibroblasts than UVA treated HSEs.

3.2.2. Proliferation

Keratinocyte proliferation was determined by the BrdU assay. UVA irradiated basal keratinocyte proliferation was less than a half of the control cells ($p \leqslant 0.05$, Fig. 3), but none of the 4 treatments alone affected proliferation. As compared to UVA-treated HSEs, medium AT pretreated tissues had significantly greater percent proliferating basal cells. The topical AP and medium Q_{gluc} pretreatments also tended to prevent UVA-induced proliferation reduction, though the differences were not significant.

3.2.3. Apoptosis

Apoptosis was assessed using the TUNEL assay. No epidermal apoptosis effects were observed from any nutrient or UVA treatment combination (data not shown). The amount of dermal apoptosis in UVA irradiated HSEs was approximately 16-fold greater than the control tissues ($p \leqslant 0.05$, Fig. 4), but none of the 4 pretreatments alone induced dermal apoptosis. Topical AT pretreatment completely protected HSEs against UVA-induced dermal

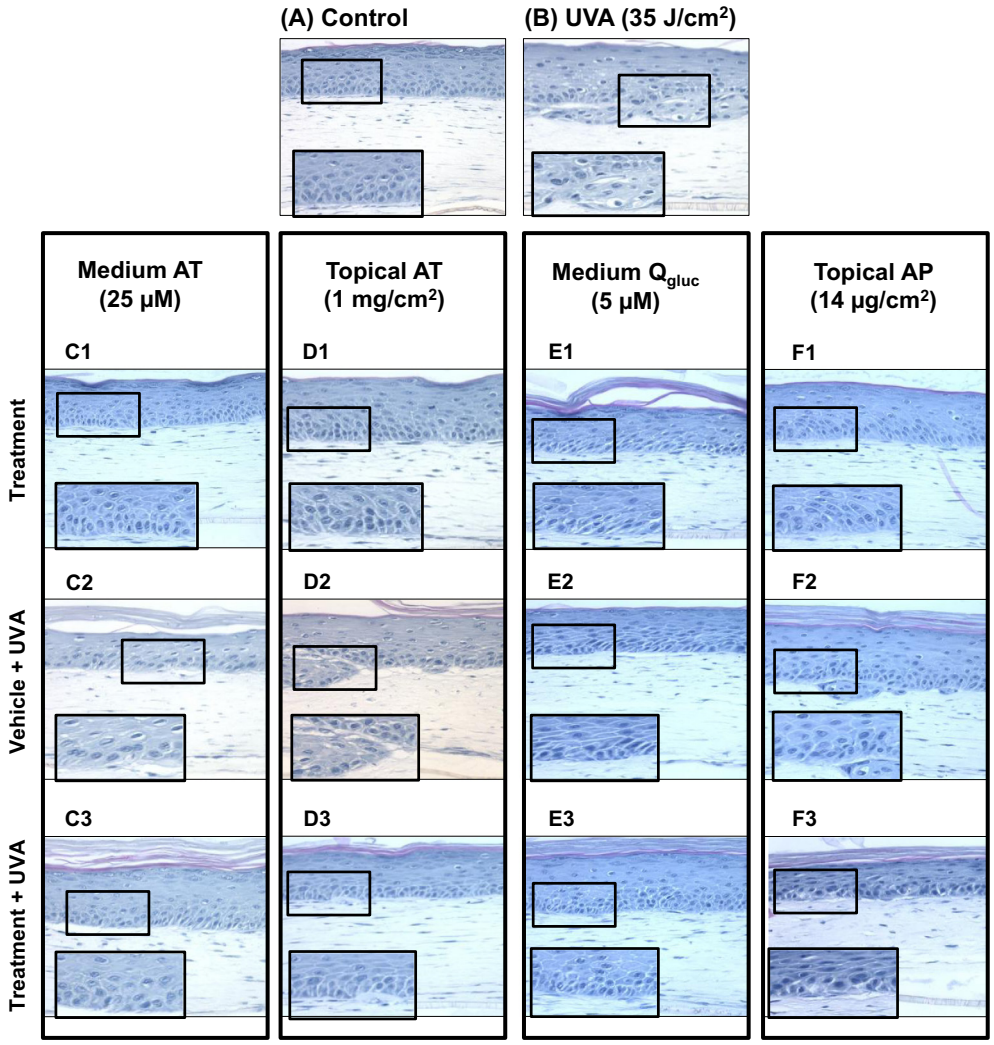

Fig. 2. Morphology of 3D HSEs assessed from H&E at 96 h after UVA (35 J/cm^2) irradiation. (A) control; (B) UVA irradiation; (C1–3) AT (25 μmol/L) in medium for 48 h, vehicle (0.1% DMSO) and UVA, AT and UVA, respectively; (D1–3) AT (1 mg/cm^2) topically for 2 h, vehicle (acetone) and UVA, AT and UVA, respectively; (E1–3) Q_{gluc} (5 μmol/L) in medium for 2 h, vehicle (blank glucuronidation reaction in PBS) and UVA, Q_{gluc} and UVA, respectively; (F1–3) AP (almond polyphenol mixture, 14 μg/cm^2) topically for 2 h, vehicle (40% acetone in PBS) and UVA, AP and UVA, respectively.

apoptosis and topical AP significantly decreased the effect by greater than 50% as compared to the UVA treated HSEs ($p \leqslant 0.05$), while AT and Q_{gluc} pretreatments delivered in the media did not affect UVA-induced dermal cell death.

3.2.4. Differentiation

The strata-specific, epidermal expression of CK10 is considered a molecular marker for the terminal differentiation of skin keratinocytes (Fig. 5). Normal CK10 patterns of expression are strictly limited to the suprabasal cells (5–10 layers right above basal cell layer), as seen in control tissues and in nutrient-treated tissues (inset of Fig. 5A, C1, D1, E1 and F1). Expression of CK10 was somewhat delayed by UVA irradiation, as seen by the greater separation between basal and CK10-positive cells (Fig. 5B, C2, D2, E2 and F2). This was most dramatically seen in tissues treated with vehicles for medium AT (Fig. 5C2) and Q_{gluc} (Fig. 5E2). Pretreatments of HSEs with AT, Q_{gluc} and AP restored the normal strata-specific pattern of differentiation in spite of the UVA exposure (Fig. 5C3, D3, E3 and F3).

3.2.5. Secretion of growth hormones and cytokine

HGF secretion to medium of UVA irradiated HSEs did not vary significantly from the control (Fig. 6). Neither alone nor in combination with UVA irradiation did medium AT, Q_{gluc} or topical AP result in any change in HGF secretion. However, topical AT alone significantly stimulated HGF secretion by 88% as compared to the control ($p \leqslant 0.05$). Combined topical AT and UVA further increased secretion by 238% as compared to the control and 82% as compared to the topical AT alone ($p \leqslant 0.05$). We also measured IL-1α and KGF in media but their concentrations were below the detection limits of the kits (9.375 and 31.25 pg/mL, respectively).

3.2.6. Photoprotection discussions

Consistent with the literature [7,32,33], we found that UVA irradiation of the HSEs induced aberrant morphologic changes, significantly increased apoptosis of dermal fibroblasts, and delayed differentiation. While beyond the scope of the current study, these changes may be mediated by UVA-derived reactive oxidant species as UVA is known to lack the energy to directly break chemical bonds [34]. Because the dermis is equipped with fewer endogenous antioxidant enzymes, e.g., catalase and superoxide dismutase, as compared to epidermis, it is likely more susceptible to oxidative damage induced by deep penetration of UVA [35].

In concordance with our positive AT results, Noel-Hudson et al. [36] reported in an epidermal model that topical AT pretreatment reduced UVA-induced cell death. While mechanism(s) for such

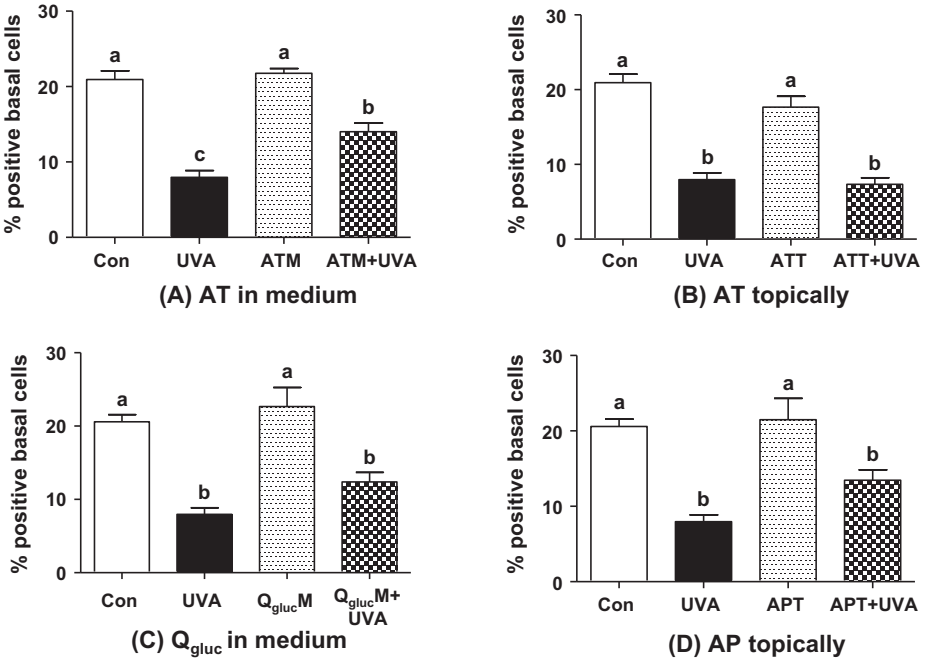

Fig. 3. Proliferation of keratinocytes (epidermal layer) determined by the BrdU assay at 96 h after UVA (35 J/cm^2) irradiation, preceded by (A) AT (25 μmol/L) in medium for 48 h, (B) AT (1 mg/cm^2) topically for 2h, (C) Q_{gluc} (5 μmol/L) in medium for 2 h, and (D) AP (almond polyphenol mixture, 14 μg/cm^2) topically for 2 h. [abc]Means with different letters in each panel are different, tested by one-way ANOVA followed by Tukey Kramer *HSD Multi-Comparison*, $p \leqslant 0.05$.

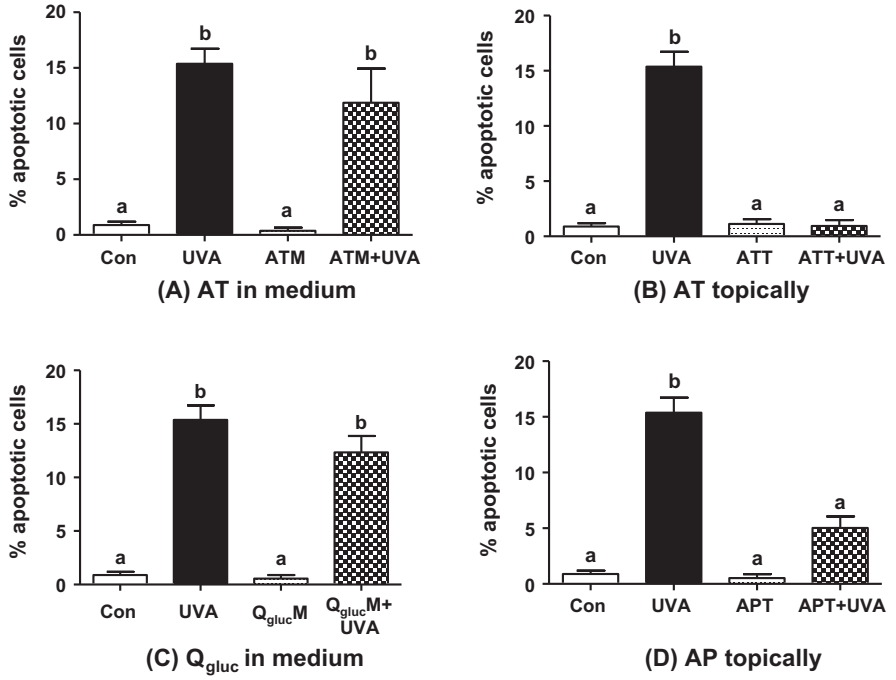

Fig. 4. Apoptosis of fibroblasts (dermal layer) determined by the TUNEL assay in 3D HSEs at 96 h after UVA (35 J/cm^2) irradiation, preceded by (A) AT (25 μmol/L) to the medium for 48 h, (B) AT (1 mg/cm^2) topically for 2 h, (C) Q_{gluc} (5 μmol/L) in medium for 2 h, and (D) AP (almond polyphenol mixture, 14 μg/cm^2) topically for 2 h. [ab]Means with different letters in each panel are different, tested by one-way ANOVA followed by Tukey Kramer *HSD Multi-Comparison*, $p \leqslant 0.05$.

photoprotection remains to be elucidated, antioxidative actions of AT might be protective of fibroblasts against UVA-induced oxidative stress, thereby protecting their survival and maintaining tissue homeostasis through cellular crosstalk modulated by growth hormones [34,37]. This speculation was supported by greater HGF secretions from fibroblasts, lower dermal apoptosis, and superior differentiation in the topical AT pretreated HSEs with UVA irradiation than the medium AT pretreated. Importantly, greater AT dermal accumulation in topically-treated HSEs than medium-treated paralleled these morphologic and biochemical differences and further supported the hypothesis. While different doses of AT application through topical and medium applications may partially explain the divergence of our results, topical application might prove more effective. Noel-Hudson et al. [36] found only topical

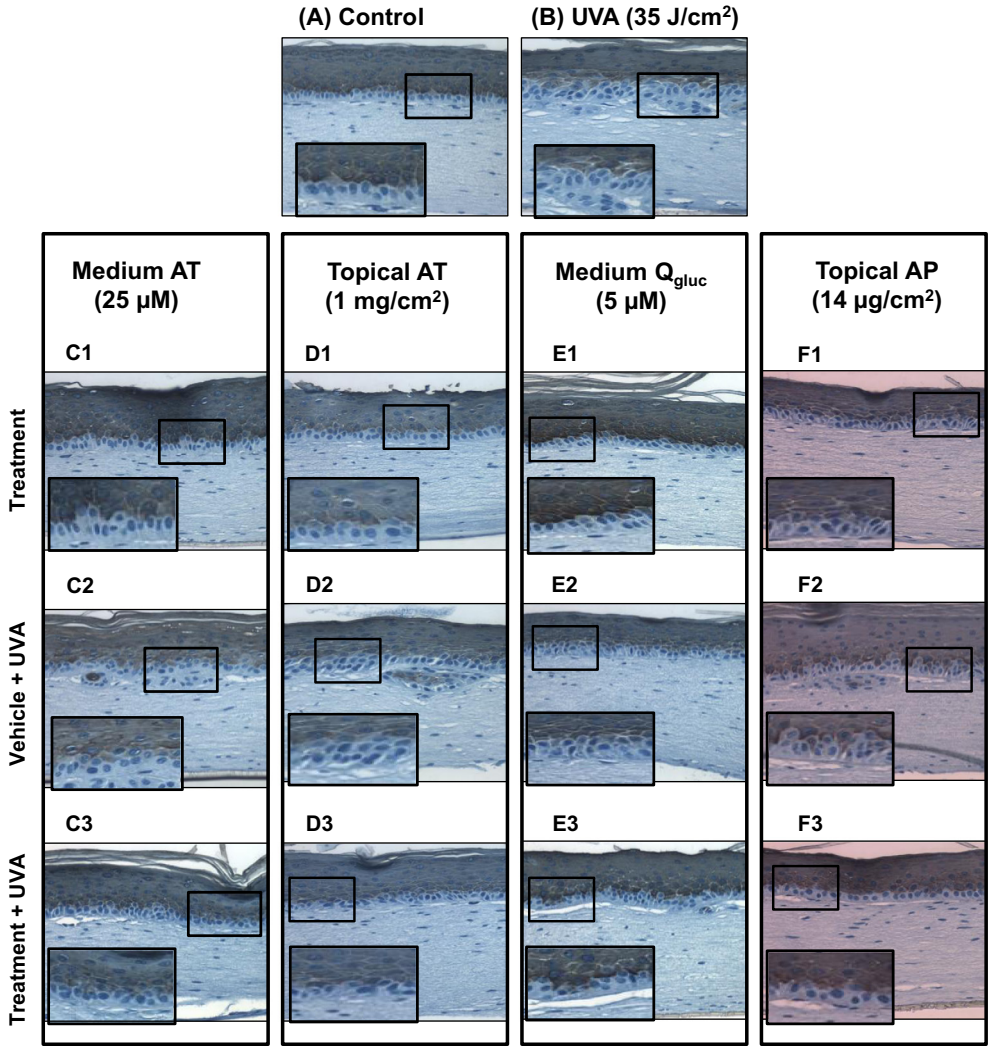

Fig. 5. Differentiation of keratinocytes determined by the cyotkeratin-10 staining assay in 3D HSEs at 96 h after UVA (35 J/cm^2) irradiation. (A) control; (B) UVA irradiation; (C1–3) AT (25 μmol/L) in medium for 48 h, vehicle and UVA, AT and UVA, respectively; (D1–3) AT (1 mg/cm^2) topically for 2 h, vehicle and UVA, AT and UVA, respectively; (E1–3) Q_{gluc} (5 μmol/L) in medium for 2 h, vehicle and UVA, Q_{gluc} and UVA, respectively; (F1–3) AP (almond polyphenol mixture, 14 μg/cm^2) topically for 2 h, vehicle and UVA, AP and UVA, respectively. Brown-stained cells are CK10 positive while blue-stained cells are CK10 negative. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AT, not medium, attenuated UVA-induced cell death in an epidermal model using equivalent doses. Interestingly, we found that topical AT did not prevent UVA-induced decrease in proliferation, which could be a result of active keratinocyte differentiation. As of yet, there is little *in vivo* evidence illustrating photoprotective efficacy of AT is more potent with topical application than from oral delivery [14]. In fact, a clinical trial investigating oral, topical, and combined application of fat-soluble carotenoids showed that combined application provided the most photoprotection [38]. Future research examining additive/synergistic photoprotective efficacy of AT, mimicking oral and topical delivery, can be readily performed in the 3D HSE. Further, the significant additive effect of topical AT and UVA on HGF secretion is intriguing, and the mechanism(s) contributing to the increase remains to be examined.

The addition of polyphenols in cosmetic formulations is burgeoning as their sun protection factor and protection factor-UVA seem comparable to or greater than some inorganic UV filters [39], and their inclusion is supported by a growing body of evidence documented in preclinical and clinical studies [40–42]. In this study, we found that topical application of a mixture of polyphenols derived from almonds significantly mitigated UVA-induced apoptosis in dermal fibroblasts, maintained healthy skin morphology and differentiation, and tended to diminish the UVA-induced reduction in keratinocyte proliferation. While more research remains to be performed to elucidate the underlying mechanism(s) for the observed photoprotection and to identify the most bioactive constituent(s) in the almond polyphenol extract, plausible mechanisms might include UVA absorbance, radical scavenging action, and modulation of cellular signaling and endogenous antioxidant defenses.

In our study, UVA irradiated HSEs pretreated with quercetin glucuronide metabolites, produced by *in vitro* human hepatic microsomes to closely emulate circulating flavonoid forms, had a more normalized tissue architecture than UVA treated tissues, demonstrating healthier and more developed epithelia. Our work supports the importance of using physiologic forms of flavonoids in such studies. Proteggente et al. [43] reported that hesperidin glucuronide metabolites, from a different flavonoid subfamily, displayed a UVA protective effect in modeled skin fibroblasts but its

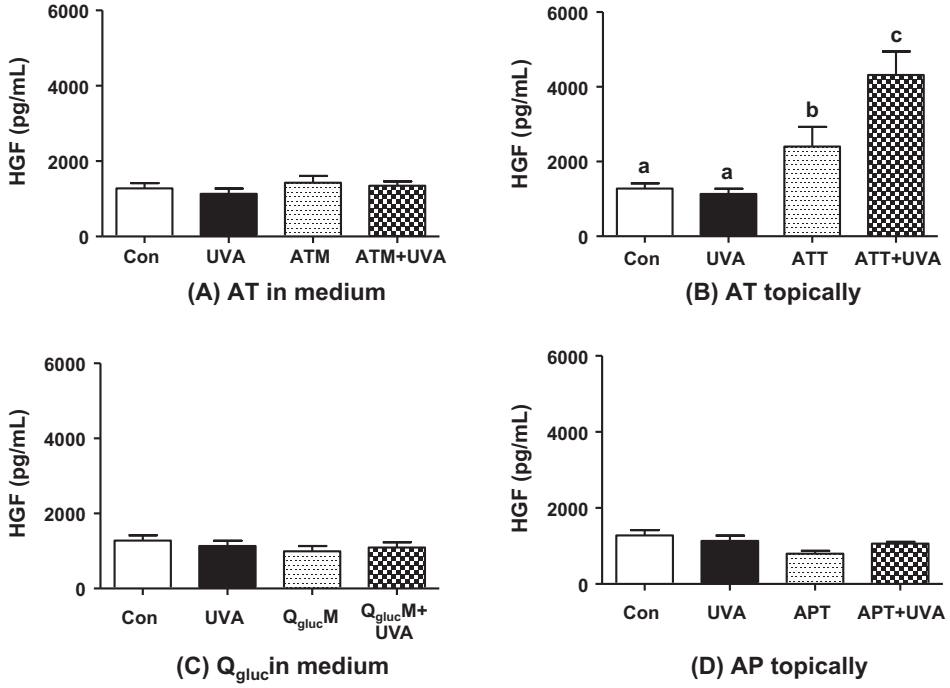

Fig. 6. Concentration of HGF in medium of 3D HSEs at 96 h after UVA (35 J/cm^2) irradiation, preceded by (A) AT (25 μmol/L) to the medium for 48 h, (B) AT (1 mg/cm^2) topically for 2 h, (C) Q_{gluc} (5 μmol/L) in medium for 2 h, and (D) AP (almond polyphenol mixture, 14 μg/cm^2) topically for 2 h. [abc]Means with different letters in each panel are different, tested by one-way ANOVA followed by Tukey Kramer *HSD Multi-Comparison*, $p \leqslant 0.05$.

cellular absorption could not be confirmed. On the other hand, hesperidin aglycone was efficiently taken up by the fibroblasts but provided no detectable UVA protection. Collectively, consumption of polyphenols could support skin health, particularly protecting skin against UV damages, even though it is well appreciated that absorbed polyphenols are generally present in a more hydrophilic form with phase II conjugates.

Our pilot *in vitro* 3D HSE study showed that AT and polyphenols applied either topically or in culture medium could protect modeled skin tissue from UVA induced damages to some extent. There are a few limitations in the study. First, our study cannot elucidate whether there was a disparity in photoprotective efficacy of topical and medium treatments because AT doses of topical and medium administration were not equal. Likewise, it was true for AP. Further, crude almond polyphenol extract was applied topically and quercetin metabolites were added to culture medium for physiological relevance. Minor Q_{gluc} vehicle effects likely result from UDP-glucuronic acid. Second, mechanism(s) by which the study treatments exerted their photoprotection were not examined. Because of our positive findings, future studies are warranted to examine the changes in *de novo* synthesis and deposition of elastin and collagen and production of metalloproteinases as these measures could provide insight into the mechanisms of action of AT and polyphenols. Finally, more work on absorption and metabolism of polyphenols in our 3D HSE shall be performed, as the information is critical to aid interpretation of photoprotective outcomes and informing design of future studies.

Because of the previously mentioned limitations of predominant models employed for nutrient photoprotection research, we investigated the feasibility of application of our novel 3D skin tissue model for such studies. We herein validate that this model can be a valuable tool to assess photoprotective capacity and nutrient absorption under conditions similar to those found in humans. Our study also demonstrates that small, but significant, photoprotection is provided by α-tocopherol and polyphenols, nutrients found in almonds and other plant foods.

4. Abbreviations

AP	almond polyphenols
AT	α-tocopherol
BrdU	Bromodeoxyuridine
CK10	cytokeratin-10
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
GAE	gallic acid equivalents
H&E	hematoxylin and eosin
HGF	hepatocyte growth factor
HPLC	high performance liquid chromatography
HDF	human dermal fibroblasts
HSE	human skin equivalent
IHS	immunohistochemical staining
KGF	keratinocyte growth factor
OCT	optimal cutting temperature
PBS	phosphate buffered saline
Qgluc	quercetin glucuronides
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling
UVA	ultraviolet A
3D	three dimensional

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