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# Clinical and *in vitro* evaluation of new anti-redness cosmetic products in subjects with winter xerosis and sensitive skin

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## Abstract

**OBJECTIVE:** To demonstrate the *in vitro* activities of panthenol, palmitoylethanolamide (PEA), and niacinamide (NAM) and determine the biophysical properties, clinical safety, tolerability together with efficacy of two developmental anti-redness (AR) formulations containing these ingredients, in alleviating facial redness associated with winter xerosis in healthy volunteers with sensitive skin.

**METHODS:** The anti-inflammatory and skin protective properties of panthenol, PEA and NAM were evaluated *in vitro*. The physical properties of the AR formulations were analysed using measurement of water vapour transport rate (WVTR) and infrared spectroscopy. Clinical studies were performed between the months of December and April (2014–2015) with efficacy assessed during the winter. Facial redness, irritation, sensitization potential, photo-irritation, and photo-sensitization were evaluated. Self-assessed adverse reactions were reported in diaries of use.

**RESULTS:** Panthenol and PEA reduced prostaglandin $E_2$, interleukin-6, and thymic stromal lymphopoietin levels *in vitro*, while NAM induced nicotinamide adenine dinucleotide (NAD) levels and the keratinocyte differentiation markers: filaggrin (2-fold increase, $P < 0.001$), loricrin (2-fold increase, $P < 0.05$), involucrin (2 fold increase, $P < 0.001$) & peroxisomal proliferator activated receptor-alpha (1.5 fold increase, $P < 0.05$). The two AR products exhibited low WVTR vs. no treatment ($P < 0.001$) and displayed an ordered lipid structure. The day cream formulation protected against ultraviolet B radiation *in vitro*. A total of 382 participants were included in clinical studies which showed the AR formulations significantly improved facial redness associated with winter xerosis (Day 29 mean change from baseline: AR day cream 0.77 ($P < 0.001$); AR serum 0.67 ($P < 0.001$)). No irritation, sensitization, photo-irritation, photo-sensitization or product-related adverse reactions were observed or reported in the clinical studies.

**CONCLUSION:** The new products significantly improved skin redness associated with winter xerosis in participants with self-perceived sensitive skin. Both products were well tolerated with a suitable safety profile for topical use in subjects with sensitive skin.

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## Résumé

**OBJECTIF:** Démontrer l'activité *in vitro* du panthénol, du palmitoyléthanolamide (PEA), et du nicotinamide (NAM) et déterminer les propriétés biophysiques, la sécurité clinique, la tolérance ainsi que l'efficacité de deux formulations anti-rougeurs (AR) en développement contenant ces ingrédients pour atténuer les rougeurs faciales associées à la xérose hivernale chez des volontaires sains présentant une peau sensible.

**MÉTHODES:** Les propriétés anti-inflammatoires et protectrices du panthénol, du PEA et du NAM ont été évaluées *in vitro*. Les propriétés physiques des formulations AR ont été analysées en mesurant le taux de transport de vapeur d'eau (WVTR) et par spectroscopie infrarouge. Des études cliniques ont été réalisées entre décembre et avril (2014-2015) et l'efficacité a été évaluée pendant l'hiver. Les rougeurs, l'irritation, le potentiel de sensibilisation, la photo-irritation et la photosensibilisation au niveau du visage ont été évalués. Des effets indésirables auto-évalués ont été signalés dans des journaux d'utilisation.

**RÉSULTATS:** Le panthénol et le PEA ont réduit les niveaux de prostaglandine $E_2$, d'interleukine-6 et de lymphopoiétine stromale thymique *in vitro*, tandis que le NAM a généré une augmentation des taux de nicotinamide adénine dinucléotide (NAD) et des marqueurs de différenciation kératinocytaire : filaggrine (multiplication des taux par 2, $P < 0{,}001$), loricrine (multiplication des taux par 2, $P < 0{,}05$), involucrine (multiplication des taux par 2, $P < 0{,}001$) et du récepteur alpha activé de la prolifération peroxysomale (multiplication des taux par 1,5, $P < 0{,}05$). Les deux produits antirétroviraux présentaient un faible taux de WVTR par rapport à l'absence de traitement ($P < 0{,}001$) et présentaient une structure lipidique ordonnée. La formulation de la crème de jour protège contre le rayonnement ultraviolet B *in vitro*. Un total de 382 participants ont été inclus dans les études cliniques qui ont montré que les formulations AR amélioraient significativement les rougeurs faciales associées à la xérose hivernale (changement moyen du jour 29 par rapport à la référence : crème de jour AR 0,77 ($P < 0{,}001$) ; sérum AR 0,67 ($P < 0{,}001$)). Aucune irritation, sensibilisation, photo-irritation, photosensibilisation ni effet indésirable lié au produit n'a été observé ou signalé dans les études cliniques.

**CONCLUSION:** Les nouveaux produits ont considérablement amélioré la rougeur de la peau associée à la xérose hivernale chez les participants présentant une peau sensible auto-perçue. Les deux produits ont été bien tolérés avec un profil de sécurité approprié pour un usage topique chez les sujets présentant une peau sensible.

## Introduction

Sensitive skin has been defined as a syndrome with unpleasant sensations (stinging, burning, pain, pruritus, and tingling) that may be associated with skin erythema [1]. Although many consumers self-diagnose their sensitive skin they can also experience adverse skin reactions [2]. It has also been reported that Asian consumers may be more chemo-sensitive than other ethnic groups [1].

Impaired barrier function, whether due to poor epidermal differentiation, compromised stratum corneum (SC) maturation, or alterations in other epidermal biochemical pathways, is thought to contribute to skin sensitivity [3–6]. Published studies have demonstrated that epidermal barrier dysfunction and the cytokine, thymic stromal lymphopoietin (TSLP), also play a role in driving the atopic march, allergen sensitization, and itch [7]. Although chronic facial erythema in sensitive skin has a complex pathophysiology, barrier impairment triggered by excess ultraviolet (UV) exposure, low humidity, and pollutants can be involved in initiating this condition [8].

While reducing the negative effects of UV irradiation is key in alleviating sensitive skin symptoms, maintaining adequate moisturization is also important [9,10]. Formulations that contain lamellar lipid bilayers, mimicking those found within the SC, are known to be more effective clinically than non-lamellar lipid formulations in treating dry skin and uremic pruritus in addition to improving barrier function and erythema in subjects with sensitive skin and atopic eczema [11–13]. The non-physiological lipid, isostearyl isostearate (ISIS), has also been shown to positively influence lipid lamellar phase behaviour in experimental skin barrier and lipid models [14,15]. Therefore, semi-occlusive formulations containing lamellar lipids and ISIS will likely increase SC water content, improve corneocyte maturation and filaggrin processing and thereby improve barrier function [16]. We have developed formulations containing a combination of the above-mentioned lipids together with xerosis-alleviating polyols such as glycerol and xylitol [17].

Herein we describe a series of *in vitro* studies designed to determine the barrier efficacy and molecular lipid structure of the phospholipid-ISIS structural lamellar formulations using water vapour transport rate (WVTR) and Fourier transform infrared (FTIR) spectroscopy, respectively. In addition, we have investigated the effects of added panthenol [18] and the putative peroxisome proliferator activated receptor alpha (PPAR-$\alpha$) agonist palmitoylethanolamide (PEA) [19] on inflammatory mediators, and the effects of niacinamide (NAM) on nicotinamide adenine dinucleotide (NAD) production and keratinocyte differentiation [20–22]. Finally, we evaluated a new anti-redness (AR) day cream and AR serum containing these ingredients, first, through *in vitro* analysis of their anti-inflammatory properties, and subsequently, in clinical studies to determine the safety, tolerability, and efficacy of the products in subjects with winter xerosis-associated facial redness and sensitive skin.

## Methods

The AR day cream contains the following ingredients: Aqua, Isoamyl p-Methoxycinnamate, Glycerin, Dicaprylyl Carbonate, Diethylamino Hydroxybenzoyl Hexyl Benzoate, Bis-Ethylhexyloxyphenol Methoxyphenyl Triazine, Isostearyl Isostearate, Niacinamide, Xylitol, Pentylene Glycol, Butyrospermum Parkii Butter, 1,2-Hexanediol, Panthenol, Caprylic/Capric Triglyceride, Hydrogenated Lecithin, Palmitamide MEA, Oryza Sativa Cera, Tocopheryl Acetate, Polyacrylate Crosspolymer-6, Squalane, Acetamide MEA, Trisodium Ethylenediamine Disuccinate, Ascorbyl Glucoside, Citric Acid, t-Butyl Alcohol, Ceramide 3.

The AR serum contains the following ingredients: Aqua, Glycerin, Niacinamide, Panthenol, Xylitol, Nylon 6/12, 1,2-Hexanediol, Isostearyl Isostearate, Pentylene Glycol, Hydroxyacetophenone, Hydrogenated Lecithin, Acetamide MEA, Palmitamide MEA, Tocopheryl Acetate, Sodium Carbomer, Acrylates/C10-30 Alkyl Acrylate Crosspolymer.

### *In vitro* studies

*Water vapour transmission rate (WVTR) measurement and FTIR analyses methodology*

Water vapour transmission rate was used to quantitatively assess the occlusive character of the phospholipid-ISIS structured lamellar formulations with white jelly paraffin (WJP) as a positive control. The WVTR is a widely accepted method for assessing occlusive behaviour of formulations *in vitro* [14]. FTIR spectroscopy was used to determine thermotropic behaviour and the lipid organisation of the phospholipid-ISIS structured lamellar formulations using methods described in Bulsara *et al.* [23].

*Reconstructed human epidermis (RHE)*

Reconstructed human epidermis were purchased from MatTek (EpiDerm, EPI-200, MatTek, Ashland, MA, USA), maintained in culture media and incubated overnight at 37°C/5% $CO_2$ according to manufacturer's instructions. The culture media were replenished with fresh media prior to the start of the study. Formulations were applied topically at 2 mg $cm^{-2}$ or 10 mg $cm^{-2}$ as indicated. All treatments were performed in duplicate or triplicate.

*UVB exposure.* After 1 h pre-treatment with formulations the RHE tissues were transferred to a sterile 6-well plate containing 1 mL of Dulbecco's phosphate buffered saline (DPBS) per well and then exposed to ultraviolet B (UVB) irradiation. The Newport Solar Simulator System (Power unit 69920, and Lamp 91192-1000, Newport Corporate, Irvine, CA, USA) was used as the UVB emitter to achieve a UVB irradiation of 150 mJ $cm^{-2}$, as measured using an ILT-1400 Handheld Portable Radiometer/Photometer with attached UVB detector (SEL240/T2ACT5, 235–307 nm) (both from International Light Technologies, Inc., Peabody, MA, USA). Topical distilled water plus UVB irradiation served as a UVB control. After UVB irradiation the RHE tissues were transferred back to the 6-well plate containing culture media and incubated at 37°C and 5% $CO_2$ for 6 h.

*Inflammatory cocktail and topical treatments.* Reconstructed human epidermis was placed into 6-well plates overnight at 37°C and 5% $CO_2$ in the hydrocortisone-free culture media (MatTek Inc.). Dexpanthenol (75w, BASF, Newport, DE, USA) and PEA (Nikko Chemicals Co. Ltd., Tochigi, Japan) were prepared in a vehicle composed of water, ethanol, and propylene glycol (50/35/15; %v/v/v), respectively. Topical dexpanthenol or PEA (6 µL, 10 mg $cm^{-2}$) was applied to the RHE surface 1 h prior to changing to a culture media containing an inflammatory cocktail containing interleukin (IL)-4 (10 ng $mL^{-1}$), IL-13 (10 ng $mL^{-1}$), tumour necrosis factor (TNF)-$\alpha$ (5 ng $mL^{-1}$) (R&D Systems, Minneapolis, MN, USA) and poly (I:C) (10 µg $mL^{-1}$) (Sigma, St. Louis, MO, USA). Control samples were treated topically with 6 µl vehicle only. Following

treatment with the inflammatory cocktail for 24 h, culture media and tissues were collected and analysed for pro-inflammatory mediators and immunohistochemistry (IHC) staining.

*HaCaT cell culture*
HaCaT cells (AddexBio, San Diego, CA, USA) were grown in Medium DMEM/GlutMax supplemented with 10% foetal bovine serum (FBS) and non-essential amino acids (NEAA) (all Life Technologies, Carlsbad, CA, USA) at a density of $2 \times 10^5$ cells per well in a 12-well plate. HaCaT cells were treated with PEA at different concentrations (prepared by mixing PEA ethanol stock solutions with a 1 mM defatted bovine serum albumin [BSA] solution [1:9, v/v] for better solubility of PEA) 1 h prior to UVB exposure at 40 mJ $cm^{-2}$ (Newport Solar Simulator system, Power unit 69920, and Lamp 91192–1000). After another 6 h incubation post UVB irradiation, cell culture media were collected for prostaglandin E2 ($PGE_2$) and IL-6 measurement.

*Measurement of inflammatory mediators*
Cell culture media were collected either from RHE or HaCaT cell cultures and analysed for $PGE_2$ or TSLP concentrations using ELISA assays (R&D Systems, Minneapolis, MN, USA), and for IL-6 and TNF-$\alpha$ measurement using Milliplex multiplex assay (EMD Millipore, Billerica, MA, USA).

*Immunohistochemistry staining*
Reconstructed human epidermis tissues were collected and processed for IHC staining of Ki67, a proliferation biomarker, using the primary Ki67 rabbit monoclonal antibody (Vector Laboratories, Burlingame, CA, USA) and a Mach2 rabbit-alkaline phosphatase polymer (Biocare Medical, Concord, CA, USA) as the secondary antibody.

*Measurement of the effects of NAM on NAD production*
HaCaT cells were cultured in Epilife medium without NAM (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS and NEAA. After overnight culture, HaCaT cells were treated with NAM (DSM, Basel, Switzerland) at different concentrations for 3 h. NAD was measured by a colorimetric assay. In brief, cells were washed twice with DPBS containing 5 mM EDTA. Acetonitrile (ACN) lysis buffer (ammonium acetate [50 mM] and 90% acetonitrile) was added to lyse the cells at room temperature (RT) for 5–10 min. The enzyme master mix including NAD substrate GW323424X, ADP ribosyl cyclase (Sigma, St. Louis, MO, USA), and a CD38 inhibitor (GSK2880268A; GlaxoSmithKline proprietary compound) in HEPES pH7 buffer, was added to the lysed cells. The enzymatic reaction was carried out at room temperature for 30 min. Supernatants were transferred to each well of a 96-well plate for colorimetric absorbance reading at 405 nm. A mixture of enzyme master mix and ACN extraction solution at 2:1 ratio was used as a blank.

*Measurement of the effects of NAM keratinocyte differentiation: RNA extraction and quantitative polymerase chain reaction (qPCR) analysis*
To assess the effect of NAM on the gene expression of keratinocyte differentiation biomarkers and peroxisome proliferator-activated receptor (PPAR)-$\alpha$, HaCaT cells were treated with NAM at 0.05% and 0.1% then harvested at Day 4 for RNA extraction and quantitative polymerase chain reaction (qPCR) analyses. Culture media and treatment were changed every second day until harvest. Total RNA was isolated from HaCaT cells using Qiagen's mini RNA isolation kit (Qiagen, Valencia, CA, USA) according to manufacturers' instructions. Total RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA). The cDNA was synthesized using Invitrogen SuperScript VILO cDNA Synthesis kit (Invitrogen, Frederick, MD, USA), followed by real-time qPCR analyses. TaqMan probes used in the study were purchased from Life Technologies (Carlsbad, CA, USA).

A one-way analysis of variance (ANOVA) with a Tukey's multiple comparison test was conducted for statistical analysis of results.

**Table I** Methodology summary for facial redness clinical study

| | |
|---|---|
| Clinical trial type | Single-centre, evaluator-blind, parallel, randomized, stratified |
| Study objective | Assessment of changes in facial redness compared to baseline |
| Main inclusion criteria | Aged 18–65 years<br>Female<br>Fitzpatrick scale I–IV<br>Self-reported sensitive facial skin<br>Facial redness associated with dry skin due to winter xerosis<br>Minimum redness and dryness grading of 2 at screening and baseline (Separate clinical grading of redness and dryness was performed) |
| Main study restrictions | No use of prohibited medications<br>No use of certain skin care products on test areas other than those provided<br>Continue use of normal make up, excluding moisturizing foundations<br>No hard physical exercise with sweating, sauna, or swimming within 24 h of a clinic visit<br>No exposure to UV light, or cosmetic procedures on the test area<br>No prolonged sun exposure. In the event of unavoidable prolonged UV exposure, participants were provided with a non-moisturizing sunscreen product. |
| Location | Thomas J Stephens & Associates, Inc., Colorado Research Center, USA |
| Assessment and criteria used | Trained examiner assessments of redness and dryness.<br>Dryness was only evaluated at baseline as part of the inclusion criteria. |
| Assessment room conditions | Prior to assessment, participants were acclimatized for 30 minutes at room temperature 20°C ± 2°C, humidity 50% ± 10% |
| Treatments | Participants were stratified by baseline redness score<br>Participants applied day cream or serum to their face twice daily for 28 days. Trained examiner visual grading of facial redness and dryness was conducted at baseline and then facial redness only at Day 15 and Day 29 |

Fitzpatrick grading: I = burns easily, never tans; II = burns easily, tans minimally; III = burns moderately; IV = tans moderately.
UV, ultraviolet.

## Clinical trials – AR day cream and AR serum

Details on methods used for the key facial redness clinical study are summarized in Table I.

Details on study design, objectives, inclusion/exclusion criteria, and assessments for the other six studies are summarized in Table S1.

The studies were conducted in accordance with the International Council for Harmonisation (ICH) Guidelines for Good Clinical Practice and the Declaration of Helsinki. All participants provided written informed consent prior to study commencement.

**Table II** Visual grading scales for trained examiner assessment of facial dryness and erythema at baseline. Facial redness was only assessed during the intervention study

| Grade | Dryness | Erythema |
|---|---|---|
| 0 | None: no dryness or scaling of the treatment area | 0 None: no erythema of the treatment area |
| 1 | Mild: slight, but definite roughness of the treatment area | 1 Mild: slight, but definite redness of the treatment area |
| 2 | Moderate: definite roughness of the treatment area | 2 Moderate: definite redness of the treatment area |
| 3 | Severe: marked roughness of the treatment area | 3 Severe: marked redness of the treatment area |

Anonymized individual participant data and study documents can be requested for further research from www.clinicalstudydata request.com.

The International Contact Dermatitis Research Group (ICDRG) criteria for irritant and allergic reactions [24] were used to classify participants' reactions. The studies also used a modification of the Draize and Kligman scale for assessment of reactions and their intensity [25,26], and a classification scheme for sensations that were self-reported by participants.

*Facial redness study*

*Study design.* This was a single-centre, evaluator-blind parallel-group, randomized study conducted in Colorado, USA. Subjects were recruited with a minimal grade of 2 for both xerosis and redness at baseline, thereafter only redness was graded in the intervention study. The stratified study (by baseline redness score; stratum 1: redness score of 2; stratum 2: redness score of 2.5 or 3) assessed the effect of the AR day cream and AR serum on moderate facial redness in participants with dry, red skin due to winter xerosis. The study included two test treatment groups (AR day cream, AR serum). The study design and assessments are detailed in Table I; the visual assessment scales for dryness and erythema are shown in Table II.

*Study objectives.* The primary objective was to evaluate the change from baseline in facial redness at Day 29 for participants who used the AR day cream. The secondary objective was to evaluate the change from baseline in facial redness at Day 15 for participants who used the AR day cream, and at Day 15 and Day 29 for those who used the AR serum.

*Adverse events.* Adverse events (AEs) were graded on a three-point scale: mild = easily tolerated, moderate = sufficiently discomforting, or severe = any event that prevents normal everyday activities.

*Statistics.* Analysis of covariance (ANCOVA) was performed to assess both the primary and secondary endpoints. All tests were performed at the two-sided 5% significance level.

Randomization was stratified according to baseline redness score (stratum 1: redness score of 2; stratum 2: redness score of 2.5 or 3). Within each stratum, participants were randomly assigned to receive one of the two test products in a 1 : 1 allocation ratio using the method of randomly permuted blocks with a block size of 4. The randomization scheme was generated by a computer program. The sample size was calculated such that a study with 29 evaluable participants in each treatment group would have 90% power to detect a change from baseline of 0.5 units in redness at the two-sided 5% significance level, assuming the standard deviation of change from baseline was 0.8.

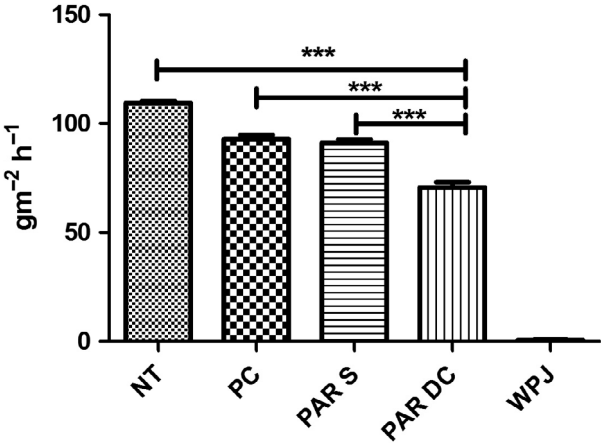



**Figure 1** WVTR of controls (NT, WPJ: 100%) and formulations (PC, PAR S, PAR DC): ($n$ = 3). ***$P < 0.001$. NT, no treatment; PAR DC, Physiogel anti-redness day cream; PAR S, Physiogel anti-redness serum; PC, Physiogel cream; WPJ, white petroleum jelly; WVTR, water vapour transmission rate.

## Results

### *In vitro* studies

*Effect of phospholipid-ISIS structured lamellar formulations on water vapour transport*

All formulations decreased WVTR values compared with no treatment ($P < 0.001$; Figure 1). The phospholipid-ISIS structured lamellar AR serum was numerically better but not significantly different to the marketed phospholipid cream. Although the marketed phospholipid cream was highly effective in lowering WVTR, the new phospholipid-ISIS structured lamellar AR day cream was more effective ($P < 0.001$). The AR day cream was also more effective than the AR serum ($P < 0.001$).

*Thermotropic behaviour of phospholipid-ISIS structured lamellar formulations studied by FTIR*

All formulations exhibited a high degree of conformational order in the lipids (Figure 2). However, the melting temperature ($T_m$) values (indicative of loss of lipid chain order) increased in descending order Physiogel® cream (45°C) > AR serum (55°C) > AR day cream (70°C). These increases relate to the formulation improvements and the presence of the phospholipid-ISIS.

*Inhibitory effect of panthenol on prostaglandin $E_2$ ($PGE_2$) release induced by an inflammatory mediator cocktail in RHE*

Exposure to the inflammatory cocktail led to significantly increased release of inflammatory mediators from RHE tissues; secretion of $PGE_2$ was significantly increased ($P < 0.001$). Topical treatment with panthenol markedly decreased cocktail-induced $PGE_2$ secretion

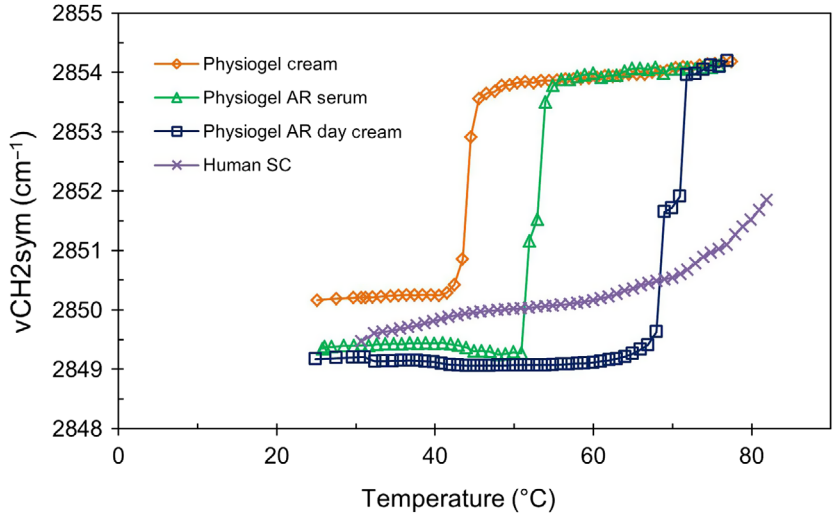



**Figure 2** Thermotropic methylene symmetric stretch (*v*CH2 conformational order) profiles for PC, PAR S and PAR DC ($n$ = 3), showing ~Tm values of 45, 55, and 70°C, respectively. AR, anti-redness; PC, Physiogel cream; PAR S, Physiogel anti-redness serum; PAR DC, Physiogel anti-redness day cream; SC, stratum corneum.

($P < 0.001$; Figure 3). IHC staining of Ki67, a proliferation biomarker, revealed a significant reduction of cell proliferation, spongiosis and tissue damage by the inflammatory cocktail (Figure 4a and b). Topical treatment with panthenol mitigated the tissue damage caused by the inflammatory cocktail and retained relatively normal cell proliferation indicated by minimally reduced positively Ki67-

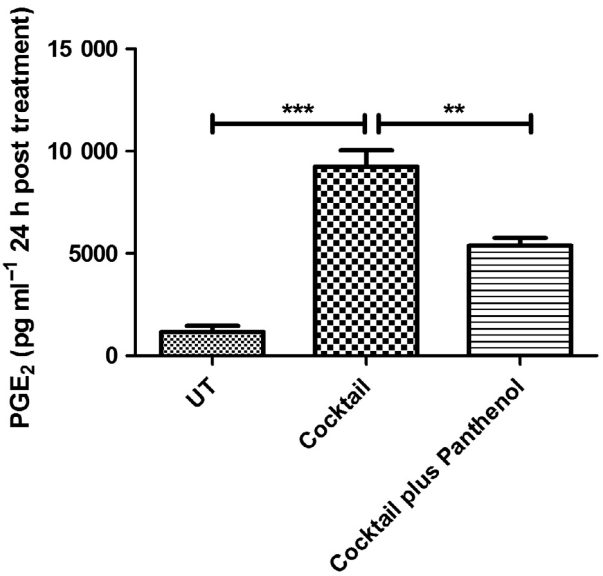



**Figure 3** Inhibitory effect of panthenol (1.4%) on $PGE_2$ release induced by an inflammatory mediator cocktail in reconstituted human epidermis. Topical dexpanthenol was applied 1 h prior to the inflammatory cocktail treatment, and $PGE_2$ release in the cell culture media was measured at 24 h post the inflammatory cocktail treatment. $^{**}P < 0.01$, $^{***}P < 0.001$. $PGE_2$, prostaglandin E2; UT, untreated.

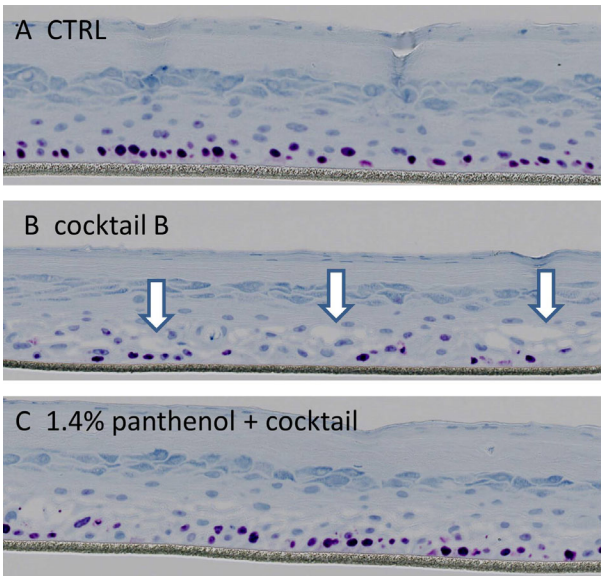



**Figure 4** Panthenol (1.4%) mitigated the detrimental impact of the inflammatory cocktail on tissue integrity and cell proliferation in reconstructed human epidermis. Topical treatment of dexpanthenol was applied 1 h prior to the inflammatory cocktail treatment in cell culture media. IHC staining of Ki67 was performed 24 h after post-cocktail treatment to determine the cell proliferation and tissue integrity. (A). RHE tissues were treated with culture media without the inflammatory cocktail. (B & C). RHE tissues were treated with an inflammatory cocktail without topical panthenol (B) and with topical panthenol (C). Topical panthenol treatment at 1.4% mitigated the tissue damage (as shown in arrows) caused by the inflammatory cocktail and retained relatively normal cell proliferation as shown by minimally reduced cell numbers with positive Ki67 staining (nuclear staining in purple color). IHC, immunohistochemistry; RHE, reconstructed human epidermis.

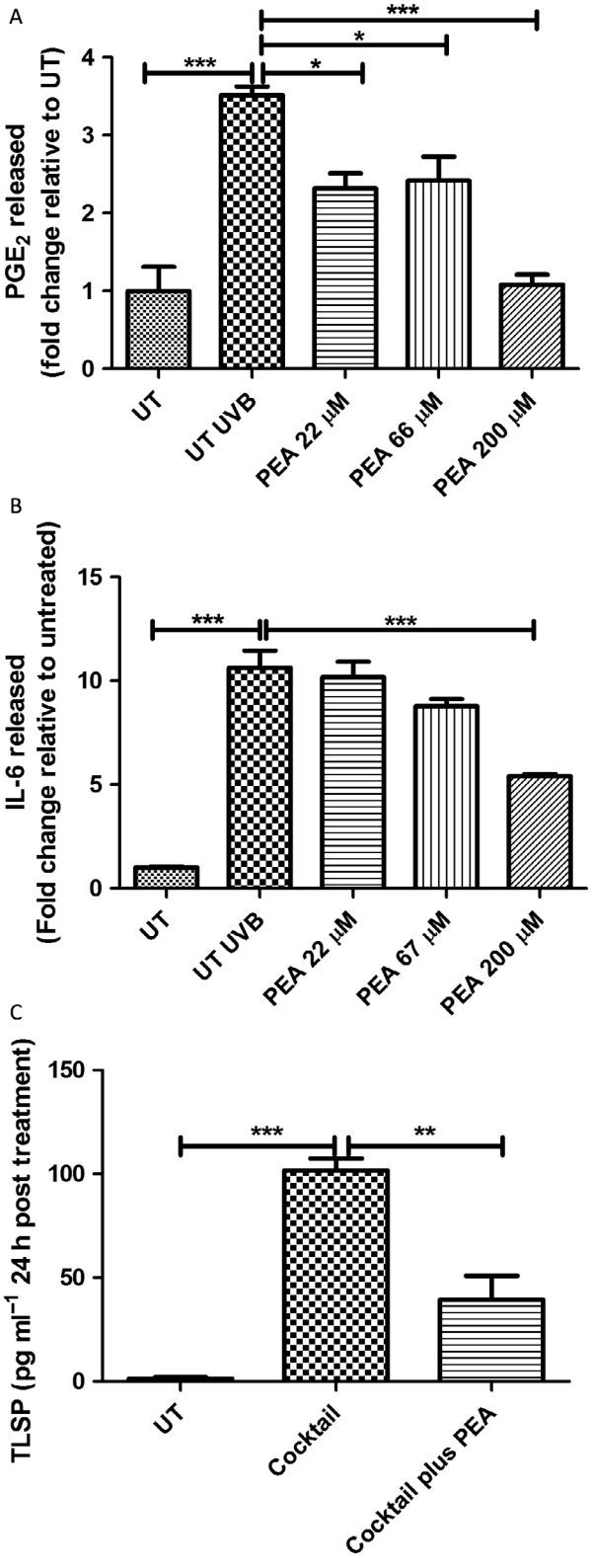



**Figure 5** Anti-inflammatory effect of PEA. (a) and (b) PEA inhibited UVB-induced inflammation in HaCaT cells. HaCaT cells were treated with PEA in the culture media 1 h prior to UVB irradiation at 40 mJ $cm^{-2}$ and culture media were collected at 6 h post-UVB for the measurement of $PGE_2$ and IL-6. PEA reduced UVB-induced $PGE_2$ release after UVB irradiation ($n$ = 3) (Figure 5a) (22 μM, $^{*}P < 0.05$; 66 μM, $^{*}P < 0.05$; 200 μM, $^{***}P < 0.001$) and reduced UVB-induced IL-6 release (Figure 5b) (200 μM, $^{***}P < 0.001$). (c) PEA reduced inflammatory cocktail-induced TSLP in reconstructed human epidermis. RHE was treated with culture media with or without an inflammatory cocktail. Topical PEA treatment at 0.3% applied 1 h prior to the inflammatory cocktail reduced cocktail-induced TSLP at 24 h post-cocktail treatment ($n$ = 3) ($^{**}P < 0.01$). IL-6, interleukin-6; PEA, palmitoylethanolamide; $PGE_2$, prostaglandin E2; RHE, reconstructed human epidermis; TSLP, thymic stromal lymphopoietin; UT, untreated.

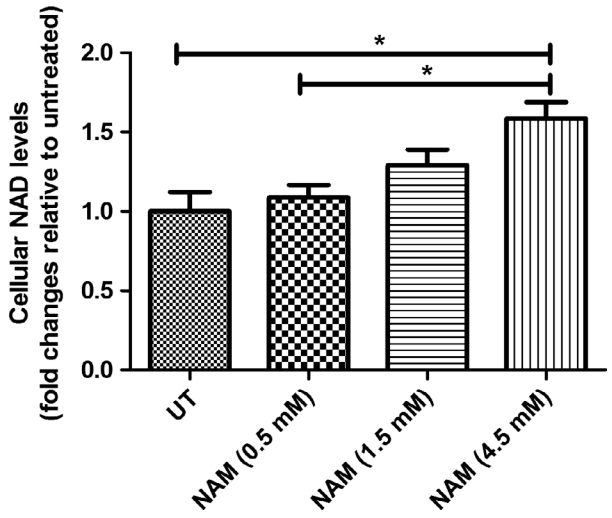



**Figure 6** NAM dose-dependently increased cellular NAD in HaCaT cells. HaCaT cells were treated with NAM in the culture media without NAM. Cellular NAD levels were measured at 3 h post treatment. NAM dose-dependently increased cellular NAD levels in HaCaT cells ($n$ = 3) ($^{*}P < 0.05$ at 4.5 mM). NAD, nicotinamide adenine dinucleotide; NAM, niacinamide; UT, untreated.

stained cell numbers compared with the control sample (Figure 4C).

*Inhibitory effect of PEA on UVB-induced inflammation in HaCaT cells and inflammatory cocktail-induced TSLP in RHE*

HaCaT keratinocytes were treated with UVB at 40 mJ $cm^{-2}$ with and without pre-treatment with PEA at different concentrations. UVB significantly increased secretion of pro-inflammatory mediators such as $PGE_2$ and IL-6 from HaCaT cells in the culture media ($P < 0.001$; Figure 5a and b). Pre-treatment with PEA dose-dependently decreased UVB-induced $PGE_2$ (Figure 5a; 22 $\mu$M, $P < 0.05$; 66 μM, $P < 0.05$; 200 μM, $P < 0.001$) and IL-6 (Figure 5b; 200 μM, $P < 0.001$).

The potential anti-inflammatory and anti-itch activity of PEA was further examined in an RHE model in which TSLP production is increased by an inflammatory cocktail. As a central inflammatory mediator and a pruritogenic molecule, TSLP has been shown

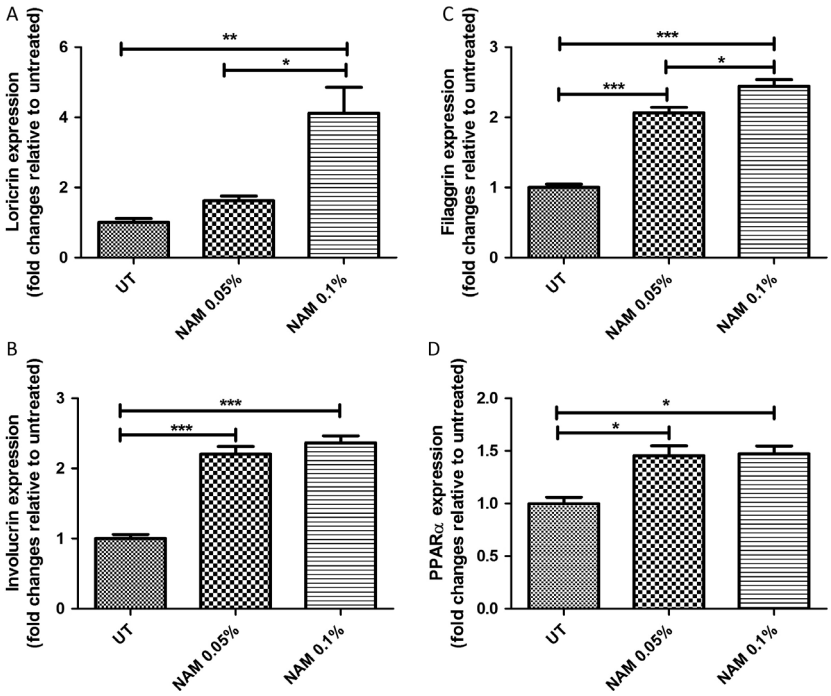



**Figure 7** NAM increased gene expressions of epidermal differentiation markers and PPARα in HaCaT cells. HaCaT cells were treated with NAM and harvested at Day 4 for qPCR analyses of epidermal differentiation biomarkers and PPAR-α. NAM increased gene expression of loricrin ($n = 3$) (a) (0.05%, $^{*}P < 0.05$; 0.1%, $^{**}P < 0.01$); involucrin (b) ($^{***}P < 0.001$ at both concentrations); filaggrin (C) ($^{***}P < 0.001$ at both concentrations and $^{*}P < 0.05$ between two niacinamide concentrations); and PPAR-α (D) ($^{*}P < 0.05$ at both concentrations). NAM, niacinamide; PPAR-α, peroxisome proliferator activated receptor alpha; UT, untreated.

to be involved in the initiation, development, and progression of atopic dermatitis in mice and humans, and plays a role in driving allergen sensitization. RHE was treated with an inflammatory cocktail prepared in the culture media with and without topical treatment with PEA. The inflammatory cocktail significantly increased TSLP release from RHE (Figure 5c, $P < 0.001$), whereas topical treatment with PEA reduced cocktail-induced TSLP (Figure 5c, $P < 0.01$).

*Effects of NAM on cellular NAD and on gene expression of epidermal differentiation markers*

Niacinamide dose-dependently increased cellular NAD in HaCaT cells cultured in a NAM-free medium, reaching significance at 4.5 mM ($P < 0.05$, Figure 6). Similar effects were shown in the regular culture medium with NAM (0.004 g $L^{-1}$, or 32.75 μM).

Niacinamide dose-dependently increased gene expression of keratinocyte differentiation biomarkers such as filaggrin (up to 2.4-fold) and loricrin (up to 4-fold), and significantly increased involucrin (up to 2.4-fold) at both concentrations tested after 4 days of treatment with NAM, as assessed by qPCR analyses (Figure 7). NAM also significantly increased PPAR-α gene expression (up to 1.5-fold), suggesting the involvement of PPAR-α in inducing keratinocyte differentiation.

*Effect of AR day cream and AR serum on UVB-induced pro-inflammatory mediators, DNA damage and apoptosis*

Anti-redness day cream and AR serum, containing panthenol, PEA, and NAM, were evaluated for their protective activity against UVB-induced damage using RHE. The exposure of RHE to UVB radiation at 150 mJ $cm^{-2}$ increased levels of IL-8, TNF-α, and $PGE_2$ compared with no radiation controls (Figure 8). Topical application of AR day cream (containing UV filters) reduced UVB-induced cytokine release of IL-8 ($P < 0.001$), TNF-α ($P < 0.001$), and $PGE_2$ ($P < 0.001$) to levels similar to untreated controls (Figure 8).

Ultraviolet B irradiation at 150 mJ $cm^{-2}$ resulted in DNA damage and apoptosis, as demonstrated by the IHC staining of cyclopyrimidine dimers (CPDs) and cleaved caspase-3 (CC3), respectively; in contrast, topical application of AR day cream blocked UVB-induced DNA damage (CPD) and apoptosis (CC3) (Figure 9a). Interestingly, application of the AR serum also reduced UVB-induced CPD but minimally reduced UVB-induced apoptosis (Figure 9b). Overnight treatment with AR serum prior to AR day cream, followed by UVB irradiation, exhibited similar UV protection to AR day cream alone (data not shown).

## Clinical studies

*Facial redness study*

Disposition of the 62 participants randomized to treatment is presented in Figure 10. Of the 59 participants included in the intention-to-treat (ITT) population and who completed the study, 44 (75%) were included in stratum 1 (redness score of 2; 22 in both the AR day cream and AR serum groups), and 15 (25%) in stratum 2 (redness score of 2.5 or 3; seven in the AR day cream group and eight in the AR serum group). All participants were female,

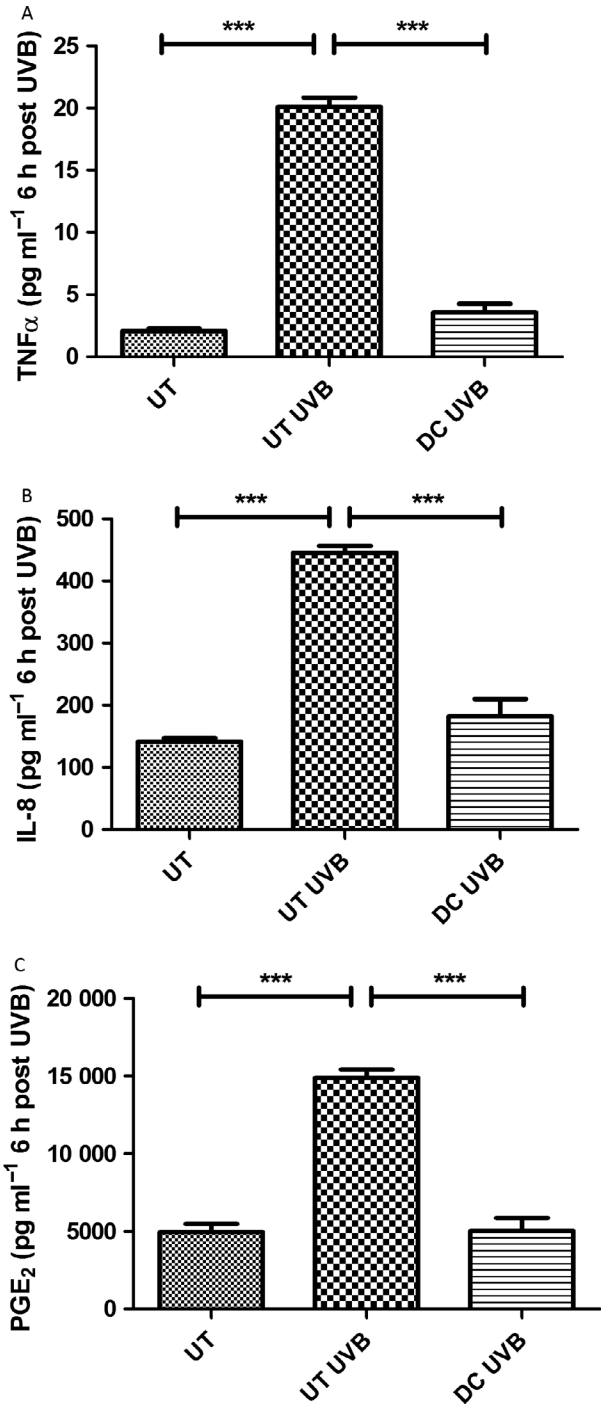



**Figure 8** AR day cream reduced UVB-induced release of TNF-α, IL-8, and $PGE_2$ from reconstructed human epidermis. AR day cream was topically applied to reconstructed human epidermis 1 h prior to UVB irradiation at 150 mJ $cm^{-2}$. UVB exposure resulted in markedly increased (a) TNF-α, (b) IL-8, and (c) $PGE_2$. AR day cream significantly reduced UVB-induced inflammatory mediators ($n = 3$) (***$P < 0.001$). AR, anti-redness; DC, day cream; IL-8, interleukin-8; $PGE_2$, prostaglandin E2; TNF-α, tumour necrosis factor alpha; UT, untreated; UVB, ultraviolet B.

with a mean age of 50.4 years (range 20–62 years) in the AR day cream group and 50.4 years (range 18–64 years) in the AR serum group (Table III). Most participants were Caucasian, and the majority had Fitzpatrick skin type II or lll (Table III).

A statistically significant mean reduction in facial redness was shown with both the AR day cream and AR serum, at Days 15 and 29 compared with baseline ($P < 0.001$) (Figure 11 and Table IV). Four AEs were recorded for four participants. Two AEs occurred in the AR day cream group (upper abdominal pain and sinusitis) and two in the AR serum group (urinary tract infection and nasopharyngitis). None were considered treatment-related and no serious AEs were reported. One participant had a change in concomitant medication during the study (nitrofurantoin for a urinary tract infection).

*Irritation and sensitisation studies*

For both studies, 235 participants were screened and 233 enrolled; 228 completed each study. Of the five withdrawals, two participants withdrew due to personal reasons unrelated to the test products and three did not receive test products. All participants were female, with a mean age of 45.1 years (range 18–70 years) and most had Fitzpatrick skin type ll or lll (Table III).

No AEs or sensations of discomfort were reported for any participant (2241 readings). The principal investigator considered that the two test products demonstrated no irritant or allergenic potential.

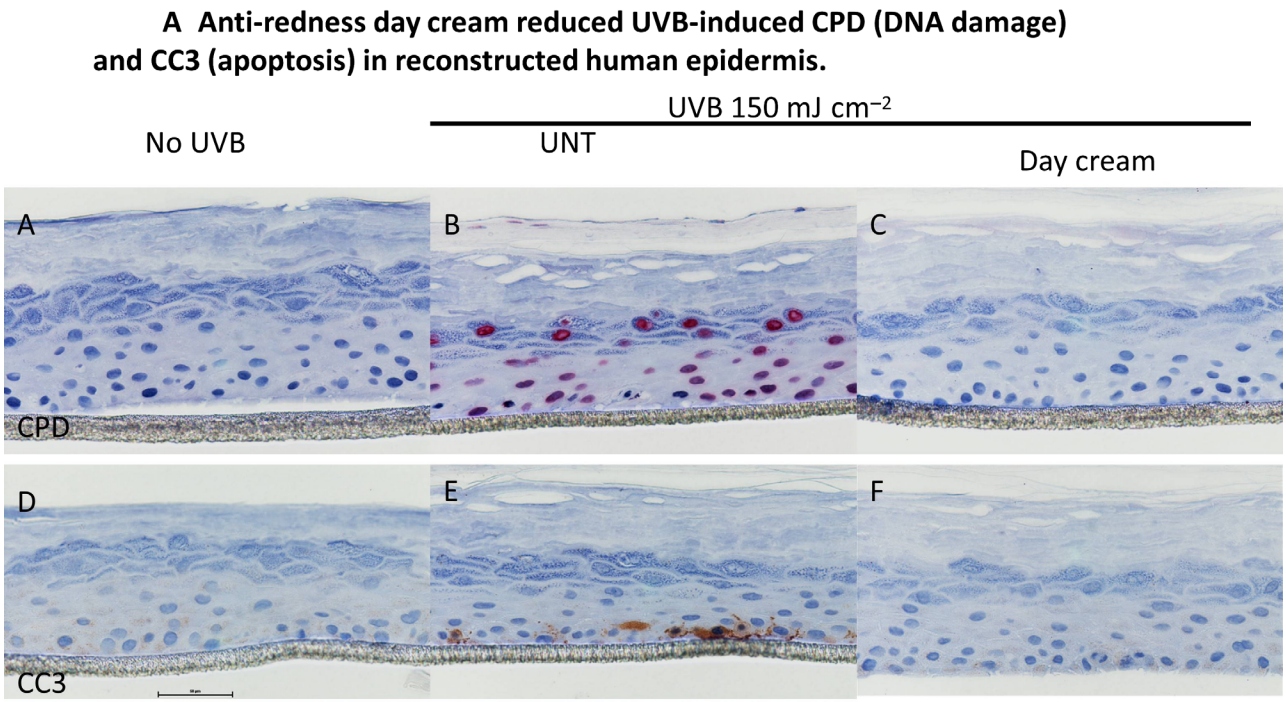
*Studies in subjects with sensitive skin*

Of the 42 participants screened for the AR day cream study, seven were excluded; five presented with a phototype of V, one had a history of allergy to cosmetic products, and one did not have sensitive skin (negative Stinging Test). Of the 35 participants enrolled, 34 completed the study; one left the study for personal reasons (not related to the test product). All participants were female, with a mean age of 42.7 years (range 19–60 years) and most had Fitzpatrick skin type ll, lll, or IV (Table III). None of the 34 participants experienced adverse reactions. During the final dermatological assessment, one participant reported that they had experienced discomfort/mild itching on the periocular area on two consecutive days after application of the AR day cream. The participant reported spontaneous remission of the symptoms within 5 min on both occasions, and no clinical signs were observed on dermatological assessment. Although the sensation was probably related to the test product, the incident was not deemed by the investigator to be relevant for inclusion in the safety analyses since it occurred 2 days after the start of product application in only one participant.

Of the 37 participants enrolled in the AR serum study, two were excluded (negative Stinging Test). All participants were female, with a mean age of 42.3 years (range 21–59 years) and most had Fitzpatrick skin type ll, lll, or IV (Table III). None of the 35 participants who completed the study reported skin lesions, adverse reactions or feelings of discomfort with the AR serum. No changes in concomitant medication were reported during the study.

*Photo-irritation and photo-sensitization studies*

For both photo-toxicity studies, 27 participants were enrolled and 26 completed the studies; one participant was excluded during the screening stage. One participant did not return after Day 29, so their data were excluded from analysis. Most participants (81%) were female, with a mean age of 46.8 years (range 21–67 years),



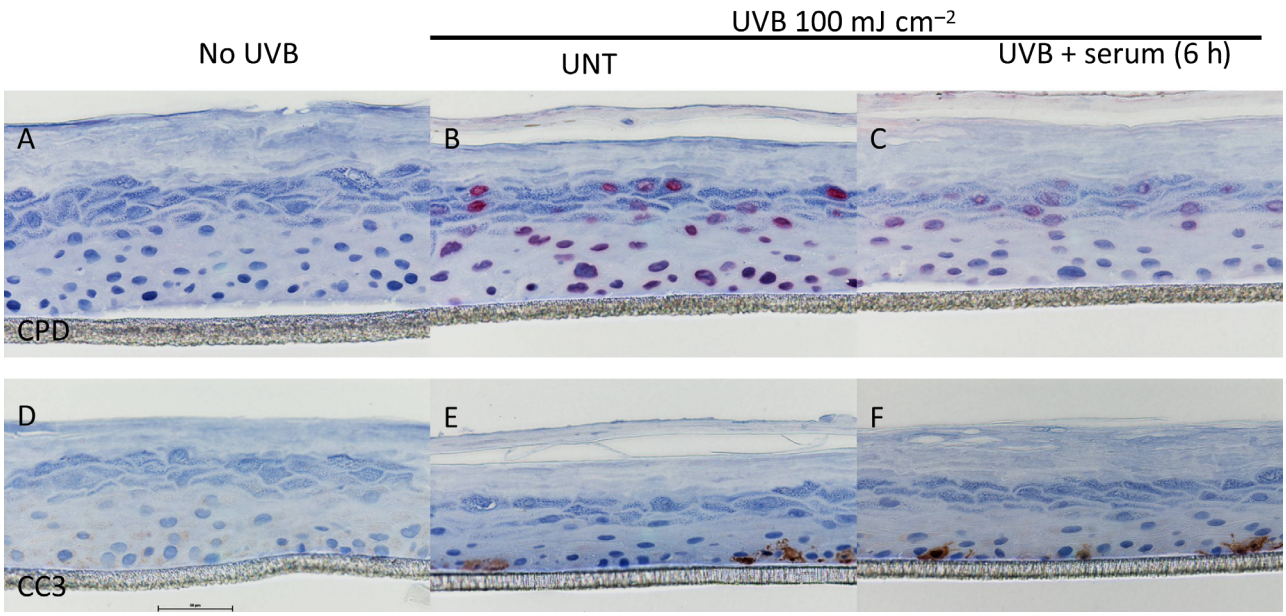




**Figure 9** AR day cream and AR serum reduced UVB-induced CPD (DNA damage) and CC3 (apoptosis). A: AR day cream or water mock treatment (UNT) was topically applied to reconstructed human epidermis at 1 h prior to UVB irradiation (150 mJ $cm^{-2}$). Tissues were collected at 6 h post-UVB for IHC staining of CPD (DNA damage, pink nuclear staining) and CC3 (apoptosis, brown cytosol staining). AR day cream blocked UVB-induced CPD (pink nuclear staining) (c) and CC3 (brown cytosol staining) (f), compared with UVB control (b&e). No UVB treatment was served as a negative control (a&d). B: AR serum or water mock treatment (UNT) was topically applied to reconstructed human epidermis at 1 h prior to UVB irradiation (100 mJ $cm^{-2}$). AR serum reduced UVB-induced CPD (c) compared with UVB alone (b), but minimally affected UVB-induced CC3 (compare e [serum] to f [UVB alone]). AR, anti-redness; CC3, cleaved caspase-3; CPD, cyclopyrimidine dimers; IHC, immunohistochemistry; UNT, untreated; UVB, ultraviolet B.

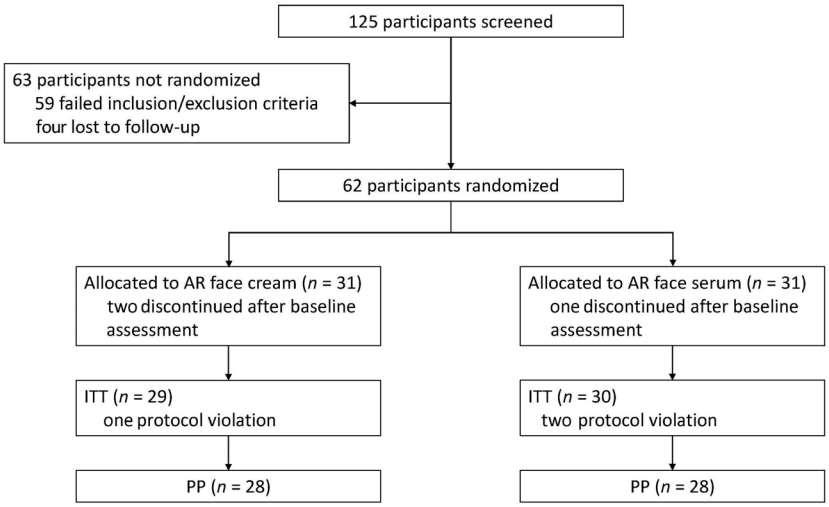



**Figure 10** CONSORT flow diagram for the anti-redness study. AR, anti-redness; ITT, intention-to-treat; PP, per protocol.

**Table III** Baseline demographics of participants included in the clinical studies

| | Facial redness study | | Assessment of irritation and sensitization HRIPT studies on day cream and serum | Effect of AR face cream and serum on subjects with sensitive skin | | Phototoxicity/photoallergy studies for day cream and serum |
|---|---|---|---|---|---|---|
| | Day Cream | Serum | | Day cream | Serum | |
| Enrolled participants (*n*) | 29 | 30 | 233 (both studies) | 35 | 35 | 27 (both studies) |
| Evaluable participants (*n*) | 29 | 30 | 228 (both studies) | 34 | 35 | 26 (both studies) |
| Mean age, years (range) | 50.4 (20–62) | 50.4 (18–64) | 45.1 (18–70) | 42.7 (19–60) | 42.3 (21–59) | 46.8 (21–67) |
| Female, *n* (%) | 29 (100) | 30 (100) | 202 (87) | 34 (100) | 35 (100) | 22 (82) |
| Race/ethnicity, *n* (%) | | | Not available | Not available | Not available | Not available |
| Caucasian | 26 (97) | 30 | | | | |
| Asian | 1 (3) | (100) | | | | |
| Fitzpatrick grade, *n* (%) | | | | | | |
| I | 0 | 0 | 8 (3) | 2 (6) | 2 (6) | 0 |
| II | 16 (55) | 14 (47) | 117 (50) | 12 (34) | 11 (31) | 6 (22) |
| III | 11 (38) | 16 (53) | 75 (32) | 11 (31) | 16 (46) | 22 (78) |
| IV | 2 (7) | 0 | 33 (14) | 10 (29) | 6 (17) | 0 |

Fitzpatrick grading: I = burns easily, never tans; II = burns easily, tans minimally; III = burns moderately; IV = tans moderately.
AR, anti-redness; HRIPT, human repeated insult patch test.

and all had Fitzpatrick skin type ll or lll (Table III). None of the participants experienced a skin reaction, photo-irritation, or photo-sensitization. No changes were reported in concomitant medication during the study.

## Discussion

The biochemistry and cellular biology of sensitive skin is complex, therefore research on topical formulations and ingredients that specifically target the problems associated with sensitive skin is limited, with very few published reports on improving facial redness associated with sensitive skin (or rosacea as an extreme example). There is limited published data describing the effect of topical serums on sensitive skin. Clearly sunscreen use will reduce skin erythema [9,27,28] but many plant extracts and oils/acids as well as beta glucans, sodium hyaluronate, glycerine, 4-t-butylcyclohexanol, tranexamic acid, oxyresveratrol, glutathione disulphide, licochalcone A and niacinamide are also known to reduce skin redness [29–38].

We sought to improve SC moisturization using novel semi-occlusive barrier mimetic lipids [11–14] together with humectants [17] and to mitigate skin redness using this moisturizer formulation supplemented with the anti-inflammatory agents panthenol, PEA and NAM. The latter was also added for their likely effects on improving keratinocyte differentiation and also boosting cellular NAD levels with NAM. Formulations were also supplemented with UV filters where appropriate to prevent UV-induced inflammation by blocking UV radiation directly.

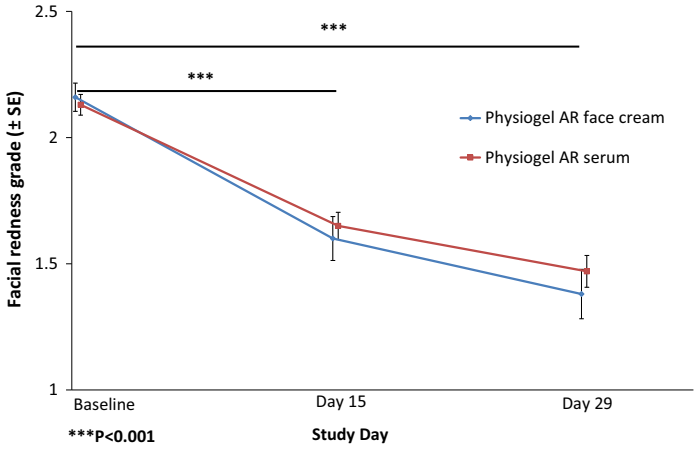



**Figure 11** Effect of AR day cream and AR serum on facial redness associated with winter xerosis in subjects with self-perceived sensitive skin. Note significant improvements for both products from baseline at Day 15 and Day 29 (***$P < 0.001$). AR, anti-redness; SE, standard error.

**Table IV** Summary of facial redness visual assessment (ITT population)

| | | Face cream | Face serum |
|---|---|---|---|
| | ***n*** | **29** | **30** |
| **Baseline** | **Mean (SD)** | **2.16 (0.300)** | **2.13 (0.23)** |
| Change from baseline | | | |
| Day 15 | *n* | 29 | 30 |
| | Adjusted mean (95% CI) | −0.55 (−0.67, −0.43) | |
| | *P* value | <0.001 | |
| Day 29 | N | 29 | 30 |
| | Adjusted mean (95% CI) | −0.78 (−0.92, −0.62) | −0.67 (−0.82, −0.52) |
| | *P* value | <0.001 | <0.001 |

CI, confidence interval; ITT, intention-to-treat; SD, standard deviation.

Isostearyl isostearate is a lipid ester that can influence SC lipid phase behaviour and computerized molecular dynamic simulations predict that it can condense the chain packing of phospholipid-lipid mixtures [15]. We therefore evaluated phospholipid-ISIS-structured lamellar formulations for their effects on WVTR. Compared with non-ISIS formulated emulsions, the new AR face cream was superior in its WVTR efficacy while the AR serum was equivalent. The former product was also superior in its WVTR performance compared to some pseudo-ceramide emulsions which have proven to be effective on sensitive skin (data not shown). FTIR studies corroborated the performance of the new phospholipid-ISIS structured emulsions by showing the AR day cream to have the most ordered lipid structure.

Panthenol is known to improve epidermal differentiation and to be an effective anti-inflammatory agent [18,39]. In these studies we demonstrated the effects of panthenol on reducing $PGE_2$ levels and tissue damage induced by a strong inflammatory cocktail in an *in vitro* model. This is relevant to the clinical setting as $PGE_2$ is known to be elevated in subjects with sensitive skin [6]. PEA also proved effective at reducing the levels of $PGE_2$ and IL-6 *in vitro*, both of which are UV-induced inflammatory biomarkers. In addition, PEA reduced the levels of TSLP, an inflammatory and pruritogenic biomarker, consistent with its reported itch-relief activity. PEA is also a putative PPAR-α agonist that may mitigate irritation via this mechanism also [19].

Niacinamide was shown to enhance the expression of epidermal differentiation genes and increase cellular NAD. The former result is contradictory to that of Blander *et al.* [40] and may reflect different testing methods, but the latter result is consistent with Rovito [21]. Moreover, topical NADH, the reduced form of beta-nicotinamide adenine dinucleotide, is reported to be effective in the treatment of rosacea and contact dermatitis [41]. Furthermore, the current work also demonstrated NAM-induced PPAR-α gene expression which is known to play an important role in regulating epidermal differentiation and reducing UV-induced erythema [42]. Although speculative at this stage, it is likely that NAM may synergise with PEA in this mechanism.

The tested formulations were shown to prevent the UV induction of inflammatory mediators as well as reduce UVB-induced DNA damage (cyclobutene dimers) and apoptosis (caspase-3). This efficacy partly results from the UV filters in the AR day cream. As the AR serum (which does not contain UV filters) also partially prevents DNA damage this highlights the beneficial effects of the ingredients described above. The *in vitro* findings indicate the potential combination of mechanisms by which the current formulations reduce skin redness and UV-induced inflammation as described in the clinical results. As the AR serum reduced the UV effects on DNA damage markers we chose not to examine inflammatory mediators.

Visual assessment data from the facial redness study showed a significant improvement in facial redness from baseline for both the AR day cream and AR serum. The level of efficacy was similar to that of products tested on subjects with rosacea [38]. Descriptive statistics for the facial redness score were presented by stratum (stratum 1: redness score of 2; stratum 2: redness score of 2.5 or 3). Although the number of subjects with a baseline redness score of 2.5 or 3 is relatively small (15 subjects), the reductions from baseline in redness at Day 29 was greater (AR day cream mean (SD) −0.86 (0.627); AR serum mean (SD) −0.88 (0.231) compared to

subjects with a baseline score of 2 (AR day cream mean (SD) −0.75 (0.401); AR serum mean (SD) −0.59 (0.366). As a result, it was valuable to stratify for baseline redness score.

The Human Repeat Insult Patch Test studies included many participants to assess the irritant/allergenic potential of the AR day cream and AR serum using repeated patch application over 3 weeks. After continuous occlusive exposure over a period of 3 weeks, neither the AR day cream nor AR serum showed irritant or allergenic potential in any participant, indicating that the products are unlikely to produce irritation under normal conditions of use.

Two clinical studies evaluated the potential of the AR day cream and AR serum to cause adverse reactions or discomfort under normal conditions of use in patients with sensitive skin. No adverse reactions or sensations of discomfort were reported by any participant and no clinical signs were observed by the dermatologist. The AR day cream and AR serum were considered by the investigator to be safe for topical use in subjects with sensitive skin. Data from two further studies using the Phototest assay showed that neither product had potential for photoirritation or photosensitization.

Data from all seven clinical studies ($n$ = 382) showed that the AR day cream and AR serum are well tolerated under normal conditions of use. Good compliance with study treatment was reported and there were only six withdrawals, none of which were related to study treatment. Only four AEs were recorded, none of which were treatment related. Consequently, data from these clinical studies support the minimal irritant potential of the products and their suitability for people with sensitive skin.

A limitation of the facial redness study was that enrolment was based on participant self-diagnosis of sensitive skin. Sensitive skin is a subjective experience with diverse sensory symptoms reported (e.g. burning, itching, or stinging), which may occur with or without clinical signs (e.g. redness, dryness, or papules) [43]. Therefore, it is likely that participants with a variety of different skin types were enrolled in the facial redness study. Other studies have shown limited correlation between self-reported sensitive skin and the Stinging Test. It has also been observed that there is little correlation between sensitivity to different substances [43]. However, the primary criteria for the study was reduction of facial redness therefore facial stinging testing was not conducted.

Another potential limitation to the study is the lack of a vehicle-control or comparative product and only changes from baseline levels were assessed. It is noted, however, many studies examining improving facial redness use a similar clinical approach [9,29,33,34,36,37].

For the studies using the Phototest assay, it should be noted that detection of photosensitization can be influenced by several factors, including anatomical test site, temperature, and seasonal variation. The Phototest assay was conducted on the skin of the back rather than the face for safety reasons, and it is likely that sensitization reactions on the face may differ to those on the body due to intrinsic differences in the skin structure and biology. Every effort was made to control environmental conditions.

## Conclusion

In conclusion, the recently developed AR day cream and AR serum significantly improved skin redness in our study. Neither the AR day cream nor the AR serum showed irritant, allergenic, photo-irritation, or photo-sensitization potential. Both cosmetic products were well tolerated under normal conditions of use. Additionally, we demonstrated using *in vitro* studies that several formulation ingredients were likely to contribute to the clinical efficacy of the topical formulations: phospholipid-ISIS-structured lipids, PEA, NAM, and panthenol.

## Acknowledgements

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## Conflicts of interest

Professor AV Rawlings is a consultant to GSK.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Methodology summary

**Table S2** Classification of the sensations reported by participants in diaries of use