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ORIGINAL ARTICLE

Retinoids in the treatment of photoageing: A histological study of topical retinoid efficacy in black skin

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Abstract

Background: Photoageing describes complex cutaneous changes that occur due to chronic exposure to solar ultraviolet radiation (UVR). The 'gold standard' for the treatment of photoaged white skin is all-*trans* retinoic acid (ATRA); however, cosmetic retinol (ROL) has also proven efficacious. Recent work has identified that black skin is susceptible to photoageing, characterized by disintegration of fibrillin-rich microfibrils (FRMs) at the dermal–epidermal junction (DEJ). However, the impact of topical retinoids for repair of black skin has not been well investigated.

Objectives: To determine the potential of retinoids to repair photoaged black skin.

Methods: An exploratory intervention study was performed using an in vivo, short-term patch test protocol. Healthy but photoaged black volunteers (>45 years) were recruited to the study, and participant extensor forearms were occluded with either 0.025% ATRA ($n = 6$; 4-day application due to irritancy) or ROL (12-day treatment protocol for a cosmetic) at concentrations of 0.3% ($n = 6$) or 1% ($n = 6$). Punch biopsies from occluded but untreated control sites and retinoid-treated sites were processed for histological analyses of epidermal characteristics, melanin distribution and dermal remodelling.

Results: Treatment with ATRA and ROL induced significant acanthosis (all $p < 0.001$) accompanied by a significant increase in keratinocyte proliferation (Ki67; all $p < 0.01$), dispersal of epidermal melanin and restoration of the FRMs at the DEJ (all $p < 0.01$), compared to untreated control.

Conclusions: This study confirms that topical ATRA has utility for the repair of photoaged black skin and that ROL induces comparable effects on epidermal and dermal remodelling, albeit over a longer timeframe. The effects of topical retinoids on black photoaged skin are similar to those reported for white photoaged skin and suggest conserved biology in relation to repair of UVR-induced damage. Further investigation of topical retinoid efficacy in daily use is warranted for black skin.

INTRODUCTION

Skin is subject to many potentially damaging factors leading to an aged appearance; chronic sun exposure is one such factor and is termed photoageing.[1] To date, the resultant phenotypic changes that arise as a consequence of chronic sun exposure have predominantly been described for white Northern European skin types, where hypertrophic photoaged skin is rough, dyspigmented and exhibits both fine and deep wrinkles.[2–4] Histological examination of white photoaged skin further reveals profound changes to the dermal extracellular matrix (ECM), with solar elastosis, the deposition of dystrophic elastic fibres in the dermis, being a prominent feature.[1] Adjacent to the dermal–epidermal junction

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(DEJ), there is a marked decrease of oxytalan fibres, forming a Grenz zone in the papillary dermis[5] and severe depletion of the fibrillin-rich microfibrils (FRM).[6] In highly pigmented black skin, photoageing manifests at a significantly slower rate and with less coarse wrinkling and laxity than is apparent in white skin.[7] However, at the histological level many similarities between photoaged black skin and white skin are apparent; both display near complete effacement of rete ridges and flattening of the DEJ, there is considerable epidermal thinning and a marked loss of FRM architecture in the papillary dermis, proximal to the DEJ.[8] In contrast, unlike white skin, black photoaged skin does not present with solar elastosis.[8]

Topical all-*trans* retinoic acid (ATRA) is used as the evidence-based 'gold standard' for the treatment of white photoaged skin.[9] Numerous studies have shown the reparative effects of topical application of ATRA, which includes the partial restoration of collagens I[10] and VII[11] and restoration of the FRM network.[12] These early studies demonstrating the efficacy of ATRA for topical skin rejuvenation were pioneering and have given rise to the widespread inclusion of cosmeceutical retinoid compounds in today's skincare products.[13] For example, retinol (ROL) is widely used in cosmeceutical products because it is not classed as a pharmaceutical drug and is thought to be better tolerated than ATRA[14,15] although local irritation including mild erythema and peeling can still occur.[16] Furthermore, there is evidence that many of the clinical and biological effects of ROL on the skin mirror those of ATRA, including improvement of skin texture, rhytids and dyschromia, regulation of keratinocyte growth and differentiation, epidermal hyperplasia, compaction of the *stratum corneum*, collagen synthesis, reduced matrix metalloproteinase (MMP) activity and deposition of FRMs.[14,17–21]

Despite the plethora of evidence for the benefits of topical retinoid treatment for photoaged white skin,[22] their efficacy for ECM repair and remodelling in black skin has not yet been established. Here, we conducted an exploratory study using the Manchester Patch Test (MPT) assay,[12] an in vivo protocol that mimics the effects of longer-term topical application, to study the effectiveness of ATRA and two concentrations of ROL (0.3% and 1%) to remodel and repair photoaged black skin.

MATERIALS AND METHODS

Participants

Participants were screened and enrolled at the Johns Hopkins Outpatient Centre, Baltimore, Maryland, USA. The inclusion criteria included African American ancestry and willingness to stop topical medication use on the forearm 2 weeks prior to study. Exclusion criteria included history of topical retinoid use within 1 year of enrolment, any skin disease that would impair evaluation of the test sites, history of use of experimental drug or experimental device in the 30 days prior to entry into the study, known allergy to any of the product ingredients, history of keloid scars, tattoos on the test sites and individuals on anti-coagulant therapies. Healthy, black African American volunteers (Fitzpatrick skin phototype V-VI; mean age ± SD: 55.4 ± 10.7 years; men: $n = 8$; women: $n = 10$) were recruited to the study. The clinical severity of photoageing was assessed using a 9-point photonumeric scale,[23] and volunteers were eligible if their photoageing grade was ≥6. Local ethical approval was obtained from The Johns Hopkins Institutional Review Board (IRB00228969). Written informed consent was obtained from the participants and the study adhered to Declaration of Helsinki principles.

In vivo Manchester patch test

Using the MPT assay, ATRA (0.025%; Retin-A® cream; Janssen-Cilag Ltd, Beerse, Belgium; 20 µL; $n = 6$) or ROL formulations consisting of either 0.3% (20 µL; $n = 6$) or 1% ROL (20 µL; $n = 6$) in a simple oil-in-water emulsion (water, glycerin, butylene glycol, dimethicone and preservative) were applied to the extensor aspect of the forearm under standard 6 mm Finn chamber occlusion. In addition, an area was left untreated but occluded to provide a control sample. For volunteers treated with ATRA, patches were left in situ for 4 days to avoid potential complications of irritancy caused by extended occlusion, whereas ROL treatment lasted 12 days, with reapplication of ROL at Days 4 and 8. At the end of the treatment period, 3 mm punch biopsies were obtained under 1% lignocaine local anaesthesia. Skin biopsies were embedded in optimal cutting temperature compound (Tissue-Tek®; Miles Laboratories, Indiana, USA), and 10 µm cryosections were prepared.

Colorimetric measurement of skin and individual typology angle determination

A Chroma Meter (CR-400; Konica Minolta Sensing Americas, Inc, New Jersey, USA) was used to measure the L^* and b^* parameters of the standard CIE $L^*a^*b^*$ colour space on the extensor forearm to assess whether any pigmentary changes had occurred as a result of retinoid treatment. Chroma Meter readings were converted to individual typology angle (ITA) values using the formula $ITA = (\arctan(L^*-50)/b^*) \times 180/\pi$. ITA values allow skin colour types to be classified into six different groups as previously described[24]: very light: >55°; light: 41° to <55°; intermediate: 28° to <41°; tan: 10° to <28°; brown: −30° to <10°; dark: <−30.

Warthin–Starry staining for melanin content

The distribution of epidermal melanin was assessed using the modified Warthin–Starry procedure.[25] Melanin content was quantified by determination of the amount of

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Warthin–Starry staining expressed as a percentage of the total epidermal area.

Immunohistochemical staining

Immunohistochemistry was performed to identify FRM (clone 11C1.3, dilution 1:1000; Merck Life Science) and the amino pro-peptide of type I collagen (pC1; clone M58, dilution 1:1000; Merck Life Science). Cryosections were fixed in ice-cold acetone or 4% paraformaldehyde and hydrated in tris-buffered saline (TBS). Primary antibody was applied for 1 h at room temperature or 4°C overnight. Sections were washed in TBS prior to incubation in appropriate biotinylated secondary antibody (Vector Laboratories, California, USA). Antibody staining was visualized using a well-characterized immunoperoxidase reaction (VectaStain *Elite* ABC system; Vector Laboratories) using Vector SG® (Vector Laboratories) as the chromogen.

Immunofluorescence staining

Immunofluorescence was performed to identify Ki67 (catalogue # ab16667, dilution 1:500; Abcam plc, Cambridge, UK), desmoglein-1 (clone EPR20382, dilution 1:100; Abcam plc), desmocollin-1 (clone Dsc1-U100, dilution 1:100; Progen, Heidelberg, Germany), keratin-10 (clone Poly19054, dilution 1:1000; BioLegend, Inc, California, USA), keratin-14 (clone LL002, dilution 1:1000; Thermo Fisher Scientific Inc, Massachusetts, USA) and collagen VII (clone LH7.2, dilution 1:100; Merck Life Science). Primary antibody was applied and incubated at 4 °C overnight. Sections were then washed in TBS prior to incubation using the appropriate VectaFluor™ Excel Amplified DyLight® IgG Kit (Vector Laboratories) or for dual labelling, the VectaFluor™ Duet Immunofluorescence Double Labelling Kit (Vector Laboratories) was used.

Microscopy, image analysis and statistical testing

Brightfield and fluorescent images were captured using a BX53 microscope (Olympus Industrial; Southend-on-Sea, UK), and image analysis was performed using ImageJ software.[26] For the morphometric analysis of the epidermis, epidermal thickness was measured using only the inter-rete ridge regions of the epidermis, from the basement membrane to the top of the granular layer at 10 randomly selected points across each section.[27] DEJ convolution index was measured using the method described previously.[27] The staining intensity of FRM and both the abundance and continuity of the arrays extending from the DEJ to the deeper dermis was quantified independently using a well-characterized 5-point ordinal scale.[12,28] The intensity and extent of staining was assessed and scored appropriately (0 indicating no staining to 4 indicating maximal staining). Statistical analysis was performed using GraphPad Prism 9.1.2 (GraphPad Software, Inc.; La Jolla, California, USA). Categorical data were analysed using Wilcoxon matched-pairs signed rank test; continuous data were analysed using paired *t*-tests. A value of $p < 0.05$ (95% confidence level) was considered significant.

RESULTS

Topical retinoid treatment induces profound changes to the epidermis of black skin

Skin biopsies obtained from extensor forearms occluded with topical retinoid—either 0.025% ATRA or ROL (at concentrations of 0.3% or 1%)—and from untreated control sites were processed for histological assessment. Treatment with ATRA and both concentrations of ROL produced significant acanthosis (all $p < 0.001$), *stratum corneum* compaction and epidermal spongiosis, as compared to control skin (Figure 1a,b). Furthermore, as a result of retinoid treatment there was a significant doubling of keratinocyte cell layers (baseline: 4 layers; retinoid-treated: 8 layers; all $p < 0.001$) and individual keratinocytes were of significantly increased area (mean ± SEM; baseline:

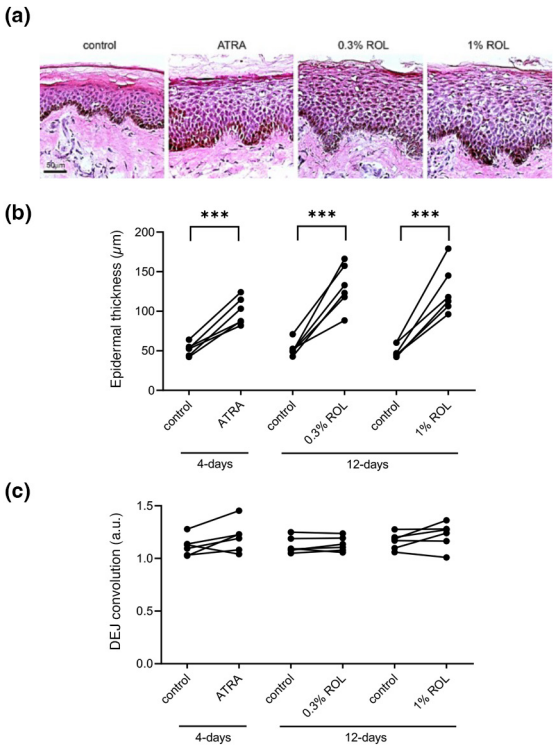

FIGURE 1 Application of topical retinoids to photoaged forearm induced significant epidermal thickening. Application of all-*trans* retinoic acid (ATRA; for 4 days) and both concentrations of retinol (ROL; for 12 days) to photoaged forearm induced significant acanthosis as compared to untreated control (a & b), yet the dermal–epidermal junction (DEJ) remained flattened (c). Scale bar = 50 μm. ***$p < 0.001$. $n = 6$ per treatment group.

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$84.37 \pm 5.89\,\mu m^2$; retinoid-treated: $115.8 \pm 3.07\,\mu m^2$ layers; $p < 0.001$). As expected for photoaged skin, the DEJ was largely flattened at control sites[8,29] and application of ATRA and ROL failed to significantly increase its convolution (Figure 1c).

Basal keratinocytes of adult epidermis express keratins 5 (K5) and K14[30,31]; however, as keratinocytes become committed to terminal differentiation, they switch expression to K1 and K10.[30,32] Therefore, we next evaluated the changes in keratinocyte behaviour that occur as a result of retinoid-induced epidermal hyperplasia. Dual immunofluorescence staining revealed significantly increased colocalization of K10 and K14 in retinoid-treated samples, as compared to control (ATRA and 1% ROL: $p < 0.01$; 0.3% ROL: $p < 0.01$; Figure 2b) and this was accompanied by a significant increase in basal keratinocyte proliferation as demonstrated by the increased number of Ki67-positive keratinocytes in retinoid-treated skin (all treatments: $p < 0.01$; Figure 2c,d).

It has previously been shown in white skin that ATRA compromises desmosome expression in the suprabasal layers of the epidermis.[33] Similarly, in black skin there was a significant reduction in suprabasal desmosome expression for desmocollin-1 (ATRA: $p < 0.05$; ROL: $p < 0.001$; Figure 3a,b) and desmoglein-1(ATRA: $p < 0.01$; ROL: $p < 0.001$; Figure 3c,d) in retinoid-treated skin, as compared to control.

Topical retinoid treatment induces epidermal melanin dispersal

Following retinoid treatment, the skin of each volunteer was also assessed for gross colourimetric changes by

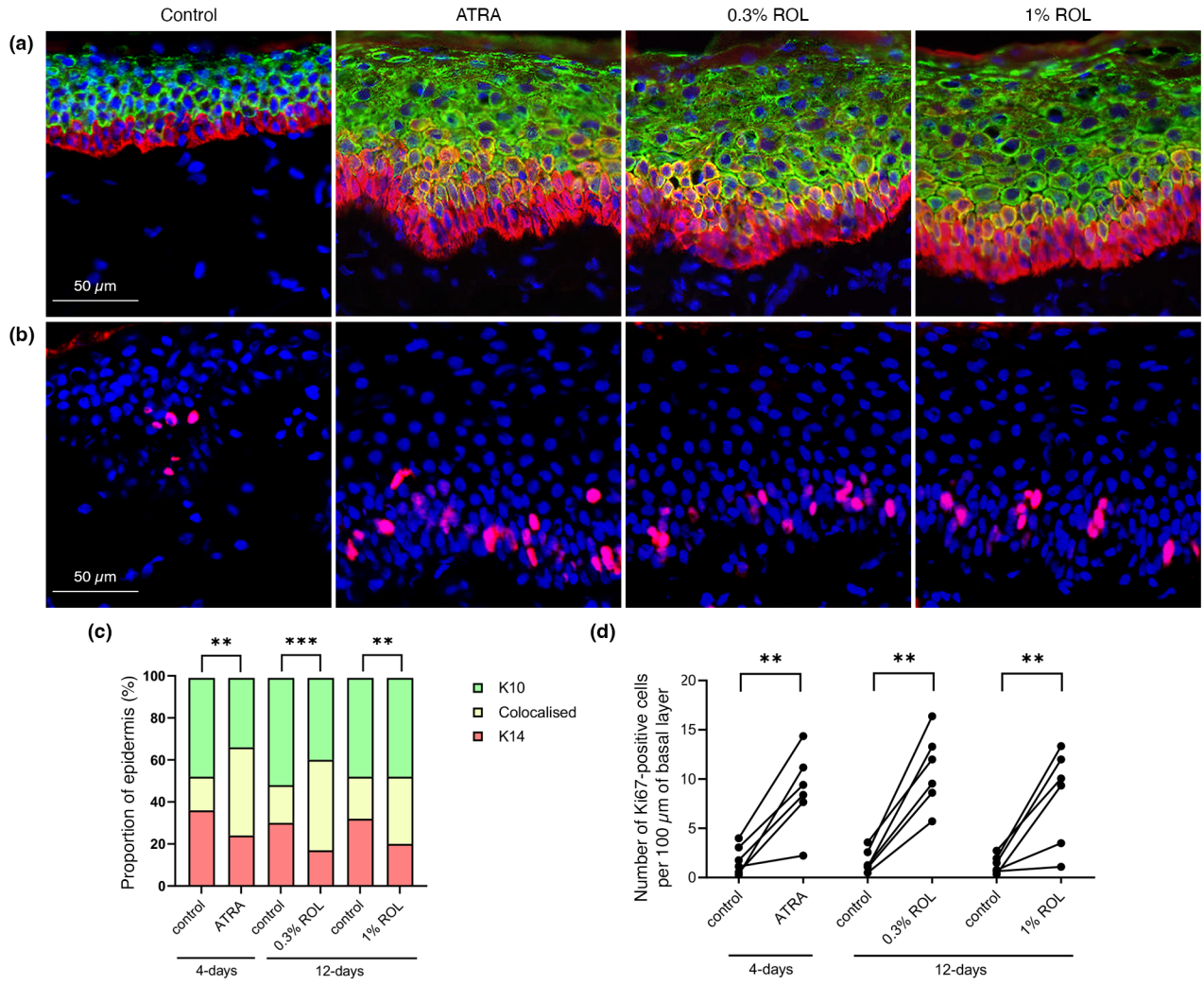

FIGURE 2 Topical retinoid treatment induces profound changes to epidermal keratinocyte behaviour. Application of all-*trans* retinoic acid (ATRA; for 4 days) and both concentrations of retinol (ROL; for 12 days) to photoaged forearm induced significant changes in keratinocyte behaviour with significantly increased colocalization of keratin 14 and 10 (a & c) and significantly increased keratinocyte proliferation (Ki67; b & d), as compared to control skin. Scale bar = 50 μm; $^{**}p < 0.01$; $^{***}p < 0.001$. $n = 6$ per treatment group.

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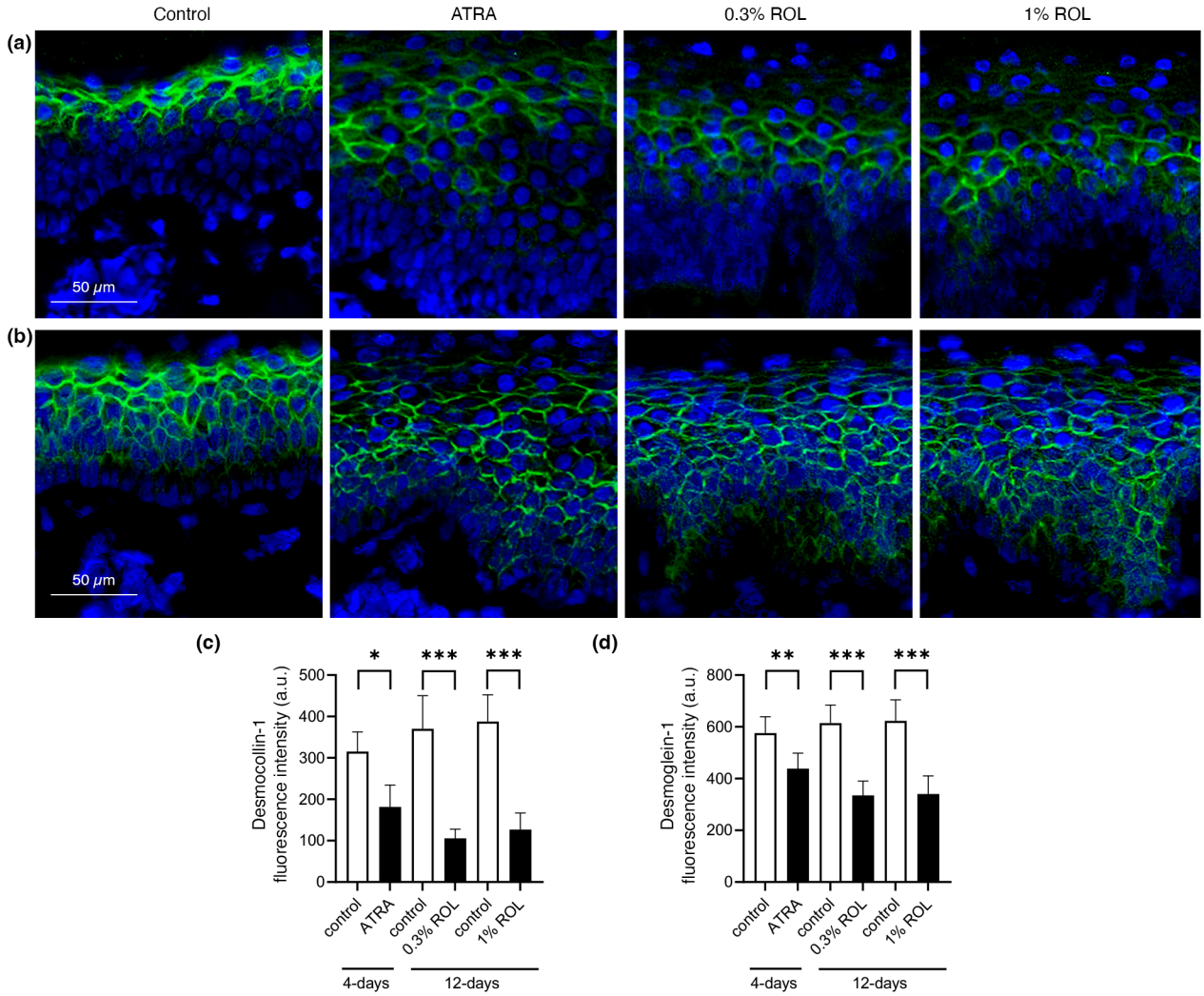

FIGURE 3 Desmosome expression is compromised following retinoid treatment. In black photoaged skin, there was a significant reduction in suprabasal desmosome expression for desmocollin-1 (a & c) and desmoglein-1 (b & d) following retinoid treatment. Scale bar = 50 μm; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. $n = 6$ per treatment group.

calculating ITA values. Colourimetry revealed that there was a minimal but statistically significant degree of skin lightening for the retinoid-treated patches, as compared to control skin (all $p < 0.05$; Figure 4a). Similarly, at the histological level, Warthin–Starry staining confirmed that epidermal melanin had dispersed throughout the thickened epidermis of retinoid-treated skin causing a significant reduction in overall melanin abundance, as compared to control skin (ATRA & 1% ROL: $p < 0.01$; 0.3% ROL: $p < 0.05$; Figure 4b,c).

Topical retinoid treatment induces dermal remodelling

Next, the effects of retinoid treatment on dermal remodelling were assessed. In control skin, and as expected for black photoaged skin, the area of papillary dermis adjacent to the DEJ displayed a loss of FRM architecture and abundance.[8] However, application of ATRA and both concentrations of ROL resulted in a significant deposition of FRMs at the DEJ and a marked improvement to the candelabra-like structure of the fibres compared with control (all $p < 0.05$; Figure 5a,b).

Reductions in fibrillar collagens are a further histological consequence of chronic photoageing in black skin.[8] In white skin, it has been demonstrated that following long-term retinoid treatment, fibrillar collagens can be partially restored.[10] Therefore, to test whether short-term application of ATRA or ROL could also induce fibrillar collagen deposition, patch test samples were stained with an antibody specific to procollagen I (pC1), a precursor of organized mature fibrillar collagen I. However, following retinoid treatment no additional pC1 immunostaining could be detected above control level (Figure 5c). Similarly, although increased collagen VII has previously been reported as a consequence of long-term

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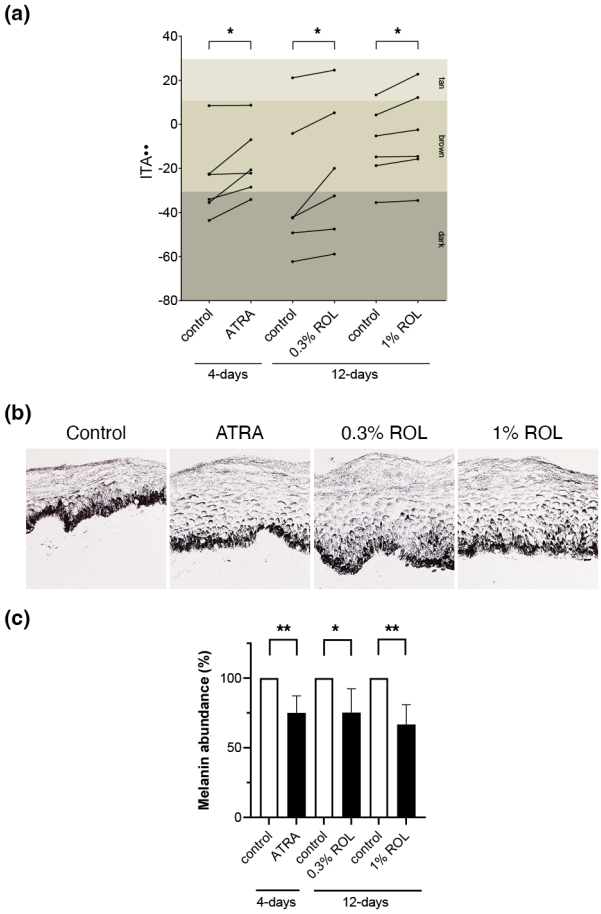

FIGURE 4 Topical retinoid treatment induces epidermal melanin dispersal. Colorimetry revealed a minimal but statistically significant degree of skin lightening for the retinoid-treated patches, as compared to control skin (a). Warthin–Starry staining confirmed dispersal of epidermal melanin throughout the thickened epidermis of retinoid-treated skin (b) and a significant reduction in overall epidermal melanin abundance in response to thickening of the epidermis (c). Scale bar = 50 μm; $*p < 0.05$; $**p < 0.01$. $n = 6$ per treatment group.

retinoid treatment[11] under patch test conditions in black skin, this was not recapitulated.

DISCUSSION

In this exploratory study using an in vivo short-term patch test protocol, we establish that ATRA and ROL induce histological remodelling of both the epidermis and dermis of photoaged black skin. This in vivo patch test protocol allows for assessment of cutaneous change in a relatively short period of time (up to 12 days) and since its inception in 2001, the assay has provided evidence that topical retinoids and some over-the-counter cosmetic 'anti-ageing' products can initiate dermal remodelling.[12,34,35] Furthermore, the results arising from the patch test are highly predictive of the clinical and histological changes observed when products are used in longer-term, clinical protocols.[36]

The effects of topical retinoids on black skin are similar to those previously reported for white photoaged skin—in particular, pronounced epidermal thickening, increased keratinocyte proliferation and FRM deposition at the DEJ.[12,20,37,38] However, in this study, we also provide a detailed analysis of the changes to epidermal architecture and keratinocyte behaviour arising from retinoid application. Retinoid treatment resulted in significant epidermal acanthosis and a doubling of keratinocyte cell layers largely driven by a significant increase in basal keratinocyte proliferation above that seen at control sites. This rapid epidermal expansion was facilitated by the downregulation of suprabasal desmosomes allowing keratinocytes to quickly transition through the *strata* of the epidermis towards the *stratum corneum*. Concomitantly, we observed increased colocalization of K14 and K10 during this acceleration of epidermal turnover. Although the functional integrity of the epidermis may be affected in the short-term by such dynamic changes in proliferation[39] and desmosome expression,[33] published literature supports its stabilization over time with prolonged retinoid use.[40]

It has previously been demonstrated that topical ATRA treatment can significantly lighten post-inflammatory hyperpigmentation in black skin and, to a clinically minimal but statistically significant degree, also lighten healthy, uninvolved black skin.[41] Consistent with this finding, we demonstrate that application of ATRA and ROL under patch test conditions causes skin lightening. Interestingly, in black skin there is no evidence that retinoids have a direct effect on the regulation of melanogenesis[42] or influence the cell–cell interactions between melanocytes, keratinocytes and fibroblasts.[43] Rather, it is thought that retinoids promote keratinocyte proliferation, leading to rapid epidermal expansion and the dispersal of melanin throughout the thickened epidermis[43–46] and our patch test data further support this concept.

Despite the photoprotective properties of melanin, chronic photoexposure is known to exacerbate the features of ageing black skin at both the histological and functional level. Furthermore, many similarities have been reported between photoaged black skin and white skin such as near complete effacement of rete ridges and flattening of the DEJ, considerable epidermal thinning and a marked loss of FRM architecture in the papillary dermis, proximal to the DEJ.[8] Application of ATRA to white skin has previously been shown to induce the de novo synthesis and deposition of the papillary dermal FRM network[12]; a process thought to be driven by the activation of basal keratinocytes.[6,47] However, this is the first time that the efficacy of topical retinoids—both ATRA and ROL—for dermal remodelling in photoaged black skin has been demonstrated and suggests that topical retinoids may be a beneficial treatment option for photoageing in black skin. The dermal elastic fibre network is the primary effector of skin elasticity, enabling it to extend and recoil many times over the lifetime of the individual. Fibrillin-rich microfibrils constitute integral components of the elastic fibre network, with their distribution showing differential deposition in the papillary dermis across

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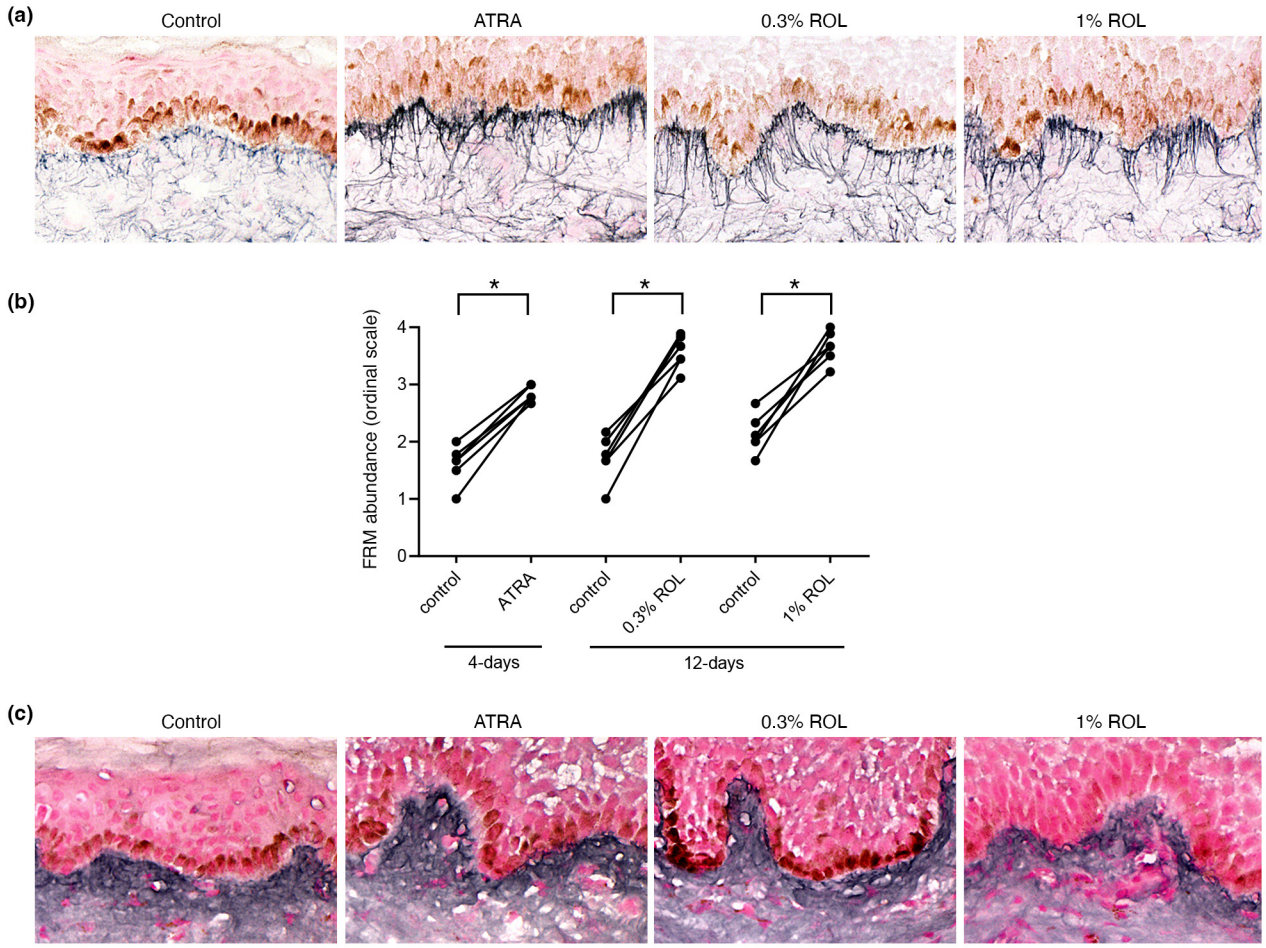

FIGURE 5 All-*trans* retinoic acid induced dermal remodelling of the elastic fibre network following 4-day application using the Manchester Patch Test assay. Application of all-*trans* retinoic acid (ATRA) and both concentrations of retinol (ROL) resulted in significant deposition of fibrillin-rich microfibrils at the dermal–epidermal junction and a marked improvement to the candelabra-like structure of the fibres (a & b). Short-term application of retinoids under patch test conditions did not induce pro-collagen 1 deposition (c). Scale bar = 50 μm; $^*p < 0.05$. $n = 6$ per treatment group.

individuals of diverse skin ethnicity.[27,48] Papillary dermal FRMs have previously been shown to be particularly susceptible to photoexposure, due in part to their chromophore composition[49] and proximity to the epidermis.[6] Excessive sun exposure, mainly in childhood, and various high-risk activities, such as intentional sunbathing, inadequate sun protection and the use of tanning lamps,[50,51] can all induce skin damage in white Northern European individuals. In contrast, in highly pigmented black African skin, melanin affords the basal keratinocytes with protection against penetrating UVR[52,53] resulting in little impact on keratinocytes or the dermal ECM. However, in common with other ECM assemblies, FRMs are thought to persist in skin for many years[54]; consequently, they are susceptible to gradual accumulation of damage and degradation. Degradation of FRMs at the DEJ causes profound structural and functional decline to overall skin health.[8,55,56] It is therefore postulated that partial restoration of these fibres in response to retinoid treatment will improve the structural integrity of the DEJ and the underlying dermis. Despite the increased FRM deposition, fibrillar collagens and collagen VII remained unchanged, possibly due to the short product application time of 4–12 days.[12,20]

Although ATRA is the most efficacious treatment option for photoaged skin, its use is often associated with the occurrence of erythema, pruritus, stinging, burning and scaling.[38,57,58] However, it is hypothesized that it is the skin irritancy associated with ATRA use that drives the active remodelling of the dermal ECM and ultimately leads to the improvement of the clinical features of photoaged skin.[38,59] In contrast, while ROL is better tolerated than ATRA,[14,15] its efficacy has been questioned as it must be converted to ATRA after application.[60–62] In the current study, analysis of our MPT biopsies found that 0.3% and 1% ROL are comparable to ATRA in terms of remodelling dermal ECM components and inducing rapid epidermal changes albeit over a longer time (12 vs. 4 days).

The present study has some limitations. Firstly, the sample numbers for this study were relatively limited and reflect the exploratory nature of this research. Secondly, the length of time that the retinoid was applied for was not consistent across the different treatments. This was because the MPT

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protocol specifies that due to irritancy ATRA can only be applied for 4 days whereas cosmetic formulations, such as ROL, should be applied for 12 days. Lastly, due to ethical constraints, each volunteer could only be patched with a single retinoid treatment and an occluded but untreated site; our preferred methodology for the Manchester Patch Test is to include an area that is untreated but occluded to provide a baseline control, an area that is occluded with a vehicle control treatment and each occluded retinoid treatment (0.3% ROL, 1% ROL and ATRA). While this may limit conclusions drawn about the relative efficacy of the different treatments, each participant acts as their own control, and therefore, the remodelling that occurs as a result of treatment can be accurately measured. Nevertheless, future studies should compare multiple treatment patches applied to each individual participant, wherever possible.

CONCLUSION

This study confirms that in photoaged black skin, topical retinoids have significant effects not only on the epidermis and keratinocyte behaviour, but also on the repair of the dermal elastic fibre network. Furthermore, the effects of topical retinoids on black skin are similar to those previously reported for white photoaged skin, suggesting that pathomechanisms of UVR-induced microfibrillar damage and repair are shared, regardless of the pigmentary status of the skin. Further investigation of their efficacy in daily use is therefore warranted.

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CONFLICT OF INTEREST STATEMENT

P Halai, Dr Kiss, Dr Wang, C O'Connor and Dr Bell have nothing to disclose. Dr Chien and Dr Kang report funding from Boots and No7 Beauty Company during the conduct of the study. Professor Griffiths reports funding and participation on an advisory board for Boots and No7 Beauty Company, during the conduct of the study. Professor Watson reports funding from Boots and No7 Beauty Company during the conduct of the study. Dr Langton reports funding from Boots and No7 Beauty Company during the conduct of the study.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Local ethical approval was obtained from The Johns Hopkins Institutional Review Board (IRB00228969). Written informed consent was obtained from the participants and the study adhered to Declaration of Helsinki principles.

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