BJD
British Journal of Dermatology

4-Hexyl-1,3-phenylenediol, a nuclear factor-κB inhibitor, improves photodamaged skin and clinical signs of ageing in a double-blinded, randomized controlled trial*

S. Kaur,[1] M. Kizoulis,[1] J. Fantasia,[1] T. Oddos,[2] N. Bigot,[3] P. Galera,[3] S. Tucker-Samaras,[1] J.J. Leyden[4] and M.D. Southall[1]

[1]Johnson and Johnson Skin Research Center, CPPW, A Division of Johnson and Johnson Consumer Companies, Inc., 199 Grandview Road, Skillman, NJ 08558, U.S.A

[2]Johnson and Johnson Skin Research Center, CPPW, A Division of Johnson and Johnson Consumer Companies, Inc., Val de Reuil, France

[3]Laboratoire Microenvironnement Cellulaire et Pathologies, (MILPAT) EA 4652, SFR ICORE 146 UCBN, UFR de Médecine, CHU Niveau 3, Avenue de la Côte de Nacre, 14032 CAEN, Cedex, France

[4]University of Pennsylvania Medical School, 415 Curie Blvd, Philadelphia, PA 19104, U.S.A.

Summary

Background The nuclear factor-κB (NF-κB) pathway is a key mediator of inflammation; however, few studies have examined the direct effects of NF-κB inhibition on the skin.

Objectives To investigate NF-κB activity in cultured human fibroblasts and to investigate the effects of 4-hexyl-1,3-phenylenediol (an NF-κB inhibitor) on elastin and collagen gene expression *in vitro* and on the clinical appearance of photodamaged skin.

Methods The amount and activity of NF-κB in human fibroblasts obtained from donors (17–78 years old) was measured after transfection with a NF-κB reporter and a luciferase promoter system. The expression of extracellular matrix (ECM) genes was determined using quantitative polymerase chain reaction. Women with moderate skin photodamage were randomized to daily treatment with a topical lotion containing 4-hexyl-1,3-phenylenediol (n = 30) or vehicle (n = 29) for 8 weeks, with clinical assessments at baseline and weeks 2, 4 and 8.

Results Fibroblasts obtained from donors older than 50 years had higher NF-κB activity compared with cells from younger donors; inhibition of the NF-κB pathway with 4-hexyl-1,3-phenylenediol enhanced the expression of ECM genes. In women, treatment for 8 weeks with 4-hexyl-1,3-phenylenediol significantly improved crow's feet fine lines, cheek wrinkles, age spots, mottled pigmentation and radiance compared with both the vehicle and baseline. Furthermore, treatment with 4-hexyl-1,3-phenylenediol resulted in a twofold greater clinical improvement in overall photodamage compared with the vehicle group.

Conclusions Inhibition of the proinflammatory NF-κB pathway resulted in increased expression of ECM proteins *in vitro* and significant clinical improvement in photodamaged skin.

Correspondence
Michael D. Southall.
E-mail: msoutha@its.jnj.com

Accepted for publication
8 February 2015

Funding sources
These studies were supported by Johnson & Johnson Consumer Companies, Inc. Skillman, New Jersey, U.S.A.

Conflicts of interest
S.K., M.K., J.F., T.O., S.T.-S. and M.D.S. are employees of Johnson & Johnson Consumer Companies, Inc. Skillman, NJ, U.S.A. N.B., P.G. and J.J.L. have consulted and/or received research funding from Johnson & Johnson Consumer Companies, Inc. Skillman, NJ, U.S.A.

*Plain language summary available online.

DOI 10.1111/bjd.13747

What's already known about this topic?

- The nuclear factor-κB (NF-κB) pathway is a key mediator of inflammation and may be involved in skin ageing.

What does this study add?

- Cultured fibroblasts from donors ≥ 50 years old had higher levels of NF-κB activity than fibroblasts obtained from donors < 50 years old.
- In humans, treatment with an NF-κB inhibitor significantly improved clinical parameters in photodamaged skin.
- These findings support the hypothesis that NF-κB activation may accelerate the skin ageing process.

Intrinsically aged skin exhibits signs of damage such as reduced elastin content and a general decline in a variety of cell populations.[1] Skin becomes increasingly thin, dry, pale and wrinkled over time as a consequence of skin ageing.[2,3] Factors in both the epidermis and the dermis contribute to the thinning of older skin, with a reduction in keratinocytes observed in the epidermis and a decrease in the amount and organisation of the extracellular matrix (ECM) in the dermis.[2,3] The ECM of the dermis is composed primarily of collagen, elastin, proteoglycans and glycosaminoglycans with embedded dermal fibroblasts. It is the degeneration of collagen and elastin over time in addition to the resulting loss of strength and elasticity that causes intrinsic skin ageing. This intrinsic skin ageing process is inherent with the passage of time; however, it is exacerbated by the presence of many extrinsic factors.

In addition to the intrinsic ageing process, the skin is under continuous assault from a variety of damaging environmental factors including ultraviolet (UV) irradiation and atmospheric pollutants. Extrinsic factors, particularly UV, have been demonstrated to accelerate the factors involved in the intrinsic ageing process, resulting in elastosis, collagen breakdown and dyspigmentation.[1,4] Multiple studies have shown that levels of ECM proteins, elastin and collagen in skin decrease with age.[5–7] The accumulation of abnormal connective tissue fibres (collagen and elastin) in the skin results in visibly damaged skin because of chronic UV exposure. Inflammation is the body's natural response to the many types of insults the body encounters everyday including infections, chemical damage (e.g. toxins, irritants, pollution) and physical damage (e.g. heat, cold, UV radiation). When cells are damaged, a complicated cascade of events takes place, ultimately resulting in the release of a large array of inflammatory mediators including reactive oxygen species (ROS). ROS can contribute to both direct oxidation damage to cells and also indirect damage via further upregulation of inflammation.[4,8] This damage often goes unnoticed at the time, for example in cases of acute UV damage to the skin, but the resulting subclinical inflammation over time may be involved in the skin ageing process.

The nuclear factor-κB (NF-κB) pathway is a signalling pathway that can be activated by interleukin (IL)-1, tumour necrosis factor (TNF)-α and ROS, and has been reported to be the final common pathway for the conversion of environmental insults into inflammation in the skin.[9–13]

In the absence of stimuli, NF-κB is normally sequestered in a cell's cytoplasm by its inhibitor (IκB) until phosphorylation of IκB frees NF-κB to translocate to the nucleus and activate target genes.[9,11–13] The UV radiation from sunlight induces IL-1 and TNF-α and creates ROS that then leads to NF-κB-mediated inflammation.[4,14] The NF-κB pathway is a key regulator of inflammatory mediators in skin cells and leads to the expression of matrix metalloproteases (MMPs), such as collagenase (MMPs 1, 3 and 9). In addition, the NF-κB pathway further induces IL-1 and TNF-α, thus upregulating its own pathway.[15] The MMPs are responsible for the degradation of the ECM fibres in connective tissue, and an excess of MMPs ultimately results in the visible signs of ageing.[16] Furthermore, the skin's ability to produce matrix proteins such as elastin and collagen also declines with age, but the aetiology of this inhibition is not well understood.

Intrinsic skin ageing has been described as the culmination of two independent processes that combine to produce skin ageing, namely the decreased expressions of ECM proteins and the increased expression of MMPs, which degrade ECM proteins. Environmental factors such as UV can significantly increase the expression of MMPs, further accelerating the ageing process. Previously, it has been demonstrated that the activation of the NF-κB pathway leads to the induction of MMPs, linking it to environmental factors, such as UV irradiation, which contribute to the extrinsic acceleration of the ageing process. However, few studies have investigated the direct effects of NF-κB activation on ECM proteins, which could suggest that NF-κB plays a role in the intrinsic ageing process. In this paper, a direct connection between NF-κB and the intrinsic ageing process was examined by demonstrating the effects of NF-κB inhibition on ECM expression and a clinical investigation was conducted in order to determine whether the topical application of an NF-κB inhibitor would improve photodamaged skin in a double-blinded, randomized controlled trial.

Patients and methods

Nuclear factor-κB luciferase reporter assays in fibroblasts

Human dermal fibroblasts were obtained from skin breast biopsies of white donors (aged 17–78 years old; $n = 11$ for donors < 50 years old and $n = 10$ for donors > 51 years old) provided by Dr M. Jolivet (Department of Reconstructive Surgery, St Martin Clinic, Caen, France) and by Johnson & Johnson Laboratories (Johnson & Johnson Santé Beauté France, Campus de Maigremont, Val de Reuil, France). The results shown are representative of three individual experiments. The passage number of cells used ranged from passage 3 to 5.

Fibroblasts were passaged by trypsin at confluency, centrifuged for 10 min at 200 *g* and transfected using the AMAXA Nucleofector® apparatus (Lonza, Köln, Germany), according to the manufacturer's instructions. The transcription activity of NF-κB was evaluated with an NF-κB-luciferase (Luc) reporter plasmid. A pSV40/β-gal plasmid was cotransfected, as a control of transfection efficiency. For transfections, 2 μg of the Luc reporter-gene constructs were mixed with 2 μg of the pSV40/β-gal plasmid and 1×10^6 cells resuspended in 100 μL of nucleofection solution. The Luc and β-galactosidase activities and the protein amount, were determined as previously described.[17] The results from several experiments were divided into two age groups: young (< 50 years old) and aged (> 51 years old) and are expressed with arbitrary units. The results were obtained from four independent experiments.

Nuclear factor-κB luciferase reporter assay in keratinocytes

Primary human keratinocytes (from adult donors) were obtained from PromoCell (Heidelberg, Germany) and seeded in 96-well plates. Cells were transiently transfected with 0·25 μg per well total DNA containing pNF-κB-Luc reporter plasmid (Stratagene, La Jolla, CA, U.S.A.) and the internal control Renilla-Luc reporter (pRL-TK; Promega Corporation, Madison, WI, U.S.A.) using Lipofectamine® 2000 transfection reagent (Invitrogen Corporation, Carlsbad, CA, U.S.A.). At 24 h after transfection, cells were treated with doses of 4-hexyl-1,3-phenylenediol or with the pharmacological inhibitor of NF-κB, Bay 11-7082 (Calbiochem/EMD Chemicals, Gibbstown, NJ, U.S.A.), followed by treatment with 100 ng mL^{-1} TNF-α (Peprotech Inc., Rocky Hill, NJ, U.S.A.) for 24 h. The ratio of Firefly:Renilla-Luc values in relative light units was converted to NF-κB activity expressed as a percentage. The results were obtained from three independent experiments, each conducted with two to three replicates.

Quantitative polymerase chain reaction assay in dermal fibroblasts

Human dermal fibroblasts were obtained from the skin of breast biopsies as mentioned above, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Life Technologies, Saint Aubin, France) with gentamicin (4 μg mL^{-1}), fungizone (0·25 μg mL^{-1}) and ciprofloxacin (10 μg mL^{-1}) added extemporaneously, in a 5% CO_2 environment. At 85% confluence, the fibroblasts were trypsinized (0·05%) with ethylenediaminetetraacetic acid (0·25 mmol L^{-1}), and amplified at 1:3 ratio. Fibroblasts were employed between passages 3–5. Cells grown on 24-well plates were treated with the indicated doses of TNF-α added to media containing 2% serum for 48 h, followed by cell lysis and RNA isolation as described. Total RNA was extracted with TRIzol (Fisher Scientific, Loughborough, U.K.) according to the manufacturer's instructions.

A total of 1·5 μg of total RNA was treated with 1·5 U DNase I (Fischer Scientific) at room temperature for 15 min to remove any DNA contaminants. Reverse transcription was conducted using 1 μg of total RNA treated with DNase I, 20 μmol L^{-1} Oligo(dT) (Fisher Scientific), 200 U Moloney murine leukaemia virus reverse transcriptase (Fisher Scientific), 40 U RNaseOUT (Fisher Scientific) and 20 μmol L^{-1} of each dNTPs (Fisher Scientific). Real-time polymerase chain reaction (PCR) was performed in an ABI Prism SDS 7000 thermocycler (Applied Biosystems, Courtaboeuf, France). All procedures were conducted in triplicate. Controls of nontemplate cDNA were included in the PCR experiments. The sequences of the forward and reverse primers (Eurogentec Corporation, Seraing, Belgium) were designed using Primer Express software (Applied Biosystems). Amplifications were performed in 96-well plates for a total volume of 15 μL containing 5 μL of 1:100 diluted cDNA samples obtained from reverse transcription, 7·5 μL of 2X SYBR Green MasterMix (Applied Biosystems) and both primers (200 nmol L^{-1} final concentration for each primer). The amplification conditions were 40 cycles of 10 s at 95 °C and 60 s at 60 °C, followed by the protocol for the melting curve: 80 cycles of 10 s with an increase of 0·5 °C between each cycle from 55 °C to 95 °C. The melting curve was used to check whether the amplification products exhibited the expected thermal melt values. The mRNA amount was normalized to ribosomal protein L13A mRNA, and analysis of relative gene expression was performed by using the $2^{-\Delta\Delta Ct}$ method.

Collagen 1a enzyme-linked immunosorbent assay

Collagen 1a protein levels were measured by using a CICP/PICP MicroVue enzyme-linked immunosorbent assay (ELISA) kit from Quidel (San Diego, CA, U.S.A.), which measures type I C-terminal collagen propeptide.

Primary human fibroblasts grown on 96-well plates were treated with TNF-α (1 ng mL^{-1}) alone or after pretreatment (2 h) with 4-hexyl-1,-3-phenylenediol (1 μg mL^{-1}) in media containing 2% serum. After 48 h of treatment, media was collected and analysed for collagen 1a ELISA following the manufacturer's instructions for the kit. Collagen 1a levels were normalized using total protein (bicinchoninic acid) and expressed as a percentage.

Test products

A topical lotion formulation containing 1% (w/v) 4-hexyl-1,3-phenylenediol was evaluated in these studies, together with a vehicle formulation. The lotion is an oil-in-water emulsion with glycerine and a low concentration of petrolatum and other emollients. The vehicle was of identical composition, but without 4-hexyl-1,3-phenylenediol.

Randomized controlled trial

This was an 8-week, double-blind, vehicle-controlled, randomized controlled trial conducted between March 2011 and May 2011 near Philadelphia, PA, U.S.A. A total of 61 women, aged 40–65 years, Fitzpatrick phototype I–III, were enrolled in the study. For inclusion in the study, subjects were required to present with the following: coarse wrinkles in crow's feet and upper cheek areas, facial pigmentation and overall photodamage on both sides of the face. Subjects were excluded if they had used topical antiageing treatments and/or topical retinoids within 30 days prior to the start of the study. On the first day of the treatment period (day 1/visit 1), subjects were randomly assigned either the vehicle formulation or test product using a randomization chart and given instructions on how to apply the assigned cream to the whole face twice daily for 8 weeks. The subjects, care providers and those assessing outcomes were all blinded to the interventions. Of the total study population, 30 subjects using the lotion containing 4-hexyl-1,3-phenylenediol completed the 8-week study and 29 subjects using the vehicle completed the study.

Two subjects were discontinued for reasons unrelated to the study.

The clinical evaluations were performed by a dermatologist (J.J.L.) at baseline and at weeks 2, 4 and 8. The clinical assessments were scored on a scale ranging from 0 (none) to 9 (severe) and included the following: crow's feet fine lines, mottled pigmentation, discrete pigmentation (age spots), skin radiance and overall photodamage severity using a validated clinical grading scale as described previously.[18]

Overall cutaneous irritation was evaluated for safety using a scale ranging from 0 (none) to 3 (severe). Safety was measured by the incidence and severity of both cutaneous irritation and adverse events. No adverse effects related to the vehicle or lotion containing 4-hexyl-1,3-phenylenediol were reported in the study. The study was approved by Allendale Investigational Review Board (Old Lyme, CT, U.S.A.) and the subjects' written informed consent was obtained prior to initiation of the investigation.

Digital images were obtained in order to document the results and the camera set-up, lighting conditions and subject's position were standardized during the study. The imaging system consisted of a digital SLR camera (Canon Digital Rebel XTI, Lake Success, NY, U.S.A.) equipped with a Canon 35 f2.0 lens and a flash light (Broncolor Picolite 1600W, model 12-5003, Allschwil, Switzerland). Both the camera and the light were enhanced with linear polarizing laminated films (Edmund Optics, model NT38-493, Barrington, NJ, U.S.A.), mounted on rotating filter wheels. The camera, light source and rotating filter wheels were controlled by a personal computer.

Statistical analysis

In vitro studies

A Student's t-test was used for comparisons between *in vitro* treatment groups; $P < 0{\cdot}05$ was considered significant. All data are presented as mean ± SD.

Randomized controlled trial

Primary data for efficacy analysis were the dermatologist assessment data at week 8. The analysis of data at weeks 2 and 4 was considered secondary. At each postbaseline visit (weeks 2, 4 and 8), descriptive statistics indicated how much the subjects within each cell had changed since the baseline for each clinical parameter. Overall, 29 of 30 subjects in the vehicle group and 30 of 31 subjects in the test-product group completed the study and were included in each analysis according to the originally assigned groups. The sample mean, SD, quartiles and other key percentile values were calculated for the change from baseline scores, in addition to the mean percentage change from baseline. The proportion of subjects who improved from baseline, corresponding to a negative change, was also calculated for each visit. A paired t-test was used to determine whether there was a statistically significant change from baseline for each product. Differences between the products were indicated based on the mean change score via a t-test and the proportion of responders via Fisher's exact test.

Results

In vitro assessment

The assessment of NF-κB activity in skin showed that fibroblasts from aged donors displayed a higher NF-κB activity compared with fibroblasts from younger subjects. The quantification of the results from several experiments ($n = 10$) demonstrated that there was a clear and significant increase in the NF-κB activity in fibroblasts from the older age group (> 50 years old) (Fig. 1a). These results are consistent with a recent and more in-depth study using NF-κB DNA-binding activity reported by Bigot *et al.*[19]

The inhibition of NF-κB by 4-hexyl-1,3-phenylenediol was demonstrated in an NF-κB-Luc assay along with the pharmacological inhibitor of NF-κB (Bay 11-7082). NF-κB activity was inhibited by 4-hexyl-1,3-phenylenediol in a dose-dependent manner with a 50% inhibition observed at a dose of approximately 0·05 μg mL^{-1} (Fig. 1b). The effect of TNF-α on ECM production was then investigated in primary human dermal fibroblasts obtained from young (< 50 years old; $n = 3$) and older (> 51 years old; $n = 3$) donors. Treatment with TNF-α in order to induce activation of NF-κB resulted in a dose-dependent downregulation of collagen 1a and elastin gene expression as measured by quantitative PCR (Fig. 1c). Pretreatment of the fibroblasts with 4-hexyl-1,3-phenylenediol directly induced the expression of elastin and collagen 1a genes, and restored the levels of these ECM genes that were inhibited by TNF-α treatment in cells from young and older donors (Fig. 2a,b).

In order to confirm the effects of 4-hexyl-1,3-phenylenediol on TNF-α-mediated downregulation of collagen at a protein level, normal human fibroblasts that were treated with TNF-α in the absence or presence of 4-hexyl-1,3-phenylenediol were analysed using collagen 1a ELISA. In line with previous findings, TNF-α (1 ng mL^{-1}) treatment lowered collagen 1a protein levels, and pretreatment of the cells with 4-hexyl-1,-3-phenylenediol (1 μg mL^{-1}) reversed the effects of TNF-α (Fig. 2c).

Clinical assessment

At 8 weeks, the product containing 4-hexyl-1,3-phenylenediol produced a statistically significant ($P < 0{\cdot}05$) improvement in crow's feet fine lines, cheek wrinkles and forehead wrinkles compared with both the vehicle and the baseline, whereas vehicle-treated skin was not significantly improved compared with the baseline. Skin radiance, mottled pigmentation and age spots were also significantly improved compared with both the vehicle and the baseline. However, the vehicle also

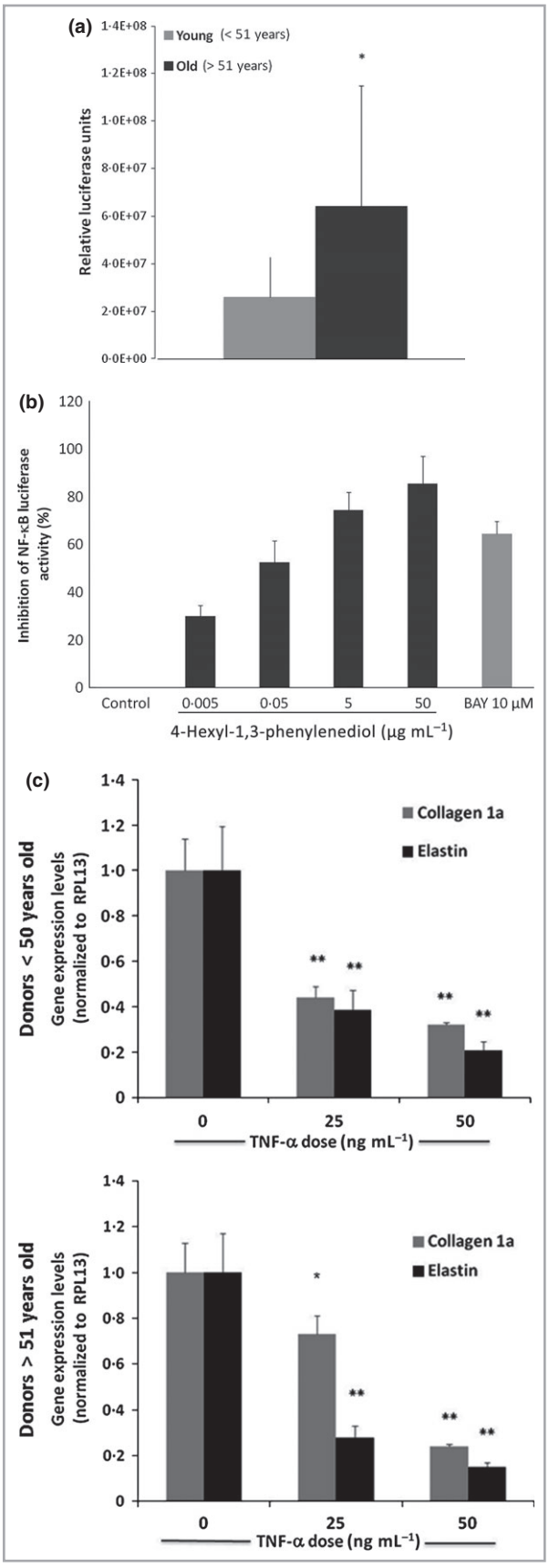

Fig 1. Aged fibroblasts display higher amounts and binding activity of nuclear factor-κB (NF-κB) than young individuals. (a) Human dermal fibroblasts derived from young (< 50 years old; n = 11) and older (> 51 years old; n = 10) donors were transiently transfected with pSV40-β-gal, and the NF-κB luciferase reporter construct. Values are mean ± SD expressed as relative luciferase units (*P < 0·05 using Student's t-test). (b) Primary human skin cells transiently transfected with the NF-κB luciferase reporter were pretreated with the indicated doses of 4-hexyl-1,3-phenylenediol or BAY 11-7082 prior to tumour necrosis factor (TNF)-α treatment for 24 h, followed by cell lysis and luciferase assay. Results are mean ± SD expressed as percentage inhibition. (c) Primary dermal fibroblasts obtained from young (< 50 years old; n = 3) and older (> 51 years old; n = 3) donors were treated with multiple doses of TNF-α for 48 h, and gene expression of collagen 1a and elastin was measured using quantitative polymerase chain reaction. Results shown are representative of three donors and are expressed as fold-change over control [normalized to ribosomal protein L (RPL)13], with the control gene expression levels set to 1 (*P < 0·05, **P < 0·005 using Student's t-test).

showed significant improvement compared with the baseline, although not as great an improvement compared with the 4-hexyl-1,3-phenylenediol treatment.

Subjects using 4-hexyl-1,3-phenylenediol showed a clinical improvement in skin radiance of 49% by 8 weeks, whereas parameters such as crow's feet fine lines improved by 28%, mottled pigmentation improved by 65% and age spots showed a 67% improvement in subjects treated with 4-hexyl-1,3-phenylenediol (Table 1). When compared with the vehicle, a higher percentage of subjects using the product containing 4-hexyl-1,3-phenylenediol responded to treatments, with 90% of subjects showing a clinical improvement in the grading of crow's feet fine lines after 8 weeks (Fig. 3a). The use of either formulation produced a significant improvement in overall photodamage compared with the baseline (vehicle, $P < 0{\cdot}05$; test product, $P < 0{\cdot}05$). However, the subjects who used the product containing 4-hexyl-1,3-phenylenediol had a twofold greater clinical improvement in overall photodamage compared with the vehicle, with 72% of the women who used 4-hexyl-1,3-phenylenediol showing an improvement compared with 30% of women who used the vehicle. The visual effect of 8 weeks treatment with 4-hexyl-1,3-phenylenediol on improvement of crow's feet fine lines is shown in Figure 3b.

Discussion

The skin is a crucial interface that protects the body from environmental insults and performs multiple complex functions, including providing an epithelial barrier, homeostasis, sensory capabilities and immunological protection.[20–22] However, the skin is constantly under stress from both natural intrinsic factors and environmental extrinsic factors that adversely affect skin health by decreasing skin cell populations and reducing the connective tissue fibres (collagen, elastin, etc.) of the supporting stroma.[15–17] The cumulative damage to

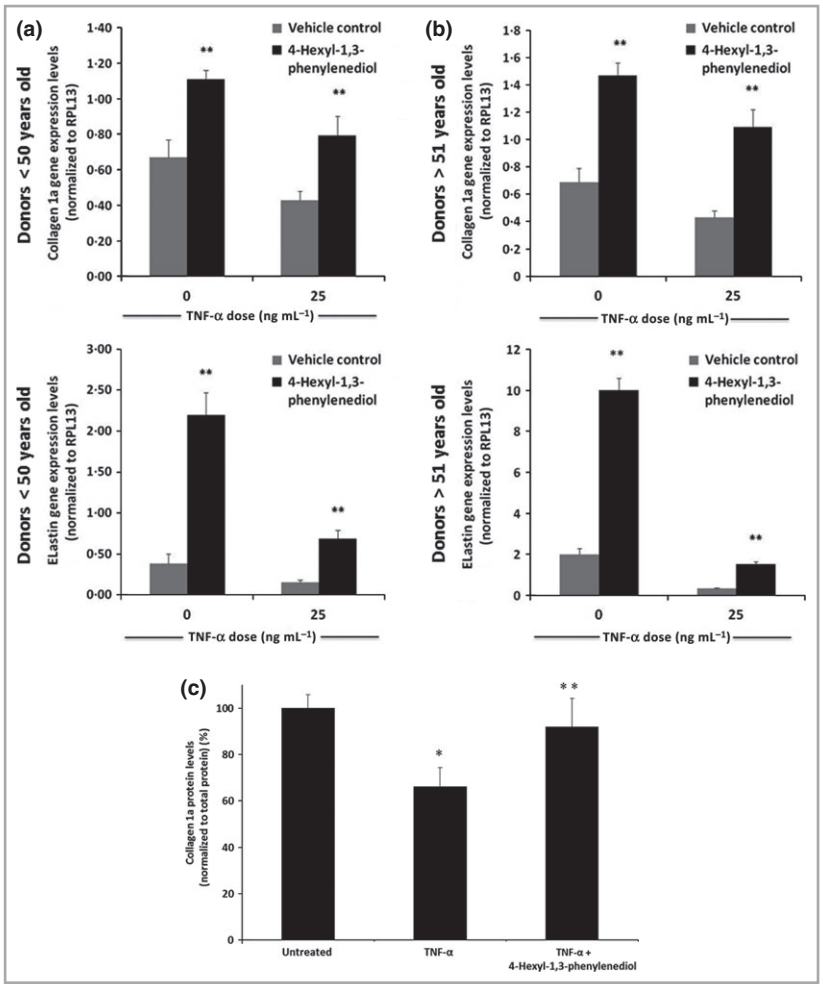

Fig 2. 4-Hexyl-1,3-phenylenediol reverses extracellular matrix downregulation caused by nuclear factor-κB activation. Primary dermal fibroblasts from (a) young and (b) older donors described in Figure 1c above were treated with 4-hexyl-1,3-phenylenediol (0·005 μg mL^{-1}) in the presence tumour necrosis factor (TNF)-α (25 ng mL^{-1}) for 48 h. Gene expression of collagen 1a and elastin was measured by quantitative polymerase chain reaction. Results shown are representative of three donors and are expressed as fold-change over control (normalized to ribosomal protein L13). (c) Treatment of primary human fibroblasts with TNF-α (1 ng mL^{-1}) lowered collagen 1a protein levels, and pretreatment of the cells with 4-hexyl-1,-3-phenylenediol (1 μg mL^{-1}) reversed the effects of TNF-α. *$P < 0·05$ compared with untreated control; **$P < 0·05$ compared with TNF-α alone (using Student's paired *t*-test).

Table 1 Percentage of subjects with clinical improvement in the following parameters when using 4-hexyl-1,3-phenylenediol

Percentage of women with clinical improvement	Time point		
Parameter	2 weeks	4 weeks	8 weeks
Crow's feet fine lines (%)	24	89	90
Age spots (%)	17	43	55
Overall photodamage (%)	13	68	72

the skin from these environmental aggressors, which include UV radiation and atmospheric pollutants, increases with age and can result in chronic inflammation and premature ageing.[21,23,24]

Inflammation is the body's natural response to the many kinds of insults that the body encounters on a daily basis and is essential to mediate the appropriate response to these insults and begin the body's natural repair processes. However, although some transient inflammatory responses may be needed to restore tissue properly, chronic activation of inflammatory pathways can result in irreversible damage to the ECM and supporting connective tissue.[4] In cases of UV and other environmental aggressors, constant exposure can cause excess damage of the skin via the induction of inflammatory processes, which can lead to the accelerated breakdown of matrix proteins in the skin.[25] The activation of the NF-κB pathway in skin keratinocytes by UV irradiation has been shown to induce various genes including IL-1 and TNF-α that subsequently further activate NF-κB in a vicious cycle, leading to premature degradation of the ECM via the production of MMPs.[25–29]

Although the link between NF-κB activation and induction of MMPs is well established, few studies have examined whether there are changes in NF-κB activation in skin during ageing. The quantification of NF-κB activity in the skin of aged subjects showed that NF-κB was considerably more active in the skin of older subjects (> 51 years) compared with that of their younger counterparts (< 50 years; Fig. 1a). These results suggest that as we age, subclinical levels of inflammation in the skin are elevated, as demonstrated by the increase in NF-κB levels with age. These results also support the hypothesis that NF-κB activation in the skin may accelerate the skin-ageing process.[30,31]

Having established a direct link between NF-κB activation and the suppression of matrix proteins (elastin and collagen), a specific inhibitor of the NF-κB pathway was analysed. We found that 4-hexyl-1,3-phenylenediol inhibited the TNF-α-induced NF-κB activation in a dose-dependent manner was

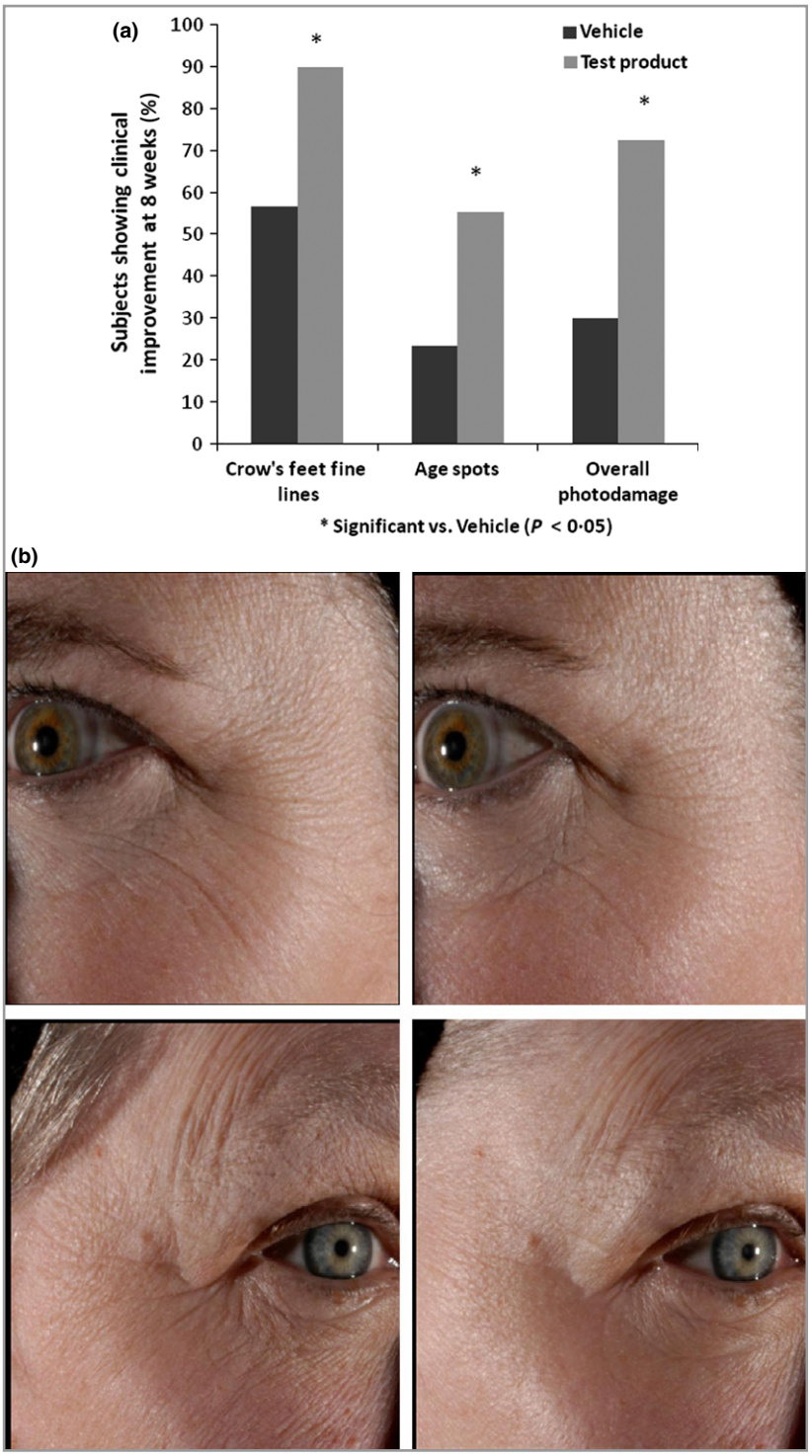

Fig 3. The use of 4-hexyl-1,3-phenylenediol leads to improvements in skin health and appearance in a clinical study. (a) Application of the test product containing 4-hexyl-1,3-phenylenediol resulted in significant clinical improvement in crow's feet fine lines, age spots and overall photodamage compared with the placebo at 8 weeks. Changes were determined by dermatologist grading. (b) Digital photographs of the faces of two panellists at baseline and after 8 weeks' application of the test product show an improvement in crow's feet fine lines and wrinkles. Top, Panellist 1; bottom, Panellist 2. Left, baseline; right, after 8 weeks of treatment with 4-hexyl-1,3-phenylenediol.

comparable with Bay 11-7082, a pharmacological inhibitor of NF-κB (Fig. 1b). This finding is consistent with a recent report showing that the compound inhibits NF-κB by blocking phosphorylation of NF-κB.[32] As phosphorylation is required for the activation of NF-κB, this finding suggests that NF-κB would be inhibited. In the current study, 4-hexyl-1,3-phenylenediol was shown to inhibit NF-κB activation using a functional assay. The combination of these results provides clear evidence that the compound can inhibit NF-κB activation. Similar observations were made in primary human fibroblasts, confirming the induction of NF-κB promoter activity by TNF-α treatment, and the ability of 4-hexyl-1,3-phenylenediol to block this activation (data not shown).

In order to confirm the downstream effects of NF-κB inhibition on ECM genes, primary human dermal fibroblasts obtained from young (< 50 years old; n = 3) and older (> 51 years old; n = 3) donors were treated with TNF-α, which led to a dose-dependent downregulation of collagen 1a

and elastin gene expression (Fig. 1c). Pretreatment of the fibroblasts with 4-hexyl-1,3-phenylenediol directly induced the expression of elastin and collagen 1a genes, and restored the levels of these ECM genes inhibited by TNF-α treatment in cells from young and older donors (Fig. 2a,b). Additional studies conducted in normal fibroblasts showed that treatment of the cells with TNF-α lowered collagen 1a protein levels (as measured by ELISA), and this effect was reversed by pretreatment with 4-hexyl-1,3-phenylenediol (Fig. 2c).

To provide further confirmation of the ECM restoration effects of 4-hexyl-1,3-phenylenediol, studies were performed using full-thickness human skin explants, which better represent the physiological complexity of the skin. Human skin biopsies, obtained with informed consent from healthy donors undergoing abdominal surgeries, were topically treated with TNF-α in the absence or presence of 4-hexyl-1,3-phenylenediol at days 2 and 3, or remained untreated. Explants were harvested at day 4, followed by the mechanical separation of the epidermis and dermis. The dermal tissues were processed for quantitative real-time PCR. As expected, treatment of human skin explants with TNF-α resulted in the inhibition of collagen 1a and elastin genes, while the presence of 4-hexyl-1,3-phenylenediol reversed the downregulation of these ECM genes (data not shown). It is also noteworthy that this NF-κB inhibitor, when used alone, significantly enhanced the levels of elastin and collagen 1a genes in primary dermal fibroblasts obtained from young and older donors. Taken together, these results demonstrate that NF-κB activation in the skin increases as a function of age and that an NF-κB inhibitor can reverse the NF-κB-mediated loss of ECM gene expression. These findings suggest that treatment of the skin with 4-hexyl-1,3-phenylenediol *in vivo* may also result in a sustained level of matrix protein production even in the presence of harmful stimuli, such as UV, which activate the NF-κB pathway. In addition, studies carried out by other researchers have linked NF-κB inhibition to epidermal proliferation,[33–35] so it is possible that topical treatment with 4-hexyl-1,3-phenylenediol might also lead to epidermal proliferation.

Although the exact mechanism by which NF-κB directly downregulates ECM proteins is not completely understood, other studies have shown that low levels of type 1 collagen in aged skin fibroblasts can be attributed to the inhibition genes such as *COL1A1*, and that the NF-κB pathway downregulates *COL1A1* expression via transcriptional control of the -112/-61-bp sequence.[36] Chromatin immunoprecipitation (ChIP) and re-ChIP assays showed that NF-κB and other common transcription factors (Sp1/Sp3/hc-Krox) bind and/or are recruited on the proximal promoter of *COL1A1*.[36] Furthermore, it seems that Sp1/Sp3/hc-Krox and p65 interact in order for NF-κB to demonstrate an inhibitory effect on *COL1A1*.[36]

To determine whether inhibition of NF-κB could affect the appearance of photodamaged skin, the efficacy of a topical lotion containing 4-hexyl-1,3-phenylenediol was assessed in a clinical study. On average, the subjects who used 4-hexyl-1,3-phenylenediol experienced a 2-point increase in skin radiance after 8 weeks of treatment with 4-hexyl-1,3-phenylenediol. On average, a 1-point improvement in the appearance of crow's feet fine lines, mottled pigmentation and age spots was also gained by subjects who used 4-hexyl-1,3-phenylenediol. The percentage improvement for each of the aforementioned parameters was also shown to be significantly higher in the 4-hexyl-1,3-phenylenediol group when compared with the vehicle control group. In addition, the clinical parameters of crow's fine lines and cheek wrinkles also showed a significant clinical improvement when compared with the control group. Finally, the percentage of subjects who responded to treatment within the above parameters was also significant ($P < 0{\cdot}05$). These conclusions are supported by two additional double-blind, placebo-controlled studies, which have been recently presented and concluded that 4-hexyl-1,3-phenylenediol significantly improved photodamaged skin and clinical signs of ageing.[37,38] Taken together, these data demonstrate that 4-hexyl-1,3-phenylenediol reduced the signs of skin ageing, while improving overall skin health, by inhibiting the NF-κB pathway and restoring the production of skin ECM proteins to a normal level.

As UV from the sun is continually bombarding the skin, it is constantly under stress, resulting in inflammation. Although some of this inflammation is necessary to maintain and repair the skin, much of it can be associated with long-term photodamage and premature signs of skin ageing. While some of these signs may be relieved by taking precautions to minimize exposure to UV, it is nearly impossible to avoid the damaging effects of UV in their entirety. While collagen and elastin expression by fibroblasts has been shown to be regulated by NF-κB, preliminary evidence suggests that other ECM proteins, such as hyaluronic acid, may similarly be regulated by NF-κB (data not shown). Furthermore, NF-κB inhibition has been reported to induce epidermal proliferation,[33–35] which suggests that NF-κB may regulate multiple epidermal targets of photoageing.

It could be speculated that inflammation may shunt epidermal keratinocytes from a 'healthy state', characterized by proliferation and ECM protein expression, to an 'aged state', characterized by decreased proliferation, decreased ECM production and increased ECM degradation, through the activation of NF-κB. Furthermore, the inflammation process initiated by activation of NF-κB may deprive the cells of the energy needed for cell proliferation and alternatively cause cells to induce inflammatory protein expression. By inhibiting a major pathway responsible for the inflammatory response in the skin and for the loss of matrix proteins, 4-hexyl-1,3-phenylenediol has been shown to improve several of the signs of photoaged skin. As such, 4-hexyl-1,3-phenylenediol has demonstrated great potential as a dermatological tool that can mitigate the signs of premature ageing in patients.

Acknowledgments

The authors would like to thank Dr Paul Khavari of the Department of Dermatology, Stanford University for discus-

sions on NF-κB, and Kristopher DeYoung of Kaztronix, LLC, for writing and editorial assistance in the preparation of this manuscript. The authors would also like to thank David Lewin, of Statistically Speaking Consulting LLC, for statistical analysis of the study data. The authors are especially grateful to Warren Wallo, of Johnson & Johnson for guidance and discussions on this project.

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