Epidermal and Dermal Hallmarks of Photoaging are Prevented by Treatment with Night Serum Containing Melatonin, Bakuchiol, and Ascorbyl Tetraisopalmitate: In Vitro and Ex Vivo Studies

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ABSTRACT

Introduction: Photoaging is a complex process that is chiefly the result of oxidative stress caused by ultraviolet (UV)-generated reactive oxygen species. To counter this process, we developed a 3-in-1 night facial serum (3-in-1 NFS) containing a combination of direct and indirect antioxidants and polyphenols that is designed to attenuate UV-generated free radicals and stimulate dermal protein synthesis. In clinical trials 3-in-1 NFS improved the appearance of photoaged skin. In this study we sought to identify some of the main histologic changes responsible for this.

Methods: We performed an immunolabeling analysis of some of the salient epidermal and dermal proteins in 3-in-1 NFS-treated primary epidermal keratinocytes (HEKs) and dermal fibroblasts (HDFs) in vitro, and in UV-exposed skin explants ex vivo. Numbers of apoptotic sunburn cells following exposure of 3-in-1 NFS-treated skin explants to UV radiation were also determined.

Results: We demonstrate that 3-in-1 NFS increases levels of filaggrin and aquaporin 3 in HEKs, and levels of collagen I and collagen III in HDFs in vitro. In clinical trials 3-in-1 NFS improved the appearance of photoaged skin. In this study we sought to identify some of the main histologic changes responsible for this.

Conclusion: Our results show that some of the histological hallmarks of photoaging are improved with the use of 3-in-1 NFS.

Keywords: Antioxidant; Ascorbyl tetraisopalmitate; Bakuchiol; Melatonin; Polyphenol; Photoaging; Skin aging; Ultraviolet

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Key Summary Points

Why carry out this study?
Facial photoaging significantly impacts quality of life and self-esteem. Effective and well-tolerated anti-photoaging treatments are therefore needed.

To identify some of the main histologic changes responsible for the clinical improvement seen in photoaged skin following 3-in-1 NFS treatment.

What was learned from the study?
Use of 3-in-1 NFS increases the expression of proteins that restore the skin’s barrier function, hydration levels, and help reverse dermal protein atrophy.

The components of 3-in-1 NFS synergize to uniquely protect the skin from the effects of UV radiation.

INTRODUCTION

Skin serves as interface between the body’s internal structures and its external environment, and is the site upon which the cumulative effects of age and its external environment visibly converge. These extrinsic factors cause gross morphological and physiological changes in skin that superimpose upon the natural aging process. The most important environmental factor leading to extrinsically aged skin is solar ultraviolet radiation (UVR). Chronic exposure of skin to solar UVR leads to a premature aging phenotype characterized by deep wrinkles, laxity, and a leathery appearance known as photoaging [1].

Photoaged skin is characterized by massive loss of dermal collagen that is the net result of reduced type I and III procollagen synthesis [2] and increased degradation of mature collagen by metalloproteinases (MMPs) [3]. This results in irregular distribution of collagen within the dermis with areas completely devoid of collagen fibers adjacent to dense clumps of collagenous material [4]. UVR exposure also reduces the synthesis of elastin and increases elastic fiber degradation [5]. Additionally, disorganized and non-functional elastic fibers accumulate within the dermis, making photoaged skin appear yellow and leathery [6]. Together the breakdown of the normal collagen and elastin reduces skin’s elasticity and tensile strength, resulting in sagging and wrinkling [7, 8]. Chronic sun exposure also perturbs the structural integrity of the top layer of the epidermis, the stratum corneum (SC), and alters its hydration and lipid properties, as well as its thickness, color, and light-absorbing properties [9–11].

Crucially both the epidermis and dermis are capable of self-repair and this natural process can be augmented by application of exogenous substances that stimulate the skin’s homeostatic responses. To date, only retinoids have been shown to unequivocally improve the appearance of photoaged skin [12]. Their use, however, is compromised by undesirable side effects such as pruritus, burning, erythema, peeling, and photosensitivity [12]. To address this, we developed a serum-in-oil night facial serum (3-in-1 NFS) that incorporates three ingredients which actively support the biological processes compromised in photoaged skin. Melatonin (5-acetyl-5-methoxytryptamine) is a pineal hormone that acts as both an indirect antioxidant, by inducing antioxidative gene expression, and a free radical scavenger [13, 14]. Bakuchiol (4-[(1E,3S)-3-ethenyl-3,7-dimethyl-1,6-octadien-1-yl]phenol) is a naturally occurring phenolic compound with retinol-like properties [15]. Bakuchiol induces collagen expression in cultured fibroblasts and activates the expression of genes involved in antioxidant defense [15, 16]. Bakuchiol induces collagen expression in cultured fibroblasts and activates the expression of genes involved in antioxidant defense [15, 16]. Bakuchiol is also thought to act as a direct free radical scavenger [17]. Clinically, bakuchiol was shown to improve signs of photoaging, including wrinkling and hyperpigmentation, to a degree comparable to retinol [18]. Ascorbyl tetraisopalmitate (ATIP) is a lipophilic and non-oxidizable form of vitamin C [19]. As well as possessing antioxidant and anti-inflammatory properties, ATIP also increases the skin’s hydration and smoothness [19, 20]. Clinically these three components resulted in skin that...
was firmer, less wrinkled, and more hydrated in subjects after nightly application of the serum for 3 months [21]. In order to identify some of the histological changes likely responsible for this, we performed an immunocytochemical analysis of primary human keratinocytes and fibroblasts cultured in the presence of different concentrations of 3-in-1 NFS, and an immunohistochemical analysis of ultraviolet (UV)-exposed skin explants topically treated with 3-in-1 NFS and each of its constituents in isolation.

METHODS

In Vitro Study

Cell Culture and Immunocytochemistry

Primary adult human epithelial keratinocytes (HEKs) and primary human dermal face fibroblasts (HDFs) were incubated with 500 µL/well of 3-in-1 NFS-containing media (0.05%, 0.1%, 0.5%, and 1%) for 24 h at 37 °C. Following 3-in-1 NFS treatment, cells were fixed and stained with the following primary antibodies: rabbit anti-collagen I (#20111, Novotec, Bron, France), rabbit anti-collagen III (#20311, Novotec, Bron, France), rabbit anti-laminin (#24811, Novotec, Bron, France), rabbit anti-fibronectin (#24911, Novotec, Bron, France), rabbit anti-aquaporin 3 (#ab153694, Abcam, Cambridge, UK), and rabbit anti-filaggrin (#PAJ103Hu01, Cloud-Clone Corp, Katy, TX) overnight at 37 °C. Cells were washed and then incubated with secondary antibody (Alexa Fluor 488 goat anti-rabbit IgG [#A-11008, Thermo Fisher Scientific, Waltham, MA]) at room temperature for 2 h. Finally, cells were incubated with DAPI (for nucleic acid staining) at room temperature for 10 min. Five independent experiments were performed for each condition.

Image Acquisition and Analysis

Fluorescence images were obtained using a Nikon Ti-S microscope (Nikon, Tokyo, Japan) and the fluorescent signal was quantified using MetaMorph® software (Molecular Devices, San Jose, CA, USA). For collagen I/III, laminin, and fibronectin, values were calculated as fluorescence intensity of the protein/nucleus number. For aquaporin 3 (AQP3) and filaggrin (FLG) it was calculated as fluorescence intensity of the protein/fluorescence intensity of DAPI (nucleus). Differences between untreated control and treated cells were determined by means of a two-tailed Student’s t test. A p value less than 0.05 was considered significant.

Ex Vivo Study

Antiaging Treatment

Abdominal skin explants (NativeSkin®, Genoskin, Toulouse, France) were generated from two healthy Caucasian women (30 and 32 years of age, Fitzpatrick type II) undergoing abdominoplasty. Two explants per donor were used for subsequent studies.

Anti-photoaging effects of the following formulations were assessed: (1) 3-in-1 NFS, (2) vehicle, (3) ATIP, (4) melatonin, (5) bakuchiol, and (6) the combination of melatonin and bakuchiol (Mel/Bak). ATIP, melatonin, bakuchiol, and Mel/Bak were used at the same concentrations as those in 3-in-1 NFS and in the same vehicle (see Table 1 for full vehicle composition). Briefly, 10 µL of each test item was evenly applied to the surface of NativeSkin® units 1 h after UV radiation (12.5 J/cm² UVA and 50 mJ/cm² UVB) for 4 consecutive days. A silicone ring was firmly fixed onto the biopsy to prevent topically applied formulations from leaking into the culture medium. Before each application, a cotton swab was used to remove the previous day’s treatment and the surface of the explant was washed twice with PBS. Following the fourth UV treatment cycle, formulations were left on the surface of the skin for 2 h prior to fixation and paraffin embedding.

| Composition |
|--------------|
| Vehicle: Caprylic/capric triglyceride, dicaprylyl carbonate, squalane, alcohol denat., caprylyl glycol, 1,2-hexanediol, PEG-8; aqua, tocopherol, ascorbyl palmitate, citric acid, ascorbic acid |

Table 1  Vehicle composition
Quantification of Sunburn Cells

Five-micron skin sections were stained with hematoxylin and eosin (H&E). Representative images were then captured using a NanoZoomer S360 Digital Slide Scanner (Hamamatsu Photonics, Hamamatsu, Japan). The number of sunburn cells (SBCs) (keratinocytes with pyknotic nuclei) in each section was visually determined. A total of 40 images were analyzed per experimental condition (10 images per replicate; 2 replicates per donor; 2 donors per experimental condition). For each replicate, donor and experimental condition, the mean ± standard error (SEM) was calculated. Data was analyzed by Dunnett’s multiple comparisons test. A p value less than 0.05 was considered significant.

Immunohistochemistry

Five-micron skin sections were stained with rat anti-procollagen type I (#ab64409, Abcam, Cambridge, UK) and rabbit anti-tropoelastin (#ab21600, Abcam, Cambridge, UK) antibodies overnight at 4 °C. Slides were washed and then incubated with secondary antibodies (goat anti-rat Alexa 546 [#A-11081, Invitrogen, Carlsbad, CA] or goat anti-rabbit Alexa 488 [#ab150077, Abcam, Cambridge, UK]) for 1 h at room temperature and counterstaining of cell nuclei was performed using DAPI. For all experimental conditions, a control incubated without primary antibodies was included.

Image Acquisition and Analysis

Immunostained slides were mounted with anti-fading medium (Fluoromount™, Sigma Aldrich, St. Louis, MO) and imaged with a Nanozoomer S360 Digital Slide Scanner (Hamamatsu Photonics, Hamamatsu, Japan) and NDP.view2 software (Hamamatsu Photonics, Hamamatsu, Japan). Procollagen type I and tropoelastin expression was determined by calculating the percentage area of the dermis using Image J software (NIH, Bethesda, MD). A total of 64 images per experimental condition were analyzed (2 donors per experimental condition; 2 skin explants per donor; 2 tissue sections per explant; 8 images per tissue section). Statistical analysis was performed considering the mean values of both donors using a Games-Howell post hoc test to compare between conditions. A p value less than 0.05 was considered significant.

Ethical Approval and Informed Consent

All human skin explants used in this study were obtained from abdominal surgical residues after written informed consent from the donors and in full respect of the Declaration of Helsinki and article L.1245 of the French Public Health Code [22]. The latter does not require any prior authorization by an ethics committee for use of surgical waste.

RESULTS

In Vitro Study

Effect of 3-in-1 NFS on FLG and AQP3 Expression

In clinical trials, 3-in-1 NFS was shown to reduce transepidermal water loss (TEWL) and increase hydration levels [21]. We therefore suspected that 3-in-1 NFS may influence the expression of key molecules involved in skin barrier formation and epidermal water transport, such as FLG [23] and AQP3 [24]. Expression levels of FLG and AQP3 were examined in primary adult HEKs cultured in media supplemented with 0.05%, 0.1%, 0.5%, and 1% 3-in-1 NFS. FLG expression was significantly increased in the presence of 3-in-1 NFS at all concentrations tested, with the greatest effects observed at 0.05% and 0.1% (Fig. 1a). A dose-dependent increase in AQP3 expression was observed (Fig. 1b).

Effect of 3-in-1 NFS on Dermal Proteins

Clinically, 3-in-1 NFS reduced facial wrinkles [21], suggesting it may influence dermal protein levels in photoaged skin. We therefore examined the effect of 3-in-1 NFS on the expression of dermal proteins by culturing primary HDFs in...
the presence of 3-in-1 NFS. Expression of collagen I and III, the most abundant dermal proteins, was higher in HDFs treated with 0.05% and 0.1% 3-in-1 NFS, but was inhibited at higher concentrations (Fig. 1c, d). Laminin, a major component of basement membranes that

**Fig. 1** Effect of 3-in-1 NFS on keratinocytes and fibroblasts. a FLG expression, b AQP3 expression, c collagen I expression, d collagen III expression, e laminin expression, and f fibronectin expression in fibroblasts treated with 3-in-1 NFS (0.05%, 0.1%, 0.5%, 1%). Graphs show mean protein expression levels (± SEM) of 5 independent experiments relative to those of untreated control cells (100%). *p < 0.05; **p < 0.01
separates the epidermis from the dermis [25], was only increased at 0.5% (Fig. 1e). Fibronectin, a large multicomponent glycoprotein that plays an important role in the organization and maintenance of the extracellular matrix (ECM) [26], was inhibited at both low (0.05% and 0.1%) and high concentrations (1%) of 3-in-1 NFS. Only 0.5% 3-in-1 NFS had no impact upon fibronectin expression (Fig. 1f).

**Ex Vivo Study**

**Effect of 3-in-1 NFS on Sunburn Cell Formation**

Explants treated with 3-in-1 NFS presented with significantly fewer SBCs following UV exposure than untreated skin explants (4.75 SBCs/donor vs. 8.5 SBCs/donor, respectively; \( p < 0.05 \)). Indeed, numbers of SBCs in 3-in-1 NFS-treated skin were similar to those in non-irradiated skin (3.5 SBCs/donor; \( p = \) not significant [n.s.]). Conversely, the number of SBCs in vehicle-treated skin (9.75 SBC/donor; \( p = \) n.s.), and in skin treated with the individual components of 3-in-1 NFS alone (melatonin 9.25 SBC/donor; bakuchiol 10 SBC/donor; ATIP 9 SBC/donor; \( p = \) n.s., all), or the combination of melatonin and bakuchiol (9.25 SBC/donor; \( p = \) n.s.) were similar to those of untreated UV-exposed skin (Fig. 2).

**Effect of 3-in-1 NFS on Collagen Biosynthesis**

Our in vitro study suggested that 3-in-1 NFS may help restore collagen deficit in photoaged skin by stimulating new collagen synthesis in dermal fibroblasts. To determine whether this effect was also observed in photoaged skin when topically applied, we sought to examine the effect of 3-in-1 NFS on the precursor of collagen I, procollagen type I, in skin explants chronically exposed to UVR.

Treatment with 3-in-1 NFS increased the expression of procollagen type I in irradiated skin explants by 78.8% (\( p < 0.01 \)) relative to untreated irradiated skin explants (Fig. 3a, b). Interestingly, neither the individual components of 3-in-1 NFS (ATIP + 1.88%; bakuchiol – 9.27%; melatonin – 4.10%; \( p = \) n.s., all) nor the combination of melatonin and bakuchiol (+ 11.0%, \( p = \) n.s.) had an effect on procollagen expression (Fig. 3a). Moreover, only 3-in-1 NFS was able to increase procollagen type I levels beyond those in unirradiated skin (+ 45.5%, \( p = \) n.s.).

**Effect of 3-in-1 NFS on Tropoelastin Levels**

Treatment with 3-in-1 NFS also increased expression levels of tropoelastin in the dermis of irradiated skin explants. Compared to untreated irradiated skin, tropoelastin expression was increased by 95.8% (\( p < 0.05 \)) (Fig. 4a, b). As observed for procollagen type I, the individual components of 3-in-1 NFS had no effect on tropoelastin expression (ATIP + 5.92%; bakuchiol – 3.97%; melatonin – 6.90%; \( p = \) n.s., all) (Fig. 4a). Tropoelastin expression in skin treated with the combination of melatonin and bakuchiol was increased with respect to untreated irradiated skin (+ 35.1%, \( p < 0.05 \)), but not to the same extent as 3-in-1 NFS (Fig. 4a). Moreover, only 3-in-1 NFS increased dermal tropoelastin levels beyond those in unirradiated control skin (+ 69.8%, \( p < 0.05 \)).

**DISCUSSION**

In clinical studies, 3-in-1 NFS, a night facial serum, comprising a direct antioxidant (ATIP), an indirect antioxidant (melatonin), and a polyphenol (bakuchiol), was shown to reduce facial wrinkles and improve the barrier properties and hydration levels of photoaged skin [21]. In this study we sought to understand the histological events responsible for this.

Skin serves a vital role in maintaining homeostasis by limiting passive water loss from the body and reducing the impact of chemical, physical, and microbial insults in its environment. These defensive functions are an inherent property of the SC [27, 28]. Thus, proper development and maintenance of the SC is paramount. By increasing FLG expression, a key molecule in SC formation [29], our results suggest that 3-in-1 NFS may help restore epidermal skin barrier integrity in photoaged skin. The clinically reduced TEWL following 3-in-1 NFS treatment is presumably a direct reflection of this. FLG also plays an important role in
maintaining proper epidermal hydration levels following its proteolysis to produce natural moisturizing factors such as urocanic acid (UCA) and pyrrolidone carboxylic acid [30]. The upregulation of both FLG and AQP3, an aquaglyceroporin that transports water, urea, and glycerol into the cells of the epidermis [31], observed in vitro upon 3-in-1 NFS treatment may thus account for the clinically improved hydration levels.

A hallmark event of UV exposure is the occurrence of SBCs within the epidermis [32, 33]. SBCs are keratinocytes undergoing apoptosis as a result of irreparable damage to their DNA [34]. The fact that 3-in-1 NFS significantly reduced their formation in the epidermis of UV-exposed skin explants suggests that it limits the damaging effects of UVR. Whether this protection is a direct consequence of the improved barrier function of 3-in-1 NFS-treated skin or an indirect consequence of higher antioxidant levels [35], modulation of apoptotic signaling pathways [36], or greater expression of UVB-absorbing UCA within the SC [23] remains to be established. What is clear, however, is that this photoprotective effect is a unique
consequence of the combination of melatonin, bakuchiol, and ATIP, since none of these components individually had an effect on SBC formation. Antioxidant synergism between melatonin and vitamin C has previously been reported, with melatonin thought to reverse the pro-oxidant activity of vitamin C [37]; however further studies are warranted to better understand the synergistic photoprotection afforded by this combination of ingredients.

After 3 months of nightly use, 3-in-1 NFS significantly reduced facial wrinkles [21]. The wrinkling and reduced elasticity typical of photoaged skin are the result of a reduction in the amount of dermal ECM [38, 39]. Here we demonstrate that 3-in-1 NFS helps reverse this decline. Expression of both of collagen I and III was increased in HDFs, indicating that 3-in-1 NFS induces new collagen synthesis. This was confirmed in photo-irradiated skin explants, in which significant increases in precursor procollagen type I levels were observed when treated topically with 3-in-1 NFS. Additionally, we observed an increase in levels of tropoelastin, an elastin precursor, in this photoaged skin model. Notably, levels of both procollagen type I and control, UV UV-exposed untreated skin. b Representative images showing fluorescence staining of procollagen type I (red) in skin dermis from non-irradiated (left), UV-exposed skin (center), and 3-in-1 NFS-treated (right) skin. All images were taken at ×10 magnification.

Fig. 3 Effect of 3-in-1 NFS on procollagen type I expression in UV-exposed skin explants. a Mean area of the dermis positive for procollagen type I expression. Error bars correspond to the mean area (± SEM) of 64 images. Significance levels are calculated versus untreated UV-exposed skin (UV). **p < 0.01. NIC non-irradiated control, UV UV-exposed untreated skin. b Representative images showing fluorescence staining of procollagen type I (red) in skin dermis from non-irradiated skin (left), UV-exposed skin (center), and 3-in-1 NFS-treated skin (right). All images were taken at ×10 magnification.
tropoelastin were only significantly increased in ex vivo skin explants upon 3-in-1 NFS treatment and not by its individual components, alluding again to synergism between these ingredients. Moreover, its effects on dermal proteins appeared to be limited to stimulating collagen and elastin expression, since other ECM proteins, including laminin and fibronectin, were largely unaffected by 3-in-1 NFS.

Although induction of collagen and elastin synthesis is essential for increasing dermal collagen and elastin levels, it is not sufficient on its own for the formation of functional collagen and elastic fibers. Hence it would therefore be necessary to examine whether functional collagen and elastin fibers are formed upon 3-in-1 NFS treatment. The fact that facial wrinkling is reduced by 3-in-1 NFS, however, suggests that it does improve the mechanical and structural integrity of the skin, alluding to the formation of functional collagen and elastin fibers. Likewise, whilst the photo-irradiated skin explant

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**Fig. 4** Effect of 3-in-1 NFS on tropoelastin expression in UV-exposed skin explants. **a** Mean area of the dermis positive for tropoelastin expression. Error bars correspond to the mean area (± SEM) in 64 images. Significance levels are calculated versus untreated UV-exposed skin (UV); *p < 0.05. NIC non-irradiated control, UV UV-exposed untreated skin. **b** Representative images showing fluorescence staining of tropoelastin (green) in skin dermis/epidermis from non-irradiated skin (left), UV-exposed skin (center), and 3-in-1 NFS-treated (right) skin. All images were taken at ×10 magnification.
model used in this study effectively recapitulates a number of the principle histologic changes of photoaging, including modulating genes involved in epidermis development and ECM homeostasis (data not shown), the anti-photoaging effects of 3-in-1 NFS can only truly be gauged by determining its effects in vivo using histological samples from photoaged patients treated with 3-in-1 NFS.

CONCLUSION

Our immunocytochemical analyses of keratinocytes and fibroblasts, in combination with our histologic analysis of skin explants, suggests that some of the characteristic hallmarks of photoaging can be restored with regular use of 3-in-1 NFS. The increased expression of proteins that improve the skin’s barrier function and hydration levels, and upregulation of collagen and elastin expression within the dermis, in turn accounts for the positive effects on skin appearance and function clinically. Moreover, our data suggests that the combination of melatonin, bakuchiol, and ATIP together exerts a synergistic effect on skin biology in reversing signs of aging acquired as a result of UV exposure.

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Disclosures. Mridvika Narda and Corinne Granger are employees of ISDIN, the manufacturer of 3-in-1 NFS. Anthony Brown is a paid consultant to ISDIN. Béatrice Muscatelli-Groux and Jean A Grimaud are employees of Matriscience who were paid by ISDIN to perform the experimental work in this study.

Compliance with Ethics Guidelines. All human skin explants used in this study were obtained from abdominal surgical residues after written informed consent from the donors and in full respect of the Declaration of Helsinki and article L.1245 of the French Public Health Code [22]. The latter does not require any prior authorization by an ethics committee for use of surgical waste.

Data Availability. Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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