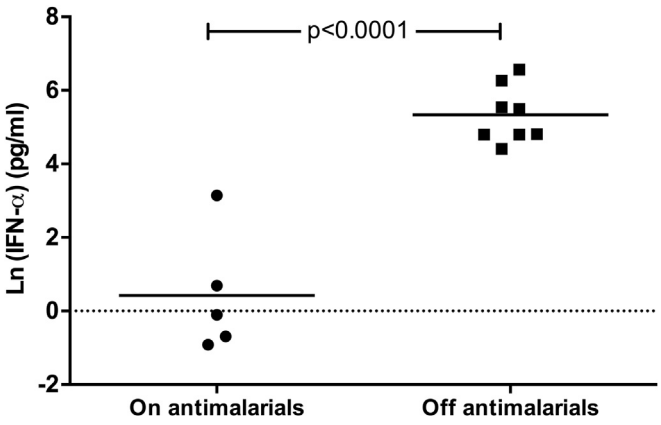

Figure 2. IFN-α secretion was reduced in the peripheral blood mononuclear cells of subjects taking antimalarial medications. Ln(IFN-α) secretion from peripheral blood mononuclear cells of dermatomyositis subjects receiving antimalarial medications and dermatomyositis subjects not taking antimalarial medications. Line represents the mean.

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Lipofuscin Generated by UVA Turns Keratinocytes Photosensitive to Visible Light

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TO THE EDITOR

Our society still faces challenges in the field of sun care. For instance, despite the massive campaign to promote the use of sunscreen in Australia, the rates of melanoma of Australians continue to rise (Czarnecki, 2016). Some studies point to the fact that exposition to solar wavelengths other than UV also affects human skin (Chiarelli-Neto et al., 2014; Mahmoud et al., 2010; Schieke et al., 2003). We report the combined damaging effects of UVA and visible light in immortalized human epidermal keratinocytes (HaCaT) and in Normal Human Primary Epidermal Keratinocytes isolated from Neonatal Foreskin (NHK). We aim to prove that UVA stimulates the accumulation of the age-pigment lipofuscin, which then acts as a visible-light photosensitizer.

Harmful effects caused by UVA radiation to eukaryotic cells have been thoroughly described (Ziegler et al., 1994). Both tested cell lines experienced a decrease in cell viability under increasing UVA doses (Supplementary Figure S1 online). Besides the usual mechanisms of cell death, UVA causes lysosomal damage and inhibits autophagy (Lamore and Wondrak, 2012, 2013).

Autophagy inhibition results in accumulation of lipofuscin (Brunk and Terman, 2002; Terman et al., 2010), which is a heterogeneous polymer made of oxidized biomolecules and traces of metals. Lipofuscin has light absorption extending to the red portion of the visible spectrum. Its accumulation has been correlated with deleterious effects of solar exposition in the eyes, but its role in skin phototoxicity has not been described (Gray and Woulfe, 2005; Haralampus-Grynaviski et al., 2003; Schweitzer et al., 2005). Transmission electron

Abbreviations: HaCaT, human epidermal keratinocytes; NHK, Normal Human Primary Epidermal Keratinocytes isolated from Neonatal Foreskin

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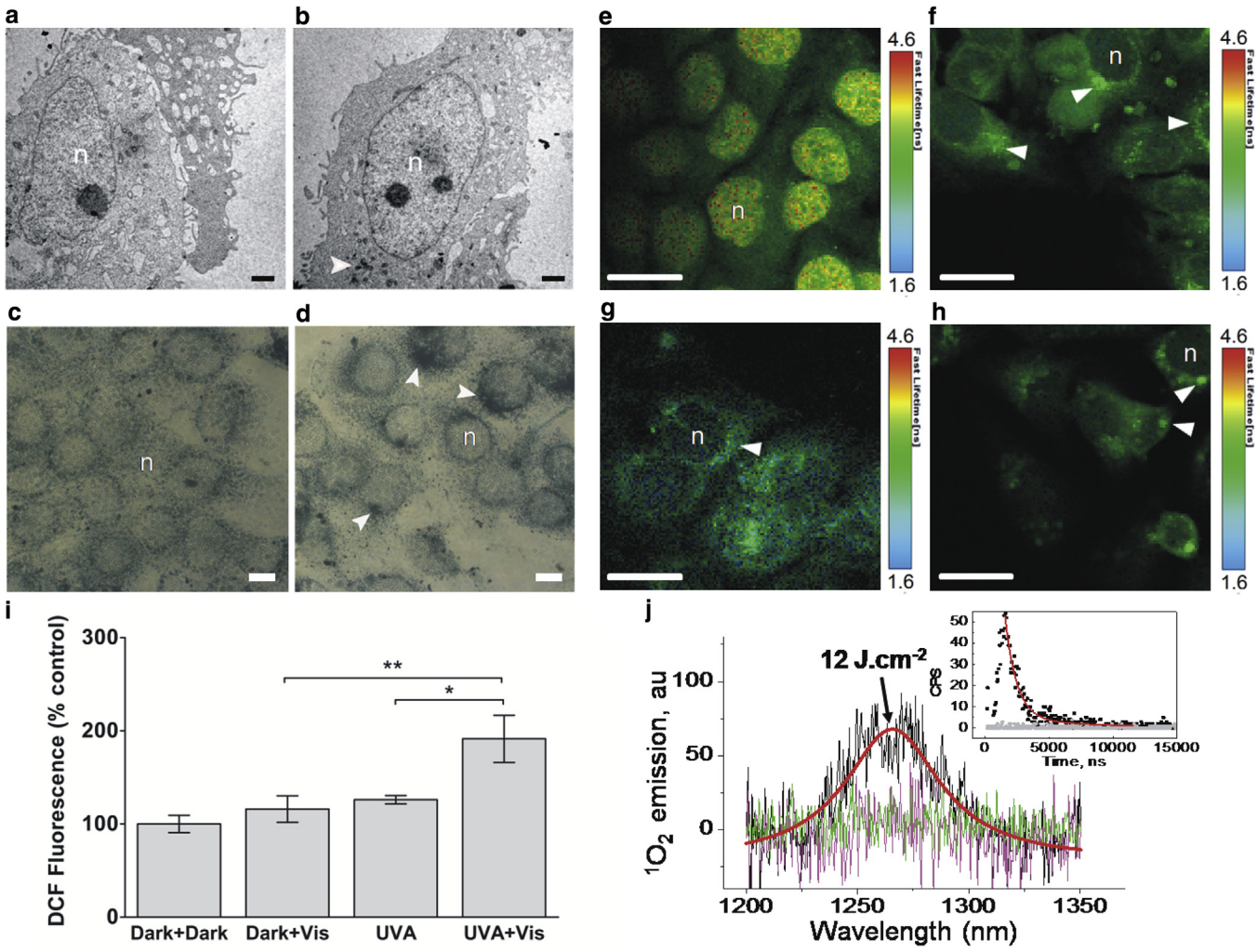

Figure 1. UVA (12 J cm^{-2}) induces the accumulation of lipofuscin that acts as a visible-light photosensitizer in HaCaT cells. Transmission electron microscopy of cells left in (**a**) the dark and (**b**) 48 hours after UVA; the arrowhead shows lipofuscin granules around the nucleus (n). Sudan black B staining of (**c**) dark control and (**d**) after UVA. Fluorescence lifetime images of (**e**) dark control, (**f**) after UVA, (**g**) after UVA with the subsequent incubation with 30 μM of deferiprone, and (**h**) 48 hours after treatment with 60 μM of chloroquine. (**i**) Dichlorofluorescein (DCF) emission (λ_{exc} = 485 nm and λ_{emi} > 510 nm) after irradiation protocols. *$P < 0.05$; **$P < 0.01$ (**j**) $^1O_2$ emission spectra by control cells (green), and by cells irradiated with visible light (pink) and with UVA (black); the inset shows emission decay at 1,270 nm from UVA-treated cells. CPS, counts per second.

microscopy, Sudan black B staining, and time-resolved fluorescence images (Figure 1a–h, Supplementary Figures S2–S6 online) show a considerable lipofuscin accumulation in both NHK and HaCaT cells 48 hours after irradiation with UVA. The percentage of cells presenting red fluorescence, which is typical of lipofuscin, increased from 6% in the control to 38% in cells pretreated with UVA (Supplementary Figure S2). The fluorescence lifetime obtained from lipofuscin granules was around 1.7 ns (Figure 1f), which is in agreement with previous reports (Schweitzer et al., 2005). The lipofuscinogenesis pattern was also observed in HaCaT cells upon the addition of chloroquine (Figure 1h), which is a classical autophagy inhibitor. This pattern was successfully inhibited by the addition of deferiprone (Figure 1g), which prevents formation of lipofuscin by chelating iron (Brunk and Terman, 2002). There is also a clear dose-dependent increase in lipofuscin accumulation in HaCaT and NHK cells treated with UVA (Supplementary Figures S4 and S5).

Lipofuscin acts as a photosensitizer, absorbing visible light and generating considerable amounts of triplet species and singlet oxygen ($^1O_2$) (Rózanowska et al., 1998). In fact, we observed an increase in dichlorofluorescein fluorescence after exposing UVA-irradiated HaCaT (Figure 1i) and NHK (Supplementary Figure S7 online) cells to visible light. Only HaCaT cells pretreated with UVA (neither dark control nor visible-irradiated cells) showed the characteristic $^1O_2$ emission (Chiarelli-Neto et al., 2011) (Figure 1j).

Both NHK and HaCaT cells were irradiated with 36 J cm^{-2} of visible light 48 hours after UVA (12 J cm^{-2}). Note that visible light becomes a lot more toxic to cells previously treated with UVA (Figure 2a and b). Note also that UVA plus visible is more toxic than visible plus UVA (Figure 2a and b), strengthening the hypothesis that UVA generates a sensitizer that enhances the effect of visible light. In addition, a considerable increase in DNA strand break was observed in HaCaT cells that

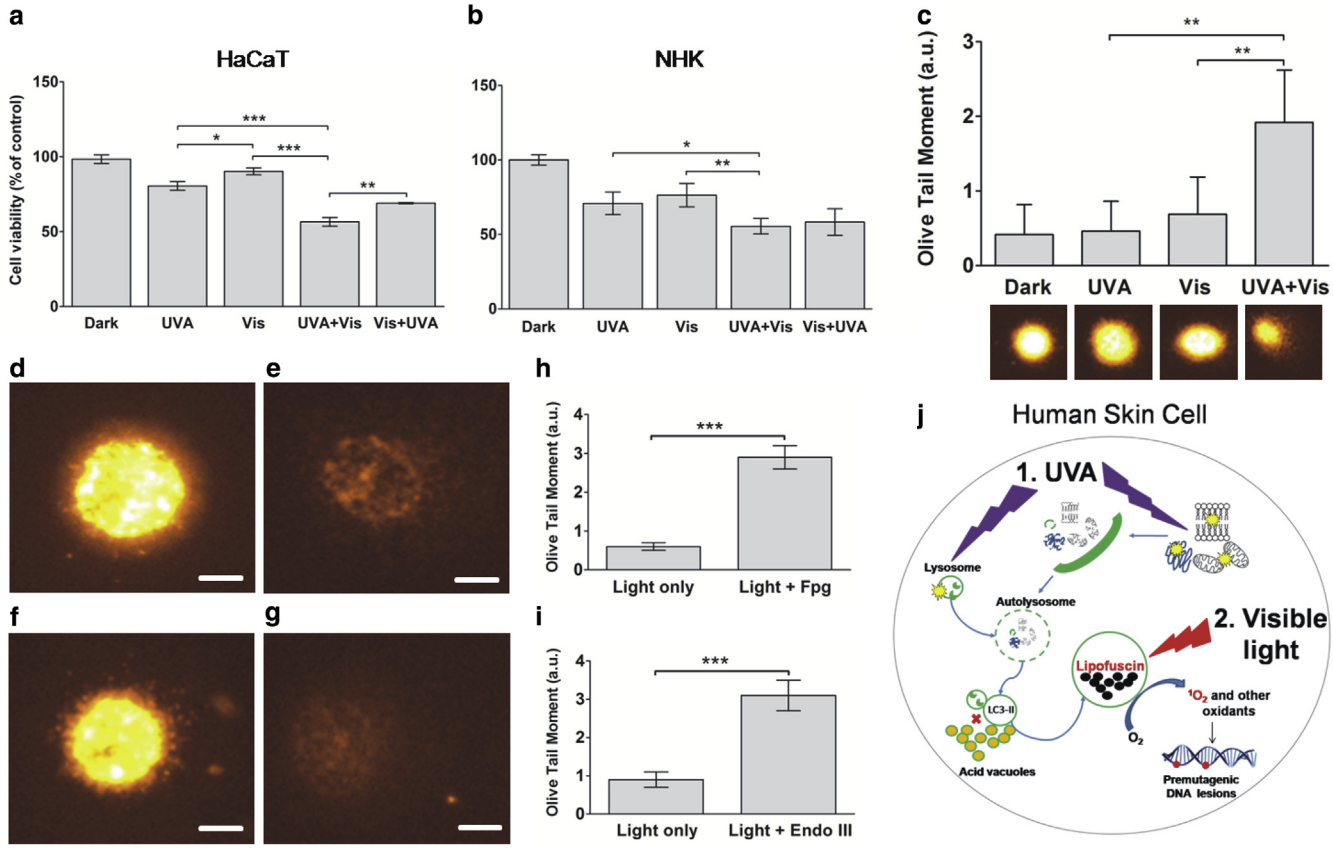

Figure 2. Visible light is phototoxic to lipofuscin-accumulating keratinocytes and causes oxidative DNA damage. Viability of (**a**) HaCaT and (**b**) NHK cells after different light treatments based on 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (visible = 36 J cm^{-2}, UVA = 12 J cm^{-2}). (**c**) DNA fragmentation (comet assay) after irradiation protocols in HaCaT cells with light doses as above. (**d**–**i**) Comet assays performed after the light treatment (UVA 6 J cm^{-2} and visible 8 J cm^{-2}) in the (**d, f**) absence and presence of (**e**) Fpg and (**g**) Endo III enzymes. Right: quantification of olive tail moment (OTM) in the experimental conditions shown above. (**h**) Quantification of (**d, e**) and (**i**) quantification of (**f, g**). (**j**) Schematic representation of our working hypothesis: UVA is absorbed by natural photosensitizers causing oxidative damage and hurting key organelles such as lysosome, inhibiting autophagy flux and favoring lipofuscin accumulation, which acts as a photosensitizer in the visible, releasing $^1O_2$ and damaging nuclear DNA. $^*P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$. Endo III, endonuclease-III; Fpg, formamidopyrimidine [fapy]-DNA glycosylase; HaCaT, human epidermal keratinocytes; NHK, Normal Human Primary Epidermal Keratinocytes isolated from Neonatal Foreskin.

were treated with both UVA and visible light (Figure 2c).

Further controls prove the role of lipofuscin in this increased toxicity with respect to visible light. As shown in Figure 1g and h, respectively, formation of lipofuscin is avoided by chelating intracellular iron or, alternatively, can be induced by chloroquine treatment. Indeed, the toxicity of visible light increased for cells previously treated with chloroquine (even though they had not been exposed to UVA), whereas cells treated with deferiprone no longer showed increased visible-light phototoxicity (even though they had been previously exposed to UVA; Supplementary Figure S8 online). The effect of deferiprone cannot be attributed to $^1O_2$ suppression, because deferiprone was added to the cell media at low concentration (30 μM) and only after cells were challenged with UVA. In addition, deferiprone is not exceptional in suppressing $^1O_2$, because the rate constant of $^1O_2$ suppression by deferiprone ($k \sim 10^6$ l mol^{-1} s^{-1}) is similar to that of other double bonds.

Although photodamage can have multiple intracellular targets, we selected for this study direct oxidation of nuclear DNA. After treating double-irradiated cells (subdoses of UVA and visible light were used; Supplementary Figure S9 online) with enzymes that recognize oxidation DNA lesions (Figure 2d–g), a clear increase in the Olive Tail Moment was observed, indicating the presence of premutagenic formamidopyrimidine [fapy]-DNA glycosylase- and endonuclease-III-sensitive DNA lesions (Figure 2h and i). Consequently, a more dangerous consequence of the synergistic action of UVA and visible light is the accumulation of premutagenic DNA lesions. The observed formamidopyrimidine [fapy]-DNA glycosylase:endonuclease-III ratio was close to 1:1 (compare Figure 2h with i). Formamidopyrimidine [fapy]-DNA glycosylase recognizes mainly $^1O_2$ products, that is, 8-oxo-7,8-dihydroguanine, whereas endonuclease-III recognizes strand breaks, abasic sites, and additional oxidative modifications (Hatahet et al., 1994). An oxidation process driven only by $^1O_2$ should provide a larger ratio of formamidopyrimidine [fapy]-DNA glycosylase- to endonuclease-III-sensitive modifications. Therefore, our results suggest that the oxidative damage in DNA is likely to involve not only $^1O_2$ but also radical-based reactions (Chiarelli-Neto et al., 2014).

Photoprotection is traditionally centered on the prevention of skin damage caused by exposure to UV (Czarnecki, 2016; Schieke et al., 2003; Ziegler et al., 1994). However, visible light can also act as an etiologic factor for skin photodamage (Chiarelli-Neto et al., 2014; Mahmoud et al., 2010). After exposition to UVA, skin cells can generate lipofuscin, which can in turn act as a potent visible-light photosensitizer, causing premutagenic lesions in nuclear DNA (Figure 2j). It is evident that this effect urgently demands the development of novel strategies for sun protection. At the same time, regulatory agencies should reconsider their current sun-protection policies to take into account the necessity for protection against a wider spectral range of sunlight. We hope our work will also stimulate new mechanistic investigations to further understand the combined effects of UVA and visible light (Ziegler et al., 1994).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.06.018.

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Spindle-Cell Variants of Primary Cutaneous Follicle Center B-Cell Lymphomas Are Germinal Center B-Cell Lymphomas by Gene Expression Profiling Using a Formalin-Fixed Paraffin-Embedded Specimen

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TO THE EDITOR

Spindle-cell variant of primary cutaneous follicle center B-cell lymphoma (PCFCL, sc-variant) is a rare cutaneous B-cell lymphoma that presents

Abbreviations: PCFCL, sc-variant, spindle-cell variant of primary cutaneous follicle center B-cell lymphoma; PCLBCL, leg type, primary cutaneous diffuse large B-cell lymphomas, leg type

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