Chapter 5

Photochemistry of Lipofuscin and the Interplay of UVA and Visible Light in Skin Photosensitivity

Carolina Santacruz-Perez, Paulo Newton Tonolli, Felipe Gustavo Ravagnani and Maurício S. Baptista

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Abstract

The topics about prevention against sunlight-induced damages and a secure threshold to light exposition have reached a bigger number of specialists in basic science and medical care. It has been accepted that ultraviolet light is very hazardous and visible light is safe, but recent studies from our group has shown that human keratinocytes exposed previously to ultraviolet A (UVA) light can generate an endogenous visible light-sensitive photosensitizer (lipofuscin), leading to higher levels of singlet oxygen, DNA damages and a wide-range of cellular insults due to intracellular lipofuscin accumulation. Disruption of cell death pathways and on essential metabolic processes, as autophagy and redox signaling, can collaborate to increase light-induced damages. We also discuss the importance of considering not only UVA but visible light too in protection against solar exposure as a way to prevent future pretumoral lesions.

Keywords: sun care, UVA radiation, visible light, lipofuscin, singlet oxygen, DNA damage

1. Introduction

The solar electromagnetic spectrum is composed of three main regions: ultraviolet (UV) (100–400 nm), visible (400–800 nm), and infrared (>800 nm). The UV range is subdivided into UVC (100–280 nm), UVB (280–315 nm), and UVA (315–400 nm), which show uneven ability to penetrate through the skin layers. UVC is broadly blocked by the ozone layer and has low penetration in epidermis rather than UVB and UVA. The UVB photons are directly absorbed by DNA, generating pyrimidine dimers which are well-known to promote carcinoma skin [1]. UVA and visible light are able to reach the deepest skin layers, as melanocytes layer. UVA has

been thoroughly studied and implicated in carcinogenesis, especially, melanomas [2]. UVA and visible light are well-known to promote the oxidative stress by photosensitization reactions, generating reactive oxygen species, and oxidatively damaging biomolecules and organelles [3]. Among these biomolecules is the DNA, which exposed to photosensitization, can induce premutagenic lesions, as 8-oxo-dG, leading to mutagenesis if not repaired by DNA repair system [4]. Visible light has similar photosensitization mechanisms to UVA and high penetrability in the skin, but poor attention has been given to this light source in relation to skin carcinogenesis processes.

The harmful effects of exposure to ultraviolet (UV) radiation are widely known nowadays and it depends on the time of exposure (chronic or acute) [5]. The excessive exposure to ultraviolet radiation reflects much more serious effects: premature aging of the skin, immunosuppression, damages to the eyes, and it can be also associated to different types of skin cancer [6–8]. Our society is becoming aware that sun exposition to other wavelengths of light (not UV only) has important consequences to the skin health [9–11]. However, the molecular mechanisms involved are only starting to be uncovered.

The Scientific Committee on Emerging and Newly Identified Health Risks published an opinion on light sensitivity, which identified ultraviolet radiation as a risk factor for the aggravation of the light-sensitive symptoms in some patients bearing the chronic diseases actinic dermatitis and solar urticaria [12]. Moreover, scientific evidence relies on the potential impacts on public health caused by the artificial light, including the visible light spectrum, like in jaundice and other photosensitizing conditions [13, 14]. It is necessary to study further the impacts of visible and invisible ranges of light as well as characterizing some molecules that can act as photosensitizing agents and reduce side-effects of UV radiation from sunlight or from another light source. Additionally, several parts of the known visible spectrum can generate photosensitivity. However, the severity of the effect depends on the wavelength, intensity, and time of exposure.

Lipofuscin is a subproduct of cross-link reactions among oxidized lipids, proteins, and organelles, accumulated mainly in lysosomal compartment during oxidative stress [15]. Lysosomal hydrolases cannot digest lipofuscin that accumulates, progressively, inside the cells as electron-dense granules around the nucleus, especially during the post-mitotic period. Besides that, lipofuscin granules can incorporate transition metals, such as iron and copper, catalyzing Fenton or like-Fenton reactions, which generate the hydroxyl radical, a highly reactive oxygen specie that attacks DNA, protein, and lipids [16]. Many studies have investigated the properties and photochemistry of lipofuscin in retinal pigment epithelium (RPE) cells [17–21]. The lipofuscin of RPE cells has absorption peaks in the ultraviolet B range (280–330 nm) with emission in 570–605 nm [19, 20] and also absorbs blue light (λ = 420 nm), generating singlet oxygen [21]. However, the cellular composition of lipids and proteins change among different cell types, assigning different photochemistry properties. Few studies have been done about lipofuscin in the human skin cells and its exact role in phototoxicity has never been described for that tissue.

In a recent publication by our group [22], we described that lipofuscin can act as an endogenous photosensitizer and lead to a higher sensitivity of human epidermal keratinocytes (HaCaT) to visible light. We observed that UVA dose of 12 J.cm^{-2} stimulates autophagy inhibition and lipofuscin accumulation in keratinocytes, which promote singlet oxygen production and induce

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photodamage in cells when exposed to the visible light source. Here, we revised the main publications in this area and we show why lipofuscin indeed act as an additional photosensitizer to visible light in primary and immortalized human skin keratinocytes (NHK and HaCaT, respectively). This photosensitization of lipofuscin was able to increase the photooxidative processes inside the cell, generating singlet oxygen and promoting important damages in nucleic acids (FPG and Endo III in comet assays to identify DNA lesions).

1.1. UVA radiation followed by visible light causes higher cytotoxicity

Harmful effects caused by UVA radiation to eukaryotic cells have been thoroughly described [23]. Here, we quantified cell viability based on the reduction of 3 (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent, using different UVA radiation doses over immortalized human epidermal keratinocytes (HaCaT) and keratinocytes isolated from neonatal foreskin (NHK). Cells were exposed to increasing doses of UVA light and 48 h after irradiation we observed that HaCaT and NHK cell survival were around 50% at doses of 12 J.cm^{-2} and 6 J.cm^{-2}, respectively (**Figure 1**). Depending on the light dose and cellular properties, such as the level of labile iron, cells will die mainly by necrosis and/or apoptosis [24]. However, live cells previously irradiated by UVA were then exposed to visible light and their survival rates were even lower than those ones obtained when we employed single light sources or inverted irradiation protocol (visible light – UVA) (**Figure 2**). This result led us to investigate whether UVA radiation could turn keratinocytes highly sensitive to visible light.

1.2. UVA radiation causes inhibition of autophagic flux and consequent lipofuscin accumulation

Profiles of apoptotic and necrotic cell death are very common in response to UVA exposure and they occur due to the prooxidant condition that severely decreases cell viability [25, 26].

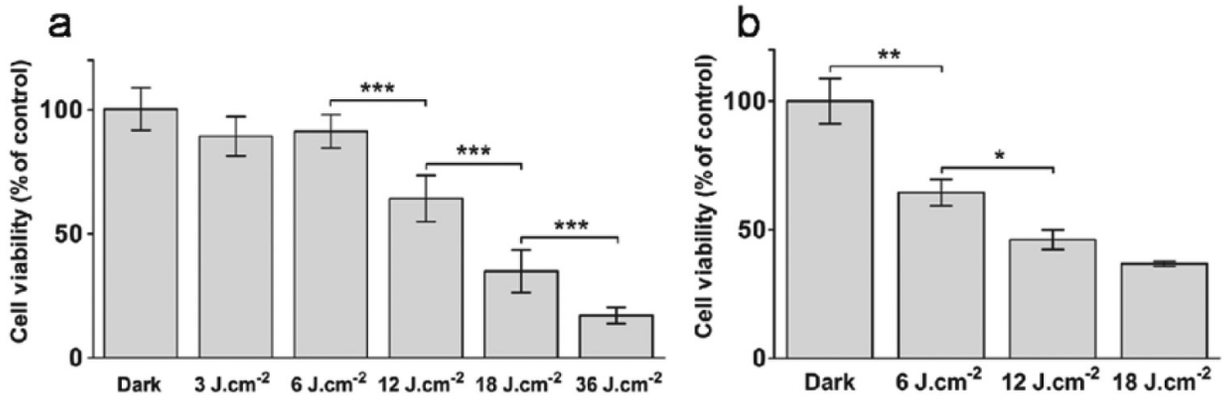

Figure 1. Viability curves as a function of UVA dose. Cell viability was measured 48 h after irradiation and is expressed as a percentage of MTT reduction by HaCaT (a) and NHK (b) cells. HaCaT and NHK cells reached survival of 50% at doses of 12 J.cm^{-2} and 6 J.cm^{-2}, respectively. Bars indicate mean ± standard deviation (SD) for three independent experiments. Statistical analysis was performed using SigmaStat v.3.5 (Abacus Concepts, Berkeley, CA), performing one-way ANOVA test followed by Holm-Sidak posttest. Statistically significant differences are shown for $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$(***). From [22] with permission.

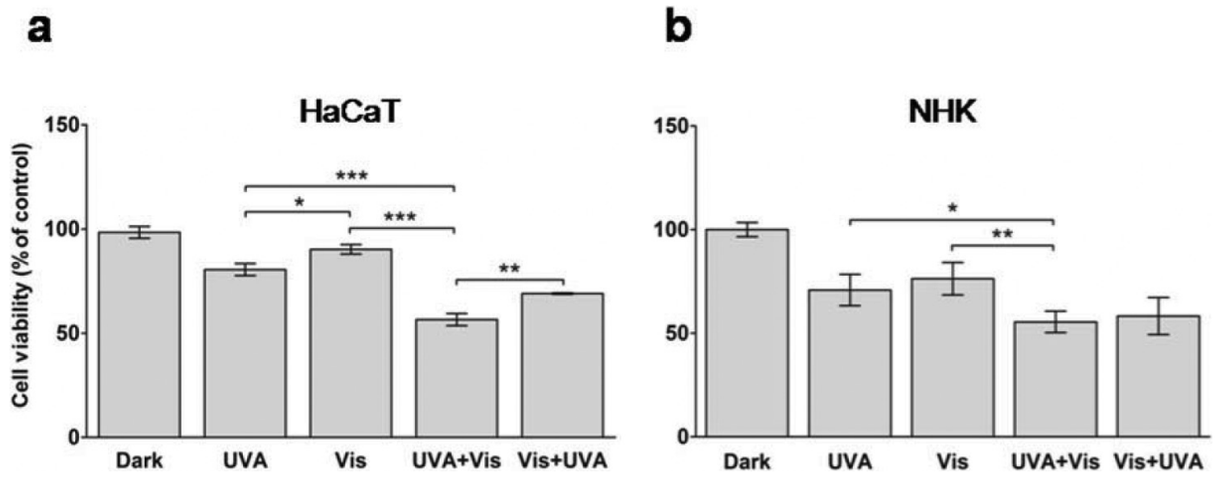

Figure 2. Viability of HaCaT (a) and NHK (b) cells after different light treatments based on MTT reduction (Visible = 36 J. cm^{-2}, UVA = 12 J.cm^{-2}). They were considered statistically significant differences for p-values <0.05 (*), <0.01(**) and < 0.001(***) in one-way ANOVA analysis. From [22] with permission.

There are few reports describing that redox misbalance and the formation of oxidized biomolecules can activate autophagy in normal and autophagy-deficient cells [27]. Lamore and co-workers showed that inactivation of lysosomal enzymes, such as cathepsins B and L, causes autophagic inhibition, with a consecutive accumulation of lipofuscin in dermal fibroblasts [28]. Therefore, UVA can lead to an efficient cell death concomitantly with autophagy or a sequential and temporal accumulation of lipofuscin in the survival cells, as previously suggested by Terman et al. [29] and Lamore and Wondrak [30].

We confirmed that lipofuscin accumulation in HaCaT cells after 48 h from exposure to UVA radiation at 12 J.cm^{-2}. It was possible to observe a five-fold higher values to the autofluorescence emission of lipofuscin in UVA-treated HaCaT. **Figure 3** shows this increment in both populations of cells (small and large ones). The scatter-plot contour of lipofuscin autofluorescence (FL1 Log) according to the cell size (FSC), obtained by flow cytometry in control cells and irradiated ones with UVA light. Average values of the population are represented in quadrant Q2 that characterize lipofuscin accumulation in UVA-treated cells (18 J.cm^{-2}) (**Figure 3b**) in relation to non-irradiated ones (dark) (**Figure 3a**).

The light absorption of lipofuscin in human skin keratinocytes extends from blue to green range of the visible spectrum. In fact, we detected a typical perinuclear fluorescence of lipofuscin (> 515 nm), when we excitate live cells with 450–490 nm. This fluorescence increased when primary and immortalized keratinocytes were exposed to growing doses of UVA, in a dose-dependent manner. Besides, lipofuscin-loaded keratinocytes showed a higher level of singlet oxygen generation when excitated at 490 nm instead of another wavelengths (**Figure 8**). Therefore, lipofuscin granules from human skin keratinocytes seem more sensitive to blue light. Performing the time-resolved fluorescence microscopy, we detected the fluorescence lifetime (FLT) for lipofuscin granules, indicating a fluorescence emission lifetime around to 1.7 ns (**Figure 4**).

Lipofuscin FLT was already related as the fastest lifetime component of typically 190 ps in the retinal pigment epithelium [31]. Considering histograms of lifetimes after fluorescence excitation

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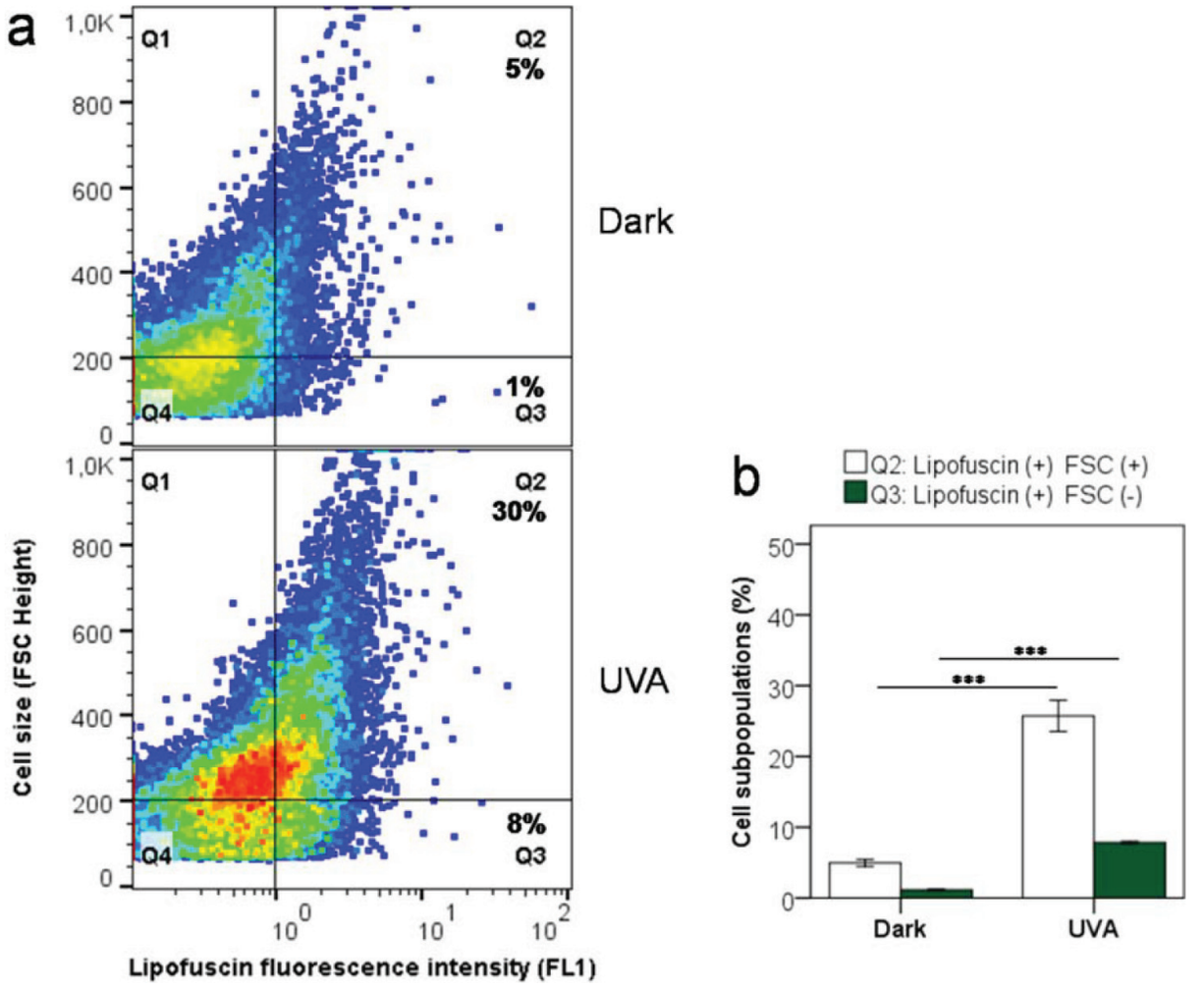

Figure 3. UVA radiation induces lipofuscin accumulation. (a) Scatter-plot contour of lipofuscin autofluorescence (FL1) versus forward-scattered light (FSC height), obtained by flow cytometry for dark control (upper diagram) and UVA-irradiated cells (18 J.cm^{-2}, lower diagram). (b) Mean values ± SD of cell subpopulations for dark control and UVA-irradiated represented in the quadrant Q2 {lipofuscin was accumulated [Lipofuscin (+)] and increased cell size [FSC (+)]}, and quadrant Q3 {lipofuscin was accumulated without increasing cell size [FSC (−)]}. Lipofuscin was excited at 488 nm and emission detected at 630 nm. Statistically significant differences were considered for $p < 0.001$(***). From reference [22] with permission.

and determined at the living human eye ground in the parapapillary region, a very low lifetime was calculated most frequently in the long-wavelength emission range ($\lambda > 500$ nm) [32]. Our experiments showed that lipofuscin-loaded HaCaT cells (irradiated with UVA 12 J.cm^{-2}) can accumulate higher and perinuclear lipofuscin granules than those cells experimenting apoptosis (irradiated with 18 J.cm^{-2}, data not shown). Different lipofuscin composition can be conceived from FLT analysis since three lifetimes were obtained from cells irradiated at 6 and 12 J.cm^{-2}, and four lifetimes from irradiated cells at 18 J.cm^{-2} of UVA light. A similar decrease of fluorescence lifetime has been observed for fixed and live cells [33]. No differences were found between FLT in artificially induced lipofuscin by another photosensitizer or by previous UVA light exposition (data not shown).

Accumulation of lipofuscin was also reported in dermal fibroblasts after UVA exposition [28]. We quantified lipofuscin fluorescence in HaCaT and NHK cells 48 h after the exposure to UVA

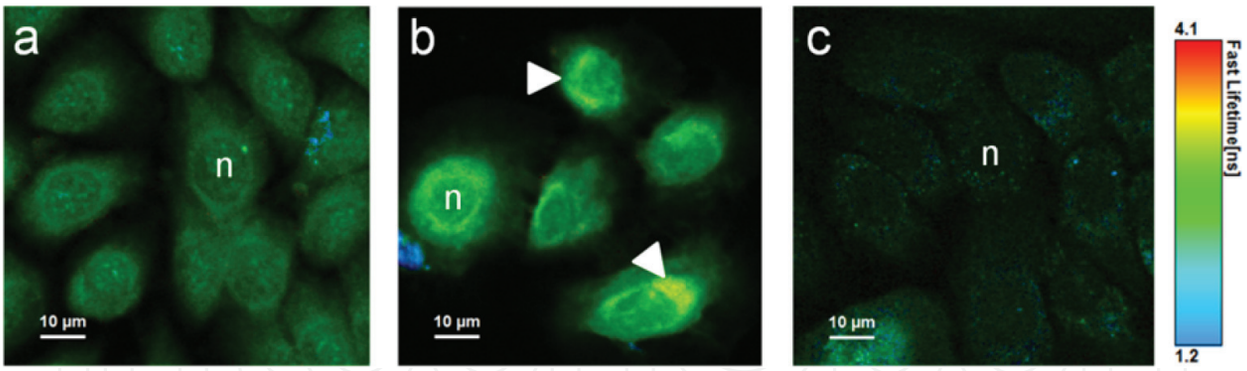

Figure 4. Lipofuscin accumulation and fluorescence lifetime in NHK cells. (a) Dark control. (b) NHK UVA-irradiated cells (6 J.cm^{-2}). White arrowheads indicate perinuclear aggregates of lipofuscin. (c) Visible light-irradiated cells (36 J.cm^{-2}). Images were obtained using a single molecule lifetime confocal microscope (Picoquant's Microtime 200). Samples were excited at 509 nm and emission was captured with a long-pass filter at 519 nm. Fluorescence decay curves of lipofuscin fluorophores were recorded by TCSPC mode. n = nucleus. From [22] with permission.

radiation (**Figure 5**), and we identified lipofuscin accumulation in the perinuclear region using Sudan Black B staining in both cell lineages (**Figure 6**).

Additionally, using transmission electron microscopy, we visualized electron-dense lipofuscin granules in the perinuclear region of HaCaT cells after 48 h from exposition to UVA radiation (**Figure 7**).

Although the accumulation of lipofuscin after UVA is evident, we needed further controls to definitively prove that it was lipofuscin photosensitization that caused an extra reduction in cell viability and not another mechanism induced by UVA. Note that UVA without formation of lipofuscin (inhibited by iron chelator) does not lead to a higher reduction in cell viability by visible light. The chemical induction of lipofuscin, employing a chemical

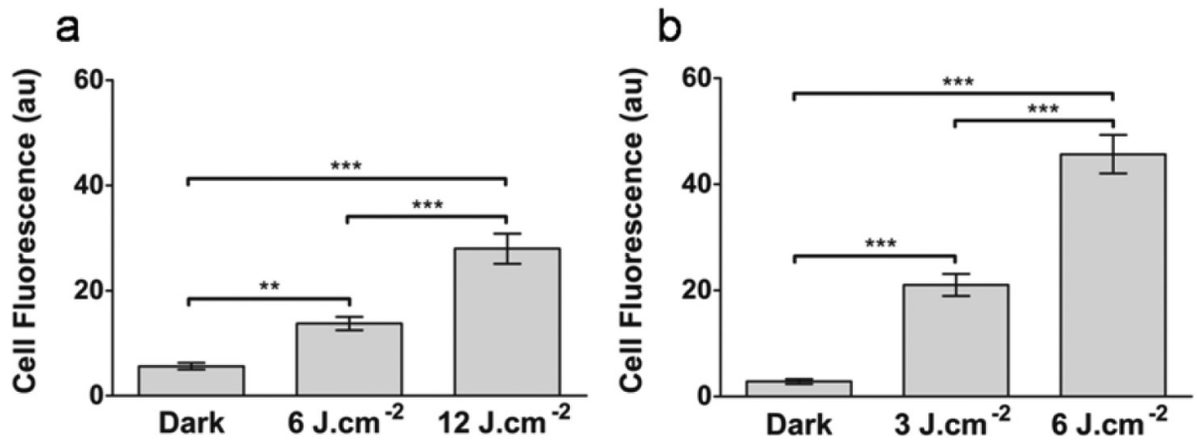

Figure 5. Lipofuscin fluorescence in HaCaT and NHK cells. Autofluorescence of HaCaT (a) and NHK (b) cells 48 h after exposure to UVA. Cell fluorescence was measured from TIF images, converted to 16-bit, and delimiting fluorescent area with polygonal selection, obtaining data per area, mean gray value, and integrated density from different images for each sample (HaCaT dark, 6 and 12 J.cm^{-2}; n = 11, n = 25, n = 20, respectively; NHK dark, 3 and 6 J.cm^{-2}; n = 5, n = 8, n = 8, respectively). Total cell fluorescence (TCF) was normalized using the following formula: TCF = integrated density – (selected area x mean fluorescence of background readings). Statistical analysis was performed using SigmaStat v.3.5. (Abacus Concepts, Berkeley, CA), performing one-way ANOVA test and Holm-Sidak posttest, considering statistically significant difference for $p < 0.01$ (**) and $p < 0.001$(***). From [22] with permission.

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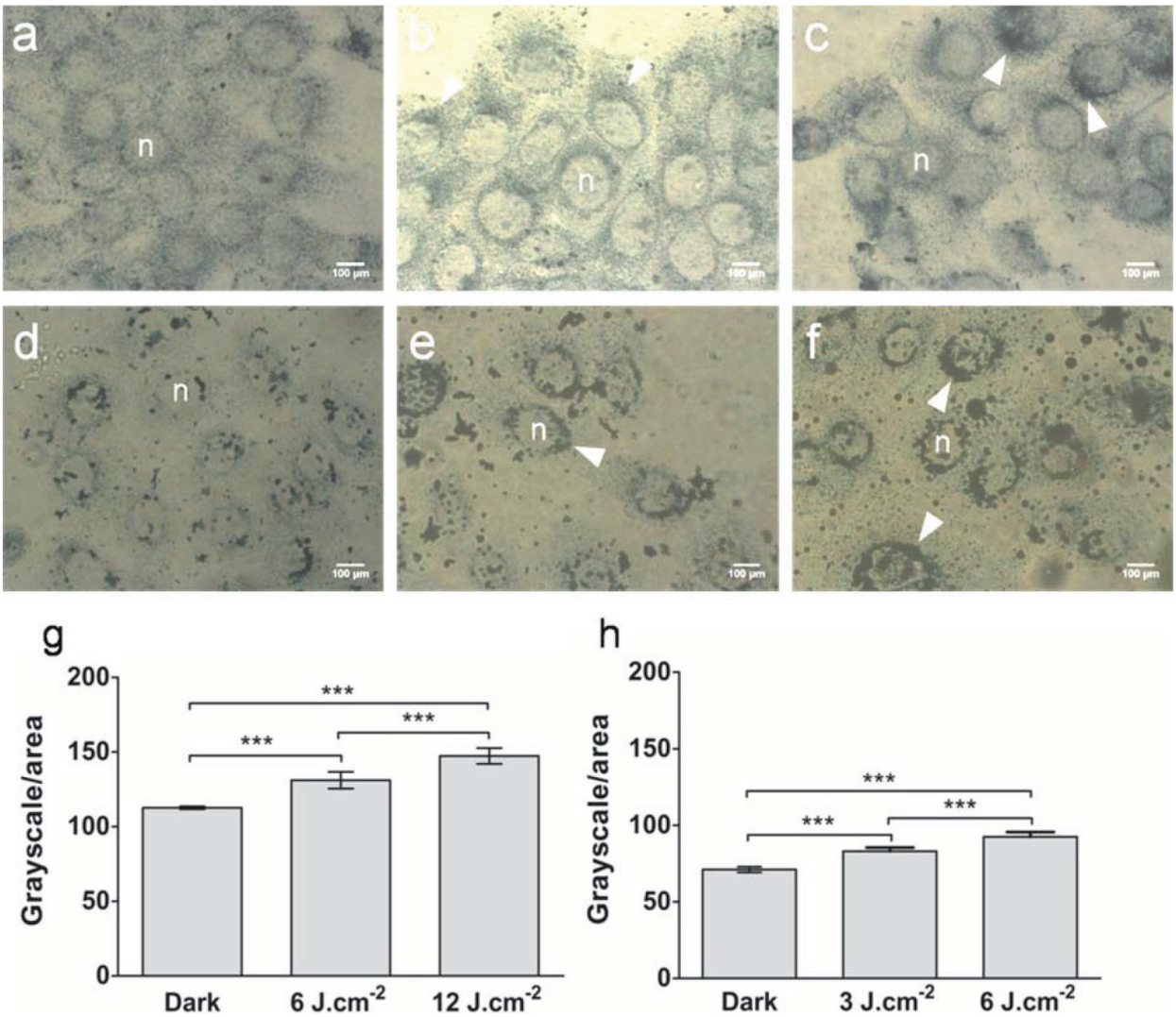

Figure 6. Lipofuscin identification by Sudan Black B staining in HaCaT and NHK cells. Both HaCaT and NHK cells were stained with Sudan Black B (SBB) 48 h after UVA radiation. Images were obtained using bright-field transmitted light microscopy. Whitehead arrows indicate lipofuscin stained by SBB. n = nucleus. For HaCaT cells are shown (a) Dark, (b) UVA 6 J.cm^{-2}, (c) 12 J.cm^{-2} and for NHK cells (d) Dark, (e) 3 J.cm^{-2} and (f) 6 J.cm^{-2}. Quantification of SBB staining in HaCaT (g) and NHK (h) cells. Images were converted to 8-bit grayscale. The sbb-stained area was delimited by adjusting cutoff levels of gray values in order to obtain area and mean gray value. Integrated intensity was divided by area from different images for each sample (HaCaT dark, 6 and 12 J.cm^{-2}; n = 3, n = 10, n = 10, respectively; NHK dark, 3 and 6 J.cm^{-2}; n = 6, n = 7, n = 4, respectively). One-way ANOVA analysis followed by Holm-Sidak posttest was performed in SigmaStat (v.3.5). Statistically significant differences are shown for $p < 0.001$ (***). From [22] with permission.

inhibitor of autophagy (chloroquine), causes this additional cell death, even without UVA irradiation (**Figure 8**).

1.3. Lipofuscin acts as a photosensitizer and causes pre-mutagenic lesions after the visible light exposition

Recently, we have shown that DNA of melanocytes suffers direct oxidative damage by melanin photosensitization with visible light [34]. We tested other skin lineages to verify if these damages can also occur from excessive exposition to visible light in response to the presence of

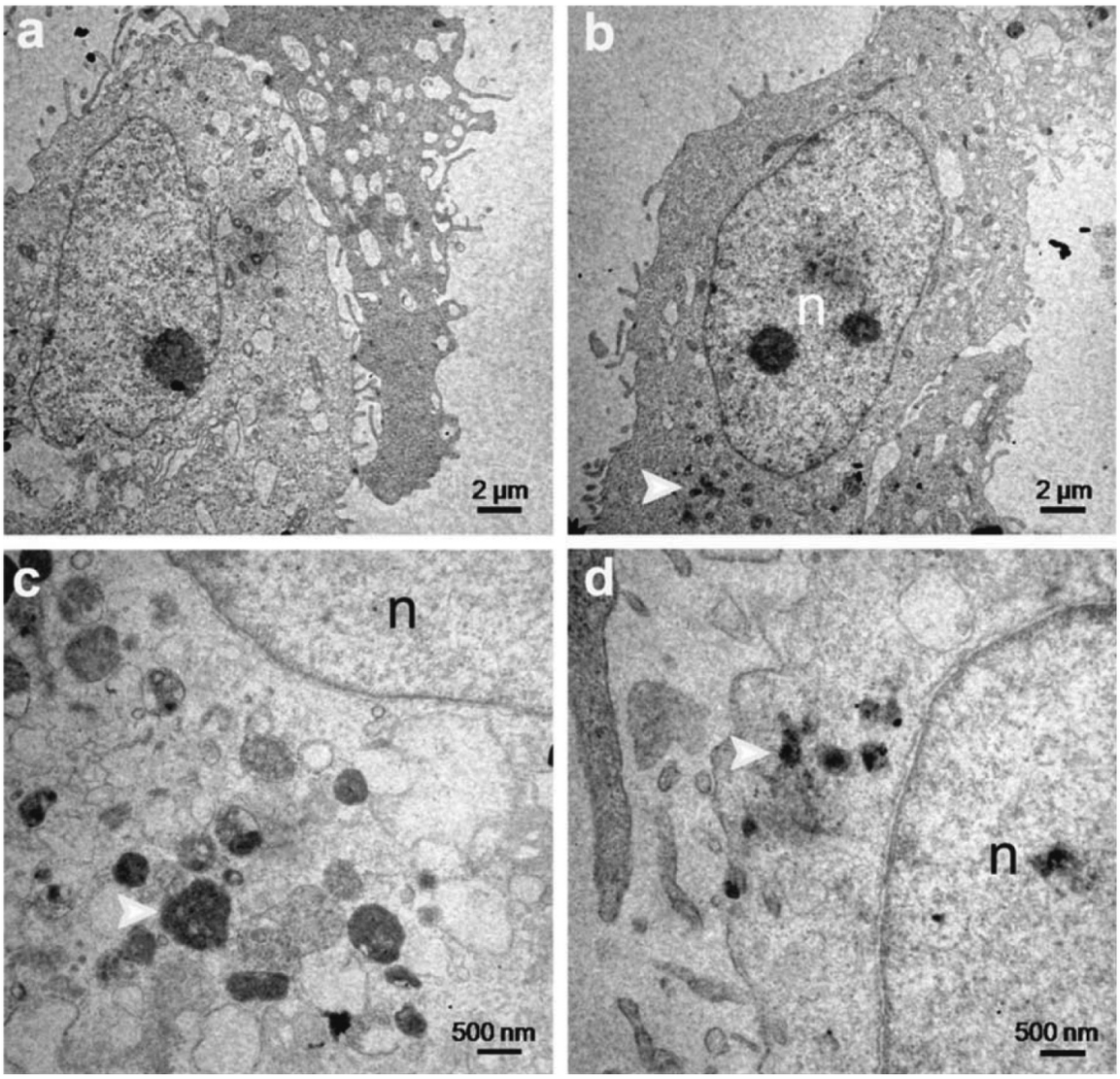

Figure 7. Lipofuscin accumulation in HaCaT cells after exposure to UVA by TEM. Lipofuscin was identified in the perinuclear region of HaCaT cells using transmission electron microscopy 48 h after exposition to UVA radiation. (a) Dark control. (b) UVA-irradiated cells (6 J.cm^{-2}) showing the typical accumulation of electron-dense granules of lipofuscin. (c) and (d) show lipofuscin in details. Whitehead arrows indicate lipofuscin granules. (n = nucleus). From [22] with permission.

natural photosensitizers as lipofuscin. To perform that, we calculated singlet oxygen production in HaCaT cells. For these cells, it was possible to identify that accumulated lipofuscin presented singlet oxygen phosphorescence emission after exposition to UVA light (data not shown) and increasing in DCF oxidation, indicating higher levels of chemical oxidizing species in UVA + Vis group (**Figure 9**). Moreover, if we treat previously HaCaT keratinocytes with a known UVA dose able to induce autophagy inhibition and lipofuscin accumulation (i.e. 12 J.cm^{-2}), after sequential irradiation with visible light (36 J.cm^{-2}), it is only possible to observe significant reduction on cell viability and concomitant higher levels of DNA damage after the second irradiation step (increase in olive tail moment—OTM) (**Figures 10** and **11**). Control cells were irradiated with 36 J.cm^{-2} of visible light only, and they did not show phototoxicity.

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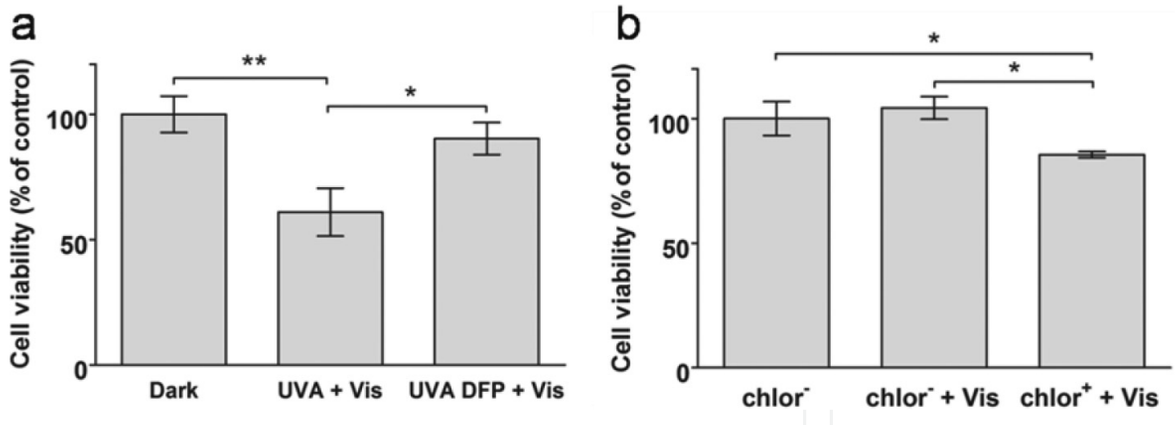

Figure 8. Cell viability based on the reduction of MTT in HaCaT cells. UVA radiation or by treatment with 60 μM chloroquine (a) UVA-treated cells (12 J.cm^{-2}) were immediately incubated with 30 μM deferiprone (DFP), an iron-chelator, avoiding lipofuscinogenesis. Forty-eight hours after initial challenge, cells were photosensitized with visible light (Vis) (36 J.cm^{-2}). (b) Cells incubated in the presence (chlor^{+}) or absence (chlor^{-}) of chloroquine (an autophagic inhibitor). Forty-eight hours after cells were photosensitized with visible light (Vis). MTT assay was measured after 48 h from the last irradiation. Bars indicate mean ± standard deviation. Statistical analysis was performed using SigmaStat v.3.5 (one-way ANOVA followed by Holm-Sidak posttest). Statistically significant differences are shown for $p < 0.05$ (*) and $p < 0.01$ (**). From [22] with permission.

Lipofuscin is a pigment that absorbs visible light and generates enough amounts of triplet and singlet oxygen [21]; therefore, it is likely that this pigment will engage in photosensitization reactions if cells were exposed to specific light sources, as sunlight. Interestingly, lipofuscin that has accumulated in keratinocytes-induced DNA damage, trigging type I and II photosensitization reactions when we employed visible light (see FPG and Endo III data in **Figure 10**).

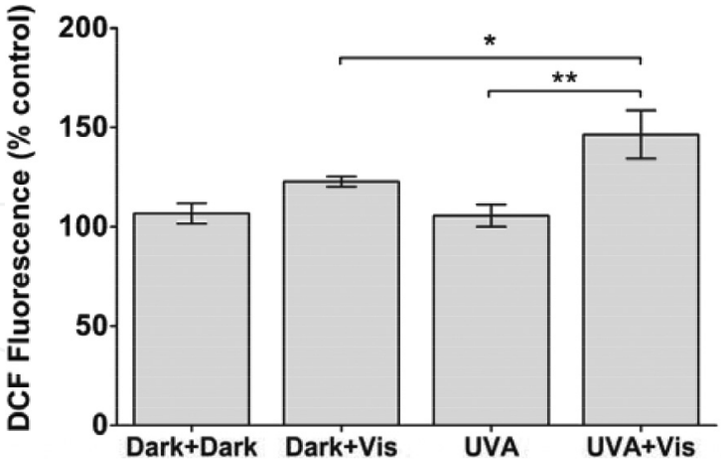

Figure 9. Dichlorofluorescein (DCF) assay for detection of oxidizing species in NHK cells measured immediately after the exposure to visible light. The increasing percentage of fluorescence per cell was calculated by $\%Fluorescence = [(Ft_{30} - Ft_0)/(Ft_0 * 100)]$, where Ft_{30} is the fluorescence at 30 min and Ft_0 is fluorescence at 0 min. After 48 h from the first irradiation, cells were exposed to visible light (Dark+Vis and UVA + Vis). Bars indicate mean ± SD obtained from three independent cell culture experiments (n = 3). Statistical analysis was performed using SigmaStat v.3.5 (one-way ANOVA followed by Holm-Sidak posttest). Statistically significant differences are shown for $p < 0.05$ (*) and $p < 0.01$ (**). From [22] with permission.

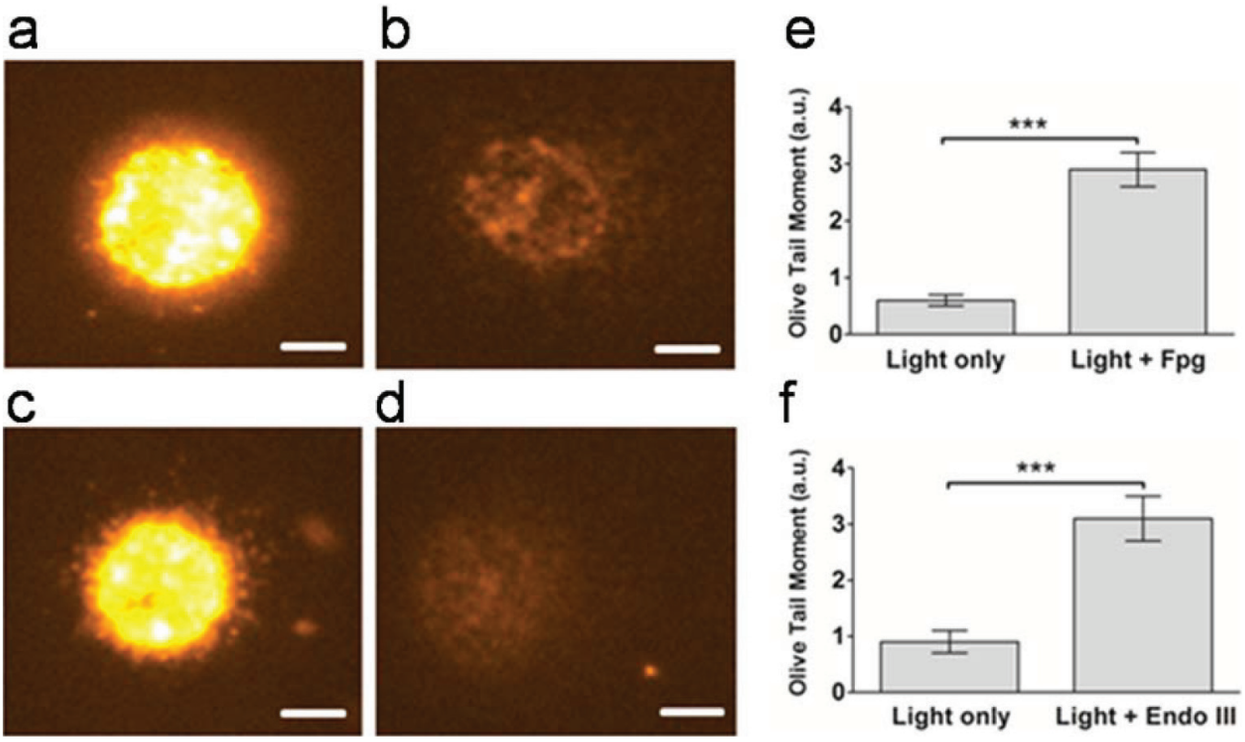

Figure 10. Visible light is phototoxic to lipofuscin-accumulating keratinocytes and causes oxidative DNA damage. Comet assays were performed after light treatment (UVA 6 J.cm^{-2} and visible light 8 J.cm^{-2}) in the absence (a and c) and presence of FPG (b) and Endo III (d) enzymes. Quantification of olive tail moment (OTM) for (a) and (b) are shown in (e) and for (c) and (d) in (f). Statistically significant differences are shown for $p < 0.001$ (**). From [22] with permission.

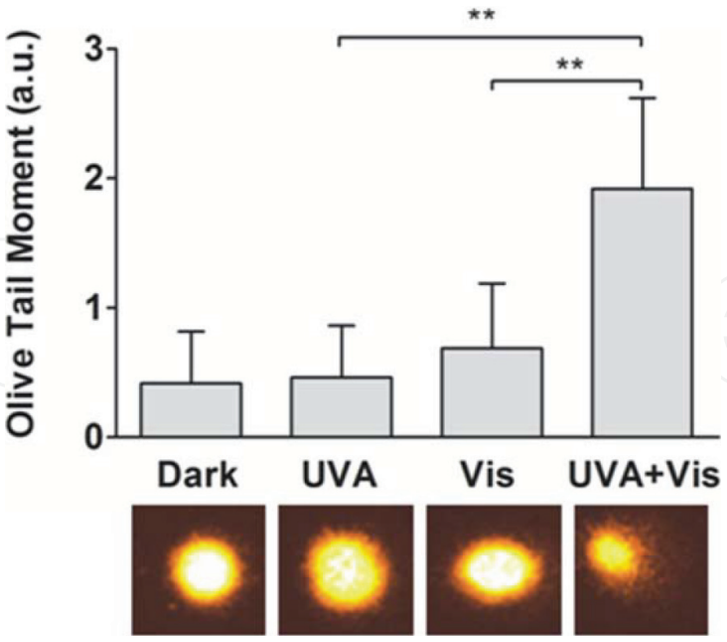

Figure 11. DNA fragmentation (comet assay) after irradiation protocols employing UVA and visible light sources. HaCaT cells were exposed to protocols with isolated light sources (UVA or visible light only) and a sequential protocol of irradiation (previous irradiation with UVA followed by visible light exposure). The graph shows the quantification of cumulative DNA damage when light sources are combined (see the figures right below the graph). Statistically significant differences were considered after one-way ANOVA analysis and Holm-Sidak posttest (**$p < 0.01$). From [22] with permission.

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2. Conclusions

In this chapter, we reported that lipofuscin-loaded human skin keratinocytes presented higher sensitivity to visible light after their exposure to UVA radiation. Photosensitization of lipofuscin by visible light reduces cell viability, generating singlet oxygen and premutagenic lesions in nuclear DNA, as 8-oxo-dG. Here, we have indicated that lipofuscin can absorb the blue light more than other wavelengths, emitting fluorescence above 515 nm.

We consider that the exposure to risk factors like sunlight, which contain UVA and visible light, might have more attention as a public health problem and as a medical/dermatological alert for consumers, especially children. UVA radiation from not only sunlight can potentially increase the risk and incidence of skin cancer. UVA fingerprints are present in the majority of deeper-layer skin tumors and nowadays we have a similar situation occurring with the visible part of the light spectrum. The scientific community is clearly showing that this region of the solar spectra can have hazardous effects on the skin, but there are still few mechanistic explanations. Our results indicate that effects of UVA and visible light can amplify each other, and therefore, it is critical to start considering visible light in terms of human sun protection.

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Conflict of interest

The authors state no conflict of interest.

Acronyms and abbreviations

Endonuclease-III (EndoIII); formamidopyrimidine [fapy]-DNA glycosylase (Fpg); 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); human epidermal keratinocytes (HaCaT); Normal Human Primary Epidermal Keratinocytes isolated from Neonatal Foreskin (NHK).

Author details

Carolina Santacruz-Perez, Paulo Newton Tonolli, Felipe Gustavo Ravagnani and Maurício S. Baptista*

*Address all correspondence to: baptista@iq.usp.br

Biochemistry Department, Institute of Chemistry, University of São Paulo, São Paulo, Brazil

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