The evaluation of the detrimental impact of High Energy Visible Light on human skin: An *ex vivo* and *in vivo* approach

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Abstract

Background

The skin plays a crucial role in protecting against exposomes such as blue light and air pollutants. Blue light is naturally present in sunlight and emitted from digital devices. It can penetrate the deeper layers of the skin to induce an oxidative stress, skin ageing acceleration and hyperpigmentation. While more and more cosmetic products are launched which claim to ward off damage from blue light, no standardized methodologies have till date been set up to substantiate the blue light protection efficacy of cosmetic products on the physiology and biochemistry of human skin.

Materials and Methods

Using two different wavelengths of blue light (412 and 450 nm), either human skin explants (*ex vivo*) or human volunteers (*in vivo*) were exposed and monitored. The oxidative stress, the inflammatory potential (Interleukin-6), the ageing effect (MMP-1 induction, pro-collagen I and degraded collagen) and pigmenting effect induced were assessed.

Results

An increase in the oxidative stress as monitored by induction of reactive oxygen species (ROS), protein carbonylation or squalene mono-hydroperoxide was reported. We also show that pigmentation induced

by blue light is differentially expressed depending on skin phototype. Blue light at both wavelengths had a significant effect on different biomarkers both *ex vivo* and *in vivo*. Depending on the skin phototype, there were differences in the *ex vivo* melanin content as well as differences in the colorimetric measurements *in vivo* after blue exposure.

Conclusions

These results point towards a possible underlying mechanism in skin pigmentation (whether immediate or persistent pigmentation) following blue light exposure, differing depending on skin phototype.

Keywords: Blue light, skin ageing, pigmentation, oxidative stress, *ex vivo* and *in vivo*.

Introduction

Besides UV radiation (UV-R), the visible spectrum of the sunlight plays a role in oxidative stress, cutaneous pigmentation, and cutaneous ageing [1]. Within the visible light spectrum, cutaneous pigmentation and skin ageing are induced by visible light between 400-470 nm, also known as blue light or High Energy Visible (HEV) light [2]. It was additionally reported that blue light penetrates more deeply in the skin compared to UV-A/UV-B [3]. In turn, this induces conditions such as erythema in lighter skin types [4,5] while in darker skin type, exposure to blue light induced more intense and prolonged pigmentation [6-8].

Blue light was reported to affect the molecular structure of skin by inducing hyperpigmentation, lipid peroxidation, protein carbonylation and inflammation resulting in marked photo-ageing [2,9,10]. Carbonylated proteins (CPs) generated following lipid peroxidation leads to DNA damage in sun-exposed skin [11]. Consequently, accumulate CPs in the dermis and epidermis disrupt the dermal matrix and exacerbates MMP-1 levels [12]. These lead to collagen degradation, expedited skin ageing, increased inflammatory hyperpigmentation and melasma [13-16].

Through scientific evidence on the detrimental cutaneous effects of blue light, cosmetic products purporting sunscreen filters and antioxidant actives with blue light protection claims are increasingly commercially available. However, a major setback is the lack of standardized methodology to substantiate the efficacy of the products objectively and accurately on human skin [10]. To address this challenge, we engineered two monochromatic sources of blue light: 412 nm and 450 nm. These faithfully mimics natural sunlight and the screen of electronic devices, respectively.

The aim of this study is to setup an *ex vivo* and *in vivo* methodologies that monitor detrimental effects such as pigmentation, oxidative stress, ageing and inflammation as a result of exposure to two wavelengths of blue light (412 nm and 450 nm). Data obtained from this study would help identity optimal biomarkers for blue light claim substantiation for cosmetics products.

Materials and Methods

1. General overview and study design

Human skin explants were obtained, with research consent (EC-18-PCL-001), from aesthetic surgery. Eight-millimeter skin biopsies composed of both epidermis and dermis were taken using sterile biopsy punches (Kai Medical, Dallas, TX, USA) and rapidly placed in a 24-well plate. The skin was maintained under air–liquid interface culture conditions in skin culture medium, Gibco™ DMEM, without glutamine or phenol red (Thermo Fisher, Waltham, MA, USA).

For *in vivo* clinical study, six healthy subjects from Mauritius participated in the determination of the effect of pigmentation of blue light. The volunteers were either of skin phototype III, IV or of skin phototype V, VI according to Fitzpatrick classification. Another sixteen healthy subjects were recruited from Mauritius to participate in the determination of squalene. The volunteers were of skin phototype III, IV and V according to Fitzpatrick classification. All subjects in this study were dermatologically examined to ensure that there were no constraints.

2. Blue light sources

Blue light was simulated using monochromatic LEDs (Honglitronics Guangzhou, PRC) and the Output Intensity was measured by a broadband thermopile detector (ScienceTech, Ontario, Canada). This unique setup allows the use of monochromatic blue light sources with narrow spectrum (low to no emission in the UV-A spectrum) and to accurately monitor the intensity and thus the dose of blue light that the skin is exposed to.

3. Intracellular Reactive Oxygen Species (ROS) scavenging activity assay – Ex vivo

Human skin punches were treated with DCFH-DA (Sigma, St. Louis, Missouri, USA) prior to exposure to blue light 412 nm or 450 nm at a dose of 40J/cm^2. Following exposure, lysis solution (with 2% Triton X-100) are added to all wells. The fluorescence (wavelength of excitation of 485/88 nm and emission 528/30 nm) was measured using the Synergy HTX multimode microplate reader (BioTek, Winooski, VT, USA).

4. Quantification of Squalene – Ex vivo

Skin discs punches were exposed to blue light 412 nm (1.5 J/cm^2, 20 J/cm^2 and 40 J/cm^2). Quantification of squalene was then determined following the instructions of Curpen et al. [17].

5. Quantification of Procollagen type I C-Peptide (PIP) – Ex vivo

Skin discs punches were exposed to blue light 412 nm or 450 nm for three consecutive days at a dosage of 40 J/cm^2 per day. Twenty-four hours after the last exposure, the cell culture supernatant was collected, and the level of PIP was measured by using the Procollagen Type I C-Peptide (PIP) ELISA Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions.

6. Immunostaining of degraded/unwound collagen – Ex vivo

The skin discs were exposed to blue light 412 nm or 450 nm at a total dosage of 40 J/cm^2 per day for three consecutive days. Twenty-four hours after exposure, the skin disc punches were fixed, and immunostaining was performed using a specific probe recognizing a sequence visible in unwound collagen only.

7. Melanin content assay (MCA) – Ex vivo

The skin discs punches were exposed to blue light 412 nm or 450 nm for three consecutive days at a total dosage of 120 J/cm^2. The melanin in human skin explants were solubilized by adding hot NaOH (1 M) containing 10% DMSO. The melanin content was then evaluated by measuring the absorbance of the lysates at 405 nm.

8. Determination of squalene from sebum of tape strips – In vivo

The tape strip (D-squam) of each volunteer was cut into two. Half was left unexposed while the second half was exposed to 15 J/cm^2 of blue light. Neutral lipids were extracted from the tape strips and analyzed according to Curpen et al. [17].

9. Oxiproteome viewing and scoring – In vivo

The collected corneocytes (on D-squame tape strips) were transferred on slide and fixed. The carbonyl groups were labelled by using a specific fluorescent probe. Images were collected by an epifluorescence microscope (Evos M5000; 40x objective) and analysed with the software ImageJ (US National Institutes of Health, Bethesda, Maryland, USA). Extracted carbonylated proteins were labeled with specific fluorescent probe and separated by electrophoresis (SDS-PAGE). Digital image acquisition of carbonyl proteins and total proteins was performed using the "Ettan DIGE imager" system (GE Healthcare). Image processing and analysis were performed using the "ImageJ" software. The signal of the carbonylated proteins (fluorescence units) was normalized with respect to the signal obtained with the total proteins for each sample (Ratio) to obtain the Carbonyl Score.

10. Determination of the pigmenting effect of blue light – In vivo

Volunteers came to the study site without any product on the upper back and were acclimatized at room temperature, back uncovered, for 15 minutes. Eight different zones were identified. One zone which was left unexposed (control zone) and the remaining zones were exposed to three different doses of either blue light 412 nm or 450 nm: 60 J/cm^2, 75 J/cm^2 and 87.5 J/cm^2 over a period of four consecutive days. Each day, prior to exposure to blue light, colorimetric readings of the four zones was performed. A last reading was collected 24 hours after the last exposure (D5). Colorimetric measurements using a Chromameter Minolta CR400 (Konica Minolta, Tokyo, Japan) were taken in the middle of the zones. The recorded L*a*b* values (CIELab colour system) were used to calculate the Individual Typological Angle (ITA°).

11. Statistical analysis

Measurements generated from the parameters of interest, mainly numerical in nature, are summarized in the form of mean ± SEM in graphical illustrations, provided by treatment and timepoint. All statistical tests were conducted at 5% level of significance, while normality tests were capped at 1% level. SPSS 19.0 (IBM Inc.) and GraphPad (GraphPad Software Inc., CA, USA) were used for analysis purposes. For the *ex vivo* studies, statistical analysis was conducted through one-way ANOVA followed by pairwise comparison. Oxidative stress *in vivo* was assessed through a paired *t*-test. Pigmentation *in vivo* included Pairwise comparisons among zones were conducted at each timepoint, on the difference data with respect to baseline, following a Univariate ANOVA procedure, with 'zone' as fixed factor and 'subject' as random component. The analysis was conducted on rank transformed data whenever the normality assumption of the standardized residuals produced by the model was violated at 1% level of the Shapiro-Wilk test

Results

12. *Ex vivo* trials

12.1. Quantification of Intracellular ROS Assay

The oxidative stress was measured by monitoring the ROS production in human skin explants whereby the unexposed and untreated skin samples demonstrated very low levels of ROS induction while blue light 412 nm demonstrated the highest (**Figure 1a**). A 15-fold induction in the level of ROS can be observed in the human skin explants after exposure to blue light 412 nm at a dosage of 40 J/cm^2 while a 14-fold induction in the level of ROS was observed when the human skin explants were subjected to blue light 450 nm (40 J/cm^2).

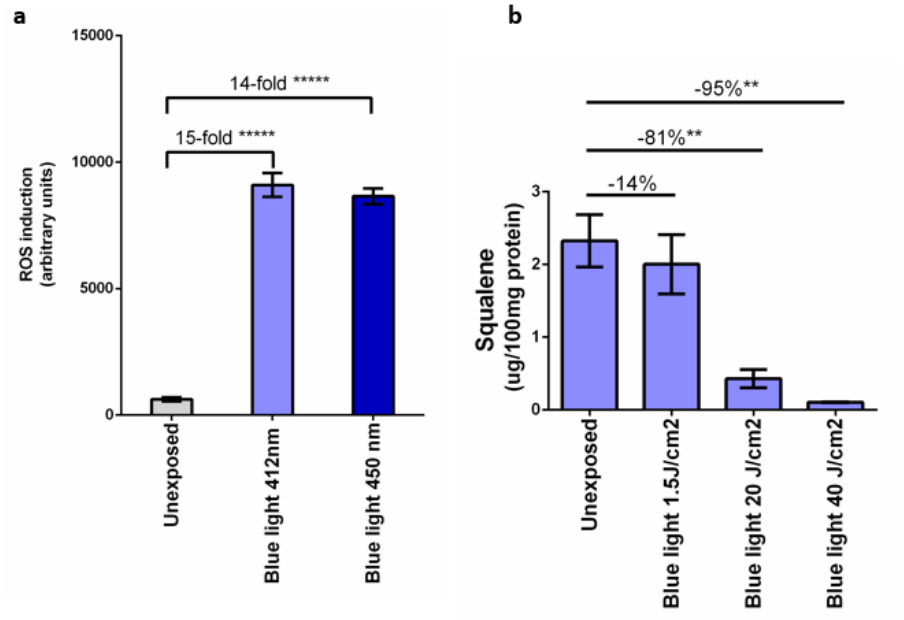

Figure 1(a): The level of oxidation in the human skin explants monitored by measuring the induction of ROS following exposure to blue light 412 nm and 450 nm. **Figure 1(b):** The level of squalene in the human skin explants monitored by quantification of squalene following exposure to blue light 412 nm and 450 nm.

12.2. Quantification of squalene

Exposure of human skin explants to increasing doses of blue light induced a corresponding dose dependent decrease in the level of squalene (**Figure 1b**). Indeed, a 14%, 81% and 95% decrease in the level of squalene was observed after exposure to 1.5 J/cm^2, 20 J/cm^2 and 40 J/cm^2 respectively. The highest dosage of blue light at 40 J/cm^2 provided the highest decrease of 95% in the level of squalene.

12.3. Detection of unbound collagen

It was investigated whether blue light promoted the degradation of the extracellular matrix by affecting the induction of the precursor of collagen I (pro-collagen I) and accelerating degradation and unwinding of the key collagen molecules present in the skin dermis. The level of pro-collagen I released in the cell culture media was first assessed in skin explants that were repeatedly exposed to blue light 412 nm and

450 nm (**Figure 2a**). Both wavelengths of blue light were capable of decreasing the level of the pro-collagen I in the cell media. A significant decrease of 65% and 49% was observed after exposure to blue light 412 nm and 450 nm respectively.

Using a fluorescent labelled probe targeted at unwound and degraded collagen, we demonstrate that human skin explants exposed for 3 consecutive days to blue light (total of 120 J/cm^2) exhibited increased red staining compared to untreated skin explants (**Figure 2b**). These results confirmed that an exposure to blue light triggered degradation of the collagen either directly by destruction of the collagen bundles and unwinding of the collagen fibers or by inducing enzymes implicated in the degradation of the extracellular matrix.

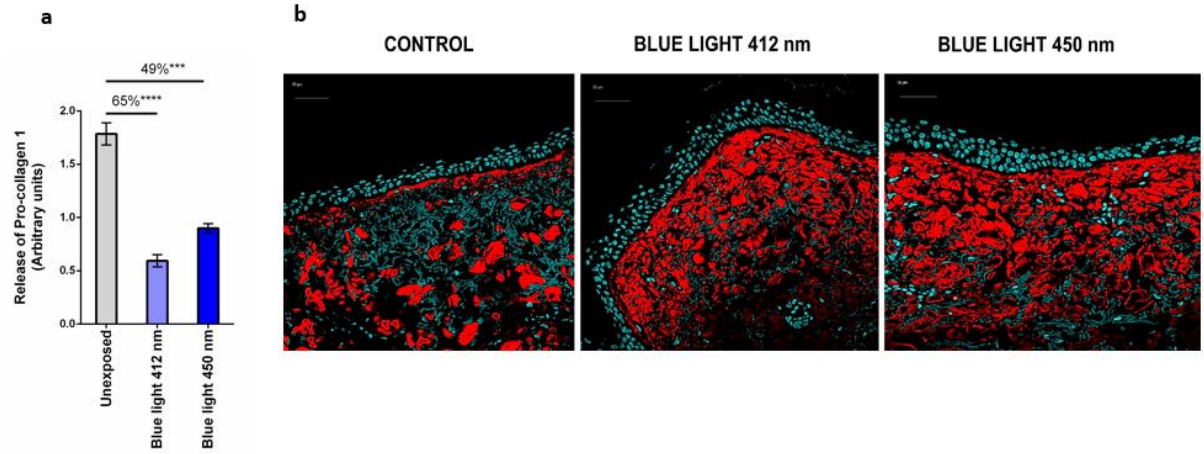

Figure 2(a): The degradation of the extracellular matrix by affecting the induction of the precursor of collagen I (pro-collagen I) **Figure 2(b):** Using a fluorescent labelled probe which targeted specifically unwound collagen and thus degraded collagen, we showed that human skin explants exposed for 3 consecutive days with blue light

12.4. Skin pigmentation

We then monitored whether blue light, 412 nm, or 450 nm induced pigmentation in human skin explants of phototype III (**Figure 3a**) or phototype VI (**Figure 3b**) after 3 consecutive days of exposure totaling to 120 J/cm^2. In **Figure 3a**, blue light 412 nm significantly induced the level of melanin by 50% in skin explants of phototype III. However, no induction in the melanin content was reported following exposure to blue light 450 nm. In skin explants of phototype VI (**Figure 3b**), a significant induction was observed for both blue light 412 nm and 450 nm. Interestingly, this induction in the melanin content was equal after exposure to the two different wavelengths of blue light whereby an induction of 47% in melanin content was observed after exposure to either blue light 412 nm or 450 nm.

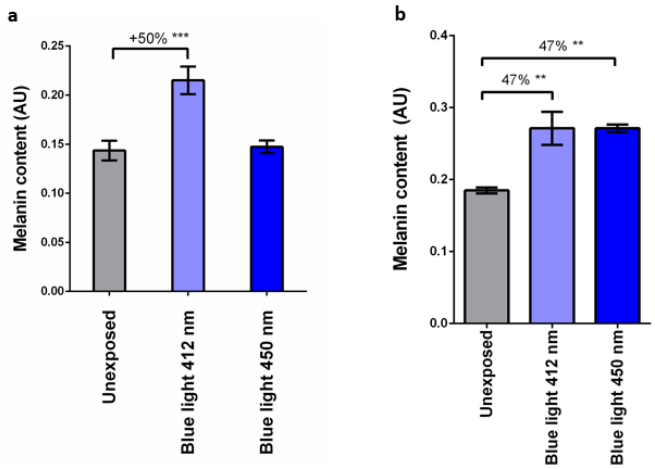

Figure 3: Melanin content in human skin explants from donor of either **(a)** phototype VI or **(b)** phototype III after exposure to different sources of blue light

13. *In vivo* trials

13.1. Oxidative stress induced following blue light exposure

In Vivo, exposure to blue light revealed an increased amount of carbonylated protein in the corneocytes compared to the control sample which was not irradiated with blue light (**Figure 4a**). This data was further corroborated by the visualization of the in-situ oxidative level (carbonylation). Indeed, an intensification of the carbonylation signal is observed in red (**Figure 4b**) following an exposure to the stressor (blue light) and compared to the non-exposed zone.

13.2. Oxidative stress induction after exposure to blue light – Combination of *In Vivo* and *Ex Vivo* techniques

As shown in **Figure 4c**, exposure of the tape strips to blue light induced an oxidative stress as shown by the significant increase in the level of Squalene monohydroperoxide (SQOOH). Indeed, a 2-fold increase, as compared to the basal level, was observed after exposure to blue light when compared to the unexposed samples (**Figure 4d**).

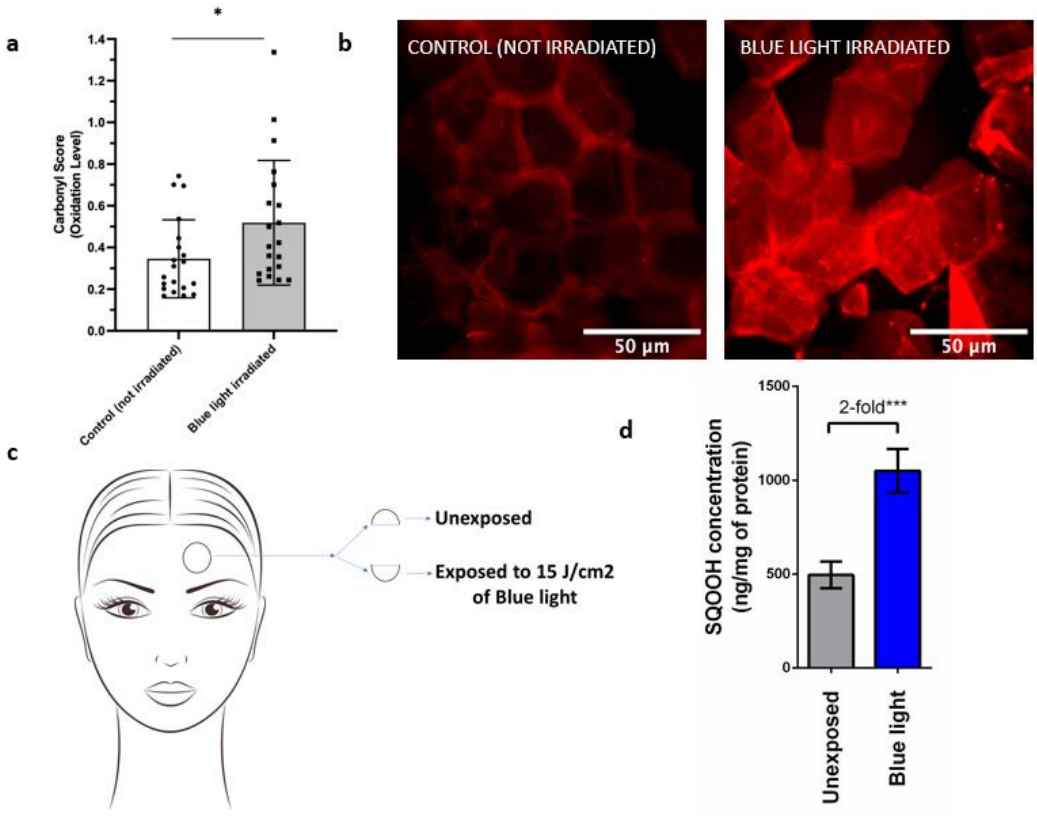

Figure 4a: Quantification of carbonylated proteins and distribution of individual values. The evolution of the oxidation rate shows a statistically significant increase (**p <0.05**) of **50%** between control and blue light exposure. **Figure 4(b)** the visualization of the in-situ oxidative level (carbonylation) is presented as intensity of carbonylation signal (in red). Increased levels of oxidative damage (carbonylation) have been observed upon stress (blue light) exposure in comparison to the not exposed zone. **Figure 4(c):** Tape strips were performed at the level of the forehead of the volunteers. The first tape strip was discarded, and the second tape strip were halved where the first half was left un-exposed, and the second half was exposed to blue light **Figure (d)** The level of oxidized squalene (SQOOH) was monitored in the un-exposed and exposed tape strips.

13.3. Skin pigmentation in light skinned individuals

As observed in Figure 5a and 5b, an exposure to blue light in light-skinned individuals caused a decrease in luminosity and fairness as reported by the L* parameter and ITA°, respectively. The decrease observed in these parameters corresponded to an increase in skin pigmentation. In human subjects with skin phototype III-IV, the maximum pigmentation was observed on Day 4 before reaching a plateau on Day 5 with constant values in L* parameter and the ITA° values. Interestingly, no significant differences in the L* parameter or ITA° values were observed when the skin was exposed to either 60 J/cm^2, 75 J/cm^2 or 87.5 J/cm^2 across all the time points. These results suggest that at 60 J/cm^2, a threshold was reached whereby the pigmentation cannot go any higher at that given time point.

Interestingly, while no changes in the b* parameter was reported across the time points, an induction in the a* parameter was observed following exposure to the blue light. This contributed to an increased reddening of the skin (**Figure 5a**). Reminiscent to what was observed in the L* parameter and the ITA values, a maximum skin redness was reported on Day 4 before reaching a plateau. Furthermore, no significant differences in the a* parameter was observed when skin was exposed to either 60 J/cm^2, 75 J/cm^2 or 87.5 J/cm^2 across all the time points investigated.

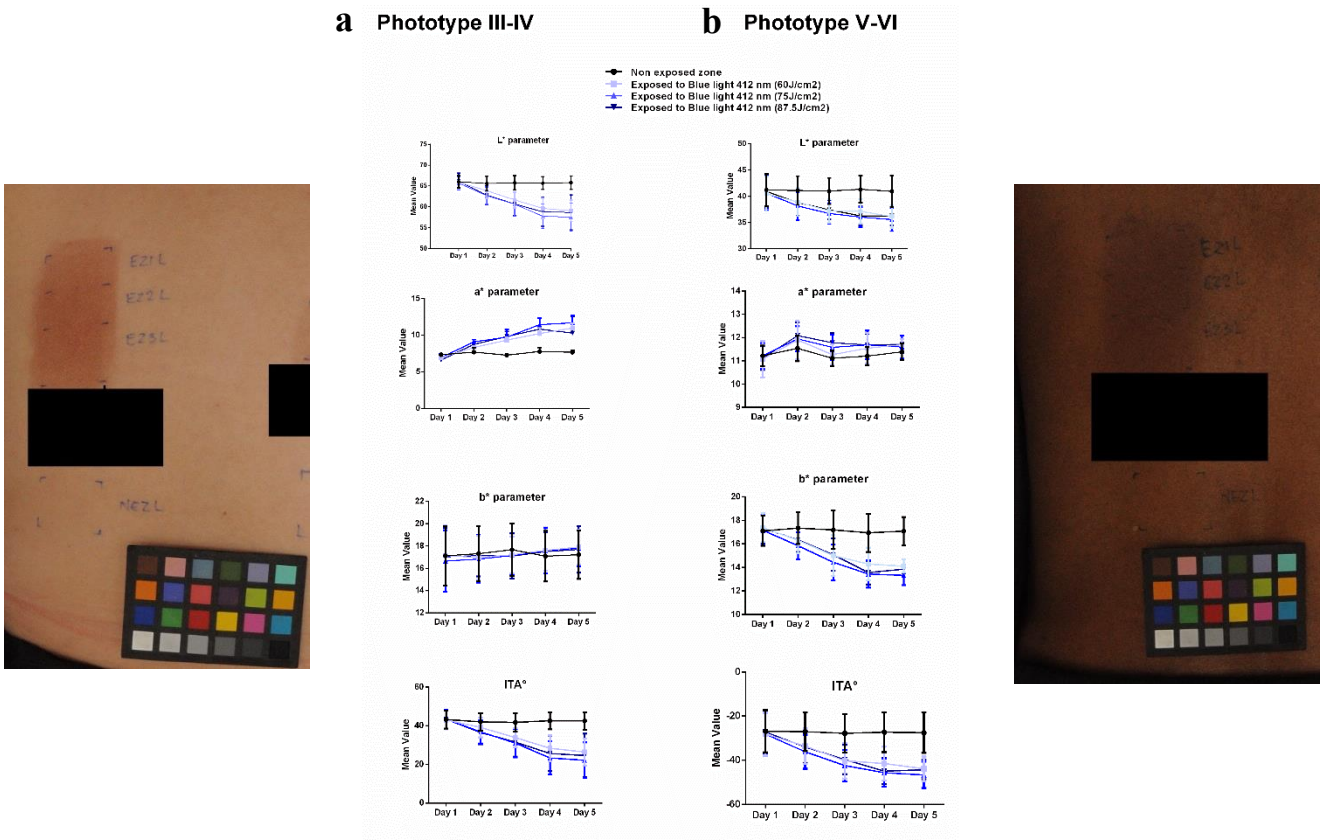

Figure 5: Determination of the L*a*b* and ITA parameters in **(a)** light skinned (phototype III-IV) or **(b)** dark-skinned (phototype V-VI) exposed to different doses of blue light 412 nm

13.4. Skin pigmentation in dark skinned individuals

The L*, a*, b* parameters and ITA values were then evaluated in dark-skinned volunteers of phototype V-VI that were subjected to either 60 J/cm^2, 75 J/cm^2 or 87.5 J/cm^2 of blue light 412 nm (**Figure 5b**).

Akin to what was reported in light-skinned individuals, a decrease in the L* parameters and the ITA^o values was observed after exposure to blue light 412 nm from Day 1-Day 3 before reaching a plateau on Day 4 for both parameters. Moreover, no significant differences in the L* parameter or ITA values was reported when the skin was exposed to either 60J/cm^2, 75J/cm^2 or 87.5 J/cm^2 across all the time

points investigated. These results suggested that similar to observations recorded in pale-skinned individuals, a threshold was reached at 60J/cm^2 in dark-skinned individuals whereby the pigmentation for that time point could not go higher (**Figure 5b**). As observed from the a* parameter, minor differences in erythema on darker-skinned human subjects were reported following exposure to blue light 412. However, a drop in the b* parameter was revealed which correlated with the observable bluish tint of the skin visible in the photographs (**Figure 5b**). Contrary to what was reported in light-skinned individuals, no increased erythema was reported but rather an increase in the bluish colour of the skin as supported by the decrease in the L* parameter. Similar to what was reported in *ex vivo* human skin explants, these results indicated that the pigmenting effect of blue light differed depending on the phototype of the skin. This investigation was repeated using blue light 450 nm at three different doses (60 J/cm^2, 75 J/cm^2 or 87.5 J/cm^2) on the same human subjects and identical results to blue light 412 nm were revealed in both light-skinned and dark-skinned individuals (**Figure 6a and 6b**).

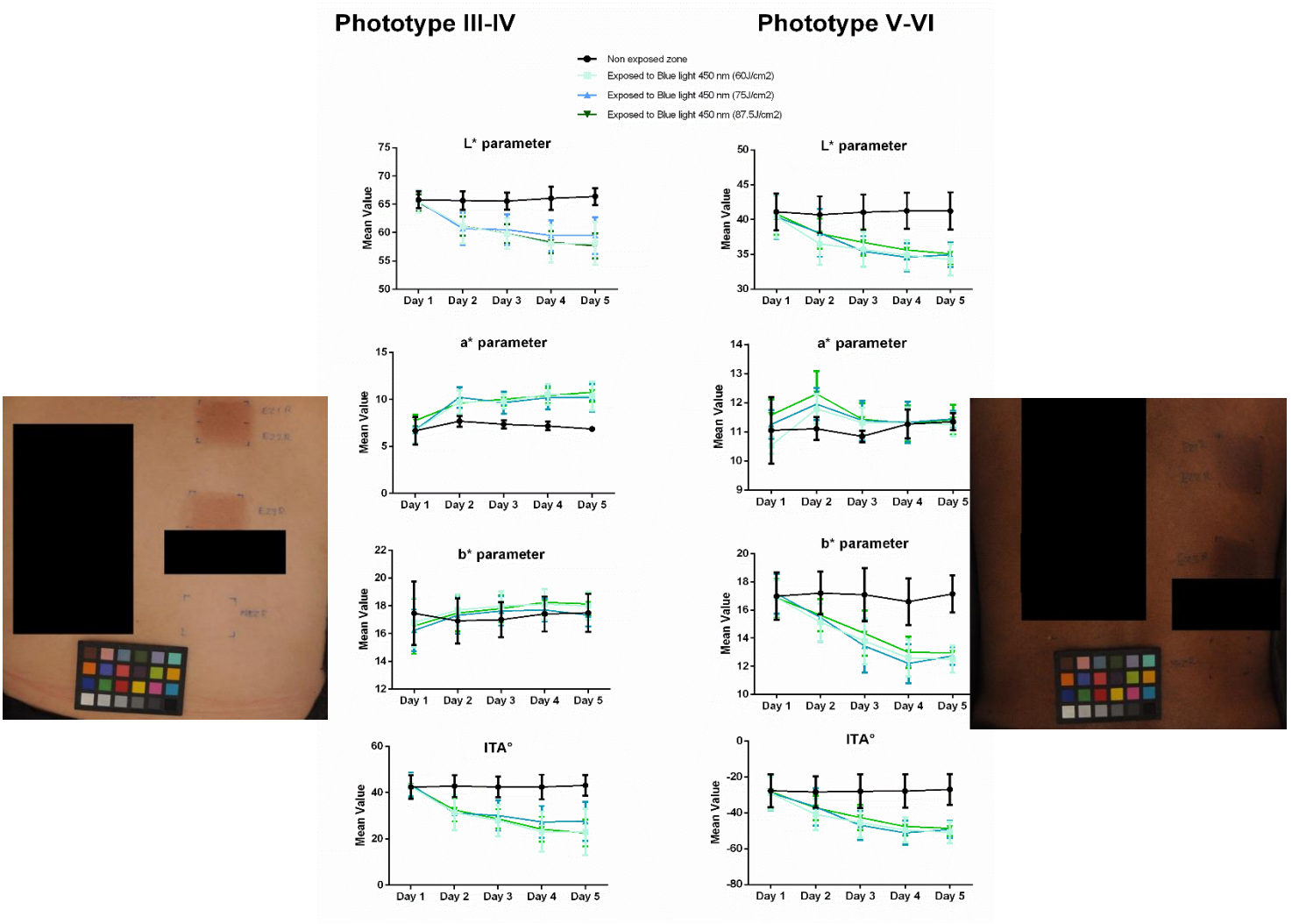

Figure 6: Determination of the L*a*b* and ITA parameters **(a)** in pale skinned (phototype III-IV) or **(b)** dark-skinned (phototype V-VI) exposed to different doses of blue light 450 nm

Discussion

It is well documented that blue light greatly influences extrinsic skin ageing [18-23]. Various cosmetic products are hence marketed to exhibit properties that potentially counteracts the effects of blue light on skin ageing and pigmentation. However, these claims remain mostly speculative as they are yet to be fully scientifically validated using a robust standardized method. While techniques of claim substantiation studies exist to assess blue light protection such as imaging devices, visual evaluations, and spectrophotometers, these are not expected to fill the gap in addressing the lack of standardized methods when more and more ingredients are commercially launched with blue light protection properties [24].

Here, we implemented a novel protocol to determine the effect of blue light on the physiology and biochemistry of the human skin using monochromatic light sources that englobes both an *ex vivo* and *in vivo* approach. The data collected revealed that both wavelength of blue light induced oxidative stress *ex vivo* and *in vivo*. We also put forward confirmation that blue light causes irreversible damage to the collagen content of the skin following exposure at two wavelengths: 412 nm and 450 nm.

Both *ex vivo* and *in vivo* results showed that blue light had a pigmenting effect on the skin which varied depending on the skin phototype. An induction in the melanin content was reported in human skin explants of phototype III when exposed to blue light 412 nm but not to 450 nm. *In vivo*, change in parameters between D1 and D2, D2 and D3 and D3 and D4 suggested that an additional pool of melanocytes was being oxidized daily till D4 where the maximum level was reached. In contrast, human skin explants of phototype VI showed an increase melanin content when exposed to both wavelengths of blue light. This variation in melanin content was further confirmed *in vivo* whereby an induction in a* parameter associated with skin redness was observed only in light skinned human subjects while no changes in the b* parameters was reported.

Liu et al., previously reported that exposure to UV-A was associated with a slight drop in the systemic blood pressure while exposure to UV-B was partly associated with skin erythema. Both of these phenomena were related to epidermal nitric oxide release and its translocation following exposure to the stressor [25]. We hypothesized that the onset of redness associated with erythema reported here might have induced the same physiological response on a smaller scale in the skin of the subjects following exposure to two wavelengths of blue light. However, this remains to be confirmed via further assessments.

On the other hand, we reported a drop in the b* parameters associated with an increase in the bluish color of the skin of dark-skinned individuals while no changes in the a* parameter was

reported. Across all volunteers, this decrease in the L* parameter and ITA was associated with an increased pigmenting effect following exposure to blue light. These observations are supported by previous studies exploring the effects of UV-R on the different ethnic skin [26]. The difference in pigmentation between the two skin phototypes depended on the amount of melanin and relative ratio of eumelanin, as well as the size, quantity, and distribution of melanosomes within the epidermis of the skin samples [26,27]. Indeed, lighter skin types was reported to contain smaller and more clustered melanosomes.

Growing evidence suggest that ROS as a result of oxidative stress induced by blue light is not linked to hyperpigmentation [10,28]. Instead, immediate pigmentation induced after blue light exposure is linked to photo-oxidized melanin [9] while persistent pigmentation in melano-competent subjects stems from neo-melanogenesis leading the formation of a protein complex in dark-skinned melanocytes [29-30]. A resulting prolonged melanogenic enzyme activity such as tyrosinase would explain the persistent pigmentation observed in darker skin type following blue light. [29].

Interestingly, the skin of all human test subjects in this study showed no significant changes between the three doses of blue light 412 nm tested while changes in the L*, a*, b* and ITA values could be detected across all time-points. Similar observations were made with blue light 450 nm. The data collected suggest that at 60 J/cm2 of blue light, a threshold is reached where further pigmentation cannot be further detected. Subsequently, a continuous increase in pigmentation was observed until a plateau on Day 4 although no significant difference between each dose was noted.

Here, for the first time we investigated the immediate and persistent pigmentation induced by blue light. Previous studies have assessed the impact of immediate and persistent pigmentation on the skin against UV-R [30]. We posit that although both immediate pigmentation and persistent pigment method has previously been used to measure the protection effect of UV-R, the strength and standardization of similar studies with blue light has not yet been explored. In order to observe a more discernible difference in skin pigmentation, long-lasting effects of blue light exposure on pigmentation such as 14 days to 1 month remains to be assessed. Moreover, since differences in the a* and b* parameters can be observed in the light and dark-skinned individuals, future studies assessing changes in the micro-circulation of different individuals following light exposure could be explored.

Conclusion

The results presented in this study demonstrate the suitability of our developed monochromatic blue light sources as a standardized method to investigate the effect of blue light on the skin in terms of oxidative stress, inflammation, extracellular matrix degradation and skin pigmentation. We put forth confirmation that a variation in the expression of pigmentation due to blue light 412 nm and 450 nm exists in skins of different phototypes. Ultimately, our data points towards a possible underlaying mechanism of pigmentation following blue light exposure which is dependent on the skin phototype.

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Conflict of Interest Statement. Authors declare no conflict of interest.

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